Behavioural and neural studies of male singing and female phonotaxis behaviour in crickets

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Preface

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the preface and specified in the text.

It is not substantially the same as any work that has already been submitted before for any degree or other qualification except as declared in the preface and specified in the text.

The total length of the text (excluding references, figure legends, and tables) does not exceed the 60,000 words limit for the Degree Committee for the Faculty of Biology.

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1 April 2021

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Summary

Acoustic communication in crickets is achieved by the production of calling, courtship, and rivalry songs in male crickets and the recognition of the songs by conspecifics. Male crickets sing by rhythmically opening and closing the forewings to generate sound pulses and song patterns. The central command for singing is controlled by the brain. The song structure is under control of the abdominal central pattern generator (CPG) and acts as a behavioural barrier to prevent courtship with females of closely related species. Therefore, an analysis of the song structure, the central command, and the organisation of the CPG in different cricket species could reveal how evolution shaped cricket singing behaviour.

In *G. bimaculatus*, I recorded different song types and corresponding wing movement of individual males. I discovered a small amplitude oscillation of the forewings during courtship song that was not reported before. The similar pulse parameters and wing movements during calling and rivalry song could imply a shared neural network for the two song types in *G. bimaculatus*.

I analysed rivalry and courtship behaviour before and after applying specific lesions to the abdominal nerve cord in *G. bimaculatus*. Most elements of rivalry and courtship behaviour are not affected by lesions, except for rivalry song, courtship song, and copulation. For generation of the rivalry song the central nerve cord from the brain to A4 is sufficient (same as calling song), whereas the whole nerve cord without the terminal abdominal ganglion is required for generation of courtship song.

I compared the calling song before and after applying specific lesions to the abdominal

nerve cord in four cricket species: *G. rubens*, *G. assimilis*, *Teleogryllus oceanicus*, and *T. commodus*. The four species show similar effects of the lesions on the generation of sound pulses besides a species-specific control of song structure, suggesting they share a conserved organisation of the CPG network for calling song.

Following the discovery of the calling song command neuron in *G. bimaculatus*, I carried out intracellular recording in the brain of males of different species. I found the putative command neurons for calling song in *G. bimaculatus*, *G. assimilis*, and *T. commodus* and characterize the physiological and functional properties of these neurons. The results suggest the command neurons in the three species could be homologues by showing a similar control on generating calling song.

Furthermore, based on female cricket phonotaxis preference I developed an animal acoustic selecting system, in which the females have to navigate through a complex parkour to reach the acoustic stimulus presenting speaker. I tested the system with different selecting experiments and proved the system can be applied to select females attracted to certain song pattern.

Overall, these findings broaden our understanding of cricket singing in terms of neural control of different song types and evolution of singing behaviour in different species, and provide a new tool to study phonotactic behaviour.

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1   Chapter One: General introduction

1.1   Acoustic communication in insects

In some groups of insects acoustic signals are commonly used for (1) intraspecific communication like attracting mates, courtship behaviour, and rivalry behaviour, (2) locating hosts in parasitoid insects, and (3) avoiding potential predators (Strauß and Lakes-Harlan 2014). These signals feature a wide frequency range from 15 Hz to over 100 kHz and cover a range of sound intensities depending on their function (Noda et al. 2019). Specialised sound production apparatus with different size and structure have evolved in different taxa for the generation of species-specific sound signals (Bennet-Clark 1998). In response to acoustic signal from the sender, tympanal ears of various kinds tuned to the sound frequency of the acoustic signal evolved in different species. Most tympanal hearing organs share three basic structures: (1) tympanal membranes or ear drums which vibrate in response to the sound waves, (2) an air filled space that backs the tympanal membrane, and (3) sensory organs responding to the tympanum oscillations, functioning as auditory receptor neurons (Strauß and Lakes-Harlan 2014). The production and recognition of acoustic signal allow insects to communicate with each other. In many species like crickets and cicadas only males possess sound producing structures and the communication is uni-directional (Fonseca 1996; Itoh and Murakami 2002), namely, only individuals of one sex produce sound and individuals of the other sex approach the signaller, in a behaviour called phonotaxis. While other species like some bush crickets implement bidirectional communication (Robinson 1980), in which sex-specific acoustic signals are produced by both sexes and individuals respond acoustically to the sound produced by the opposite sex.

In insects, sound production mainly relies on rhythmically grating a specialised apparatus,

which may have evolved on different body parts, e.g. wing and wing in crickets and bush crickets (Dumortier 1963), leg and wing in grasshoppers and some moths (Elsner 1974; Surlykke and Gogala 1986), wing and abdomen in cockroaches and beetles (Hartman and Roth 1967; Roth and Hartman 1967; Michael and Rudinsky 1972) and leg and abdomen in bladder grasshoppers (Van Staaden and Römer 1997). A different way of sound production occurs in cicadas which deform the springs in an timbal apparatus during timbal muscle contraction (Fonseca 1996). Besides these far-field acoustic signals based on pressure changes, by fanning the wings fruit flies generate near-field sounds which are picked up by their antennal velocity receivers. In the following paragraphs the sound production machinery in different insect species are reviewed.

In bush crickets (*Tettigoniidae*), acoustic signals are generated by rubbing together the forewings, during which teeth of the file on one wing are swept against the scraper on the other wing; sound is produced by resonant vibrations of the wing membrane structure called mirror (Bailey and Broughton 1970; Montealegre-Z and Mason 2005). The sound frequency generated by bush crickets ranges from 600 Hz to over 100 kHz in about 7000 different species (Heller 1995; Chivers et al. 2017), and some species in tropical forest were reported to produce sound up to 150 kHz (Montealegre-Z et al. 2006; Sarria-S et al. 2014).

Sound generated by contact of leg and wing was described in grasshopper and some moth species. In gomphocerine grasshoppers, the calling, courtship and rivalry song are generated by rubbing a file on the inner side of the hindleg with rhythmic up and down movements against a tegminal vein (Elsner 1974; Ostrowski et al. 2009). In the noctuid moth *Thecophora fovea*, sound is produced by rubbing the hind leg against the hind wing and the song appears as pulse trains with a frequency peak at 32 kHz (Surlykke and Gogala 1986).

The cockroach *Nauphoeta cinera* and *Dendroctonus* beetles are two examples that produce sound by rubbing the body against the wing. In the cockroach *Nauphoeta cinera*,

sound is generated by forward-backward or side to side movements of the pronotum against the costal veins that rubbing the parallel striae of the body parts (Hartman and Roth 1967; Roth and Hartman 1967). In *Dendroctonus* beetles, rubbing of the plectrum on the median posterior margin of 7th tergite against teeth file at the bottom of elytra is used to produce songs for courtship.

A very different way of sound production not depending on friction has evolved in cicada, with a specialised timbal apparatus containing two ribbed integument membranes (Fonseca 1991, 1996). Upon contraction of the timbal muscle, the membranes are deformed and sound is generated. The frequency of the sound falls in the range of 5 kHz to 15 kHz. Different from many acoustic communication insects, songs of many cicada species are characterized by frequency modulation (Fonseca et al. 2000).

All these species have in common that they generate far field acoustic signals based on changes in the sound pressure. Although the sound producing apparatus is very similar in each of the different taxa, it is the underlying motor pattern that controls the generation of species-specific sound signals.

Other species like *Drosophila* and mosquitoes may generate near field sound signals based on changes in the particle velocity for intimate communication over a few millimetres. These near field sound signals generated by vibration of air particles decay much faster than far field signals and are suitable for intimate communication. One of the example is courtship song generated by *Drosophila melanogaster* (Tauber and Eberl 2003; Zanini et al. 2014). As part of courtship display, males generate two kinds of songs, sine songs and pulse songs by one raised wing. Sine songs were hums with a dominant frequency between 140 and 170 Hz produced by continuous fanning of the wings, and pulse songs were generated by regular wing strokes with 35 ms interval that produce pulse trains with carrier frequency of 150-200 Hz. In mosquitoes, flying tones are produced during flapping of the wings (Arthur et al. 2014). The tone frequency

depends on the flapping speed, ranges from several hundred to over 1000 Hz (Cator et al. 2009; Arthur et al. 2014). These tones were used during courtship and the frequency of the tones are changing to communicate with other conspecifics (Belton 1994; Gibson and Russell 2006; Cator et al. 2009).

1.2   *Acoustic communication and social behaviour in crickets*

In cricket stridulation, males raise both the forewings and generate acoustic signals by rubbing together the two raised forewings, sweeping the plectrum on the left wing over the teeth of the file on the right wing (Koch et al. 1988; Pfau and Koch 1994). Each opening-closing movement of the forewings generates a sound pulse and rhythmic opening and closing cycles give rise to song structure (Kutsch and Franz 1989). A cuticular membrane in the front wing called harp serves as mechanical resonator driven by the generated wing vibrations, and the harp is tuned to the carrier frequency of the calling song for efficient sound production (Nocke 1971). The rhythmic opening and closing movements of the forewings are controlled by closer (e.g. M90) and opener wing muscles (e.g. M99), and the strict timing of their activity defines the temporal pattern of the acoustic signals (Kutsch 1969). To communicate with conspecifics, three types of species-specific songs are generated (Alexander 1961). Calling songs are produced to attract distant females and females respond to calling songs by directional steering to the sound source called phonotaxis, both during walking and flight. Courtship songs are generated to trigger female mounting and copulation. Rivalry songs are produced when males confront each other. Both courtship and rivalry song are part of complex courtship and rivalry behaviour sequences.

Courtship behaviour refers to series of behaviour when males try to court the females (Adamo and Hoy 1994). It begins when males recognise females by their antennae upon contact

with female antennae or body. The males then turn around and reveal the posterior body to the front of females in preparation for following acoustic display. After that, courtship songs are generated continuously until the females express interests in mating or walk away. If ready to mate, females touch the cerci or posterior abdomen which makes the male to move backward, and the females mount the males, after which transfer of a spermatophore begins.

Rivalry behaviour describes a sequences of behaviour when two males encounter, confront, and show aggressiveness to each other (Adamo and Hoy 1995; Stevenson et al. 2000; Sakura et al. 2012). It always starts by rapid antennae contact called antennae fencing. Then both males raise their anterior body by standing with the forelegs straight and open their mandible widely while antennating is still going on. They start to fight by biting, wrestling and grappling if no one flees. Rivalry songs are generated by both males during any of the behaviour components, but once winner and loser are decided, only the winner will produce a rivalry song to claim the territory. Rivalry behaviour can cease at any point after antennae contact as soon as a male retreats.

To study complex behaviour sequences the application of ethograms is a fundamental method as it provides a description of behaviour components, information about the order of behaviour sequences, and the frequency of each behaviour components. It was widely adapted to describe complex behaviour in different animals (Adamo and Hoy 1994, 1995; Mather and Alupay 2016; Smith and Wassmer 2016), and will be used in analysing courtship and rivalry behaviour in the study of lesion effects.

1.3   *Evolution of cricket stridulation*

Based on the song structure, the basic elements of cricket songs can be categorized into chirps, i.e. song units that include short pulse sequences with a fixed number of pulses; trills

which are song units including long pulse sequences with a variable pulse number, and phrases, song units containing both chirps and trills. Calling song as the acoustic signal used to attract females, provides the first prezygotic isolation barrier to filter out conspecifics. For example, in two closely related *Teleogryllus* species, *T. commodus* and *T. oceanicus*, the preference for temporal pattern and carrier frequency of the calling song are different in the females of the two species (Bailey et al. 2017). Members of the Hawaiian cricket genus *Laupala* show similar morphology among species while each species show specific pulse rate in the trills (Shaw 1996; Mendelson and Shaw 2002). Two closely related *Gryllus* species, *G. texensis* and *G. rubens*, show song structure differences in both calling song and courtship song (Fitzpatrick and Gray 2001). The genetic background for song structure and female preference was tested by hybridization experiments. Crossing of *T. commodus* and *T. oceanicus* produced male offspring that generate a song structure in between of the two parent species, and females that prefer the songs produced by the male hybrid offspring (Hoy and Paul 1973; Hoy 1974; Hoy et al. 1977). Similar results were also retrieved by using two *Gryllus* species, *G. armatus* and *G. rubens* (Bentley and Hoy 1972), or *Laupala* species, *L. paranigra* and *L. kohalensis* (Shaw 1996). These suggest a common genetic background and co-evolution of male song structures and female preferences. The species-specific song structures like chirps, trills, complex songs (phrases), and temporal parameters like pulse number, pulse rate, chirp rate are also used as traits for generating phylogenic trees (Otte 1992; Desutter-Grandcolas and Robillard 2003).

1.4 Neurobiology underlying cricket stridulation

Although the sound producing apparatus is very similar in the different crickets, it is the underlying motor pattern that controls the generation of species-specific temporal patterns of sound pulses composed to chirps and/or trills in the songs. Thus the motor activity generated

by the central nervous system is a crucial factor defining species-specific signalling. In crickets, key functional properties have been described for the neural organisation of acoustic signalling behaviour.

1.4.1 Command neuron

A key question for all animals is the control of behavioural sequences. How does the nervous system decide what behavioural pattern to initiate and how is this achieved? As the brain processes a range of sensory information, it is key to the control of behaviour. One influential concept for invertebrates was the control of rhythmic behaviour by command neurons developed by Wiersma and Ikeda (1964). The command neuron concept refined by Kupfermann and Weiss (1978) proposed a command neuron should fulfil the criteria of sufficiency and necessity and show tonic activity for controlling a particular behaviour. To conform to the criterion of sufficiency, the neuron's spike activity should elicit the whole behaviour or behavioural sequences when activating over threshold. To conform to the criterion of necessity, the ongoing behaviour should be interrupted when the neuron activity is suppressed or removed. Several neurons controlling different behaviour in many animal models were reported as command neurons. In fish and amphibian, Mauthner cells are sufficient to elicit fast escape response, but the neuron is not necessary for the behaviour and therefore was defined as "command-like" neuron (Rock et al. 1981; Korn and Faber 2005). In the mollusc *Pleurobranchaea californica*, command neurons called paracerebral neurons are controlling feeding behaviour when the animals senses food stimuli (Gillette et al. 1978, 1982). Egg-laying behaviour in *C. elegans* is controlled by a pair of command neuron called serotonergic hermaphrodite specific neurons (Trent et al. 1983; Leifer et al. 2011; Brewer et al. 2019). In *Drosophila*, command neurons for courtship song, feeding and walking were identified by thermogenetics and mass screening (von Philipsborn et al. 2011; Flood et al. 2013; Bidaye et al. 2014). In grasshopper *Omocestus viridulus*, three different stridulatory movement

patterns are controlled by three separate identified command neurons (Hedwig and Heinrich 1997).

The existence of command neurons for cricket songs was implied by extracellular current stimulation experiments of the brain and the cervical connectives (Huber 1960, 1963; Otto 1971; Bentley 1977). Calling, courtship, and rivalry song were elicited by electrical stimulation of different areas of the protocerebrum in *G. campestris* (Huber 1960, 1963). Subsequent connective stimulation experiments suggested descending brain neurons control the calling song stridulation by pure tonic activity in *G. campestris*, *G. bimaculatus*, and *T. oceanicus* (Otto 1971; Bentley 1977). The identification of a descending interneuron as command neuron for calling song stridulation was then reported in *G. bimaculatus* by intracellular recording (Hedwig 1996, 2000). This neuron showed only tonic activity when recorded. By applying depolarising and hyperpolarising current, the neuron was proven to initiate calling song when activated, while ongoing calling song activity was abolished after the neuronal activity was inhibited. Besides these experimental data, very little is known about the control of singing behaviour in other cricket species, which generate very different song patterns.

1.4.2   *Central pattern generator (CPG)*

The generation of acoustic signals is generally based on rhythmic movements of appendages, which are driven by well-timed rhythmic motor patterns, generated by the central nervous system with specific sets of neurons, called Central Pattern Generator (CPG). These refer to neural circuits that can drive rhythmic motor pattern without any phasic input (Marder and Bucher 2001). CPGs are underlying various behaviour based on rhythmic movements, such as breathing, swallowing, sucking and locomotion like walking, swimming, flying, and can be found in both invertebrates and vertebrates (Dick et al. 1993; Ijspeert and Kodjabachian 1999; Miller and Sigvardt 2000; Barlow and Estep 2006; Pirtle and Satterlie 2007; Bicanski et al.

2013; Olivares et al. 2018). The neuronal mechanism and basic principles of CPGs were described, and due to the ease of recording and quantitative data analysis, CPGs was extensively studied in neuroscience (Marder and Bucher 2001; Katz 2016). Many insect behaviours are driven by rhythmic activity of the CPGs. In an iconic experiment studying flying in desert locust *Schistocerca gregaria*, the central flight motor pattern generated was recorded when all movement-related sensory feedback was removed (Wilson 1961). After that, recording fictive motor pattern (rhythmic neuronal activity for generating motor pattern) enable a convenient and effective method in studying CPGs, and is commonly applied in motor pattern studies like in crawling, walking, flying, and rhythmic singing (Marder and Bucher 2001; Buhl et al. 2008; Schöneich and Hedwig 2012; Pulver et al. 2015; Mantziaris et al. 2017).

The CPG in crickets generating the motor activity for singing was long thought to be located in the mesothoracic ganglion, which houses the motor neurons innervating the stridulation muscles. This conclusion was support by lesion experiment severing the thoracic and abdominal nerve cord in *G. campestris* (Huber 1963). However, in *G. campestris* males with the cervical connectives cut could produce songs a few days after lesion, while males with both the cervical and the abdominal connectives cut stopped singing (Kutsch and Otto 1972). Differential heating experiments in *G. firmus* also suggested the involvement of abdominal ganglia in the control of song patterns (Pires and Hoy 1992). Systematic lesion experiments of the abdominal nerve cord in *G. bimaculatus* demonstrated the requirement of abdominal ganglia in generating song patterns and moreover the control of pulse timing and chirp timing by different abdominal ganglia (Schöneich and Hedwig 2011; Jacob and Hedwig 2016). Intracellular recording in abdominal ganglia revealed CPG neurons that can be categorised into pulse timer and chirp timer neurons (Schöneich and Hedwig 2011, 2012; Jacob and Hedwig 2019, 2020).

1.5   *Phonotactic Behaviour and Assay*

At the receiver side the processing of the conspecific calling songs initiates response songs or phonotactic behaviour. Phonotaxis describes the directional steering toward a sound source of preferred acoustic signals and it is commonly observed in insect acoustic communication (Schul et al. 1998; Verburgt et al. 2008; Kong et al. 2015). The study of phonotaxis involves topics related to acoustic signal recognition, to localisation of the sound source, and motor activity underlying directional steering (Rheinlaender and Blätgen 1982; Petrou and Webb 2012; Hedwig and Sarmiento-Ponce 2017). To investigate phonotaxis behaviour several devices have been developed to track animal movements or test acoustic signal preferences. Treadmill or trackball systems are used to record animal movements during phonotaxis (Hedwig and Poulet 2005; Verburgt et al. 2008). It features a light weight ball-like structure on which tethered insects can walk continuously while rotating the trackball with their legs. The forward-backward and left-right movements of the trackball are recorded by a sensor below the track-ball (Hedwig 2017) while the insects orient to loud speakers presenting acoustic test patterns. Systematic testing allows a quantitative analysis of the behavioural preferences. A Y-maze, as the name implies, is a Y-shaped device used to test animal choice, not only for phonotaxis (Rheinlaender and Blätgen 1982; Simões et al. 2011; Erregger et al. 2018). The contestant is placed at the long end of "Y", two different attractants are placed at the two short ends of the "Y" and during its approach the walking animal makes choice according to its preference. To record detailed movements of female crickets during phonotaxis high-speed video recordings have been obtained to study the details of leg movements and body parts involved in phonotactic steering (Witney and Hedwig 2011; Petrou and Webb 2012).

2 Chapter Two: Sound and wing recordings of three song types in free moving *Gryllus bimaculatus*

Abstract

Male crickets produce sound pulses and structured songs by rhythmic movements of the forewings. Sound amplitude, carrier frequency, and temporal parameters of songs in different species or different song types result from different motor pattern used for generating songs. By using opto-electronic camera and microphone, we recorded the wing movements and sound signals simultaneously during the production of calling, rivalry and courtship song in the field cricket *Gryllus bimaculatus*. Recordings confirm that salient sound pulses during calling and rivalry song are generated during the closing movements of the wings. Wing movements for calling and rivalry song start from an elevated wing position, and are performed with a very similar opening-closing movement, indicating that both types of songs may be generated by the same neuronal network. Wing movements for courtship song start from a low wing position; rapid closing movements generate high frequency ticks and low-amplitude wing oscillations lead to low–amplitude pulses, generated during the opening and closing movements with a frequency corresponding to the calling song. The two types of wing movements underlying courtship song indicate a different motor control as compared to calling song and suggest another pattern generation system for courtship song.

2.1 Introduction

Acoustic communication in animals displays multiple mechanisms of sound production, but in all cases particular motor patterns are required to control movements coupled to sound producing organs. In amphibians, vocalization relies on the cooperation of lung, larynx, and vocal sac (Roy 1996). In birds the syrinx, an avian specialized organ, is responsible for singing (Elemans et al. 2015; Riede et al. 2019). In human and other primates, voice production involves the larynx and vocal-tract airways (Ghazanfar and Rendall 2008). While in most vertebrates sound generation is coupled to vibrations of a specialized organ in the larynx during the respiratory cycle, in insects sound is mainly generated by rhythmically grating or rubbing the structures of a specialized apparatus against each other. This form of sound production is called stridulation and generally involves a rough cuticular structure like a set of pegs or striations which are scratched against a scraper or plectrum. For sound production the cockroach *Nauphoeta cinerea* and *Dendroctonus* beetles, rub the abdomen against the wings (Hartman and Roth 1967; Roth and Hartman 1967; Michael and Rudinsky 1972). In moths, the hawk moth *Cechenena lineosa* stridulates by modified scales on the genital valves against the abdomen tergum (Barber and Kawahara 2013) and the noctuid moth *Thecophora fovea* use a tarsal segment on the hindleg and hind wing for sound production (Surlykke and Gogala 1986). Sound production is most prominent in the insect group of Orthoptera. In acridid grasshoppers, songs are produced by rubbing the hindlegs against the tegminal vein (Elsner 1974; Vedenina and von Helversen 2003; Ostrowski et al. 2009) and in bush crickets and crickets sound is generated by rubbing specialized structures on the forewings against each other (Dumortier 1963). As cricket songs are simple in terms of temporal structure, it provides a good model to study the wing movements and motor activity underlying singing behaviour (Dumortier 1963).

Many cricket species communicate by generating a species-specific calling song, rivalry song, and courtship song (Alexander 1961). They produce a loud calling song to attract distant

females, a soft courtship song to initiate sexual behaviour with nearby females, and a rivalry song as part of aggressive behaviour when fighting for mates and territories. While the females are mute, male crickets generate songs by elevating the forewings into a raised singing position and rhythmically opening and closing the wings. A sound pulse is produced during the closing phase, when the plectrum on the left wing contacts the teeth of the file on the ventral side of the right wing (Elliott and Koch 1985; Koch et al. 1988). Upon sweeping of the plectrum over the file, a specialized region on the forewing called harp serves as primary resonator and radiates the sound (Nocke 1971). The temporal structure of generated pulses relies on the timing of the contact between the plectrum and the file; thus the precise control of the wing movements is key to consistent species-specific song patterns and the basis for successful song recognition by conspecific females.

In cricket songs, a pulse or syllable refers to the sound signal generated by a complete movement of the plectrum sweeping the file during the closing movement of the wings. In different species the pulses are grouped into species-specific song units such as chirps with a stable number of few pulses, long lasting trills consisting of more pulses and a variable pulse number, or phrases containing both chirps and trills. These song units are repeated to constitute the calling song and the song unit duration can range from 100 ms like in *G. assimilis* to more than 5 seconds like in *G. rubens* (see Chapter 4).

In the calling song of *Teleogryllus* species, phrases are found in *T. commodus, T. oceanicus, T. emma,* and *T. Taiwanemma* (Honda-Sumi 2005; Simmons et al. 2005; Zuk et al. 2008), while only chirps occur in the calling song of *T. leo* and *T. yezoemma* (Honda-Sumi 2005; Rothbart and Hennig 2012). During courtship song, *T. oceanicus* generates softer phrases compared to calling song while still containing chirp and trills (Libersat et al. 1994; Balakrishnan and Pollack 1996; Rebar et al. 2009).

In *Gryllus* species, chirps consisting of several pulses are reported for the calling song of *G. bimaculatus, G. campestris, G. assimilis, G. lineaticeps, G. firmus, G. pennsylvanicus,* and *G. veletis* (Bentley and Kutsch 1966; Kutsch 1969; Nocke 1972; Rheinlaender et al. 1976; Wagner and Reiser 2000; Pollack and Kim 2013). Rivalry song of *Gryllus* species generally contain chirps with more pulses compared to calling song (Huber 1963; Kutsch 1969). Courtship song of *Gryllus* species, are different from calling song and rivalry song, and contain one high amplitude tick sound and several low amplitude pulses. (Nagao and Shimozawa 1987; Libersat et al. 1994; Wagner and Reiser 2000; Fitzpatrick and Gray 2001; Rantala and Kortet 2003; Vedenina and Pollack 2012; Shestakov and Vedenina 2015). While calling song in *Gryllus* is centred around 5 kHz, courtship songs are much higher and in the range of 12-16 kHz. *Gryllus bimaculatus*, a *Gryllus* species widely studied in terms of acoustic signal production and recognition, have a carrier frequency of 4.8 kHz for calling song and 13.5 kHz for courtship song (Nocke 1971; Libersat et al. 1994).

Calling song, rivalry song, and courtship song of crickets and the underlying muscle activities were extensively studied by sound and electromyographic recordings. In the mesothoracic segment wing opener muscles, which contract to drive the wing opening movements and closer muscles which initiate the wing closing were identified and recorded in *Acheta domesticus, G. firmus, G. pennsylvanicus, G. veletis, G. campestris,* and *G. bimaculatus,* and these cricket species share a homologous muscle sets for singing behaviour (Ewing and Hoyle 1965; Bentley and Kutsch 1966; Kutsch 1969; Pfau and Koch 1994). However, the wing movement underlying cricket stridulation has scarcely been studied.

In this study, I report the relationship between wing movements and three song types in *G. bimaculatus*. Taking advantage of an opto-electronic system to monitor moving wings (Hedwig 2000), I recorded the three types of songs and the underlying wing movements simultaneously in freely moving males. By doing so, I could compare the timing of sound

production with the phase of wing movement and record the relative height of the wings in singing position when males generate the three song types.

2.2 Material and methods

2.2.1 Experimental animals

G. bimaculatus were reared and bred in the Department of Zoology, University of Cambridge. Crickets were kept in large boxes (52.5 x 36.5 x 28 cm) with a 12hr-12hr light:dark cycle and given *ad libitum* access to fish food, muesli, and water. Last instar male nymphs were isolated and kept in small boxes (17.5 x 11.5 x 13 cm) to monitor their age after final moulting and prevent contact to females prior to the experiments. Adults one to two weeks after imaginal eclosion were selected for experiments. Treatments and experiments complied with the principles of Laboratory Animal Care (ASAB Ethics Committee and ABS Animal Care Committee 2021).

2.2.2 Sound and wing movement recording for three song types

Sound of calling, rivalry, and courtship song was captured by two microphones. One microphone (Teisco Sound Research UEM-83) was connected to a micro 1401 data interface and recorded with CED Spike2 software (CED, Cambridge, UK) at a sampling rate of 16.6 kHz. The other microphone (Omni type; Maplin Electronics, Rotherham, UK) was recording the sound at a sampling rate of 44 kHz using Cool Edit 2000 software (Syntrillium Software Corporation, Phoenix, AZ, USA). As males were freely moving in the arena and the main purpose of the study is to correlate wing movements to produced sound, the sound intensity was not calibrated.

To monitor wing movements during stridulation, a 1.5 mm diameter round reflective sticker (Scotchlite foil type 7610, 3 M Laboratories, Germany) was attached to the left forewing of the male specimen (**Figure 2.1**). The males showed normal movements and singing activity after labelled with the sticker. When illuminated with a DC light source, the signal from the reflective foil was picked up by a linear position sensitive diode (Laser Components, Type 1L30-UV) built into the film plane of a reflex camera (Hedwig 2000). Wing movements were recorded from the side of the cricket, thus the up and down direction in the wing recording correspond to closing and opening movements of forewings during stridulation, respectively. The range of amplitudes of the recorded wing movements during stridulation were within 2.5-3 mm. Small oscillations in the wing recording are due to 50 Hz hum from power supplies.
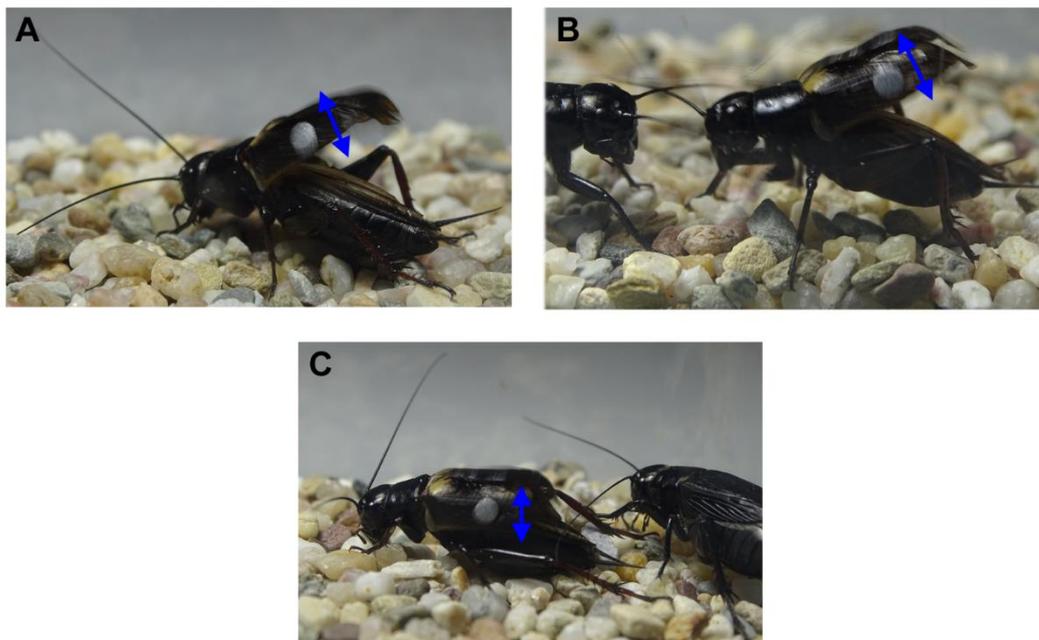
Figure 2.1 Singing *G. bimaculatus* males during stridulation of three song types
Blue arrows indicate the up (wing closing movement) and down (wing opening movement) direction of wing movements during stridulation of (A) Calling song, (B) Rivalry song, and (C) Courtship song.

Males were freely moving during the experiments, which would interfere with the wing recordings when males changed their position and made it difficult to obtain long lasting stable

recordings. To trigger rivalry and courtship song, another male or female was introduced to the arena along with the target male. In the case of rivalry song recording, the introduced male was silenced by removing one forewing beforehand. A total of 10 males were used for recordings of the calling song, in 7 males recordings of the rivalry song were obtained and in 10 males the courtship song was recorded. Experiments were performed at a room temperature of 22-24°C.

2.2.3   Data analysis

Data were analysed with Cooledit 2000 software for relative sound intensity and frequency, and with Neurolab (Hedwig and Knepper 1992; Knepper and Hedwig 1997) to measure the temporal parameters of the wing movements, like cycle periods and intervals of events. Sound signals and wing movements were averaged to analyse the timing and temporal relationship between movements and sounds. Sound signals were full wave rectified prior to averaging. T-test was performed to test significance between song parameters by SigmaPlot 11.0 (Systat Software, San Jose, CA).

2.3   Results

2.3.1   Sound and wing recording of calling song

Male *G. bimaculatus* produce calling song to attract females for courtship. As the females do not provide any sensory feedback to males until they closely approached the singer, remaining stationary males may continuously call for many hours. To generate calling song, they raised their forewings to a singing position to about 40-50 degrees relative to the long axis of the body **(Figure 2.1A)**, and began stridulating by rhythmic opening and closing the forewings. In **Figure 2.2A** the wings were already in singing position and four consecutive chirps are displayed together with the wing movements underlying sound production. The

number of wing up and down movement cycles was the same as the number of pulses generated, as one pulse was produced in one opening-closing cycle of the wings. After a complete chirp, the wings remained in the raised position and were prepared for the next one. In the first two chirps, the first closing movements were not completed so the first sound pulses of these chirps were missing, while normal chirps like the third and the fourth chirp were generated by four complete wing open-close movement cycles that constituted four sound pulses. In the calling songs recorded (10 males, 255 chirps) the chirps contained three to five pulses (average 3.6 ± 0.4 pulses) and had an average duration of 138.9 ± 19.0 ms, a chirp interval of 369.5 ± 70.7 ms, and a chirp period of 508.3 ± 71.7 ms. The average pulse parameters (N=967) were as follows: pulse duration was 21.0 ± 4.5 ms; pulse interval 23.8 ± 3.7 ms; and pulse period 44.2 ± 3.2 ms. One chirp is enlarged along with the wing movement to demonstrate the timing of the sound pulse generation in phase of the wing movement (**Figure 2.2B**). The start and end of pulses were closely related to start and end of the closing movement of the wing, as shown by dashed lines in **Figure 2.2B**. Pulses of calling song were generated during the closing phase of the wing movements (indicated by the arrow in **Figure 2.2B**). Sometimes soft sound pulses were also generated during opening phase of wing movements as shown in **Figure 2.2A** and **2.2B**, but the sound intensity was very low compared to sound pulse generated in wing closing phase.

Averaging sound and wing recording of 35 pulses further demonstrated the relationship between phases of wing movements and sound production (**Figure 2.2C**). For averaging, trigger points were set at every start of the opening movements, the averaged wing movement and averaged pulse envelope are shown over the pulse period. As the wing went through the opening and closing cycle, the opening phase produced no sound at all, while the pulses were generated soon after the wing movement entered closing phase, peaked in the middle of closing phase, and ended as the closing phase terminated. During the closing movement, sound production started at 5.0 ± 1.4 ms after the wing started to rise. The sound amplitude increased

and the pulse reached the highest amplitude at 14.1 ± 2.4 ms, and the pulse ended at 24.9 ± 4.2 ms in closing phase. The carrier frequency of calling song was in the range of 4.6 to 4.9 kHz. The sound and wing movement recording during calling song stridulation demonstrated that generation of each sound pulse in calling song chirps corresponds to each wing open-close movement cycle, and the sound production is temporally tightly coupled to the wing closing phase.

A

chirp

pulse

Calling song

Wing movement

wing raised
in singing position

500 ms

B

Calling song

closing

Wing movement

opening

50 ms

C

Sound intensity (rel. Units)

Wing position (mm)

3

1.5

opening

closing

0   10   20   30   (ms)

Pulse Period

20
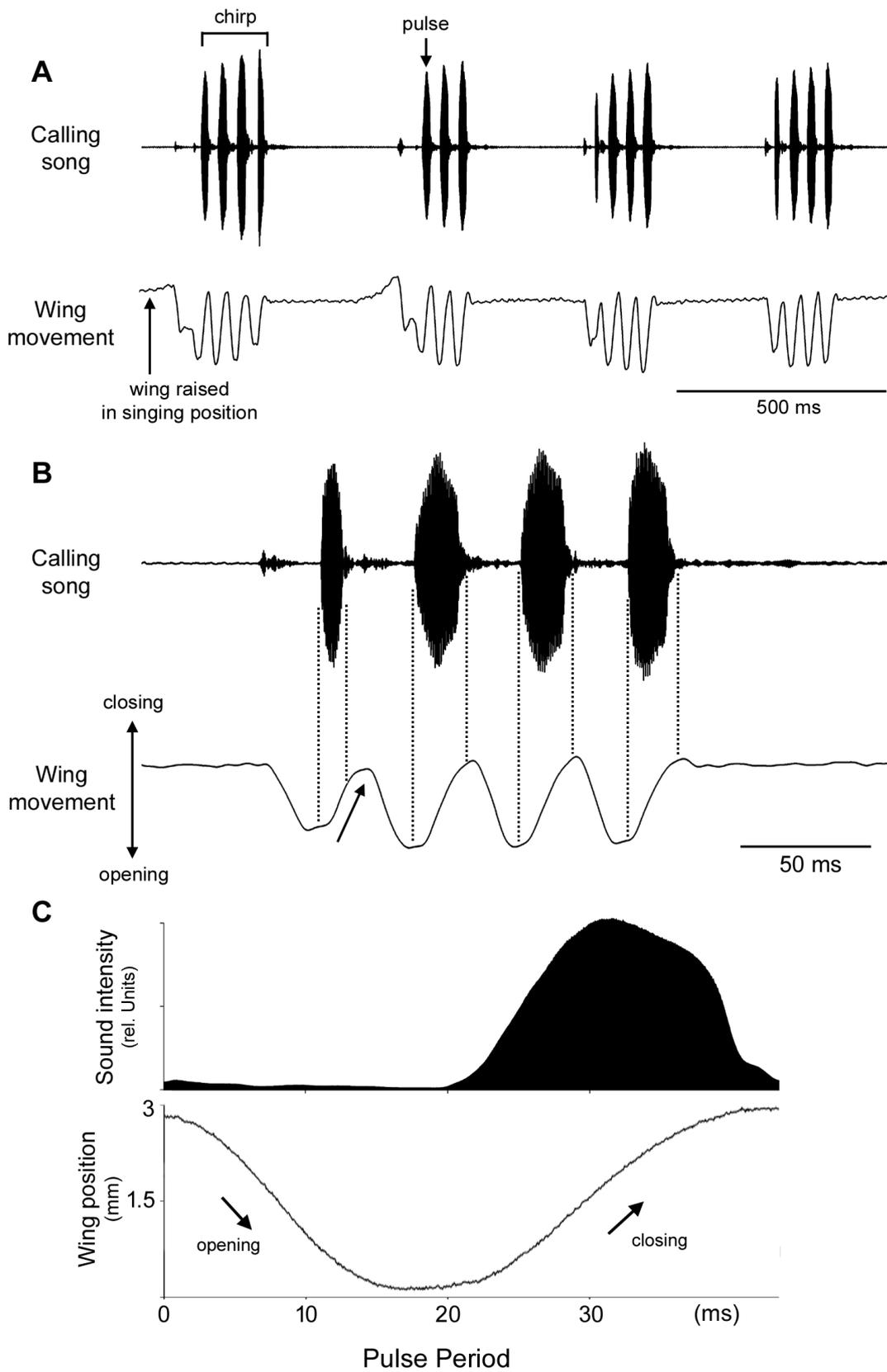
Figure 2.2 Sound and wing recording during calling song in *G. bimaculatus.*

(A) Four chirps of calling song and the underlying wing movement. (B) One chirp of calling song and wing movement displayed at higher temporal resolution. Dashed lines align start and end time of sound pulses to wing positions. Sound is generated during the closing movement of the open-close cycle, when the wing is moving from the open/lower position to the close/higher position. (C) Averaging of sound and wing recording of 35 pulses starting from the raised wing position. Forewings of the male moved within a 3 mm range during stridulation.

2.3.2   Sound and wing recording of rivalry song

When encountering another conspecific male, male *G. bimaculatus* initiate a series of aggressive behaviour (Adamo and Hoy 1995; Stevenson et al. 2000) (see also Chapter 3) in a fast and dynamic situation, in which the fighting males rapidly change position. Rivalry song is generated during confrontation of the two males or immediately after the fight. One or both of the males raise the forewings to 40-60 degrees relative to the long axis of the body and produce rivalry song (**Figure 2.1B**). The duration of rivalry song depends on the length of fights and aggressiveness of the males, but mostly terminates within few seconds. Rivalry songs were recorded from 7 males, the range of rivalry song duration was 2.1-14.5 sec containing 3-20 chirps, after the rivalry display songs generally changed to calling song. The pulse number in one chirp ranged from 5 to 21 pulses. The first 2-3 chirps generally contained more pulses than the following chirps, on average there were 7 ± 0.8 pulses per chirp. Of the 60 chirps analysed, these chirps have an average duration of 280.5 ± 38.3 ms, an interval of 333.6 ± 66.1 ms, and a period of 631.6 ± 77.7 ms. The averaged pulse parameters of 429 pulses were as follows: pulse duration 20.2 ± 4.6 ms; pulse interval 23.9 ± 4.7 ms; and pulse period 44.0 ± 2.7 ms. **Figure 2.3A** shows two sequences of rivalry songs and corresponding wing movements. In the chirps of rivalry song, pulses were produced by rhythmic wing open-close movements. Each pulse corresponds to one closing movement of the wing no matter how long the chirp was. Each chirp started with the wing in the high raised position and continuous

uniform wing open-close movements gave rise to sound pulses. When the wing oscillations stopped and the chirp ceased the wing returned to the raised position.

A

Rivalry song

Wing movement

Rivalry song

Wing movement

500 ms

B

Rivalry song

closing

Wing movement

opening

100 ms

C

Sound intensity (rel. Units)

Wing position (mm)

3

1.5

opening

closing

0   10   20   30   (ms)

Pulse Period
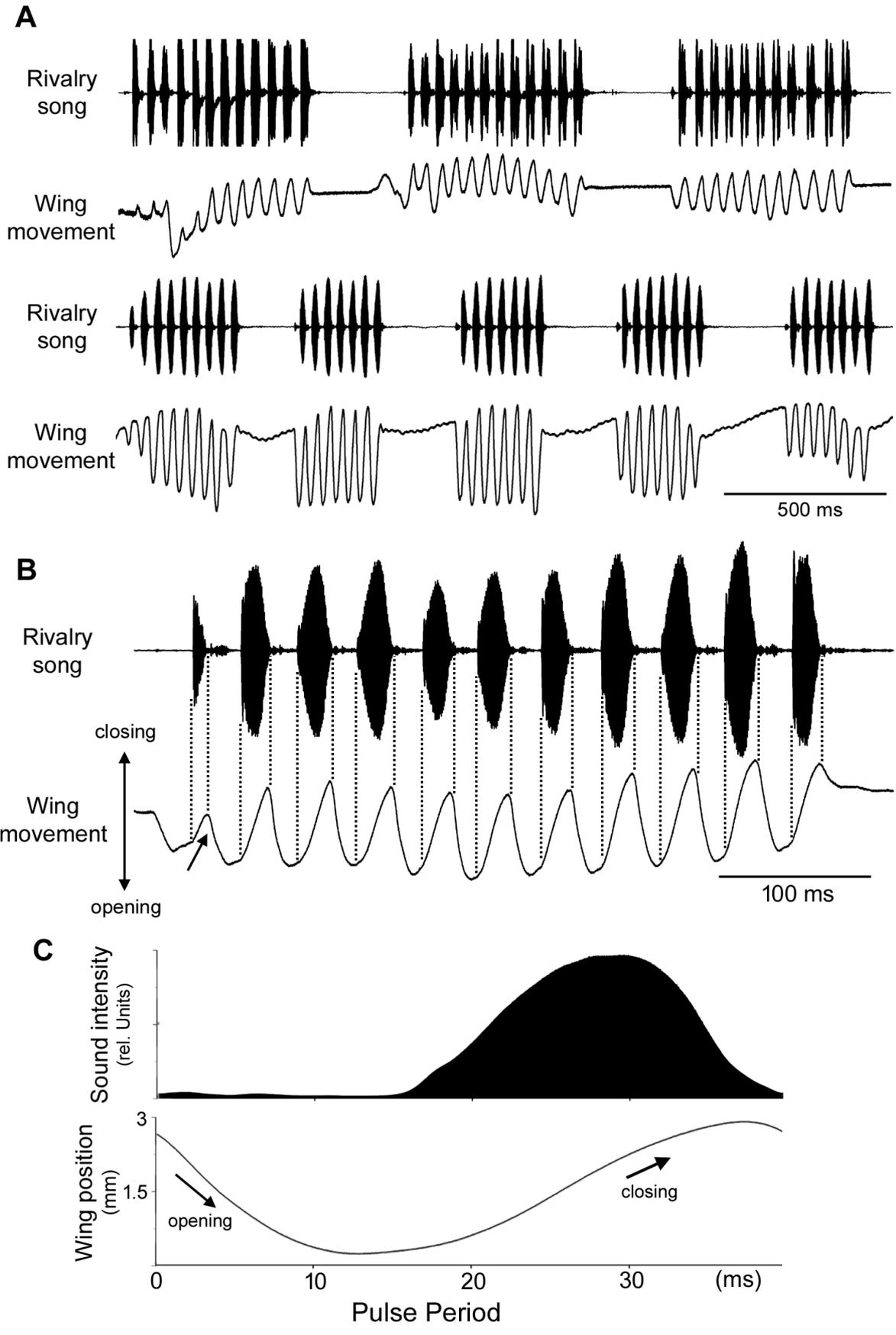
Figure 2.3 Sound and wing recording of rivalry song in *G. bimaculatus*.
(A) Two sections of rivalry song and wing movement recorded in two freely moving males. The wing movements look slightly different as distortions in the recordings occurred when the males changed their position, while the sound recording remained stable. The upper recording shows long chirps, the lower male was better aligned to the camera. The wings were in a high position and moved downward during the opening movement, and moved upward during the closing movement when the wings were in a low position. (B) One rivalry chirp and corresponding wing movement. Dashed lines align start and end of sound pulses to wing positions. (C) Averaging of sound and wing recording of 119 pulses starting from the raised wing position. The wings moved within a 3 mm range.

A rivalry song chirp displayed on a high resolution time scale in **Figure 2.3B** provides details of the relationship between wing movement and pulse generation. It is obvious that all of the pulses were produced during the closing phase indicated by dashed lines. In **Figure 2.3C** averaging of 119 pulses with the wing movement demonstrate a complete cycle of pulse generation. Triggered by the start of opening phase, the average displays the opening-closing cycle. When the wings were in opening phase, no sound was generated. When the wings closed, pulses were produced, and reached the highest amplitude just before the end of the closing phase. The sound pulse average demonstrates that the sound pulse amplitude gradually built up before it decayed and the time ratio between increasing phase and decreasing phase was about 2:1. In detailed analysis of temporal relationship between pulse and wing closing phase, pulses started 5.0 ± 0.8 ms after the wing entered the closing phase, peaked at 14.0 ± 1.6 ms, and ended at 26.1 ± 4.2 ms of the closing phase. The carrier frequency was in the range of 4.6 to 4.9 kHz.

Simultaneous sound and wing movement recording for rivalry song in *G. bimaculatus* indicate that generation of each sound pulse is linked to one wing open-close movement cycle and that sound was only produced during the closing phase of the movements.

2.3.3 Sound and wing recording of courtship song

Males produce courtship song in the presence of nearby females to facilitate female mounting and copulation. Courtship song in *G. bimaculatus* is different from calling song and rivalry song by having two components in a courtship song unit: a loud high-frequency tick sound and several soft low-frequency pulses (Nagao and Shimozawa 1987; Libersat et al. 1994; Rantala and Kortet 2003) (**Figure 2.4**). The wing movements during courtship song reveal a more complex motor pattern than for calling and rivalry song. Overall wings were kept in a considerably lower position and were close to the abdomen during courtship singing (**Figure 2.1C**). Due to the low wing position, during courtship song, wings were closed and raised to a higher position for the generation of ticks, and different to calling song based on a closing-opening cycle. The tick duration was 13.8 ± 4.0 ms (10 males, 241 ticks), the complete wing close-open cycle underlying a tick was 64.8 ± 12.0 ms, and the time ratio for closing phase and opening phase was between 1:3 and 1:4. A whole song unit from tick to tick lasted 344.8 ± 64.5 ms (241 song units). After the generation of a tick the wings were opened and lowered again into the initial low wing position and began to oscillate with a small movement amplitude, which was 8 to 10 times smaller than the movement amplitude for generating ticks. The cycle period of the oscillations was 30 ± 3.1 ms, and 8.0 ± 1.3 low amplitude oscillations occurred in one courtship song unit (1891 oscillations, 241 courtship song units). The oscillating wings generated soft sound pulses when the wing went upward and downward and two pulses were produced over one oscillation period. Therefore, each tick was followed by an average of 15.7 ± 2.3 low-amplitude pulses with a pulse period of 15.1 ± 1.5 ms (n=3676 pulses). The carrier frequency was 13.0 to 13.5 kHz for ticks and 4.6 to 4.9 kHz for the low amplitude pulses.

A closer inspection of a courtship song unit reveals the relationship of the wing movements and the sound produced (**Figure 2.4B**). The ticks were generated during the closing movement of the wings and ended as soon as the wings began to move downward. During the

downward movement, instead of lowering directly to the initial wing position, the movement was slowed down and a shoulder occurred after a tick (indicated by arrowhead in Figure **2.4B**), low-amplitude sound pulses were also generated in this period. The 2:1 ratio of the low-amplitude pulses and the wing oscillation cycle is demonstrated by the dashed lines and arrows in **Figure 2.4B,** each up and down oscillation is related to one soft sound pulse. Averaging of ticks and pulses along with wing recordings (**Figure 2.4C)** shows the characteristics of courtship song. A total of 41 ticks were averaged, triggered by the start of the closing movement. The ticks were generated while the wing went up and ended as the wing reached the highest peak of the closing movement. The downward movement was slower, the slope during the wing downward movement gradually flattened before it entered the oscillation phase generating the low-amplitude pulses. For the low-amplitude pulses, 60 oscillations were averaged triggered by the highest wing position. Pulses were generated during both the up and down movement and separated by silent intervals at the highest and lowest wing position, when the wing changed movement direction.

