# The role of sensory influences in the control of motor activity of a stepping insect leg

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# Zusammenfassung

Im Diskurs um die Erforschung der Bewegungskontrolle standen lange Zeit stereotype Bewegungsabläufe im Mittelpunkt, wie zum Beispiel Vorwärtslaufen. In dieser Dissertation wird beleuchtet, wie verschiedene Ebenen der neuronalen Bewegungskontrolle zur Bewegungsveränderungen beitragen. Daraus ergaben sich drei Hauptfragen. Erstens, wie sensorische Rückkopplung die Aktivierung und Aktivität von Muskeln im Allgemeinen beeinflusst, zweitens, wie diese Rückkopplung bei aufgabenspezifischen Bewegungsveränderungen verarbeitet wird und drittens, welche Mechanismen im neuronalen Netzwerk genutzt werden, um flexible Bewegungsaktivität zu gewährleisten.

Zentrale Mustergeneratoren erzeugen eine rhythmisch alternierende Motoraktivität, welche durch sensorische Rückkopplung zeitlich und in der Aktivitätsstärke moduliert wird. Im Mittelbein der Stabheuschrecke *Carausius morosus* messen zwei Hauptgruppen von Sinnesorganen entweder die Belastung oder die Bewegungs- und Positionsparameter des Beins. Die Rolle dieser Sinnesorgane wurde in dieser Arbeit untersucht.

In der ersten Studie wurde der Einfluss von campaniformen Sensillen (CS) auf die Stärke der Aktivität und den Aktivierungszeitpunkt von Stemmphasenmuskeln herausgestellt. Hierfür wurde ein Versuchsaufbau mit einer absenkbaren Glitschplatte genutzt. Während des Experiments lief das Tier entweder mit Bodenkontakt auf dieser Glitschplatte oder trat in ein Loch, wenn die Platte während der Laufsequenz abgesenkt wurde. Durch den Bodenkontakt wurden die Beine belastet und die sensorische Rückkopplung durch CS aktiviert. Diese Rückkopplung fehlte bei dem Schritt ins Loch. Durch Ablationsexperimente konnte ich neben CS ein weiteres Sinnesorgan identifizieren, die durch ihre sensorische Information über den Bodenkontakt den *Flexor tibiae* aktivierten. In allen Stemmphasenmuskeln, mit Ausnahme des *Depressor trochanteris*, erhöhte sich die Stärke der Muskelaktivität durch den Bodenkontakt.

In der zweiten Studie wurde die Kontrolle durch die Rückkopplung des femoralen Chordotonalorgans auf die Aktivierung, insbesondere des *Extensor tibiae* Muskels, untersucht. Hierbei wurde ein Fokus auf die Verarbeitung dieser Rückkopplung bei Bewegungsveränderungen, insbesondere beim Kurvenlaufen, gelegt. Das fCO misst verschiedene Parameter der Kniegelenkbewegung und –position (Femur-Tibia [FT] Gelenk). Durch die Rückkopplung vom fCO werden zum einen Widerstandsreflexe generiert, die zur Aufrechterhaltung der Positur genutzt werden. Zum anderen, wird die Reflexumkehr beobachtet, die im aktiven Tier zur Unterstützung der Stemmphase beiträgt. Während des Kurvenlaufens haben die Beine auf jeder Seite des Tieres eine unterschiedliche Kinematik. Das Innenbein wird hauptsächlich im FT Gelenk bewegt, während das Außenbein

größtenteils im Hüftgelenk (Thorax-Coxa Gelenk) bewegt wird. Die Aktivierung von *Extensor tibiae* Motorneuronen (MN) während einer Reflexumkehr wurde bei einer bestimmten Winkeldifferenz ausgelöst. Dies geschah unabhängig von der Funktion des Beines als Innen- oder Außenbein. Darüber hinaus induzierte die fCO Stimulation häufiger AR, wenn das Bein als Innenbein fungierte. Beim Außenbein traten hingegen RR häufiger auf. Außerdem wurden mehr AR ausgelöst, wenn das fCO mit langsameren Geschwindigkeiten und größeren Startwinkeln stimuliert wurde. Der *Protraktor coxae* zeigte im Außenbein eine erhöhte Aktivierung durch die fCO-Stimulation, während der Levator coxae keinen Unterschied in der Reaktion zwischen den beiden Laufsituationen zeigte. Somit konnte in diesem Teil meiner Arbeit gezeigt werden, dass Rückkopplung vom fCO aufgabenspezifische Motoraktivität hervorrufen kann.

In einer letzten Studie wurden die Mechanismen untersucht, die für die Abnahme der Reaktion auf die fCO Stimulation verantwortlich sein könnten, wenn das Bein als Außenbein genutzt wird. Die direkte Verbindung von fCO Afferenzen zu ExtTi MN, sowie zu Nicht-Spikenden-Interneuronen (NSI), war abgeschwächt. Ein ähnliches Bild zeigte sich über die gesamte Länge der fCO Stimulation in ExtTi MN. Ferner erhielten die ExtTi MN eine größere tonische Depolarisation und der Membran Eingangswiderstand wurde verringert.

In meiner Dissertation konnte ich somit zeigen, dass Motoraktivität durch verschiedene Sinnesorgane beeinflusst wird. Diese Beeinflussung kann an Bewegungsveränderungen angepasst werden, wobei ein Mechanismus aufdeckt werden konnte, der zu dieser Anpassung beiträgt. Welche Einflüsse NSI oder eine präsynaptische Inhibierung auf aufgabenspezifische Motoraktivitäten haben, bleibt offen.

# Abstract

For a long time the focus of the discourse in motor control research was on stereotypical movements, such as forward walking. My thesis emphasizes how different levels of neuronal motor control contribute to the processing of task-dependent locomotor behavior. This resulted in three main questions. First, how sensory feedback affects the timing and magnitude of muscle activity in general. Second, how this feedback is processed in changes of task-dependent movement behavior. And third, what mechanisms are used in the neural network to evoke an adaptive capability.

Central pattern generators (CPGs) generate a rhythmic, alternating motor activity that is in turn modulated by sensory feedback in timing and in magnitude. In the middle leg of the stick insect *Carausius morosus*, two major groups of sense organs measure either the load or the movement and positional parameters of the leg. The role of these sense organs was examined in my work.

In the first study, the influence of campaniform sensilla (CS) on magnitude and the timing of stance phase muscles was examined. For this purpose, a trapdoor setup with a slippery surface was used. The animal either stepped on a slippery surface with ground contact or they stepped into a hole when the trapdoor was lowered (SIH). Through ground contact, the legs are loaded and thereby activate the leg CS. During SIH this sensory feedback is missing. Through ablation experiments, I was able to show, in addition to CS, an additional sense organ that activated the *flexor tibiae* (FlxTi) through their sensory information of the touchdown (TD). In all stance phase muscles, except for the *depressor trochanteris* (DepTr), the strength of muscle activity increased through the TD.

In the second study, the control on the timing of activation, especially of the *extensor tibiae* (ExtTi) muscle by sensory feedback of the femoral chordotonal organ (fCO) was investigated. Here, a focus was set on the processing of this feedback to movement changes, in particular curve walking. The fCO measures various parameters of the knee joint movement (femorotibial [FT] joint) and position. Feedback from the fCO generates resistance reflexes (RR), which are used to maintain the posture. Assistance reflex or reflex reversals (AR), which assist stance phase activity in the active animal, were also observed. During curve walking, the legs on each side of the animal have different kinematics. The leg inside of a curve (inside) is mainly moved around the FT joint, while the leg outside of the curve (outside) is mostly moved in the hip joint (thoracocoxal [ThC] joint). ExtTi motor neurons (MN) were activated during an AR at a certain angular difference. This was independent of the function as inside or outside leg. Further, the fCO stimulation induced ARs more often in the inside leg while RRs occurred more frequently in the outside leg. In addition, more ARs were generated through fCO stimulation at slower velocities and larger starting angles. The *protractor coxae* (ProCx) showed increased activation through fCO stimulation in the outside leg, while the *levator coxae* (LevTr) showed no difference in the reaction between the two leg

functions. Thus, it could be shown in this part of my thesis that feedback from the fCO can cause task-specific motor activity.

In the third study, the mechanisms that might be responsible for the decrease in the response to fCO stimulation when the leg is used as the outside leg were investigated. The monosynaptic connection of fCO afferents to ExtTi MNs, as well as to nonspiking interneurons (NSIs), was reduced. Similar observations were made in ExtTi MNs throughout the complete fCO stimulation. Furthermore, ExtTi MNs received greater tonic depolarization and their membrane input resistance was reduced.

In my dissertation I could show that motor activity is influenced by various sense organs. These influences can be adapted to changes in movement, whereby I could reveal one mechanism leading to task-dependent motor activity. The influence of NSIs or presynaptic inhibition on changes in task dependent motor output remains unclear.

# 1. General Introduction

Every animal navigates its environment using locomotion. Depending on the habitat, this can be swimming, crawling, flying or walking. Each of these behaviors consists not only of one type of locomotion, but it can be adapted to the environmental needs. An animal can not only move straight forward, but can change the pattern in a task-dependent manner to backwards locomotion (*lamprey*: Islam et al., 2006; *salamander*: Ashley-Ross and Lauder, 1997; *humans*: van Deursen et al., 1998; Pang and Yang, 2002; Choi et al., 2008; *crayfish*: Ayers and Davis, 1977; *stick insect*: Rosenbaum et al., 2010), climbing (*cockroach*: Watson et al., 2002 a,b; *fruit fly*: Pick and Strauss, 2005; *stick insect*: Bläsing and Cruse, 2004a, b) and turning (*fish*: Orger et al., 2008; *human*: Lamb and Yang, 2000; *cockroach*: Mu and Ritzmann, 2005; *fruit fly*: Frye and Dickinson, 2001; 2004; Bender and Dickinson, 2006; *stick insect*: Dürr and Ebeling, 2005; Gruhn et al., 2009 a, 2016). This behavioral plasticity, not only in walking, but also in the other forms of locomotion, emerges from an interaction of neuronal networks, sense organs and muscles (Orlovskiĭ et al., 1999). The neuronal networks consist of rhythm generating networks that activate motor neurons (MN) in a rhythmical manner, which then initiate alternating muscle activity. The timing and magnitude of the rhythmic output of this neuronal network, is modulated by sensory feedback. The sensory feedback measures position, load and movement parameters, and originates from sense organs that correspond to the locomotor organs.

In vertebrates, the command centers for locomotion are located in the higher brain centers. Signals are transmitted from the basal ganglia, via the motor cortex, the cerebellum and the brainstem to the local networks in the spinal cord, where central pattern generators (CPGs) generate a rhythmic motor output (c.f. Grillner et al., 1995, Grillner, 2003, Kiehn, 2006). For invertebrates, especially insects, it is known that the signals, initiating locomotion descend from the central complex (Strauss, 2002) to the ventral nerve cord, where local networks that contain CPGs generate the motor output (Büschges and Bässler, 1998).

Another adaptation to stereotyped locomotion is the use of different coordination patterns between the legs. In vertebrates these so called gaits, like walking, trot, gallop in horses, are correlated with a distinct movement speed (Alexander, 1989, Hoyt and Taylor, 1981), to decrease energy costs (Hreljac, 1993). Invertebrates, like crustaceans, arachnids or insects, on the other hand, show continuous transition between gaits, which are thus here better described as coordination patterns (Cruse et al., 2009, Grabowska et al., 2012, Graham, 1985, Hughes, 1952, Wendler, 1964, Wosnitza et al., 2013). Insects use three major coordination patterns. For fast walking speeds, this is a tripod, for intermediate velocities a tetrapod, and for slow velocities a metachronal coordination (Cruse et al., 2009,

Delcomyn, 1971, Grabowska et al., 2012, Graham, 1972, 1985, Hughes, 1952, Wendler, 1964, 1966, Wosnitza et al., 2013). During the tripod coordination, three legs are in stance phase, whereas, during the tetrapod coordination, four legs are in stance phase at the same time (Cruse et al., 2009, Graham, 1985). An increase in walking speed is not only marked by change in the coordination pattern, but also by changes in the step cycle.

One step cycle consists of two phases. The first is the stance phase, during which the leg has ground contact and generates propulsion to push the body forward. The second is the swing phase, during which the leg is moved through the air to a new position to generate the next stance phase (Büschges and Gruhn, 2008, Epstein and Graham, 1983, 1985).

In stick insect, the local control of a step cycle in a single leg is well described, partially down to the level of single contributing neurons (reviews in Bässler and Büschges, 1998, Büschges et al., 2008, Büschges and Gruhn, 2008). The single leg is moved by antagonistic muscles around three mayor leg joints. Around the thoraco-coxal (ThC) joint the leg is moved posteriorly by the *retractor coxae* (RetCx) and anteriorly by the *protractor coxae* (ProCx), respectively. The next leg segment, the fused trochantero-femur is moved around the coxa-trochanter (CTr) joint. Here, the *depressor trochanteris* (DepTr), lowers the leg and the *levator trochanteris* (LevTr) lifts it The last mayor leg joint is the femoro-tibial (FT) joint. Through it, the *flexor tibiae* (FlxTi), and the *extensor tibiae* (ExtTi) flex, respectively extend the tibia of the leg (Büschges et al 2008). When the stick insect is walking forward, the RetCx, the DepTr and the FlxTi are used as stance phase muscles, coordinated in a specific activity pattern (Graham, 1985, 1985). During backward walking the same muscles are used, only the RetCx and ProCx are switched as respective stance and swing, as the leg is moved anteriorly during stance (Rosenbaum et al., 2010).

## 1.1. Central pattern generating networks

The activity of the antagonistic muscles in a leg is often controlled and timed by the activity of CPGs (Grillner and Zangger, 1979, Jordan et al., 1979, Matsushima and Grillner, 1992). These networks are located in the spinal cord of vertebrates or the ventral nerve chord of invertebrates. Examples for the importance of CPGs in locomotion has been found in vertebrates (review in Grillner, 2011, Rossignol et al., 2006), such as cats (Grillner and Zangger, 1979, Pearson and Rossignol, 1991), mouse (Kiehn, 2006), tadpole (Roberts et al., 1998) and lamprey (Grillner et al., 1981, Wallén and Williams, 1984) or invertebrates (Bässler and Büschges, 1998, Pearson, 1993), such as crayfish (Chrachri and Clarac, 1987), cockroach (Pearson and Iles, 1970, Zill, 1986), in locust (Ryckebusch and Laurent, 1993), hawk moth (Johnston and Levine, 1996) and stick insect (Büschges, 1995c). In stick insects, the neurons of the CPG have not yet been identified, although single nonspiking interneurons (NSIs) were found, which contribute to the function of the CPG (Berg et al., 2015, Büschges,

1995a). In other invertebrates, such as the stomatogastric nervous system of crustaceans (Marder and Bucher, 2007), the leech heartbeat (Kristan et al., 2005) or the swimmeret system of the crayfish (Mulloney and Smarandache-Wellmann, 2012), the cellular basis of the CPG is known better. The cells of the CPG express particular intrinsic properties, like endogenous bursting, spike frequency adaptation, postinhibitory rebound, plateau potentials and synaptic interaction between the cells of the CPG, which allow them to show rhythmic behavior (Marder and Calabrese, 1996, Marder and Bucher, 2001). Two types of networks are known, the pacemaker driven CPG, as it is found in the pyloric system of the stomatogastric nervous system (Marder and Eisen, 1984) and the half center oscillator, like it is found in the lamprey spinal cord, which oscillates through reciprocal inhibition between network components (Grillner et al., 1991).

In stick insects, it has been shown though pharmacological activation with the muscarinic agonist pilocarpine that each leg joint is driven by it's own CPG (Büschges, 1995c). In the stick insect, the rhythmic motor activity is the joint result of tonic descending excitatory drive, inhibitory patterning influence from the CPG and sensory inputs, and excitatory patterning influences from sensory inputs. (Büschges, 1998, Büschges et al., 2004, Ludwar et al., 2005a, Ludwar et al., 2005b).

## 1.2. Summary of the leg sense organs of the stick insect

As alluded to above, the activity pattern of the CPGs for each joint in a stick insect leg are strongly modulated by sensory feedback from the leg sense organs. Campaniform sensilla (CS) are known to activate and even entrain the CPG activity (FT joint: (Akay et al., 2001, Berendes et al., 2013, Zill et al., 2011, Zill et al., 2013, Zill et al., 2015); ThC joint: (Akay et al., 2004, Zill et al., 2004); CTr joint: (Borgmann et al., 2011, Zill et al., 2011, Zill et al., 2012, Zill et al., 2015). The femoral chordotonal organ (fCO) in the stick insects femur has been shown to induce phase transition in the CTr joint, from LevTr activity to DepTr (Bucher et al., 2003, Hess and Büschges, 1999). In the active behavioral state of the stick insect, fCO activation also induces a transition of motor activity in the FT joint. During extension of the tibia, feedback of the fCO is first increasing the FlxTi activity, which is at a certain position of the tibia, switched to ExtTi activity. This reaction to extension of the tibia in the active animal is called “reflex reversal” or "active reaction" (AR) (Bässler, 1976, 1988). However, depending on the behavioral state, in the inactive animal, the same fCO feedback, measuring an extension of the tibia, directly initiates a strong activation of the ExtTi muscle, which is thought to maintain posture and is thus called “resistance reflex” (RR) (Bässler, 1977b, 1988). Moreover, the sense organs of the leg can also provide sensory feedback to establish an accurate coordination of the leg joints.

### 1.2.1. Tactile Hairs

**Hair rows**

Four hair rows have been described on the coxa of the stick insect *Carausius morosus* (Bässler, 1965) that react to coxal movement in a posterior direction with increasing activity (Cruse et al., 1984). Bässler (1977a, b) described additional tactile hairs on the femur, which show a phasic response to a bending stimulus, but without a preferred directional response. In some animals, the slow extensor tibia (SETi) was increasing its firing rate slightly as a result of the stimulation of these tactile hairs (Bässler, 1977b). The physiology was similar to tactile hairs found on the tarsi of the locust (Runion and Usherwood, 1968) and trochanteral hair receptors in cockroach (Spencer, 1974).Tactile hairs have also been described on the tarsus of the stick insect (c.f. Bässler, 1983).

**Hair plates**

Several hairplates (HPs) are located on the stick insect leg. Two HPs (ventral and dorsal coxal HP) are located on the coxa (Büschges and Schmitz, 1991, Wendler, 1964) that both react to an anteriorly directed bending of the coxa (Bässler, 1965, Bässler, 1977b, Cruse et al., 1984, Wendler, 1964). The ventral coxal HP (vcxHP) was also found to presynaptically inhibit afferents of the fCO (Stein and Schmitz, 1999). One HP is located anteriorly on dorsal surface of the trochanter (trHP), close to the CTr joint, and one HP is located on the ventral trochanter (Schmitz, 1986b). The trHP has proprioceptive function (Wendler, 1964) and reacts to lifting of the leg (Schmitz, 1986a, b). Ablation experiments demonstrated the importance of the trHP for the feedback about the ThC angle, as the activation of DepTr MNs failed after ablation (Schmitz, 1986a).

### 1.2.2. Campaniform sensilla

Campaniform sensilla (CS) are bipolar sensory cells with a dendritic ending in caps on the cuticle of insects (Zill et al., 2004), first described by Pringle on the cockroach palps (Pringle, 1938a) and legs (Pringle, 1938b). The CS react to resisted force which is applied to the cuticle of the animal as well as to resisted muscle contractions. Both produce strains of the cuticle, which deform the cuticular cap and thereby activate the sensory cell beneath (Barth and Blickhan, 1984, Moran and Rowley III, 1975, Zill et al., 2004). Most of the CS have a preferred direction, in which the CS are easier to activate, depending on the oval shape of the cuticular cap. The direction, the CS is easiest to activate is the axis perpendicular to the longitudinal axis of the oval cap (Spinola and Chapman, 1975) .The Cs measure load of the leg and muscle forces (Zill et al., 2012).

**Trochanteral campaniform sensilla**

The trochanteral campaniform sensilla (trCS) consist of four groups of CS (Groups 1-4). Group 1 is located on the posterior, group 2 on the anterior trochanter, and groups 3-4 are located on the dorsal side of the trochanter (Hofmann and Bässler, 1982, Zill et al., 2012). Groups 1 and 2 were found to measure load of the leg mainly in anterior and posterior direction (Delcomyn, 1991, Hofmann and Bässler, 1982, 1986, Schmitz, 1993), whereas groups 3 and 4 are found to measure load mainly in dorsal and ventral direction (Zill et al., 2012).

Stimulation of the trCS can reset the timing of the ThC joint CPG and switch the MN activity from RetCx to ProCx MN, or vice versa (Akay et al., 2004). This, however, depends on the walking direction. The trCS could provide either excitatory or inhibitory drive to ThC MN (Schmitz and Stein, 2000). Sensory feedback from group 3 trCS was also described to be dependent on the behavioral state of the animal, in that lifting of the leg activates slow DepTr MN in the resting animal, and LevTr MNs when the animal is in the active state (Akay et al., 2007, Zill et al., 2012).

**Femoral campaniform sensilla**

The femoral campaniform sensilla (feCS) are located on the posterior side of the proximal femur (c.f. Bässler, 1983, Petryszak and Fudalewicz-Niemczyk, 1994, Zill et al., 2017). During stance phase, when the posterior femur is loaded in an upward direction, the feCS initiate FlxTi and terminate ExtTi activity and thereby induce a transition from ExtTi to FlxTi activity (Akay et al., 2001, Berendes et al., 2013, Zill et al., 2015). The caps and forms of the feCS can be divided into three groups, one ventral (caps' orientation with great angles to the autotomy plane of the trochantero-femoral joint, one dorsal group (caps' orientation with angles in line to the autotomy plane) and one central group with round caps (Zill et al., 2017). Through this, the feCS are capable of detecting forces at the CTr as well as at the trochantero-femoral joint, whereas the last joint is immobilized in stick insects and forms one plane from the CTr to the FT joint (Cruse and Bartling, 1995, Zill et al., 2017).