Song and wing movement recordings of courtship song in *G. bimaculatus* indicate that the two components of courtship song, i.e. ticks and low-amplitude pulses, were generated by two different wing movement patterns. Ticks were produced during the closing phase of wing close-open movements, and low-amplitude pulses were produced by both the upward and downward movements during low-amplitude wing oscillations.

A

Courtship song

Wing movement

closing (up)   opening (down)

oscillating

200 ms

B

Tick

Low-amplitude pulses

Courtship song

closing

Wing movement

opening

Low-amplitude oscillations

50 ms

C

Tick

Sound intensity (rel. Units)

Wing position (mm)

3

1.5

0

0   20   40   60   80   (ms)

Time after Tick

Low-amplitude pulses

0.3

0.15

0   5   10   15   20   (ms)

Oscillation Period

27
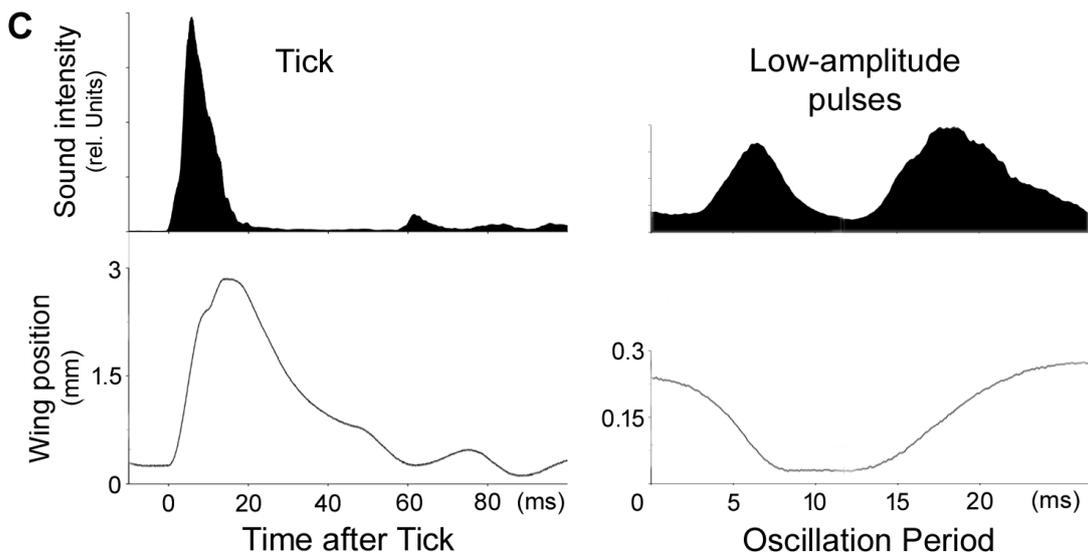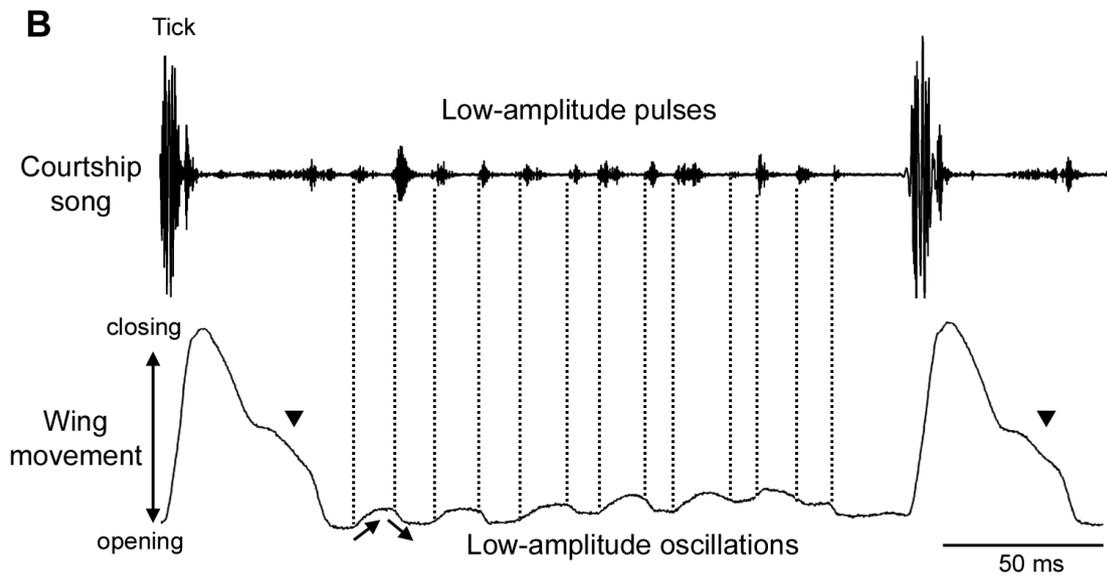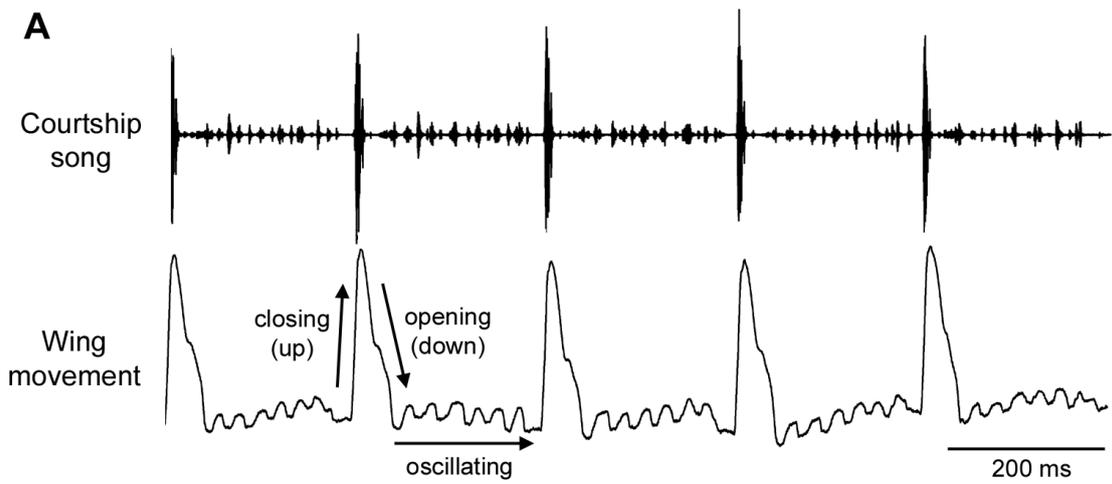
Figure 2.4 Sound and wing recording during courtship song in *G. bimaculatus.*

(A) Courtship song and underlying wing movement in a freely moving male. (B) One courtship song unit and underlying wing movement at higher temporal resolution. Note that the wing is in a lowered position and moves upward during the generation of a tick. Dashed lines align start of soft sound pulses to wing positions. Arrowhead: "shoulder" observed during opening movement of the wings at the end of a tick. (C) Left panel: averaging of sound and wing recording of 41 ticks starting from the closing movement generating the tick. Right panel: averaging of sound and wing recording of 60 wing oscillation cycles starting from the high wing position. The wing moved within a 3 mm range for ticks and 0.3 mm for low amplitude pulses.

2.3.4 *Sound and wing recording during song type transition*

Transitions between song types have been reported before in experiments electrically stimulating the brain or connectives to release singing behaviour (Huber 1960; Otto 1971), but the underling wing movements had never been captured. In this study the wing movements for two types of song transition were recorded.

A transition from rivalry song to calling song was common in all male encounters (**Figure 2.5A**), when the high number of 6-20 pulses of the chirps generated at the very beginning of rivalry singing, gradually decreased to match the structure of chirps in calling song composed of 3-5 pulses. As the wing movements for generating rivalry and calling song are similar, the change in pulse number and thus chirp duration is the only parameter altered during the transition, while the carrier frequency did not change and stayed between 4.6 and 4.9 kHz. Generally, the wing position during rivalry song was higher during aggressive display and was lowered when the song was gradually changed to calling song.

On one occasion a clear transition from calling song to courtship song could be recorded (**Figure 2.5B**). The two songs can be easily distinguished by the song structure, wing position, and wing movement pattern. While calling song includes only chirps and pulses generated by simple opening-closing wing movements starting from a raised wing position, the courtship song was produced from a low wing position by at least two motor patterns generating ticks and low-amplitude pulses. These are all reflected in the transition from calling

to courtship song. During the transition, the wing position was gradually lowered over the duration of at least three courtship song units. Also the amplitude of ticks became lower which is common when the animals generate courtship song after calling song. The carrier frequency was 4.9 kHz for the calling song pulses and 4.7 kHz for the first two ticks and then changed to 13.0 kHz starting with the third tick of the courtship song. The carrier frequency of the low-amplitude pulses was in the range of 4.5 to 5.5 kHz. The most interesting change is the sudden transition from calling song to courtship song (**Fig. 2.5B**, arrow), when during a chirp the wing movements for calling song suddenly changed into a wing movement for generating ticks of courtship song, which was followed by low-amplitude wing oscillations and a further lowering of the wings. With this, the male had switched from calling song to courtship song.
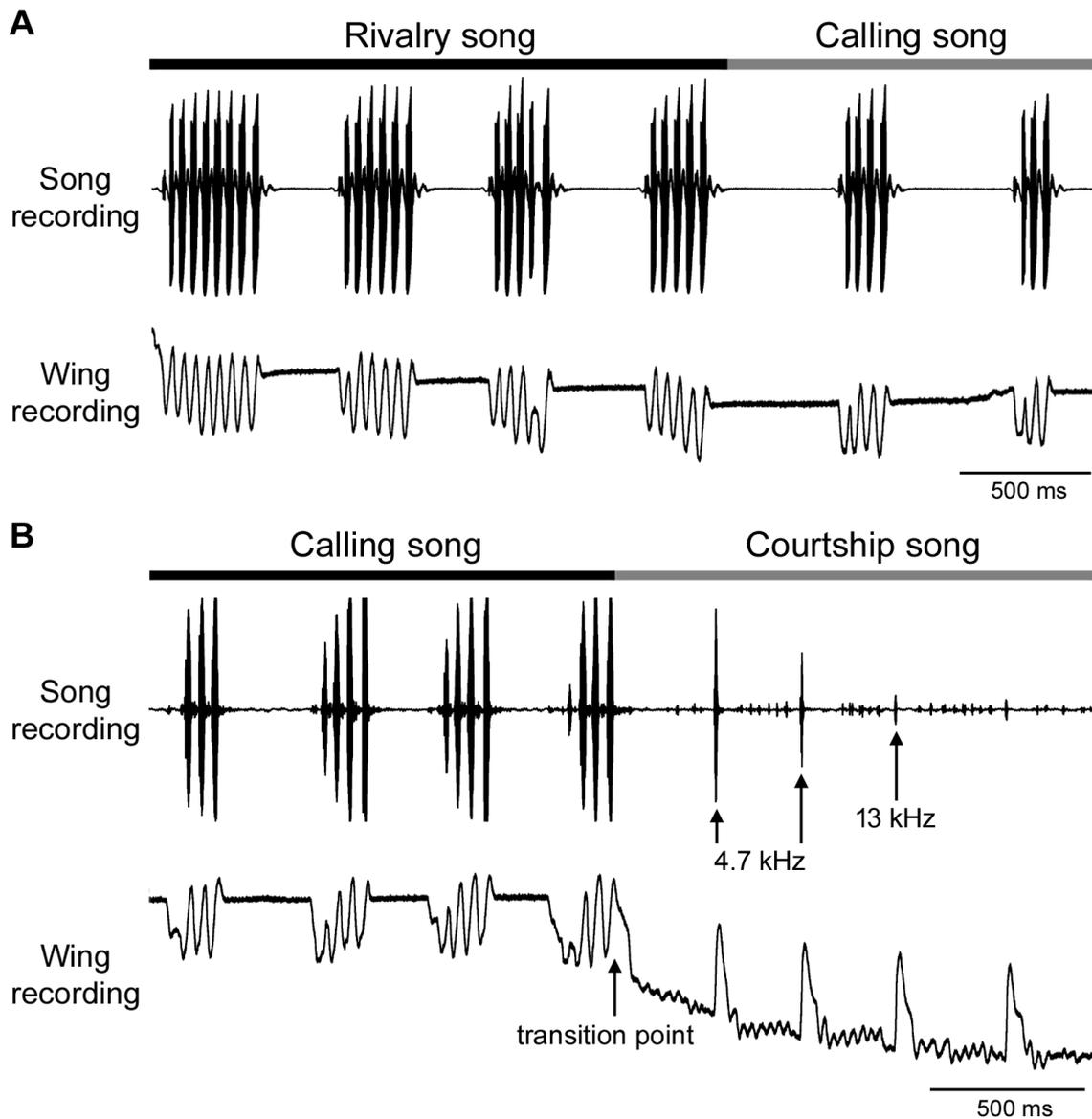
Figure 2.5 Sound and wing recording during song type transition

(A) Transition of rivalry song to calling song, note the pulse number decreases over the chirps. (B) Transition of calling song to courtship song, note the lowered wing position during the transition, and the decrease in sound intensity of ticks. The opening-closing movement for the last pulse of the last calling song chirp, is similar to the movement generating a courtship song tick. The carrier frequency was 4.9 kHz for calling song pulses, 4.7 kHz in the first two ticks, and was 13.0 kHz from the third tick onwards.

2.4 Discussion

In current study, wing movement recordings along with sound recordings during calling, rivalry, and courtship song in *G. bimaculatus* were obtained. These experiments provided for the first time a systematic study of wing movements and sound recordings in a cricket species, and the wing movement during song type transition was first time revealed. Wing movement pattern as output of neuromuscular control, provide information of motor pattern for different song types. The data indicate there are multiple pattern generation network for different song types. The same methods could be apply to other cricket species and stridulating insects to provide evidences for unravelling singing pattern generation network.

Similar experiments had been done on gomphocerine grasshoppers, where the leg movements during stridulation were recorded by a Hall-generator system (Elsner 1974). In the system, a Hall-generator was fixed on the back of the animal and a permanent magnet was attached to the hindleg in a way that the stridulatory movements of the hindleg was correspond to the voltage generated by the generator based on the position of the magnet.

As the recordings were performed on freely moving males, the overall locomotor activity of the males could interfere with the wing recordings, which was particular the case during rivalry behaviour. In these cases, the recording of the wing movements became distorted, but still reflected the temporal pattern. Measurement of sound intensity and amplitude of wing movement was also restricted. Wing movement recordings could be easily interrupted when a male turned its body or moved during rivalry behaviour and courtship behaviour, but stable sequences could be captured.

2.4.1 Wing movement pattern underlying calling song and rivalry song in G.bimaculatus

The simultaneous recordings of sound and wing movements reveal that in calling song and rivalry song, sound production starts at the beginning of the closing movements, sound

intensity reaches the highest amplitude during the steepest/fastest movement of the wings and stops at the upper reversal point, while the opening movements occasionally generated soft sound pulses with considerably lower amplitude compared to calling song pulses. The timing of calling song pulse generation corresponds to the proposed cricket singing mechanism that during closing movement the plectrum on left wing is rubbing against the teeth of the file on right wing (Pfau and Koch 1994).

In *G. bimaculatus* and *G. campestris* calling and rivalry songs were initiated when the wings were in a raised position. There are a few fundamental differences in the performance of the song types. While calling song can be maintained for many hours, rivalry song occurs only transiently during male encounters. In addition, chirps in calling song and in rivalry song differ in the number of pulses in chirps which is 3.6 ± 0.4 pulses and 7 ± 0.8 pulses (P<0.001), respectively, and hence the longer chirp duration in rivalry song (280.5 ± 38.3 ms) as compared to calling song (138.9 ± 19.0 ms, P<0.001). Otherwise, the wing movements during calling song (**Figure 2.2**) and rivalry song (**Figure 2.3**) show a very similar pattern in both types of songs. During both songs pulses are produced during the closing phase of the movement. (Koch et al. 1988). Pulse parameters are very much alike in calling song and rivalry song, respectively: pulse duration: 21.0 ± 4.5 ms and 20.2 ± 4.6 ms (P=0.732); pulse interval: 23.8 ± 3.7 ms and 23.9 ± 4.7 ms (P=0.975); pulse period: 44.2 ± 3.2 ms and 44.0 ± 2.7 ms (P=0.889), respectively. In addition, it was commonly observed in aggressive behaviour that rivalry song lasted for a few seconds, gradually shorten in terms of pulse number, and eventually became indistinguishable from calling song (**Figure 2.5A**). Furthermore, experiments of systematic severing abdominal nerve cord in *G. bimaculatus* exhibited similar effect on the control of calling song and rivalry song when the same site of the CNS was incised (Jacob and Hedwig 2016) (see Chapter 3). These findings suggest a similar underlying motor pattern for calling song and rivalry song in *G. bimaculatus*.

2.4.2 Wing movement pattern underlying courtship song in G. bimaculatus

Details of the courtship sound signals of *Gryllus* species have been extensively described. Courtship songs display two components, a high-amplitude tick with 13.5 kHz followed by low-amplitude 4.5 kHz pulses (Nocke 1972; Rheinlaender et al. 1976; Libersat et al. 1994; Wagner and Reiser 2000; Fitzpatrick and Gray 2001; Vedenina and Pollack 2012). This is different to the one-component calling song in *Gryllus* species. Although the low-amplitude wing oscillations had been observed previously, no experiments have systematically studied the underlying wing movement pattern, some preliminary recordings by Innenmoser (1974) are reported in Elsner and Popov (1978).

While many cricket species generate low frequency (2-8 kHz) pure tone calling songs, high-frequency communication in the subfamily Eneopterinae was reported (Robillard et al. 2007). The specialisations of short file, high tooth density, and high tooth strike rate contribute to the generation of high-frequency songs (Robillard et al. 2013). In this study, by comparing wing movements and sound pulse production during calling song and courtship song, the difference of dominant frequency in calling song pulse (4.6-4.9 kHz) and courtship song tick (13 kHz) might be a result of different speed during closing movement. The shorter closing movement duration during tick generation might give rise to high carrier frequency of the tick. Further functional morphology study is required to provide insights on courtship song generation machinery.

In the current study the wing movements were recorded during the courtship song. The recordings demonstrate two modes of movements when generating ticks and low-amplitude pulses, both starting from a low wing position. Wings were raised high and generated one tick in a courtship song unit, while the low-amplitude pulses were produced multiple times by continuous low-amplitude oscillations when the wings were lowered again.

Unlike pulses in calling or rivalry song, where an opening (downward) movement occurs first and subsequently a sound pulse is generated during the closing (upward) movement, the tick sound is generated by a closing movement starting from a low wing position followed by the opening movement. The recordings therefore reveal two very different motor patterns. Sound production underlying calling and rivalry song is driven by *opening-closing* movements of the wings, whereas the production of ticks is driven by *closing-opening* movements. In both cases the closing movement generates the sound, but due to different overall wing positions different motor programs are required. Besides, the ticks were generated almost at the same time as the wings began to rise while the onset of sound pulse in calling song and rivalry song was roughly 5 ms after the wings entered the closing phase. Together with the fact that courtship song ticks have a carrier frequency of 13.0 to 13.5 kHz compared to 4.6 to 4.9 kHz for pulses of calling song, these support the assumption that the motor patterns controlling the wing movements in calling song and courtship song use different pathways.

During opening movement after generation of ticks, there was a decrease in the speed (indicated by arrowhead in **Figure 2.4B**) that prolonged the opening movement of the ticks. This could imply the low amplitude wing oscillation was superimposed with the opening and lowering of the wing. Considering that the duration of the tick wing movement was twice as long as the low-amplitude wing oscillation period, it seems that the wing oscillations for pulses were always ongoing, and that the wing movement generating ticks was superimposed and initiated by another motor system.

2.4.3   *The transition from calling song to courtship song in G. bimaculatus*

In current study the transition from calling song to courtship song for the first time is demonstrated with simultaneous recordings of the sound and wing movements (**Figure 2.5B**). The recording demonstrates the different baselines of wing position for calling song and

courtship song, which is not obvious in the recording of each song type alone. A raised wing position was typical when the command neurons for calling song were activated, and wings were gradually lowered, when the command activity decreased or was abolished (see Chapter 5). A low wing position therefore may indicate that the command neurons for calling song were no longer activated. The transition happened as the male gradually lowered the wings and switched to the generation of soft sound pulses. The most striking discovery during the transition is the sudden change of the wing movement mode from the calling song wing movement pattern to the courtship song wing movement pattern with the last opening-closing movement of a chirp. This demonstrates that a switch can occur within one pulse period. This may be supported by the organization of the singing networks, as the network for calling song and courtship song are located in different ganglia of the abdomen (see Chapter 3).

Evolution has shaped the species-specific songs in different cricket species (Otte 1992). These include trill-producing, chirp-producing, and phrase (complex song)-producing species. As the simultaneous recording of acoustic signal and wing movements in freely moving males is feasible, the method provides opportunities to examine wing movements during generation of three song types in different species. The wing movement recordings in different species, together with recorded acoustic signals, could provide information on how different song structures are generated by the wing movements, how three song types differ in the way they are produced, and the similarities and differences of wing movements during stridulation in different species that may explain the evolution of cricket stridulation.

3 Chapter Three: The impact of lesions of abdominal connectives on the courtship and rivalry behaviour in *Gryllus bimaculatus*

Abstract

Neural circuits responsible for cricket song pattern generation have long been believed to be housed in the thoracic ganglia until recently, when systematic lesion experiments and identification of central pattern generator neurons for singing confirm that abdominal nervous system contribute to calling song pattern generation. However, it is not clear where motor patterns for courtship and rivalry song are generated. In current study, a combination of systematic lesion application to abdominal nerve cord and video recording of courtship and rivalry behaviour revealed the contribution of abdominal nervous system to both behaviour. The results suggest most of the behaviour components were not affected by the lesions, except for courtship song, rivalry song, and copulation with female. For generation of courtship song abdominal ganglion A6 (+A5) is important, while a central nerve cord extends to A4 is sufficient for rivalry song production. Any lesion on abdominal nerve cord abolished copulation. These findings indicate that the pattern generation network for courtship song is different from calling and rivalry song, while calling song and courtship song might share a common network for generating sound pulses.

3.1 Introduction

Courtship and rivalry behaviour are common behaviour performed to communicate with conspecifics and occur in both vertebrates and invertebrates. In zebrafish, males interact with other males by nipping, chasing, and circling, and pursue females by chasing, swimming along females, and spawning (Colman et al. 2009; Zabegalov et al. 2019). In bird species, visual display and acoustic signals are commonly used for courtship and rivalry behaviour (Fusani et al. 2007; Akçay et al. 2015; Scholes et al. 2017). In insects, presenting food gift in scorpionfly (Engqvist and Sauer 2003), bioluminescence communication in firefly (Michaelidis et al. 2006), visual display and substrate-borne signal in Drosophila (Fabre et al. 2012), and acoustic communication in grasshoppers and crickets (Adamo and Hoy 1994; Ostrowski et al. 2009) are examples demonstrating the diversity of insect mating behaviour. Rivalry behaviour also happens in various forms, such as fatal combat in parasitoid wasp (Reece et al. 2007), competing for shelter in shelter-building caterpillars (Sigmon 2015), inter-colonial aggression in the Formosan subterranean termite (Florane et al. 2004), vibratory display in caterpillar when defending territory (Yack et al. 2014), and confrontation between male crickets when fighting for resources or mates (Adamo and Hoy 1995).

In cricket courtship and rivalry behaviour, species-specific courtship and rivalry songs are generated as part of the display (Alexander 1961). Along with calling song for attracting distant females, the three song types are generated by sweeping of the plectrum on the left wing against the teeth of the file on the right wing during rhythmic movements of the forewings (Elliott and Koch 1985; Koch et al. 1988). Wing muscle activities during three song types were characterised by EMG recordings (Kutsch 1969).

Different from calling song, courtship song and rivalry song are produced while interacting with other conspecifics and accompanied by other components of courtship and rivalry behaviour. In *Gryllus bimaculatus* the two behaviour were extensively studied and

described by ethograms (Adamo and Hoy 1994, 1995; Sakura et al. 2012). Courtship behaviour includes physical contact with and identification of a female, the production of courtship song, mounting by the female, and finally copulation (Adamo and Hoy 1994). Rivalry behaviour refers to a sequence of confrontational behaviour between two males, with antenna fencing, threat posture, generation of the rivalry song, and fighting, to establish a dominant-subordinate hierarchy (Adamo and Hoy 1995; Stevenson et al. 2000; Sakura et al. 2012). In both behaviour, courtship or rivalry songs are generated by the male as part of the interaction.

Extracellular current stimulation in the brain of *G. campestris* elicited calling song, rivalry song, and courtship song when targeting different area (Huber 1960, 1963). Components of rivalry behaviour like antennae fencing, strong body shaking, and increased respiration have been described during rivalry song stridulation. Also microinjection of acetylcholine and cholinergic agonists into these brain areas could elicit all three song types in *G. bimaculatus* (Wenzel and Hedwig 1999). These experiments revealed the central role of the brain in controlling the three song types, but localising the CPG networks controlling the singing behaviour has been less straight forward.

Initial lesion experiments in *G. campestris* suggested that the abdominal nerve cord was not necessary for the generation of calling, rivalry, and courtship song (Huber 1963). However, the exact lesion site to the abdominal nerve cord was not specified in these studies. The requirement of at least abdominal ganglion 3 (A3) and abdominal ganglion 4 (A4) for the generation of calling song was demonstrated by systematic lesions applied to the abdominal nerve cord in *G. bimaculatus* (Jacob and Hedwig 2016). Neurons of the calling song pattern generator were also identified by intracellular recordings in the abdominal ganglia in *G. bimaculatus* (Schöneich and Hedwig 2011, 2012; Jacob and Hedwig 2019, 2020). While these studies towards the generation of the motor pattern underlying singing revealed the organization of the network controlling calling song, it is still unclear where the pattern

generators responsible for courtship and rivalry songs are located in the nerve cord.

In the current study, systematic lesions were applied to connectives between abdominal ganglia in the field cricket *Gryllus bimaculatus*, and the ability of the males to produce courtship song and rivalry song was monitored before and after each lesion. Furthermore, all other components of courtship and rivalry behaviour, before and after each lesion were recorded and used to generate an ethogram for the behaviour after each lesion. By doing so, this study provides information about the contribution and relevance of the abdominal nerve cord for the generation of courtship and rivalry behaviour and provides strong evidence for the location of the corresponding song pattern generators.

3.2 Material and methods

3.2.1 Experimental animals

All crickets were bred and raised in insect colonies of the Department of Zoology, University of Cambridge. Colonies were kept in large boxes (52.5 x 36.5 x 28 cm) at 28°C with a 12hr-12hr light:dark cycle and given *ad libitum* access to fish food, muesli, and water. Last instar male nymphs were isolated and kept in small boxes (17.5 x 11.5 x 13 cm) to monitor their age after final moulting and prevent contact to females prior to the experiments. Adults one to two weeks old after imaginal eclosion were selected for video recording, sound recording, and lesion experiments. All animal treatments and experiments complied with the principles of Laboratory Animal Care (ASAB Ethics Committee and ABS Animal Care Committee 2021).

3.2.2 Selective lesions of connectives in the abdominal CNS

After video recording and sound recording of courtship and rivalry behaviour in intact males, the crickets were placed into a 4°C fridge for 15 minutes to reduce motor activity.

Immobilized males were then carefully mounted ventral side up on a plasticine covered stand with the body and legs fixed by metal clamps produced from bent staples. To expose the abdominal nerve cord, the appropriate sternum and intersegmental membrane were incised and the wound was held open by forceps. Fat body surrounding the target abdominal ganglia and connectives was carefully removed without damaging tracheae, tracheoles, and nerves. Exposed tissue was covered in insect saline (in $mmoll^{-1}$: NaCl 140; KCl 10; CaCl2 7; NaHCO3 8; MgCl2 1; N-trismethyl-2-aminoethanesulfonic acid 5; D-trehalose dehydrate 4, pH 7.4) at all times. Lesions were applied with small scissors to the bilateral connectives between target abdominal ganglia, specifically either to the connectives between the metathoracic ganglion complex T3 and the first free abdominal ganglion A3 (T3-A3), or the connectives between A3 and A4 (A3-A4), or the connectives between A4 and A5 (A4-A5), or between A5 and A6 (A5-A6), or between A6 and the terminal abdominal ganglion (A6-TAG). After the lesion, the ventral cuticle was folded back, the wound sealed by drying haemolymph, and the insect recovered for one day before subsequent video recording and sound recording of courtship and rivalry behaviour over the time course of up to 14 days. Once the animal had died, the abdominal nervous system was dissected to confirm the site of the lesion.

3.2.3 *Video and sound recording of the behaviour*

Before and after applying lesions to the abdominal nerve cord, the courtship and rivalry behaviour of the male crickets were video recorded and sound recorded. Videos were taken with a Cyber-shot DSC-HX400V compact camera (Sony Corporation, Tokyo, Japan). Sound was recorded at the same time by Cooledit 2000 software (Syntrillium Software Corporation, Phoenix, AZ, USA) running under a Windows 7 system, with a standard PC microphone (Omni type, Maplin Electronics, Rotherham, UK) at a sampling rate of 44 kHz. For courtship behaviour, the target male and a female were placed in a box (17.5 x 11.5 x 13 cm) with clear

transparent walls. Each male was recorded once before applying the lesion to ensure that normal courtship behaviour occurred and was recorded at least four times after applying the lesion (one day, four days, one week, and two weeks after the operation). For studies of rivalry behaviour, the target male and another male were placed in the same box. One forewing of the other male was removed to stop it from making any sound while retaining all the rivalry behaviour including rhythmic wing movements implicating the generation of "silent" rivalry song.

3.2.4 Ethograms

All behaviour components in courtship and rivalry behaviour can be identified and separated easily as described by Adamo and Hoy (1994, 1995), and are listed in **Table 3.1**. Number of crickets performing each behaviour components were summarized in **Table 3.2**. One component of the behaviour followed by another component was counted as one behaviour transition event and is shown as an arrow connecting the two components in the ethograms. Recurring behaviour components are indicated by curved arrows if the same component repeatedly occurred as separate events. Ethograms were constructed by including all behavioural transitions in the period of analysis. For establishing the courtship ethograms, 60 minutes of video recording was selected for each lesion group. Each individual contributed equal time to the video selection, for example, if n=6, each animal contributed 10 min. For rivalry behaviour, 30 minutes of video recording was selected for each lesion group. Behaviour that is not related to courtship or rivalry behaviour, like free moving and breathing, is not considered in the ethograms. All ethograms start when the target male contacts the other male or the female. Only the behaviour of the target male was analysed and presented. A total of 53 male crickets were used in the experiments. Two animals did not show normal courtship and rivalry behaviour before the lesion application. Six animals died within three days after the

lesion. Seven males did not perform any courtship or rivalry behaviour after the lesion, they showed no response to females and kept running away upon encounter of another male. 4 animals were sham operated without applying a lesion and served as controls to check possible effects derived from creating an injury to the abdomen. All ethograms start at the moment of contact of the target male with the female or male, respectively. Number of transition events are shown in ethograms and the percentage of transition events after specific behaviour components were used to describe animal behaviour in the text.

3.3 Results

3.3.1 General effects of applying lesion

Behavioural data was collected from 38 animals. These males did not show any defects in mobility and lived for more than two weeks after the lesion was applied. 31 animals contributed both courtship and rivalry behaviour data, 3 males only presented courtship behaviour, and 4 males only performed rivalry behaviour after the lesion.

3.3.2 Courtship behaviour in intact males

Courtship behaviour of male *G. bimaculatus* is composed of a series of behavioural components related to identifying, attracting, and copulating with a female. The behaviour can be simplified as occurring in three stages. It begins with the male recognising a nearby female by making antennal contact, followed by the male stridulating courtship song. This is a species-specific nearfield high-frequency song generated as part of the courtship display before copulation. The courtship behaviour finishes as the female mounts the male for copulation (Adamo and Hoy 1994). The whole courtship behaviour lasts from a few seconds if the female

mounts the male right after courtship song, to more than 10 minutes when the female is unwilling to court with the male and keeps running away, in which case the male will approach the female and repeat the courtship behaviour over and over.

In video recording of courtship behaviour in intact animals, males initiated courtship behaviour once they encountered and recognized a female (**Figure 3.1 and Table 3.3**). The two most common reactions of a male after contact with a female were back and forth rocking movements of the body (42.1%), and turning its body to present the abdomen as preparation for courtship song (40.8%); this depended on the relative position to the female. These two behavioural transitions could happen in any order. Rapid shaking of antennae was observed in one male after it recognized a female (1.3%). This was not common in intact males but happened vigorously after lesions of the abdominal nerve cord (see **Figure 3.2-3.6** and **Table 3.4-3.8**).
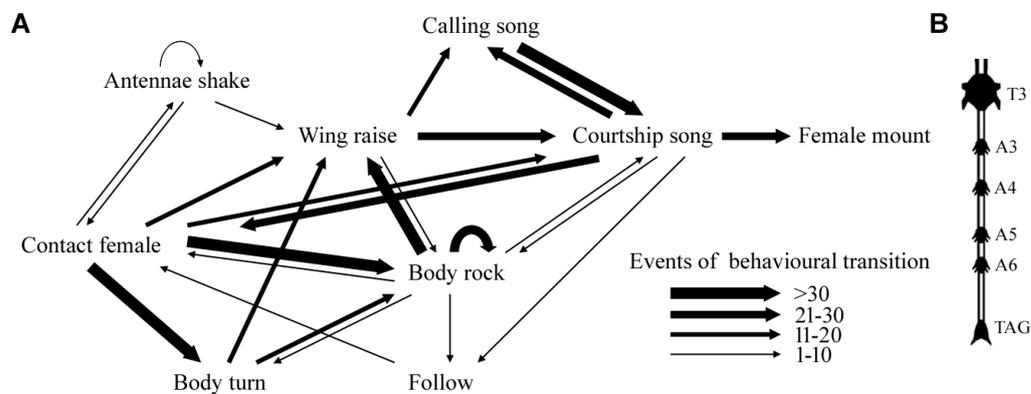
Figure 3.1 Ethogram of courtship behaviour in intact male *G. bimaculatus.*
(A) The ethogram starts at the moment of contact with the female. Thickness of arrows denotes the transition between components of the behaviour. Statistics of the transitions is shown in **Table 3.3**. (B) Schematic illustration of intact central nervous system from the metathoracic ganglion complex (T3) to the terminal abdominal ganglion (TAG).

When males were in a good position to advertise themselves, or after turning (48.1%) and the rocking movements (49.2%) they raised their wings and generated calling song (38.1%) or courtship song (50%). Calling songs then soon switched to courtship songs (100%). Courtship

songs may switch back to calling songs (32.4%), or pause upon female contact (30.9%) and restart again, or lead to the female mounting and to copulation (35.4%). While stridulating calling or courtship songs, males adjusted their position to the female by stepping slowly to the female. If they noticed the female had changed its position and was no longer behind, they would approach the female and start all over again.

Females attracted by courtship song followed the males and touched the males' wings and posterior abdomen with the forelegs and antennae in the lead up to copulation. Males responded by stopping the courtship song and moving backwards to facilitate and allow the female mounting. Once mounted, the males bent their abdomen upward to transfer a spermatophore, which could take one to several minutes. During copulation, males vibrated their antennae rapidly toward the female.

All 4 sham operated animals presented courtship behaviour in the same way as intact animals and successfully attracted and copulated with females.

3.3.3 Courtship behaviour after A6-TAG lesion

Animals with a lesion of the connectives between the 6th abdominal ganglion and the terminal abdominal ganglion (A6-TAG, n=8, **Figure 3.2** and **Table 3.4**) showed only few changes in courtship behaviour. After contact with a female, they initiated courtship behaviour mainly by rocking their body (35.2%) or turning their body towards the female (36.2%). 83.3 percent events of body turning toward females lead to rocking of the body. Rocking of the body could be followed by another contact with the female (38.1%), raising the forewings to prepare for courtship singing (22.6%), or rapid shaking of antennae (19%). During the antennae shaking, males stood still, pointed their antennae upward and slightly anteriorly, and rapidly shook antennae in a small angle for less than one second with an interval of two to ten seconds. The repeated antennae shakings and pauses could last for several minutes. Antennae shake

(11.4%), body rock (22.6%), and body turn (16.7%) could lead to raising of the forewings. Unlike in intact animals, raising of the wings was not always followed by calling song or courtship song. Males could raise and lower wings repeatedly (33.3%), fall into a loop of raising wings, lowering wings and shaking antennae (30.4%), or start to generate calling song (17.4%) or courtship song (8.7%). Calling songs almost always (91.3%) switched to courtship song.
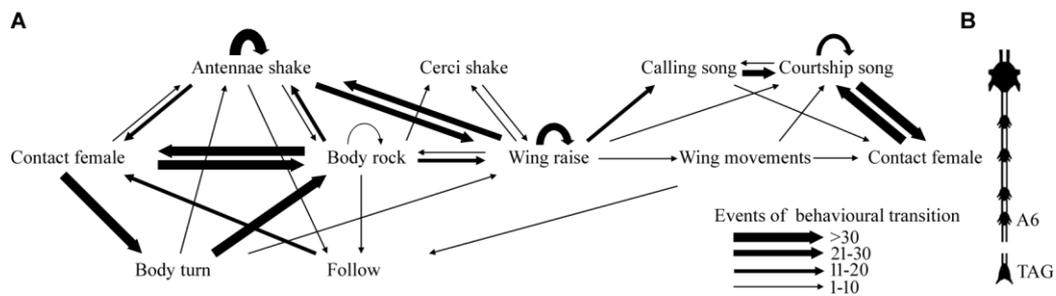
Figure 3.2 Ethogram of courtship behaviour in A6-TAG male *G. bimaculatus*.
(A) Thickness of arrows denotes the transition between components of the behaviour. Statistics of the transitions is shown in **Table 3.4**. (B) Schematic illustration of central nervous system after A6-TAG lesion.

A total of six out of eight lesioned animals were recorded singing courtship song. Two males were able to generate courtship song one day after the lesion, two males started to produce courtship song four days after the operation, and two animals sang courtship song one and two weeks after surgery, respectively. The initiation and build-up of motor activity of the very first courtship song after the lesion was slower than in intact animals. The raised wings were closed and opened with increasing frequency and gradually produced the complete courtship song with low amplitude wing vibrations and the typical large wing movements leading to single high frequency sound pulses. Once the males had fully recovered, they could generate the song in the normal way for the rest of their lives. Females were attracted and tried to mount them. However, without sensory feedback from the posterior abdomen and the genital apparatus, these males couldn't properly adjust their position to the females, they failed to

cooperate with the mounting female and no copulation occurred (Ritz and Sakaluk 2002). It was common (61.7%) that singing males stopped their courtship song as females touched their abdomen, and following failed female mounting the male produced courtship song again. Some tried to copulate by raising their abdomen, but always in vain, and as the control over releasing the spermatophore was lost it lead to failure in copulating with females. In two males the cerci were even bitten off and eaten by the female during courtship, indicating the loss of cercal sensory information.

3.3.4 Courtship behaviour after A5-A6 lesion

Males with a lesion of the connectives between abdominal ganglion 5 and abdominal ganglion 6 (A5-A6, n=6, **Figure 3.3** and **Table 3.5**) showed a further reduction in the number of crickets generating courtship song compared to A6-TAG males, while the display of other behaviour components was similar. Upon encounter with a female, they rocked their bodies (71%), presented the posterior part toward the female (26.6%) or did both. After rocking of their body, they may contact the female again (24.2%) and rock again. They could spend minutes with shaking the antennae near the female after contacting the female (2.4%), rocking their body (9.4%), turning their body (15.8%) and adjusted their position and direction in response to female movements. When a female touched the male while walking, the male started body rocking or turned again towards the female. If a female moved away they followed her to a suitable position relative to the female and continued courtship behaviour. It was common (25%) that males raised and lowered the wings continuously if the female was stationary. Antennae shaking and wing raising could happen one after another (antennae shaking to wing raising: 12.4%, wing raising to antennae shaking: 46.7%).

5 out of 6 males did not generate courtship song after the lesion. One male was observed singing courtship song one week after the lesion had been applied. The initiation and build-up

of motor activity for generating courtship song took about a minute. The male gradually increased the frequency of opening and closing the wings and in the end generated the complete courtship song pattern. All A5-A6 males failed to copulate with the female, as described before.
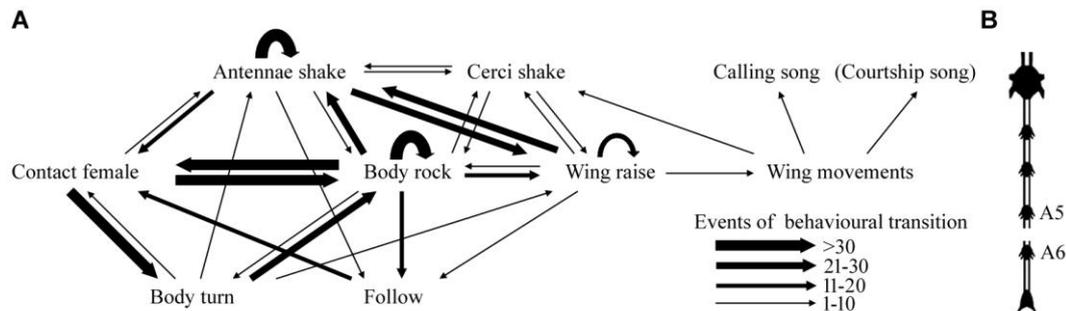
Figure 3.3 Ethogram of courtship behaviour in A5-A6 male *G. bimaculatus.*

(A) Thickness of arrows denotes the transitions between components of the behaviour. Statistics of the transitions is shown in **Table 3.5**. (B) Schematic illustration of central nervous system after A5-A6 lesion.

3.3.5   Courtship behaviour after A4-A5 lesion

All males with lesioned connectives between abdominal ganglion 4 and abdominal ganglion 5 (A4-A5, n=5, **Figure 3.4** and **Table 3.6**) initiated courtship behaviour in a way similar to A5-A6 animals but showed no courtship song and no copulations occurred. Males rocked their body (61.8%) or turned their body to expose the rear of their abdomen (34.2%) when they contacted the female. Antennae shaking occurred after almost all other behaviour (3.9% after contacting female, 8.3% after body rocking, 11.9% after body turning, 40.3% after wing raising, and 28.6% cerci shaking). The males could stand still near the female and shake antennae repetitively (64.5%) for minutes. Raising of forewings mainly happened after body rocking (15.8%) and antennae shaking (17.1%). It was common that males episodically raised and lowered wings repeatedly (22.6%) or raised and lowered their wings together with antennae shaking. Males could be stuck at this stage of wing and antennae movement, but did not progress to generate courtship song as it normally would occur. If the female moved away,

the males followed the female and repeated the whole sequences of courtship behaviour. In A4-A5 males, 9 events of silent episodic wing movements and 2 events of calling song were recorded.

Figure 3.4 Ethogram of courtship behaviour in A4-A5 male *G. bimaculatus.*
(A) Thickness of arrows denotes the transition between components of the behaviour. Statistics of the transitions is shown in **Table 3.6**. (B) Schematic illustration of central nervous system after A4-A5 lesion.

3.3.6 *Courtship behaviour after A3-A4 lesion*

After the connectives between abdominal ganglion 3 and abdominal ganglion 4 were cut (A3-A4, n=5, **Figure 3.5** and **Table 3.7**), male crickets displayed reduced courtship behaviour, no calling song or courtship song, and no copulations occurred. These animals turned their body to present their back to females (42.3%) or rocked their body (55.8%) after they contacted the female. They may contact the female again (30.1% after body rock) and rock their body once again, stand and shake antennae rapidly over and over again (56.8%), continuously raise and lower forewings (31.3%), or shake antennae together with wing movements. It was common that males raised and lowered their wings, however they did not generate the typical opening and closing movements as required for sound production. Occasionally a "scratchy" sound pulse was generated when the wings were lowered again, but no structured sound pattern like calling, courtship, or rivalry song was ever recorded in these males.

Figure 3.5 Ethogram of courtship behaviour in A3-A4 male *G. bimaculatus.*
(A) Thickness of arrows denotes the transitions between components of the behaviour. Statistics of the transitions is shown in **Table 3.7**. (B) Schematic illustration of central nervous system after A3-A4 lesion.

3.3.7 Courtship behaviour after T3-A3 lesion

In males with a lesion of the connectives between metathoracic ganglion complex T3 and abdominal ganglion 3 (T3-A3, n=6, **Figure 3.6** and **Table 3.8**) the occurrence of courtship behaviour decreased dramatically. While behaviour like rocking (8%) or turning the body (48%) still occurred upon encounter of the female, males generally were standing still and shaking their antennae over and over again (86.7%). These males raised and lowered their wings (75%) but only occasionally low amplitude scratchy sound pulses occurred upon lowering the wings (Jacob and Hedwig 2016). The males did not generate any calling, courtship, or rivalry song over the period of recovery and even thereafter, and no copulations occurred.

Figure 3.6 Ethogram of courtship behaviour in T3-A3 male *G. bimaculatus.*

(A) Thickness of arrows denotes the transitions between components of the behaviour. Statistics of the transitions is shown in **Table 3.8**. (B) Schematic illustration of central nervous system after T3-A3 lesion.

3.3.8 *Rivalry behaviour in intact males*

Male *G. bimaculatus* display a well-organized rivalry behaviour when fighting with conspecific males for resources such as food, shelter, or a mate. The rivalry behaviour can be simplified into three stages. It starts by antennae contact of two males, and once they identify each other as male, both males initiate a series of threatening, rivalry singing and fighting behaviour until one male surrender and runs away. The winner of the fight then banishes the loser with continuous threatening or biting. When the loser moves away from the winner and stays at a distance the rivalry behaviour finishes. The whole rivalry behaviour could finish in seconds or last for more than one minute depending on the timing when one of the males decides to stop rivalry behaviour and flee.

Figure 3.7 Ethogram of rivalry behaviour in intact male *G. bimaculatus*.

(A) Thickness of arrows denotes the events of transition between components of the behaviour. Statistics of the transitions is shown in **Table 3.9**. (B) Schematic illustration of intact central nervous system from metathoracic ganglion complex (T3) to terminal abdominal ganglion (TAG).

In the experiments the intact target male and a one-winged opponent male (**Figure 3.7** and

Table 3.9) recognized each other through antennae contact and began antennae fencing (64.7%) right away. The first contact of two males was always followed by antennae fencing. Body rocking after contacting a male as indicated in the ethograms of rivalry behaviour only happened when one male won the fight and tried to banish the loser. This also applies to operated animals. During antennae fencing they adjusted their position to face the opponent head-on. Antennae fencing lasted throughout the initial confrontation of the two males. After few seconds of fencing they demonstrated the threat posture (100%), during which the males raised their bodies with extended forelegs and opened their mandibles widely. During or after the threat posture, the males may raise the front wings (54.5%) and stridulate the rivalry song (100%), otherwise the male could bite the opponent (22.7%) or grapple with the opponent (18.2%) after the threat posture. In the latter case, two males wrestled with their mandibles and sometimes fought to the ground. The competition of two males could end at any point during antennae fencing, demonstration of the threat posture, singing of the rivalry song, biting, or grappling when one male surrendered and ran away. The winner then continued chasing the loser and threatening it by singing the rivalry song (10%), biting (47.5%), or rocking the body (32.5%). The winner also rocked the body whenever it physically encountered the loser (35.3%) or during rivalry song (45.5%). Chasing and rivalry behaviour stopped when the loser was no longer nearby. The 4 sham operated males showed the same rivalry behaviour sequences as intact males did.

3.3.9 Rivalry behaviour after A6-TAG lesion

Males with a lesion to the connectives between abdominal ganglion 6 and terminal abdominal ganglion (A6-TAG, n=7, **Figure 3.8** and **Table 3.10**) performed normal rivalry behaviour like intact animals. Upon encounter of the other male they started antennae fencing (57.1%) and then threat posture (100%). After the threat posture the male may rock the body

(12.5%), bite the opponent (6.3%), grapple with the opponent (31.3%), or raise the wings (50%) and started to generate the rivalry song (100%). Once the male won the fight and the loser ran away, the winner kept chasing and threatening the loser by rocking the body, producing rivalry song, or biting. The winner also rocked the body whenever the loser passed by and had physical contact with it (39.3%). The winner stopped rivalry behaviour when the loser was far away enough.
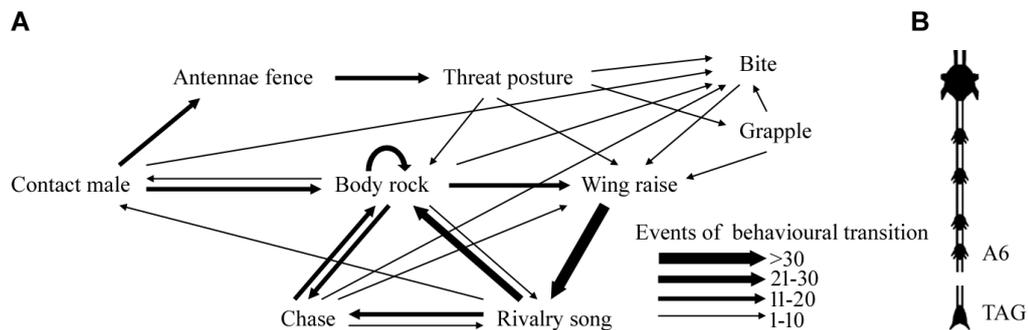
Figure 3.8 Ethogram of rivalry behaviour in A6-TAG male *G. bimaculatus*.
(A) Thickness of arrows denotes the events of transition between components of the behaviour. Statistics of the transitions is shown in **Table 3.10**. (B) Schematic illustration of central nervous system after A6-TAG lesion.