**Tibial campaniform sensilla**

The two main groups (6A and 6B) of tibial campaniform sensilla (tiCS) are located on the dorsal tibia, where group 6A consists of two subgroups, located more proximal to the FT joint, one anteriorly and one posteriorly, and group 6B is located more distally on the central tibia (Zill et al., 2011). The tiCS respond to muscle contractions of the tibial muscles and also to forces applied onto the plane of the FT joint, however, tiCS also react to unloading of the leg with increasing firing rate and could thereby contribute to the stance-swing transition (Zill et al., 2011, Zill et al., 2013). The different tiCS groups have different effects on tibial and trochanteral MN. Group 6B stimulation increases the activity in ExtTi and DepTr MN, while

the anterior group 6A stimulation opposes this effect by decreasing the activity in ExtTi and DepTr MN (Zill et al., 2011, Zill et al., 2013).

**Tarsal campaniform sensilla**

Tarsal campaniform sensilla (taCS) are found on tarsal segments 1 - 4 and on the Arolium. They are located dorsally at the distal part of each segment and the Arolium (Zill et al., 2014). By application of force on the taCS, the FlxTi, the DepTr and the *retractor unguis* (RetUng) are activated. taCS could also be activated by applying force to the RetUng tendon (Zill et al., 2014, Zill et al., 2015). The latencies until the activation of the different muscles were between 35 ms in the FlxTi to 43 ms in the RetUng (Zill et al., 2015). Functionally, by activation of the FlxTi, which occurs in parallel to an inactivation of the ExtTi, the leg is pulled towards the body and by activation of the RetUng, the claw is bent, which both lead to a higher adhesion to the ground (Zill et al., 2014, Zill et al., 2015).

## 1.2.3. Chordotonal organs

**Femoral chordotonal organ**

A chordotonal organ in the femur was first mentioned in the work of Borchard (1927) about heteromorphism in *Dixippus* (today *Carausius*) *morosus*. The fCO lies in the dorsal part of the proximal femur and is connected to the proximal tibia with a receptor apodeme (Bässler, 1965, Field and Matheson, 1998, Füller and Ernst, 1973). A single scolopidium within the fCO consists of two sensory neurons, one fiber cell and one or more sheet cells (Füller and Ernst, 1973). The fCO consists of a ventral scoloparium with 80 sensory neurons and a dorsal scoloparium with 420 sensory neurons (Kittmann and Schmitz, 1992). The sensory cells of the dorsal scoloparium measure manly vibration (Büschges, 1994, Field and Pflüger, 1989, Sauer and Stein, 1999, Stein and Sauer, 1999) and the cells of the ventral scoloparium measure velocity, position and acceleration of the tibia, or a combination of these three parameters (Büschges, 1994, Hofmann and Koch, 1985, Hofmann et al., 1985). The sensory feedback of the ventral scoloparium, is used in the FT control loop (Kittmann and Schmitz, 1992). In the inactive animal, the feedback about extension of the tibiae, measured by the fCO, elicits a RR, to maintain posture (Bässler, 1972a, b, Büschges, 1989, Büschges, 1990, Kittmann, 1991), while in the active animal, a behavioral state elicited by stimulation or by walking, the same feedback can generate a AR that assists FlxTi activity (Bässler, 1974, Bässler, 1983, 1988, Cruse and Schmitz, 1983, Nothof and Bässler, 1990, Schmitz, 1985, Weiland and Koch, 1987). In addition to the influence on the FT joint, the feedback of the fCO also produces inter-joint influences. The elongation of the fCO increases the activity of the LevTr with a phasic and a tonic component, and also entrains LevTr MN activity, while the opposite was found for DepTr (Bucher et al., 2003, Hess and Büschges,

1997, Hess and Büschges, 1999). RetUng activity is increased by the fCO elongation, eliciting a AR (Bässler, 1988). The ProCx MN are only found to be weakly affected by fCO feedback (Bässler, 1986, 1988). The fCO was also shown to have intersegmental influences. Stimulation of the front leg fCO could elicit an increase in FlxTi and RetCx, and simultaneously a decrease ProCx activity (Ludwar et al., 2005a).

**Subgenual and distal organ**

The subgenual organ is located as a hemicircle in the proximal tibia and consists of 41 to 44 sensory neurons. The distal organ is located distally to the subgenual organ in the tibia and consists of 16-17 sensory neurons. The subgenual organ is thought to be sensitive to substrate vibrations (Field and Matheson, 1998, Strauß and Lakes-Harlan, 2013).

### 1.2.4. Other sense organs

**Apodeme receptor**

The apodeme receptor is connected to the distal part of the ExtTi muscle tendon, and reacts to both flexion and extension of the tibia. Its minimum firing frequency is reached at a FT joint angle of around 90° (Bässler, 1977a, 1983), but the reaction was only found in *Cuniculina impigra*.

**Tension receptor**

One sense organ, reacting to tension of muscle fibers of the FlxTi muscle was found by Bässler (1977a).

**Récepteur dorso-antéro-latéral**

The Récepteur dorso-antéro-latéral has first been described by Coillot and Boistel (1968) in the locust. In the stick insect, the Récepteur dorso-antéro-latéral was described by Bässler (1977a). It's firing frequency increases through extension of the tibia and putting pressure onto the membrane of the FT joint. It's effect on SETi was reported to be either increasing or decreasing SETi firing frequency (Bässler, 1977a).

**Récepteur dorso-postéro-latéral**

The Récepteur dorso-postéro-latéral has first been described by Coillot and Boistel (1968) in the locust. In the stick insect, the Récepteur dorso-postéro-latéral firing frequency is increased by a fast extension of the tibia, and by putting pressure onto the membrane of the FT joint. Stimulation of this receptor decreased the firing frequency of the SETi in most of the experiments (Bässler, 1977a).

**Récepteur ventro-postéro-latéral**

The Récepteur ventro-postéro-latéral has first been described by Coillot and Boistel (1968) in the locust. Bässler (1977a) described the Récepteur ventro-postéro-latéral to possibly react to deformation of the FT joint membrane, if the leg is completely flexed.

In summary, there are many sense organs in and on the stick insect leg, however, for the control loop of the legs motor network, the main sense organs are probably the hair plates, responsible for giving positional information, CS, reporting on load, and the fCO, giving feedback about movement and position of the tibia.

## 1.3. Femoro-tibial control network

The FT control network consists of five levels of parallel and antagonistic interaction. The first level is the information of the sense organ itself. Bässler (1993) described the FT control network with fCO as afferents, but also the CS and HP sensory feedback is processed in the same fashion (review see Büschges and Gruhn, 2008, Schmitz and Stein, 2000). As described above, the fCO has sensory cells measuring position and movement of the FT joint in the ventral scoloparium (Büschges, 1994, Hofmann and Koch, 1985, Hofmann et al., 1985). The afferents receive presynaptic inhibition at their terminals from afferents of the same type (Sauer et al., 1997). Additionally, the fCO afferents also receive presynaptic inhibition from other leg sense organs, like CS and HP (Stein and Schmitz, 1999). The second level of processing is the synaptic connection from the fCO afferents onto NSI, spiking interneurons (SI) and ExtTi and FlxTi (as well as MN of the other leg joints). The NSI receive inputs from fCO afferents with the same parameter, which are acceleration, velocity and position, or a combination of these parameters. The postsynaptic cells can either receive direct excitatory or delayed inhibitory inputs (review in Bässler and Büschges, 1998, Büschges, 1990, Sauer et al., 1995, Sauer et al., 1996). The third level is the transmission of information from the NSI onto the ExtTi MN. The NSI provide parallel excitatory and inhibitory drive to the ExtTi MN (Büschges, 1990, Driesang and Büschges, 1996, Sauer et al., 1996). The fourth level is the force produced by the ExtTi muscles. The muscle is innervated by the common inhibitor 1, inhibiting the ExtTi muscle, as well as the fast extensor tibiae (FETi) and the SETi MN, which excite the ExtTi (Bässler and Storrer, 1980). The last level is the movement of the tibia as a result of ExtTi muscle contraction, and that of its antagonist, the FlxTi muscle (Bässler and Stein, 1996). As this neuronal network is not only capable of producing the RR, which was analyzed in the present study, but also its reversal, it's FT joint control was described by Bässler (1993) as one that was controlled through distributed processing in a parliamentary manner, consisting of these five levels that act in parallel and also antagonistically (cf. Kristan, 2000, Morton and Chiel, 1994).

## 1.4. Sensory processing during curve walking

The kinematics of the stick insects middle leg on the inside of the turning animal differs strongly from the one on the outside. The leg on the inside is mostly moved around the FT joint, while the movements of other leg joints are often rather small. The outside middle leg, on the other hand is retracted and protracted strongly, while the FT joint is only moved very little (Dürr and Ebeling, 2005, Gruhn et al., 2009 a). Moreover, when the leg functions as inside leg (inside) the frequency of ARs is increased over straight walking, while ARs in leg functioning as outside leg (outside) are rare (Hellekes et al., 2011, Hellekes, 2012).

A mechanism underlying this change in motor activity is likely to be the relative weighting of excitatory and inhibitory synaptic inputs to NSI, which is known to change during the generation of the AR (Bässler and Büschges, 1990, Driesang and Büschges, 1996). Hellekes (2012) gave some insight into the task specificity of fCO afferent feedback processing in NSI during curve walking. She could show a change in weighting of synaptic inputs to some NSI in the mesothoracic ganglion also during turning. During outside stepping the NSI E2/3 was inhibited less, whereas a strong inhibition was visible during inside-stepping, when the fCO was stimulated. For an another NSI, E5/6, the membrane potential changed from a slight hyperpolarization during inside-stepping to a slight depolarization during outside-stepping (Hellekes, 2012). NSI E8, did show a hyperpolarization for outside-stepping during fCO stimulation, which was not found for the inside-stepping leg. However, for this neuron, only a qualitative analysis was possible (Hellekes, 2012). For other NSI (E4, E9/10, I2 and I) no differences were found between the outside- and inside-stepping leg (Hellekes, 2012). Even though this was not tested during curve walking, the NSI E4 shows three different reactions during generation of the AR, where in two out of three possibilities also APs of the SETi were shown during the inactive phase (Driesang and Büschges, 1996).

## 1.5. Interleg and central influences

In addition to local network activity, also influences from other leg joints, neighboring legs, and other descending central influences have an impact on the motor output of the FT joint motor network.

In the stick insect, stepping of a single front leg can elicit alternating activity in the MN pools of the middle leg, and also increase general activity in MN of the hind leg. The MN in the mesothoracic segment receive a tonic depolarization, which is then phasically modulated by front leg stepping (Borgmann et al., 2007, Borgmann et al., 2009, Gabriel, 2005, Ludwar et al., 2005a, Ludwar et al., 2005b). With pharmacological experiments, Westmark et al. (2009) could show that the tonic depolarization is evoked by acetylcholine (ACh) and enhanced by the neuromodulator octopamine (OA). OA was found to modulate motor output and arouse motor activity in locust as well as stick insects (Büschges et al., 1993, Sombati

and Hoyle, 1984). A source for OA in the stick insect is likely to be dorsal unpaired median (DUM) neurons, which originate in the suboesophageal ganglion. By stimulation of these neurons, two classes of neurons were found that influence the gain of the ExtTi MN activity during fCO stimulation (Stolz el al., in prep).

## 1.6. Experimental Approach

The stick insects used in the present study, *Carausius morosus*, *Cuniculina impigra* and *Aretaon asperrimus* have the advantage to be relatively large in size for an insect, and that they are easy to breed and to keep.

In the present study, different approaches have been used to investigate the influence of leg sense organs on the activity of the motor control network of phasmid species. In all experiments, a slippery surface setup (Berendes et al., 2013, Gruhn et al., 2006) has been used.

The slippery surface setup has been used to study backward walking (Graham and Epstein, 1985, Rosenbaum et al., 2010), escape responses of cockroach (Camhi and Nolen, 1981), curve walking of cockroach and stick insects (Gruhn et al., 2009 a, Hellekes et al., 2011, Tryba and Ritzmann, 2000), and changes in velocity of stick insects (Gruhn et al., 2009 b). These setups have the advantage that the animal is free to move its legs, and the legs are not coupled mechanically via the substrate, the animal is walking on. This reduces sensory load feedback and unmasks the influence of central output during walking.

The first study used a trapdoor setup, to reveal the influence of load on the timing and magnitude of motor output in the middle leg. The investigated leg is stepping on a platform, which is giving the exact time of ground contact via a current circuit. The platform then can be lowered, while the animal is in swing-phase. The next ground contact is then measured by a laser light sheet which the leg passes through (Berendes et al., 2013). Through this method, the impact of the missing ground on the motor output of this step could be investigated.

In the second and third study, a slippery surface setup was used without measuring the ground contact. The slippery surface was used to allow stationary animals to freely move their legs and be able to perform turning movements (Gruhn et al., 2009 a, Hellekes et al., 2011, Hellekes, 2012). Here, I used mechanical (Bässler, 1977b, Hellekes et al., 2011) as well as electrical stimulation (Sauer et al., 1995) of the fCO to investigate the task-dependent processing of fCO feedback. In the different approaches, neuronal and muscle activity was monitored by electromyographic, extracellular and intracellular recordings.

## 1.7. Aim of the study

Sensory feedback is known to influence the muscle activity and phase transitions in stick insects. For the FT motor network, the fCO and the CS of the leg have an mayor influence on the MN output. However, only little was known about the influence of load sensors on the timing and the magnitude of muscle activity. By using a trapdoor setup, the impact of load sensing on muscle activation and activity could was to be compared for steps with and without loading of the leg. In addition, by ablating load sensing CS selectively, experiments should reveal the source FlxTi activation and modulation in the middle leg.

Furthermore, the influence of leg sense organs seems to be task-dependent (Hellekes et al., 2011). The influence of the parameters of the tibial movement and position could influence the likelihood of occurrence of ARs and RRs, depending on the task, the leg is performing. It was an additional aim to test this and to reveal the mechanisms, underlying the task-dependent changes of fCO afferent processing.

# 2. The influence of touchdown in the middle leg of the walking stick insect for the control of the local locomotor activity[1]

## 2.1. Abstract

A lot is known about how the activation of MN pools in the locomotor control system of stick insects is influenced by sensory input. However, the neuronal connectivity of the sensory feedback as well as the exact origin is unknown. In the present study, I make use of a trap door setup to expose the role of ground contact for the intensity and timing of the activation of stance phase muscles in different stick insect species. I also investigate the afferent inputs that contribute to the respective changes. When the animal steps into the trap door, only the timing of the activation of the FlxTi muscle was changed. In addition, the magnitude of the activity of all stance phase muscles (RetCx, DepTr, FlxTi and RetUng) is changed, if the leg unexpectedly lacks ground contact. Ablations of single and multiple force sensors on the leg were performed to reveal the source of sensory feedback. The Ablations show that the feCS have a major role in timing of the activation of the FlxTi, by increasing the latencies to activation during ground and air steps significantly. Our results show that the swing-to-stance transition of the FlxTi is timed by load feedback. However, the ablation experiments also show that additional sensory feedback may be involved in FlxTi muscle activation. With respect to timing, the DepTr, RetUng and RetCx are controlled by sensory feedback other than that elicited through ground contact.

## 2.2. Introduction

Movement of legs during stepping in vertebrates as well as invertebrates is the joint result of central rhythmic activity influenced by sensory feedback. Central rhythmic activity and the sensory feedback are also responsible for the activation of all muscles, which then cause movements of limbs and the body (for review see: Kiehn and Kjaerulff, 1998, Pearson, 2008), for example in mammals (Brown, 1911), in turtle (Robertson et al., 1985) and in insects (Büschges, 1995a, 2005). The discussion about the relative importance of central output over sensory feedback for the timing of the coordinated muscle movements has been

[1] The chapter is already published: Schmitz J, Gruhn M, Büschges A. The role of leg touchdown for the control of locomotor activity in the walking stick insect. J Neurophysiol. 2015 Apr 1;113(7):2309-20. doi: 10.1152/jn.00956.2014. Epub 2015 Feb 4. The authors contributions to the paper are as follows: JS, MG, and AB designed research; JS performed experiments, analyzed data and prepared figures; JS, MG, and AB wrote manuscript. The chapter is a modified version of the original paper.

discussed since the early days of neuroscience, starting with Brown and Sherrington (Brown, 1911, Prochazka et al., 2000, Sherrington and Sowton, 1911). Since then, it has been shown that proprioceptive feedback from sense organs of the leg is shaping the CPG activity by modifying the magnitude of motor output as well as the timing of phase transitions between muscle antagonists in each individual leg joint (for detailed reviews, see Büschges, 2005, Büschges and Gruhn, 2008, Duysens et al., 2000, Ekeberg et al., 2004, Orlovskiĭ et al., 1999). In addition, much is known about the phase transition of a step cycle, between the stance and swing phases, and how it is affected by load and position feedback measured by sense organs in the same and also in neighboring legs (Bässler, 1967, 1993, Conway et al., 1987, Cruse, 1985, Cruse, 1990, Duysens and Pearson, 1980, Gorassini et al., 1994, Hiebert and Pearson, 1999, Wendler, 1964, Zill et al., 2009). In vertebrates, an experiment with cats used a trap door setup to reveal the relative contribution of peripheral over central influences during transitions between step cycles. In this experiment, a cat was walking on a tread wheel for several steps until the wheel was taken away mimicking a step into a hole in the ground. This demonstrated an effect of sensory feedback on the leg ExtTi activity later than 30 ms after touchdown (TD), however, before that the leg ExtTi activity was driven centrally (Gorassini et al., 1994, Hiebert et al., 1994). In the present study, only the magnitude of the muscle activity was influenced by loading of the leg, not the timing. In invertebrates, namely the stick insect, there is knowledge about several sense organs that provide input and thus contribute to phase transitions of muscle antagonists (for review, see Büschges, 2005). It is known that the transition from stance to swing phase is affected by load and position feedback, on top of to coordinating influences from the neighboring legs (Bässler, 1967, 1977b, Cruse, 1985, Cruse, 1990, Wendler, 1964). On the other hand, during the transition from swing to stance, the timing of the FlxTi muscle was found to be linked to leg TD (Bässler et al., 1991, Berendes et al., 2013, Cruse, 1985, Gruhn et al., 2006, Rosenbaum et al., 2010). However, in the case of the FlxTi coupling to TD, it is unresolved to what extent this is affected by afferent feedback or by central drive. Therefore, Berendes et al (2013) developed a laser-assisted trap door setup, which is similar to the one used by Gorassini et al. (1994) for the cat, to study how local sensory feedback influences the activation of different leg muscles of the stick insect (Berendes et al., 2013). With this experimental setup it was shown that the timing of the activation of the FlxTi in stick insects was strongly delayed, or in some cases absent when ground contact was unexpectedly missing for one stance cycle. Berendes et al. (2013) proposed that the absence of load feedback due to the missing ground contact might be the reason of the delayed or missing FlxTi activation (Berendes et al., 2013). In vertebrates, tendon organs can provide load feedback (Prochazka et al., 1997). In insects the load sensing organs are CS (Büschges and Gruhn, 2008, Zill et al., 2004). In the cockroach as well as in the stick insect, ground contact could be signaled by

feCS and also trCS. These CS have been shown to play a crucial role for the stance initiation and its maintenance by influencing the MN activity (Akay et al., 2001, Akay et al., 2004, Rosenbaum et al., 2010, Zill et al., 2004, Zill et al., 2009). An increase in load on the leg has been demonstrated to initiate and increase the activity of DepTr MN (Borgmann et al., 2011, Cruse et al., 1993, Rosenbaum et al., 2010, Watson et al., 2002a, Zill et al., 2004, Zill et al., 2009), and the duration of the activity (Pearson and Bradley, 1972, Rosenbaum et al., 2010, Watson and Ritzmann, 1997, Zill et al., 1999). Also, the FlxTi muscle electromyogram (EMG) magnitude was reduced after ablation of feCS (Akay et al., 2001). However, until now, there is neither knowledge on how the activation of stance phase muscles is controlled through the TD signal in general, nor on the influence of CS as load sensors or other leg sense organs with regard to TD in particular (for review, see Büschges, 2005).

In the present study, I have used the trap door setup to systematically investigate the influence of the missing ground contact on the activity of the stick insect´s major stance phase muscles. The investigated muscles were RetCx, DepTr, FlxTi as described above, and the RetUng, all during forward stepping and the ProCx during backward stepping. Moreover, I analyzed the influence of the leg sense organs on FlxTi muscle activation, by selectively ablating either single or multiple sense organs, and also amputated parts of the leg, to remove multiple sense organs. I compared the latencies to the onset of the FlxTi activity between normal steps on ground (SG) and steps into the hole (SIH). Finally, to determine if the findings from Carausius morosus are transferrable to other phasmid species *Aretaon asperrimus* and *Cuniculina impigra*, which are often used interchangeably, I compared the response in all three species.

# 2.3. Material and Methods

## 2.3.1. Experimental animals

For the experiments in the present study adult, female stick insects of three species *C. morosus*, *A. asperrimus* and *C. impigra* were used. The animals were kept in a colony at the Biocenter of the University of Cologne under a 12:12-h light-dark cycle at 20–22°C and were fed with blackberry leaves (*Rubus fructiosus*) *ad libitum*.