3.3.10 Rivalry behaviour after A5-A6 lesion

Males with a lesion between abdominal ganglion 5 and abdominal ganglion 6 (A5-A6, n=5, **Figure 3.9** and **Table 3.11**) showed all the rivalry behaviour like intact males. After contact with the other male, they interacted with their opponent by antennae fencing (36.6%) and threat posture demonstration (100%). They proceeded to rock their body (66.7%), bite (26.7%) and grapple (6.7%) after the threat posture if their rival did not give up. Rivalry song was generated if the confrontation lasted long. Upon winning the fight, the male kept chasing the loser until the loser stayed away at a considerable distance. The winning males may rock their body repeatedly (35.1%) whenever they had contact with the loser (61%), chase and rock the body when the loser was nearby (53.7%), produce rivalry song after body rocking (19.1%) or during chasing (19.5%), or even try to bite the loser upon physical contact (2.4%).
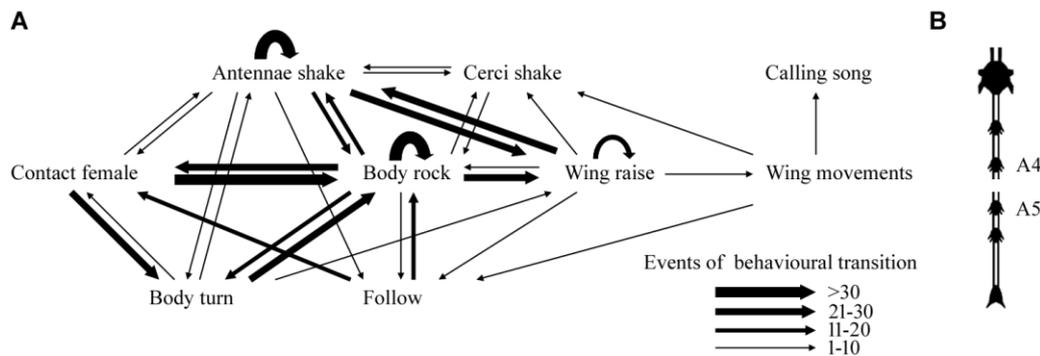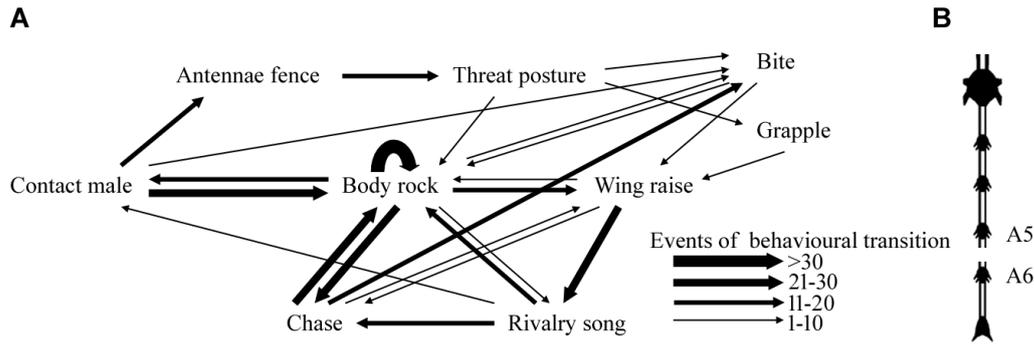
Figure 3.9 Ethogram of rivalry behaviour in A5-A6 male *G. bimaculatus*.

(A) Thickness of arrows denotes the transitions between components of the behaviour. Statistics of the transitions is shown in **Table 3.11**. (B) Schematic illustration of central nervous system after A5-A6 lesion.

3.3.11 Rivalry behaviour after A4-A5 lesion

Males with a lesion between abdominal ganglion 4 and abdominal ganglion 5 (A4-A5, n=6, **Figure 3.10** and **Table 3.12**) still performed rivalry behaviour like intact males. They initiated rivalry behaviour after identifying another male by antennae fencing (36%) and following threat posture (72.2%). After threat posture, they could rock their body (53.3%), bite their opponent (6.7%), grapple with the opponent (13.3%), or raise their wings (26.7%) and generate the rivalry song (89.1%). After winning the fight, the males continued threatening their opponent by producing the rivalry song, chasing, or rocking their body. When the loser was close by or had physical contact (64%) with them, and when the loser tried to escape, the winner rocked its body repetitively (31%). Rivalry song occurred after body rocking of the males (35%). When the loser was at a proper distance the winner stopped chasing and threatening, and the rivalry behaviour ceased.
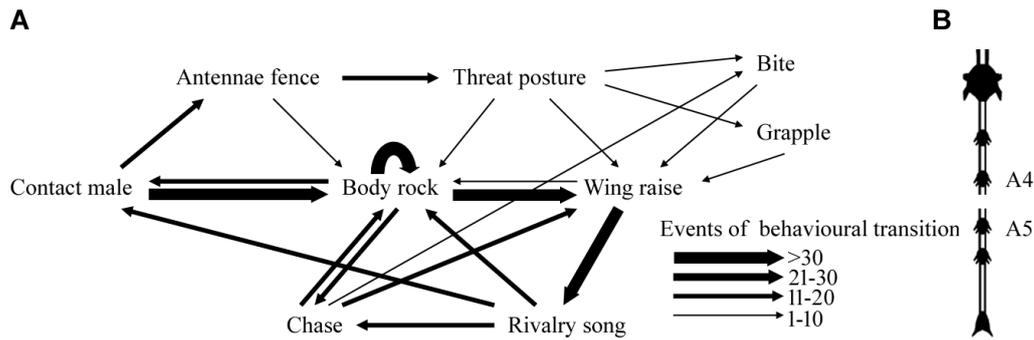
Figure 3.10 Ethogram of rivalry behaviour in A4-A5 male *G. bimaculatus*.
(A) Thickness of arrows denotes the transitions between components of the behaviour. Statistics of the transitions is shown in **Table 3.12**. (B) Schematic illustration of central nervous system after A4-A5 lesion.

3.3.12 Rivalry behaviour after A3-A4 lesion

Males with lesion between abdominal ganglion 3 and abdominal ganglion 4 (A3-A4, n=6, **Figure 3.11** and **Table 3.13**) performed most components of the rivalry behaviour while no rivalry song was generated during the encounter. The males began rivalry behaviour with antennae fencing (53.8%) after recognizing the other male. Then they demonstrated the threat posture to their opponent (100%). Threat posture was followed by body rocking (28.6%), biting (35.7%), or grappling (35.7%). These three behaviour components could all lead to raising of the wings, with 45.7% after body rocking, 4.8% after biting, and 33.3% after grappling. However different to intact animals, these males did not produce the rivalry song as no proper opening and closing movements of the front wings occurred, although they raised and lowered the wings. The lesioned males could still win the fight without rivalry song. Once their opponent fled, they may chase and rock their body (56.4%) when they caught up with the loser, or rock their body after physical contact with the loser (42.3%), or raise the wings without generating any sound after rocking their body (45.7%). Rivalry behaviour ceased when the winner stopped chasing and threatening as the loser ran away.
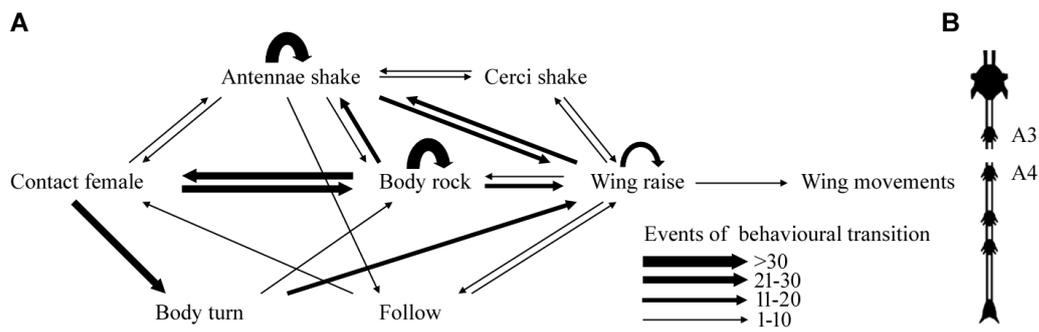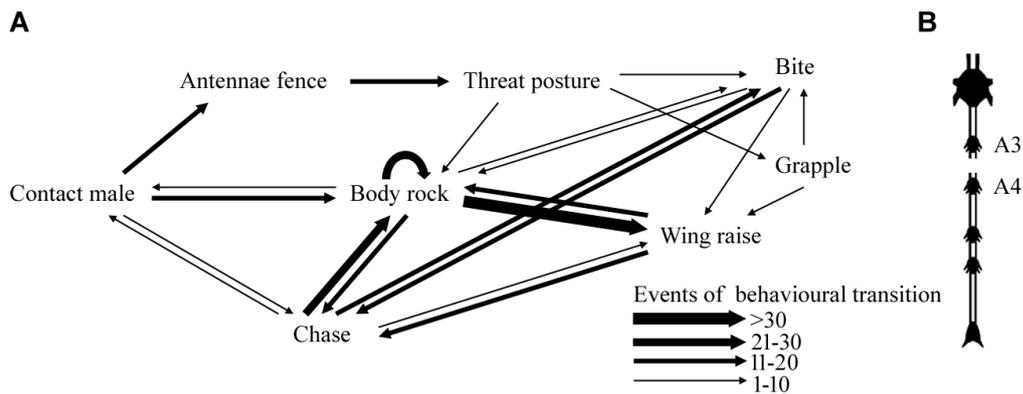
Figure 3.11 Ethogram of rivalry behaviour in A3-A4 male *G. bimaculatus*.
(A) Thickness of arrows denotes the transitions between components of the behaviour. Statistics of the transitions is shown in **Table 3.13**. (B) Schematic illustration of central nervous system after A3-A4 lesion.

3.3.13 Rivalry behaviour after T3-A3 lesion

Males with a lesion of the connectives between metathoracic ganglion complex T3 and abdominal ganglion 3 (T3-A3, n=8, **Figure 3.12** and **Table 3.14**) showed decreased rivalry behaviour activity compared to intact males, and displayed all the components of the rivalry behaviour except for the rivalry song. After antennal contact and identification of the other male, these males began antennae fencing with their opponent (69.2%), followed by demonstrating the threat posture (100%). They bit (61.1%), rocked their body (16.7%), or grappled with their opponent (22.2%) after the threat posture. They could raise their wings after rocking their body (36.4%) or biting (20%), however without proper opening and closing movements of the wings, no rivalry song was generated in these males. After winning the fight, they chased and threatened their opponent. They rocked their body when the opponent was nearby or after contact with the opponent (23.1%). They may even bite the opponent after rocking their body (3%) or during chasing (37.5%). When the opponent was far away the winner stopped chasing, and rivalry behaviour came to an end.
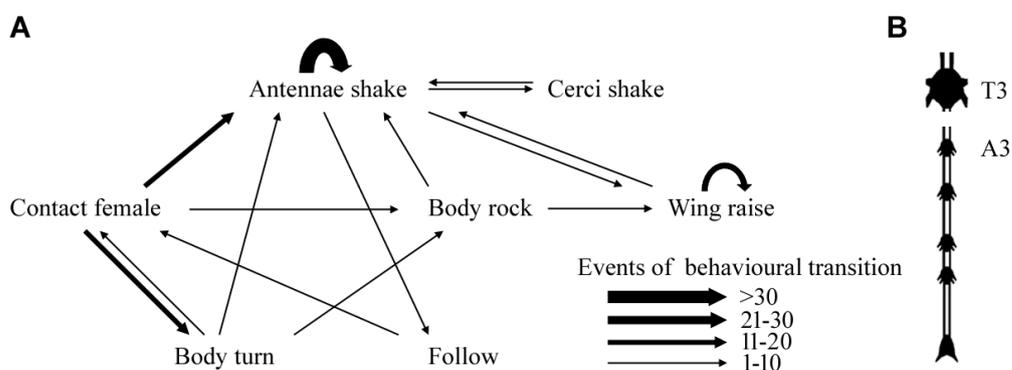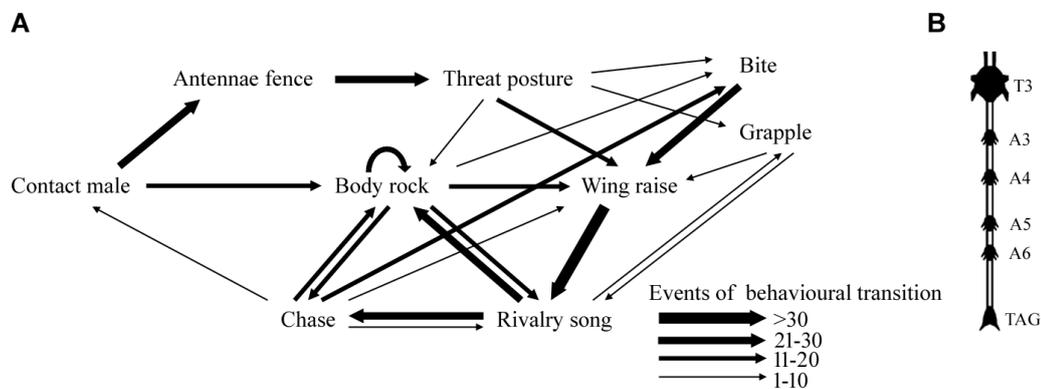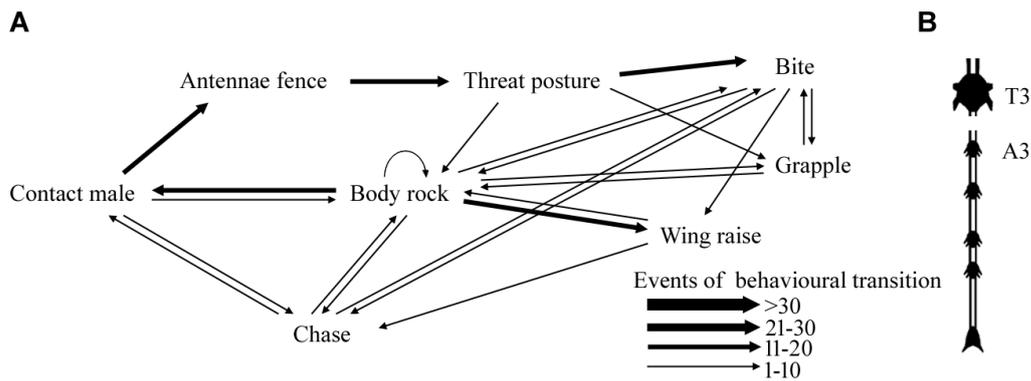
Figure 3.12 Ethogram of rivalry behaviour in T3-A3 male _G. bimaculatus_.
(A) Thickness of arrows denotes the transitions between components of the behaviour. Statistics of the transitions is shown in **Table 3.14**. (B) Schematic illustration of central nervous system after T3-A3 lesion.

3.4 Discussion

In the current study, behaviour sequences of courtship and rivalry behaviour in _G. bimaculatus_ were examined under intact and abdominal nerve cord lesioned conditions. The results suggest only courtship song, rivalry song, and copulation require involvement of abdominal nervous system, while the other behaviour components were controlled by the brain and thoracic nervous system.

The combination of lesion application and ethograms provides opportunities to study the organization of neural network organization underlying behaviour. For fixed action patterns like courtship and rivalry behaviour in crickets, the altered behaviour after lesions to specific connectives suggests the involvement of the separated parts of the nervous system, while behaviour components that are not affected may not require the separated part of the nervous system.

After the pioneering studies severing different connectives in the CNS of _G. campestris_ Huber (1960, 1963) proposed that an intact connection between the brain and the mesothoracic

ganglion is sufficient for the males to generate all three song types. Recently the song pattern generator for calling song was proved to be distributed along the A3 to A6 ganglia of the abdominal nerve cord by systematic lesion experiments (Jacob and Hedwig 2016). Furthermore, intracellular recording identified neurons of the song pattern generator in the abdominal ganglia A3, A4, A5, and A6 in *G. bimaculatus* (Schöneich and Hedwig 2011, 2012; Jacob and Hedwig 2019, 2020). While these studies were focused on pattern generation for calling song, in the present study, the importance of abdominal nerve cord for production of courtship song and rivalry song was evaluated for the first time.

Courtship song was observed in males with an A6-TAG lesion indicating that the terminal abdominal ganglion may not be relevant for courtship song motor pattern generation, and that the sensory information from the genitalia is not necessary for this song to occur. This contradicts the claim made by Huber (1963) that courtship song does not normally occur when the connectives are interrupted anywhere between the mesothoracic ganglion and the TAG. One of the reasons for the different conclusions might result from the different sites of lesions applied then and in this study.

Only one male after an A5-A6 lesion produced courtship song a week after the lesion, while the rest A5-A6, A4-A5, A3-A4, T3-A3 lesioned males failed to generate courtship song after the lesions. Depending on how the single evidence of courtship song generation after an A5-A6 lesion should be weighted, the experiments point to the crucial importance of the A6 (and maybe A5 ganglion), in generating the courtship song motor pattern. There are several possibilities how the courtship song pattern generating network could be organized. One possibility is that the pattern generating network is confined to the A6 (+A5) ganglia. These ganglia would be completely sufficient to generate the neural activity that would drive the motor neurons and wing muscles in the mesothoracic segment by some ascending interneurons, which run through the more anterior ganglia (A4, A3 and T3 complex) without further

interaction. Another possibility, which cannot be ruled out based on the lesion experiments, is that the generation of courtship song additionally requires interneuron networks in any of the more anterior ganglia e.g. A3, A4, T3, or any combination of these ganglia, which would be under the control of the activity patterns generated in A6 (+A5). These networks might contribute to the pattern generated in A6 (+A5) to make it a fully functional courtship motor pattern. For generation of the pulse pattern of calling song, the neural network is distributed along the A3 to A6 ganglia, and it may be recruited for courtship song. However, calling song sound pulses and underlying wing movements are very different from the courtship song wing movements (see Chapter 2). Interneurons in A3 involved in calling song motor pattern generation have a very strong effect on mesothoracic motoneurons (Schöneich and Hedwig 2012, Jacob and Hedwig 2019, 2020); this may make it unlikely that they could contribute to the courtship motor pattern. Thus, although the current experimental evidence suggests that the network for courtship song generation is housed in the A6 (+A5) ganglia further evidence will be required. Adapting lesion methods such as hemisection of the A3, A4, or both ganglia would destroy the ascending interneurons of the calling song pattern generating network and might allow to solve this question (Ronacher et al. 1988; Ronacher 1989; Jacob and Hedwig 2016). Furthermore, intracellular recordings to identify interneurons of the courtship network in A6 (+A5) would provide further insight into the organization of the system.

Rivalry song was observed in males with lesions at A6-TAG, A5-A6, and A4-A5, and no rivalry song was generated when connectives were severed between A3 and A4, or T3 and A3. Also calling song requires intact T3-A3 connectives and A3-A4 connectives (Jacob and Hedwig 2016), and intracellular recordings point to a neuronal network generating the motor activity for wing opening-closing movements in these ganglia (Jacob and Hedwig 2019, 2020). For calling song, after cutting the A4-A5 connective and removing a timing signal from the posterior ganglia, males will generate chirps with a larger number of pulse, a typical feature of

the rivalry song. Moreover, the duration of suprathreshold intracellular current injection to the ascending opener neuron in A3 will determine the duration of chirps (Schöneich and Hedwig 2011, 2012; Jacob and Hedwig 2019, 2020). There is a high similarity between calling song and rivalry song regarding sound pulse parameters, wing movements and underlying muscle activity (Kutsch 1969) (see Chapter 2). Also the short bouts of rivalry song with a high number of 8-12 pulses/chirp will always gradually shift to calling song with 3-5 pulses/chirp (Kutsch 1969) (see Chapter 2). These evidences indicate that the generation of the motor patterns for rivalry song and calling song may use the same pattern generating network. The different song types would just require a different command to the singing-CPG, which could be provided by a command neuron controlling rivalry song or by combining a rivalry command with the activity of the calling song command neuron. Intracellular stimulation experiments of calling song command neuron, demonstrated that its enhanced spike activity can increase the calling song chirp rate, but the release of rivalry song has never been observed. (see Chapter 5)

This study provides further insight into the organization of the song pattern generators for courtship and rivalry song. It reveals the central role of the abdominal ganglia for generating the underlying motor patterns, which has only recently been acknowledged. In combination with the lesion experiments elucidating the network for calling song (Jacob and Hedwig 2016), the abdominal ganglia required for pattern generation of three song types were identified in *G. bimaculatus* (**Figure 3.13** and **Table 3.15**). Calling and rivalry songs require at least the ganglia A3 and A4 to produce complete chirps, and A6 and maybe A5 ganglia are essential for courtship song generation. Interestingly courtship singing involves the more posterior ganglion A6 (+A5) while calling and rivalry singing depend on networks in A3 and A4. In all three cases of song patterns the abdominal CPG networks are distant to the motoneurons in the mesothoracic ganglion, assembling the final motor output. This raises the question how the systems may have evolved and comparative studies with different species may provide further

insight. In Chapter 4, I addressed this question by performing systematic lesions to abdominal nerve cord and compared the changes of species-specific calling songs after each lesion in different cricket species. The results suggest the control of pulse timing is universally housed in A3 and A4, while higher song structure is determined mainly by A5. Applying the same methodology for courtship song and rivalry song in other cricket species might provide further insights toward the organisation and evolution of the cricket song pattern generation system.
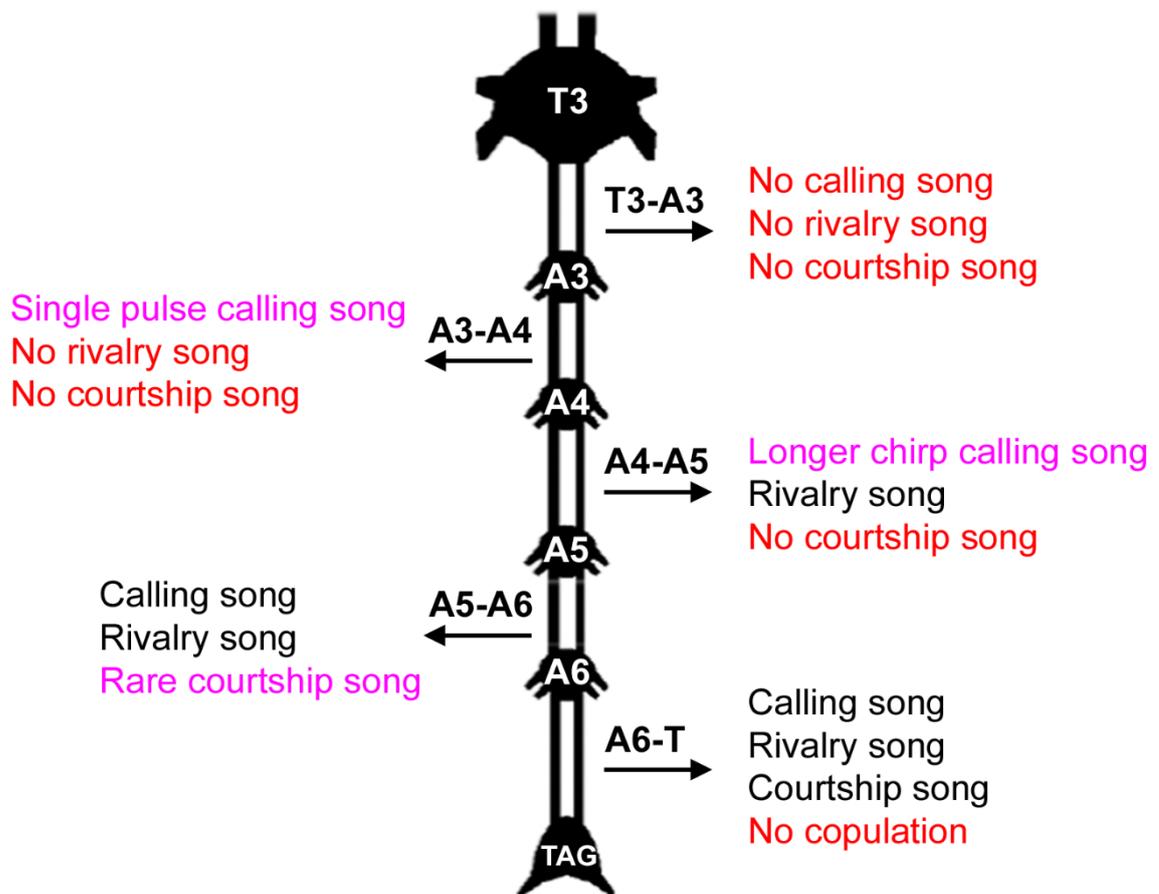
Figure 3.13 Summary of effects on three song types and behaviour by different lesions to abdominal nerve cord.

Central nerve cord from metathoracic ganglion complex (T3) to terminal abdominal ganglion (TAG) is illustrated. Arrows connect lesions between specific ganglia and the effects after each lesion. Observations of song types and behaviour change after lesions are listed in the following order: calling song, rivalry song, courtship song, and behaviour change. Black colour represents no change to the song type after lesion, red colour represents abolished song type and behaviour after lesion, and magenta colour represents change of the song type after lesion.

In courtship behaviour, separation of the terminal abdominal ganglion from the anterior central nerve cord at any point of the male's nervous system always led to failure of copulation due to lack of sensory information from the genitalia which prevented the male to adjust its abdomen in order to allow female mounting. Similar results was also reported in tropical house cricket *Gryllodes sigillatus* that cercectomized males significantly reduced mating success compared to control males (Ritz and Sakaluk 2002). Huber (1963) concluded that after severing of the connectives connecting the TAG, males responded to the female antennae contact as if the copulation had already finished, with antennae lowering sidewards and vibration. This was not observed in the current study and the A6-TAG lesioned males almost always turned around to reveal their back to the females after antennae contact. Production of a spermatophore still functioned normally and in some lesioned males secreted spermatopores were observed sticking to the genital apparatus.

The video-recording of courtship behaviour revealed two behaviours that were not commonly observed in intact animals, antennae shaking and cerci shaking, were frequently found in all lesioned animals. This could result from the loss of contact with the terminal abdominal ganglion. In the case of antennae shaking, after physical contact with a female and usually when the abdomen of the male was facing female, the antennae were pointing vertically upright and vibrating intermittently. This could be a compensation for the loss of terminal sensory information. The importance of cerci in courtship behaviour was reported in different cricket species. In *Teleogryllus commodus*, cerci of males performed strong oscillatory searching movements during backward movements toward females (Loher and Rence 1978). In *G. bimaculatus*, backward movements were shown to be elicited by contact stimuli to the cerci (Sakai and Ootsubo 1988). Cercal sensory feedback was also shown to be necessary for spermatophore transfer in *Acheta domesticus* (Snell and Killian 2000). These evidences indicate the crucial role of cercal sensory information for the proceeding of courtship behaviour.

The antennae shaking behaviour might be compensation for the loss of cercal sensory feedback after connectives were severed. Cerci shaking, on the other hand, could happen without direct contact with the females. Since the behaviour was only observed when the female was presented nearby, this could be an autonomous response upon detecting a female-specific compound mixture (Tregenza and Wedell 1997; Nagamoto et al. 2005), even though the presence of chemoreceptor on cerci was not proved in cricket, while possible chemoreceptors were described in locust and earwig by light and electron microscopy (Al-Dosry 2010; Yu et al. 2011).

Courtship behaviour in male crickets contains a sequence of interaction with females including sexual recognition, courtship song acoustic display, female mounting, and copulation (Loher and Rence 1978; Adamo and Hoy 1994). Multiple sensory inputs and responses are involved in the process. For sexual recognition, visual signals and the reception of species-specific chemical compounds by the antennae are processed in the brain to determine the sex of the other cricket and to initiate subsequent behavioural sequences (Tregenza and Wedell 1997; Nagamoto et al. 2005). Visual signals provide information on the female position and guide the males to turn at a correct angle. Once the posterior abdomen is facing the female and courtship song begins, cercal sensory input notifies the male when the female shows intention to mate by touching the cerci (Sakai and Ootsubo 1988). The following female mounting and copulation also require integration of mechanosensory inputs from the wings, cerci and tergites (Sakai and Ootsubo 1988; Snell and Killian 2000). In this study, lesions at different sites of abdominal nerve cord abolished the sensory information from and motor commands to the cerci. Thus the behaviour requiring sensory information from the posterior body parts such as backward movements for female mounting was not properly controlled. However, the other behaviour sequences, such as sexual recognition which is processed in the brain, and turning behaviour which requires corporation of the brain and the thoracic ganglia was normally

expressed. These behavioural data suggest that courtship behaviour, as fixed action pattern, will occur in a fixed order until finished or interrupted, at which point the whole sequence will start over again in the same order.

The complex courtship behaviour sequences involving species-specific traits demonstrated that antennae contact and conspecific courtship song prevent the possibility of hybridization with closely related species. Elaborate courtship behaviour containing four phases of courtship songs was observed in the grasshopper *Stenobothrus clavatus* (Ostrowski et al. 2009). Different song patterns generated during each phase and visual display in the third phase ensure the mating with conspecifics. In *Drosophila*, courtship behaviour also appears as a behaviour sequence involving olfactory/visual recognition of the female, tapping, wing extension, generation of courtship song, licking, attempted copulation, and final sperm transfer (Billeter et al. 2006). The complex behaviour sequences provide chances for the males to display and time for the females to select. These courtship rituals in different animal models pose different mechanisms in performing mating but all point to the same purpose, that is, to court with conspecifics. Considering the similar sensory and motor pathway required for final copulation in these species, application of systematic lesions on these animal models might also interfere the behaviour components that require the control of the genital apparatus and cerci, while the rest of the behaviour components controlled only by the brain and the thoracic ganglia will function normally.

For the purpose of excluding rivalry song produced by the opponent male in the rivalry behaviour experiments, one of its forewings was removed to prevent sound production. Consequently, the target males with intact wings won over 80% of all fights. This suggests rivalry song signal is an influencing factor in winning the fights, but even males which could no longer generate the rivalry song could be winners. This is also supported by a study in *Acheta domesticus*, where the rivalry song conveying information about resource holding

potential of the song producer (Brown et al. 2006). Since such manipulation favoured the intact males in the fights and led to fast determination of the dominant-subordinate hierarchy based on a proposed assessment strategy of the males during aggressive behaviour (Hack 1997; Hofmann and Schildberger 2001; Rillich et al. 2007), in my experiments the occurrence of more aggressive behaviour components like biting or grappling might be reduced and the frequency of these components as given in the ethograms may be lower than in the natural condition.

Rivalry behaviour in *G. bimaculatus* shows at least 7 different elements. Even after lesions to the abdominal nerve cord posterior to the A4 ganglion, all the typical components of rivalry behaviour: antennal fencing, threat posture, body rocking, rivalry singing, chasing, biting, and grappling were observed. This indicates the nervous control of rivalry behaviour is mainly carried out by the brain and the thoracic ganglia. While sex recognition and antenna fencing might only require processing in the brain, the other behaviour components require the integration of chemical and visual cues in the brain, central command transmitted from the brain to control the mandibles, likely via the SOG, and to the thoracic ganglia to control the body and the legs to achieve each behaviour component. The only component that requires the abdominal nerve cord is singing of the rivalry song. Since extracellular current stimulation could elicit not only rivalry song but also rivalry behaviour like antennae beating (fencing), body shaking, raising body (threat posture) (Huber 1963), there might be a central command that initiates the whole behaviour sequences upon recognition of another male, and subsequent components of rivalry behaviour are mediated in response to visual, acoustic, tactile signals from the opponent during confrontation. The decision to continue fighting or flee is then made by self-evaluation and judging strength of the opponent (Hofmann and Schildberger 2001; Rillich et al. 2007).

Table 3.1 Behaviour components described in ethograms

Behaviour	Description
Courtship behaviour	
Antennae shake	Male rapidly shakes vertically upright antennae
Body rock	Male rocks body back and forth
Body turn	Male turns and presents posterior abdomen to female
Calling song	Male stridulates calling song
Cerci shake	Male shakes both cerci
Courtship song	Male stridulates courtship song
Contact female	Male contacts female
Female mount	Female mounts male
Follow	Male searches and follows female
Wing raise	Male raises wings
Wing movements	Male episodically opens and closes wings, no sound generated
Rivalry behaviour	
Antennae fence	Male makes rapid antennae contact with another male
Bite	Male bites another male
Body rock	Male rocks body back and forth
Contact male	Male contacts another male
Chase	Male chases other male after winning a fight
Grapple	Males fight with mandibles wrestling
Rivalry song	Male stridulates rivalry song
Threat posture	Male threats other male by raising itself on forelegs and widely open mandibles
Wing raise	Male raises wings

Table 3.2 Courtship and rivalry behaviour components in each lesion group

	T3-A3	A3-A4	A4-A5	A5-A6	A6-T
Courtship behaviour					
Antenna shake	6/6	5/5	5/5	6/6	7/8
Follow	4/6	4/5	4/5	5/6	5/8
Body rock	1/6	2/5	5/5	6/6	8/8
Body turn	3/6	4/5	5/5	6/6	8/8
Cerci shake	1/6	2/5	4/5	5/6	6/8
Wing raise	1/6	5/5	5/5	6/6	8/8
Courtship song	0/6	0/5	0/5	1/6	6/8
Rivalry behaviour					
Antenna fence	8/8	6/6	6/6	5/5	7/7
Body rock	3/8	5/6	6/6	5/5	3/7
Threat pose	8/8	6/6	6/6	4/5	6/7
Wing raise	6/8	6/6	5/6	5/5	7/7
Chase	6/8	5/6	5/6	4/5	4/7
Bite	3/8	2/6	3/6	2/5	4/7
Grapple	2/8	1/6	1/6	1/5	2/7
Rivalry song	0/8	0/6	5/6	5/5	7/7

Table 3.3 Behaviour transitions in intact males during courtship

Behaviour A	Behaviour B	Percentage
Antennae shake (4 transitions)	Antennae shake	2/4 (50%)
	Contact female	1/4 (25%)
	Wing raise	1/4 (25%)
Body rock (63 transitions)	Body rock	21/63 (33.3%)
	Body turn	1/63 (1.6%)
	Contact female	2/63 (3.2%)
	Courtship song	7/63 (11.1%)
	Follow	1/63 (1.6%)
	Wing raise	31/63 (49.2%)
Body turn (27 transitions)	Body rock	14/27 (51.9%)
	Wing raise	13/27 (48.1%)
Calling song (35 transitions)	Courtship song	35/35 (100%)
Contact female (76 transitions)	Antennae shake	1/76 (1.3%)
	Body rock	32/76 (42.1%)
	Body turn	31/76 (40.8%)
	Courtship song	12/76 (15.8%)
Courtship song (68 transitions)	Body rock	1/68 (1.5%)
	Calling song	22/68 (32.4%)
	Contact female	21/68 (30.9%)
	Female mount	23/68 (35.4%)
	Follow	1/68 (1.5%)
Follow (2 transitions)	Contact female	2/2 (100%)
Wing raise (42 transitions)	Body rock	5/42 (11.9%)
	Calling song	16/42 (38.1%)
	Courtship sing	21/42 (50%)

Table 3.4 Behaviour transitions in A6-TAG males during courtship

Behaviour A	Behaviour B	Percentage
Antennae shake (185 transitions)	Antennae shake	143/185 (77.3%)
	Body rock	2/185 (1.1%)
	Contact female	16/185 (8.6%)
	Follow	3/185 (1.6%)
	Wing raise	21/185 (11.4%)
Body rock (84 transitions)	Antennae shake	16/84 (19%)
	Body rock	8/84 (9.5%)
	Cerci shake	3/84 (3.6%)
	Contact female	32/84 (38.1%)
	Follow	6/84 (7.1%)
	Wing raise	19/84 (22.6%)
Body turn (42 transitions)	Body rock	35/42 (83.3%)
	Wing raise	7/42 (16.7%)
Calling song (23 transitions)	Contact female	2/23 (8.7%)
	Courtship song	21/23 (91.3%)
Cerci shake (4 transitions)	Wing raise	4/4 (100%)
Contact female (122 transitions)	Antennae shake	4/122 (3.3%)
	Body rock	43/122 (35.2%)
	Body turn	44/122 (36.1%)
	Courtship song	31/122 (25.4%)
Courtship song (60 transitions)	Calling song	6/60 (10%)
	Contact female	37/60 (61.7%)
	Courtship song	17/60 (28.3%)
Follow (13 transitions)	Contact female	13/13 (100%)
Wing oscillation (2 transitions)	Contact female	1/2 (50%)
	Courtship song	1/2 (50%)
Wing raise (69 transitions)	Antennae shake	21/69 (30.4%)
	Body rock	4/69 (5.8%)
	Calling song	12/69 (17.4%)
	Cerci shake	1/69 (1.4%)
	Courtship song	6/69 (8.7%)
	Wing movements	2/69 (2.9%)
	Wing raise	23/69 (33.3%)

Table 3.5 Behaviour transitions in A5-A6 males during courtship

Behaviour A	Behaviour B	Percentage
Antennae shake (226 transitions)	Antennae shake	171/226 (75.7%)
	Body rock	4/226 (1.8%)
	Cerci shake	1/226 (0.4%)
	Contact female	19/226 (8.4%)
	Follow	3/226 (1.3%)
	Wing raise	28/226 (12.4)
Body rock (265 transitions)	Antennae shake	25/265 (9.4%)
	Body rock	143/265 (54%)
	Body turn	5/265 (1.9%)
	Cerci shake	4/265 (1.5%)
	Contact female	64/265 (24.2%)
	Follow	11/265 (4.2%)
	Wing raise	13/265 (4.9%)
Body turn (38 transitions)	Antennae shake	6/38 (15.8%)
	Body rock	27/38 (71.1%)
	Contact female	2/38 (5.3%)
	Wing raise	3/38 (7.9%)
Cerci shake (10 transitions)	Antennae shake	6/10 (60%)
	Body rock	2/10 (20%)
	Wing raise	2/10 (20%)
Contact female (124 transitions)	Antennae shake	3/124 (2.4%)
	Body rock	88/124 (71%)
	Body turn	33/124 (26.6%)
Follow (20 transitions)	Contact female	20/20 (100%)
Wing oscillation (8 transitions)	Calling song	3/8 (37.5%)
	Cerci shake	1/8 (12.5%)
	Courtship song	4/8 (50%)
Wing raise (60 transitions)	Antennae shake	28/60 (46.7%)
	Body rock	3/60 (5%)
	Cerci shake	3/60 (5%)
	Follow	1/60 (1.7%)
	Wing movements	10/60 (16.7%)
	Wing raise	15/60 (25%)

Table 3.6 Behaviour transitions in A4-A5 males during courtship

Behaviour A	Behaviour B	Percentage
Antennae shake (152 transitions)	Antennae shake	98/152 (64.5%)
	Body rock	11/152 (7.2%)
	Body turn	2/152 (1.3%)
	Cerci shake	9/152 (5.9%)
	Contact female	4/152 (2.6%)
	Follow	2/152 (1.3%)
	Wing raise	26/152 (17.1%)
Body rock (133 transitions)	Antennae shake	11/133 (8.3%)
	Body rock	42/133 (31.6%)
	Body turn	18/133 (13.5%)
	Cerci shake	4/133 (3%)
	Contact female	27/133 (20.3%)
	Follow	10/133 (7.5%)
	Wing raise	21/133 (15.8%)
Body turn (42 transitions)	Antennae shake	5/42 (11.9%)
	Body rock	23/42 (54.8%)
	Contact female	4/42 (9.5%)
	Wing raise	10/42 (23.8%)
Cerci shake (7 transitions)	Antennae shake	2/7 (28.6%)
	Body rock	5/7 (71.4%)
Contact female (76 transitions)	Antennae shake	3/76 (3.9%)
	Body rock	47/76 (61.8%)
	Body turn	26/76 (34.2%)
Follow (27 transitions)	Body rock	12/27 (44.4%)
	Contact female	15/27 (55.6%)
Wing oscillation (8 transitions)	Calling song	2/8 (25%)
	Cerci shake	5/8 (62.5%)
	Follow	1/8 (12.5%)
Wing raise (62 transitions)	Antennae shake	25/62 (40.3%)
	Body rock	7/62 (11.3%)
	Cerci shake	3/62 (4.8%)
	Follow	4/62 (6.5%)
	Wing movements	9/62 (14.5%)
	Wing raise	14/62 (22.6%)

Table 3.7 Behaviour transitions in A3-A4 males during courtship

Behaviour A	Behaviour B	Percentage
Antennae shake (74 transitions)	Antennae shake	42/74 (56.8%)
	Body rock	4/74 (5.4%)
	Cerci shake	1/74 (1.4%)
	Contact female	6/74 (8.1%)
	Follow	2/74 (2.7%)
	Wing raise	19/74 (25.7)
Body rock (83 transitions)	Antennae shake	11/83 (13.3%)
	Body rock	35/83 (42.2%)
	Contact female	25/83 (30.1)
	Wing raise	12/83 (14.5%)
Body turn (12 transitions)	Body rock	1/12 (8.3%)
	Wing raise	11/12 (91.7%)
Cerci shake (2 transitions)	Antennae shake	1/2 (50%)
	Wing raise	1/2 (50%)
Contact female (52 transitions)	Antennae shake	1/52 (1.9%)
	Body rock	29/52 (55.8%)
	Body turn	22/52 (42.3%)
Follow (2 transitions)	Contact female	1/2 (50%)
	Wing raise	1/2 (50%)
Wing raise (48 transitions)	Antennae shake	19/48 (39.6%)
	Body rock	3/48 (6.3%)
	Cerci shake	1/48 (2.1%)
	Follow	1/48 (2.1%)
	Wing movements	9/48 (18.8%)
	Wing raise	15/48 (31.3%)

Table 3.8 Behaviour transitions in T3-A3 males during courtship

Behaviour A	Behaviour B	Percentage
Antennae shake (45 transitions)	Antennae shake	39/45 (86.7%)
	Cerci shake	2/45 (4.4%)
	Follow	2/45 (4.4%)
	Wing raise	2/45 (4.4%)
Body rock (6 transitions)	Antennae shake	3/6 (50%)
	Wing raise	3/6 (50%)
Body turn (11 transitions)	Antennae shake	8/11 (72.7%)
	Body rock	2/11 (18.2%)
	Contact female	1/11 (9.1%)
Cerci shake (2 transitions)	Antennae shake	2/2 (100%)
Contact female (25 transitions)	Antennae shake	11/25 (44%)
	Body rock	2/25 (8%)
	Body turn	12/25 (48%)
Follow (2 transitions)	Contact female	2/2 (100%)
Wing raise (20 transitions)	Antennae shake	15/20 (75%)
	Wing raise	5/20 (25%)

Table 3.9 Behaviour transitions in intact males during rivalry behaviour

Behaviour A	Behaviour B	Percentage
Antennae fence (22 transitions)	Threat posture	22/22 (100%)
Bite (21 transitions)	Wing raise	21/21 (100%)
Body rock (52 transitions)	Bite	1/52 (1.9%)
	Body rock	11/52 (21.2%)
	Chase	13/52 (25%)
	Rivalry song	15/52 (28.8%)
	Wing raise	12/52 (23.1%)
Chase (40 transitions)	Bite	19/40 (47.5%)
	Body rock	13/40 (32.5%)
	Contact male	1/40 (2.5%)
	Rivalry song	4/40 (10%)
	Wing raise	3/40 (7.5%)
Contact male (34 transitions)	Antennae fence	22/34 (64.7%)
	Body rock	12/34 (35.3%)
Grapple (8 transitions)	Rivalry song	1/8 (12.5%)
	Wing raise	7/8 (87.5%)
Rivalry song (55 transitions)	Body rock	25/55 (45.5%)
	Chase	27/55 (49.1%)
	Grapple	3/55 (5.5%)
Threat posture (22 transitions)	Bite	5/22 (22.7%)
	Body rock	1/22 (4.5%)
	Grapple	4/22 (18.2%)
	Wing raise	12/22 (54.5%)
Wing raise (55 transitions)	Rivalry song	55/55 (100%)

Table 3.10 Behaviour transitions in A6-TAG males during rivalry behaviour

Behaviour A	Behaviour B	Percentage
Antennae fence (16 transitions)	Threat posture	16/16 (100%)
Bite (8 transitions)	Wing raise	8/8 (100%)
Body rock (52 transitions)	Bite	3/52 (5.8%)
	Body rock	13/52 (25%)
	Chase	11/52 (21.2%)
	Contact male	2/52 (3.8%)
	Rivalry song	6/52 (11.5%)
	Wing raise	17/52 (32.7%)
Chase (23 transitions)	Bite	2/23 (8.7%)
	Body rock	12/23 (52.2%)
	Rivalry song	4/23 (17.4%)
	Wing raise	5/23 (21.7%)
Contact male (28 transitions)	Antennae fence	16/28 (57.1%)
	Bite	1/28 (3.6%)
	Body rock	11/28 (39.3%)
Grapple (4 transitions)	Bite	1/4 (25%)
	Wing raise	3/4 (75%)
Rivalry song (43 transitions)	Body rock	21/43 (48.8%)
	Chase	15/43 (34.9%)
	Contact male	7/43 (16.3%)
Threat posture (16 transitions)	Bite	1/16 (6.3%)
	Body rock	2/16 (12.5%)
	Grapple	5/16 (31.3%)
	Wing raise	8/16 (50%)
Wing raise (33 transitions)	Rivalry song	33/33 (100%)

Table 3.11 Behaviour transitions in A5-A6 males during rivalry behaviour

Behaviour A	Behaviour B	Percentage
Antennae fence (15 transitions)	Threat posture	15/15 (100%)
Bite (16 transitions)	Body rock	9/16 (56.3%)
	Wing raise	7/16 (43.8%)
Body rock (94 transitions)	Bite	3/94 (3.2%)
	Body rock	33/94 (35.1%)
	Chase	22/94 (23.4%)
	Contact male	13/94 (13.8%)
	Rivalry song	5/94 (5.3%)
	Wing raise	18/94 (19.1%)
Chase (41 transitions)	Bite	11/41 (26.8%)
	Body rock	22/41 (53.7%)
	Wing raise	8/41 (19.5%)
Contact male (41 transitions)	Antennae fence	15/41 (36.6%)
	Bite	1/41 (2.4%)
	Body rock	25/41 (61%)
Grapple (1 transition)	Wing raise	1/1 (100%)
Rivalry song (29 transitions)	Body rock	11/29 (37.9%)
	Chase	14/29 (48.3%)
	Contact male	4/29 (13.8%)
Threat posture (15 transitions)	Bite	4/15 (26.7%)
	Body rock	10/15 (66.7%)
	Grapple	1/15 (6.7%)
Wing raise (26 transitions)	Body rock	1/26 (3.8%)
	Chase	1/26 (3.8%)
	Rivalry song	24/26 (82.3%)

Table 3.12 Behaviour transitions in A4-A5 males during rivalry behaviour

Behaviour A	Behaviour B	Percentage
Antennae fence (18 transitions)	Body rock	5/18 (27.8%)
	Threat posture	13/18 (72.2%)
Bite (6 transitions)	Wing raise	6/6 (100%)
Body rock (100 transitions)	Body rock	31/100 (31%)
	Chase	16/100 (16%)
	Contact male	18/100 (18%)
	Wing raise	35/100 (35%)
Chase (32 transitions)	Bite	5/32 (15.6%)
	Body rock	15/32 (46.9%)
	Wing raise	12/32 (37.5%)
Contact male (50 transitions)	Antennae fence	18/50 (36%)
	Body rock	32/50 (64%)
Grapple (2 transitions)	Wing raise	2/2 (100%)
Rivalry song (41 transitions)	Body rock	14/41 (34.1%)
	Chase	16/41 (39%)
	Contact male	11/41 (26.8%)
Threat posture (15 transitions)	Bite	1/15 (6.7%)
	Body rock	8/15 (53.3%)
	Grapple	2/15 (13.3%)
	Wing raise	4/15 (26.7%)
Wing raise (46 transitions)	Body rock	5/46 (10.9%)
	Rivalry song	41/46 (89.1%)

Table 3.13 Behaviour transitions in A3-A4 males during rivalry behaviour

Behaviour A	Behaviour B	Percentage
Antennae fence (14 transitions)	Threat posture	14/14 (100%)
Bite (21 transitions)	Body rock	6/21 (28.6%)
	Chase	14/21 (66.7%)
	Wing raise	1/21 (4.8%)
Body rock (70 transitions)	Bite	2/70 (2.9%)
	Body rock	23/70 (32.9%)
	Chase	12/70 (17.1%)
	Contact male	1/70 (1.4%)
	Wing raise	32/70 (45.7%)
Chase (39 transitions)	Bite	14/39 (35.9%)
	Body rock	22/39 (56.4%)
	Contact male	1/39 (2.6%)
	Wing raise	2/39 (5.1%)
Contact male (26 transitions)	Antennae fence	14/26 (53.8%)
	Body rock	11/26 (42.3%)
	Chase	1/26 (3.8%)
Grapple (3 transitions)	Bite	2/3 (66.7%)
	Wing raise	1/3 (33.3%)
Threat posture (14 transitions)	Bite	5/14 (35.7%)
	Body rock	4/14 (28.6%)
	Grapple	5/14 (35.7%)
Wing raise (26 transitions)	Body rock	11/26 (42.3%)
	Chase	15/26 (57.7%)

Table 3.14 Behaviour transitions in T3-A3 males during rivalry behaviour

Behaviour A	Behaviour B	Percentage
Antennae fence (18 transitions)	Threat posture	18/18 (100%)
Bite (10 transitions)	Body rock	5/10 (50%)
	Chase	2/10 (20%)
	Grapple	1/10 (10%)
	Wing raise	2/10 (20%)
Body rock (33 transitions)	Bite	1/33 (3%)
	Body rock	3/33 (9.1%)
	Chase	4/33 (12.1%)
	Contact male	12/33 (36.4%)
	Grapple	1/33 (3%)
	Wing raise	12/33 (36.4%)
Chase (8 transitions)	Bite	3/8 (37.5%)
	Body rock	4/8 (50%)
	Contact male	1/8 (12.5%)
Contact male (26 transitions)	Antennae fence	18/26 (69.2%)
	Body rock	6/26 (23.1%)
	Chase	2/26 (7.7%)
Grapple (4 transitions)	Bite	1/4 (25%)
	Body rock	3/4 (75%)
Threat posture (18 transitions)	Bite	11/18 (61.1%)
	Body rock	3/18 (16.7%)
	Grapple	4/18 (22.2%)
Wing raise (14 transitions)	Body rock	9/14 (64.3%)
	Chase	5/14 (35.7%)

Table 3.15 Effects of lesions on calling, rivalry, and courtship song in *G. bimaculatus*

	Calling song	**Rivalry song**	**Courtship song**
T3-A3	No calling song	No rivalry song	No courtship song
A3-A4	No calling song	No rivalry song	No courtship song
A4-A5	Calling song with more sound pulses in chirps	Rivalry song	No courtship song
A5-A6	Calling song	Rivalry song	One animal produced courtship song
A6-TAG	Calling song	Rivalry song	Courtship song

4   Chapter Four: Lesions of abdominal connectives and their impact on the calling song pattern in different cricket species

Abstract

While crickets use movements of their front wings for sound production, the abdominal ganglia house the network of the singing central pattern generator. We compared the effects on calling song activity of specific lesions to the connectives of the abdominal ganglion chain in four different species of crickets, generating very different pulse patterns in their calling songs. In all species singing activity was abolished after the connectives between the metathoracic ganglion complex and the first abdominal ganglion A3 were severed. The song structure was lost and males generated only single sound pulses when connectives between A3 and A4 were cut. Severing connectives between A4 and A5 had no effect in the trilling species, it led to an extension of chirps in a chirping species and to a loss of the phrase structure in two *Teleogryllus* species. Cutting the connectives between A5 and A6 caused no or minor changes in singing activity. In spite the species-specific pulse patterns of calling songs, our data indicate a conserved lay-out of the calling song motor pattern generating network with the generation of pulses controlled by ganglia A3 and A4 while A4 and A5 provide the timing information for the chirp and/or phrase structure of the song.

4.1 Introduction

Rhythmic movements can be observed in behaviours such as breathing, swallowing, sucking, and in locomotion like swimming, crawling, flying, walking in a variety of animal models from invertebrates to vertebrates (Dick et al. 1993; Ijspeert and Kodjabachian 1999; Miller and Sigvardt 2000; Barlow and Estep 2006; Pirtle and Satterlie 2007; Bicanski et al. 2013; Katz 2016; Olivares et al. 2017). These movements are controlled by neural circuits called central pattern generators (CPGs) that once activated, produce the neural activity driving the underlying rhythmic motor pattern (Katz 2016). These neural circuits are in the focus of neuroscience (Marder and Bucher 2001), and the general principles and functions of these circuits have been unraveled (Bucher et al. 2015). In insects, CPGs were studied for different behaviour, such as flying behaviour in locust and moth (Wilson 1961; Buhl et al. 2008; Vierk et al. 2009), locomotion of stick insect (Borgmann et al. 2009; Harischandra et al. 2015; Mantziaris et al. 2017; Goldammer et al. 2018) and cockroach (Kukillaya et al. 2009; Fuchs et al. 2011), oviposition in locust and grasshopper (Facciponte and Lange 1992; Silva and Lange 2011; Wong and Lange 2014; Thompson 2018), feeding in the fruit fly (Hückesfeld et al. 2015), and leg pattern generators and ventilatory control in locust (Armstrong et al. 2006; Knebel et al. 2019). Central questions in these studies are: Where are the CPG networks located within the CNS and how are they organized at a systems and cellular level?

The species-specific singing behaviour of different crickets serves as an interesting model for studying CPGs. In order to attract females, male crickets sing a calling song by opening and closing forewings in a rhythmic manner with specialised structures on the forewings, plectrum and file, sweeping against each other (Ewing and Hoyle 1965; Elliott and Koch 1985; Koch et al. 1988). Each closing movement of the elevated wings generates a sound pulse, and several pulses may be grouped in trills or chirps. The muscle activity underlying the rhythmic

wing movements was easily accessed with electromyographic recordings and was studied extensively (Ewing and Hoyle 1965; Bentley and Kutsch 1966; Kutsch 1969; Pfau and Koch 1994). Species-specific carrier frequency, song structure, and temporal parameters of the calling song result from the precise control of the wing muscles and allow prezygotic isolation in closely related cricket species (Shaw 1996; Gray and Cade 2000; Mendelson and Shaw 2002; Honda-Sumi 2005; Bailey et al. 2017). Calling song patterns in crickets likely evolved from simple trilling patterns to rhythmic chirp patterns and more complex compound songs, with at least 2-3 rhythms involved in shaping the song pattern (Otte 1992). Therefore, studying the CPG driving the neural activity underlying calling song generation will improve the understanding of motor control mechanism and also provide clues on cricket species evolution.

It was assumed that the CPG for cricket calling song is located in the mesothoracic ganglion, where the motor neurons innervating the wing muscles are housed (Huber 1960, 1963; Elepfandt 1980). However, accumulating experimental evidences subsequently pointed to the involvement of the abdominal nervous system in shaping the calling song structure. The mesothoracic ganglion in *Gryllus bimaculatus* can be split along its midline, without altering the calling song pattern (Hennig and Otto 1996). In G*ryllus campestris*, male crickets failed to sing once their cervical connectives *and* the nerve cord posterior to the thoracic ganglia was cut (Kutsch and Otto 1972). In *Gryllus firmus*, recording of songs under differential heating conditions of the body suggested the involvement of abdominal ganglia in the control of singing motor activity (Pires and Hoy 1992). These results triggered a new set of experiments analyzing the organization of the singing network with acute lesion experiments (Schöneich and Hedwig 2011) and combining lesions with a quantitative approach using continuous sound recordings of crickets' calling songs (Jacob and Hedwig 2016). In *Gryllus bimaculatus*, a comprehensive study by systematic lesions of the abdominal nerve cord implicated a different role of each abdominal ganglion for the generation of pulses and chirps defining the calling

song structure (Jacob and Hedwig 2016). These findings showcase the importance of the abdominal ganglia in cricket song pattern generation and have been supported by intracellular studies of singing interneurons recorded and identified in the abdominal ganglia (Schöneich and Hedwig 2011, 2012; Jacob and Hedwig 2019, 2020).

I aim to evaluate the possible contribution of each abdominal ganglion to the calling song structure and temporal organization of the pulse pattern in different species, and to compare the similarities and differences among cricket species. In the current study, systematic lesions were applied to the abdominal nerve cord of four cricket species with different calling song types: *Gryllus rubens* as trill-producing species, *Gryllus assimilis* as chirp-producing species, and *Teleogryllus oceanicus* and *Teleogryllus commodus* as phrase-producing species. The change of the calling song after each lesion was compared to the normal singing behaviour and provides evidence on the organization and evolution of the cricket song pattern generating system.

4.2   Material and methods

4.2.1   Experimental animals

Colonies of four cricket species (*G. rubens, G. assimilis, T. oceanicus* and *T. commodus*) were bred and raised in the Department of Zoology, University of Cambridge. Crickets were kept in large boxes (52.5 x 36.5 x 28 cm) and at the last instar male nymphs were separated and kept solitary in 17.5 x 11.5 x 13 cm boxes to monitor their development and age until sexual maturity. Fish food, muesli, potatoes and water were provided on a daily basis. Crickets were housed at 26-28°C with a 12hr-12hr light:dark cycle. Adults seven to fourteen days old after imaginal eclosion were selected for sound recording and lesion experiments. All animal treatments and experiments complied with the principles of Laboratory Animal Care (ASAB

Ethics Committee and ABS Animal Care Committee 2021).

4.2.2 *Selective lesions of the abdominal CNS*

In preparation to the experiments male crickets were placed in a 4°C fridge for 15 minutes to reduce activity. Immobilized animals were mounted ventral side up on a plasticine block with the body and legs fixed by metal hooks made out of staples. To expose the connectives between specific abdominal ganglia, the appropriate sterna and intersegmental membranes were incised and the wound was temporarily held open by a forceps. Fat body surrounding the target ganglia and connectives was carefully removed to avoid damaging tracheae and tracheoles. Exposed tissue was covered in insect saline (in mmoll^{-1}: NaCl 140; KCl 10; CaCl$_2$ 7; NaHCO$_3$ 8; MgCl$_2$ 1; *N*-trimethyl-2-aminoethanesulfonic acid 5; *D*-trehalose dehydrate 4, pH 7.4) at all times. All lesions were applied with micro scissors to the bilateral connectives between target abdominal ganglia. After lesioning the connectives, the ventral cuticle was folded back, the wound sealed by drying hemolymph, and the insect recovered within one to few days. Following several days of sound recording under the same condition as before the lesion, the crickets were sacrificed, and the nervous system was dissected and inspected to confirm the site of the applied lesion. In rare cases that the severed connectives were reconnected by connective tissue, data were treated as invalid and discarded. In crickets the first 2 abdominal neuromeres are fused with the metathoracic ganglion T3. Lesions were applied to the connectives between the metathoracic ganglion complex T3 and the first free abdominal ganglion A3 (T3-A3) and between the posterior abdominal ganglia, i.e. the A3-A4, the A4-A5 and the A5-A6 connectives (**Figure 4.1B**). The connectives between A6 and the terminal ganglion (TAG) were not severed as such lesions do not alter the calling song pattern (Jacob and Hedwig 2016).

4.2.3 Sound recording

Due to the nocturnal behaviour of crickets and to reduce noise from the surrounding environment, most recordings were retrieved at night for continuous eight hours. Singing activity of male crickets was recorded one day before applying a lesion, and one to seven days after lesion to study acute effect (less than a week) of the lesion on calling song structure, to avoid effect of possible regrowth of the nervous system. A standard PC microphone (Omni type; Maplin Electronics, Rotherham, UK) was placed in the box of the cricket and sound was recorded with Cooledit 2000 software (Syntrillium Software Corporation, Phoenix, AZ, USA) running under Windows 7, at a sampling rate of 22 kHz, at 22-24°C.

4.2.4 Data analysis and Statistics

For each lesion experiment the songs of at least five animals were recorded and for each animal recordings of at least one-minute duration before and after the lesion were analysed in terms of song structure and temporal parameters. Analysis of song pattern was carried out with NEUROLAB software (Hedwig and Knepper 1992; Knepper and Hedwig 1997). For songs the duration, interval and period of pulses, chirps, trills and phrases (**Figure 4.1A**) were determined by labeling the start and the end of each pulse, chirp, trill and phrase, respectively. Sound pulses were detected by a threshold algorithm after full-wave rectifying the sound signal. Latency histogram and interval histogram functions were used to calculate the average and standard deviation (X±SD) for each parameter. The PST-histogram function was applied to calculate the pulse number in chirps or trills, or trill number in phrases.

To define and selectively detect each pulse, I set minimum pulse duration for each species (*G. assimilis*: 7 ms; *G. rubens*: 20 ms; *T. oceanicus* and *T. commodus*: 30 ms) and manually checked whether the start of each pulse was correctly marked. For the description of the calling

song structure in *Teleogryllus* species, previous studies have used the terminology of "long chirps" and "short chirps" for the two components of the song of *T. oceanicus, T.emma,* and *T. taiwanemma* (Honda-Sumi 2005; Zuk et al. 2008), while other studies used "chirp" and "trill" in describing the songs of *T. oceanicus* and *T. commodus* (Simmons et al. 2005; Bailey et al. 2017). In the current study, the terms "chirp" and "trill" were used to describe the two components in *T. oceanicus* and *T. commodus* for consistency and also due to the fact that the trills in *T. oceanicus* and *T. commodus* share a similar genetic control (Bentley and Hoy 1972; Hoy and Paul 1973; Hoy 1974).

A definition of terms used in this study is given in **Figure 4.1A** and is as follows:

(1) Pulse: sound pulse as basic unit of cricket songs corresponding to one closing movement of the forewings;

(2) Chirp: a short sequence with a fixed number of pulses, like a complete song unit in *G. assimilis*, the leading pulse sequence in a song unit in *T. oceanicus* and *T. commodus*;

(3) Trill: a sequence of pulses with variable pulse number, i.e. a song unit in *G. rubens*, or a sequence of pulses with a fixed pulse number, but repeated multiple times after chirps in *T. oceanicus* and *T. commodus*;

(4) Phrase: a song unit in *Teleogryllus* species consisting of one chirp and several trills;

(5) Duration: the time a pulse, chirp, or trill lasts;

(6) Interval: the time in between pulses, chirps, or trills;

(7) Period: the time from the start of a pulse, chirp, trill or phrase to the next start of the unit.

For a qualitative analysis of song patterns, 10 min (5 min for *G. rubens*) of recording was selected to create raster plots and cross correlograms. Events of sound pulses were plotted as raster plots in a way that the first pulse of each chirp, trill, phrase, or sequence was used as a reference and aligned to time zero of the plot. The timing of pulses before and after the reference pulse was plotted over a selected time window along the x-axis. In this way the timing

of pulses of the reference song unit (chirp, trill, phrase, or sequence) and also of pulses of the preceding and subsequent song units (chirp, trill, phrase, or sequence) were displayed. The y-axis position of the plot was marginally shifted to a higher value for each new reference event.

In each species, I used a different time window to provide a proper resolution of the timing of sound pulses and used for *G. rubens*: -5000 to 5000 ms; *G. assimilis*: -50 to 250 ms, -2000 to 2000 ms, and -3000 to 3000 ms; *T. oceanicus*: -500 to 1000 ms; *T. commodus*: -500 to 2500 ms. To demonstrate the frequency of events, cross-correlograms corresponding to the time window of each raster plot were calculated, using again the first pulse of the chirps, trills, phrases or sequences as reference time. The black (control) or light grey (lesion group) marked areas in the cross-correlograms indicate the pulse events of the reference chirps, trills, phrases or sequences, respectively, while dark grey shaded areas indicate events from previous or subsequent chirps, trills, phrases or sequences. This procedure is not required in *G. assimilis.*

Statistical significance of differences in song parameters before and after applying lesions was tested by paired sample T-Test applying a two-tailed hypothesis when comparing two groups or one-way analysis of variance (ANOVA) when comparing three groups. Average timing of song parameters in each individual was calculated by NEUROLAB and was exported to SigmaPlot 11.0 (Systat Software, San Jose, CA) for statistical analysis and for drawing bar graphs.

4.3   Results

4.3.1   Normal calling song pattern of the four cricket species

All males were recorded before a lesion to the abdominal connectives was applied. In the following an overview of the normal song patterns is given. The calling song of *G. rubens* consist of long trills (Gray and Cade 2000) (**Figure 4.1A**). A total of 284 trills with 24415

pulses in 13 males were analysed. The trill duration (2170.4 ± 647.4ms) and trill interval (1361.6 ± 2110.8ms) are very variable and so is the pulse number within trills (91.2 ± 27.1). On average pulses in trills have a duration of 13.9 ± 4.3ms, an interval of 10.5 ± 6.1ms, and a period of 23.9 ± 2.6ms.

The calling song of *G. assimilis* contains chirps repeated at rather long intervals (Pollack and Kim 2013) (**Figure 4.1A**). Analysis of 4742 chirps with a total of 34158 pulses in 11 males shows that one chirp contains six to nine sound pulses (7.4 ± 0.5 pulses). In this species some chirps showed clear pulse interval (see **Figure 4.6A**) while other chirps showed no or only a very short pulse interval (**Figure 4.1A** and **4.5A**). I therefore report only the pulse period (14.0 ± 2.8ms). The chirps of *G. assimilis* have an average duration of 93.5 ± 16.3ms, while the chirp intervals are long and come with high variability (1447.6 ± 772.4ms).

The calling song of *T. oceanicus* contains phrases composed of one chirp and several trills (Zuk et al. 2008; Bailey et al. 2017) (**Figure 4.1A**). In 10 males, 401 phrases containing 401 chirps with 1855 chirp-pulses, and 3008 trills with 6597 trill-pulses were recorded and analysed. The phrase period is 1606.8 ± 395.2 ms, chirp duration is 283.4 ± 62.8 ms, trill duration is 80.3 ± 23.3 ms, trill interval is 74.5 ± 15.5 ms, and trill period is 153.3 ± 17.3 ms. Each phrase consists of 8.1 ± 2.4 trills on average. In chirps, the pulse duration is 35.3 ± 7.4 ms and similar in length to the pulse interval (31.2 ± 10.8 ms), while in trills the pulse duration is 28.8 ± 7.1 ms and is considerably longer than the pulse interval (13.0 ± 7.4ms). The difference in chirps and trills is indicated by the pulse period which is 66.5 ± 5.3 ms in chirps and 41.6 ± 5.8 ms in trills, therefore in the oscillogram chirp-pulses appear to be more loosely packed than trill-pulses. On average chirps contain 4.7 ± 0.6 pulses and trills contain two or three pulses (2.2 ± 0.4 pulses), but occasionally also more pulses can occur.
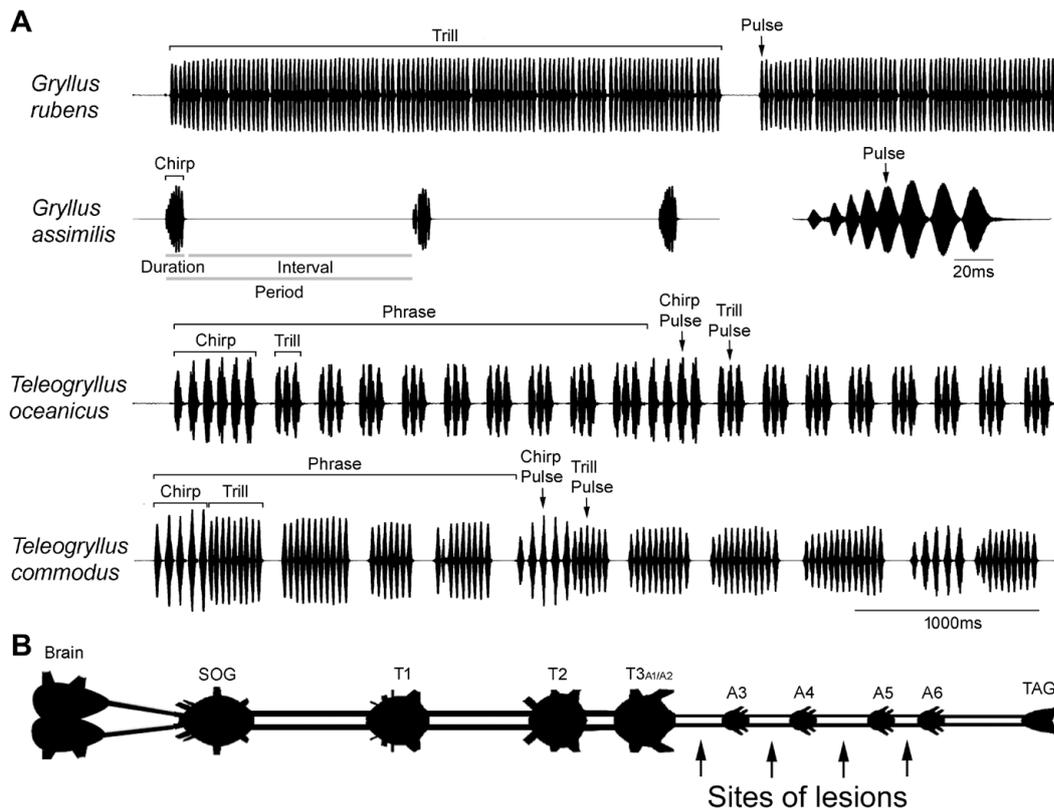
Figure 4.1 Calling song recordings of four cricket species and diagram of cricket central nervous system
(A) Calling songs of *G. rubens, G. assimilis, T. oceanicus* and *T. commodus* with terminology used for song structure (pulse, chirp, trill, and phrase) and temporal parameters (duration, interval, and period). (B) Schematic representation of a cricket central nervous system and sites of applied lesions, the first 2 abdominal neuromeres are fused with the metathoracic ganglion T3 (modified from Jacob and Hedwig 2016).

In *T. commodus* the calling song is composed of phrases with one chirp followed by a few trills (Simmons et al. 2005; Bailey et al. 2017) (**Figure 4.1A**). A total of 1234 phrases containing 1234 chirps with 6929 chirp-pulses and 2606 trills with a total of 31087 trill-pulses in 10 males were analysed. The phrase period is 1822.8 ± 509.6 ms, chirp duration is 337.7 ± 62.6 ms, trill duration is 553.6 ± 431.6 ms, trill interval is 236.4 ± 130.7 ms, and trill period is 824.6 ± 539.1 ms. Each phrase includes one chirp and 2.2 ± 1.1 trills. Similar to *T. oceanicus*, chirps in *T. commodus* have a pulse duration of 31.5 ± 3.4 ms and a pulse interval of 30.6 ± 4.6 ms; whereas trills have a longer pulse duration (25.4 ± 3.8 ms) than the pulse interval (13.0

± 2.7 ms). This is reflected in the difference of the chirp pulse period (61.9 ± 5.5 ms) and the trill pulse period (38.4 ± 3.9 ms), and in the dense pattern of trill-pulses in the oscillogram. Each chirp contains 5.5 ± 1.0 pulses and each trill contains 14.7 ± 10.2 pulses on average.

4.3.2 General effects after lesions

A total of 164 male crickets (42 *G. assimilis*, 30 *G. rubens*, 51 *T. oceanicus*, and 41 *T. commodus*) were used in this study. Seven males died within three days after applying a lesion (5 *G. assimilis* and 2 *T. commodus*). In these males, severe reduction of mobility and a constantly low position of the antennae was observed. 32 males (8 *G. assimilis*, 3 *G. rubens*, 13 *T. oceanicus*, and 8 *T. commodus*) never sang again after a lesion, even some of them lived for another three weeks. After a lesion to the abdominal nerve cord males did no longer respond to direct cercal contact of a pen brush with escape behaviour and failed to copulate. Many of the males showed an accumulation of feces at their anus. The rest of the cohort of crickets did not show any obvious defects in walking and ingestion, and their post-lesion calling songs were recorded successfully. Life span after lesion ranged from a week to two months (68 days the longest). In all four species the impact of the T3-A3 lesion and of the A3-A4 lesions was in each case very similar, these data will be presented together. Lesions of A4-A5 and A5-A6 had more specific effects and will be presented separately for each species.

4.3.3 Loss of calling song activity after T3-A3 lesion

Crickets with their connective cut between the metathoracic ganglion complex T3 and first free abdominal ganglion A3 stopped singing. Without exceptions, the sound recordings did not demonstrate any sign of singing activity. All the males of the four species (5 *G. rubens*, 6 *G. assimilis*, 8 *T. oceanicus*, and 5 *T. commodus*) no longer produced identifiable sound pulses

once the T3-A3 connectives were severed (**Figure 4.2A**). Males still raised their forewings into singing position, but no coordinated opening-closing movements of the forewings ever occurred while watching the animals for extended periods of time. Most T3-A3cut males lived for another one to two weeks after the lesion. These males showed no deficiency in locomotor activity before they died.
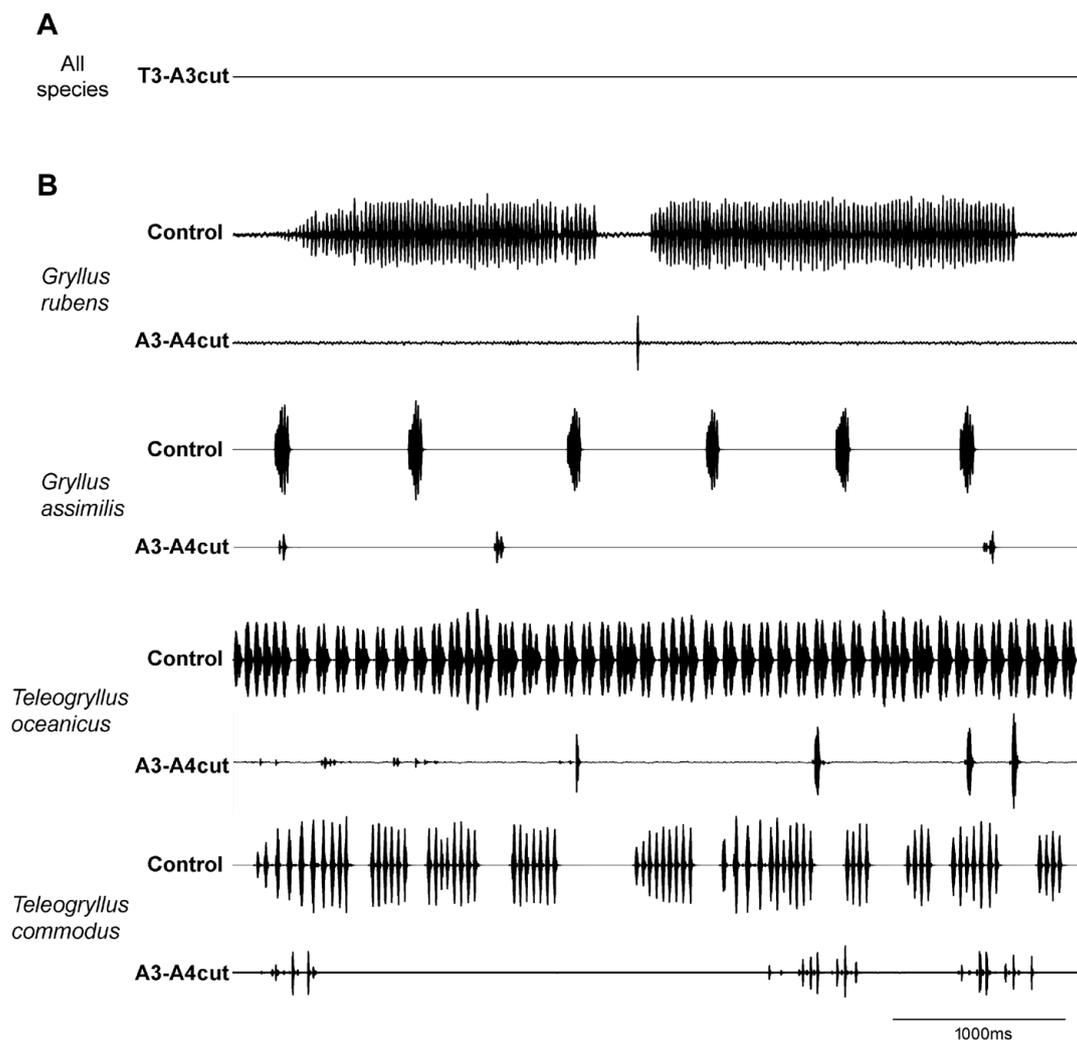
Figure 4.2 Representative sound recording of *G. rubens, G. assimilis, T. oceanicus* and *T. commodus* for males with an T3-A3 or A3-A4 lesion.

(A) Recording indicates failure of calling song production in the four cricket species after T3-A3 lesion. (B) Calling song recordings before and after A3-A4 lesion in *G. rubens, G. assimilis, T. oceanicus* and *T. commodus*.

4.3.4 Generation of single pulses and loss of song structure after A3-A4 lesion

All four cricket species showed similar effects on the calling song structure after the connectives between A3 and A4 were sectioned (A3-A4cut, 7 *G. rubens*, 6 *G. assimilis*, 9 *T. oceanicus*, and 6 *T. commodus*). Overall, the A3-A4 cut animals strongly reduced their singing activity and generated less than 100 sound pulses in an overnight recording (*G. rubens*: 37.1 ± 36.2 pulses n=7, *G. assimilis*: 58.7 ± 19.7 pulses n=6, *T. oceanicus*: 83.9 ± 113.7 pulses n=9, *T. commodus*: 26.7 ± 21.7 pulses n=6). These males either produced scratchy sound or single pulses only without a higher song structure (**Figure 4.2B**). These pulses, due to quivering or lack of proper coordination of the wing movements, were usually incomplete and clearly lower in sound amplitude compared to normal pulses. One *G. assimilis* male produced only single pulses for the first week, after which it began to sing normal calling songs. Dissection of this animal revealed a tissue connection of the separated connectives, either due to an incomplete lesion or a possible regrowth.

4.3.5 No change in calling song pattern after A4-A5 lesion in G. rubens

Male *G. rubens* exhibited normal singing activity after an A4-A5 or A5-A6 lesion was applied. They produced normal trills after the connectives between A4 and A5 were incised (A4-A5cut, **Figure 4.3**). Calling songs of 8 males before (155 trills, 14349 pulses) and after an A4-A5 lesion (177 trills, 15625 pulses) were recorded and analysed. As trills produced by intact males were already variable in terms of trill parameters, there was no significant difference between the control and operated group in trill duration (control: 2260.2 ± 696.2 ms, A4-A5 cut: 3104.6 ± 2157.2 ms, P=0.310), trill interval (control: 1814.0 ± 2647.5 ms, A4-A5 cut: 1544.8 ± 1292.0 ms, P=0.800), trill period (control: 4147.3 ± 2746.0 ms, A4-A5 cut: 4643.9 ± 2553.7, P=0.714), and pulse number in trills (control: 94.2 ± 29.6 pulses/trill, A4-A5 cut: 131.1

± 93.3 pulses/trill, P=0.304) (**Figure 4.3C**). Although in one male some trills showed substantially more pulses after the A4-A5cut, this was not observed in other males and overall this did not lead to a significant difference in the mean pulse number of trills (**Figure 4.3C**, Pulse Number/Trill, dot graph).

Pulse parameters in A4-A5cut males also showed no significant difference compared to intact males (**Figure 4.3C**). Pulse duration was 14.3 ± 4.8 ms in the control group and 11.7 ± 2.7 ms in the A4-A5cut group (P=0.206). Pulse interval was 9.9 ± 6.69 ms in the control group and 11.9 ± 2.7 ms in the A4-A5cut group (P=0.439). Pulse period was similar between control (24.2 ± 3.3 ms) and A4-A5cut animals (23.8 ± 2.4 ms, P=0.780). In summary, there was no obvious change after the A4-A5 lesion.

Cross-correlogram and raster plot were drawn based on a 5-minute recording of one male (**Figure 4.3B**). Black (control) and light grey (A4-A5cut) shade in correlograms denotes the corresponding pulses of the reference trills, respectively. Dark grey shade denotes the pulses of previous or subsequent trills. In both groups, pulse number, trill duration, and trill interval were variable. Because of the variable pulse number and trill duration, the accumulated pulses of reference-trills gradually decline with increasing time (black and light grey shade in correlogram), while the pulses in the subsequent trill gradually increases (dark grey shade). Overall, calling song of *G. rubens* contains trills with variable temporal parameters and no significant change occurred after the A4-A5 lesion was applied.
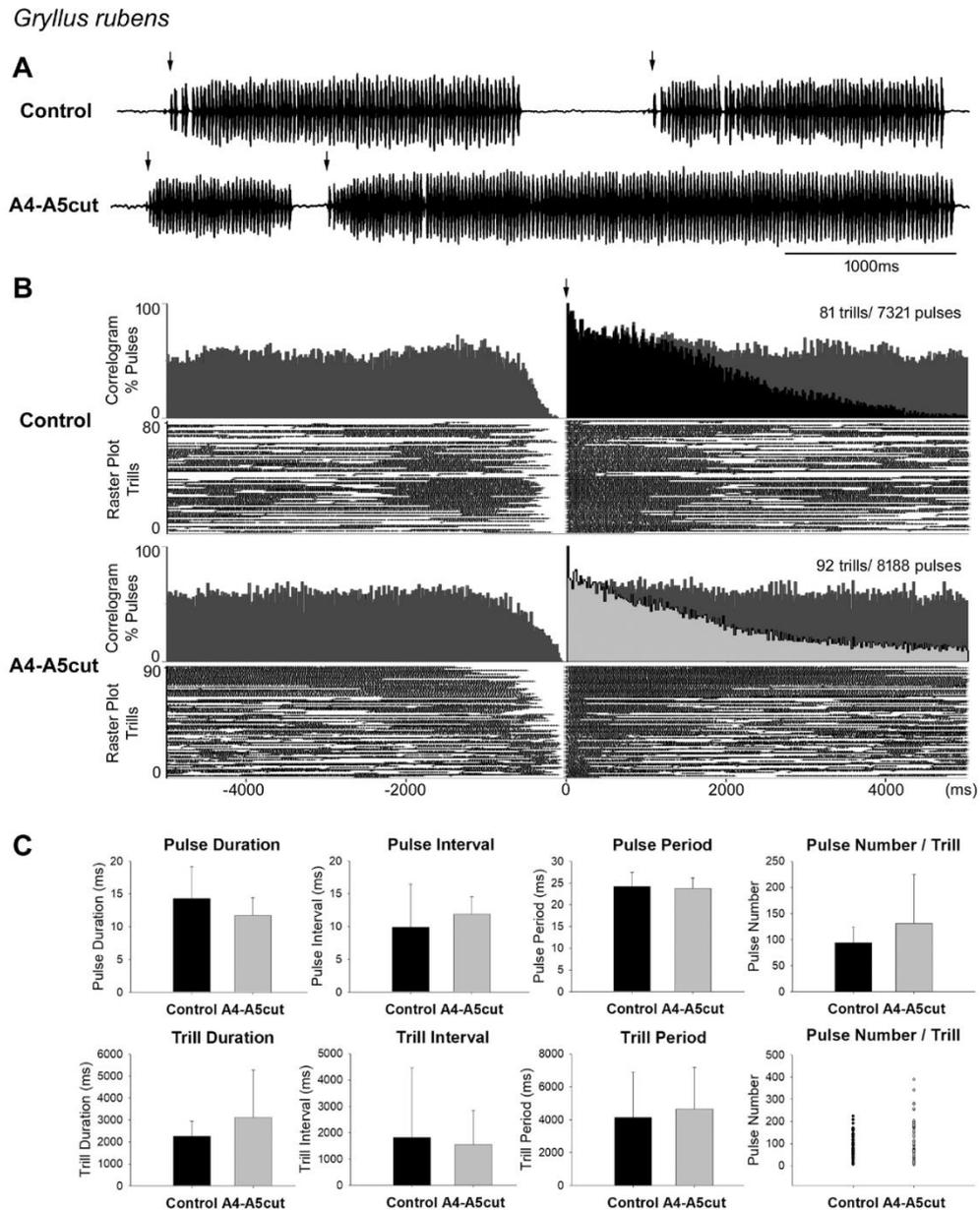
Figure 4.3 Calling song before and after the A4-A5 lesion in *G. rubens*.
(A) Song recording of *G. rubens* before (top) and after the lesion (bottom). Arrows indicate the start of each trill. (B) Cross-correlogram and raster plot of 5-minute song recording of one male before and after the lesion on a time scale from -5000 ms to 5000 ms. Black (control) and light grey (A4-A5cut) shading represent the pulses of the reference-trills in correlograms and dark grey shading indicates the pulses of the subsequent (to the right) or previous (to the left) trill. Arrow indicates the start of trills. (C) Temporal parameters of calling song before and after the lesion in 8 males.

4.3.6 *No change in calling song pattern after A5-A6 lesion in G. rubens*

Male *G. rubens* generated normal trills after the connective between A5 and A6 were severed (A5-A6cut, **Figure 4.4**). Like intact or A4-A5 lesioned animals, A5-A6cut animals showed a substantial variation in the trill parameters. In 5 males, 136 trills (9787 pulses) under intact condition and 107 trills (8790 pulses) after the A5-A6 cut were analysed. There was no significant difference between the control and the A5-A6 group in terms of trill duration (control: 2026.8 ± 605.9 ms, A5-A6 cut: 1723.7 ± 553.4 ms, P=0.433), trill interval (control: 637.8 ± 187.2 ms, A5-A6 cut: 488.3 ± 210.2 ms, P=0.269), trill period (control: 2622.4 ± 620.7 ms, A5-A6 cut: 2203.9 ± 510.3 ms, P=0.278), and pulse number in trills (control: 86.6 ± 24.9 pulses/trill, A5-A6 cut: 76.6 ± 26.6 pulses/trill, P=0.558) (**Figure 4.4C**). Even few trills showed a higher number of pulses/trill in the A5-A6cut animals, the overall pulse number was not significantly different from intact animals. (**Figure 4.4C,** Pulse Number/Trill dot graph).

Also regarding pulse parameters, there was no significant difference in pulse duration (control: 13.1 ± 3.6 ms, A5-A6 cut: 11.4 ± 4.4 ms, P=0.518), pulse interval (control: 10.2 ± 3.1 ms, A5-A6 cut: 11.4 ± 5.9 ms, P=0.685), or pulse period (control: 23.4 ± 0.8ms, A5-A6 cut: 22.8 ± 2.5ms, P=0.626) between intact males and A5-A6cut males (**Figure 4.4C**).

For a 5-minute calling song recording of one male a cross-correlogram and raster plot were generated (**Figure 4.4B**). The raster plot shows that pulse number, trill duration, and trill interval were all variable in the control and A5-A6 operated group. The variable pulse number and trill duration are reflected in the correlogram as the gradually decreasing pulse numbers of the trills over time (black and light grey shade in correlogram). Sound pulses of subsequent and preceding trills are indicated by dark grey shading, they reveal the trill interval before the reference-trills. In summary, calling songs of *G. rubens* showed no significant change in song parameters before and after A5-A6 lesion, as trills with a variable pulse number, duration, and

interval were generated by the males. This is in line with the A4-A5 lesions, which did not cause significant changes.
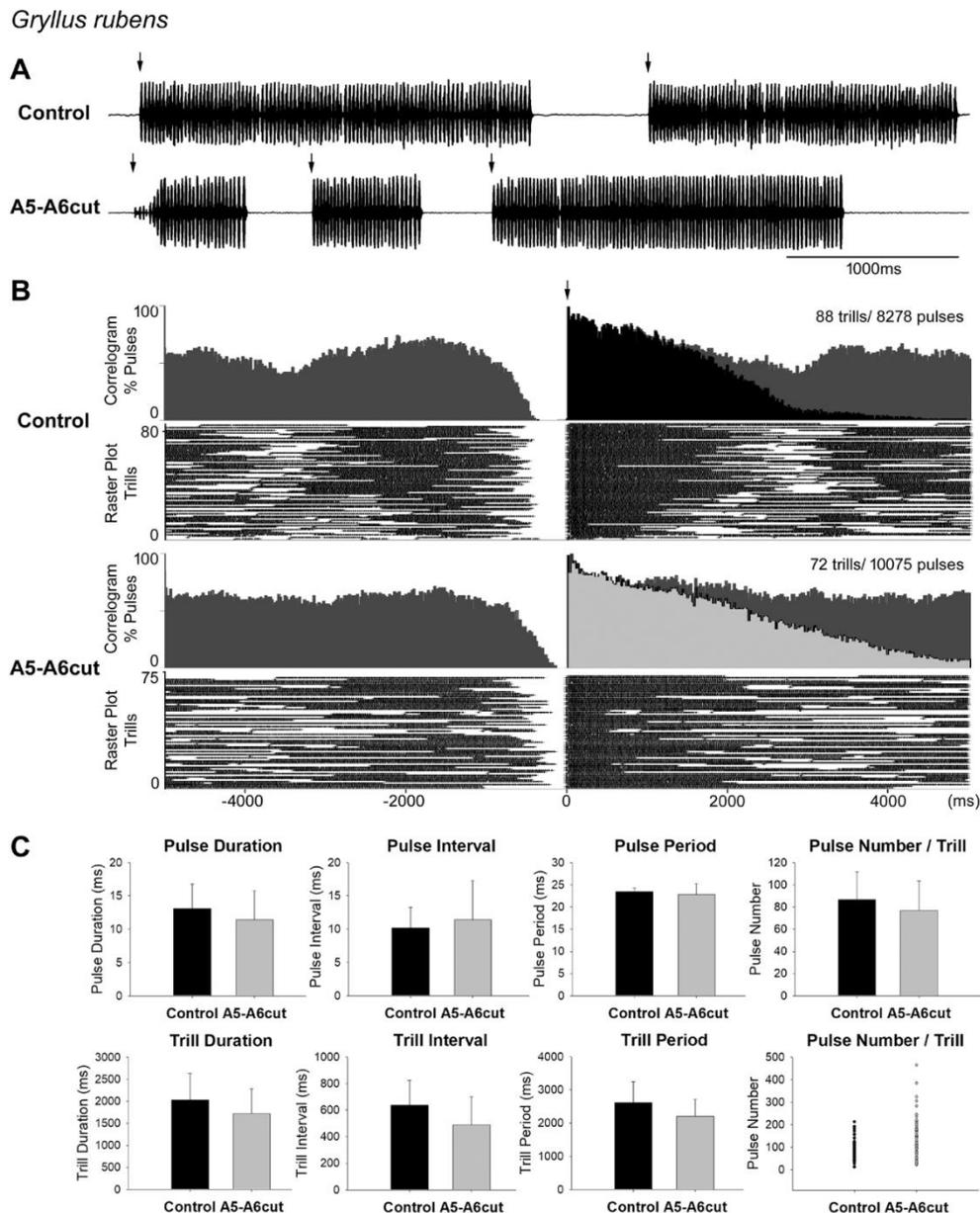
Figure 4.4 Calling song before and after the A5-A6 lesion in *G. rubens*.
(A) Song recording of *G. rubens* before (top) and after the lesion (bottom). Arrows indicate the start of each trill. (B) Cross-correlogram and raster plot of 5-minute song recording of one male before and after the lesion on a time scale from -5000 ms to 5000 ms. Black (control) and light grey (A5-A6cut) shade represent the pulses of the reference-trills in the correlograms and dark grey shading indicates the pulses of the subsequent (to the right) or previous (to the left) trill. Arrow indicates the start of trills. (C) Temporal parameters of calling song before and after lesion in 5 males.

4.3.7   Extended chirps after A4-A5 lesion in G. assimilis

G. assimilis males showed continued singing activity after an A4-A5 or A5-A6 lesion was applied. They produced calling songs with extended chirps after the connectives between A4 and A5 were incised (A4-A5cut, **Figure 4.5**). Calling songs of 6 males before (2993 chirps, 21644 pulses) and after the lesion (4799 chirps, 43703 pulses) were analysed. Chirps after the A4-A5 lesion had significant more pulses (9.6 ± 1.4 pulses/chirp, P=0.005) and showed a longer chirp duration (119.2 ± 24.2 ms, P=0.01) compared to chirps produced before the operation (7.4 ± 0.5 pulses/chirp and 86.3 ± 7.2 ms) (**Figure 4.5A** and **4.5D**). Though not significant (P=0.239), after the A4-A5 lesion 5 of 6 males showed shorter chirp intervals (708.4 ± 448.7 ms) than chirps produced by the intact males (1039.9 ± 467.6 ms) (**Figure 4.5D**). The pulse period was not significant different between the two groups (control: 12.5 ± 1.7 ms and A4-A5 cut: 13.5 ± 2.1ms, P=0.398). Overall, the A4-A5 lesion increased the pulse number in chirps and the chirp duration, showed a tendency of a reduced chirp interval, but did not alter the pulse period.

Cross correlogram and raster plot made from 10-minute calling song recording of one male demonstrate the change. Before the A4-A5 lesion, calling song chirps contained up to nine pulses (**Figure 4.5B**). After the lesion, the pulse number in chirps increased and became more variable, the raster plot shows chirps containing ten pulses while more than 20 pulses occurred in some other chirps. The longest chirp produced after A4-A5 lesion contained over 30 pulses. At higher time resolution (**Figure 4.5C**), the raster plot reveals the shorter chirp interval after the A4-A5 lesion as more chirps occur in the given time frame. This is also demonstrated by more chirps generated in the 10-minute recording after the A4-A5 lesion as compared to the control (control: 399 chirps, A4-A5 cut: 715 chirps). However, not all the males showed a reduced chirp interval after the lesion.

Figure 4.5 Calling song before and after the A4-A5 lesion in *G. assimilis*.

(A) Song recording of a *G. assimilis* male before (top) and after the lesion (bottom). The first chirp of the section is shown in high resolution on the right hand side. Arrows indicate the start of each chirp. (B) Cross-correlogram and raster plot of a 10-minute calling song recording of one male before (top) and after the lesion (bottom) over a time scale from -50 ms to 250 ms. Arrow indicates the start and first pulse of chirps, taken as reference for the data analysis and aligned to 0 ms, all subsequent pulses give rise to separate peaks in the correlogram with increasing scatter. The raster plot shows the timing of the sound pulses in the vertical rows of dots. Each dot represents one sound pulse, chirps are indicated by horizontal sequences of dots. (C) Raster plot for the same recording before and after the lesion over a time scale from -2000 ms to 2000 ms. Arrow indicates the start of chirps. Dots to the left and right end of the raster plot indicate the sound pulses of previous and subsequent chirps, respectively. (D) Temporal parameters of the calling song before and after the lesion in 6 males (*= $P < 0.05$, **= $P < 0.01$)

4.3.8 No change in calling song pattern after A5-A6 lesion in G. assimilis

G. assimilis produced calling song with normal chirps after the connectives between A5 and A6 were severed (A5-A6cut, **Figure 4.6**). Calling songs of 5 males before (1749 chirps, 12514 pulses) and after the lesion (2262 chirps, 16799 pulses) were analysed. The calling songs of lesioned males were similar to the normal calling song and showed no significant difference in temporal parameters (**Figure 4.6A** and **4.6D**). Pulse period of A5-A6cut condition (13.9 ± 1.6 ms) was close to pulse period in control recordings (15.7 ± 3.0 ms, P=0.282). Pulse number in chirps was not changed before (7.4 ± 0.5 pulses/chirp) and after (7.5 ± 0.4 pulses/chirp, P=0.715) the lesion. In terms of chirp parameters, chirp duration after lesion (90.9 ± 14.0 ms) was similar to normal calling song (102.1 ± 20.8 ms, P=0.349). There was no significant difference between chirp intervals before (1936.8 ± 818.3 ms) and after the lesion (1248.0 ± 365.1 ms, P=0.124), even 4 of 5 operated males showed a shorter chirp interval (**Figure 4.6D**).