## 2.3.2. Experimental setup

The trap door setup used in these experiments has been described in detail in Berendes et al. (2013). In brief, the animals were fixed on a wooden animal holder (see below) above a slippery surface. The slippery surface had a separate platform for the left middle leg (49 mm × 34 mm, stainless steel surface) which was integrated at the same height. This platform in the slippery surface is lowerable pneumatically (SLS-6-25-P-A; FESTO mini slide, Esslingen, Germany). It could be lowered from the original height in 2-mm intervals. The touchdown of the leg was detected electrically using a lock-in amplifier (Gruhn et al., 2006) (electronics workshop, Zoological Institute, Cologne, Germany). A small current is applied to the slippery surface and during TD a small voltage could be detected at the animal at any platform level. In addition, a sheet of laser light (LG series, 1 mW, 660 nm, Lasertechs, Aschaffenburg, Germany) was used to measure virtual touchdown (vTD) after the platform was lowered (photo detector SLCD-61N4, Silonex, Montreal, Canada). After a manual initialization of the setup control, the lowering of the platform occurred after the tarsus was lifted off from the platform the next time. The lift off was also measured by the ground contact detection circuit. After the platform was lowered, the passing of the tarsus through the level of normal ground level was detected by the laser light sheet. To allow more trials, the experimenter could bring the platform pneumatically back up to its original position at any time. The virtual and tarsal ground contact detection signals were converted by an analog-to-digital converter (Micro1401, CED, Cambridge, UK), and then it was recorded with the Spike2 software (version 7, CED, Cambridge, UK). The swing phase was marked by the period of maximum amplitude (Amax), where the tarsus is lifted off. Stance phase was marked by the minimum amplitude, where the tarsus had contact with the surface. When the tarsus crossed the laser light sheet, a digital pulse was produced, marking the first virtual ground contact. Additional deflection signals were possible, which could be caused by either a misreading of the laser signal, when the leg was changing its position within the laser light sheet or by additional leg movement through the light sheet. The moving leg on the slippery platform was recorded with a high-speed camera (AOS S-PRI, AOS Technology AG, Baden

Daettwil, Switzerland, resolution: 400 × 1,024 pixel, frame rate: 500 fps, shutter speed: 2000 µs) to ensure for correct ground contact detection.

### 2.3.3. Preparation

As described in Berendes et al. (2013), the stick insect was glued dorsal side up (two component glue, ProTemp II, ESPE, Seefeld, Germany) onto a balsa stick. Autotomy of the animals hind and front legs was induced as described in Schmidt and Grund (2003). If autotomy induction was unsuccessful, the legs were removed at the level of the coxa with scissors. Walking was elicited by tactile stimulation, either by stroking the abdomen with a brush for forward, or pulling on the antennae for backward walking. Electromyographic recordings of the stance phase muscles RetCx, DepTr, FlxTi, and the RetUng at its tibial branch for forward and the ProCx in backward walking, were done using two twisted copper wires (51 µm outer Ø) (Rosenbaum et al., 2010). The EMG recording sites were chosen according to locations described before (Radnikow and Bässler, 1991, Rosenbaum et al., 2010). The wires were freshly cut-off and the tips were inserted into the muscles through small holes in the cuticle, as described above, and fixed with ProTemp II glue. The recorded EMG signal was amplified 100-fold with an insulated preamplifier MA101 (electronics workshop, Zoological Institute, Cologne, Germany), and additionally amplified 10-fold with a signal conditioner/main amplifier MA102 (electronics workshop, Zoological Institute, Cologne, Germany). The EMG signals were band-pass filtered between 100 Hz and 1,000 Hz in all experiments. In most of the experiments ExtTi muscle cross talk was visible in FlxTi EMG recordings. However, it was easily distinguishable from FlxTi activity, based on its smaller amplitude and higher frequency. Over its length, the FlxTi muscle, which is multiply innervated, shows different innervation patterns depending on the location of the muscle recording (Debrodt and Bässler, 1989, Goldammer et al., 2012). As it has been reported previously by Berendes et al. (2013), the latencies to the activation of the FlxTi muscle can vary depending on whether the recording is proximal or distal within the femur. For the experiments in the present study, the recording wires of the EMG were always placed at the end of the proximal third of the femur. To determine the first large FlxTi unit of every stance phase and to distinguish the FlxTi from the ExtTi, a threshold was placed above the ExtTi potentials.

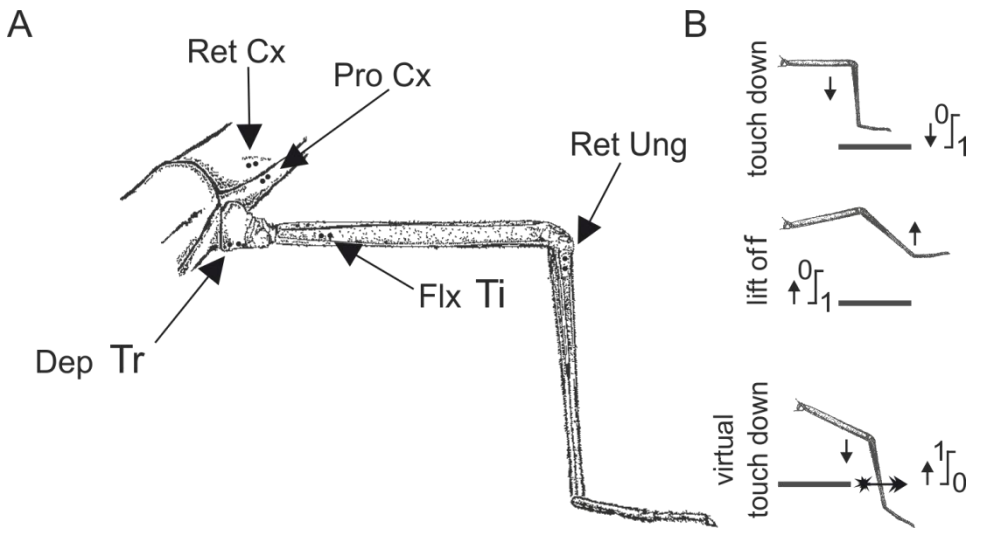



Fig. 2.1: EMG recording sites and current flow for detecting TD.
(A) Schematic drawing of the stick insects right middle leg with marked EMG recording sites for the stance phase muscles and (B) scheme of current flow during TD, lift off and the vTD; the left arrows mark leg movement and right arrows mark current flow. This is a modified figure, already published in Schmitz et al., 2015.

All experiments, in which ablations or amputations were carried out, were performed 24 h after surgery. These animals had not been used for control experiments previously, because the EMG signals could deteriorate over this period of time.

Ablations of CS were performed as described by Zill et al. (2011). The caps of the CS were destroyed with an insect pin, which was subsequently inserted into the destroyed cap. The CS, which were ablated included trCS (groups 1-4, Bässler, 1977a, Zill et al., 2012), feCS (Akay et al., 2001) and tiCS (groups 6A and B, Zill et al., 2011), together with two groups located anterior and posterior from these. After the CS ablation experiments, the tendon of the RetUng was cut. For this procedure the cuticle was opened at the tibia by cutting a window. Without damaging muscles or nerves in the tibia, the tendon was slightly lifted with an insect pin and cut with a fine pair of scissors. After cutting the RetUng tendon, the cuticle window was closed. For ablation of the tarsus, the tarsus and the distal part of the tibia were cut just above the tibia-tarsus joint. The different sense organs as well as the RetUng tendon were either destroyed in combinations or alone, as it is described below in the results section.

### 2.3.4. Evaluation of muscle latencies and magnitude of muscle activity

The latencies from TD to activation of the stance phase muscles were calculated by using the first unit in the EMG visible above noise level, TD as described above. For comparison of muscle activities between SG and SIH, the original EMG recordings were rectified (rect) and smoothed (sm) with a time constant of 20 ms and the integral of muscle

activity between the first 200 ms after TD and the vTD were compared (grey shaded area in Fig. 2.2). However, if the antagonistic muscle was activated before the time window of 200 ms ended and a second step was initiated within this time window, the steps were excluded from the analysis. The 200ms time window was chosen after the length of stance phase was compared to the time before the swing phase muscle activity started. In addition to the integral, the maximum value of the integrals was compared for the time window of 200ms. The values were exported to Origin for statistics (version 8.5, Origin Lab, Northhampton, MA).

For the analysis of the activation and activity of the stance phase muscles, 3 different experiments were performed in which I used three different species. In total, 16 animals of *C. morosus* were used. Three of them for the RetCx, ProCx and DepTr experiments, nine for the FlxTi, and four for the RetUng experiments. The FlxTi muscle activity was also analyzed in four animals of the species *A. asperrimus,* and five stick insects of *C. impigra* to compare cross-species differences. Furthermore, ablation experiments were performed to find the sensory source that influences the muscle activation and its magnitude. These experiments were carried out solely in *C. morosus*. Single ablations of the trCS and the tiCS were carried out in five animals, respectively, and the feCS was ablated in three different animals. In three experiments the trCS were ablated together with the feCS, and in five animals, the tiCS and the tarsus were ablated additionally. The RetUng tendon was cut in seven animals.

### 2.3.5. Statistics

The program Origin (version 8.5, Origin Lab, Northhampton, MA) was used for analysis and statistics. The acquired data sets were checked for normal distribution using the Shapiro-Wilk test. As none of the data sets were distributed normally, nonparametric test were used. For the dependent data sets the Wilcoxon signed-rank test, and for the independent data sets, the Mann-Whitney-U-test was used. Dependent data sets were the latencies, the integral and the amplitude compared between TD and vTD. As there were more TD before the vTD, these values were pooled for each walking sequence. Significant changes were marked in Figs. 2–5 as following: * for significance levels of $P < 0.05$; ** for significance levels of $P < 0.01$, and *** for significance levels of $P < 0.001$. The number of animals was labeled with N, the number of steps with n.

## 2.4. Results

During walking, stance phase muscles are activated in a specific sequence, which is modulated by sensory feedback (Büschges, 2005, Rosenbaum et al., 2010). In this regard, ground contact has been shown to have a major influence for the FlxTi muscle activation (Berendes et al., 2013).

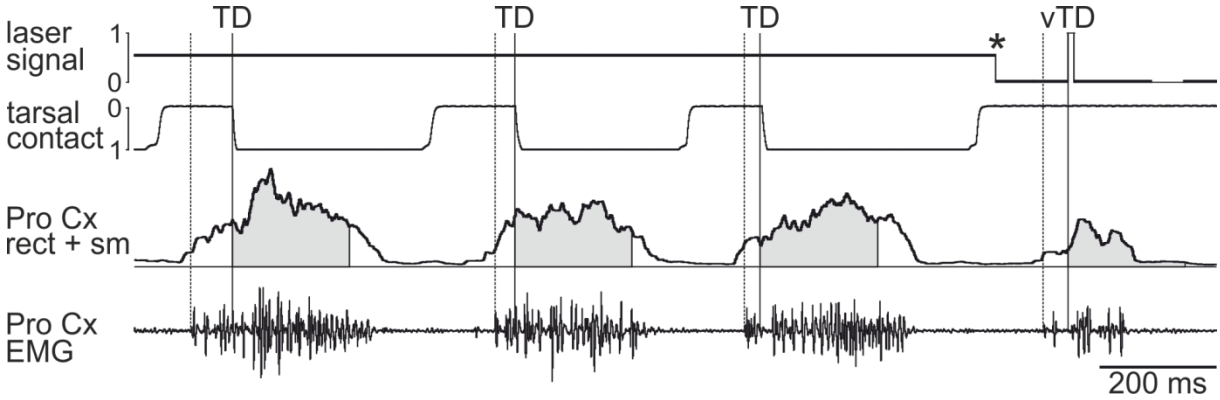



Fig. 2.2 Example of a ProCx muscle recording during backward walking.
Traces of three consecutive SG [after TD, marked with TD] and one SIH [after vTD, marked with vTD)]. *Top* trace marks laser signal [asterisk marks the activation of the laser light sheet], 2nd trace from *top* is the electrical tarsal contact trace with the slippery surface, 3rd trace from *top* is the rect and sm EMG from the 4th trace, the original EMG recording. Gray shaded area under the rectified and smoothed trace marks the window of 200-ms for the integral analysis. This is a modified figure, already published in Schmitz et al., 2015.

However, it needs to be investigated if sensory input from the ground contact also affects the activity of other stance phase muscles (ProCx and RetCx, DepTr, RetUng). Besides, it is still unclear, which sense organ signals link ground contact to the FlxTi muscle activation. For this purpose, I used the trap door setup described by Berendes et al. (2013) to investigate how ground contact influences the different stance phase muscles and to reveal sensory sources, which could affect these muscles.

First, the timing of the activation of all stance phase muscles was investigated using EMG recordings. Here, the RetCx, which was only investigated during forward walking, the ProCx, only during backward walking, and DepTr, FlxTi and RetUng were recorded. The recording sites of the EMGs are shown in Fig. 2.1. In Fig. 2.2 an example recording of a ProCx EMG is shown. Next to the original recording, the rectified and smoothed trace with the calculated integrals are depicted together with the respective tarsal contact trace for steps on the ground (SG) and one corresponding SIH (Fig. 2.2).

### 2.4.1. Ground contact influences on the latency of stance muscle activation

The RetCx, responsible for moving the leg from the back to the front by moving the coxa posteriorly was activated due to the TD. However there was no difference in activation latency of this muscle between SG and SIH (Fig. 2.3). RetCx activation latency measured in the three animals was on average -34.0 ms before SG (SD = 32.1; N = 3, n = 27), and with -33.5 ms to SIH not significantly different (SD = 21.0; N = 3, n = 9) (Fig. 2.3). During backward walking, the ProCx takes over the role of the RetCx, but moves the leg forward during stance

phase. An example recording of the activity of the ProCx is shown in Fig. 2.2 and Fig. 2.4, with a larger scale.

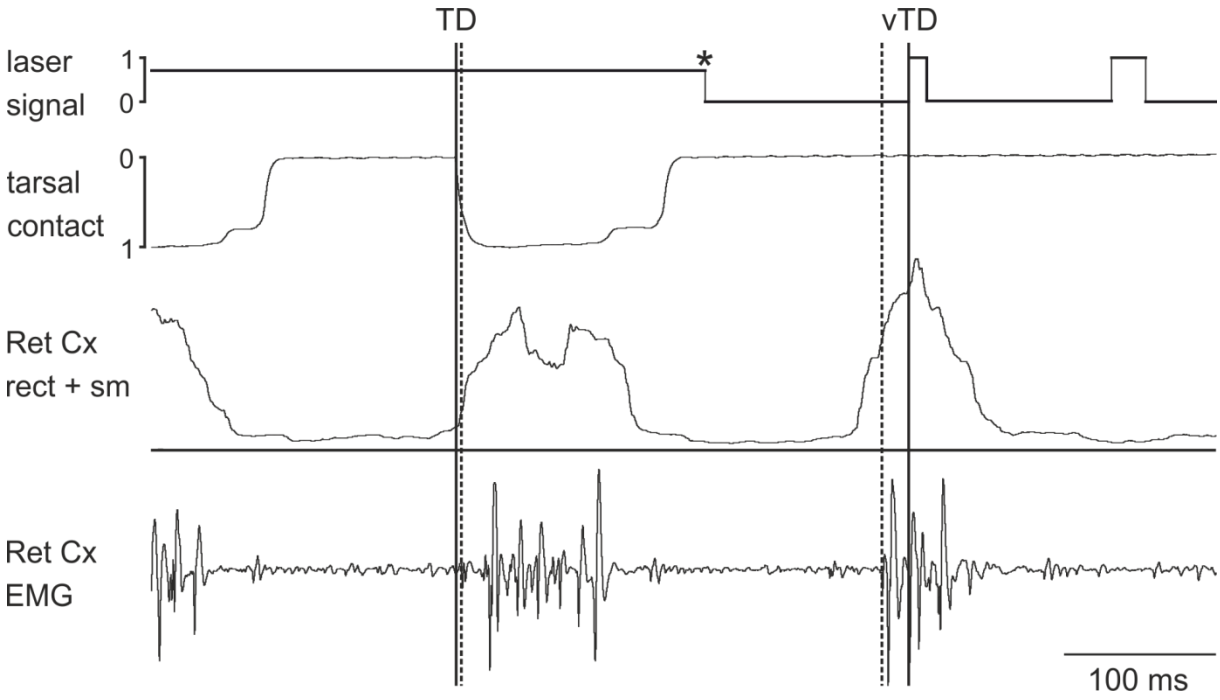



Fig. 2.3: Example of a RetCx muscle recording during forward walking.
Traces of one SG [after TD, marked with TD] and one SIH [after vTD, marked with vTD)]. *Top* trace marks laser signal [asterisk marks the activation of the laser light sheet], 2nd trace from *top* is the electrical tarsal contact trace with the slippery surface, 3rd trace from *top* is the rect and sm EMG from the 4th trace, the original EMG recording. This is a modified figure, already published in Schmitz et al., 2015.

On average, in three animals, the latency of the activation of the ProCx was −19.4 ms (SD = 24.7; N = 3, n = 27) before TD. There was no significant change to SIH with an average latency of −34.1 ms before the SIH (SD = 34.3; N = 3, n = 9). The DepTr, a stance phase muscle, which is located in the coxa and lowers the leg is activated a long time before TD and vTD (Fig. 2.5). The average latency was -93.5 ms before the SG (SD = 35.6; N = 3, n = 27) and −105.5 ms before SIH (SD = 39.9; N = 3, n = 9), which is not significantly different. In previous studies, a correlation of FlxTi activation and TD has been demonstrated (Berendes et al. 2013; Gruhn et al. 2006; Rosenbaum et al. 2010). In the present study the results of Berendes et al. (2013) could be confirmed.

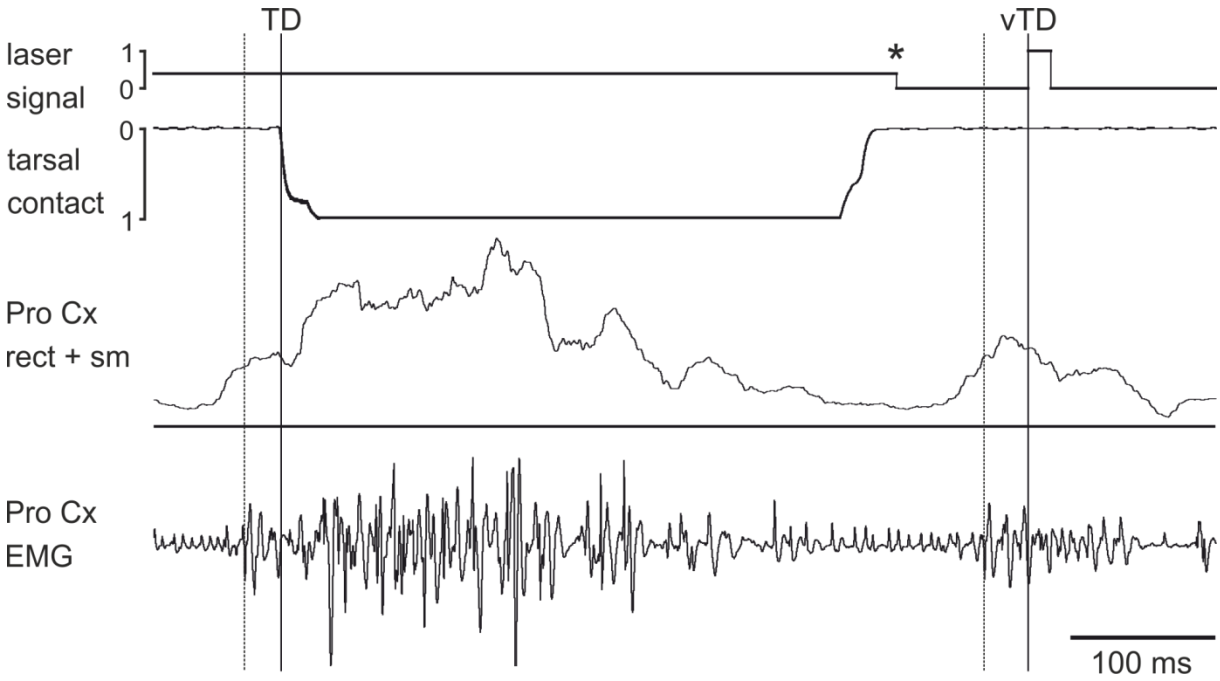



Fig. 2.4: Second example of a ProCx muscle recording during backward walking. Traces of one SG [after TD, marked with TD] and one SIH [after vTD, marked with vTD)]. Top trace marks laser signal [asterisk marks the activation of the laser light sheet], 2nd trace from top is the electrical tarsal contact trace with the slippery surface, 3rd trace from top is the rect and sm EMG from the 4th trace, the original EMG recording. This is a modified figure, already published in Schmitz et al., 2015.

The FlxTi latency during SG was significantly shorter compared to SIH ($P < 0.001$). The average latency of FlxTi to SG was 10.2 ms (SD = 3.0; N = 9, n = 171) after the TD and 36.3 ms (SD = 19.1; N = 9, n = 57) after vTD (Fig. 2.6). The last leg muscle tested, the RetUng, which is distributed over three different locations in the stick insect leg (proximal femur, proximal tibia and distal tibia), is responsible for flexing and lowering the claw. The RetUng EMG was recorded at the proximal tibia. The average latency of RetUng activation in respect to TD was −130.3 ms (SD = 63.3; N = 4, n = 36) before TD, which is shortly after the lift off. The average latency to the SIH was also around the time of the lift off with −142.1 ms (SD = 74.5; N = 4, n = 12), however, this was not significantly different from the latency of the SG (Fig. 2.7).

A summary of the results from all investigated stance muscles is shown in Fig. 2.8. Only the FlxTi had a significantly changed latency between stepping on the ground and stepping into a hole, which confirms the results of Berendes et al. (2013). The other stance phase muscles were not influenced in their activation, as they are all activated before the TD. Summarizing, these results indicate, with respect to the timing of muscle activation that only the FlxTi is affected by ground contact.