10-minute calling song recordings of one male before and after the A5-A6 lesion were selected to produce cross-correlograms and raster plots (**Figure 4.6B**). This male generated chirps with 7-9 pulses under intact condition and up to 8 pulses after the operation. In this male the pulse period was reduced after the A5-A6 lesion, while this was not observed in all of the males after the A5-A6 lesion, and the statistics of 5 males showed no significant change in pulse period (P=0.282). In the raster plot with larger time scale (**Figure 4.6C**), a tendency to shorter chirp intervals is indicated, after the A5-A6 cut more chirps are included in the given time frame. This is also shown as more chirps are produced in the 10-minute recording (Control: 218 chirps, A5-A6 cut: 511 chirps). However, this change of the chirp interval was not found in all the males and it is not significantly different compared over all males (P=0.124). To sum up, A5-A6 lesion did not change the temporal parameters of the chirp structure.
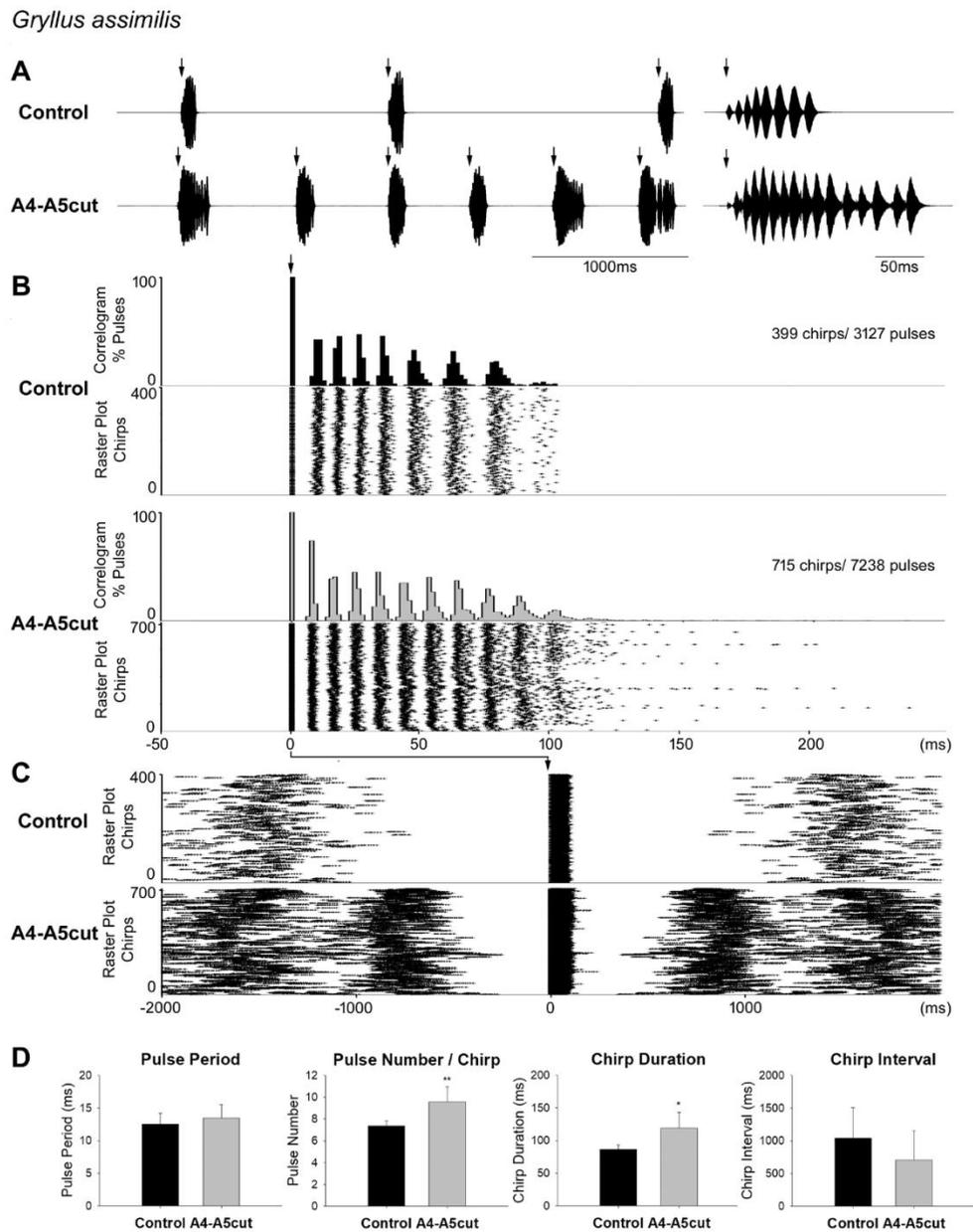
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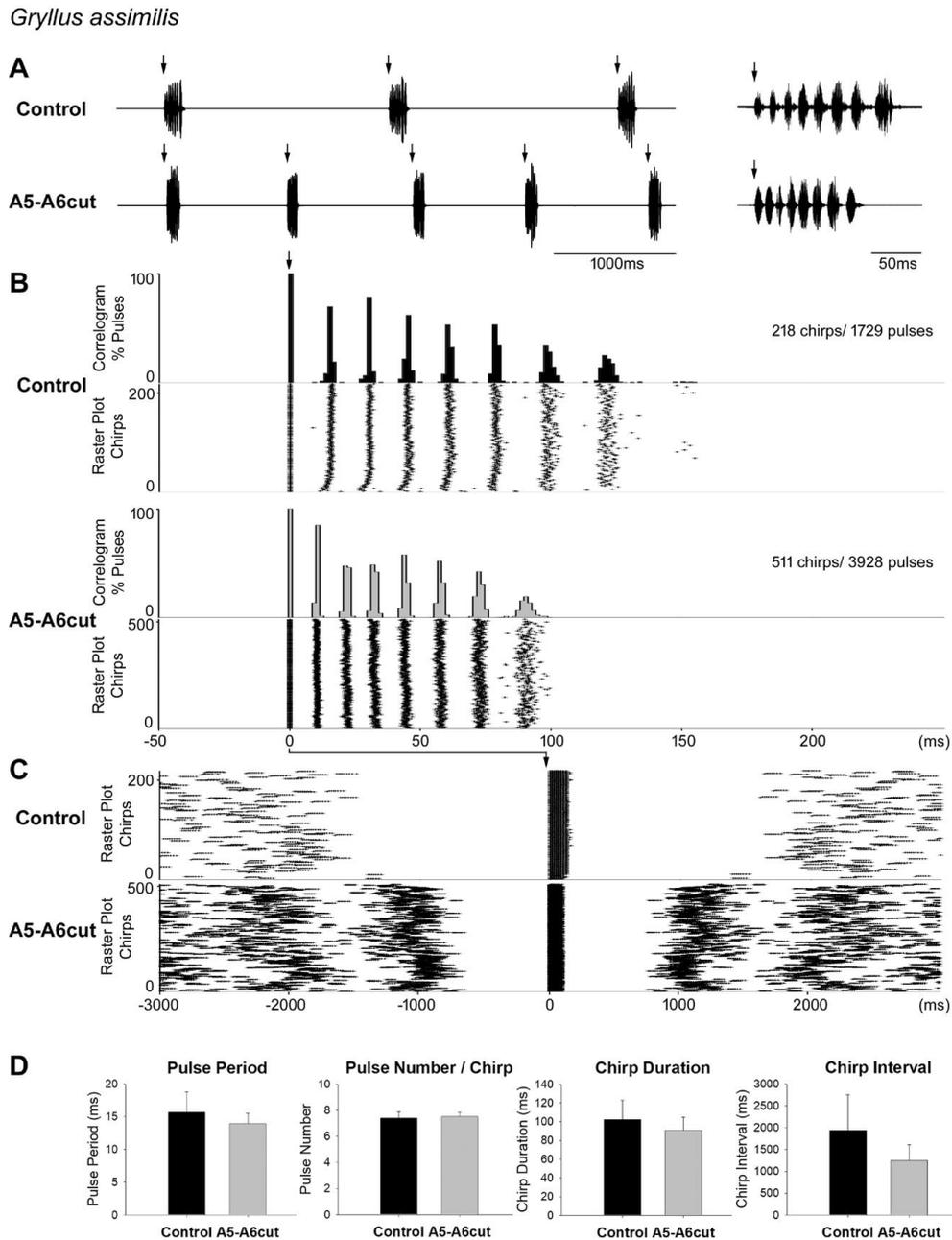
Figure 4.6 Calling song before and after the A5-A6 lesion in *G. assimilis*.
(A) Song recording of a male before (top) and after the lesion (bottom). The first chirp of the section is shown in high resolution on the right hand side. Arrows indicate the start of each chirp. (B) Cross-correlogram and raster plot for a 10-minute song recording of a male before and after the lesion over a time scale from -50 ms to 250 ms. Arrow indicates the start of chirps aligned to 0 ms. Vertical dot patterns indicate the occurrence of sound pulses over the chirps. (C) Raster plot for the same recordings over a time scale from -3000 ms to 3000 ms. Arrow indicates the start of chirps, dots to the left and right end of the raster plot indicate the sound pulses of previous and subsequent chirps, respectively. (D) Temporal parameters of calling song before and after the lesion in 5 males.

4.3.9   Loss of phrase structure after A4-A5 lesion in T. oceanicus

Male *T. oceanicus* continued to sing regularly after the connectives between A4 and A5, or between A5 and A6, were cut. The calling song after the A4-A5 lesion showed substantial changes of the song structure (A4-A5cut, **Figure 4.7**). The two song components in the phrases, the chirps and trills, were no longer generated and were replaced by a repetition of groups of sound pulses, which were intermediate between chirps and trills and here are called *sequences* (**Figure 4.7A**). Without the intermittent onset of the two song components, the calling song generated by A4-A5 cut males sounded more like the pattern of chirp-producing species.

The cross correlogram and raster plot of 10-minute song recording before and after an A4-A5 lesion in one male highlight the change in song structure after the operation (**Figure 4.7B**). In the control recording, 4 chirp-pulses with longer pulse interval occur at the start of each phrase between 0 and 200 ms, giving rise to four peaks in the correlogram, corresponding to the four vertical bands of dots indicating the chirp-pulses in the raster plot. The trill-pulses occurred before and after the chirp-pulses. In this case, the trill-pulses before 0 ms showed the typical doublet pattern of trill-pulses giving rise to bands of two coupled pulses in the raster plot and two corresponding peaks in correlograms, e.g. between -150ms and -100ms. The scatter for the timing of the trill-pulses increased however, with increasing the interval before the reference-phrase. The trill-pulses occurring after the chirps are reflected in the flat part of the correlogram from 300-1000 ms, and in the corresponding distribution of dots in the raster plot. The clear pattern of trills as seen in the control sound recording is gradually lost as the timing of the trill-pulses occurs with an increasing scatter in relation to the start of the reference-chirp and also as doublets and triplets of trill-pulses occurred after chirps.

In the A4-A5 lesion group, chirps and trills were no longer obvious, rather 2 to 4 pulses were grouped in sequences. There was only one form of sequence-pulse, it occurred with a shorter interval as chirp-pulses and a longer interval as trill-pulses in intact animals. The timing

of the sound pulses was analysed with the first pulse of each sequence taken as reference. The correlograms and raster plot reveal the characteristic temporal organization of the reference-sequences. However, with increasing time intervals the timing of subsequent sequences becomes scattered, relative to the start of the reference-sequences, because of the variable sequence durations. Looking however, at the events before the reference-sequences reveals that the interval between sequences was very stable and similar like in the normal song.

In 5 males, 945 phrases (945 chirps, 4352 chirp-pulses, 7674 trills, 17073 trill-pulses) before the lesion and 5925 sequences with 17929 sequence-pulses after the lesion were analysed. To evaluate the change in the song structure caused by the A4-A5 lesion, pulse parameters of original chirps and trills, and of the sequences were compared (**Figure 4.7C**).

Most pulse parameters of sequences were between the values of normal chirps and trills: pulse interval (chirp: 35.2 ± 11.8 ms, trill: 15.0 ± 8.7 ms, sequence: 25.1 ± 14.6 ms, P(chirp vs trill)=0.015, P(chirp vs sequence)=0.263, P(trill vs sequence) =0.222), pulse period (chirp: 67.4 ± 6.9 ms, trill: 41.4 ± 6.2 ms, sequence: 50.7 ± 5.6 ms, P(chirp vs trill)<0.001, P(chirp vs sequence)=0.003, P(trill vs sequence)=0.037), and pulse number (chirp: 4.7 ± 0.7 pulses, trill: 2.3 ± 0.5 pulses, sequence: 3.0 ± 0.6 pulses, P(chirp vs trill)<0.001, P(chirp vs sequence)=0.008, P(trill vs sequence) =0.096). However the sequence-pulse duration remained closer to the duration of pulses in trills, (chirp: 32.5 ± 6.9 ms, trill: 26.2 ± 7.4 ms, sequence: 25.0 ± 10.4 ms, P(chirp vs trill)=0.207, P(chirp vs sequence)=0.220, P(trill vs sequence)=0.840).

In terms of song structure, the sequence duration (109.4 ± 23.1 ms) was closer to the trill duration (79.3 ± 31.2 ms) than to chirp duration (286.9 ± 88.3 ms, P(chirp vs trill)<0.001, P(chirp vs sequence)<0.001, P(trill vs sequence)=0.121). Sequences however showed longer intervals and thus had longer periods than the trills (sequence interval: 145.7 ± 88.5 ms, trill interval: 78.3 ± 16.9 ms, P=0.133; sequence period: 254.3 ± 78.0 ms, trill period: 155.7 ± 22.1 ms, P=0.026).
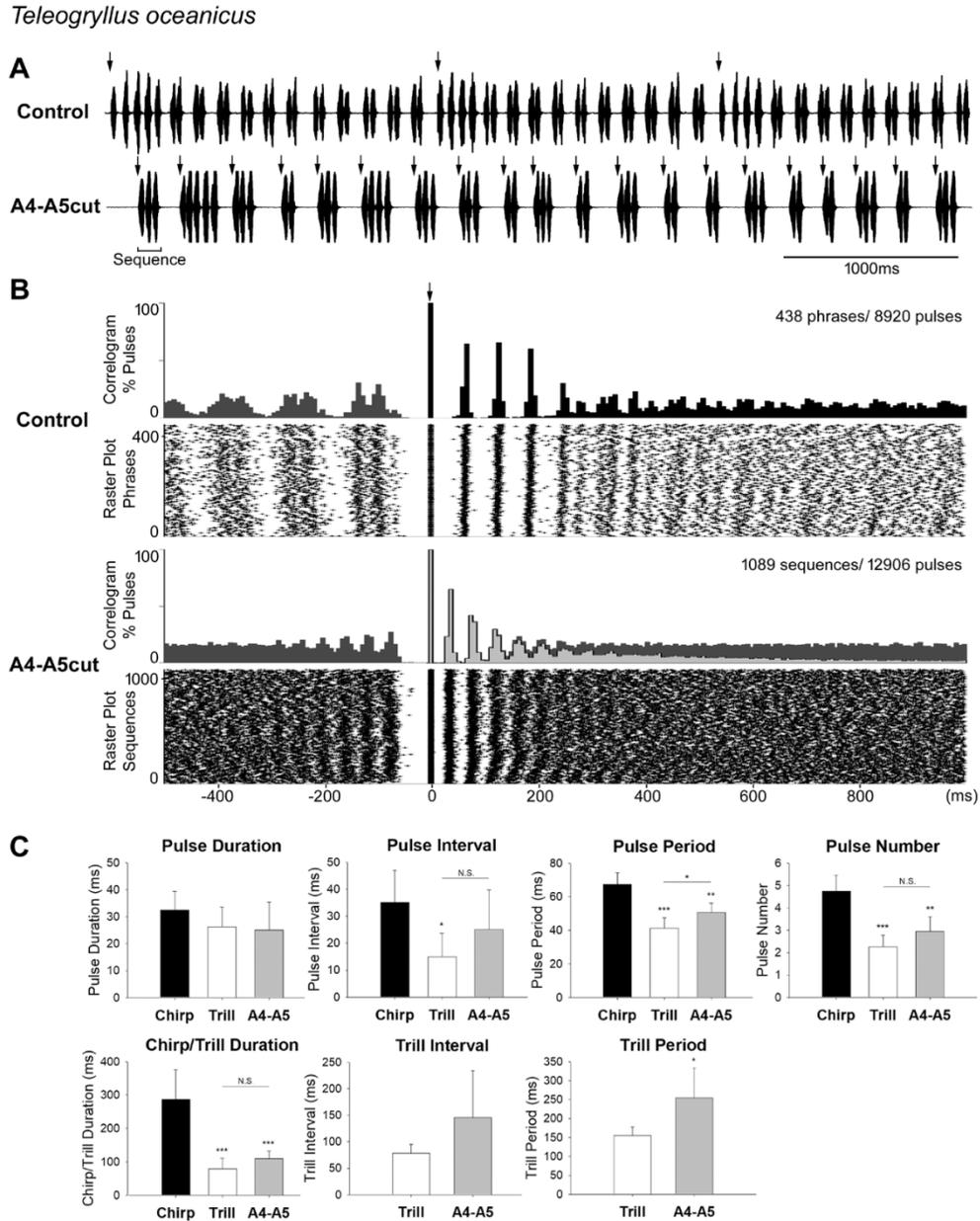
Figure 4.7 Calling song before and after the A4-A5 lesion in *T. oceanicus*.

(A) Song of *T. oceanicus* before (top) and after the lesion (bottom). Arrows indicate the start of each phrase or sequence used as reference in the data analysis. (B) Cross-correlogram and raster plot of 10-minute song recordings of one male before and after the lesion at a time scale from -500 ms to 1000 ms. Black (control) and light grey (A4-A5cut) shades represent the reference phrases or sequences in correlograms and dark grey shading indicates the pulses of the subsequent (to the right) or previous (to the left) phrase or sequence. Arrow indicates the start of reference-phrases and sequences, respectively. (C) Temporal parameters of the calling song before and after the lesion in 5 males (*= $P < 0.05$, **= $P < 0.01$, ***= $P < 0.001$).

To sum up, the A4-A5 lesion destroyed the complex chirp and trill structure of phrases found in normal *T. oceanicus* calling song, and the resulting calling song contained only short sequences with an intermediate pulse form and a variable number of 2-4 pulses.

4.3.10 No change in calling song pattern after A5-A6 lesion in T. oceanicus

Calling song generated by *T. oceanicus* after the connectives between A5 and A6 were severed contained chirps and trills as the calling song in intact males (A5-A6cut, **Figure 4.8**). Each phrase was composed of one chirp followed by several trills, which consisted of doublet or triplet sound pulses (**Figure 4.8A**). Two 10-minute song recordings of one male before and after the A5-A6 lesion were used to generate cross correlograms and raster plots (**Figure 4.8B**). In both groups, chirp-pulses showed as pronounced and sharp peaks in the correlogram and were properly aligned to the raster plot; there were 4 peaks for chirp-pulses under intact condition and 5 peaks under the A4-A5 cut condition. Chirp-pulses had longer pulse intervals than trill-pulses which followed the chirp-pulses. As the occurrence of the trill-pulses was plotted relative to the start of the chirps, and due to the variability of the pulse number in trills, the scatter of the trill-pulses gradually increased and only the trill-pulses following the chirp between 250 ms and 350 ms showed clear peaks in the correlogram. The three vertical bands in the raster plot and the three peaks in the correlogram suggest these trills were triplets, while the following trills could be doublets or triplets of pulses. In the A5-A6 cut group, there were signs of triplets at 400 ms and doublets at 600 ms. For both groups, the trills preceding the reference chirps showed doublet pulses between -150 ms and -100 ms and revealed that the interval between the end of the last trill in a phrase and the subsequent chirp remained very similar in the control and lesioned males.

In 5 males, 1060 phrases (1060 chirps, 4925 chirp-pulses, 7367 trills, and 15911 trill-pulses) before the lesion and 1120 phrases (1120 chirps, 5936 chirp-pulses, 6345 trills, and

16441 trill-pulses) after the lesion were analysed regarding the temporal song parameters. There was no significant difference between the two groups in respect to phrase period (control: 1492.3 ± 352.9 ms, A5-A6 cut: 1396.8 ± 375.5 ms, P=0.689), trill number in phrases (control: 7.4 ± 2.3 trills/phrase, A5-A6 cut: 5.9 ± 1.7 trills/phrase, P=0.292), chirp duration (control: 279.8 ± 32.3 ms, A5-A6 cut: 309.2 ± 61.9 ms, P=0.374), pulse number in chirps (control: 4.7 ± 0.6 pulses/chirp, A5-A6 cut: 5.2 ± 1.1 pulses/chirp, P=0.321), trill duration (control: 81.4 ± 15.74 ms, A5-A6 cut: 98.94 ± 14.54 ms, P=0.103), trill interval (control: 70.74 ± 14.74 ms, A5-A6 cut: 71.14 ± 19.1 ms, P=0.972), trill period (control: 150.9 ± 13.1 ms, A5-A6 cut: 170.2 ± 16.0 ms, P=0.071), and pulse number in trills (control: 2.2 ± 0.2 pulses/trill, A5-A6 cut: 2.6 ± 0.5 pulses/trill, P=0.091) (**Figure 4.8C**).

The pulse parameters of chirps and trills produced by A5-A6cut males were not significantly different from the songs of intact males. For chirps, the chirp-pulse duration (control: 38.2 ± 7.3 ms, A5-A6 cut: 37.9 ± 8.3 ms, P=0.953), chirp-pulse interval (control: 27.3 ± 9.2 ms, A5-A6 cut: 24.7 ± 10.0 ms, P=0.686), and chirp-pulse period (control: 65.6 ± 3.6 ms, A5-A6 cut: 62.7 ± 3.7 ms, P=0.240) are not different between the two groups. The trill-pulse parameters like trill-pulse duration (control: 31.3 ± 6.5 ms, A5-A6 cut: 31.4 ± 6.1 ms, P=0.972), trill-pulse interval (control: 11.0 ± 6.2 ms, A5-A6 cut: 10.7 ± 6.1 ms, P=0.944), and trill-pulse period (control: 41.9 ± 6.1 ms, A5-A6 cut: 41.7 ± 2.3 ms, P=0.956) also showed no significant difference before and after the lesion. Overall, the phrase structure and the parameters of the pulse patterns were not altered before and after the A5-A6 lesion.
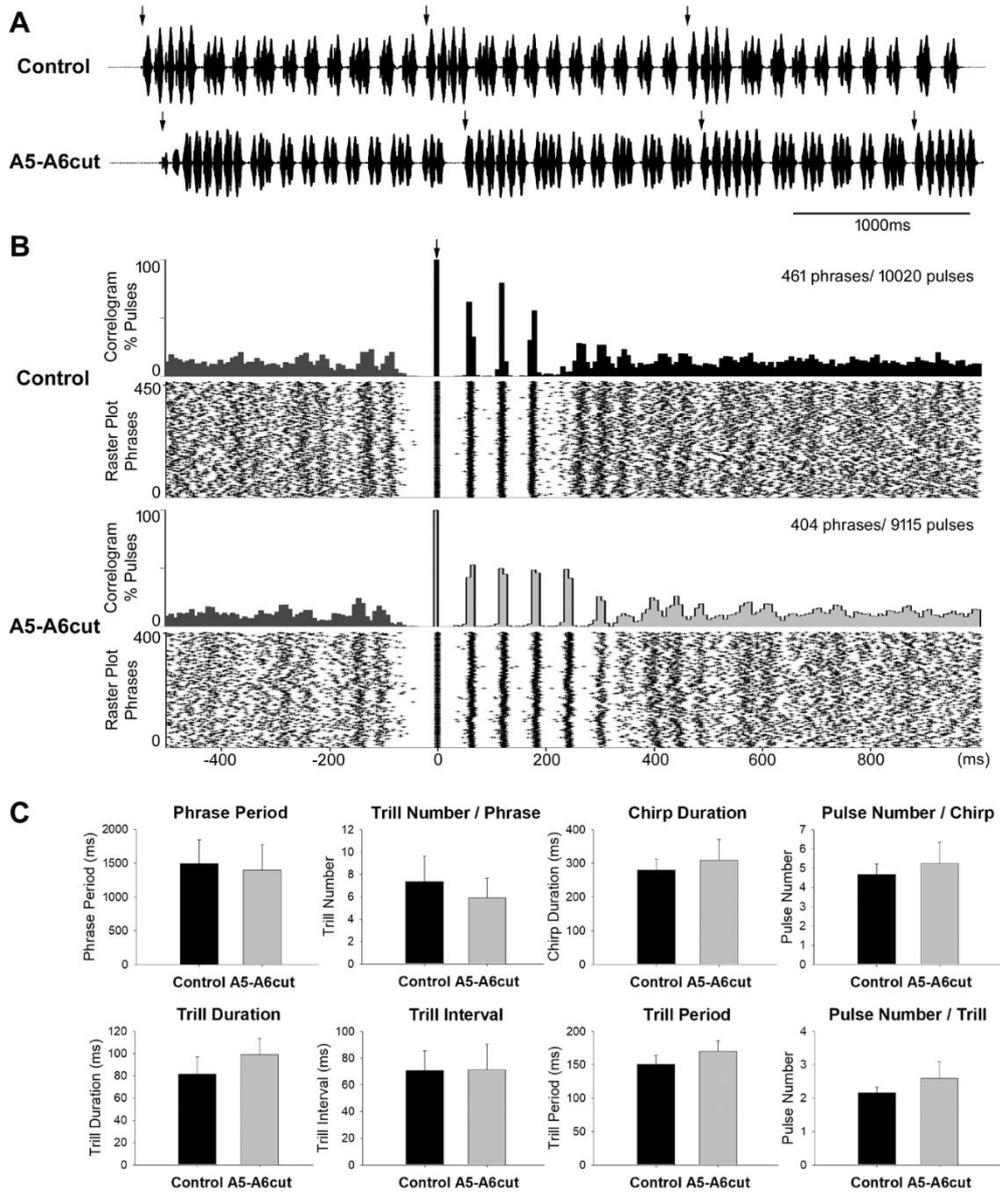
Figure 4.8 Calling song before and after the A5-A6 lesion in *T. oceanicus*.

(A) Song of *T. oceanicus* before (top) and after the lesion (bottom). Arrows indicate start of each phrase, used as reference for the calculation of correlograms and raster plots. (B) Cross-correlogram and raster plot of 10-minute song recordings of one male before and after the lesion over a time scale from -500 ms to 1000 ms. Black (control) and light grey (A4-A5cut) shade represent the reference-phrases in correlograms and dark grey shading indicates the pulses of the previous phrases (to the left). Arrow indicates the start of phrases. (C) Temporal parameters of calling song before and after lesion in 5 males. No significant differences occurred between the intact and A5-A6 cut group.

4.3.11 *Loss of phrase structure after A4-A5 lesion in T. commodus*

Male *T. commodus* continued to sing after the connectives between A4 and A5, or A5 and A6, were lesioned. Lesions to the connectives between A4 and A5 had a similar effect on the calling song of *T. commodus* (A4-A5cut, **Figure 4.9**) as in *T. oceanicus*. Phrases consisting of chirps and trills were no longer generated by A4-A5cut males, and were replaced by repeated short groups of sound pulses, called sequences (**Figure 4.9A**). These songs composed of sequences sounded like calling song from a chirp-producing species, but with a variable pulse number.

Cross correlogram and raster plot based on two 10-minute song recordings of one male show the change of song structure after the A4-A5 lesion (**Figure 4.9B**). For the control group, five peaks in the correlogram and five aligned bands in the raster plot represent the chirp-pulses. The smaller sixth peak suggests a few chirps contained six pulses. These chirp-pulses exhibited longer pulse interval compared to the following trill-pulses and pulses found in A4-A5 cut group. In *T. commodus*, trills contain a variable pulse number. As the timing of the trill-pulses is measured relative to the reference-chirps the temporal structure of trills is only obvious in the raster plot between 350 ms and 700 ms. The decrease in the black area of the histogram signifies the end of the reference-phrases between 1500 ms and 2000 ms, while the occurrence of subsequent phrases is indicated by the dark grey shaded area. The transition between the phrases is indicated by a less intense dot pattern in the raster plot.

In the A4-A5 cut group, the correlograms and raster plot are based on the start of the sequences. The sequence-pulses are reflected in the peaks of the correlograms and the corresponding vertical bands in the raster plot. Pulse interval of sequence-pulse was shorter than chirp-pulses and there was no change of pulse form. Most sequences ended before 500 ms, as indicated by the falling light grey area in the correlogram. The timing of the subsequent sequences in the correlogram and raster plot became more scattered relative to the start of the

reference-sequences, and also due to the variable pulse number in the sequences. While the song structure changed dramatically after the operation, at least in this male the interval between the last sound pulse of a trill and the start of the next chirp in the control group and the interval between the last pulse of a sequence and the start of the subsequent sequence in the lesioned group remained stable and was around 100 ms to 150 ms. This is indicated by the strict timing of the sound pulses before the reference-chirps and reference-sequences in the raster plot, between -150 ms and -100 ms.

In 5 males, 1054 phrases (1054 chirps, 5967 chirp-pulses, 2301 trills, 26372 trill-pulses) before the lesion and 6114 sequences with 43706 pulses after the lesion were analysed. Comparing the temporal parameters of sequences to control chirps and trills, revealed that all the pulse parameters of the sequences were in between those of chirps and trills: pulse duration (chirp: 31.6 ± 2.4 ms, trill: 24.9 ± 3.8 ms, sequence: 27.0 ± 5.1 ms, P(chirp vs trill)= 0.01, P(chirp vs sequence)=0.102, P(trill vs sequence)=0.493), pulse interval (chirp: 28.5 ± 3.5 ms, trill: 12.4 ± 2.3 ms, sequence: 16.4 ± 5.0 ms, P(chirp vs trill) < 0.001, P(chirp vs sequence) < 0.001, P(trill vs sequence)=0.146), pulse period (chirp: 59.9 ± 4.0 ms, trill: 37.4 ± 4.2 ms, sequence: 43.9 ± 3.5 ms, P(chirp vs trill) < 0.001, P(chirp vs sequence) < 0.001, P(trill vs sequence)= 0.031). Also the pulse number (chirp: 5.5 ± 1.0 pulses/chirp, trill: 11.5 ± 9.0 pulses/trill, sequence: 9.7 ± 7.5 pulses/sequence, P(chirp vs trill)=0.176, P(chirp vs sequence)=0.248, P(trill vs sequence)=0.739) showed an intermediate value. In terms of song structure, sequence duration (412.0 ± 335.0 ms) was not significantly different from chirp duration (356.9 ± 34.8 ms, P=0.724) or trill duration (393.8 ± 265.9 ms, P=0.927). Sequence interval (229.4 ± 46.2 ms) and period (640.2 ± 302.9 ms) was close to trill interval (268.2 ± 156.3 ms, P=0.609) and period (669.3 ± 409.9 ms, P=0.901).

In summary, as a consequence of the A4-A5 lesion in *T. commodus* the two typical song component, chirps and trills, disappeared and the calling song was composed of short sequences of sound pulses with a variable number of pulses.
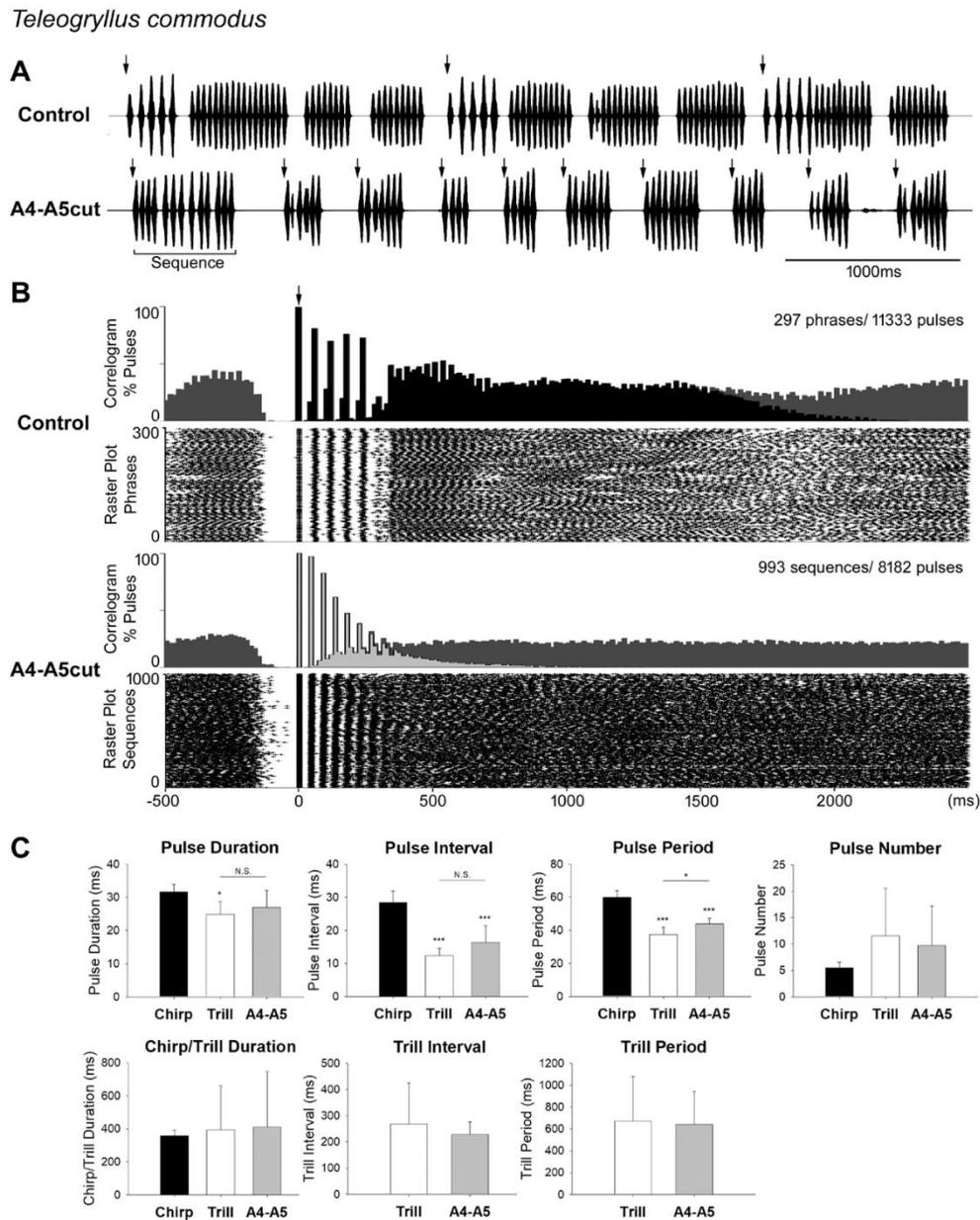
Figure 4.9 Calling song before and after the A4-A5 lesion in *T. commodus*.
(A) Song recording of *T. commodus* before (top) and after the lesion (bottom). Arrows indicate start of each phrase or sequence, taken as reference for the histograms. (B) Cross-correlogram and raster plot of 10-minute song recording of one male before and after the lesion over a time scale from -500 ms to 2500 ms. Black (control) and light grey shade (A4-A5cut) represent reference-phrases or reference-sequences in correlograms and dark grey shading indicates the pulses of the subsequent (to the right) or previous (to the left) phrases or sequences. Arrow

indicates the start of phrases and sequences. (C) Temporal parameters of calling song before and after lesion in 5 males. (*= P < 0.05, **= P < 0.01, ***= P < 0.001.)

4.3.12 *No change in calling song pattern after A5-A6 lesion in T. commodus*

Males of *T. commodus* generated normal phrases containing chirps and trills during calling song after the connectives between A5 and A6 were severed (A5-A6cut, **Figure 4.10**). The overall song structure was not changed after the operation (**Figure 4.10A**). From the cross correlogram and raster plot drawn from 10-minute recording of one male, both groups show the chirp and the trill components (**Figure 4.10B**). At 0 ms, phrases started with chirps with longer pulse interval. These chirps, with stable pulse period, align in the raster plot as five bands and show as five peaks in correlogram in both groups. The following trill-pulses show shorter pulse interval and only the bands of the reference-trills after chirps can be seen in the raster plot (between 300 ms and 1000 ms) because of the variable pulse number in trills. The reference-phrases ended around 1500 ms to 2000 ms, as indicated by the less dense scatter of the raster plot and the falling number of events in the correlogram (light grey area). In both conditions in this male, the last trills in a phrase were closely linked to the start of the next phrase, as indicated by the three light bands in the raster plot between -150 ms and 0 ms. Overall, these figures reveal no difference in calling song structure before and after A5-A6 lesion.

In 5 males, 900 phrases (900 chirps, 4810 chirp-pulses, 1526 trills, 23577 trill-pulses) before the lesion and 651 phrases (651 chirps, 4254 chirp-pulses, 2074 trills, 20922 trill-pulses) after the lesion were analysed statistically (**Figure 4.10C**). The calling song structure of A5-A6 cut males showed no significant difference in phrase period (control: 1968.5 ± 688.8 ms, A5-A6 cut: 2548.2 ± 389.7 ms, P=0.140), chirp duration (control: 318.5 ± 81.7 ms, A5-A6 cut: 367.9 ± 65.4 ms, P=0.322) and trill number in phrases (control: 1.8 ± 0.7 trills/phrase, A5-A6 cut: 3.1 ± 1.3 trills/phrase, P=0.076). The trill parameters like trill duration (control: 713.5 ± 533.4 ms, A5-A6 cut: 474.6 ± 435.2 ms, P=0.460), trill interval (control: 204.6 ± 107.0 ms,

A5-A6 cut: 255.0 ± 96.0 ms, P=0.456), trill period (control: 979.9 ± 652.3 ms, A5-A6 cut: 781.6 ± 481.3 ms, P=0.599) showed no significant difference after the A5-A6 lesion. Finally, the pulse number in chirps (control: 5.4 ± 1.1 pulses/chirp, A5-A6 cut: 6.7 ± 1.1 pulses/chirp, P=0.111) and pulse number in trills (control: 17.8 ± 11.3 pulses/trill, A5-A6 cut: 12.5 ± 10.8 pulses/trill, P=0.469) did not significantly change after the lesion.

Also the pulse parameters of chirps and trills produced by A5-A6 lesioned males were not significantly different from intact males. For chirps, chirp-pulse duration (control: 31.3 ± 4.6 ms, A5-A6 cut: 27.3 ± 5.5 ms, P=0.244), chirp-pulse interval (control: 32.7 ± 4.9 ms, A5-A6 cut: 31.7 ± 5.3 ms, P=0.767), and chirp-pulse period (control: 63.9 ± 6.6 ms, A5-A6 cut: 58.9 ± 4.7 ms, P=0.206) did not change between the two groups. Pulse parameters in trills also showed no significant difference before and after A5-A6 operation: trill pulse duration (control: 25.9 ± 4.1 ms, A5-A6 cut: 22.1 ± 4.2 ms, P=0.195), trill pulse interval (control: 13.6 ± 3.1 ms, A5-A6 cut: 16.5 ± 4.7 ms, P=0.267), and trill pulse period (control: 39.5 ± 3.7 ms, A5-A6 cut: 38.8 ± 2.9 ms, P=0.753).

To summarize, the calling song of *T. commodus* was not altered by the lesion between A5 and A6 in terms of phrase structure and the temporal pulse parameters.
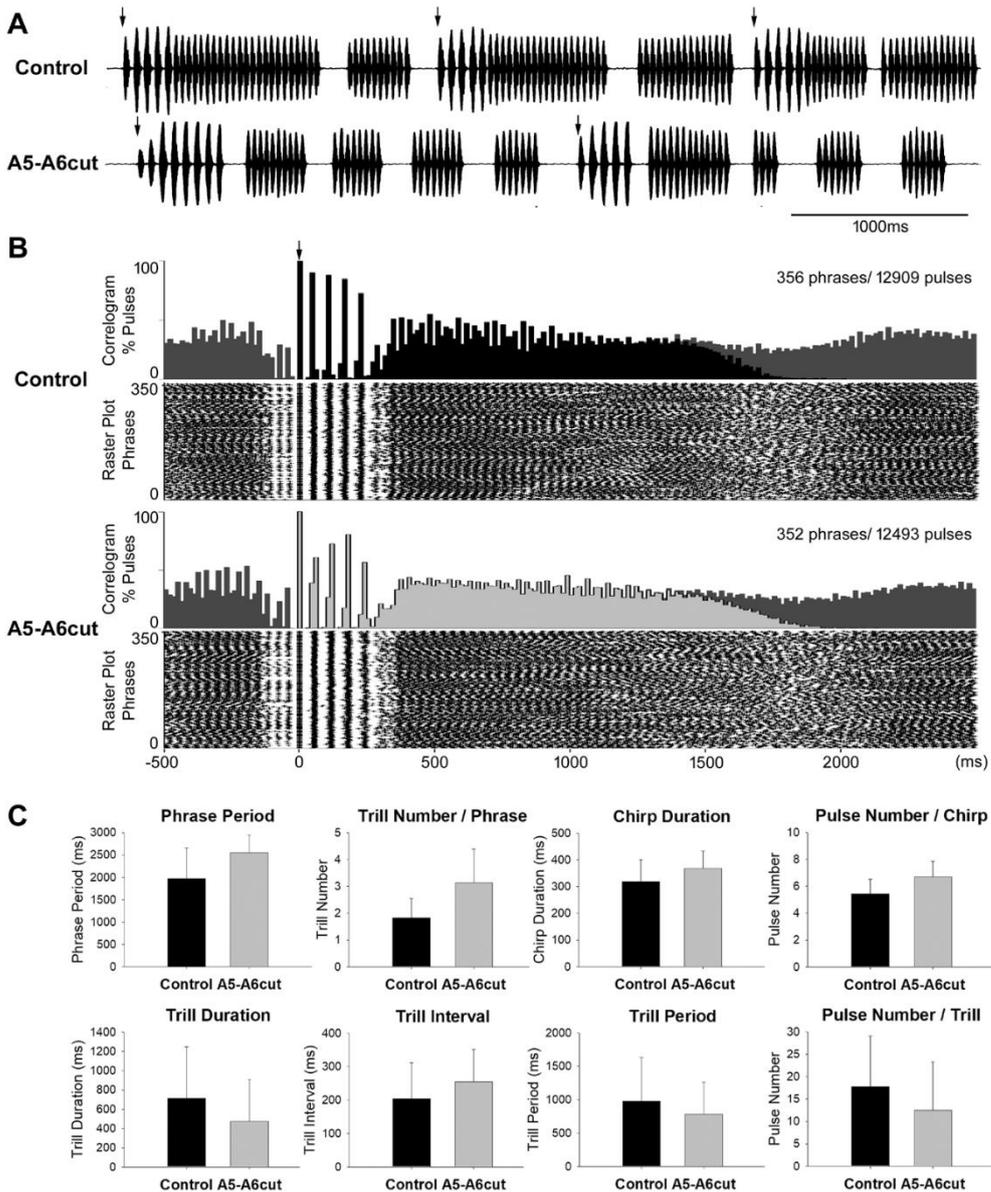
Figure 4.10 Calling song before and after the A5-A6 lesion in *T. commodus*.

(A) Song of *T. commodus* before (top) and after the lesion (bottom). Arrows indicate the start of each phrase, taken as reference for the histograms. (B) Cross-correlogram and raster plot of 10-minute song recordings of one male before and after the lesion over a time scale from -500 ms to 2500 ms. Black (control) and light grey shade (A4-A5cut) represent the reference-phrases in correlograms and dark grey shading indicates the pulses of the subsequent (to the right) or previous (to the left) phrases. Arrow indicates the start of phrases. (C) Temporal parameters of calling song before and after the lesion in 5 males.

4.4 Discussion

Recent studies (Schöneich and Hedwig 2011, 2012; Jacob and Hedwig 2016) indicated that the singing-CPG in the field cricket *G. bimaculatus* is organized along the abdominal nerve cord and not located in the thoracic ganglia as previously suggested (Huber 1960, 1963; Hennig and Otto 1996). To investigate if the neural organization for song pattern generation is conserved among cricket species, I performed lesion experiments on four species from different clades generating very different species-specific calling songs with pulses organized in chirps, trills or phrases. The data revealed conserved organization of singing pattern generation network among the four species, while species-specific features were observed in species with different calling song forms.

I focus on the acute effect of the lesions within 7 days to avoid long-term effect such as compensatory growth after lesions. In *Gryllus bimaculatus*, deafferentation of an auditory interneuron, ascending neuron 2 (AN-2), led to regrowth of dendrites toward contralateral side (Horch et al. 2011). In *Acheta domesticus*, regeneration of medium giant interneuron was monitored after lesion application to proximal and distal unilateral connective (Roederer and Cohen 1983). Their results showed that new neurite emerged 2 days after lesion and regrowth continued up to 3 to 4 weeks following the lesion, and different preparations of the same manipulation exhibited different regrowth arborization. As most of the CPG neurons for singing identified are interneurons across multiple abdominal ganglia (Jacob and Hedwig 2019, 2020), the long-term effect such regrowth of neurites and reorganization of the nervous system after lesions should be considered. Thus, only the acute effect of lesion on calling song is taken into consideration and the effect of each lesion on calling song was consistent in all the preparations.

All the four species showed the same effect after a lesion to the T3-A3 connectives was applied. All males with T3-A3 lesions retained the ability to raise the forewings into singing position but for the rest of their lives they failed to generate rhythmic wing movements for singing and no longer generated any proper sound pulses. This outcome is in line with the results of acute connective severing experiments in males induced to sing by brain stimulation (Schöneich and Hedwig 2011) and with the long term recordings of singing activity after this lesion in *G. bimaculatus* (Jacob and Hedwig 2016). It provides strong evidence that the abdominal ganglion chain is crucial for the generation of calling song in different cricket species and explains why singing activity in male *G. campestris* failed, after the cervical *and* the T3-A3 connectives were cut (Kutsch and Otto 1972). It also indicates that the control of the elevated front wing position used for singing does not depend on the abdominal ganglia but rather is under control of the cephalic and/or thoracic nervous system.

After lesions to the A3-A4 connectives overall singing activity was greatly reduced. Males could still produce single sound pulses, however in all species the typical calling song structure was lost. This suggests the abdominal ganglion in all 4 species A3 houses neural circuits that are sufficient to control the wing movements for pulse generation. This is in line with the results in *G. bimaculatus* (Jacob and Hedwig 2016), suggesting that the A3 ganglion is part of a pulse timer network. This is also supported by the discovery of an ascending opener interneuron in A3, which was identified in all five cricket species. It shows synchronized spike activity preceding wing opener motoneurons and elicits singing motor activity upon current injection (Schöneich and Hedwig 2011, 2012; Jacob and Hedwig 2019, 2020).

The effect of lesions to the A4-A5 connectives depended on the calling song structure of the species, it altered the song in *G. assimilis*, *T. oceanicus*, and *T. commodus*. In *G. bimaculatus* this lesion led to a breakdown of the calling song chirp structure with chirps considerably extended in duration coupled to a more variable chirp period (Jacob and Hedwig

2016). After the lesion also the chirps in *G. assimilis* exhibited an increased pulse number and a longer chirp duration, while the rather long chirp period was not altered.

The effect of the A4-A5 lesion on the calling song of the phrase-producing species, *T. oceanicus* and *T. commodus*, was dramatic. The organised chirp and trill components of the phrases disappeared and instead males generated repetitions of short sequences of sound pulses with variable pulse number. These newly generated pulse sequences showed almost all pulse parameters in between the normal chirp-pulses and trill-pulses. The comparison of pulse number in chirps, trills, and sequences before and after the lesion in the two species may explain the effect of the lesion on the song structure. In normal calling song both species produced 4-6 pulses in chirps (*T. oceanicus*: 4.7 ± 0.7 pulses/chirp, *T. commodus*: 5.5 ± 1.0 pulses/chirp), and the number of pulses/trill in *T. commodus* (11.5 ± 9.0 pulses/trill) was higher than in *T. oceanicus* (2.3 ± 0.5 pulses/trill). After applying the A4-A5 lesion, the chirps and trills were replaced by sequences, and the number of pulses/sequence was higher in *T. commodus* (9.7 ± 7.5) than in *T. oceanicus* (3.0 ± 0.6). Moreover, the number of pulses in sequences was always in between the normal number of pulses/chirp and pulses/trill in both species. In the case of *T. oceanicus*, the number of pulses/trill was higher than the number of pulses/chirp, and the number pulses/sequence was lower than the number of pulses/trill and higher than the number of pulses/chirp. This was opposite in the case of *T. oceanicus*, where the number pulses/trill was lower than the number pulses/chirp; here the number pulses/sequence was higher than the number of pulse/trill and lower than the number of pulses/chirp. The fact that the pulse parameters and pulse number of sequences was in between of chirps and trills suggests that after the A4-A5 lesion the sequences generated were a consequence of generating pulses with a structure between chirp-pulses and trill-pulses in a way that chirps and trills are no longer identifiable. Compared to *T. oceanicus* the number of pulses/trill was higher in *T. commodus*

before the lesion and also in sequences after lesion, this indicates that pulse number in calling song, as a species-specific feature, was retained after the A4-A5 lesion.

These findings imply that the network remaining after the lesion can no longer generate the coordinated pattern of chirps and trills, and that at least the A5 ganglion contributes to the generation of the phrases, housing a neural mechanism that drives the generation of chirps and trills, which either could be the excitation level provided by one neuron or network or could be due to two functionally separate networks. It also demonstrates that ganglia A3 and A4 together can generate sequences of sound pulses, according to the implications from the *G. bimaculatus* experiments that the pulse timer network extends over the A3 and A4 ganglion (Jacob and Hedwig 2016).

Interestingly in *G. rubens*, no significant change in the trill structure of the song or of pulse parameters occurred. Even in intact males the trill pattern was variable and not precisely structured. In the evolution of cricket calling songs a train of pulses may represent the original primitive ancestral condition from which more structured songs evolved (Otte 1992). In the light of this evolutionary background a network in A5 and beyond contributing to the timing of trills like to the timing of chirps may actually not be present in this species.

In all 4 species lesions to the A5-A6 connectives showed no effects on the structure of the calling song and implicate that the A6 and the TAG are not required for calling song pattern generation. It also implicates that the effect of the A4-A5 lesion can be attributed to a contribution of the A5 ganglion to pattern generation, corresponding to the organization of the chirp timer network along abdominal ganglia as proposed in *G. bimaculatus* (Jacob and Hedwig 2016).

The similar effects after specific lesions to the abdominal ganglion chain indicate that the overall organization of the singing-CPG network is conserved among cricket species, besides the species-specific variations. None of the males sang, when the connectives between the

thoracic and abdominal ganglia were severed, in all males the generation of pulses was possible when the A3 ganglion was intact, sequences of pulses were generated when A3 and A4 were intact, however the chirp/trill structure was impeded; and the normal song was only generated, when the A5 ganglion was included in the structure of the CNS. Minor effects may occur if the connection to the posterior abdominal ganglion A6 is removed from the nerve cord (Jacob and Hedwig 2016). The pattern generating network underlying singing stretches over the A3, A4 and A5 abdominal ganglia, while the temporal structure of the singing activity is getting more complex with more posterior ganglia contributing to motor pattern generation. This is in line with the results in *G. rubens,* which may indicate that the recruitment of network components in the more posterior abdominal ganglia may have supported the evolution of more complex song structures.

It still appears surprising that the cricket singing-CPG extends over the abdominal ganglia, and is not located in the second thoracic ganglion, which houses the motoneurons innervating the singing muscles. One possibility that receives support from behavioural studies (Kutsch 1969) and recent electrophysiological experiments (Schöneich and Hedwig 2019) is that elements of the ventilatory pattern generator network in the abdominal ganglion chain supported the evolution of the singing network in crickets.

Lesion experiments in *G. campestris* once excluded the involvement of abdominal ganglia in singing behaviour, however the exact sites of lesions to the abdominal ganglion chain was not specified (Huber 1960, 1963). As lesions to the abdominal ganglion chain show very different results and lesions posterior to the A5 ganglion have no effect on singing at all; without considering the position of a lesion the interpretation of these early experiments may have been misguided. One might assume that the singing-CPG in *G. campestris* is organized in a different way, however considering the consistent results of systematic lesions in *G. assimilis* and *G. bimaculatus*, the results by Kutsch and Otto (1972) for *G. campestris*, and the

close phylogenetic relationship between *G. bimaculatus* and *G. campestris* (Huang et al. 2000; Desutter-Grandcolas and Robillard 2003), a different organization of the abdominal singing network in *G. campestris* seems unlikely.

Lesions applied to the insect central nervous system have been an important approach to studying behaviour and the gross organization of the underlying neural networks. In the study of grasshopper stridulation it narrowed down the ganglia housing the singing CPG to the 2nd and 3rd thoracic ganglion (Hedwig 1986) while the organization of hemi-ganglionic singing-CPGs in the 3rd thoracic ganglion was revealed (Ronacher 1989, 1991; Fries and Elsner 1996). A hemiganglionic organisation of the pattern generator in thoracic ganglia was also demonstrated for the locust flight system by means of connective pair severing and hemisection of thoracic ganglia (Wolf and Pearson 1987; Ronacher et al. 1988). Lesion studies were also applied to other invertebrates and vertebrates. In leech, selective sectioning of lateral connectives and medial Faivre's nerve had separated the swimming initiation process from the intersegmental coordination during swimming (Weeks 1981). In the studies of leech heartbeat, nerve cords were denervated to analyse intersegmental and side-to-side-coordination of the hearts (Wenning et al. 2004). In *C. elegans*, a combination of optogenetic and lesion experiments revealed that forward locomotion was achieved by coordination of multiple oscillators (Fouad et al. 2018). In lamprey, the surgically hemisected and electrically activated spinal cord was used to study the locomotor CPG in swimming without reciprocal inhibition from the contralateral generators (Cangiano and Grillner 2005).

4.4.1 *Future perspective*

My comparative approach using lesion experiments provided crucial information on the organization of the calling song network in crickets and also indicates its evolutionary conserved structure. It may be interesting to apply a similar study to other acoustically

communicating insect like bush-crickets, which also use their wings for sound production. The lesion applied in the current study were incision of connectives between abdominal ganglia that demonstrate the calling song structure generated by the remaining abdominal nerve cord. To interpret the role of each abdominal ganglion, another set of lesion experiments by hemisecting specific abdominal ganglion/ganglia in these four species, as done in *G. bimaculatus* (Jacob and Hedwig 2016), might provide further information for resolving the calling song generation network. Follow up studies may also analyze the cellular organization of the singing network in the different ganglia of these species with neurophysiological methods to allow a deeper understanding of the network elements and properties. In *G. bimaculatus*, neurons with pulse-timer properties were identified in A3 and A4, chirp-timer neurons were found in A4, A5, and A6, and the activation of chirp-timer neuron triggered the pulse-timer neuron activity while the activation of pulse-timer neurons led to generation of pulses without chirp structure (Jacob and Hedwig 2020). These were in line with findings from lesion experiments (Jacob and Hedwig 2016). From the results in the current study, one could expect intracellular recordings in A4 might further reveal the pulse-timer neurons in the four species and targeting A4 and A5 could identify chirp-timer neuron in *G. assimilis* and reveal the neurons responsible for chirp and trill patterns in *Teleogryllus* species. With developing genetic tools, a combination of genetic labeling and recording techniques in studying cricket singing-CPG can also be expected in near future.

5 Chapter Five: Evidence for calling song command neurons in the brain of field crickets

Abstract

Command neuron refers to a neuron which is capable of eliciting whole behaviour sequence on its own, and necessary for sustaining the behaviour. In the study of cricket singing behaviour, one such neuron for calling song was identified in the brain of field cricket *Gryllus bimaculatus*. Nonetheless, it is not clear if this sort of command neuron exists in other cricket species. In this study, intracellular recording and current injection experiments were carried out in the brain of three cricket species, *G. bimaculatus*, *G. assimilis*, and *Teleogryllus commodus*, and discovered putative command neurons for calling song in these three species. Though no proper staining of neuron morphology was retrieved, physiological properties of the recorded neurons in the three species showed similar functions on controlling calling song. When the neurons were triggered, the forewings were raised to singing position and rhythmic wing movements were gradually building up. Additional depolarization to the neuron increased chirp repetition rate but not pulse repetition rate in *G. bimaculatus* and *G. assimilis*. Hyperpolarizing current injection was able to reduce chirp repetition rate and terminate calling song activity. These evidences support the idea that these crickets have homologous neurons for controlling calling song, while further description of neuron morphology is required.

5.1 Introduction

In neuroethology, the idea of command neurons for specific fixed action patterns in animal behaviour has been appealing ever since the term was first introduced when describing the giant fibres in crayfish escape behaviour (Wiersma and Ikeda 1964). The criteria of sufficiency and necessity for the function of command neurons was later proposed (Kupfermann and Weiss 1978): a command neuron should reliably elicit the complete motor pattern for a specific behaviour once activated over threshold (sufficiency), and the motor pattern should cease once the neuron activity is abolished or removed (necessity). Additionally, the command neuron should activate the CPG underlying the rhythmic motor pattern by tonic activity only. Command neurons for different behaviour have been reported in invertebrates and vertebrates. In fish and amphibians, the Mauthner cell (M-cell) identified to be involved in short-latency tail-flip escaping conforms to the sufficiency but not the necessity criteria and was defined as a "command-like neuron" (Rock et al. 1981; Zottoli and Faber 2000; Korn and Faber 2005; Lacoste et al. 2015). In the nematode *C. elegans*, two symmetric serotonergic hermaphrodite specific neurons (HSNs) were defined as command neurons as mutants with a defect in HSNs failed in egg-laying and optogenetic activation of HSN was sufficient to elicit egg-laying (Trent et al. 1983; Leifer et al. 2011; Brewer et al. 2019). Feeding and escaping behaviour in mollusc has been reported to be controlled by command neurons (Gillette and Davis 1977; Balaban 1979). By means of thermogenetic neuronal activation and mass screening, command neurons for walking, feeding, and courtship song were identified in *Drosophila* (von Philipsborn et al. 2011; Flood et al. 2013; Bidaye et al. 2014). In grasshopper, a command system (neurons function collectively to fulfil sufficiency and necessity criteria) for stridulation was demonstrated by intracellular recording and current injection (Hedwig 1994).

Cricket stridulation is a suitable model to study the control of rhythmic motor patterns. Male crickets sing, by rhythmically rubbing their front wings together, with the stridulatory moto-machinery of muscles and motoneurons located in the mesothoracic segment. Extracellular electric stimulation experiments in the brain of male *Gryllus campestris* indicated that its three song types, calling, courtship, and rivalry songs, can be elicited by stimulating different neuropil regions and implied the existence of command neurons for cricket singing behaviour (Huber 1960, 1963), which would carry forward the command to sing from the brain to the singing CPG. Electrical stimulation of single interneurons in the cervical connectives of *G. campestris*, *G. bimaculatus*, and *Teleogryllus oceanicus* suggested the command signal from the brain contains no temporal information of the cricket songs (Otto 1971; Bentley 1977). Intracellular recording in the brain of *G. bimaculatus* demonstrated a pair of descending command neurons for cricket calling song (Hedwig 1996, 2000). The command neuron fulfilled the criteria of sufficiency as depolarizing current applied to the neuron elicited calling song singing, and fulfilled the criteria of necessity as hyperpolarizing current applied to the neuron stopped ongoing calling song singing. Brain interneurons linked to the control of courtship and rivalry song have not been identified, but in grasshoppers evidence demonstrates that different stridulation motor programs are controlled by different descending command neurons (Hedwig and Heinrich 1997).

Cricket songs exhibit species-specific characteristics in their temporal pulse pattern and song structure. The diversity of cricket songs is a result of independent evolution and prevents closely related or sympatric species from interbreeding (Otte 1992). In the current study, I aim to search for and characterize command neurons in the brain for calling song stridulation in different cricket species by intracellular recording and current stimulation. By comparing the morphology and physiological properties of command neurons in different species, this study could reveal the similarities and differences of how command neurons control singing

behaviour and provide evidence towards the evolution of cricket singing at a cellular level.

5.2   Material and methods

5.2.1   Experimental animals

G. bimaculatus, *G. assimilis*, and *T. commodus* were reared and bred in insect colonies in the Department of Zoology, University of Cambridge. These crickets were cultured in large boxes (52.5 x 36.5 x 28 cm) in a 12hr-12hr light: dark cycle at 26-28 °C. Unlimited fish food, muesli, and water supply were provided. Last instar male nymphs without conspicuous injury were selected and isolated in individual boxes (17.5 x 11.5 x 13 cm) to prevent mating before experiments. These males were reared to adulthood and selected for experiments when they were 7-14 days old after last moulting. Completeness of the wings and capability of producing calling song were confirmed for each individual before experiments.

5.2.2   Intracellular recording of brain neurons

Male crickets were mounted dorsal side up on a plasticine block attached to a stand, with the prothorax and six legs stabilized by bent staples. The stand was tilted in a way that the head was facing upward. The head was then waxed to a U-shaped metal holder to prevent movements during dissection and intracellular recording. A 1.5-mm diameter round foil was stuck on the right forewing for recording the wing movements (see wing movement recording and sound recording). Dissection began by removing the cuticle between the compound eyes and opening a rectangle window. The ocellar nerves were removed together with the cuticle without damaging the brain. Excess fat body was removed to reveal the brain which was stabilized between a small metal platform at its dorsal side and a metal ring at its ventral/frontal

side. A light fibre powered by a DC light source was built in the platform and served as light source during the intracellular recording. The brain was constantly supplied with insect saline (in mmol^{-1}: NaCl 140; KCl 10; CaCl$_2$ 7; NaHCO$_3$ 8; MgCl$_2$ 1; N-trismethyl-2-aminoethanesulfonic acid 5; D-trehalose dehydrate 4, pH 7.4) to prevent desiccation. Capillary Glass (Clark, Hilgenberg, 1 mm OD) was pulled by a DMZ puller (Zeitz-Instruments, Germany) to micro-capillaries. These micro-capillaries were filled with either 2M potassium acetate (with 30-40Ω resistance, Alexa 568 or Alexa 555 as dye) or 1M lithium chloride (with 80-100Ω resistance, Lucifer Yellow as dye) and inserted in an electrode holder, which was attached to a micromanipulator to control the positioning of the electrode. A digital gauge (Mitutoyo, Digimatic Indicator 543; resolution, 1 μm) monitored the depth of the electrode in the brain to provide information for approaching the target neuron. The intracellular recording was monitored as audio signal by speakers during experiment. Current injection (depolarization and hyperpolarization) in each recorded neuron was used to identify the singing command neuron. Stable recordings of identified singing command neuron last for 15 to 30 minutes and contain spikes with amplitude above 10mV. All data of the experiments were recorded by CED Spike2 software (CED, Cambridge, UK). Experiments were performed on 98 *G. bimaculatus*, 57 *G. assimilis*, and 28 *T. commodus*.

5.2.3 *Wing movement and song recording*

An optoelectronic camera (Hedwig 2000) with a linear position-sensitive photodiode (Laser Components, Type 1L30-UV) was set up at a distance of 80 cm to focus on the reflective foil disk stuck on the right wing of the animal. The up-down movement during singing, representing the lateral projection of the opening and closing movement of the wing, was picked up with the diode by the light reflected from the disk when the animal was illuminated by a DC light source. The up-down wing movements during calling song stridulation was 2-3

mm. At the same time the song produced by the animal was recorded by a microphone (Teisco Sound Research UEM-83). Once singing behaviour was elicited both wing movement and sound signal were recorded in Spike2 software.

5.2.4   Data analysis

The spike rate of recorded neurons, sound pulse rate, chirp rate, and pulse number in chirps were analysed by NEUROLAB (Hedwig and Knepper 1992; Knepper and Hedwig 1997). For the calculation of spike rate, the recordings were subjected to differentiation to reduce DC changes and spikes were identified by a triggering function using changes in membrane potential as threshold criteria. The identification of spikes was manually checked and then used to generate the instantaneous spike frequency diagram. For calculating the sound pulse rate, sound pulses were full wave rectified by a gliding length filter, labelled by the triggering function, and the trigger times were used to generate the pulse frequency diagram. Chirp rate was processed in the same way based on the first pulse in the chirps as trigger points. Pulse number in chirps was calculated by a Peri-Stimulus-Time histogram function and manually checked.

5.3   Results

Intracellular recordings were carried out in the cricket anterior protocerebrum, in the region where extracellular electric stimulation elicited calling song singing in *G. campestris* (Huber 1960) and a descending interneuron was identified as command neuron for calling song in *G. bimaculatus* (Hedwig 2000) (**Figure 5.1**). The recording region was 100 μm lateral to the mid-line (left or right) and was ± 50 μm anterior/posterior to the root of the medial ocellar nerve as reference. Each neuron recorded was tested by depolarizing and hyperpolarizing

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current injection for behavioural effect. Singing-like wing movements during searching for stable recordings of the command neuron with the microelectrode were recorded in 14 experiments with *G. bimaculatus*, 15 experiments with *G. assimilis*, and 10 experiments with *T. commodus*. Neurons related to calling song stridulation were stably recorded 5 times in *G. bimaculatus*, 6 times in *G. assimilis*, and 3 times in *T. commodus*. All the experiments in the result part were repeated in all the stably recorded individuals and same results were obtained.
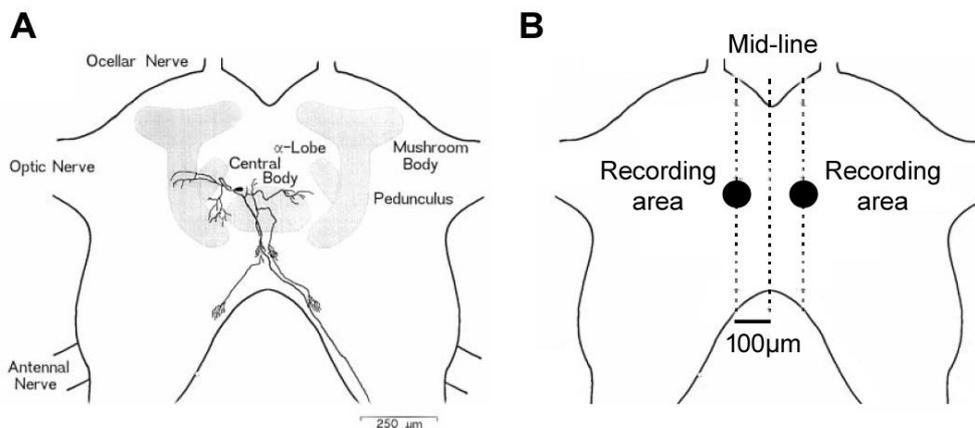
Figure 5.1 Area for recording of putative command neurons for calling song in the brains of different cricket species.

(A) Morphology of the command neuron for calling song in *G. bimaculatus*. (B) Recording area for calling song command neurons as indicated by black circles. (A modified from Hedwig 2000)

5.3.1 Calling song stridulation elicited by injury to the putative command neuron (pCN) for calling song in G. bimaculatus

Calling song of *G. bimaculatus* consists of chirps with 3-5 sound pulses. The sound pulse rate is around 30 Hz and the chirp rate 3 Hz (Kutsch and Huber 1989). In *G. bimaculatus*, a neuron related to calling song stridulation was encountered at a depth of 150-250 μm from ventral side of the brain. Sometimes the neuron was penetrated or damaged during searching for a stable recording and singing behaviour was activated, although the neuron activity was

not recorded at the same time. In some cases, the forewings of the male gradually raised and started slow opening and closing movements like stridulation with only scratchy or no sound produced. In other cases, the male rapidly raised the forewings into a high singing position and immediately began rhythmic opening and sonorous closing movements, generating the calling song (**Figure 5.2**). One opening-closing cycle generated one distinct sound pulse. The calling song began with one sound pulse per chirp and gradually increased to four sound pulses per chirp. The repetition rate of the chirps was highest at the beginning, when chirps contained only few pulses, and gradually decreased from about 4.5 Hz to 2 Hz. Without any current stimulation, singing stopped 5 to 40 seconds after the neuron was activated, the wing position as well as the chirp rate gradually decreased before the calling song stopped. The neurons successfully recorded with stable microelectrode penetrations were subjected to current injection experiments.
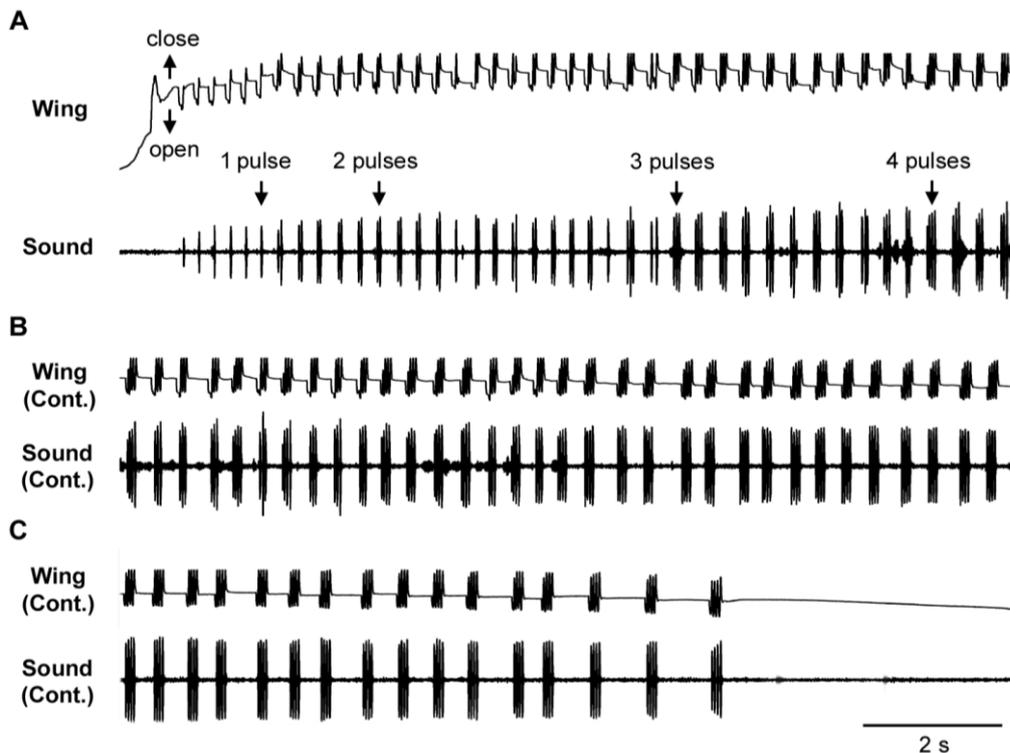
Figure 5.2 Calling song stridulation elicited by activating the pCN for calling song during intracellular recording attempts in the brain of *G. bimaculatus*.

The command neuron for calling song stridulation likely was excited during searching for a stable recording. The male started to produce calling song, the forewings raised to singing position, and rhythmic opening (downward)

and closing (upward) wing movements followed. The pulse number in chirps increased from one pulse to 4 pulses as the calling song carried on. Note the lowering of wing position and lower chirp rate before the song ended.

5.3.2 Testing the sufficiency criterion: Depolarization of the putative command neuron (pCN) for calling song initiated calling song stridulation in G. bimaculatus

The sufficiency criterion for the characterization of command neurons states that a command neuron on its own could elicit the whole fixed action pattern when activated (Kupfermann and Weiss 1978). The sufficiency of the pCN for calling song was tested by depolarization experiments.
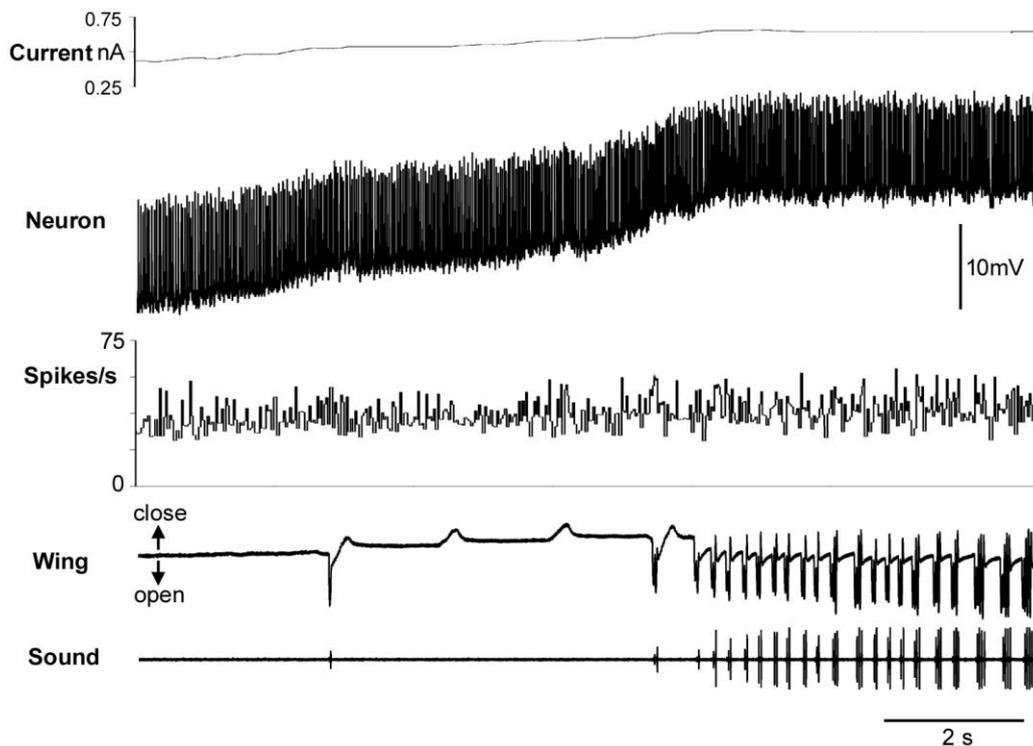
Figure 5.3 Stridulation of calling song elicited by depolarizing current to the pCN for calling song in male *G. bimaculatus*.
Depolarizing current applied to a putative command neuron was gradually increased from 0.4 nA and elicited calling song stridulation when the spike rate of the neuron reached about 50 Hz. Note the slow wing movements (bulges) before stridulation and the increasing number of pulses in chirps.

In the first experiment, depolarizing current was applied to the neuron with increasing amplitude starting from 0 nA (**Figure 5.3**). Due to the microelectrode penetration, the spike rate of the neuron was already at 10 APs/s before current was injected. When the depolarizing current increased above 0.5 nA the spike rate increased to about 50 APs/s and calling song stridulation began. The neuron exhibited tonic activity during calling song stridulation and the activity was not temporally coupled to the chirp or pulse pattern. The neuronal activity also showed no prominent EPSP during spiking. Before the rhythmic movements for stridulation, the wings slowly moved up and down for a few times revealed as bulges in the wing recording (4 times in **Figure 5.3**). The pulse number in the chirps gradually increased from one pulse to two, three, and four pulses. Once the calling song was stable with three to four pulses per chirp, no one-pulse or two-pulse chirps were generated again. The sound pulse repetition rate was between 30-35 Hz while the chirp rate decreased from 5 Hz to 2.5 Hz as the pulse number per chirp increased. The effect of further increase of the depolarizing current to the neuron is demonstrated in **Figure 5.4**. The continuous depolarizing current caused the slow changes in the recorded potential. While the current was slowly increased from 0.7 nA to 2.5 nA the spike rate of the neuron gradually increased from 50 APs/s to 100 APs/s. The effect of enhanced neuronal activity was reflected in the change of the chirp rate but not the sound pulse rate. The chirp rate increased from 2.5 Hz to 3.5 Hz in the time course of this figure (**Figure 5.4**, two 4.5 Hz peaks in the chirp rate were caused by three-pulse chirps). A further increase of the depolarizing current to 3 nA led to a spike rate of about 150 APs/s and initiated the highest chirp rate, with about 5Hz for 3-pulse chirps and 4 Hz for 4-pulse chirps. Further increase of the current injected had no further effect on the chirp rate. With increasing the neuron's spike rate the sound pulse rate was not changing and stayed at 30-35 Hz. The pulse number of 3 or 4 pulses in a chirp was not affected by increasing the neuronal activity. Overall, the experiment demonstrates that the neuron is sufficient to elicit sequences of calling song singing when

activated (Kupfermann and Weiss 1978). The activity of this neuron is related to the chirp rate, while the sound pulse rate and pulse number are independent of the neuron's spike rate.
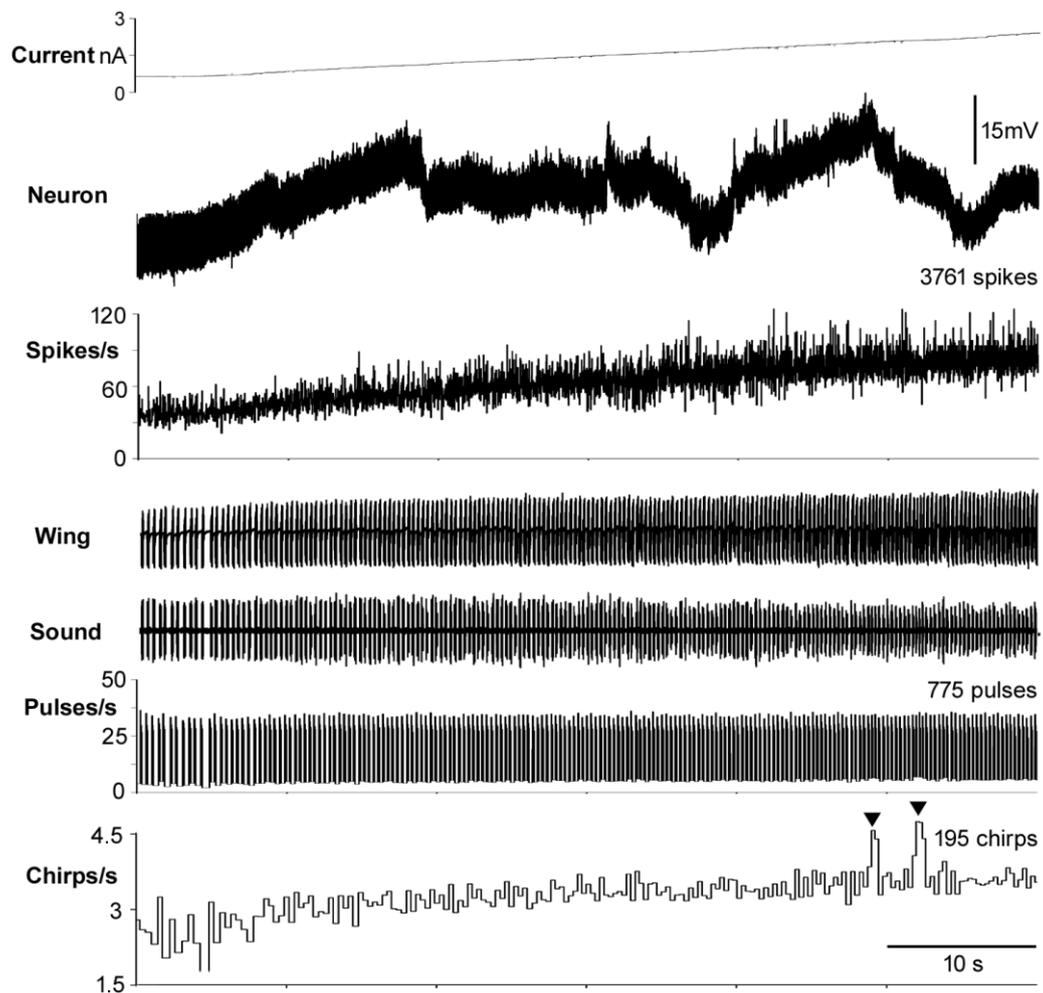
Figure 5.4 Increasing spike activity of the pCN for calling song by depolarizing current enhanced the chirp rate but not the sound pulse rate in *G. bimaculatus*.

Increasing depolarizing current from 0.7 nA to 2.5 nA was applied to the the neuron to gradually raise neuronal activity from 50 APs/s to 100 APs/s. Note the increasing chirp rate and the unaffected sound pulse rate. Arrowheads in chirp rate histogram indicate three-pulse chirps, while the others are four-pulse chirps.
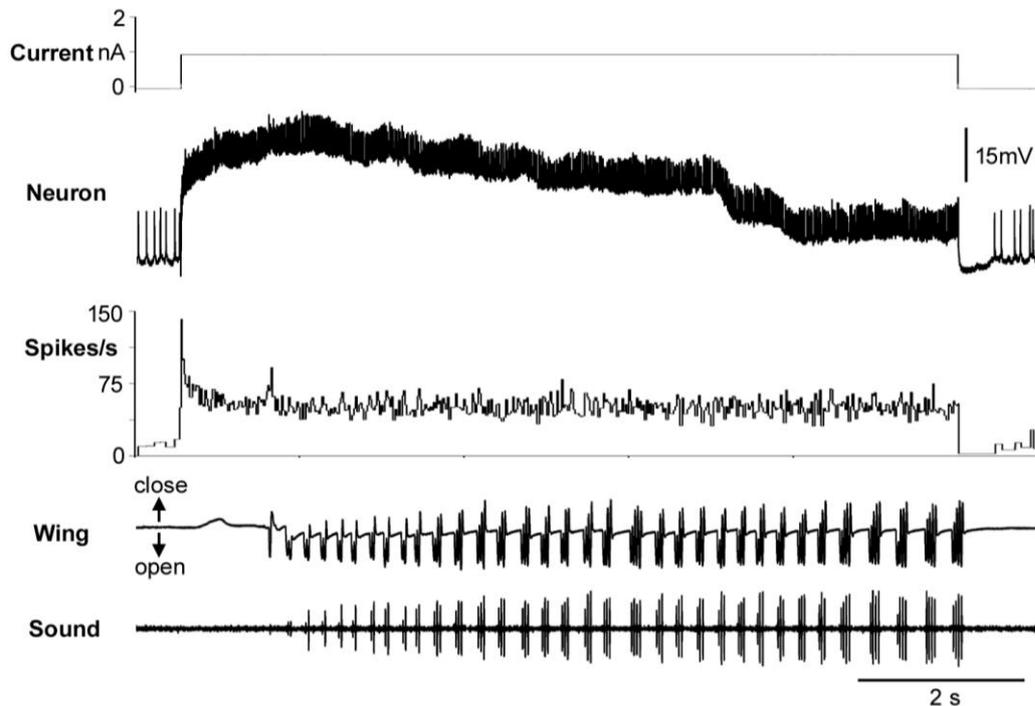
Figure 5.5 Depolarization of the pCN with current pulse initiated calling song stridulation in *G. bimaculatus*. A 1 nA depolarizing current was applied to the neuron for 9.5 s when the male was in a state with the forewings already raised. Note the delay between current injection and onset of singing activity, the increasing pulse number in chirps, the increasing sound amplitude, and the gap in spiking after removal of the depolarizing current.

In other experiments, depolarizing current was injected into the pCN when the male was either in a resting state or already stridulating calling song to investigate the immediate effect of the neuron activity in different behavioural states. A 1 nA depolarizing current was applied in a male, which had stopped generating calling song with its forewings still raised (**Figure 5.5**). The current injection at first transiently increased the neuronal activity from 10 APs/s to 140 APs/s, and then decreased and stabilized at 40-60 APs/s over the time course of 500 ms. Calling song stridulation began about 1 s after the current injection, it started with chirps with a small pulse number and a low amplitude; as stridulation went on the pulse number/chirp increased to 4 and at the same time the amplitude of the wing movements and sound intensity increased. The calling song lasted until the 9.5 s of current injection was stopped, at which point the neuron stopped firing for 450 ms and then started to spike again, at a rate of 10 APs/s. The calling song was no longer generated without any current stimulation.

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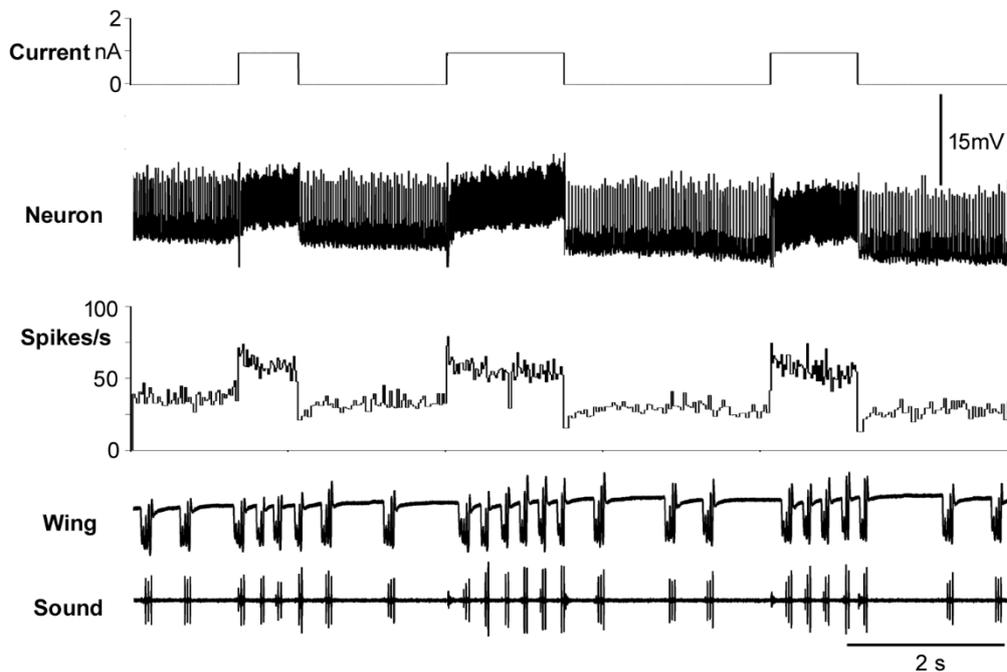
Figure 5.6 Depolarization of the pCN during ongoing calling song activity increased the chirp rate in *G. bimaculatus*.

Repetitive depolarizing current injection with 1 nA for 0.8 s, 1.5 s, and 1.1 s reliably increased the neuronal activity and the chirp rate. Note the different chirp rate before, during, and after depolarization.

In the case of a male with ongoing low level calling song activity (**Figure 5.6**), repetitive injection of 1 nA depolarizing current raised the spike rate of the neuron from 30-40 APs/s to a short transient peak of 75 APs/s and then to a tonic rate of 50-55 APs/s. The change of neuronal activity enhanced the chirp rate from 1.5-2 Hz to 4-5 Hz, while the sound pulse rate was not affected and was steady at 27-30 Hz. Upon removal of the current the neuronal spike rate initially decreased to below 25 APs/s and then stabilized at about 30 APs/s, and over the same time the chirp rate dropped to 1-1.5 Hz. Three repeats of the current injection reliably demonstrated the impact of the neuronal activity on the chirp rate of the ongoing calling song.
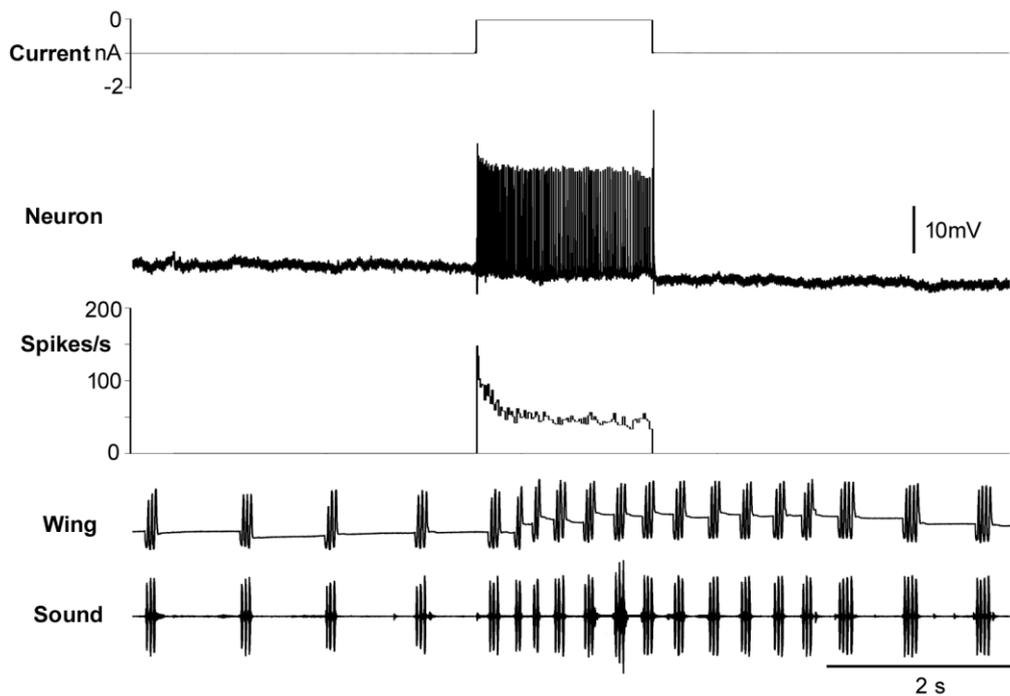
Figure 5.7 Removal of hyperpolarizing current to the pCN command neuron increased the chirp rate of ongoing calling song in *G. bimaculatus*.

A 1nA hyperpolarizing current stopped the calling song command neuron from firing and the chirp rate was decreasing. Removal of the hyperpolarizing current for 1.8 s restored the neuronal activity and transiently increased the chirp rate. The chirp rate decreased again when the hyperpolarizing current was reapplied. Note, the wing position was raising when the neuron began to fire, and was gradually lowered when the hyperpolarizing current was applied again.

A similar effect was observed in another recording, when the recorded neuron's spiking activity was abolished by a 1 nA hyperpolarizing current injection while the chirping activity decreased but still continued (**Figure 5.7**). Removal of the hyperpolarization for 1.8 s boosted the neuronal spike rate transiently to 150 APs/s which then gradually decreased and stabilized at 50 APs/s. Over the time course the chirp rate increased from 1 Hz to 5 Hz and then slowly dropped to 3 Hz, while the repetition rate of the sound pulses did not change and was between 23-30 Hz. When the 1 nA hyperpolarizing current was applied again, the neuron stopped firing and the chirp rate gradually decreased from 3 Hz to 1-1.5 Hz over a time course of 3 s. To sum

up, depolarization of the neuron can initiate calling song stridulation in resting males and increase the chirp rate during ongoing calling song activity. The chirp rate however, is not tightly coupled to the command activity, as singing activity only gradually declined after blocking the pCN spike activity, and the pulse rate is not affected by the command.

5.3.3 *Testing the necessity criterion: Hyperpolarization of the putative command neuron (pCN) for calling song slowed and terminated calling song stridulation in G. bimaculatus*

The necessity criterion for the characterization of command neurons states that the continued generation of the fixed action pattern requires the sustained activity of the command neuron (Kupfermann and Weiss 1978). The necessity of the pCN for calling song was tested by hyperpolarization experiments. In the first experiment, hyperpolarizing current was injected to the pCN when a male was stridulating calling song. The current started from -1 nA and gradually increased (**Figure 5.8**). The neuron activity was suppressed by the hyperpolarizing current and gradually declined from a spike rate of 25 APs/s when 1 nA current was applied to less than 5 APs/s when the current reached -1.7 nA over 16.5 s. The chirp rate decreased from 3.5 Hz to 1.5 Hz, while the sound pulse rate was not influenced and stayed in the range of 25-30 Hz. With the reduction of the pCN activity the male gradually lowered the wings and finally stopped generating chirps as the neuron activity was lower than 5APs/s. The number of pulse in the chirps was always 4-5 during the current injection and was not affected by reducing the neuronal activity until the male finally stopped singing. This experiment suggested the neuron's activity was necessary for the calling song stridulation as calling song was not sustained when the neuronal activity was below a certain spike rate (Kupfermann and Weiss 1978).
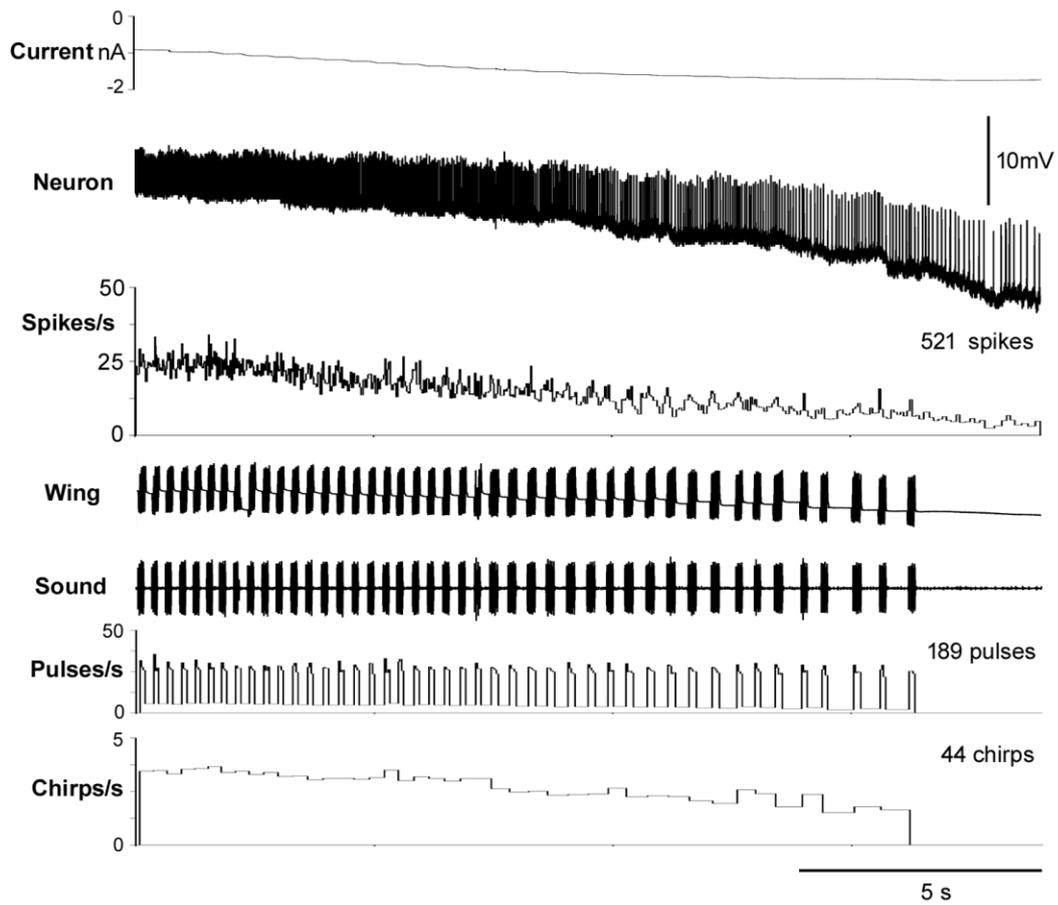
Figure 5.8 Increasing hyperpolarizing current injection to the pCN for calling song slowed and terminated the calling song in *G. bimaculatus*.

Hyperpolarizing current with increasing amplitude from -1 nA to -1.7 nA was injected to the neuron in a singing male. Note the reduction in chirp rate and the gradual lowering of the wing.
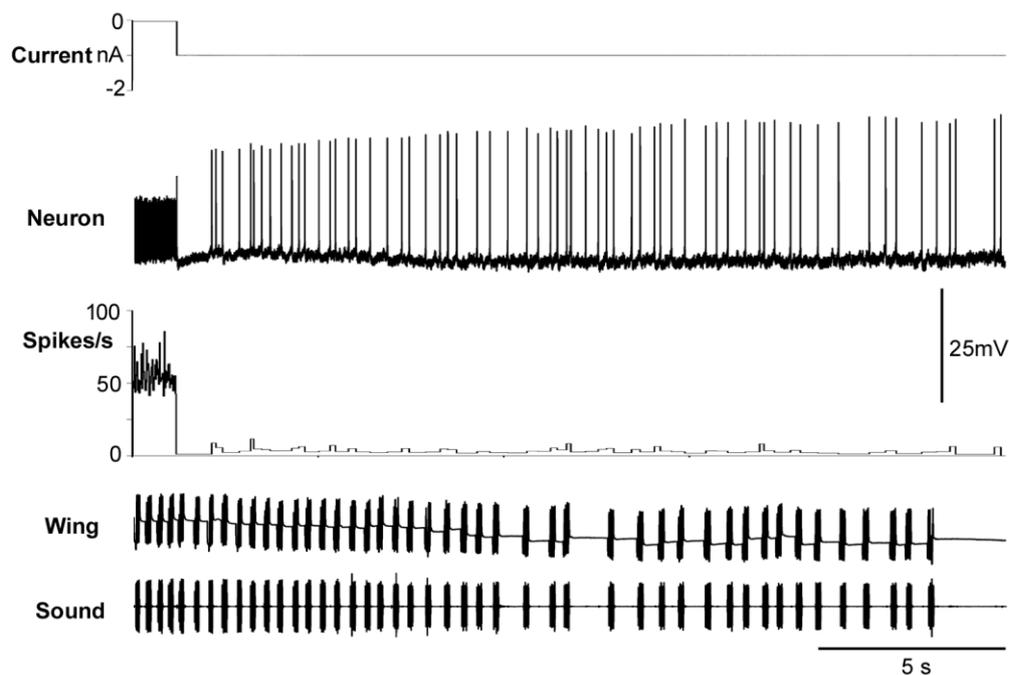
Figure 5.9 Hyperpolarization of the pCN for calling song gradually decreased the chirp rate of ongoing calling song in *G. bimaculatus*.

Injection of a 1nA hyperpolarizing current to the calling song command neuron when the male was generating calling song. Note the gradual decrease in chirp rate and the lowering of the wing during hyperpolarization.

Hyperpolarizing current was applied to the pCN when the male was generating calling song to test the effect of reduced neuronal activity on calling song activity (**Figure 5.9**). A 1 nA hyperpolarizing current reduced the spike rate of the neuron from 50-75 APs/s to less than 10 APs/s. During the hyperpolarization the activity of the neuron showed no outstanding EPSPs indicating synaptic activity. The chirp rate before hyperpolarization was 3.5 Hz, it gradually decreased to 1.5 Hz over 20 seconds; while the wing position was slowly lowered and then the calling song stopped. The sound pulse rate was not affected by the reduced neuronal activity and was 25-30 Hz, so was the pulse number in the chirps which all contained 3-4 pulses.

In another recording hyperpolarization of the neuron reliably slowed the chirp rate and terminated the calling song (**Figure 5.10**). The neuron was firing at spike rate
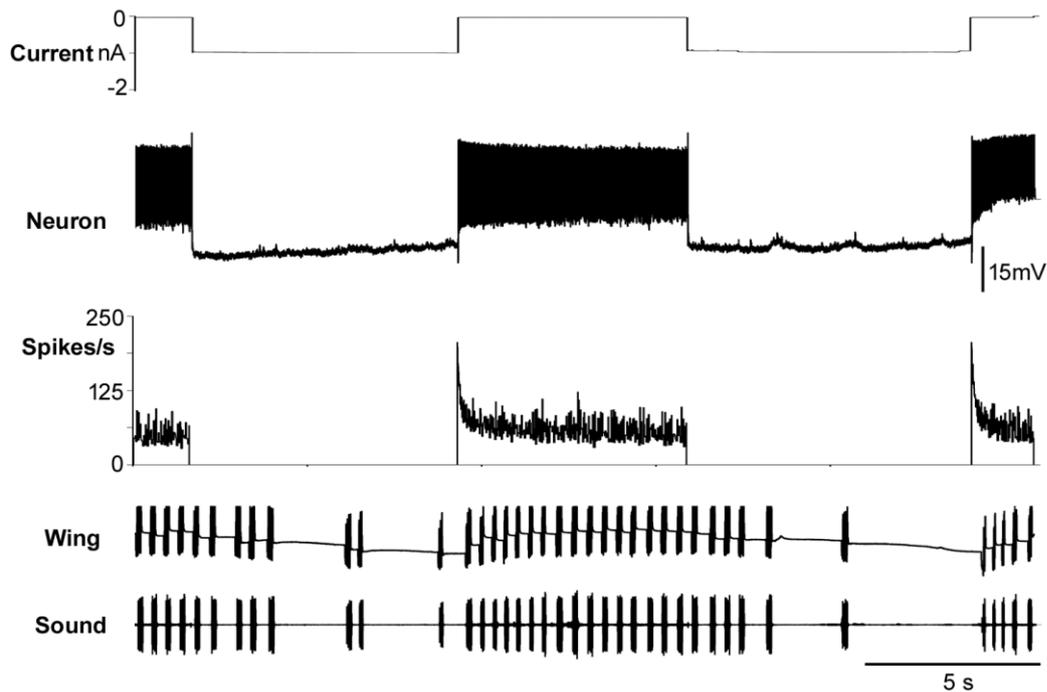
Figure 5.10 Hyperpolarization of the pCN for calling song during ongoing calling song activity reduced the chirp rate in *G. bimaculatus*.