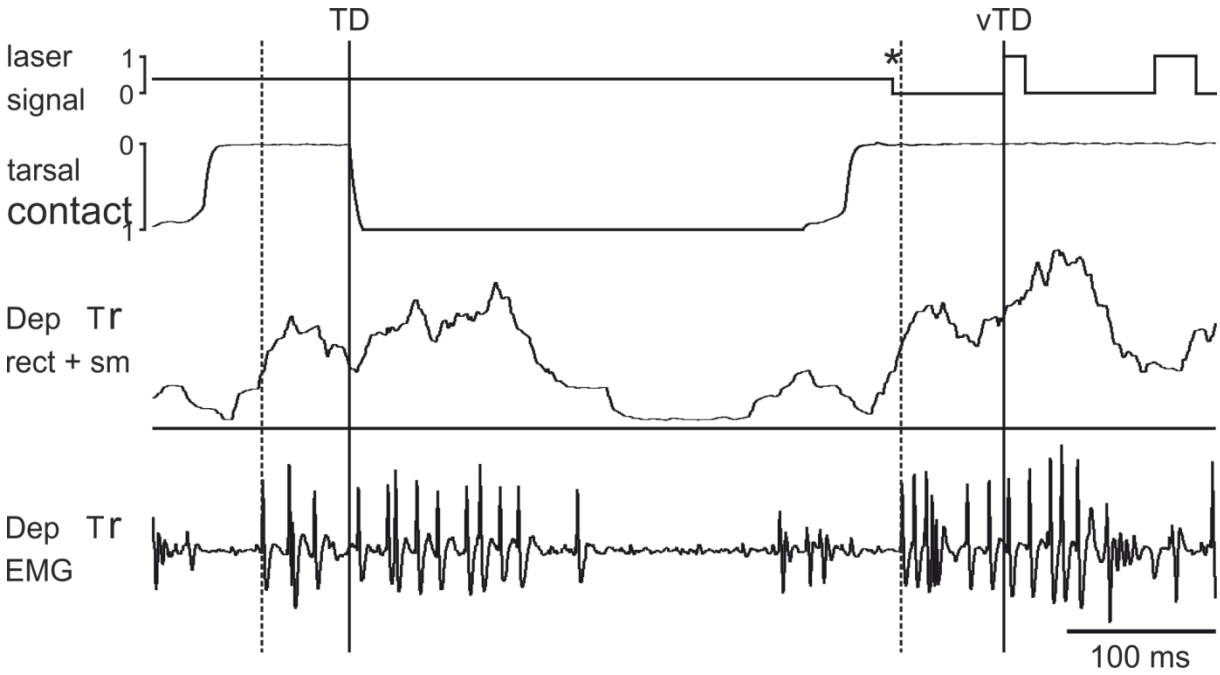



Fig. 2.5: Example of an ExtTi muscle recording during forward walking.

Traces of one SG [after TD, marked with TD] and one SIH [after vTD, marked with vTD)]. Top trace marks laser signal [asterisk marks the activation of the laser light sheet], 2nd trace from top is the electrical tarsal contact trace with the slippery surface, 3rd trace from top is the rect and sm EMG from the 4th trace, the original EMG recording. This is a modified figure, already published in Schmitz et al., 2015.

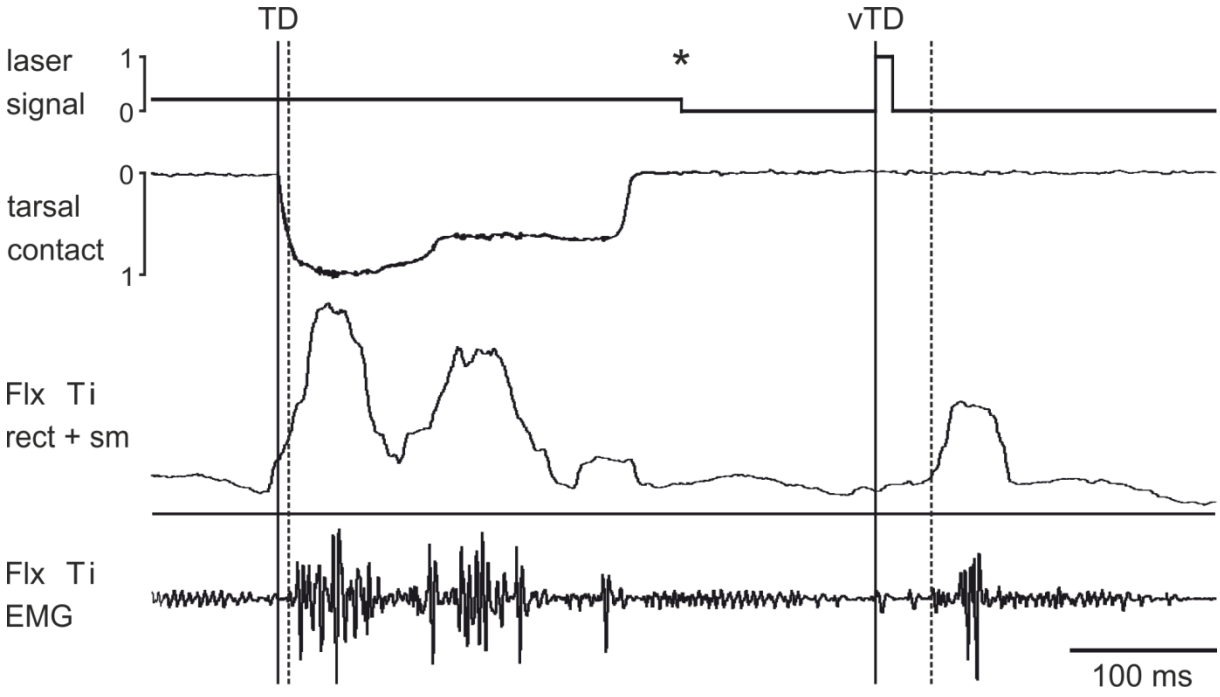



Fig. 2.6: Example of a FlxTi muscle recording during forward walking.

Traces of one step on the ground [after TD, marked with TD] and one step into the hole [after vTD, marked with vTD)]. Top trace marks laser signal [asterisk marks the activation of the laser light sheet], 2nd trace from top is the electrical tarsal contact trace with the slippery surface, 3rd trace from top is the rect and sm EMG from the 4th trace, the original EMG recording. This is a modified figure, already published in Schmitz et al., 2015.

### 2.4.2. Ground contact influences on the strength of stance muscle activation

In spite of the missing influence of the TD on the activation latency on all other stance phase muscles beside the FlxTi, there was a chance that the stance phase muscles could be influenced in their activity levels by the ground contact during the ongoing stance phase and after the leg was on the ground. Moreover, this has been described for two of the stance phase muscles, the FlxTi and the DepTr, in previous studies (Gabriel and Büschges, 2007, Rosenbaum et al., 2010).

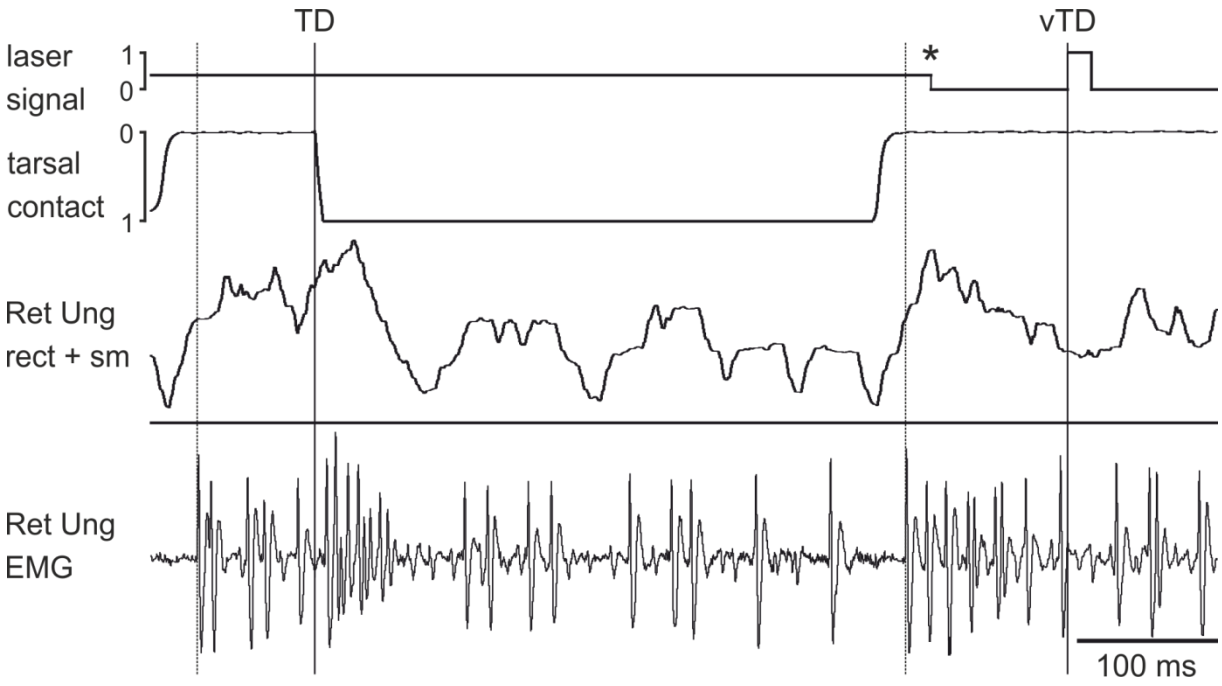



Fig. 2.7: Example of a RetUng muscle recording during forward walking.
Traces of one step on the ground [after TD, marked with TD] and one step in the hole [after vTD, marked with vTD)]. Top trace marks laser signal [asterisk marks the activation of the laser light sheet], 2nd trace from top is the electrical tarsal contact trace with the slippery surface, 3rd trace from top is the rect and sm EMG from the 4th trace, the original EMG recording. This is a modified figure, already published in Schmitz et al., 2015

To investigate the strength of the activity of the different stance phase muscles with respect to TD, I compared the strength in a time window of 200 ms after TD between SG and SIH. As a measure, I compared the ratio of the maxima between SG and SIH. Furthermore, I compared the area beneath the rect and sm EMG activity. The maximum (Amax) can give a measure about the maximal intensity of the activation, whereas the area can provide an insight about the overall strength of the activity during the time window. To better compare the two values from TD and vTD, I divided the Amax of SIH and also the integral by the ones of the SG. If the maximal amplitude as well as the integral of the SIH was smaller compared to the SG, the ratio was smaller than one. If it was the other way around, the activity during the SIH was increased, the ratio was greater than one.

Only the Amax of the RetCx activity did not show a significant difference between the SG and the SIH (Amax-ratioRetCx = 1.04, SD = 0.23). All other muscle activities differ significantly between SG and SIH. The DepTr was, however, the only muscle with an increasing ratio of the SIH divided by SG, which means an increase in activity during the SIH (Amax-ratioDepTr = 1.51, SD = 0.5, P < 0.01), whereas the three other muscles, ProCx, RetUng, and FlxTi showed a significantly greater maximal amplitude during SG, compared to

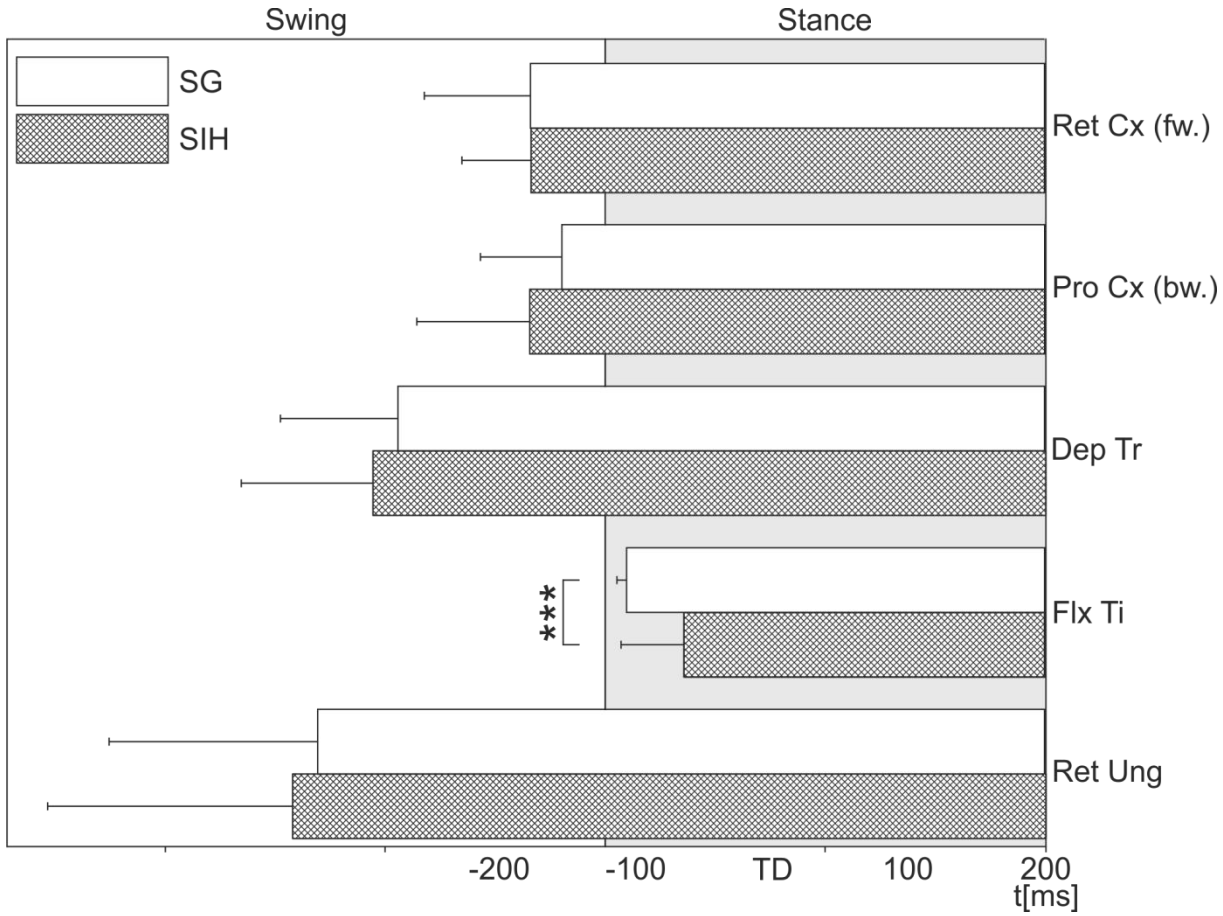



Fig. 2.8: Schematic comparing activation latencies for the different stance muscles during.
Activation latencies of stance phase muscles SG (open bars) and SIH (filled bars). fw, Forward; bw, backward. RetCx: N = 3, nSG = 27, nSIH = 9; ProCx: N = 3, nSG = 27, nSIH = 9; DepTr: N = 3, nSG = 27, nSIH = 9; FlxTi: N = 9, nSG = 171, nSIH = 57; RetUng: N = 4, nSG = 36, nSIH = 12. N = number of animals; n = number of steps; the gray shaded area marks stance. Values are means ± SD. ***Significant difference, P < 0.001. This is a modified figure, already published in Schmitz et al., 2015

SIH (Amax-ratioProCx = 0.68, SD = 0.22, P < 0.5; Amax-ratioFlxTi = 0.9, SD = 0.25, P < 0.01; Amax-ratioRetUng = 0.86, SD = 0.16, P < 0.05) (Fig. 2.9 A).

For the DepTr, also the SIH integral was significantly increased compared to SG (mean ratioDepTr = 1.75, SD = 0.63, P < 0.05). For all other stance phase muscles, RetCx, ProCx, FlxTi, and RetUng, the SIH-to-SG activity ratios were clearly decreased (mean ratioRetCx = 0.73, SD = 0.19, P < 0.05; mean ratioProCx = 0.42, SD = 0.13, P < 0.01; mean ratioFlxTi = 0.67, SD = 0.38, P < 0.001; mean ratioRetUng = 0.8, SD = 0.22, P < 0.01) (Fig. 2.9 B). The

values for the maximal amplitude and for integral of the stance phase muscle activity including the medians and interquartile ranges are summarized in Table 2.1.

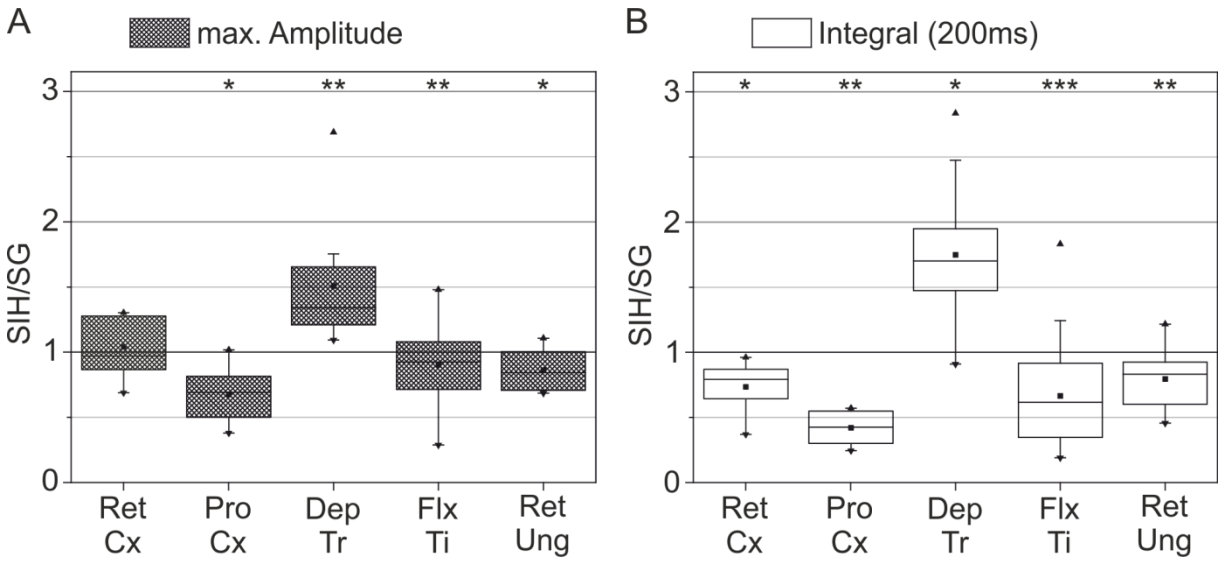



Fig. 2.9: Ratio of the Amax and integral of stance phase muscle activity
(A) The ratio of the Amax and (B) the integral of the rect and sm stance phase muscle EMG of SIH compared to the normalized activity during SG for a 200-ms period after TD. RetCx: N = 3, nSG = 21, nSIH = 7; ProCx: N = 3, nSG = 24, nSIH = 8; DepTr: N = 3, nSG = 27, nSIH = 9; FlxTi: N = 9, nSG = 171, nSIH = 57; RetUng: N = 4, nSG = 36, nSIH = 12. N = number of animals; n = number of steps. Values are means ± SD. Significant difference: *P < 0.05; **P < 0.01, and ***P < 0.001. This is a modified figure, already published in Schmitz et al., 2015.

On top of the fact that only the timing of FlxTi muscle activation is affected by touchdown, the comparison of the stance phase muscle activities demonstrates that feedback of the ground contact, does affect all stance phase muscles in the magnitude of their activity. However, the influence of the ground contact only affects the magnitude of already ongoing muscle activity. Furthermore, the influence can either be negative or positive, depending on the stance phase muscle.

### 2.4.3. Ground contact influences on FlxTi in different phasmid species

In addition to the well-known phasmid species *C. morosus*, two additional species, *C. impigra* and *A. asperrimus* were investigated, which are also often used in the research of locomotion. They have each have advantages (Berg et al., 2013, Jeck and Cruse, 2007) and data from *C. morosus* and *C. impigra* are often used interchangeably. *C. impigra* is a larger phasmid species, but with similar body proportions than *C. morosus*. *A. asperrimus* has much shorter legs, but is of similar size than *C. morosus*, with a more frequent walking activity.

Table 2.1: Ratios of the burst integrals during SIH vs. SG for the stick insect stance muscles

| SIH to SG burst integral ratio | mean | SD | Median | IRQ |
|---|---|---|---|---|
| FlxTi *total area* | 0.67 | 0.38 | 0.62 | 0.57 |
| FlxTi *maximum* burst amplitude | 0.9 | 0.25 | 0.93 | 0.37 |
| RetCx *total area* | 0.73 | 0.19 | 0.79 | 0.23 |
| RetCx *maximum* burst amplitude | 1.04 | 0.23 | 0.97 | 0.41 |
| ProCx *total area* | 0.42 | 0.13 | 0.42 | 0.25 |
| ProCx *maximum* burst amplitude | 0.68 | 0.22 | 0.69 | 0.31 |
| DepTr *total area* | 1.75 | 0.63 | 1.7 | 0.48 |
| DepTr *maximum* burst amplitude | 1.51 | 0.5 | 1.34 | 0.45 |
| RetUng *total area* | 0.8 | 0.22 | 0.83 | 0.32 |
| RetUng *maximum* burst amplitude | 0.86 | 0.16 | 0.84 | 0.3 |

Since the preparation of each phasmid species offers advantages to the experimenter (Berg et al., 2013, Jeck and Cruse, 2007), and as they are used interchangeably, I wanted to confirm if the observations in *C. morosus* were a general finding for phasmids. Since the magnitude of the activity and also the timing of the activation of the FlxTi muscle were influenced by ground contact in *C. morosus*, I investigated this muscle for a subsequent comparative analysis between the three species. The first EMG unit visible in the FlxTi EMG recording during SG had the shortest latency in *C. morosus* (mean = 10.2 ms, SD = 3.0; N = 9, n = 171) followed by the latency to the first EMG unit in *A. asperrimus* (mean = 14.9 ms, SD = 6.4; N = 4, n = 63). The longest latency was measured in *C. impigra* (mean = 21.4 ms, SD = 17.9; N = 5, n = 75). The latency to the first unit in the FlxTi EMG was significantly shorter between *C. morosus* and the other two phasmid species ($P < 0.01$ and $P < 0.001$, respectively), however, there was no significant difference in the latency between the *A. asperrimus* and *C. impigra*. This result was different for the latency to the first unit in the FlxTi EMG after the leg stepped into a hole. Here, still, the latency of *C. morosus* was the shortest, but the longest latency after SIH was measured for *A. asperrimus* (*C. morosus*: mean = 36.3 ms, SD = 19.1; N = 9, n = 57; *A. asperrimus*: mean = 190.5 ms, SD = 90.3; N = 4, n = 21; *C. impigra*: mean = 50.0 ms, SD = 28.0; N = 5, n = 25).