A 1 nA hyperpolarizing current reliably abolished the neuronal activity, it reduced the chirp rate, and terminated the calling song. Note the gradual change in wing position and the change in chirp rate before, during, and after hyperpolarization.

around 50-60 APs/s, which was abolished by a 1 nA hyperpolarizing current. The chirp rate dropped from 2.5 Hz to 0.5 Hz as the spike activity was suppressed. The wing position was also lowered during hyperpolarization. Removal of the hyperpolarizing current restored the spike activity, it first reached a peak of over 150 APs/s and then gradually stabilized at 80-100 Hz. The chirp rate raised with the recovered neuronal activity to 2.5-3 Hz, and the wing position also increased. The following hyperpolarizing current again led to a reduction in the chirp rate from 2.5 Hz to 0.5 Hz and to a lower wing position. Removal of the second hyperpolarization boosted the neuronal activity and the calling song chirp rate again. Over the course of the experiment the sound pulse rate (25-35 Hz) was not altered by the abolished or restored

neuronal activity. These hyperpolarization experiments strongly indicate that the neuron's activity was necessary to sustain calling song stridulation. In addition, however, calling song could last for a few seconds and up to 20 seconds with decreasing chirp rate after the activity of the neuron was suppressed.

5.3.4 *Correlation of spike frequency of the pCN for calling song with chirp rate and sound pulse rate in G. bimaculatus*

Statistical analysis of 182 chirps in *G. bimaculatus* calling song and the corresponding pCN spike frequency reveal the relationship between spike frequency and the chirp rate and the sound pulse rate, respectively (**Figure 5.11**). Pulse rate was calculated from the first two pulses of each chirp as pulse rate varied in a range within chirps (25-35 Hz). The range of spike frequency was 0 to 122 spikes per second, the chirp rate ranged from 2.3 to 4 chirps per second, and the range of the pulse rate was 32 to 36 pulses per second. Plotting spike frequency against chirp rate revealed a positive correlation (R = 0.7532) (**Figure 5.11A**), while no correlation was found between spike frequency and sound pulse rate (R = 0.1568) (**Figure 5.11B**). This suggests the changing neuronal activity of pCN altered chirping rate, which was also shown by the depolarization and hyperpolarization experiments (**Figure 5.4, 5.6, 5.7, 5.8, and 5.10**), while sound pulse rate was not changed by neuronal activity of pCN.
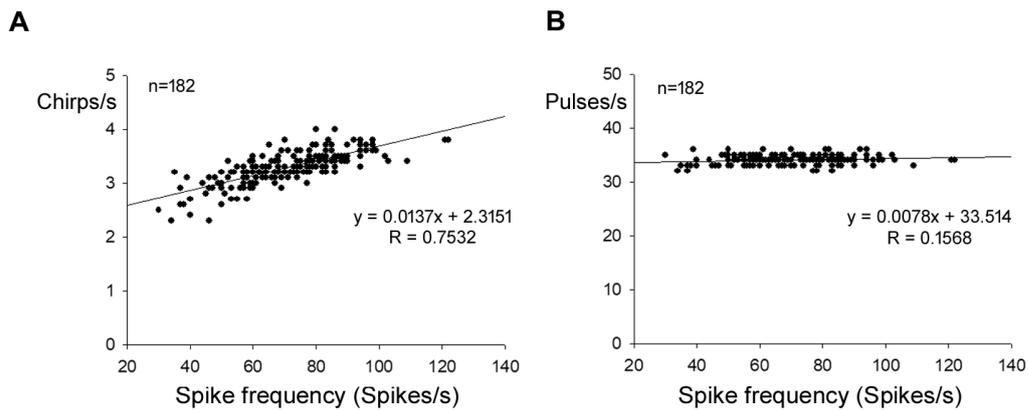
Figure 5.11 Statistical correlation between the pCN spike frequency and the chirp rate, and the sound pulse rate in *G. bimaculatus*.

(A) There is a high correlation (R = 0.7532) between the pCN spike frequency and the chirp rate. (B) There is no correlation (R = 0.1568) between the sound pulse rate and the pCN spike frequency.

5.3.5   *Loud clapping sound temporarily modulated the chirp rate of ongoing calling song*

in G. bimaculatus

Cricket singing motor activity can be interrupted temporarily by air-currents activating the cercal sensory pathway (Dambach and Rausche 1985; Hedwig 2000; Jacob and Hedwig 2015). The timing of chirps following the cercal stimulus was determined by the phase of the stimulus applied within the chirp period (Jacob and Hedwig 2015). To test if calling song stridulation was affected by an acoustic startle response, loud clapping sounds were made by hand during ongoing calling song activity (**Figure 5.12**). Spike activity of the neuron was briefly affected at 40-80 ms after the clapping sounds (**Figure 5.12A**) each clapping sound reduced the pCN activity by 1-2 spikes as compared to the normally activity and the spike rate transiently dropped from 40-50 APs/s to 20-30 APs/s after each clap (arrowheads). The calling song was transiently interrupted over the time course of the claps. In another example (**Figure 5.12B**), clapping sounds were made over the time course of 4 seconds; the calling song was

disturbed but chirps appeared intermittently in between the clapping sound. Some of the chirps were abolished and the interchirp interval of some chirps was extended. As a result, the timing of the chirps did not follow the original temporal pattern (indicated by black dots). These findings indicate the command neuron activity and the ongoing calling song singing was affected by the clap stimuli, which may activate multiple sensory pathways.
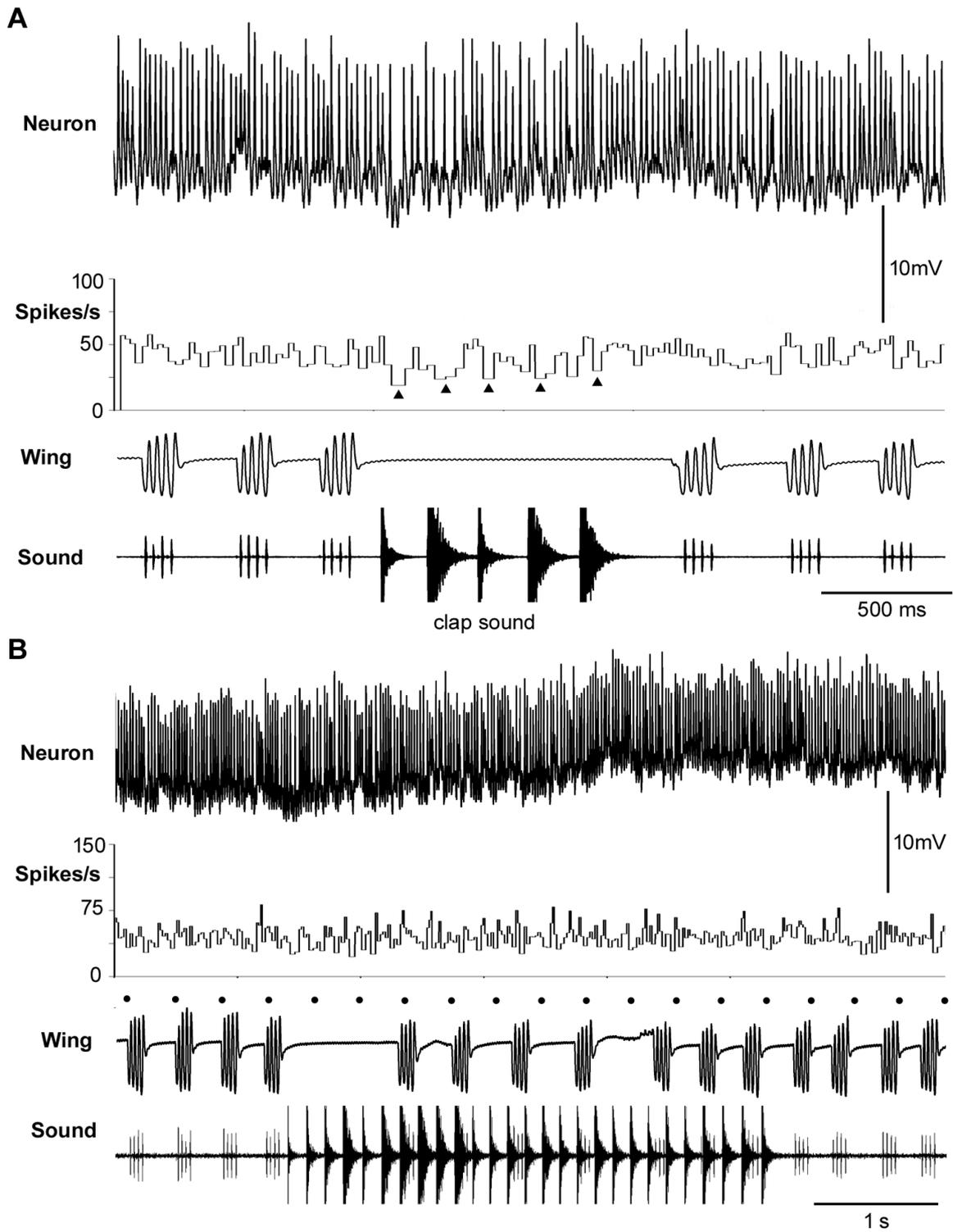
Figure 5.12 Ongoing calling song stridulation was temporarily disturbed by loud clapping sound.

(A) In response to clapping the neuronal activity of calling song command neuron was slightly reduced, indicated by arrow heads. (B) The chirp pattern disturbed by repeated clapping for 4 seconds. Dots denote anticipated start point of chirps if there were no clapping sound.

5.3.6 *Calling song stridulation elicited by injury to the putative command neuron (pCN) for calling song in G. assimilis*

The calling song of *G. assimilis* consists of chirps with a duration around 100 ms, and unusual long chirp intervals in the range from 500 ms to more than 1 s. Each chirp normally contains 7-9 sound pulses with pulse period of 10-15 ms, (Pollack and Kim 2013) (also see Chapter 4). In *G. assimilis* intracellular recordings targeting the brain region where the command neuron was identified in *G. bimaculatus* (Hedwig 2000) revealed a neuron related to calling song stridulation. The recording electrode encountered the neuron at depth of 120-220 μm from the ventral surface of the brain. Penetrating or damaging the neuron released sequences of calling song activity (**Figure 5.13**). The forewings were raised into singing position and rhythmic opening-closing wing movements generated chirps of the calling song. The chirp rate was about 5 Hz and fastest for the first two chirps and declined to 2 Hz over the next 3 s and then 1 Hz before the calling song stopped. Pulse number per chirp increased from 1 pulse at the beginning to 8 pulses as the chirping went on. In these experiments the calling song lasted for 20 seconds to a minute and then ceased. The neuron was subjected to current injection if properly penetrated.
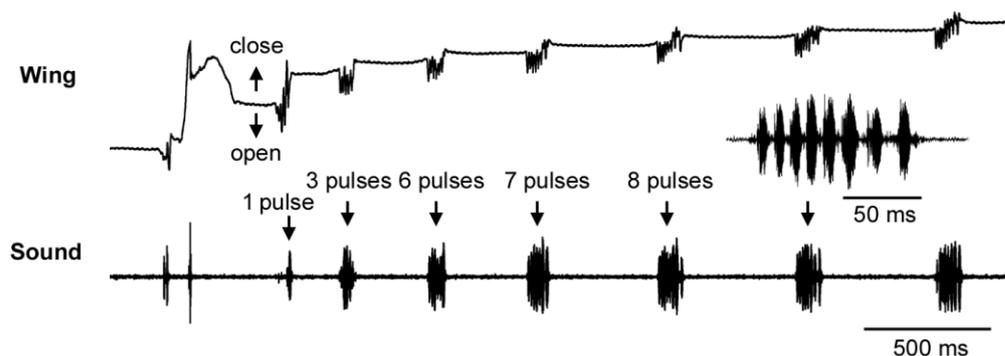
Figure 5.13 Calling song stridulation elicited by penetrating or damaging the pCN for calling song in the brain of *G. assimilis*.

A likely contact of electrode and neuron while searching for a stable recording triggered calling song activity including a raised wing position and rhythmic opening (downward)- closing (upward) wing movements. Note the overall change in wing position and the gradually increasing pulse number in chirps. Details of one chirp are shown in the inset to show details of sound pulses in chirps, note the increasing pulse period within the chirp.

5.3.7   *Testing the sufficiency criterion: Depolarization of the putative command neuron (pCN) for calling song initiated calling song stridulation in G. assimilis*

To test the sufficiency criterion a depolarizing current was gradually increased from 0.0 to 3.0 nA and applied to the pCN in *G. assimilis* to examine the effect of the neuron's spike activity on singing (**Figure 5.14**). The recording of the putative command neuron showed tonic spike activity with no evidence of prominent EPSPs. Spike activity was enhanced from less than 25 APs/s to almost 75 APs/s during the depolarization. The male began to stridulate calling song when the current reached 2.9 nA and the spike rate about 50 APs/s. The wings were raised after the first chirp. Pulse number in chirps gradually increased from 1 pulse to 7-8 pulses. Chirp rate was between 0.7 and 1 Hz. Sound pulse rate was much faster in the leading pulses (up to 100 Hz) than the following pulses (55 Hz the lowest) (**Figure 5.13** enlarged chirp) and was decreasing within the chirp. This experiment demonstrates that the activity of this neuron initiates the calling song stridulation, and thus fulfilled the criteria of sufficiency for a

command neuron for calling song (Kupfermann and Weiss 1978).
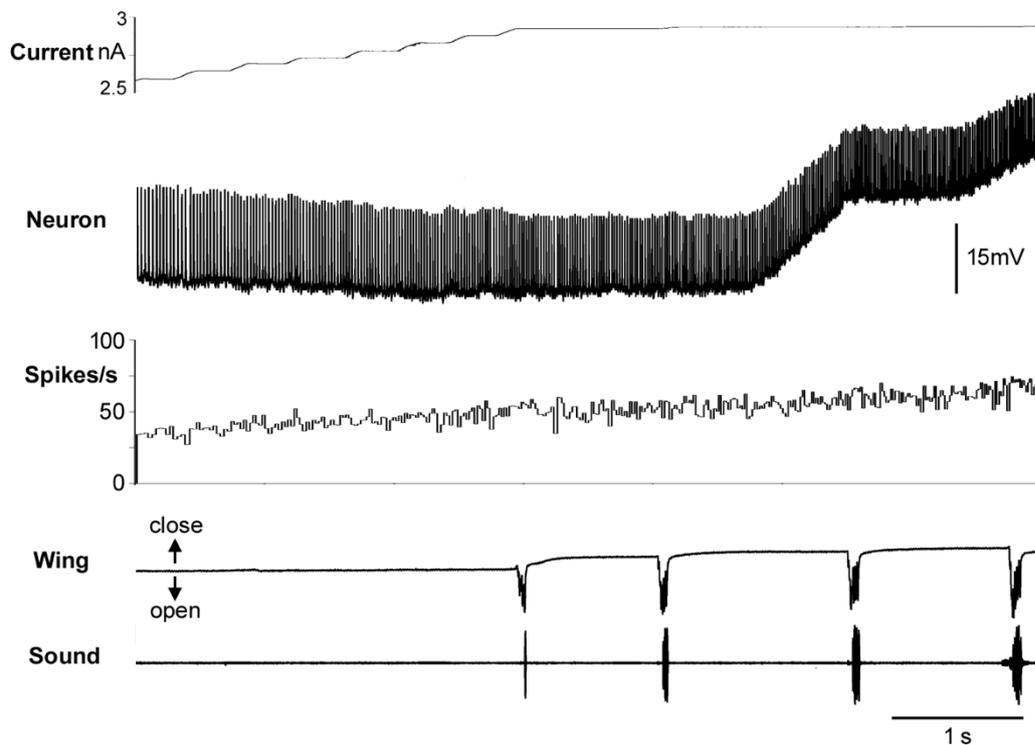
Figure 5.14 Stridulation of calling song triggered by depolarizing current to the pCN for calling song in male *G. assimilis*.

An increasing depolarizing current to the pCN for calling song elicited calling song stridulation. Note the increase in the wing position after the first chirp and the increasing pulse number in the chirps with increasing spike activity.

In order to test the immediate effect of the neuron on calling song activity, a 1.5 nA depolarizing current was injected into the pCN. The experiments were carried out under two behavioural states of the males. In the first scenario the male was in the resting state (**Figure 5.15**). The applied current increased the neuronal activity to an initial peak of about 225 APs/s which declined over 200 ms and stabilized at 180-190 APs/s. About 250 ms after the depolarization, the male began stridulation and raised its wings and kept the wings raised for several seconds until the second current pulse was delivered. The chirp rate was 1.2-1.3 Hz, and the sound pulse rate was 90-100 Hz for the leading pulses in a chirp and 60-70 Hz for the

following pulses towards the second half of the chirps. Pulse number was 3-6 pulses per chirp. In the two current injections, the second chirp was generated 300 ms and 100 ms after the depolarizing current, respectively, while the wings maintained a raised position.
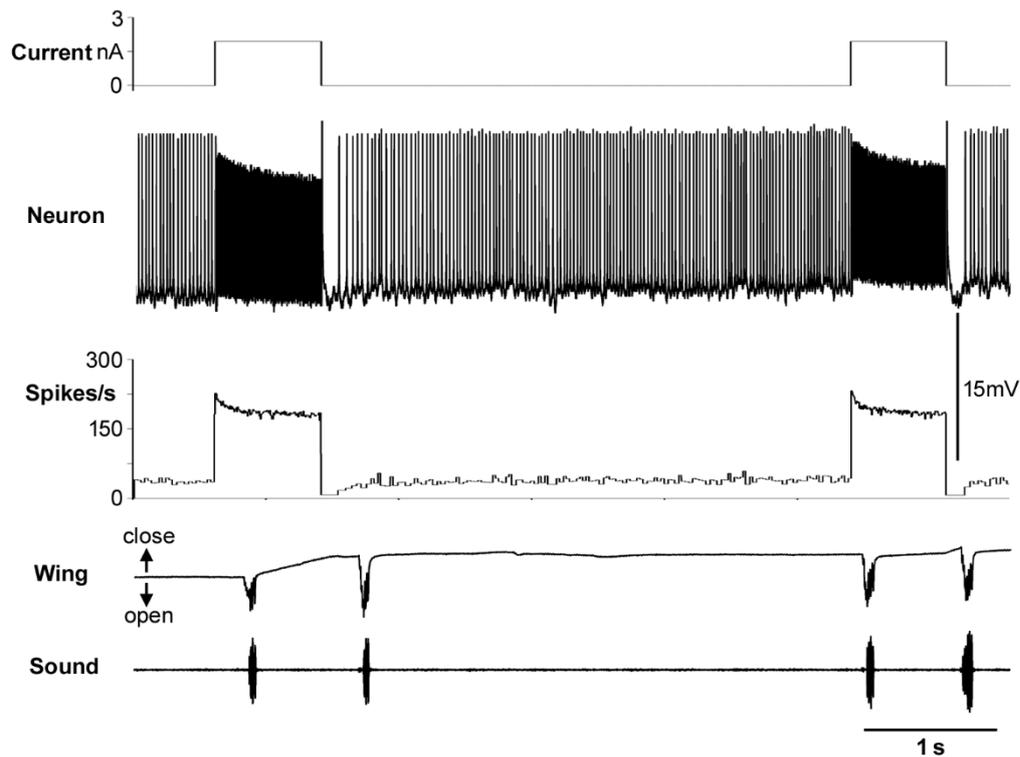
Figure 5.15 Depolarization of the pCN for calling song in *G. assimilis* elicited calling song stridulation. When the male was in a resting state a 1.5 nA depolarizing current was applied for 800 ms and for 650 ms to the neuron. The male raised the wings and generated two chirps per stimulus, while the wings were kept in the raised position. Note, the delay from depolarization to the start of the calling song stridulation, and that in both attempts the second chirps were generated after the pCN activity had declined.

In the second scenario, depolarizing current was applied during ongoing calling song activity (**Figure 5.16**). In this case, there was no obvious change in wing position. Spike rate of the neuron was enhanced from about 50 APs/s to a transient burst of 225 APs/s and then stabilized at 130-150 APs/s. Chirp rate was increased from 0.5-0.7 Hz to 0.9-1.3 Hz, while the sound pulse rate was not altered by the enhanced neuronal activity. It felled in the range of 60-

100 Hz within a chirp, with the leading pulses occurring at a higher rate (up to 100 Hz) than the following pulses (60 Hz the lowest). Pulse number per chirp did not change during the depolarization. After removal of the depolarizing current, the neuron stopped firing for about 600 ms. These depolarization tests demonstrated the neuron's spike activity could initiate calling song stridulation and increase the chirp rate of the calling song.
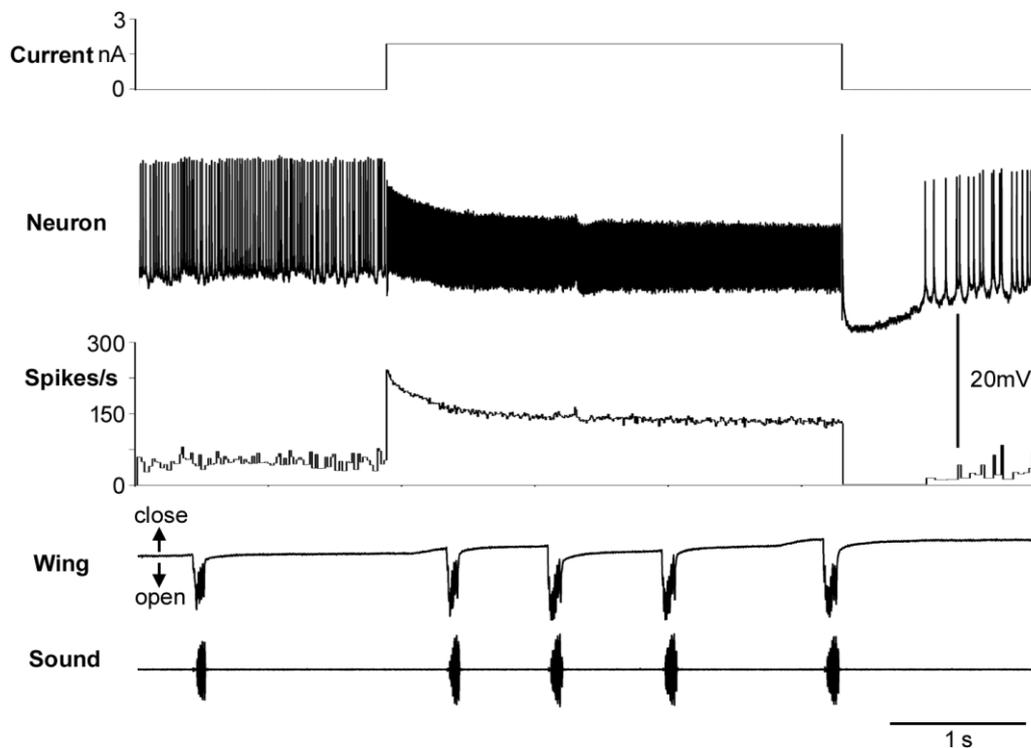
Figure 5.16 Depolarization of the pCN for calling song in *G. assimilis* increased the chirp rate of ongoing calling song.

A 1.5 nA depolarizing current was injected to the neuron while the male was already generating calling song. During the current injection the spike rate of the neuron increased and so did the chirp rate.

5.3.8 Testing the necessity criterion: Hyperpolarization of the putative command neuron (pCN) for calling song slowed and terminated the calling song in G. assimilis

To test the necessity criterion a hyperpolarizing current was applied to the putative

command neuron in *G. assimilis* to evaluate if the neuron's spike activity is necessary for ongoing calling song activity. A 2 nA hyperpolarizing current was injected to the neuron when the male was already generating calling song with chirp rate of 1.5-1.8 Hz (**Figure 5.17**). Neuronal spike activity was totally abolished by the hyperpolarization and the chirp rate was reduced to 0.7-0.8 Hz in 1 s. Removal of the hyperpolarizing current restored the neuronal activity. The spike rate of the neuron first reached a peak of 250 APs/s and then after 300 ms declined to about 100 APs/s. During the increased spike activity, the chirp rate was raised again to 1.8 Hz. The control of the chirp rate was reliably reproduced by several sequences of current injection. Pulse rate and pulse number in the chirps were not affected by the changing neuronal activity.
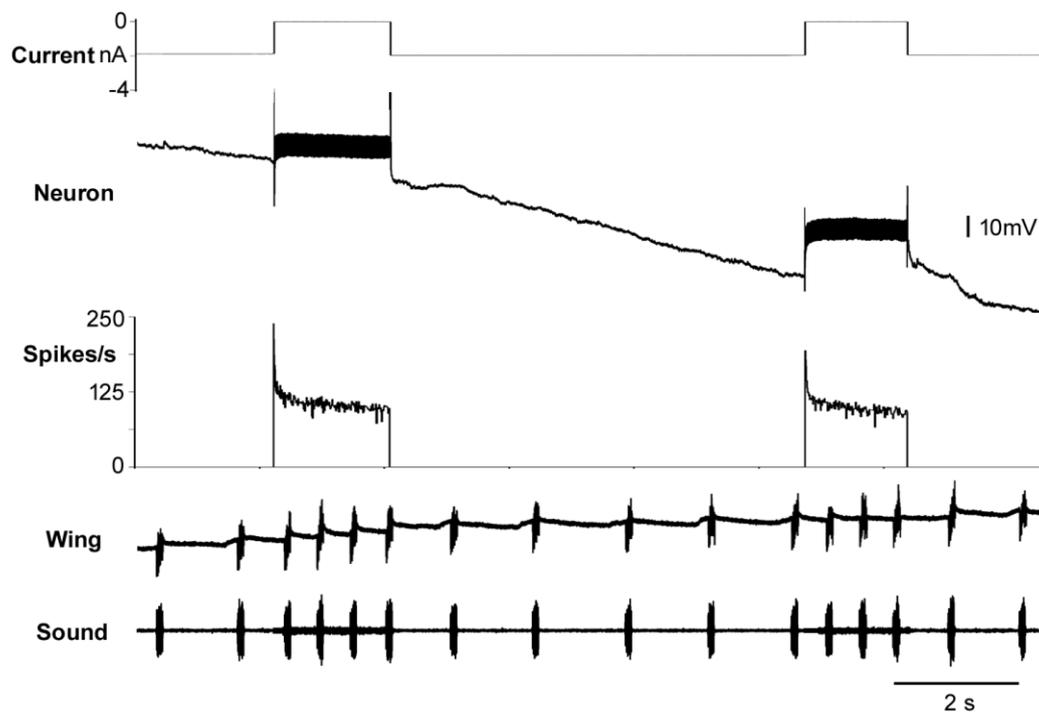
Figure 5.17 Hyperpolarization of the pCN reliably reduced the chirp rate of ongoing calling song in *G. assimilis*.

An ongoing -2 nA hyperpolarizing current was injected into the neuron and removed for 2 s and for 1.5 s. Note the increased spike rate of the neuron and the increase in chirp rate when the hyperpolarizing current is removed and the neuron is activated.

In another case, the hyperpolarizing current applied started from -1 nA and continuously increased in amplitude (**Figure 5.18**). The chirp rate declined and the male stopped to generate calling song chirps when the spike rate was below 30 APs/s. These experiments proved the putative command neuron can reduce the chirp rate or even terminate the calling song singing activity. Thus, it met the criteria of necessity for a command neuron (Kupfermann and Weiss 1978).
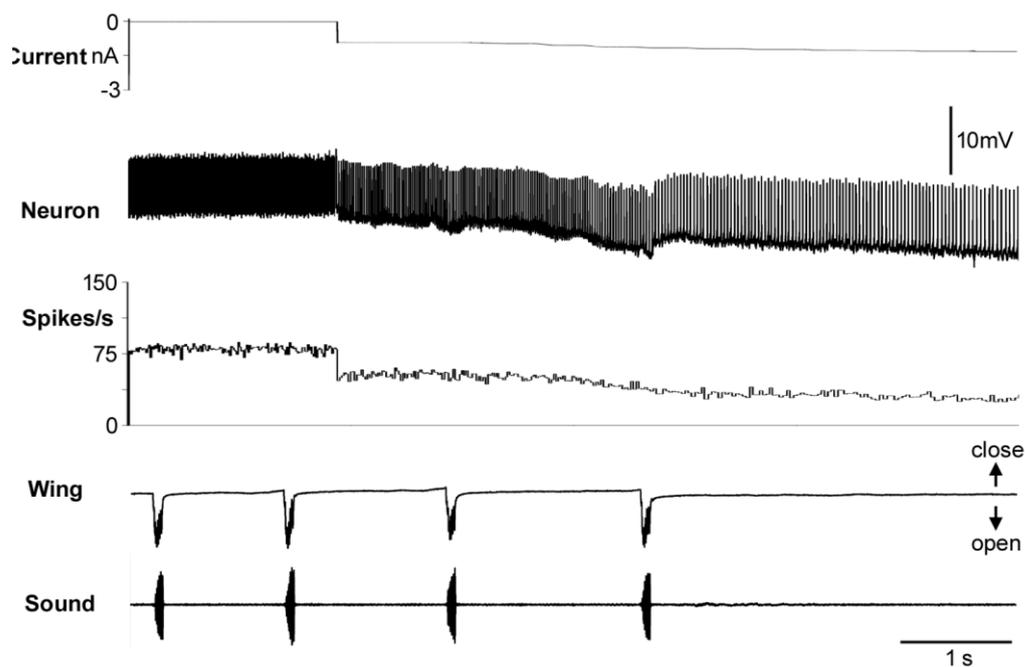
Figure 5.18 Hyperpolarization of the pCN for calling song in *G. assimilis* terminated ongoing calling song stridulation.

Hyperpolarizing current with increasing amplitude from 1 nA was applied to the putative command neuron. Note the sound pulse number in chirps was not reduced before the song stopped.

5.3.9   *Correlation of spike frequency of the pCN for calling song with chirp rate and sound pulse rate in G. assimilis*

Statistical analysis of 38 chirps in *G. assimilis* calling song and the corresponding pCN

spike frequency reveal the relationship between spike frequency and the chirp rate and the sound pulse rate, respectively (**Figure 5.19**). Pulse rate was calculated from the first two pulses of each chirp as pulse rate in *G. assimilis* calling song varied in a wide range within chirps (60-100 Hz). The range of spike frequency was 0 to 185 spikes per second, the chirp rate ranged from 0.4 to 2.25 chirps per second, and the range of the pulse rate was 75 to 100 pulses per second. Plotting spike frequency against chirp rate revealed a positive correlation (R = 0.8595) (**Figure 5.19A**), while no correlation was found between spike frequency and sound pulse rate (R = 0.1640) (**Figure 5.19B**). This suggests the changing neuronal activity of pCN affected chirping rate, which was also shown by the depolarization and hyperpolarization experiments (**Figure 5.16, 5.17, 5.18**), while sound pulse rate was not altered by neuronal activity of pCN.
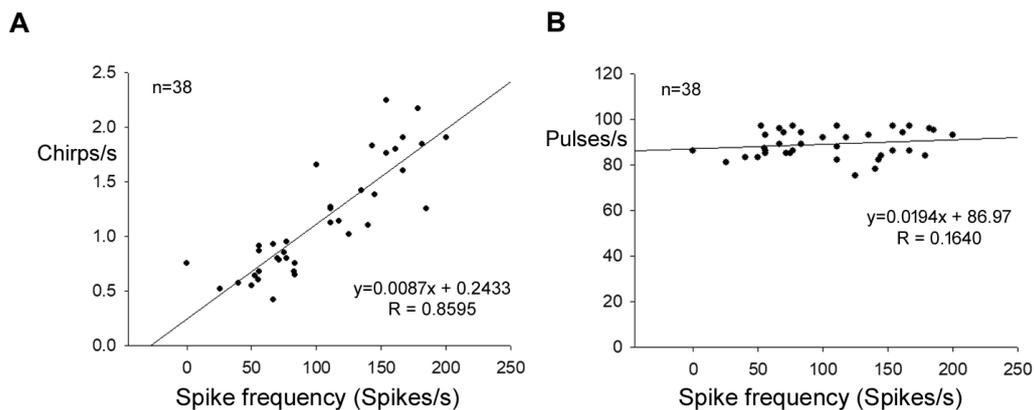
Figure 5.19 Statistical correlation between the pCN spike frequency and the chirp rate, and the sound pulse rate in *G. assimilis*.

(A) There is a high correlation (R = 0.8595) between the pCN spike frequency and the chirp rate. (B) There is no correlation (R = 0.1640) between the sound pulse rate and the pCN spike frequency.

5.3.10 Evidence for a command neuron for calling song in T. commodus

Calling song of *T. commodus* contains phrases with two components, chirps and trills, each generated with a different pulse period (chirps: 60 ms, trills: 40 ms). A normal phrase contains one chirp followed by a few trills (Simmons et al. 2005; Bailey et al. 2017) (see also

Chapter 4). An example of *T. commodus* calling song is shown in **Figure 5.20A**. While searching for intracellular recording of the command neuron in the brain of *T. commodus*, wing movements similar to calling song stridulation were triggered (**Figure 5.20B**). The wing movements can be categorized into two groups. Each group corresponded to either chirps or trills based on the relationship of the wing up-down movement cycle period to the pulse period of chirps and trills, and the number of up-down cycles compared to the number of pulses in chirps and trills. One group of wing movements contained fewer up-down cycles and was generated when the wings were in higher position (wing movements labelled by black bars in **Figure 5.20B**). The wing up-down cycle period was in the range of 50-70 ms, which is close to the pulse period in chirps. The other group of wing movements was generated when the wings were in a lower position and the number of wing up-down cycles was higher. The wing up-down cycle period in this group was in the range of 30-40 ms, which is similar to pulse period of trills. The two different wing movement patterns and their temporal relationship to the pulse period of chirps and trills suggest the wing movements recorded during intracellular recording were likely wing movements of calling song stridulation and point toward a right brain area in searching of command neuron for calling song in *T. commodus*.
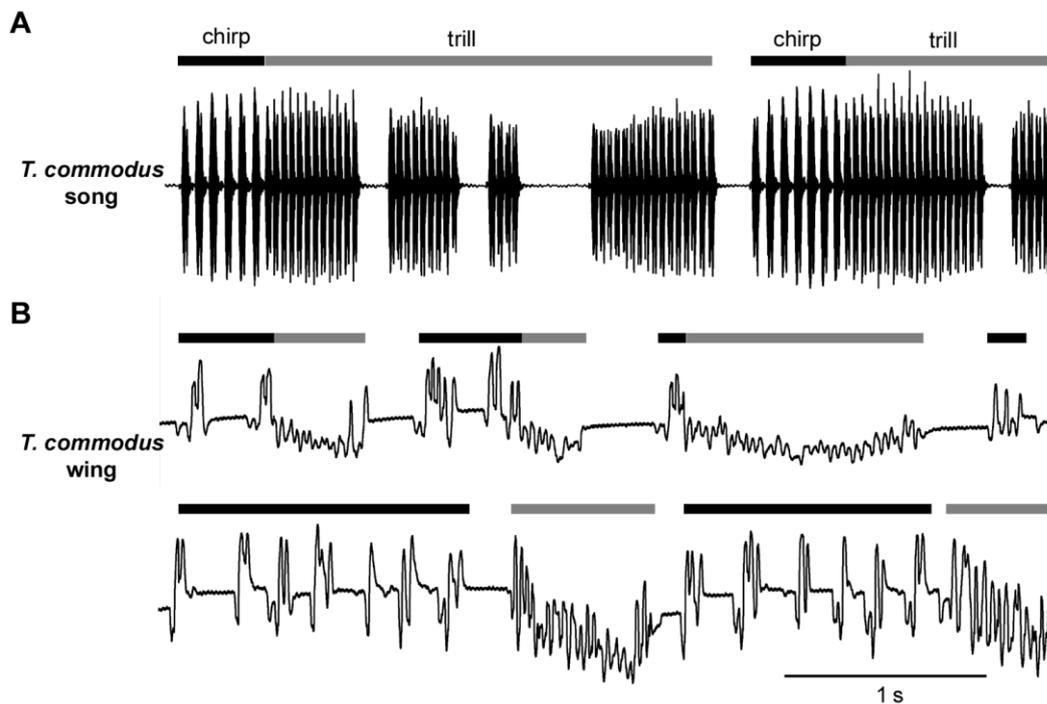
Figure 5.20 Alignment of calling song recorded from a freely moving *T. commodus* male and wing movements recorded during searching for the calling song command neuron in another *T. commodus*.
(A) Sound recording shows the chirp and trill pattern of two phrases. (B) Wing movements during neuronal recording experiments revealed two different patterns. One pattern recorded at a higher wing position with 2-5 wing up-down cycles and a cycle duration of 50-70 ms (indicated by black bars), while during the other patterns the wing was lower and exhibited 7-30 wing up-down cycles with a cycle duration of 30-40 ms (indicated by grey bars).

5.3.11 Testing the sufficiency criterion: Depolarization of the putative command neuron

(pCN) for calling song elicited stridulation-like wing movements

Intracellular recordings in the brain of *T. commodus* revealed a neuron related to calling song stridulation (**Figure 5.21**). The amplitude resolution of the neuron was restricted by a massive change on membrane potential during current injection, and this also applies to following experiments. This neuron showed tonic activity with no prominent EPSPs when firing. Activate ion of the neuron by contact with the electrode during the search phase of the

experiments led to slow up-down wing movements. Upon penetration, the spike rate reached above 150 APs/s and gradually decreased to 50 APs/s over 3 seconds. Three 2 nA depolarizing current pulses were applied to the neuron and repeatedly raised the spike rate to 80-100 APs/s. The wing position was raising before and during the depolarization and slow wing up-down movements appeared over the whole process. During the third depolarization, the wing recording showed three sets of up-down movements similar to wing movements for stridulation, however, no sound was generated. Removal of the third depolarizing current stopped the singing-like wing movements while the slow wing up-down movement still occurred. After each depolarization, the neuronal activity dropped below 40 APs/s.
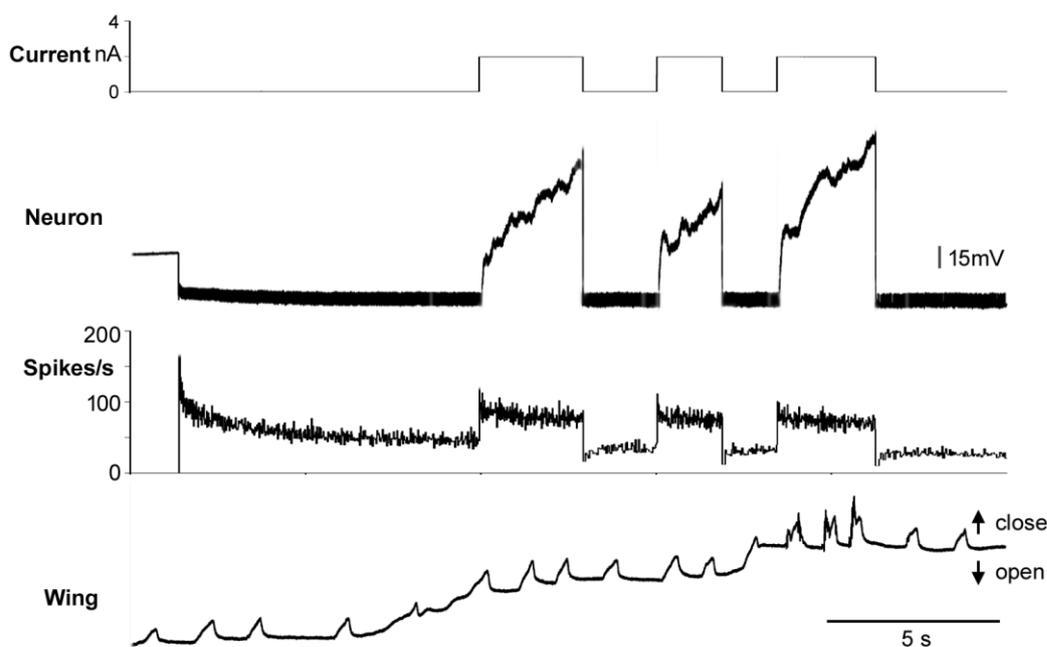
Figure 5.21 Depolarization of the pCN for calling song raised the forewings and elicited stridulation-like wing movements in *T. commodus*.

The neuron was stably recorded and stimulated with three 2 nA depolarizing current pulses. Note the raising of the wing position, the stridulation-like wing movements during the third depolarization, and the slow wing up-down movements.

In another depolarization experiment, 2 nA depolarizing current was injected to the neuron

when the spike rate was around 20 Aps/s and the male was not showing any stridulation-like or slow wing movements (**Figure 5.22**). The neuronal activity was transiently increased to 100 Aps/s and stabilized at 70 Aps/s after 300 ms. The male started slow wing up-down movements 100 ms after depolarization and stridulation-like wing movements 1.5 seconds after depolarization. The stridulation-like wing movements were irregular regarding up-down cycle period and superimposed with slow wing up-down movements, and there was no sound generated during the wing movements.

Thus, it is difficult to tell if the wing movements relate to chirps or trills. The depolarization lasted 4.5 seconds and after that the male stopped any wing movements and the neuron stopped firing for 200 ms.
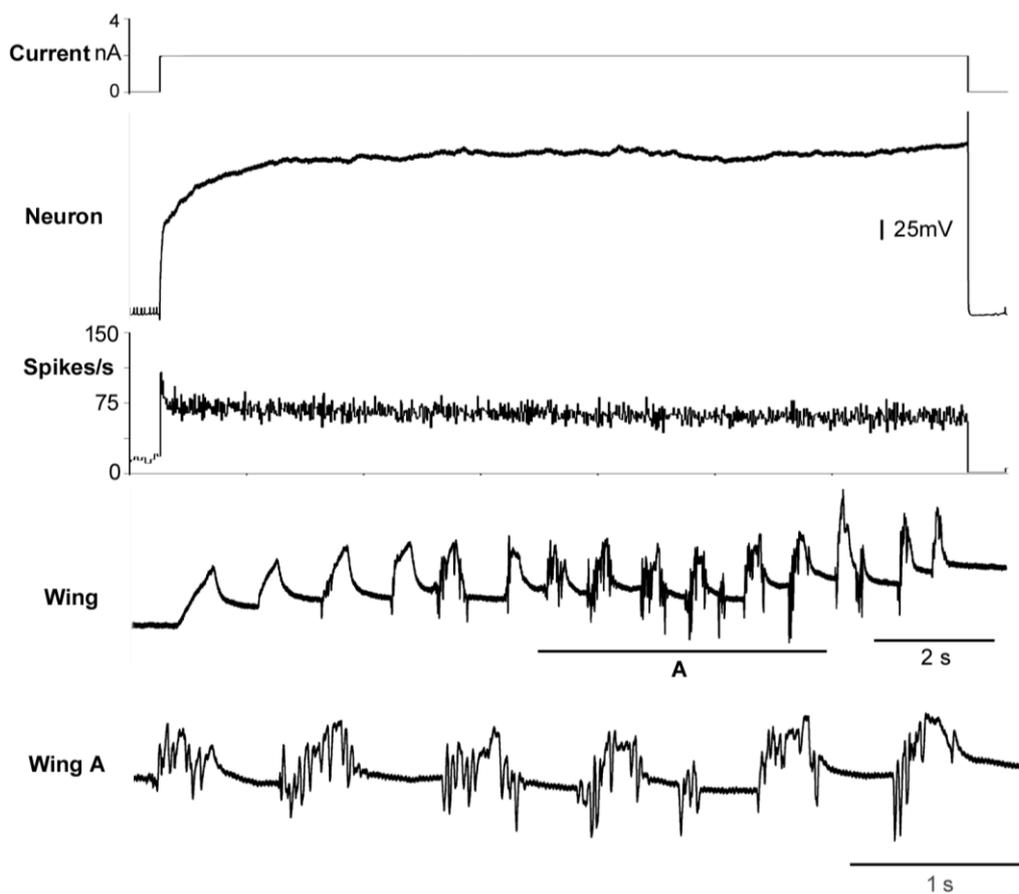
Figure 5.22 Depolarization of the pCN for calling song initiated both slow wing up-down movements and

stridulation-like wing activities in *T. commodus.*

A 2 nA depolarizing current was applied to the putative command neuron for 14 seconds. Note the slow wing up-down movements and stridulation-like wing activities during depolarization.

5.3.12 Testing the necessity criterion: Hyperpolarization of the putative command neuron

(pCN) for calling song terminated stridulation-like wing movements

A 2 nA depolarizing current and 2 nA hyperpolarizing current was sequentially applied to the putative command neuron to relate the neuron's activity to stridulation (**Figure 5.23**). The 2 nA depolarization increased the spike rate to 150 APs/s instantly and stabilized at 70 APs/s after 300 ms. Slow wing up-down movements started 150 ms after depolarization and stridulation-like wing movements began 2 seconds after depolarization. Though no sound was generated, two groups of wing movements can be identified. One group of wing movements recorded when the wings were in higher position with fewer wing up-down cycles and longer wing up-down period (50-60 ms) were likely for generation of chirp pulses (**Figure 5.23**, black bars in Wing A recording). The other group of wing movements were recorded at lower wing position with more wing up-down cycles and shorter wing up-down period (30-40 ms) were likely for the production of trill pulses (grey bars in Wing A recording).

The stridulation-like wing movements stopped as the depolarizing current was removed, while the slow wing up-down movement still appeared. The neuronal activity dropped to 30 APs/s without the depolarizing current. A following 2 nA hyperpolarizing current injection abolished the activity of the neuron and the slow wing up-down movement stopped. The depolarization and hyperpolarization test suggest the stridulation-like wing movements can be triggered and abolished by controlling the activity of putative command neuron. Together these findings imply the existence of a command neuron for calling song stridulation in the brain of *T. commodus*.
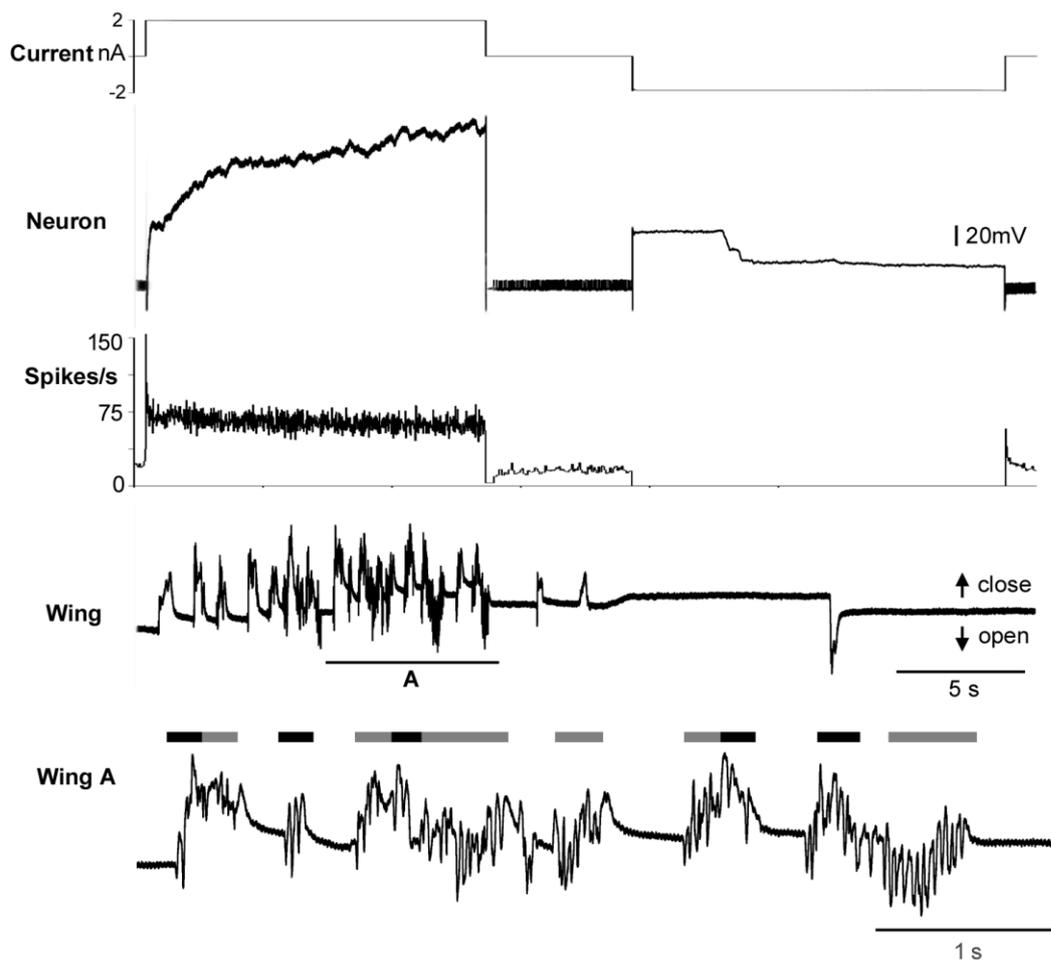
Figure 5.23 Depolarization and hyperpolarization of the pCN for calling song controlled both slow wing up-down movements and stridulation-like wing movements in *T. commodus*.

A 2 nA depolarizing current and 2 nA hyperpolarizing current were sequentially applied to the putative command neuron. Black and grey bars indicate the wing recordings likely for generation of chirp pulses (Black) and trill pulses (Grey). Note the slow up-down movements and stridulation-like wing movements.