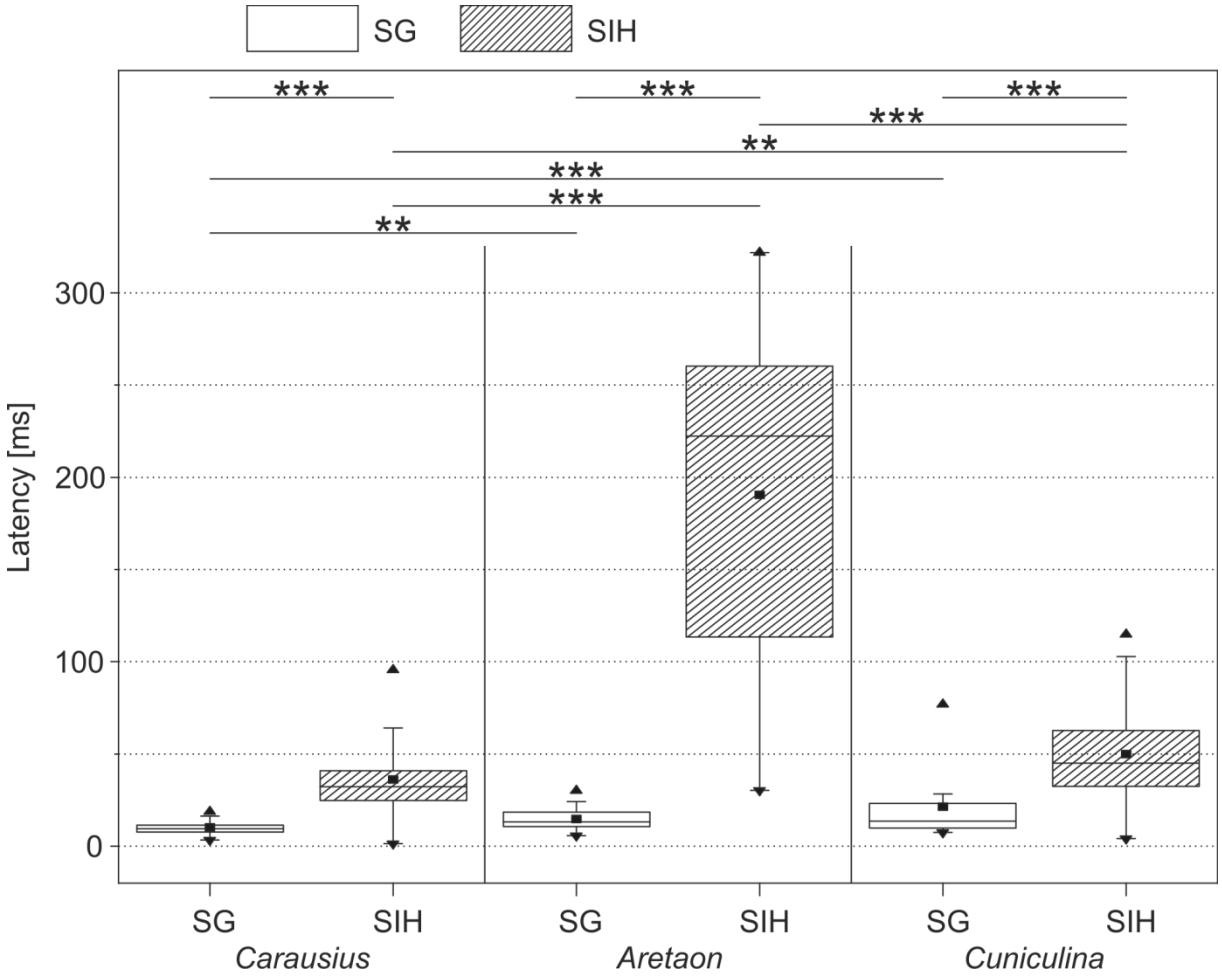



Fig. 2.10: Comparison of latency of FlxTi activation for three phasmid species
The FlxTi activation latencies during SG and SIH for the phasmid species Carausius morosus, Cuniculina impigra and Aretaon asperrimus; Carausius morosus: N = 9, nSG = 171, nSIH = 57; Aretaon asperrimus: N = 4, nSG = 63, nSIH = 21; Cuniculina impigra: N = 5, nSG = 75, nSIH = 25. This is a modified figure, already published in Schmitz et al., 2015.

All latencies to the first unit in the FlxTi EMG after SIH were significantly different to each other in the different phasmid species ($P < 0.001$, except *A. asperrimus* to *C. impigra* SIH: $P < 0.01$) (Fig. 2.10). In the three different species, the latency to the first unit in the FlxTi EMG after SIH was significantly longer than the latencies after SG for all three species ($P < 0.001$).

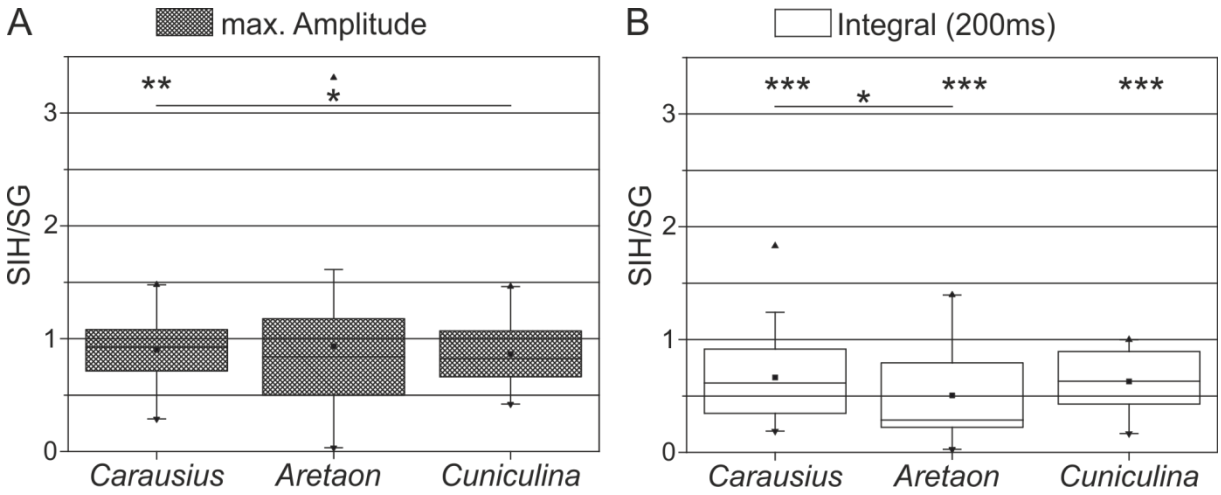


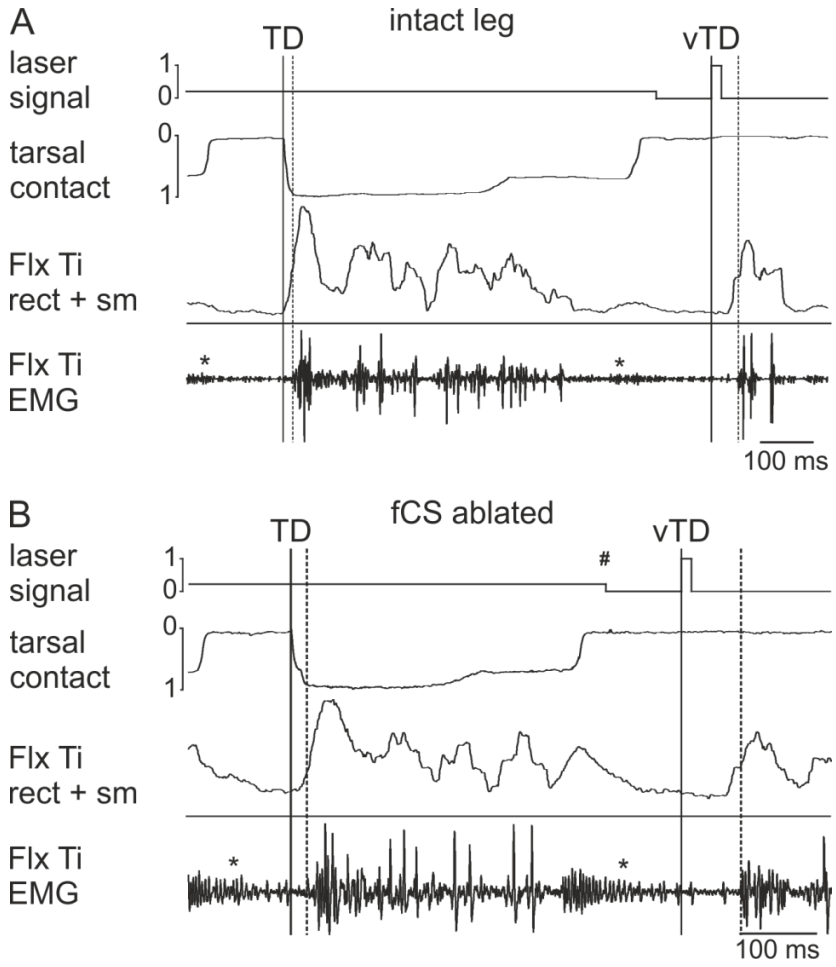

Fig. 2.11: Ratio of the maximal amplitude and integral of FlxTi activity for three phasmid species

(A) The ratio of the maximal amplitude and (B) the integral of the rect and sm FlxTi muscle EMG of SIH compared to the normalized activity during SG for a 200-ms period after TD. Carausius morosus: N = 9, nSG = 171, nSIH = 57; Aretaon asperrimus: N = 4, nSG = 63, nSIH = 21; Cuniculina impigra: N = 3, nSG = 39, nSIH = 13. N = number of animals; n = number of steps. Values are means ± SD. Significant difference: *P < 0.05; **P < 0.01, and ***P < 0.001. This is a modified figure, already published in Schmitz et al., 2015.

Not just the timing also the magnitude of the FlxTi activity was compared between the three species. Similarly to the FlxTi Amax of *C. morosus*, the Amax of the rect and sm FlxTi EMG was smaller in *A. asperrimus*. (93.3%, SD = 71.1) as well as *C. impigra* (86.4%, SD = 29.1), however, these changes were not significantly different (Fig. 2.11 A). Between the three phasmid species, only the ratio of the maximal amplitude of SIH to SG of *C. morosus* and *C. impigra* was significantly different ($P < 0.05$).

However, also here, the FlxTi activity ratio in the 200 ms window after the TD, vTD respectively, was significantly lower for *C. morosus* than for the other two species ($P < 0.001$). While the FlxTi activity in *C. morosus* during SIH dropped to 67% of its value compared to steps with ground contact (SG), it even dropped to 50.5% (SD = 38.5) in *A. asperrimus*. This lead to a significantly lower activity ratio compared to *C. morosus* ($P < 0.05$), while FlxTi activity in *C. impigra* dropped to 62.8% (SD = 28.4), which was not significantly different to the other phasmid species (Fig. 2.11 B).

In summary, in the presence of ground contact, the dependence of the strength of activity in the FlxTi muscle, and also of its activation was the same in the different phasmid species. For *A. asperrimus* the TD signal has the largest impact on the FlxTi activation. However, as there are also significant differences between the species, the three species offer themselves to the investigation of the relationship between activation of the FlxTi muscle and the sensory feedback caused by ground contact.




### 2.4.4. Potential sources of TD feedback for FlxTi activation

Next, I investigated potential sense organs that produce the relevant sensory feedback that affects the timing of the FlxTi activation in *C morosus*. These putative sense organs influence the activity of the FlxTi regarding the timing or magnitude by sensing the TD of the leg on the ground. Potential sense organs include the trCS, with the groups 1-4 (Bässler, 1977b, Zill et al., 2012), the feCS (Akay et al., 2001), and also the tiCS with the groups 6A and B (Zill et al., 2011). On the tibia, next to the tiCS groups 6A and B, there are two additional groups anterior and posterior. In a subset of the ablation experiments, the tendon of the RetUng was cut to remove feedback, which would be associated with activity in this muscle, measured by, for example, a multipolar stretch receptor connected to the RetUng tendon. Multipolar stretch receptors are known to be found in more proximal muscles (Bässler, 1977a, Bräunig et al., 1981, Matheson and Field, 1995). The ablation experiments were done either individually or in combinations. The recordings were made 24 h after the ablation procedure. In

Fig. 2.12: Example recording of FlxTi for intact and feCS ablation
Activation of FlxTi during SG and SIH in an (A) intact animal and (B) after ablation of the feCS. Solid vertical lines mark the TD and the vTD, vertical dashed lines mark muscle activation in the EMG recording, the *extensor tibiae* crosstalk marked by asterisks, *Top* trace marks laser signal [numbers sign marks the activation of the laser light sheet], 2nd trace from *top* is the electrical tarsal contact trace with the slippery surface, 3rd trace from *top* is the rect and sm EMG from the 4th trace, the original EMG recording. This is a modified figure, already published in Schmitz et al., 2015.

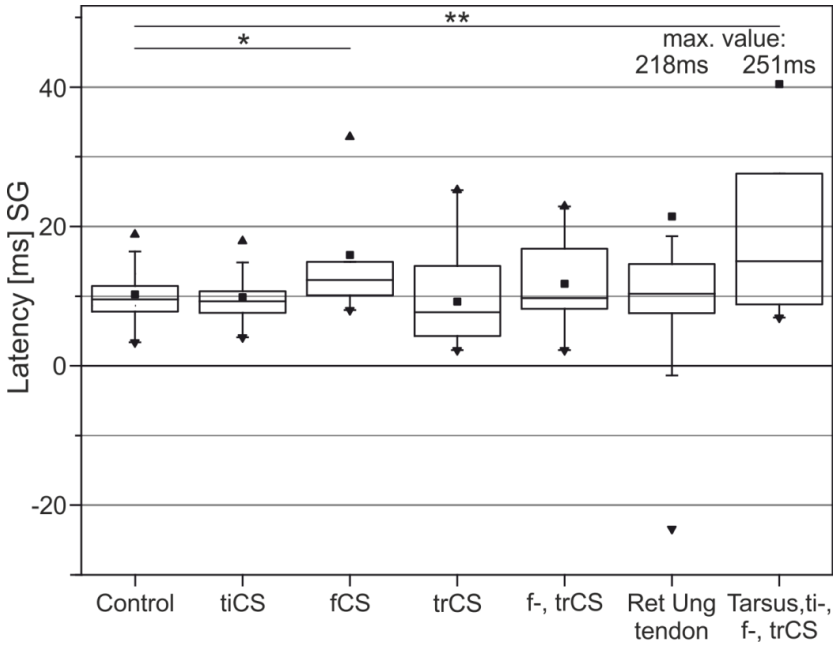



Fig. 2.13: FlxTi activation latencies after ablation of different sense organs during SG

FlxTi activation latencies after ablation of different sense organs during SG; the median and interquartile range of the SG values are shown. Control: $N$ = 9, $n_{SG}$ = 171; tiCS ablated: $N$ = 5, $n_{SG}$ = 90; feCS ablated: $N$ = 3, $n_{SG}$ = 27; feCS and trCS ablated: $N$ = 3, $n_{SG}$ = 54; trCS ablated: $N$ = 5, $n_{SG}$ = 45; RetUng tendon cut: $N$ = 7, $n_{SG}$ = 93; taCS, tiCS, feCS, and trCS ablated: $N$ = 5, $n_{SG}$ = 69. $N$ = number of animals; $n$ = number of steps. Values are means ± SD. Significant difference: *$P$ < 0.05; **$P$ < 0.01, and ***$P$ < 0.001. This is a modified figure, already published in Schmitz et al., 2015.

Fig. 2.12 a recording of the FlxTi is shown during SG and SIH in a control animal (A) and in another animal 24 h after the feCS had been ablated (B). There was no effect on the latency of FlxTi activation during SG in comparison to the control, after all trCS or tiCS were ablated (trCSabl: SGmean = 12.9 ms, SD = 6.2, N = 5, n = 15; tiCSabl: SGmean = 9.9 ms, SD = 3.2, N = 5, n = 90) (Fig. 2.13). However, the feCS ablation lead to a significant prolongation of the FlxTi latency (P < 0.05; feCSabl SGmean = 15.9 ms, SD = 9.2 ms, N = 3, n = 27). Interestingly, if the ablation of the feCS and the tiCS were combined, this did not leed to a significant change in the latency of FlxTi activation (feCStrCSabl SGmean = 11.8 ms, SD = 5.6, N = 3, n = 54). Before TD, the tarsus is flexed by the RetUng, also the TD itself leads to a passive stretching of the tendon of the RetUng. This stretch is prevented by cutting the tendon at the level of the mid-tibia, through which proper feedback from a putative stretch receptor would be removed.

Cutting the RetUng tendon, lead to a large increase in the variability of FlxTi latencies after TD (Variability tendon intact (Vartendon|int): 15.5 ms; Variability tendon cut (Vartendon|cut): 240.9 ms). However, the mean of the latencies in this ablation experiments was not different from the control experiment (SGmean = 21.4 ms, SD = 43.5, N = 7, n = 93) (Fig. 2.13).

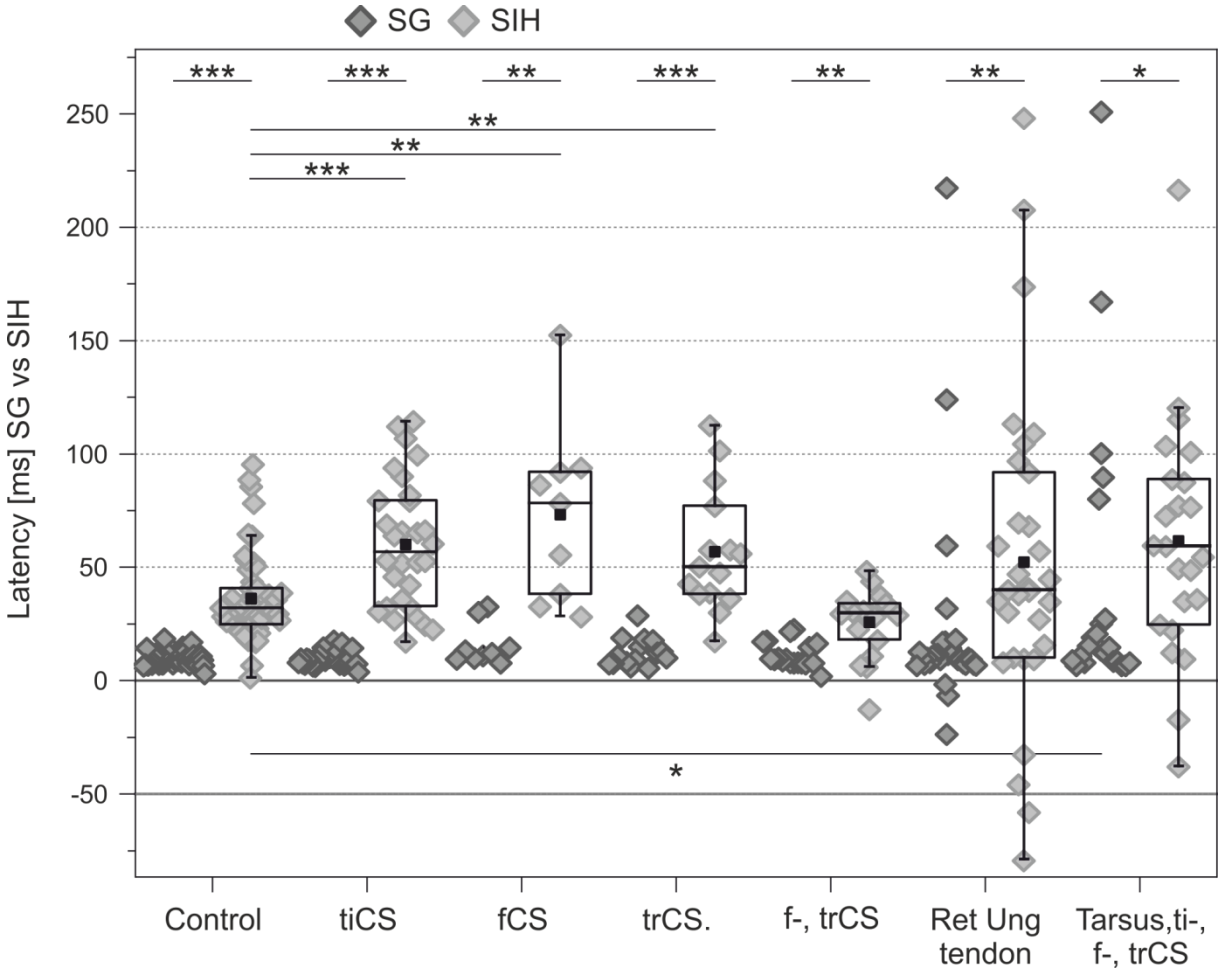



Fig. 2.14: FlxTi activation latencies after ablation of different sense organs during SG and SIH

Scatter plot for FlxTi activation latencies after ablation of different sense organs during SG and SIH; the median and interquartile range of the SIH values are shown (see Fig. 2.13 for median and interquartile range of SG values). Rhombuses in dark grey for SG and light grey for SIH. Control: N = 9, nSG = 171; tiCS ablated: N = 5, nSG = 90; feCS ablated: N = 3, nSG = 27; feCS and trCS ablated: N = 3, nSG = 54; trCS ablated: N = 5, nSG = 45; RetUng tendon cut: N = 7, nSG = 93; taCS, tiCS, feCS, and trCS ablated: N = 5, nSG = 69. N = number of animals; n = number of steps. Values are means ± SD. Significant difference: *P < 0.05; **P < 0.01, and ***P < 0.001. This is a modified figure, already published in Schmitz et al., 2015.

During the ablation experiments not only the stepping on the ground was examined, but also the activation latencies of the FlxTi after vTD. For all ablation possibilities I tested as it was also seen for the control, the latencies of FlxTi activation increased significantly after vTD compared to SG (Fig. 2.14). Furthermore, the ablation of individual parts of CS groups on the leg led to a significant increase of the latency in FlxTi response to SIH in comparison to control (trCSabl: SIHmean = 57.0 ms, SD = 27.0; N = 5, n = 15, P < 0.01; tiCSabl SIHmean = 60.1 ms, SD = 28.3, N = 5, n = 30, P < 0.001; feCSabl: SIHmean = 73.2 ms, SD = 39.4, N = 3, n = 9, P < 0.01). Interestingly, as well as for SG, for a combined ablation of feCS and trCS the latency of FlxTi activation after SIH was not different to control (feCStrCSabl SIHmean = 25.7 ms, SD = 14.9, N = 3, n = 18). Also, the cutting of the RetUng tendon had a similar effect on SIH latencies as described above for SG and did not differ

(tendon cut SIHmean = 52.2 ms, SD = 70.3, N = 7; n = 31), while the variability was greatly increased (Vartendon|int: SIH = 94.1 ms; Vartendon|cut: SIH = 326.8 ms).

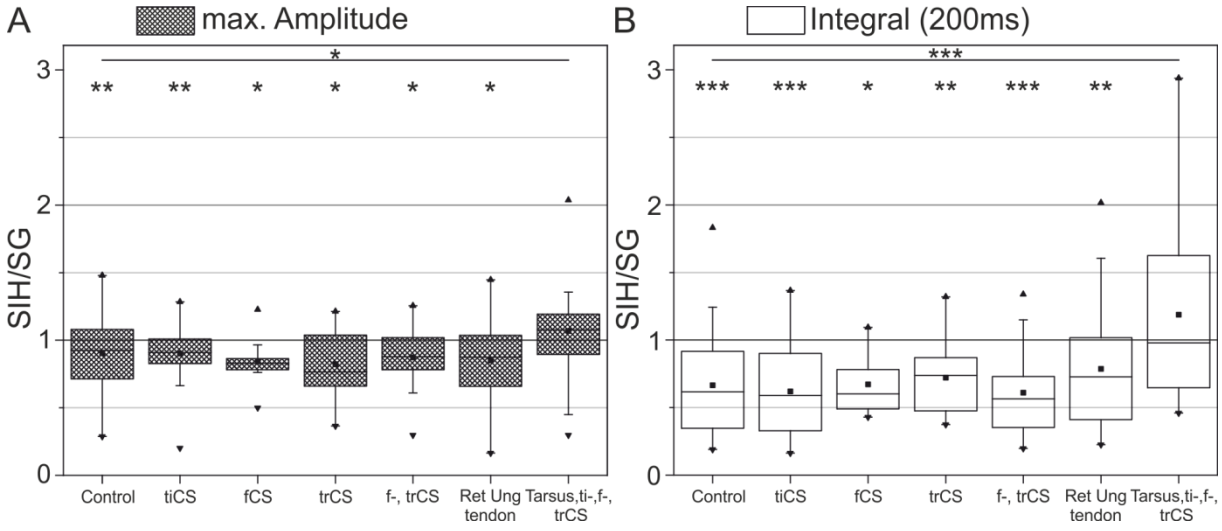



Fig. 2.15: Ratio of the maximal amplitude and integral of FlxTi activity after ablation of different sense organs

(A) The ratio of the maximal amplitude and (B) the integral of the rect and sm FlxTi muscle EMG of SIH compared to the normalized activity during SG for a 200-ms period after TD. Control: N = 9, nSG = 171; tiCS ablated: N = 5, nSG = 90; femoral feCS ablated: N = 3, nSG = 27; feCS and trCS ablated: N = 3, nSG = 54; trCS ablated: N = 5, nSG = 45; RetUng tendon cut: N = 7, nSG = 93; taCS, tiCS, feCS, and trCS ablated: N = 5, nSG = 69. N = number of animals; n = number of steps. Values are means ± SD. Significant difference: *P < 0.05; **P < 0.01, and ***P < 0.001. This is a modified figure, already published in Schmitz et al., 2015.

To see if an interplay of different sense organs, either ablated individually or in combinations, were responsible for the absence of effects during SG, I performed an experiment, where all CS groups were ablated together with the tarsus. The animals, on which this ablation experiments were performed, showed a great reluctance to perform continuous stepping with the investigated leg. In addition, the variability of the latencies of FlxTi activation after SG and SIH was greatly increased (VarSG = 243.9 ms; VarSIH = 254.2). In addition, the mean latency of FlxTi activation for both SG and SIH were increased as well (SGmean = 40.4 ms, SD = 61.0, P < 0.05; SIHmean = 61.7 ms, SD = 52.8, P < 0.05, N = 5, nSG = 69, nSIH = 23; (Fig. 2.13 and Fig. 2.14)).

Finally, after the effect of the ablation experiments on the activation of FlxTi was investigated, I wanted to know if there was also an effect of the ablations on the magnitude of FlxTi activity. As described above, the FlxTi activity during SIH was drastically reduced for both the Amax and total activity, measured by the integral of the rect and sm EMG (see above and Fig. 2.15 A and B). The maximal value of the integral for the SIH compared to the SG was significantly reduced in all ablation experiments, except for the one, where all CS and also the tarsus were ablated (summary of all values, see Table 2.2). However, for the same ablation experiments the ratio of the maximal amplitude between SIH and SG was

never significantly different from that in the intact animal (Fig. 2.15 B). The Amax values of SIH were only 82–90% of the respective SG value, while the total integrals of the SIH were reduced to between 61–78% (summary of all values, see Table 2.3).

Table 2.2: Burst integral percentage of FlxTi muscle during SIH compared with SG for the intact leg and for different ablation scenarios

| Burst Integral % of FlxTi activity in SIH compared to SG | | | | |
|---|---|---|---|---|
| | mean | SD | median | IRQ |
| Control | 66.513 | 38.359 | 61.582 | 57.03 |
| trCS abl. | 72.163 | 27.531 | 73.731 | 39.347 |
| RU tendon cut | 78.739 | 41.844 | 72.729 | 60.608 |
| fCS abl. | 67.147 | 22.96 | 60.186 | 29.023 |
| fCS, trCS abl. | 60.967 | 32.096 | 56.497 | 37.74 |
| tiCS abl. | 61.981 | 33.512 | 59.022 | 57.247 |
| Tarsus, tiCS, fCS, trCS abl. | 118.767 | 65.525 | 97.796 | 97.754 |

Table 2.3: Burst maximal amplitude percentage of FlxTi muscle during SIH compared with SG for the intact leg and for different ablation scenarios

| Maximum Burst Amplitude % of FlxTi SIH compared to SG | | | | |
|---|---|---|---|---|
| | mean | SD | median | IRQ |
| Control | 90.209 | 25.275 | 92.557 | 36.607 |
| trCS abl. | 82.17 | 24.943 | 76.424 | 37.807 |
| RU tendon cut | 85.144 | 27.115 | 87.457 | 37.787 |
| fCS abl. | 84.246 | 19.082 | 82.846 | 8.113 |
| fCS, trCS abl. | 87.246 | 22.483 | 87.721 | 23.658 |
| tiCS abl. | 90.072 | 22.778 | 90.918 | 18.317 |
| Tarsus, tiCS, fCS, trCS abl. | 106.828 | 37.488 | 107.608 | 29.781 |

The observed changes in the integral of the smoothed and rectified FlxTi EMG in the single or double ablation experiments, however, are not caused by qualitative changes of the EMG recording, like a delayed recruitment of single units or changes in the occurrence of small or large units (compare Fig. 2.6 and

Fig. 2.12 A and B). Only in case of the ablation of all CS fields together with the tarsus, was the integral of the rect and sm EMG significantly different compared to the control animals. As described above, these animals showed a reluctance to move the investigated leg. The variability was increased for both the Amax as well as the total integral. However, neither the mean of the total activity nor of the Amax were different between SG and SIH.

Instead, the ratio of both was significantly different from the control animals. Unlike in the other ablation experiments, the animals with the ablation of all CS and the tarsus, often lacked the large FlxTi units (data is not shown).

In summary, the only individual group of CS, where ablation had an effect on the magnitude of the FlxTi activation during stepping on the ground is that of the feCS. However, the ablation of the feCS as well as the ablation of all other CS on an individual leg part did change the latency of FlxTi activation during SIH.

A potential sense organ, connected to the RetUng tendon only increased the variability of the activation latency. While the ablation of all CS groups and the tarsus, which also ablates the anchor point of the RetUng tendon, also affected the latencies itself during SG and SIH. This was also the only procedure, which induced a change in the activity ratio of the FlxTi response between SG and SIH, which was absent after this particular procedure only.

## 2.5. Discussion

Using a trap door setup, in the present study, I systematically investigated, which leg muscles depend on TD for their activation during stepping, and to what extent. I found out that the only muscle whose activation depends on TD was the FlxTi. By comparing three different phasmid species, I could show that this muscle is equally dependent on TD in all three species, albeit to a different extent. To single out the sense organs responsible for the sensory feedback that contributes to the dependence of the FlxTi on the TD, I performed ablation experiments of load sensitive sense organs. The only sense organ that is having a direct effect on FlxTi activation is the feCS. However, a putative sense organ connected to the tendon of the RetUng led to a greatly increased variability in the latency of FlxTi activation after cutting the tendon. This suggests that this sense organ may also be involved in activation of the FlxTi.

### 2.5.1. Dependence of stance muscle latency on TD

The influence of feedback about TD on the activation of the stance phase muscles in stick insects are only partly known for *C. morosus* (Berendes et al., 2013). To gain further insight into the role of sensory feedback on timing and magnitude of muscle activation, I analyzed the latencies of all important stance phase muscles with respect to electrically determined TD in a two-middle-leg preparation in *C. morosus* during regular stepping on a slippery surface. The activation latencies of the DepTr and the FlxTi were identical to the values reported in previous studies for the stance phase of intact, six-legged animals (Gruhn et al., 2006, Rosenbaum et al., 2010). The RetUng, the FlxTi of the tarsus, was activated during swing as reported by Fischer et al. (2001). The only activation latencies with respect to TD, which were different from the ones of the intact, six legged animal, were the ones of

the RetCx and ProCx, during forward and backward stepping, respectively (Rosenbaum et al., 2010). However, in the study of Rosenbaum et al. (2010), it was reported that the RetCx activation occurred significant earlier, when the animal walked with two legs. This shift in activation was well before TD in any walking direction, and thus unrelated to TD or any signaling connected to it. The difference for the activation latencies of these two muscles between the two- and the six-legged animal could be a result of the general large variability in the activation latency of these muscles. However, it could also suggest that adjacent legs have an influence on the timing of these particular muscles. This had already been postulated previously. Borgmann et al. (2007, 2009) found influences by front leg stepping on the middle leg RetCx MN activation, however, it remains unclear, by which sense organ such an interleg sensory influences could be detected or how they are processed. A sense organ which could be responsible for the sensory feedback from the front to the middle leg could be the front leg fCO (Ludwar et al., 2005a).

### 2.5.2. Effects of lack of ground support on stance muscles

The reason to use the trap door setup was, to reveal if the lack of ground contact shifts the activation latency of stance phase muscles. This approach was first used in cats that walked on a tread wheel, and where the cat suddenly stepped into a hole (Gorassini et al., 1994, Hiebert et al., 1994). Here, the experiments revealed that ground contact influenced only the magnitude of *gastrocnemius lateralis* activity and only during the first 30 ms after TD (Gorassini et al., 1994, Hiebert et al., 1994). In the stick insect, the SIH affected both, the activation strength, and also the latency of selected stance muscles to a different extent. The FlxTi was the only muscle where the activation latency was affected in a way that it was significant greater for SIH compared to SG. This result confirmed an earlier study of Berendes et al. (2013). The FlxTi seems to be the only muscle where the timing the TD signal is influenced. In contrast to muscle activation, the intensity or magnitude was affected in all muscles by ground contact, however, not always with the same sign. In most of the stance phase muscles, the RetCx during forward, the ProCx during backward walking, the FlxTi, as well as the RetUng the activity was drastically reduced as a result of a lack of ground contact. In contrast, the activity of the DepTr increased significantly in this case. This result stresses the importance of local sensory feedback for the control of the magnitude of muscle activity.

For each stance phase muscles investigated in the present study, at least one single local sense organ is known to activate the muscle. The extension of the FT joint, measured by the fCO, is known to facilitate the activation of the RetCx (Bässler, 1986). In addition to the influence of the fCO, the vcxHP, which reports the position of the coxa, is capable of initiating and sustaining the activity of the RetCx (Bässler, 1977b, Cruse et al., 1984).

The antagonistic muscle, the ProCx, which is a stance phase muscle active during backward walking, is influenced by coxal hair rows (Cruse et al., 1984), but not investigated in detail. It could be that their feedback helps to initiate protraction stance movements. On top of the HPs and the fCO, both muscles are known to be influenced by local load feedback. The trCS are known to support the termination of the ProCx and initiation of RetCx activity (Akay et al., 2007). Additionally, the trCS are known to increase in RetCx activity itself (Schmitz, 1993), and, if the ProCx is acting as stance phase muscle, the trCS are also increasing ProCx activity (Akay et al., 2007). With a stimulation of the trCS it is also possible to sustain the stance phase (Bässler, 1977b). Hence, it is interesting that I found no change in the SIH latency of the RetCx activation during forward and also no change in the latency of the ProCx activation during backward walking. This result indicates that feedback about TD is only of little importance for timing of the RetCx and ProCx in the two-legged-preparation.

DepTr activation latency does also not change when the animal SIH, which also supports earlier studies (Bässler, 1967, Bucher et al., 2003, Graham and Bässler, 1981, Hess and Büschges, 1999). In these, it was shown that DepTr activation is strongly affected by the extension of the tibia, measured, again, by the fCO. Furthermore, as described before, during ongoing stance phase, sustained load from ground contact, measured by trCS, leads to an increase in DepTr activity (Rosenbaum et al., 2010, Zill et al., 2012, Zill et al., 2013). However, in our study, I also found that the lack of ground contact during the SIH leads to an increase in DepTr activity. This increase is probably caused by another mechanism, providing the feedback to the DepTr. Its sensory source is likely to be the trHP, located at the trochanter and giving feedback about the position of the joint (Schmitz, 1986b). In an ablation experiment, it could be shown that downward directed force is strongly reduced when this HP is dysfunctional. From this, one can conclude that the activation of this HP during the lowering of the leg during SIH could increase the activity of the DepTr (Schmitz, 1986b). RetUng activation latency did not change during SIH, but the activity of this muscle decreased below a certain leg position after the leg passed through the laser sheet. As sense organs that give feedback to the RetUng of locust, several sense organs on the tarsi have been described (Laurent and Hustert, 1988). Additionally, in stick insects, forces generated by the tarsal flexion, contribute to RetUng activity. This has been shown to enhance the muscle activity, however, with an average delay of 35 ms from force detection to muscle activation (Zill et al., 2010, Zill et al., 2014). This could explain reduction of mean activity when the animal steps into a hole.

Finally, the only muscle that is clearly influenced in its timing and magnitude was the FlxTi muscle. Its activation latency was strongly increased during SIH, accompanied by a reduction in the overall activity, confirming earlier results by Berendes et al. (2013). Both effects were seen in all three phasmid species investigated, however, to a different degree.

While the FlxTi activation latencies in the morphologically similar species *C. morosus* and *C impigra* were similar during SIH, the FlxTi activation latency was more dependent on the TD in *A. asperrimus*. In any case, however, all three species are suitable for further investigation of this phenomenon.

The effects on the magnitude in muscle activation were most likely underestimated in comparison to the effect, leg loading would have in an intact freely walking animal (Dallmann et al., 2016). Under our experimental conditions the animals had not to carry their own weight because they were tethered above a slippery surface. The reduction of magnitude during SIH in all investigated stance phase muscles, but the DepTr, fits to the reduction of amplitude of the *gastrocnemius lateralis* and *vastus lateralis* muscles in cats, when lacking ground contact (Gorassini et al., 1994). As described above, the increase in DepTr activity could be explained by a corrective response elicited by a further stimulation of the trHP, the more the leg is lowered (Schmitz, 1986b). The studies on the EMG response in cats with lack of ground contact have shown a strong dependence of the state and the posture of the contralateral leg on the magnitude control, which I did not investigate, and which does not play a role in a tethered animal (Gorassini et al., 1994). In cats, compared to control steps, the muscle activity of the ExtTi (which is the stance muscle in cat, was increased during SIH, when the contralateral leg was in swing phase (Gorassini et al., 1994). However, I did not observe a similar change in the response during SIH in stick insects, which suggests either a rather stereotypical response, or little or no influence of the state of the contralateral leg in this context. Nevertheless, the influence of the state of the ipsilateral front leg and also hind leg cannot be excluded, and could be subject of additional experiments.

### 2.5.3. Sensory influences on FlxTi activation

The close correlation of FlxTi activation to the TD discovered in previous studies, as well as mine, indicates a strong influence of leg loading upon TD, signaled by the CS (Berendes et al., 2013, Gruhn et al., 2006). Since I would like to understand the relative importance of peripheral and central influences on a phase transition in a step cycle, I performed ablation experiments to find potential candidate sources for this peripheral feedback. In our study, I excluded the tarsus as an additional ablation, as it had previously been shown not to change the FlxTi muscle activation (Berendes et al., 2013). The FlxTi muscle is known to receive input from sensory organs measuring the movement and load. The fCO, a movement sensitive sensory organ measuring the FT joint movements, is known to enhance and support the FlxTi activity at the beginning of the stance movement and also during stance (for review, see Büschges and Gruhn, 2008). However, the FlxTi muscle is most probably not activated by this sensory feedback. Furthermore, the FlxTi muscle has been shown to be influenced by load feedback from various CS groups on the leg (Akay et

al., 2001, Akay et al., 2004, Zill et al., 2014). Until now it has not been shown that these or other CS are causal for the activation of the FlxTi muscle at the beginning of stance phase (for review, see Büschges 2005). During the ablation experiments of the individual groups of one leg part, I only found feCS to cause a significant increase in FlxTi activation latency during steps on the ground. This is the response, which would be expected for feedback from a sense organ if it were largely involved in the activation of the muscle. In addition to an ablation of the feCS, the only ablation of any group of an individual leg part, which also caused an increase in latency was the ablation of the trCS, which, however, was not significant.

These results indicate that the local feCS of the same leg provide the main timing information for the FlxTi muscle activation in the two-legged preparation. In previous studies by Akay et al. (2001, 2004), it has been demonstrated that the feCS provide important feedback for the FlxTi muscle for magnitude control of its activity. So far, there has been no evidence for the trCS to influence the FlxTi muscle at all (Büschges and Gruhn, 2008), and intracellular recordings are needed to verify such a possible influence. Finally, any multipolar receptor, which is connected to the RetUng tendon in the tibia, is not directly involved in the activation of the FlxTi muscle, as cutting the tendon only increased the variability.

### 2.5.4. FlxTi activation during SIH

The trap door setup was not only used to investigate the influence of sensory organs during stepping on the ground, but also for experiments, where the animals stepped into a hole. In summary, during SIH the FlxTi activation latency was increased, accompanied by a decrease in FlxTi overall activity. However, in 19% of all cases, the FlxTi was not activated at all (Berendes et al., 2013). This result could indicate that the activation of the FlxTi muscle is a centrally controlled, but only weakly, and this would be not completely unlike the findings in cats (Gorassini et al., 1994). In the stick insect, a central neural network that generates rhythmic activity has been demonstrated by the application of the muscarinic agonist pilocarpine onto a deafferented nervous system (Büschges, 1995a, c). One of the mechanisms, activating the FlxTi without ground contact was found by Berg et al. (2015), who described the NSI I4 to be sufficient to initiate searching movements, and which is gated by sensory feedback about the ground contact. However, the influence of this network does not seem to be very strong, if the animal is not deafferented, as the FlxTi becomes active without ground contact in only in 81% of the cases. In addition, it is plausible that, in the two-legged preparation, not only the sensory feedback about the TD signaled by the feCS is used to activate the FlxTi, but also other feedback, which ultimately adds up to elicit the FlxTi activation during SIH.

To compensate for the lack of feedback from the feCS, a possible sensory mechanism could be a passive flexion of the FT joint through passive forces at the end of ExtTi activity. This movement could cause sufficient flexion movements in the FT joint (Hooper et al., 2009) to stimulate the fCO during SIH to assist the activation of the FlxTi. The fCO is known to be involved in the support and also reinforcement of flexion movements produced by the FlxTi during stance (Bässler, 1973, Bässler, 1977b). During stepping on the ground, the time course of this passive flexion is not sufficiently fast to act as feedback on the FlxTi, because the ExtTi activity often ends around the beginning of FlxTi MN activity (Hooper et al., 2009, Rosenbaum et al., 2010). However, for SIH, feedback from the passive movement could help to activate the belated FlxTi. During ablations of individual groups of CS, the latency to the activation of the FlxTi during SIH was longer compared to the latencies measured with the intact leg, but the magnitude of the FlxTi activity during SIH did not change as a result of these ablation experiments. This suggests that all investigated CS have an influence on the FlxTi MN, the trCS or the feCS, together with the tarsal CS (Zill et al., 2014), as well as the tiCS. Such a sensory influence from the tiCS onto the FlxTi has not been demonstrated until now. For these effects two reasons are conceivable. The first reason could be that by ablation of any single group of CS the inherent tonic activity would be silenced (Zill et al., 2012, Zill et al., 2013, Zill et al., 2014), and thereby the likelihood of a FlxTi activation would be decreased. The other reason could be that CS feeding continuous or transient load back, which is applied externally for example as load through TD, also detect cuticular strain, which is self-generated by muscle contractions (Zill et al., 2012, Zill et al., 2014). In this scenario, the DepTr and the RetUng could play a major role, as these muscles are activated way before the TD and therefore independent of it. It is possible to activate the TrCS, and also the taCS, by the strains developed by the muscle movements of the DepTr and RetUng, which could in turn activate the FlxTi. Up to now, it has not been demonstrated that a muscle could be activated by such an indirect effect. It has only been shown that the activity of the CS, which is induced by muscle contractions, is used in resisted movements (Zill et al., 2011, Zill et al., 2012, Zill et al., 2014). However, both of these mechanisms could explain our results, where ablations of CS groups prolonged the latency of the FlxTi during SIH compared to those in the intact leg. Finally, it is possible that sensory feedback coming from other legs, which I cut off in the two-legged preparation, may also play a role in an intact, freely walking animal. This is especially true if the substrate is not a slippery surface, which prevents mechanical coupling of the legs. Such interleg sensory influences might add up to the local sensory influences, described by the present study, and together support activating the FlxTi in the intact animal. It remains unclear, why the ablation of all groups of CS together with the ablation of the tarsus had an increasing effect on the latency of FlxTi muscle activation and,

at the same time, prevented the reduction of the amplitude of the FlxTi amplitude, which is seen in the intact animal.