5.4   Discussion

In this study putative command neurons for calling song stridulation were physiologically identified by intracellular recording in the same region of the brain in three cricket species, *G. bimaculatus*, *G. assimilis*, and *T. commodus*. These neurons showed tonic activity without

temporal information related to song parameters, and no prominent synaptic potentials were observed during activity. The control of calling song stridulation by the pCNs was similar. In all three species, depolarization of the neurons was sufficient to trigger raising of the wings into singing position and calling song stridulation, or stridulation-like wing movements in *T. commodus*. Hyperpolarization of the neurons could abolish ongoing calling song stridulation, and stridulation-like wing movements in *T. commodus*. Thus, the neurons discovered in *G. bimaculatus* and *G. assimilis* fulfilled the criteria sufficiency and necessity for the characterization of calling song command neurons, and the neuron found in *T. commodus* is a likely candidate for a calling song command neuron. The same recording area, similar control of calling song stridulation, and similar physiological properties of the neurons in three cricket species suggest the calling song command neurons are a conserved cellular system to elicit and control the species-specific calling song by tonic activity. However, in this study labelling of these neurons was not successful and revealing the morphology of these neurons requires further investigation. The command neuron discovered in *G. bimaculatus* was recorded in the region where extracellular current stimulation elicited calling song (Huber 1960, 1963) and a command neuron for calling song was identified (Hedwig 1996, 2000). The similar physiological and functional properties of the neuron described in this study and the command neuron reported before highly suggests these two neurons are the same one.

In *G. bimaculatus* and *G. assimilis*, higher neuronal activity of the command neuron for calling song corresponded to a higher chirp rate, while sound pulse rate and pulse number in chirps were not affected by changing spike rates. Together with the tonic activity of the pCN this indicates that the species-specific pulse pattern is controlled by the singing-CPG located in the abdominal ganglia. In *G. bimaculatus*, CPG neurons acting as pulse timers (pulse opener and pulse closer) and chirp timers (chirp start neuron and chirp interval neuron) were identified along the abdominal nerve cord (Jacob and Hedwig 2020). The pulse timer neurons housed in

abdominal ganglia A3 and A4 define the pulse period and thus pulse rate, and the chirp timer neurons in A4-A6 determine start and stop of chirps by controlling activity of pulse timer neurons. In *G. bimaculatus*, *G. assimilis*, *G. rubens*, *T. commodus*, and *T. oceanicus*, homologous pulse opener interneurons were identified in A3, they were similar in morphology and in the way driving the pulse patterns, yet demonstrated different membrane depolarization patterns during singing (Jacob and Hedwig 2019). These findings regarding CPG neurons, combined with current results of command neurons controlling calling song, suggest in different species a shared conserved neural network for singing behaviour in which command neurons with tonic activity elicit calling song stridulation and the CPG neurons provide the temporal information for the species-specific song pattern. The relationship of command neuron activity and chirp rate might imply that the command neurons, directly or indirectly connect with chirp timer neurons and drive the chirp period. In addition, in hyperpolarization experiments in *G. bimaculatus* and *G. assimilis* which suppressed pCN activity, the males continued generating chirps for 5 to 25 seconds. This indicates the singing-CPG network, once activated by the command neuron, can sustain singing activity for a short period of time without an input from the command neuron. After lesion of the cervical connectives, abolishing any link between the pCN and the singing-CPG, the singing-CPG can even generate the calling song autonomously, given sufficient time for reorganization (Kutsch and Otto 1972).

Male crickets produce long-range calling song to attract conspecific females, courtship song to initiate courtship behaviour with females, and rivalry song during confrontation with another male (Alexander 1961). Calling song, courtship song, and rivalry song were shown to be elicited by extracellular electrical stimulation of different locations in the brain of *G. campestris* or microinjection of neuroactive substances into the brain of *G. bimaculatus* (Huber 1960, 1963; Wenzel and Hedwig 1999). Transitions between the calling song and courtship song, and between the calling song and rivalry song were reported during extracellular brain

stimulation and connective stimulation experiments (Huber 1960; Otto 1971). Antennae contact with isolated antenna from conspecific males or females containing sex-specific pheromone triggered generation of rivalry song or courtship song in *G. bimaculatus*, respectively (Nagamoto et al. 2005). These experiments suggest the generation of courtship song and rivalry song is controlled by the brain. In *Aplysia*, a multifunctional interneuron CC5 was described involved in head lifting, head turning, head withdrawal, tentacle withdrawal, feeding, and locomotion (Xin et al. 1996). In this study and in Hedwig (2000) current injection into the command neurons of *G. bimaculatus* and *G. assimilis* never elicited courtship song or rivalry song even when the spike rate reached over 150 APs/s. This does not support a multifunctional role of these command neurons, they are rather labelled lines for specific motor programs. In the acridid grasshopper *Omocestus viridulus* three stridulatory hindleg movement patterns constitute a courtship sequence, and each movement pattern is elicited by activation of one of three different descending brain interneurons(Hedwig and Heinrich 1997). In the cricket stridulation system, there may be two other command neurons for courtship song and rivalry song, and further electrophysiology experiments are required to identify the neural control of these two song types.

Clapping sound made during ongoing calling song stridulation transiently stopped the generation of calling song chirps and slightly reduced the activity of the command neuron in *G. bimaculatus*. The hyperpolarization experiments demonstrated that the generation of chirps could continue for 5 to 25 seconds without spike activity of the command neuron; therefore, the stop of chirps during clapping likely was not caused by the reduced activity of the command neuron. While clapping could activate multiple sensory pathways like acoustic signalling and substrate-born vibration; the cercal escape pathway triggered by the loud sound and air currents generated during clapping might be the explanation for the transient interruption of the chirp pattern. Previous studies delivering air puffs to the cerci had effectively stopped ongoing

calling song (Dambach and Rausche 1985; Jacob and Hedwig 2015)without interrupting the calling song CN activity (Hedwig 2000). Application of air puffs during different phases of the calling song chirp cycle stopped the song prematurely or lengthened the interchirp interval by affecting the opener interneuron in the A3, which is one of the crucial pulse timer neurons of the singing-CPG (Jacob and Hedwig 2015). The continuous clapping (**Figure 5.12B**) contained different stimulation times in relation to the chirp cycle and thus the chirp pattern was interrupted and some chirps were generated with extended intervals. As for the command neuron activity, with the many possible sensory inputs caused by clapping, it is not clear how the clapping transiently reduced the neuron's activity, but the pathway likely acted via the brain.

Examples of rhythmic activity driven by tonic neuronal activity of command neurons or command-like neuron were reported in different animal behaviour models. For feeding behaviour, in *Aplysia*, activity of a command-like neuron, the cerebral buccal interneuron-2 (CBI-2), was elicited by food stimuli and trigger rhythmic protraction and retraction movements during feeding. CBI-2 received inhibitory input during the retraction phase, and further depolarization and restoration of CBI-2 spike activity did not interfere with ongoing feeding rhythm (Hurwitz et al. 2005). In *Pleurobranchaea*, paracerebral neurons (PCNs) are sufficient to elicited feeding behaviour when the animal is presented with food stimuli but the neurons are not necessary for the behaviour to occur. The tonic activity was receiving cyclic inhibitory feedback during feeding and depolarization of PCNs enhanced the spike rate but did not remove the inhibition (Gillette et al. 1978, 1982). For swimming behaviour, in the marine mollusk *Tritonia diomedea*, activation of the dorsal ramp interneuron (DRI) was shown to excite the CPG neurons, cerebral cell 2 (C2) and the dorsal swim interneuron (DSI). The rhythm of DSIs was determined by C2 but not by the dorsal ramp interneuron (DRI) activity (Frost and Katz 1996). In *G. bimaculatus*, depolarization of four swimming initiating neurons (SINs) in subesophageal ganglion triggered synchronized motion of the legs (Matsuura et al.

2002). Different from swimming and feeding behaviour, the tonic discharging rate of command neurons for stridulation in grasshopper and cricket is related to the behaviour output. In the acridid grasshopper *O. viridulus*, the enhanced spike rate of the command interneuron for stridulation (B-DC-3) led to a higher amplitude of leg movements during stridulation (Hedwig 1994). In the current study, depolarization of the command neurons for calling song in *G. bimaculatus* and *G. assimilis* increased both the neuronal activity and the chirp rate. These neurons in charge of different behaviour demonstrate a similar control of rhythmic movements by tonic activity, while for some behaviour the tonic discharge rate might also affect the amplitude of the behaviour output.

5.4.1 *Future perspective*

Physiological evidences provided in the current study suggest the existence of command neurons for calling song in a particular area of the brain not only in *G. bimaculatus,* but also in *G. assimilis* and *T. commodus*. Revealing the morphology of the neurons would be essential to provide a complete picture of the similarities between the species. *T. commodus* as phrase-producing species, presents an opportunity to study to what extent the command neurons are controlling the timing of phrases, chirps, or trills. The staining of command neuron in *G. bimaculatus* revealed a descending interneuron morphology and indicated the axon extends at least to the prothoracic ganglion, while the exact terminal arborisations of the neuron are not clear so far (Hedwig 1996, 2000). A complete morphology of the command neurons could reveal the site of possible synaptic connections to singing-CPG neurons, which subsequently would need to be identified by simultaneous intracellular recordings. This would further reveal the control mechanism of calling song stridulation by identified command neurons.

6 Chapter Six: A system for bulk selection of female crickets based on phonotaxis preferences

Abstract

Acoustic communication in crickets includes male song production and female phonotactic response to sound signals. To study female phonotaxis, treadmill and trackball system were developed to track female walking in response to acoustic signal, Y-maze experiments were used to test female preferences, and high-speed camera and video-recording were adapted to capture movements during female phonotaxis. In current study, a new three-chamber system was created for animal selection based on female phonotaxis preferences. The three chambers in the system represent start point (start chamber), buffering (buffer chamber), and sound source (sound chamber), and three chambers were connected by two bridges to form an enclosed system. Females were introduced to start chamber and allowed to move freely inside the system. Different acoustic signals were played through a loudspeaker installed in sound chamber for 24 hr duration and female number in each chamber was recorded after each acoustic signal. By presenting different acoustic stimuli, the system could sort out the individuals that favoured the given sound signals. Preliminary results suggest the system is capable of filtering out specific species from mixture of two species by playing conspecific songs, and can be used to test theoretically attractive and non-attractive acoustic signals.

6.1 Introduction

Selection and breeding of animals and plants has been an effective strategy to improve the quality, productivity, or other properties that meet human needs in various species, and were extensively applied to filter out individuals which express specific traits (Dugatkin 2018). The genetic basis of the selected traits was thus preserved during the process of selection (Parker and Ostrander 2005). In experimental biology, screening of animals with specific traits or induced mutations provides opportunities to study genetic background of animal behaviour (Funato 2020). In cricket acoustic communication, male crickets generate stereotyped calling song and female crickets respond by phonotaxis. The calling songs produced by males and the preference to calling songs in females were demonstrated to share a coupled genetic basis by series of hybridization experiments of two *Teleogryllus* species, *T. commodus* and *T. oceanicus* (Bentley and Hoy 1972; Hoy and Paul 1973; Hoy 1974; Hoy et al. 1977). Calling songs generated by males of each species or reciprocal hybrids were used to test the phonoresponse of females from each group, and the results suggested the females of different groups prefer songs generated by males from the same groups. Same results were drawn by using two closely related *Gryllus* species, *G. armatus* and *G. rubens* (Bentley and Hoy 1972). These experiments concluded that, the song structure of male crickets and preference to song structure in female crickets are traits decided by genetic background, and thus implied these two traits can be manipulated toward specific direction by means of selection and breeding.

The process of female phonotaxis can be separated into acoustic signal recognition and traveling towards the signal source (Hoy 1978; Weber and Thorson 2019). Successful phonotaxis requires attractiveness of the acoustic signal to the receiver and a feasible route for the animal to reach the destination. This study aims to build a device to filter out from a group of females, the females performing phonotaxis toward given acoustic signals and to separate

them from the remaining females showing different preferences. The prototype device developed based on these criteria consists of three chambers connected by two narrow tunnels. Female crickets were put into one chamber that serves as start point and a loudspeaker playing acoustic signal was placed in the chamber at the other end that served as destination. By playing sound stimuli with different attractiveness to the test group, only animals with a specific matching signal preference will make it to the destination. This allows selecting animals with particular behavioural features from a large group of individuals. Preliminary experiments include sorting out females responding to particular sound patterns, sorting out one species of females from a group of mixed species females, and testing the attractiveness of song patterns. Results suggest the design and performance of this system is feasible but it could further be improved. The ultimate goal is to use the device to sort out females with good phonotaxis responses for further behavioural tests, to select females with preference to specific acoustic signal that subsequently could be used to analyse the genetics underlying the preference, or to choose specific females for breeding to generate a cricket line with a specific phonotaxis preference.

6.2 Material and Methods

6.2.1 Experimental animals

G. bimaculatus, *T. oceanicus*, and *T. commodus* were reared and bred in the Department of Zoology, University of Cambridge. Crickets were kept in large boxes (52.5 x 36.5 x 28 cm) with a 12hr-12hr light:dark cycle and given *ad libitum* access to fish food, muesli, and water. Last instar female nymphs were isolated and kept in small boxes (17.5 x 11.5 x 13 cm) to monitor their age after final moulting and prevent contact to males prior to the experiments. Adults one to two weeks old after imaginal eclosion were selected for experiments. Treatments

and experiments complied with the principles of Laboratory Animal Care (ASAB Ethics Committee and ABS Animal Care Committee 2021).

6.2.2 The phonotaxis selecting system and experimental design

A prototype of the animal selecting system was built with three cardboard boxes (chambers, 30.5 x 21.5 x 23 cm) and two cardboard tubes (bridges, length 11 cm, diameter 5 cm) (**Figure 6.1**). A three-chamber system was built by connecting box 1 and 2 and box 2 and 3 with the cardboard tubes, which were placed 15 cm above the ground of the boxes. The three chambers were named as start chamber (1st chamber), buffer chamber (2nd chamber), and the sound chamber with a loudspeaker playing acoustic stimuli (3rd chamber). A rectangle was cut into the sides of the cardboard boxes and covered with transparent film to create observation windows. The three-chamber complex was placed on a table with the viewing windows facing the observer. Crickets were capable of moving freely on the ground and walls, but not on the ceiling of the chambers. For each experiment, two egg cartons, muesli, fish food, and a water-soaked cotton pad were placed in each of the chambers. The distance from the centre of the start chamber to the centre of the sound chamber was about 80 cm, and females had to negotiate the cardboard obstacles and elevated tubes in order to reach the sound chamber.

For each experiment a group of 10 or 20 female crickets depends on different experiments was introduced into the start chamber. The experiment started without presenting an acoustic stimulus (silence) for a day as a control reference. For each following day scheduled acoustic stimuli were played for entire day from the speaker in the sound chamber. The number of females in each chamber was then recorded after each acoustic stimulus. Females that moved to the sound chamber were scored as attracted by the acoustic stimulus. After counting the crickets in each box for each acoustic stimulus, all crickets were put back to the start chamber. For each experiment every sound stimulus was played once, so one experiment last (Number

of sound stimuli + 1 (for silence)) days. And then another batch of females were introduced into the system for another experiment.

After each experiment the chambers and the bridges were cleaned with 70% ethanol, and egg boxes, cotton pads, and remaining food were removed. Acoustic stimuli schedule for each experiment will be described in the Results section.
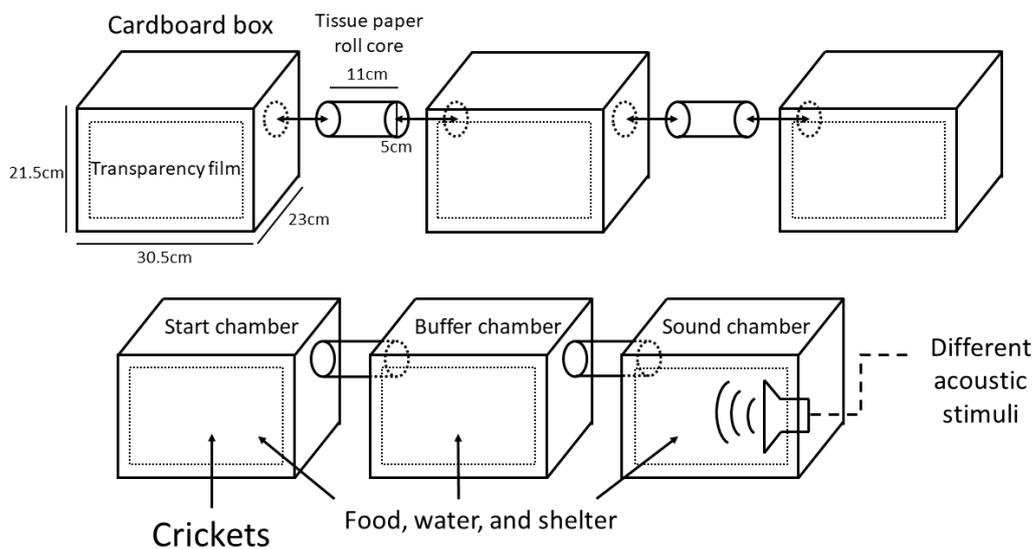
Figure 6.1 System for selecting female crickets based on phonotactic behaviour.
The system contains three chambers connected by two elevated bridges. Crickets were introduced into the start chamber and acoustic stimuli were presented in the sound chamber. Food, water, and shelter were placed in all the chambers. Different acoustic stimuli were played according to experimental design and changed on a daily basis. The number of crickets in the three chambers were recorded after each acoustic stimulus.

6.2.3 Data analysis

Statistical significance of differences of number of females in each chamber was tested by paired sample T-Test applying a two-tailed hypothesis or one-way analysis of variance (ANOVA) using SigmaPlot 11.0 (Systat Software, San Jose, CA). Bar graphs were generated by the average and standard deviation (X±SD) of each group.

6.3 Results

Several tests with a different number of crickets introduced into the selecting system under the silent control condition indicated that about 20 crickets is the capacity of the system. When the cricket number was lower than or equal to 20, less than two females moved to the buffer chamber or sound chamber. When 25, 30, or 40 crickets were introduced, 3 or more females were counted in the buffer chamber and sound chamber. This suggests the crickets moved to these chambers because of density issues in the start chamber and that a high number crickets introduced to the start chamber might influence the results of the selection experiments. Therefore, cricket groups of less or equal 20 were used in the following experiments.

6.3.1 Separation of a mixed group of two cricket species

To test the selecting potential of the system, 20 females from two cricket species (10 crickets for each species) were introduced into the start chamber. Each experiment started with one day silent control, followed by calling song presentation of one species for 24 hours, and then calling song presentation of another species on the next day. The order of presenting the calling song of the two species was random to avoid experimental bias. The female number in each chamber was recorded after each sound stimulus. Each experiment consists of silence, calling song from one species, calling song from another species was repeated five times, and the average and standard deviation (X±SD) of crickets in the different chambers are presented.

Separation experiments were carried out using groups of *G. bimaculatus* and *T. commodus*, and groups of *G. bimaculatus* and *T. oceanicus*. In the experiment of *G. bimaculatus* and *T. commodus* (**Figure 6.2B** upper trace), 0.6 ± 0.5 *G. bimaculatus* and 0.2 ± 0.4 *T. commodus* had moved to the sound chamber (3rd chamber) when exposed to the control experiment.
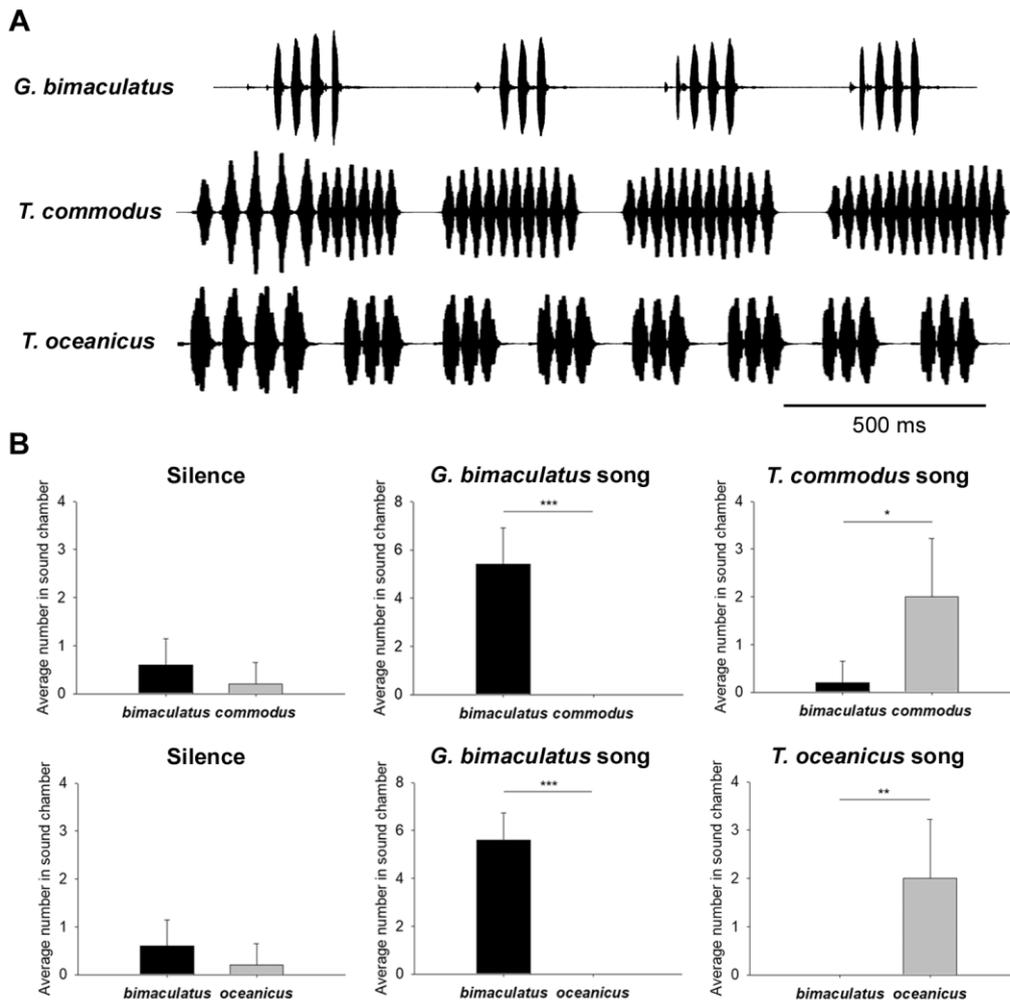
Figure 6.2 Separation of females of two cricket species by the selecting system.

(A) Calling song recordings of *G. bimaculatus*, *T. commodus*, and *T. oceanicus*. (B) Two separation experiments with mixed groups of *G. bimaculatus* and *T. commodus* (upper diagrams), and *G. bimaculatus* and *T. oceanicus* (lower diagrams). Number of crickets found in the sound chamber after different acoustic stimuli, averaged over 5 experiments. (*= P < 0.05, **= P < 0.01, ***= P < 0.001).

After a calling song recording of *G. bimaculatus* was played for one day, in the mean 5.4 ± 1.5 *G. bimaculatus* and 0 ± 0 *T. commodus* were recorded in the sound chamber (P<0.001). After calling song of *T. commodus* was played for one day, in the mean 0.2 ± 0.4 *G. bimaculatus* and 2 ± 1.2 *T. commodus* had move toward the sound chamber (P=0.015). In the test of *G. bimaculatus* and *T. oceanicus* (**Figure 6.2B** lower trace), 0.6 ± 0.5 *G. bimaculatus* and $0.2 \pm$

0.4 *T. oceanicus* were found in the sound chamber when no sound was played. After one-day playing of *G. bimaculatus* calling song, 5.6 ± 1.1 *G. bimaculatus* and 0 ± 0 *T. oceanicus* were recorded in the sound chamber (P<0.001). When *T. oceanicus* calling song was played for a day, 0 ± 0 *G. bimaculatus* and 2 ± 1.2 *T. oceanicus* were found in the sound chamber (P=0.006). Detailed cricket number recorded in each chamber after different acoustic stimuli are shown in **Table 6.1** and **6.2**. These experiments proved the separation function of the selecting system, while not all of the females were attracted to the sound chamber when playing conspecific songs.

6.3.2   *Test of attractive and non-attractive song patterns with the system*

By tracking the number of females attracted to the sound chamber, the system allows to evaluate the relative attractiveness of different acoustic stimuli. To validate this assumption, three different song patterns were played to female *G. bimaculatus* in order to test their attractiveness. These song patterns were characterised to be attractive or non-attractive based on a delay-line and coincidence detector mechanism (Hedwig and Sarmiento-Ponce 2017). In this mechanism, the neural response to a sound pulse is forwarded to a coincidence detector and also to a delay-line. If the delay of the neural delay-line matches the sound pulse period, the delay-line neural signal from first pulse will overlap with direct neural signal from the next sound pulse and boost the response of the coincidence detector (**Figure 6.3A**). Based on this hypothesis, the pulse duration of the first, second, or third sound pulse in standard *G. bimaculatus* three-pulse chirps (20ms duration-20ms interval) were modified and these chirps were used to generate phonotaxis response tuning curves. The tuning curves revealed that three-pulse chirps with a short first pulse and a long third pulse are attractive to *G. bimaculatus*, while chirps with a long first pulse and short third pulse are non-attractive (Hedwig and Sarmiento-Ponce 2017). According to these data, two modified three-pulse chirps were designed (**Figure**

6.3B). One with pulse duration of 5ms-20ms-80ms, and the other 80ms-20ms-5ms, both with pulse intervals fixed at 20ms. These two song patterns, along with standard *G. bimaculatus* chirps (20ms-20ms-20ms), were used to test female *G. bimaculatus* responses in the selecting system. 10 females were used for each experiment. The experiments started with one-day silence, then each of the three song pattern was played for 24 hours over the next three days. The number of crickets in each chamber was recorded after each acoustic stimulation (**Figure 6.3C**). The cricket number in the sound chamber was 4.2 ± 0.8 for 20ms-20ms-20ms chirps, 4.8 ± 0.8 for 5ms-20ms-80ms chirps, and 1.8 ± 0.8 for 80ms-20ms-5ms chirps, and 0.2 ± 0.4 in the control run. All the numbers of cricket encountered in the sound chamber after chirps were significantly higher than in the control (P(20-20-20 vs Silence)<0.001), P(5-20-80 vs Silence)<0.001, P(80-20-5 vs Silence)=0.005), and numbers for the 20ms-20ms-20ms group and 5ms-20ms-80ms group were significantly higher than for the 80ms-20ms-5ms group (P(20-20-20 VS 80-20-5)= 0.002, P(5-20-80 VS 80-20-5)<0.001). These results suggest the 5ms-20ms-80ms song pattern is more attractive to female *G. bimaculatus* than the 80ms-20ms-5ms song pattern and it showed a similar attractiveness as the standard 20ms-20ms-20ms chirp. This indicates that the selecting system can be used to screen attractive acoustic stimuli from non-attractive ones, and it should be noticed that not all the females were attracted to the sound chamber when playing attractive patterns.
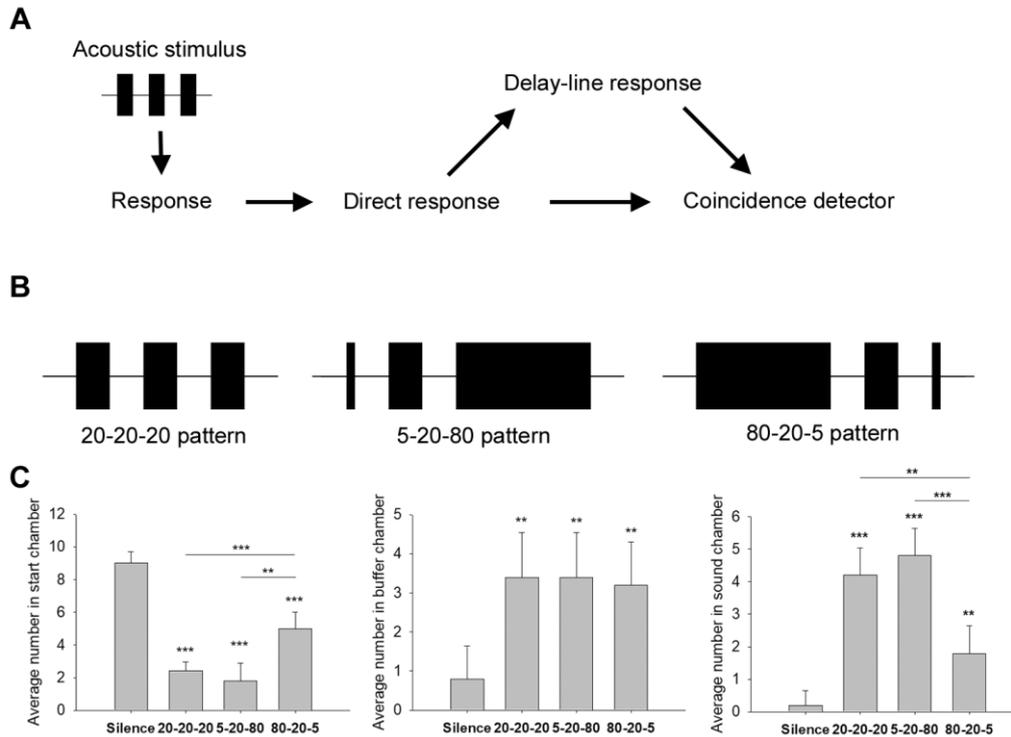
Figure 6.3 Females selecting attractive and non-attractive song patterns as tested with the system. (A) Schematic representation of delay-line and coincidence detector mechanism. (B) Putative attractive and non-attractive song patterns used in selecting system. Pulse interval was fixed at 20ms (C) Number of crickets recorded in each chamber after each song pattern. (**= $P < 0.01$, ***= $P < 0.001$).

6.4 Discussion

In this study, the function of newly generated selecting system was tested by species separation experiments and an experiment including attractive and non-attractive sound signal. The results of these experiments proved the filtering function of this system based on female phonotaxis. Further experiments applying the system could be combined with trackball system to see if the females sorted by the system really show higher response to given acoustic signals. In the long run, the system can be used to selecting females with special phonotaxis preferences. One could develop a cricket line with selected females and use the line to study the genetic background of female phonotaxis preference.

The selecting system aims for a group screening method that takes advantage of the

phonotactic steering behaviour. With the device, female crickets with different phonotactic preferences could be identified and separated from the test group. This implies a selection of individuals with preferences toward specific song properties such as carrier frequency and temporal pattern is possible, which can be used to breed and establish animal lines to study the genetics underlying phonotactic choices. Combining with understandings of animal phonotactic preferences, such a selection system could also be used as an acoustic trap for pest control (Mankin 2012). In the study of phonotaxis, this system can sort out desired individuals or test song patterns with different attractiveness as demonstrated in current study. Unlike a Y-maze commonly used for forced choice experiments (Rheinlaender and Blätgen 1982; Simões et al. 2011; Erregger et al. 2018), this system is based on a long-term attracting process and allows screening of multiple animals simultaneously. In addition, the system requires the animals overcoming the gravity and obstacles before reaching the sound chamber. It simulates the natural condition by having both attractiveness from acoustic signals and resistance from the geographical obstacles.

However, as a prototype, there are several points that should be taken into account or require further refinement. First, reuse of the system with the same batch of animals might affect the outcome of the tests due to spatial memory of the animals (Santos-Pata et al. 2017). Second, the current size of the device can only accommodate about 20 animals. Third, there is still chance that animals were freely moving or following other individuals (group effect), but not attracted, to the sound chamber, and some animals may have walked back to previous chambers during the experiments. These flaws can be solved to certain extent by enlarging the size of the device and increasing the complexity of internal environment by adding more obstacles. Furthermore, expanding the chamber numbers could increase the buffer capacity and enhance the accuracy of selecting females. If another loudspeaker is placed in the opposite end the system could serve as a complex version of a Y-maze to test animal preference. To optimise

the system, one could also consider the texture of the device so that female crickets could walk and climb in accordance with the insects' physical structure, as different texture might influence the locomotor performance of the insects (Sarmiento-Ponce et al. 2018).

Different acoustic communicating species display species-specific song preferences that contribute to prezygotic isolation. In cricket and bush cricket, calling song and courtship song of closely related species were shown recognised only by the conspecifics (Schul et al. 1998; Fitzpatrick and Gray 2001; Bailey et al. 2017). The recognition of conspecific songs relies on the identification of song properties such as sound intensity, frequency, and song structure. In this study, experiments using a mixture of two species has proved the capability of the selecting system to allow the separation of females with different song preferences. In addition, test of attractive and non-attractive sound stimuli showed similar results as retrieved by using a trackball system (Hedwig and Sarmiento-Ponce 2017). This demonstrates the potential of the selecting system in studying phonotactic behaviour.

Physiological and physical status influence the performance of phonotaxis. Previous studies suggest the age after final moulting and the mating status of female crickets affect responsiveness to songs (Prosser et al. 1997; Pacheco et al. 2013; Sarmiento-Ponce 2018; Tanner et al. 2019). Intactness of the sensory (eyes and ears) and motor system (legs) define the physical ability of recognition and locomotion required for phonotactic steering. By applying the selecting system, comprehensive phonotactic performance of contestants were evaluated, and only the females recognising the sound pattern and attracted to the acoustic stimuli, which overcame the obstacles, and reached the destination were defined as phonotactic responsive to the acoustic stimuli. This method selects the animals with phonotaxis performance regardless of age, previous life history and can be used as pre-selection method for further more detailed behavioural experiments.

Treadmill or trackball systems are powerful methods for measuring phonotacic steering

of the animals and are widely adapted (Hedwig and Poulet 2005; Ofner et al. 2007; Verburgt et al. 2008; Kong et al. 2015). These methods generate real-time recordings of phonotacic steering and thus provide details of how animal respond to acoustic stimuli. The selecting system proposed in current study provides another way of studying phonotaxis behaviour. Instead of describing phonotacic movements of individuals on restricted platforms, this system provides a complex environment in which freely moving female crickets could approach the acoustic stimuli over a long time course. By expanding the space of the device and introducing a larger number of animals, a high throughput scoring for the attractiveness of acoustic stimuli should be possible. Furthermore, a combination of the selecting system and video recordings might allow to track how and when the animals are responding to the acoustic stimuli.

Table 6.1 Number of crickets recorded in each chamber after presented with calling song of *G. bimaculatus* and *T. commodus.*

1st trial	1st chamber	2nd chamber	3rd chamber
Silence	8 B + 9 C	1 B + 0 C	1 B + 1 C
G .bimaculatus song	3 B + 9 C	1 B + 1 C	6 B + 0 C
T. commodus song	10 B + 8 C	0 B + 0 C	0 B + 2 C
2nd trial	**1st chamber**	**2nd chamber**	**3rd chamber**
Silence	9 B + 10 C	1 B + 0 C	0 B + 0 C
G .bimaculatus song	3 B + 10 C	0 B + 0 C	7 B + 0 C
T. commodus song	9 B + 6 C	0 B + 0 C	1 B + 4 C
3rd trial	**1st chamber**	**2nd chamber**	**3rd chamber**
Silence	9 B + 10 C	0 B + 0 C	1 B + 0 C
G .bimaculatus song	6 B + 10 C	1 B + 0 C	3 B + 0 C
T. commodus song	10 B + 9 C	0 B + 0 C	0 B + 1 C
4th trial	**1st chamber**	**2nd chamber**	**3rd chamber**
Silence	9 B + 10 C	0 B + 0 C	1 B + 0 C
G .bimaculatus song	5 B + 10 C	0 B + 0 C	5 B + 0 C
T. commodus song	10 B + 8 C	0 B + 0 C	0 B + 2 C
5th trial	**1st chamber**	**2nd chamber**	**3rd chamber**
Silence	9 B + 10 C	1 B + 0 C	0 B + 0 C
G .bimaculatus song	4 B + 10 C	0 B + 0 C	6 B + 0 C
T. commodus song	10 B + 9 C	0 B + 0 C	0 B + 1 C

B= *G. bimaculatus*, C= *T. commodus*

Table 6.2 Number of crickets recorded in each chamber after presented with calling song of *G. bimaculatus* and *T. oceanicus*.

1st trial	1st chamber	2nd chamber	3rd chamber
Silence	9 B + 10 O	1 B + 0 O	0 B + 0 O
G. bimaculatus song	5 B + 10 O	0 B + 0 O	5 B + 0 O
T. oceanicus song	10 B + 8 O	0 B + 0 O	0 B + 2 O
2nd trial	**1st chamber**	**2nd chamber**	**3rd chamber**
Silence	9 B + 10 O	0 B + 0 O	1 B + 0 O
G. bimaculatus song	3 B + 10 O	1 B + 0 O	6 B + 0 O
T. oceanicus song	10 B + 10 O	0 B + 0 C	0 B + 0 O
3rd trial	**1st chamber**	**2nd chamber**	**3rd chamber**
Silence	8 B + 9 O	1 B + 0 O	1 B + 1 O
G. bimaculatus song	4 B + 10 O	2 B + 0 O	4 B + 0 O
T. oceanicus song	10 B + 6 O	0 B + 1 O	0 B + 3 O
4th trial	**1st chamber**	**2nd chamber**	**3rd chamber**
Silence	9 B + 9 O	1 B + 1 O	0 B + 0 O
G. bimaculatus song	4 B + 10 O	0 B + 0 O	6 B + 0 O
T. oceanicus song	10 B + 8 O	0 B + 0 O	0 B + 2 O
5th trial	**1st chamber**	**2nd chamber**	**3rd chamber**
Silence	9 B + 9 O	0 B + 1 O	1 B + 0 O
G. bimaculatus song	3 B + 10 O	0 B + 0 O	7 B + 0 O
T. oceanicus song	9 B + 5 O	1 B + 2 O	0 B + 3 O

B= *G. bimaculatus*, O= *T. oceanicus*

Table 6.3 Number of female *G. bimaculatus* recorded in each chamber after presented with theoretically attractive and non-attractive song pattern.

1st trial	1st chamber	2nd chamber	3rd chamber
Silence	9	1	0
20-20-20	3	2	5
5-20-80	1	3	6
80-20-5	4	5	1
2nd trial	1st chamber	2nd chamber	3rd chamber
Silence	8	2	0
20-20-20	2	4	4
5-20-80	1	5	4
80-20-5	6	2	2
3rd trial	1st chamber	2nd chamber	3rd chamber
Silence	9	0	1
20-20-20	3	3	4
5-20-80	3	2	5
80-20-5	5	3	2
4th trial	1st chamber	2nd chamber	3rd chamber
Silence	9	1	0
20-20-20	2	3	5
5-20-80	1	4	5
80-20-5	4	3	3
5th trial	1st chamber	2nd chamber	3rd chamber
Silence	10	0	0
20-20-20	2	5	3
5-20-80	3	3	4
80-20-5	6	3	1

7 Chapter Seven: General Discussion

In the current study, I addressed different aspects of male cricket singing behaviour from wing movements during stridulation to the neural control of different song types and behaviour in different cricket species. I also proposed a selecting system for females based on their phonotactic preference to acoustic signals. In Chapter 2, the three song types generated by *G. bimaculatus* were recorded along with the corresponding wing movements. These recordings provide clues on the machinery controlling the wing for the different song types. In Chapter 3, the involvement of abdominal ganglia in courtship and rivalry behaviour was addressed by systematic lesion experiments in *G. bimaculatus*. A different contribution of specific abdominal ganglia to the control of courtship and rivalry song was revealed. Furthermore, copulation behaviour was lost after lsevering the connection to the terminal abdominal ganglion, while most components of the rivalry behaviour were not affected by lesions of the abdominal connectives. In Chapter 4, calling songs of different cricket species were analysed after systematic lesions to the abdominal nerve cord. The results suggest a conserved basic CPG organization for calling song across the abdominal nervous system in different cricket species. Differences among the species producing, trills, chirps, and phrases could imply species-specific traits shaped by evolution. In Chapter 5, the intracellular recordings of a physiologically identified command neuron for calling song in three cricket species revealed a similar control of calling song activity. In Chapter 6, a novel animal selecting system was described and the capabilities to screen female crickets with specific acoustic preference was verified, while further improvements are required to increase the capability and specificity of the selecting system. These experiments provide a deeper understanding of the neural circuits and mechanics of cricket song production and a new method to study cricket acoustic communication. However, more studies are required to unravel the whole picture of cricket singing in terms of machinery, neurobiology, and evolution.

7.1 Neuromuscular control and functional morphology of stridulation in crickets

Wing muscle activity during generation of three song type was determined by EMG studies in *G. campestris* (Kutsch 1969). The activity of closer muscle M90 and opener muscle M99 are in phase with closing and opening movements of the wings during calling song and rivalry song. During courtship song only the closer muscle M90 was activated when producing high-amplitude ticks while the opener M99 was firing rhythmically in between the ticks. A combination of sound, wing, and muscle recording in courtship song of *G. bimaculatus* (Innenmoser 1974) revealed a high amplitude upward/closing movement for ticks along with closer M90 activity while the opener M90 fired in the interval between the ticks and no obvious sound nor wing movements was described. The high frequency ticks and intermitting low frequency pulses with low amplitude were observed in courtship song of *G. bimaculatus* but were not linked to wing movements or muscle activity (Libersat et al. 1994). My current study recorded courtship song and corresponding wing movements with a high amplitude resolution so that ticks as well as low-amplitude pulses in the tick intervals were revealed together with the low amplitude wing oscillations underlying the low-amplitude pulses. These, together with previous EMG recordings, complete the description of courtship song regarding sound, wing, and muscle activity in *G. bimaculatus*: courtship song contains high frequency (13-13.5 kHz) ticks and low-amplitude pulses with low frequency (4.5-5.5 kHz). During the generation of ticks closer M90 is activated and the wings are raised to high position, and during generation of low-amplitude pulses, the wings are lowered and oscillate rhythmically with activity of opener M99. Besides, the transition from calling song to courtship song demonstrate the change in frequency from low-frequency calling song pulses to high frequency ticks is coupled to lowering of the wings. This suggests the low wing position is required for the generation of high frequency ticks. This opens questions about the functional morphology for courtship song

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generation. What are the structures (file teeth) responsible for generating the sound of ticks and low-amplitude pulses? How do the structures function when the wings are oscillating and the opener M99 is firing? These demand further investigation including revisits of the cricket wing structure by high-speed video recording combined with recordings of sound and wing movements.

While the neuromuscular system for stridulation was extensively studied in chirping species like *G. campestris* and *G. bimaculatus*, it is less clear in phrase-producing species like *Teleogryllus* (Hennig 1989; Honda-Sumi 2005; Bailey et al. 2017). How do the wing muscles cooperate and how does the wing position change during stridulation of chirps and trills in calling songs. Also, since both *Gryllus* species and *Teleogryllus* species show two motor patterns when generating the courtship song, it might be interesting to compare the underlying machinery used in both groups of crickets.

7.2 *Toward a comprehensive understanding of the neurobiology underlying cricket stridulation*

The neural circuit underlying cricket calling song stridulation is now believed to consist of a command neuron descending from the brain, CPG neurons within the abdominal ganglion chain to generate the temporal motor pattern, and wing motor neurons in the mesothoracic ganglion that innervate the wing muscles used for stridulation, based on series of studies mainly in *G. bimaculatus* and *G. campestris* (Huber 1960, 1963; Kutsch 1969; Hedwig 1996, 2000; Schöneich and Hedwig 2011, 2012; Jacob and Hedwig 2016, 2019, 2020). In the current study, I extend the understanding of the function of command neurons and the singing CPGs to other cricket species with different calling song structures. I also compared the similarities and differences of the neural organisation underlying song generation among cricket species and provided evidence regarding the evolution of cricket stridulation.

7.2.1 *Command neuron for calling song in different cricket species*

The strict definition described in command neuron concept (Kupfermann and Weiss 1978) make it difficult for a neuron to be categorized as a command neuron. Many neurons are defined as command-like neurons as they fulfilled some of the criteria (Rock et al. 1981; Zottoli and Faber 2000; Hurwitz et al. 2005; Korn and Faber 2005; Lacoste et al. 2015). In a complex nervous system where neural circuits work together to carry out coordinated behaviour, the idea of a single command neuron controlling whole behaviour seems impractical, and only very few neurons were qualified throughout the history of searching command neurons. Indeed, some behaviours are controlled by command neurons, but it should be recognised as one of the many neural controls for behaviours. On the other hand, the sufficiency and necessity criteria stated in the concept provide a way to describe relationship between neuron and behaviour, and the concept is still useful in certain circumstances as command neuron or command-like neuron express the function and role of the neuron.

In the study, I intracellularly recorded putative command neurons (pCN) for calling song in three cricket species. Though no morphology of the pCNs was revealed, the physiological properties of the neurons are similar across the three species. All of them conform to the criteria of sufficiency and necessity in controlling the calling song stridulation by pure tonic activity. These neurons could be homologous neurons to the command neuron described in *G. bimaculatus* (Hedwig 1996, 2000), while the possibility of upstream neurons, presynaptic to the command neurons, could not be ruled out as no morphology of the recorded neurons was revealed. Still, the similar recording sites, physiological properties, and functionality, indicate that the three neurons found in the three species are homologous and pose an example of a conserved cellular component for cricket calling song stridulation. As the control of the calling song in the chirping species *G. bimaculatus* and *G. assimilis* is better understood, it is interesting to see how command neurons control calling song in trilling and phrase-producing

species. In *T. oceanicus*, cervical connective stimulation had elicited calling song containing chirps and trills driven by tonic activity of the pCN (Bentley 1977). Together with pCN found in *T. commodus*, these evidences imply the existence of command neuron for calling song also in the phrase-producing species and indicate that the brain activates the singing CPG by the tonic activity of a specific set of descending interneurons. The decision to sing is likely reflected in the enhanced activity of these neurons.

7.2.2   *Command neurons controlling the different song types*

Extracellular current stimulation of the brain released calling, courtship, and rivalry songs in *G. campestris* (Huber 1960, 1963), and cervical connective stimulation experiments triggered three song types, and also the transition from courtship song to rivalry song (Otto 1971). In the grasshopper *Omocestus viridulus* three command neurons for three different stridulatory movement patterns were identified (Hedwig and Heinrich 1997). These clues point to the possible existence of different command neurons for courtship song and rivalry song in the brain of crickets. However, so far no rivalry or courtship song was ever elicited during intracellular recording attempts, though wings held at low position similar to the wing position for courtship song was discovered once without any sound generated. The initiation of courtship song and rivalry song might be controlled by other specific command neurons or in corporation with the command neuron for calling song.

7.2.3   *Synaptic connection of the command neuron to the singing CPG*

The projection of the command neuron for calling song in *G. bimaculatus* was not fully revealed but the axon can be traced at least to the prothoracic ganglion (Hedwig 2000). As the command neuron activity is related to the chirp rate but not to the sound pulse rate and as the

chirp timer neurons are at a higher hierarchy than the pulse timer neurons (Jacob and Hedwig 2020), it is more likely that the command neuron for calling song makes connections to the chirp timer neurons rather than the pulse timer neurons, while connections to both CPGs is possible as well. A complete staining of the command neuron morphology or search for the command neuron in the abdominal ganglia which houses the singing CPG might provide further insights into the synaptic connection between the command neuron and CPG neurons.

7.2.4 *CPG organisation in different species provides insight into the evolution of species-specific calling songs*

In this study, a set of lesion experiments was performed on four different species, where trill-producing species, chirp-producing species, and phrase-producing species were taken into consideration. The calling song recorded after applying lesions between T3-A3, and A3-A4 demonstrate similar changes on song structure in all four species, as T3-A3 lesion abolish the songs and only single pulses were observed after an A3-A4 lesion. The A4-A5 lesion showed different effects on the song patterns depending on the original calling song type: no significant change in trill-producing species, longer chirps occurred in chirp-producing species, and the chirp and trill components were destroyed in phrase-producing species. The similarities in four species after T3-A3 and A3-A4 lesions suggest a shared organisation for generating pulses commonly retained in different cricket species. This is also proved by recordings of homologous A3-AO opener interneurons in five species (Jacob and Hedwig 2019). The differences in four species after an A4-A5 lesion further indicate species-specific differences in the organisation of the singing network that shape the species-specific calling song in each species. The different effects after an A4-A5 lesion and no obvious change after an A5-A6 lesion suggest the neural circuits in A5 participate in song structure evolution: trill-producing

species might be the most ancestral form of song as no change happened, chirp-producing species had alteration that fixed the length of pulse sequences and group pulses into chirps, phrase-producing species show alterations that group the pulses into chirps and trills. Thus, a detailed investigation of the singing-CPGs in different cricket species might unravel the principles for generating the chirp and trill structure, and how these are arranged as phrases. This might further shed light on the evolution of singin-CPGs that shape the species-specific songs in crickets.

7.2.5   *CPGs for courtship song and rivalry song*

While the CPG for calling song has been well studied, little is known about the organisation of the CPGs for courtship song and rivalry song in crickets. Previous lesion experiments in *G. campestris* had concluded that the males stopped producing courtship song after the connectives to the TAG was severed (Huber 1960, 1963). In the current study, lesion experiments in *G. bimaculatus* suggest the TAG is not necessary for the production of courtship song, while an intact abdominal nerve cord extending to A6(A5) is important.

Rivalry song, on the other hand, required an abdominal nerve cord containing A3 and A4. Results from lesion experiments and wing recording during calling song and rivalry song suggest an overlapping pattern generation system used for the two song types. However, it cannot be decided if generation of courtship song will also require A3 and A4 as for calling and rivalry song. Characterization of CPGs for courtship song in A6 (A5) might provide further information to this question.

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