Summarizing this chapter, I could demonstrate for the middle leg of stick insects that only the activation latency of the FlxTi is influenced by feedback about ground contact, while the magnitude of activity of all stance phase muscles is influenced. This result proved the current assumption wrong, which claims that the activation of RetCx is dependent of load signaling onset (Ekeberg et al., 2004). However, the source for the feedback influencing the activation of the RetCx MN remains unknown. The activation of the FlxTi muscle is mostly dependent on the sensory feedback of the feCS, which had previously only been thought to be important for the control of the magnitude of the FlxTi activity. When the stick insect is lacking ground support, the phasic activity from the CS becomes absent, which delays the FlxTi activation. This could be responsible for the initiation of searching movements following the lack of ground support, to regain ground contact.

# 3. Task dependent changes in sensorimotor processing of movement feedback

## 3.1. Introduction

Animals perform movements to survive, like eating, grabbing, or walking. Task-dependent locomotion is essential to find mates and resources, escape predators, or to explore the environment. Therefore, basic locomotor rhythms are produced by nervous system networks consisting of CPGs. These are, in turn, modified by feedback from sensory organs located in the peripheral nervous system. Feedback from load, force, and movement sensors can modify the timing of motor output as well as the magnitude of muscle activity (Bässler, 1983, Büschges, 2012, Grillner, 1981, Pearson, 2000). In vertebrates, muscle spindles are present in each muscle and are organized in parallel to the muscle fibers. They serve as both stretch receptors and motor output modulators and are essential for the stretch reflex (for review see Clarac et al., 2000, Pearson, 1993). In invertebrates, stretch reception is mainly performed by chordotonal organs such as the coxo–basipodite chordotonal organ in crayfish (Clarac et al., 2000, DiCaprio and Clarac, 1981) or the fCO in the stick insect (Bässler, 1983, Büschges, 2012). During postural control in a standing animal, the leg joints need to resist movements, which are mainly measured by stretch receptors. Therefore, the stretch receptors monosynaptically innervate antagonistic MNs in order to quickly oppose movements at the leg joint (Clarac et al., 2000, Pearson, 1993). In a walking animal, on the other hand, the same sense organs can produce AR and thereby support the stance muscles. This has been demonstrated in vertebrates (cat: Forssberg et al., 1975; rat: Fouad and Pearson, 1997; human: Duysens et al., 1990) and invertebrates (crayfish: DiCaprio and Clarac 1981; locust: Zill, 1985; stick insect: Bässler 1983, Büschges 2012).

The fCO in stick insects is a stretch receptor in the proximal femur that is connected to the tibia with a receptor apodeme. It consists of 480 sensory neurons (Hofmann et al., 1985), which encode various parameters of leg movement, such as the position of the tibia relative to the femur (FT joint angle), the velocity of the change in angle of the FT joint the acceleration of movement around the FT joint. (Hofmann and Koch, 1985, Hofmann et al., 1985). Hofmann et al. (1985) examined the functional role of various units in two behavioral states, during activity and inactivity. In the inactive stick insect, stimulation of the fCO leads to a RR, which is comparable to a stretch reflex in vertebrates and used to stabilize the animal's posture (Bässler, 1988, 1993, Clarac et al., 2000). The RR is a reaction to movement of the leg when it is on the ground and not actively moving. During passive tibial

flexion of the immobile leg, fCO activity excites ExtTi MNs, which, in turn, activate the ExtTi muscle to oppose the passive movement and retain the posture of the leg (Bässler, 1974, Bässler, 1993). If the stick insect starts moving, the behavioral state changes from inactive to active (Bässler, 1976, 1988). During active movement, feedback from the fCO can also evoke reactions other than the AR (Bässler, 1988). Bässler (1988) described different categories of reactions to stimulation of the fCO, which can be summarized in three groups: inactive reactions (RR), active reactions (AR), and undefined reactions. During ARs, the effects of the RR are reversed; shortly after the onset of fCO stimulation, FlxTi activity is increased, while the ExtTi muscle is inhibited (Bässler, 1986, 1988). In the end of a ramp stimulus of the fCO, the stance-to-swing transition takes place; the FlxTi muscle is inactivated, and ExtTi activity is increased again (Bässler, 1986, 1988, Büschges, 1995b). This transition takes place in a certain FT joint angle, independent of the starting angle of the fCO in the front legs (Bässler, 1986). In the case of undefined reactions, all intermediate cases are summarized and even include reactions that showed no change. A typical example would be flexion signal measured by the fCO that triggers a typical AR, but shows activity in ExtTi MNs before the stance-to-swing transition (see: Bässler, 1988). Due to the inhibition in the beginning of the stimulation, this would not lead to a RR. However, little is known about the influence of movement feedback on the motor output while the animal is performing different tasks, such as during curve stepping.

During curve stepping, the kinematics of the legs on each side of the body are changed (Gruhn et al., 2009 b). In the stick insect, middle legs functioning as the legs on the outside of the curve are moved by large longitudinal movements with only minor tibial flexion. Middle legs functioning as legs on the inside of the curve mostly move the FT joint, and longitudinal movement is reduced to only small but flexible forward and backward movements (Gruhn et al., 2006, Gruhn et al., 2009 a, Gruhn et al., 2009 b, Gruhn et al., 2016). Hellekes and colleagues (2011), found a difference in movement-related feedback processing between inside and outside legs. For inside-stepping middle legs, the likelihood of an AR is higher compared to outside-stepping middle legs, while the outside-stepping middle legs have high tibial MN activity (Hellekes et al., 2011). As the likelihood of an AR changes due to the task, pathways from the fCO to the MNs need to be modulated. A monosynaptic pathway was described by Driesang and Büschges (1996) to the FETi, which is activated at the onset of every ramp stimulus (RS). They found an early-latency depolarization generated by monosynaptic input from acceleration-sensitive units of the fCO. This depolarization was visible in the inactive animal, during RR, as well as in the active animal during the AR (Driesang and Büschges, 1996). However, fCO feedback does not only influence the FT joint, but also the other leg joints. Activity of the LevTr, the swing-phase muscle of the CTh joint, increases due to fCO feedback in active animals (Hess and Büschges, 1997).

Furthermore, feedback from the fCO, which leads to an AR in the ExtTi, also increases activity in the ProCx. The ProCx is the swing-phase muscle of the ThC joint during forward walking (Bässler, 1988). However, in 20% of all trails, ProCx activity did not coincide with ExtTi activity, demonstrating an AR (Bässler, 1988). In both CoTh and ThC joints, fCO feedback leads to changes in motor neuron activity, but the question remains of how fCO feedback is processed in a task-dependent manner.

In the present study, I investigated the task-dependency of changes in motor output caused by fCO feedback in the individual leg joints. Moreover, I looked at the influence of three movement parameters measured by the fCO. I analyzed effects on the motor output of the FT joint in middle legs during stepping on the inside and outside of a curve.

## 3.2. Materials and Methods

Adult female stick insects (*Carausius morosus*) were used for all experiments, which were carried out at room temperature (20–22°C) under reduced light conditions. The animals were taken from a colony at the Biocenter of the University of Cologne (Cologne, Germany), where they were kept at 25 °C and 75-85% humidity before May 2016 and 60-70% humidity thereafter. Animals were kept under a 12:12 h light-dark cycle and were fed blackberry (*Rubus fructiosus*) leaves *ad libitum* collected from various sites in Cologne, Germany. All experimental procedures reported here comply with the German national and state regulations for animal welfare and animal experiments.

### 3.2.1. Positioning of the experimental animal for walking

Animals were glued with dental cement (Protemp II; 3M ESPE, St. Paul, MN) dorsal side up on a foam- and wax-covered metal rod (animal holder), which was placed above a slippery surface (Gruhn et al., 2006). The animal holder was placed at a height so that the resting angle of the FT joint in the legs was approximately 90° (Epstein and Graham, 1983, Graham and Bässler, 1981, Gruhn et al., 2006). The middle leg, the leg I focused on for our experiments, was glued in a well of wax on an extension of the foam. The coxa and the FT joint were immobilized with dental cement (Hellekes et al., 2011), and the tarsus was ablated. The angle of this fixed FT joint was around 110° (Fig. 3.1). Furthermore, the ipsilateral hind leg was removed in all experiments.

### 3.2.2. Preparation for fCO stimulation and recording of motor activity

The femoral cuticle was opened on the dorsal side to allow mechanical stimulation of the fCO and extracellular recordings from the ExtTi motor nerve (Büschges, 1989).

Additionally, electrodes for recording FlxTi activity were placed into small holes in the cuticle on the posterior proximal femur. The holes were made with an insect pin (Schmitz et al., 2015). The well and the leg cavity were filled with saline (Weidler and Diecke, 1969), and a movable clamp was attached to the receptor apodeme of the fCO. The position of the clamp was controlled by a linear motor (Hellekes et al., 2011). Mechanical displacement was produced by applying voltage to the motor with a stimulus generator (Electronics Workshop; Zoological Institute, University of Cologne, Cologne, Germany). All stimulations were ramp-and-hold-stimuli (RaHS). These displacements of the apodeme of 100 µm induce a 20° change in FT joint (Weiland et al., 1986). To monitor the activity of ExtTi MNs, I recorded the F2 nerve extracellularly with a hook electrode (modified from Schmitz et al., 1988). An EMG of the FlxTi was also recorded (EMG; e.g., Rosenbaum et al., 2010, Schmitz et al., 2015). Walking activity was monitored by video recordings, with an high speed camera (AOS S-PRI, AOS Technology AG, Baden Daettwil, Switzerland, resolution: 400 × 1,024 pixel, frame rate: 7 fps, shutter speed: 2000 µs) from the dorsal side of the animal. The recorded EMG and extracellular signals were amplified 100-fold with an insulated preamplifier MA101 (electronics workshop, Zoological Institute, Cologne, Germany), and additionally amplified 10-fold with a signal conditioner/main amplifier MA102 (electronics workshop, Zoological Institute, Cologne, Germany). The EMG signals were band-pass filtered between 100 Hz and 1,000 Hz in all experiments. The extracellular recording signals were band-pass filtered between 100 Hz and 3500 Hz in all experiments.

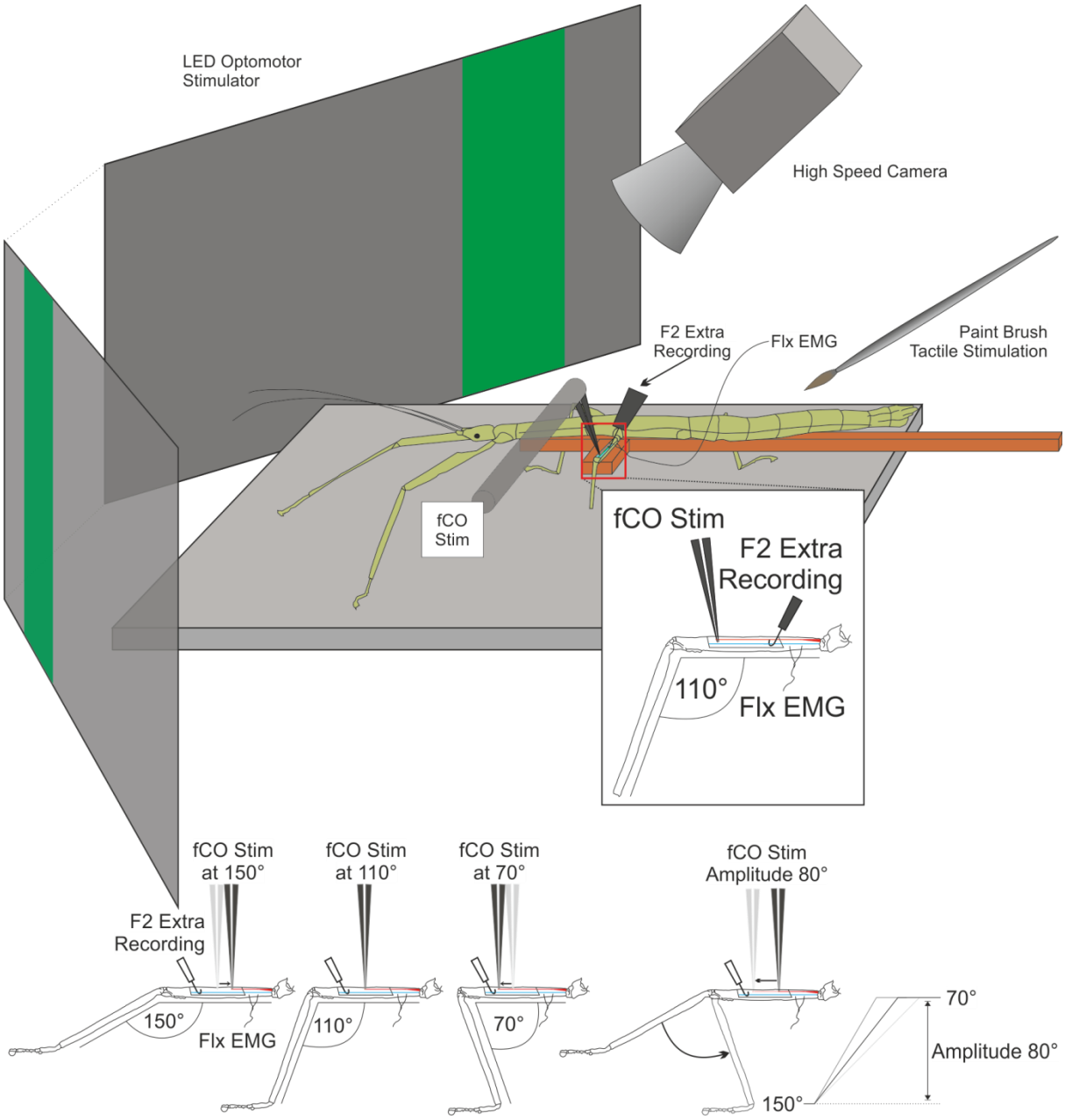



Fig. 3.1: Curve walking setup to reveal differences processing of fCO parameters.
The animal glued to an animal holder, walking on a slippery surface (Gruhn. 2006). The fCO is clamed and stimulated, while FlxTi EMG´s are recorded and the F2 nerve, containing ExtTi MN, is recorded extracellularly. Different Starting angles (150°, 110° and 70°) were applied, by pre-stretching or pre-relaxing the receptor apodeme; Changes in amplitude could be applied by changing the ramp of the stimulation, velocities could be varied by changing the slope of the ramp. The animal was optically and tactilely stimulated to induce curve walking, which was monitored by a high speed camera.

### 3.2.3. Curve walking on a slippery surface

Tactile stimulation with a paintbrush on the abdomen of the animal was used to elicit walking movements (Graham and Epstein 1985). To induce curve walking, visual stimulation was applied in front of the walking animal. Vertical stripes were presented on two LED screens (panels of 8 x 8 green light emitting diodes [LEDs]; Electronics Workshop, University

of Cologne, Cologne, Germany) with one stripe per LED screen. By moving the pattern to the left or to the right, directional curve walking can be elicited (comp. Gruhn et al., 2006, Gruhn et al., 2009 a, Hellekes et al., 2011). This setup was used to elicit curve walking in all experiments described herein.

### 3.2.4. Curve walking on a ball

The Setup consists of a Rohacell-ball (diameter: 246 mm, material: polymethyl methacrylimide, density: 0.052 g/cm$^3$, weight 32.6 g), which was placed in a PVC bowl. The ball was floating on compressed air, to eliminate friction (Dürr and Ebeling, 2005). The animal was placed above the ball and was filmed with a high speed video camera.

### 3.2.5. Experiments with different movement parameter stimulations

Different sets of fCO ramp-and-hold stimulus parameters were combined with different FT joint movement parameters:

1) Ramp velocities of 150°/s, 300°/s, and 700°/s
2) Starting angles of 150°, 110°, and 70°
3) Hold-amplitudes of 40°, 60°, 80°, and 100°

The apodeme of the fCO was moved with the linear motor for at least 10 ramp-and-hold stimuli for every movement parameter combination.

### 3.2.6. Extracellular recordings of motor nerves to investigate intraleg influences of fCO feedback

To perform extracellular recording of motor nerves, the body was opened dorsally between the pro- and metathoracic segments. The cut cuticle was then bent back with insect pins to form a well, which was sealed with petroleum jelly and filled with saline. To investigate influences of the fCO on the LevTr and ProCx muscles, activity was recorded with hook electrodes in the C1 and nl2 nerves, respectively (modified after Schmitz et al., 1988).

### 3.2.7. Data acquisition

Physiological signals were amplified and filtered (e.g., von Uckermann and Büschges, 2009). Signals were digitized using a Micro 1401 A/D converter (sampling rate, 12.5 kHz) and recorded with Spike2 software (version 5.04; Cambridge Electronic Design, Cambridge, UK), which was also used to record video data, which were used only to evaluate the stepping direction. Data were tested for normal distribution using the Shapiro-Wilk test.

Depending on the result of the Shapiro-Wilk test, the data were handled with parametric or non-parametric tests. The latency to the first spike was tested for significance with a paired t-test; data of the about the different movement parameters and the recordings from the nl2 and C1 were tested for significance with the Kruskal–Wallis test and Dunn´s multiple comparison test. Statistical significance was set at a level of $P < 0.05$. To determine the reliability of the frequency of occurrence of the AR, I defined 95% confidence intervals for the different experimental situations. Evaluation of the data and production of graphs were performed with OriginPro 8.5 (OriginLab Corp., Northhampton, MA, USA), Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA), CorelDRAW X6 (Corel Corporation, Ottawa, Ontario, Canada) and MATLAB R2015b (MathWorks, Inc., Natick, MA, USA). In the text and figures, "*N*" designates the number of animals, and "*n*" designates the number of observations per experiment.

## 3.3. Results

In the present study, the intention is to examine whether sensory feedback is processed in a task-dependent manner. Therefore, I recorded FT MN activity while the fCO was stimulated in a curve stepping insect, so that the investigated leg functions as inside and outside leg. During curve stepping the inside- and outside-stepping legs show different kinematics. In stick insect, the middle leg on the inside of a turn is mostly moved around the FT joint, while the middle leg on the outside of the turn is mostly retracted during stance phase, and the FT joint is moved minimally (Gruhn et al., 2006, Gruhn et al., 2009 a, Gruhn et al., 2016).

Two experimental approaches were used as curve walking setups. The slippery surface setup, shown in Fig. 4.1 and a ball setup. The major difference is the mechanical coupling of the legs on the ball setup, which is missing while the animal walks on the slippery surface. On the ball setup the animal was able to walk with six and five legs, so that the ball was moved by the legs in the path of walking (compare Simon, 2014). The trajectories for four and two legs increased in its length (Fig. S. 2) in three tested animals. Also, the animal could not move the ball stably with less than five legs. Often, it was observed that the ball was moving to its center of gravity. With four and two legs, it was not possible for the animal to move the ball away from the center of gravity. With two legs, and sometimes with four legs, the center of gravity was at the bottom of the ball and the animal could just turn the ball around this center. When the ball was not turned around the center of gravity, it was often observed that it was pulled towards the animal during stance phase and rolled back to the initial position during swing phase. Additionally, if the ball started rolling the legs of the animal were pulled in the rotation of the movement during stance phase. Hence, the slippery surface was used as experimental approach for the curve walking experiments in this study.

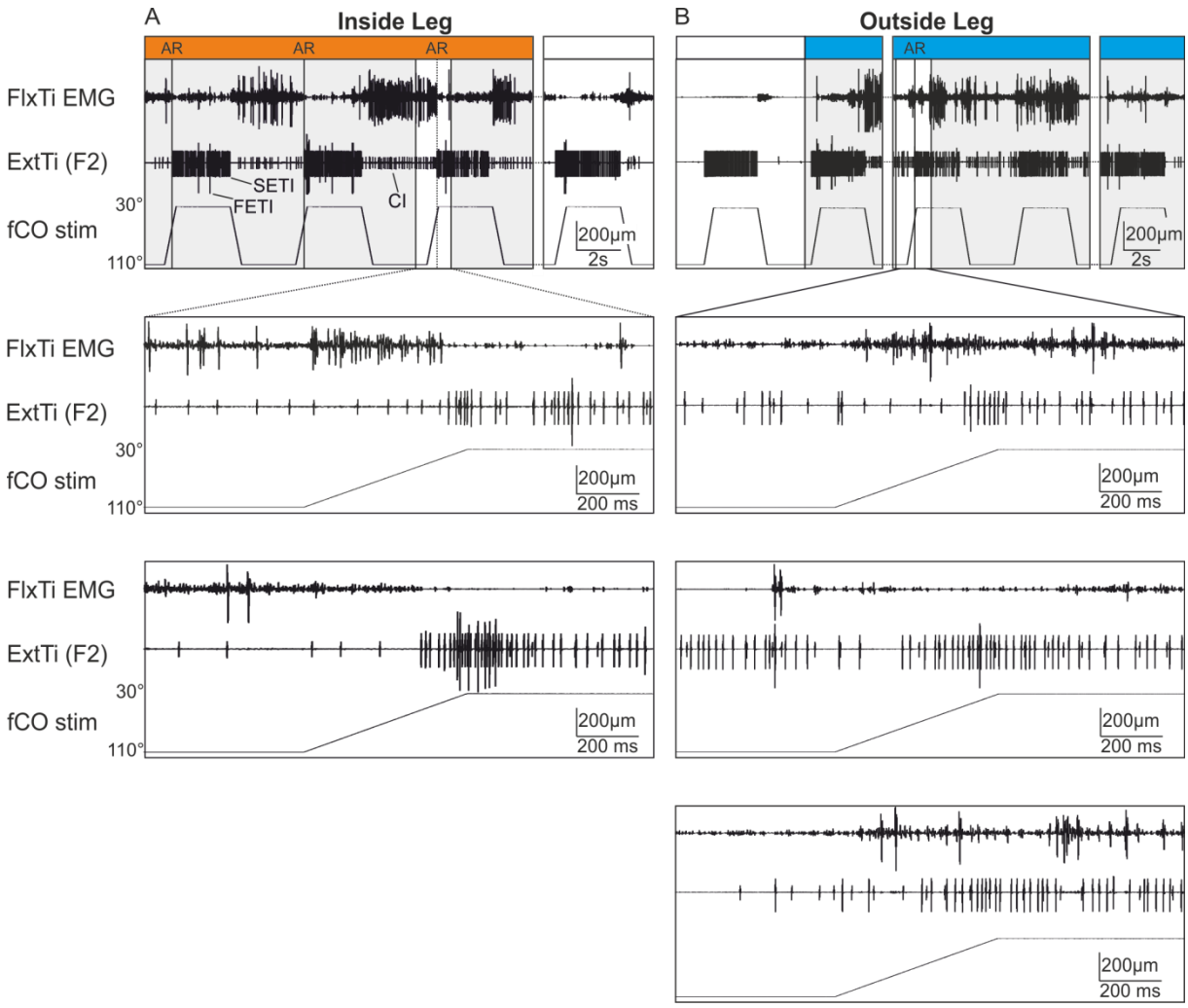



Fig. 3.2: Example ExtTi nerve and FlxTi muscle recordings of inside and outside leg stepping wit fCO stimulation

(A, B) Recording of the ExtTi nerve (F2) and FlxTi EMG during ongoing (i) or during a single (ii) fCO stimulation, when the recorded side of the animal served as (A) “inside” or (B) “outside”. (ii) Recording showing a reflex reversal. Grey shaded area: actively walking animal; White area: resting animal; ARs mark the stimulations, when a reflex reversal was generated; inside leg condition (orange) and outside leg condition (blue).

Activity of the ExtTi MNs, recorded extracellularly via the F2 nerve and the FlxTi muscle, was lower for an inside-stepping middle leg compared to an outside-stepping middle leg (Fig. 3.2) (Hellekes et al., 2011). In the present study, the fCO of the left middle leg was stimulated with RaHS, while all contralateral legs and the ipsilateral front leg were allowed to move freely. Additionally, the animal was stimulated mechanically to induce walking and optically to induce turning. Looking at differences in the processing of movement feedback between the inside and outside leg, I compared stepping sequences of both conditions with fCO stimulation.

As observed by Hellekes et al. (2011), stick insects show very stereotypic RR during rest, which increase in response to RaHS (**Fig. 3.3**i). During curve walking, activity of the muscles modifying FT joint angle is higher depending on the function of the leg (Hellekes et al., 2011). The AP discharge from the FETi and the SETi seemed to decrease, while the

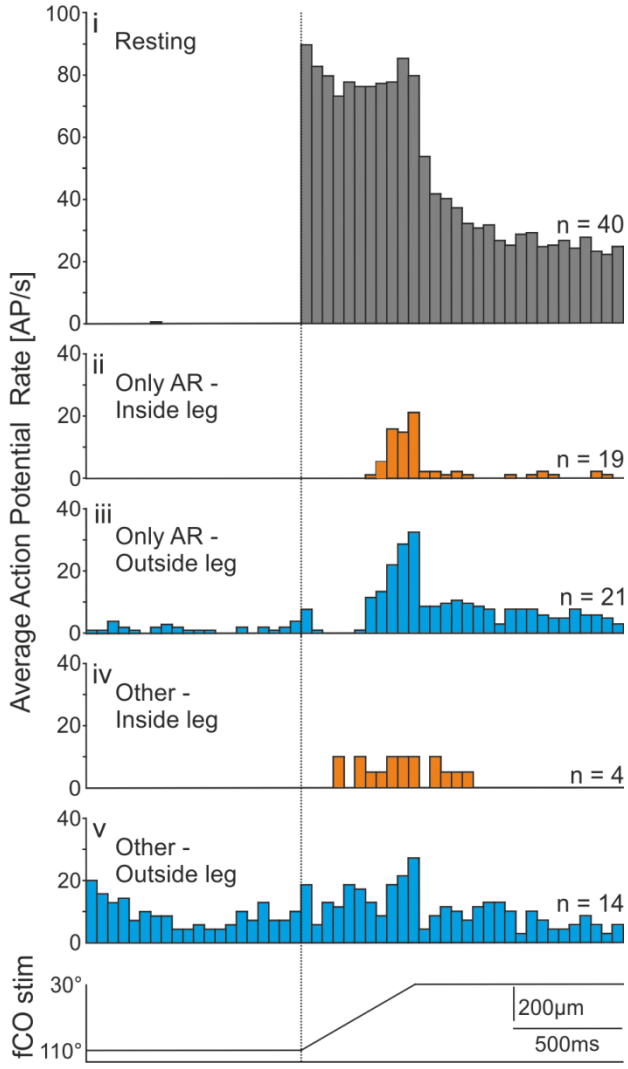



Fig. 3.3: Stimulus time histogram activity of the ExtTi MNs during fCO elongation

(i) Resting animal; (ii,iii) Undefined activity during (ii) inside leg condition and (iii) outside leg condition (iv,v) Active: selected ARs for (iv) the inside leg condition (orange) and (v) the outside leg condition (blue).

activity of the common inhibitor did not seem to differ (Fig. 3.2). The likelihood of an AR was higher for the inside legs, as illustrated in the example where only ARs could be observed (Fig. 3.2 A). In addition to the RR and the AR, the third category (undefined responses) was also observed. Here, motor output could not be allocated to one of the two other categories due to activity between the 50 ms after the beginning of the stimulation and the middle of the RS. In this time window, the ExtTi MN was not silent, so this activity was not categorized as an AR. Also, the ExtTi MN activity was not high enough to count as an RR. For the outside leg in this example, RRs as well as intermediate reflexes (undefined responses) were identified (Fig. 3.2 B). The AR was defined inside and outside leg by an absence of ExtTi MN activity. The ExtTi MN activity was decreasing during the RS while the flexor was active. This was followed by a switch in activity between the ExtTi and FlxTi. This effect was observed in both, the inside and outside stepping leg. During inside-stepping of a leg, the first APs during an AR were visible at the transition from FlxTi to ExtTi activity in the last third of the RS, whereas APs of the SETi were already recorded at the onset of RS. In addition to this, no APs of the SETi and FETi were visible before the RS started during the inside-leg condition.

To summarize these findings for one animal, I calculated the AP rate for several RaHS. When the motor output of the FT joint could be discriminated as RR, AR, or undefined the AP rate per second for stimulations was compared between rest and inside vs. outside walking leg condition (Fig. 3.3). For the RR in the resting animal there was a huge increase in AP

rate in response to the fCO stimulation (Fig. 3.3 i). For the inside and outside leg (RR and undefined), this increase in activity was not as high as it was or the RR in the standing animal (Fig. 3.3 ii and iii). However, the AP rate was higher for the outside leg compared to the inside. This was also true, if one compares only the cases from the inside and outside leg, where AR were found. Here, a decrease in activity could be observed during the outside leg condition shortly after the onset of the fCO stimulation, while there was no activity during inside leg (Fig. 3.3 v and iv). In the last third of the RS, the AP rate of the ExtTi increased again for both inside and outside leg.

In Fig. 3.4 (A), a recording of the FlxTi EMG, an extracellular recording of the F2 nerve containing all ExtTi MN, and an intracellular recording of a SETi MN is shown. The MN activity is illustrated in response to fCO RaHS. The recording in Fig. 3.4 (A) shows an AR: Shortly after the onset of the stimulation, the SETi and FETi MNs in the F2 nerve recording became silent and the activity of the FlxTi muscle increased. At the end of the ramp stimulation, the ExtTi MNs started firing APs again. In the intracellular recording, the membrane potential hyperpolarized, while the SETi MN was silent in the F2 nerve recording. The decrease in SETi MN firing frequency, at the beginning of the RaHS correlates with the hyperpolarization of its membrane potential.

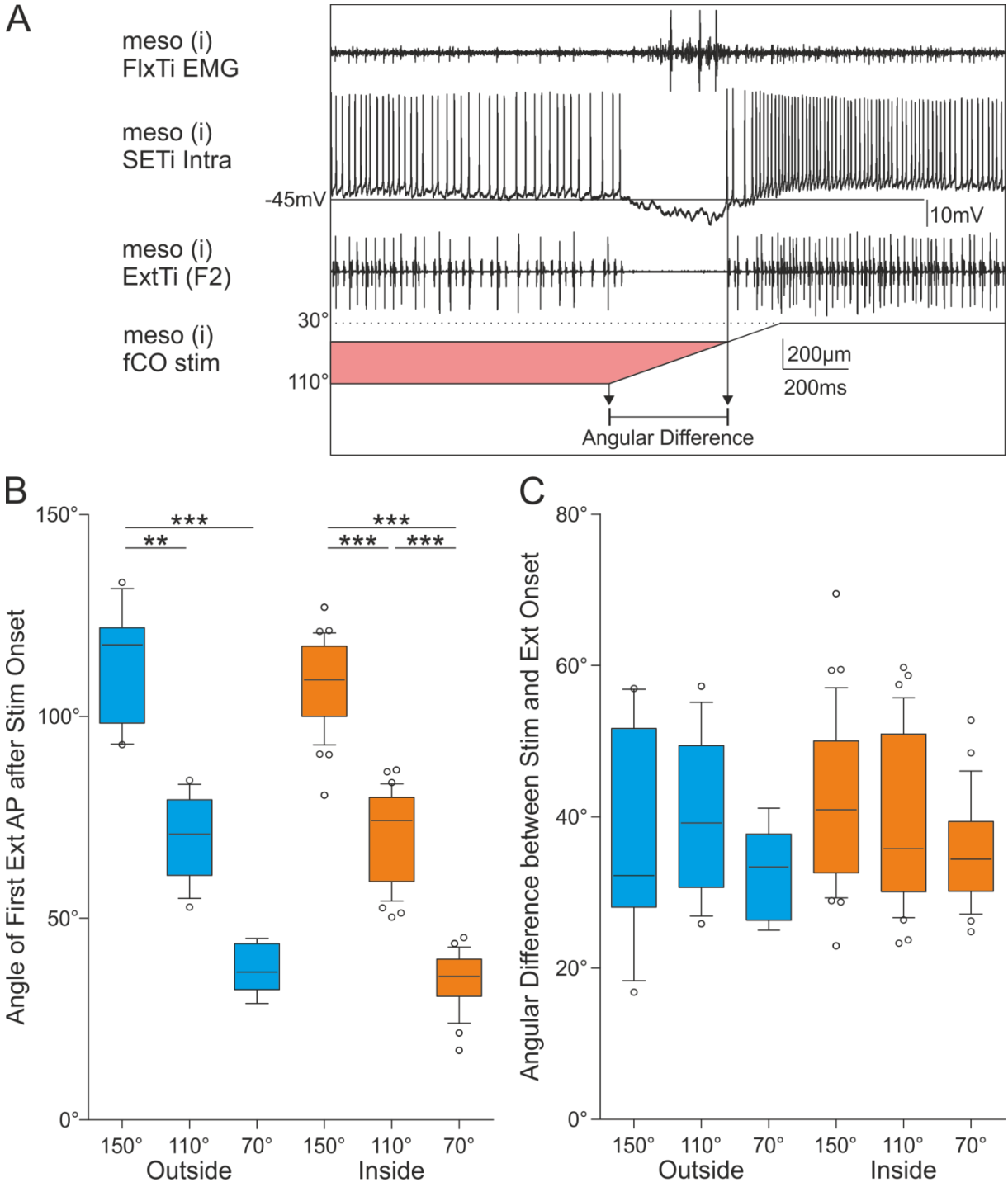



Fig. 3.4: Transition between FlxTi and ExtTi MN activation during AR

(A) Recording of the slow extensor tibiae motor neuron (SETi), ExtTi nerve and FlxTi muscle showing an AR, (B) Angle of the first ExtTi AP after stimulation onset for AR, (C) Angular Difference between the stimulus onset and the first ExtTi AP for AR, outside: 150°: N = 4, n = 11; 110°: N = 5, n = 14; 70°: N = 3, n = 5; inside: 150°: N = 6, n = 35; 110°: N = 5, n = 34; 70°: N = 4, n = 24. Angular difference to the first AP marked with box in light red (i), Onset and first ExtTi AP are marked by arrows; inside leg condition (orange); outside leg condition (blue).

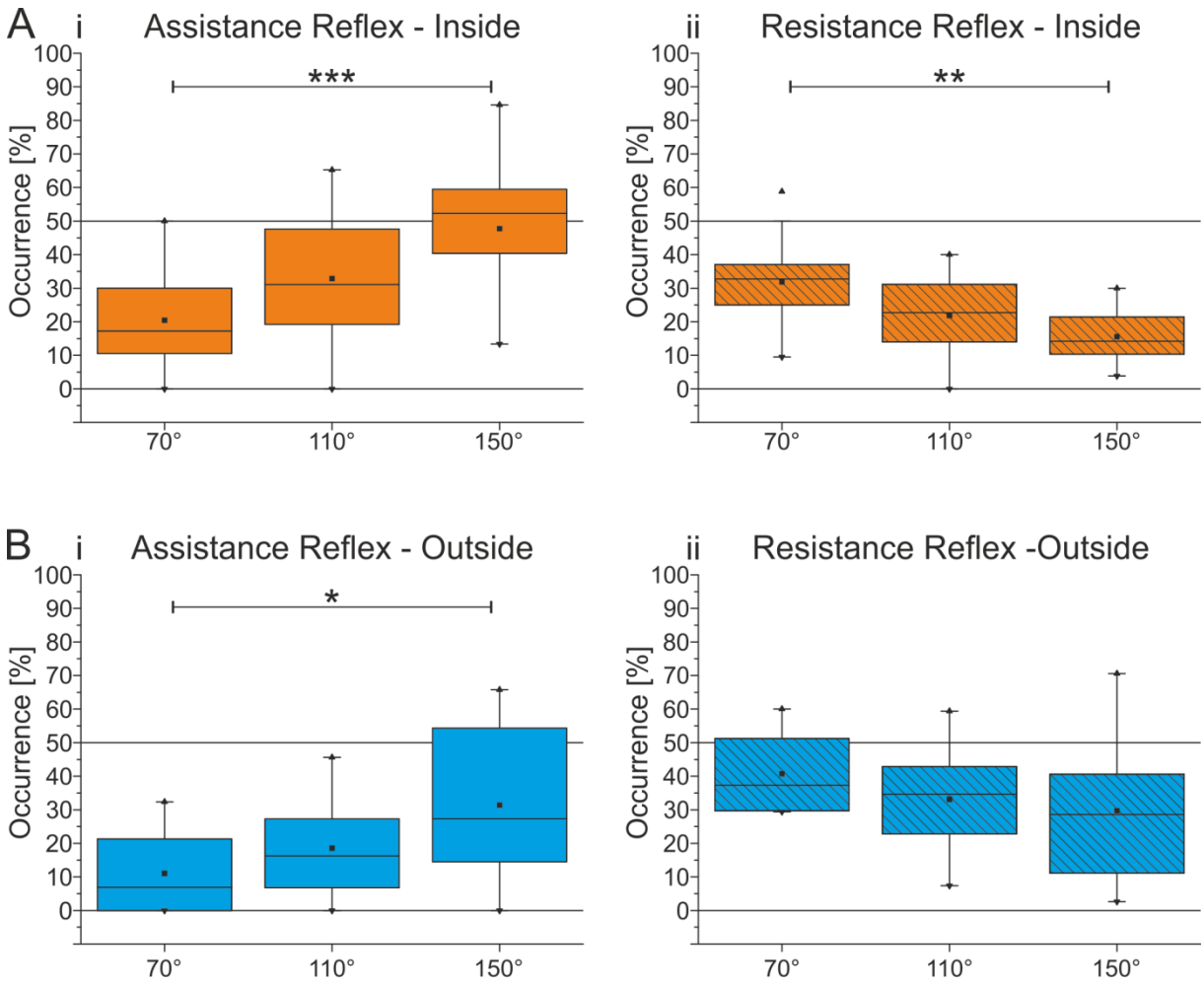



Fig. 3.5: Occurrence of AR and RR due to different starting angles
A i-ii: Inside middle leg: Occurrence of (i) AR and (ii) RR for different starting angles: 70° (N = 13, n = 441), 110° (N = 21, n = 851), 150° (N = 18, n = 919). B i-ii: Outside middle leg: Occurrence of (i) AR and (ii) RR for different starting angles: 70° (N = 8, n = 242), 110° (N = 18, n = 627), 150° (N = 15, n = 529); inside leg condition (orange); outside leg condition (blue); RR: lined box plots.

For the purpose of comparing the angle of transition from stance phase to swing phase between inside and outside legs, the angle was calculated from the stimulus onset to the first AP occurred in the F2 nerve recording during three different starting angles (70°, 110° and 150°) (Fig. 3.4B). The angle at which the first ExtTi AP occurred after the stimulus onset was significantly smaller with decreasing starting angle. However, there was no difference between the inside- and outside-stepping leg when comparing the different starting angles. This is summarized in Fig. S. 1 for an extended RaHS of 100°, to include all transition angles of different amplitudes, compared to all amplitudes pooled. By calculating the angular difference between the starting angle and the angle of ExtTi AP onset, it could be seen that this angular difference was similar for all starting angles at each amplitude. For the RaHS

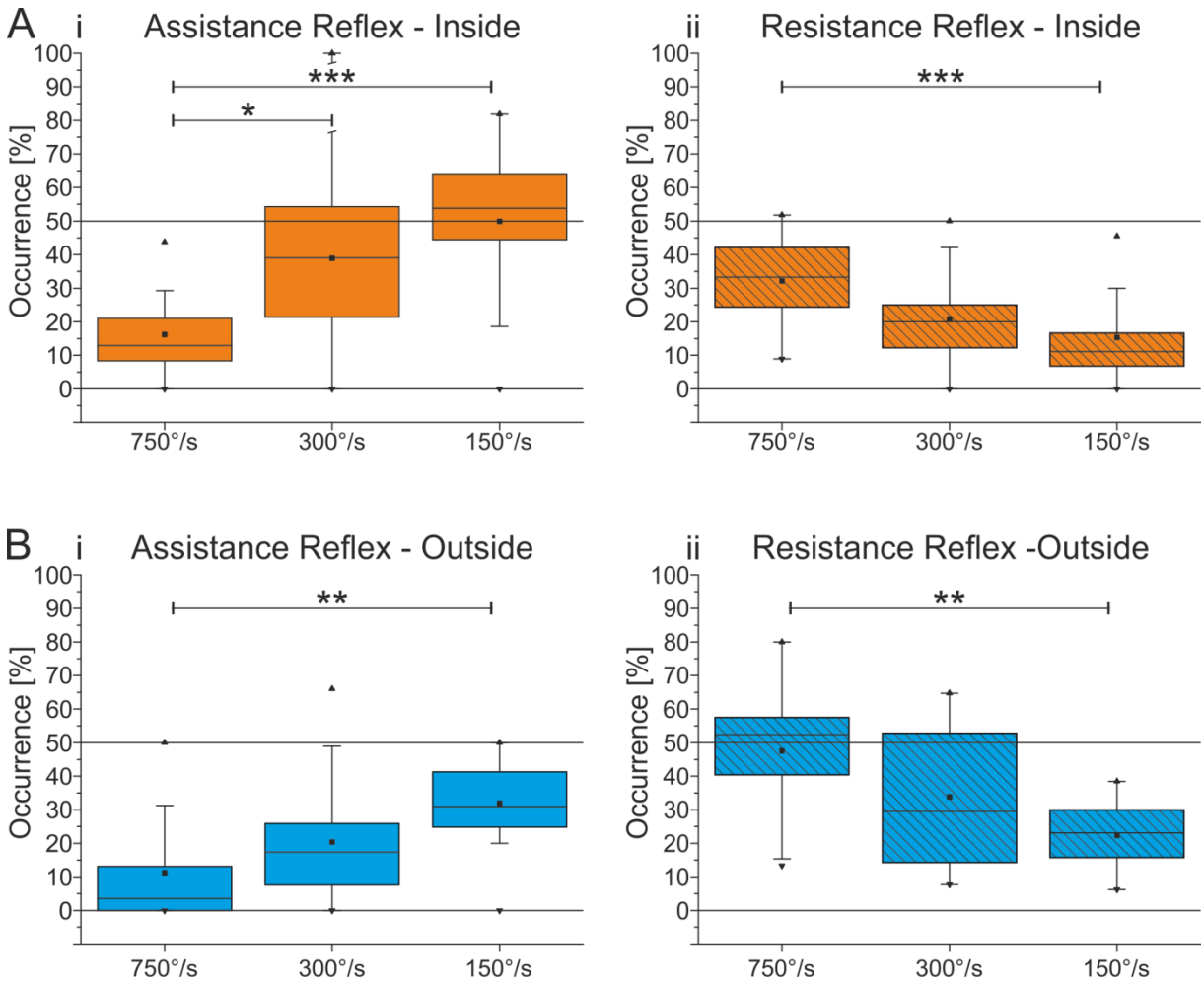



Fig. 3.6: Occurrence of AR and RR due to different stimulus velocities
A i-ii: Inside middle leg: Occurrence of (i) AR and (ii) RR different stimulus velocities: 150°/s (N = 17, n = 656), 300°/s (N = 19, n = 731), 750°/s (N = 21, n = 843). B i-ii: Outside middle leg: Occurrence of (i) AR and (ii) RR different stimulus velocities: 150°/s (N = 16, n = 448), 300°/s (N = 14, n = 447), 750°/s (N = 16, n = 481); inside leg condition (orange); outside leg condition (blue); RR: lined box plots.

with an amplitude of 60° as an example, the angular difference between stimulus and ExtTi onset was around 35° for all starting angles. There was no difference observed between the inside and outside leg. Summarizing this, I found the transition from stance to swing phase to take place always at a certain angular difference relative to the onset of the stimulation and not at a fixed leg position.

Due to my findings, I classified the reaction to an elongation of the fCO into three categories: 1) RRs, always occurring in inactive animals but also in active animals; 2) ARs, an inhibition of the ExtTi MNs and an excitation of the FlxTi MNs due to the elongation stimulus. 3) The undefined category, which was between RR and AR in firing activity of the ExtTi MNs.

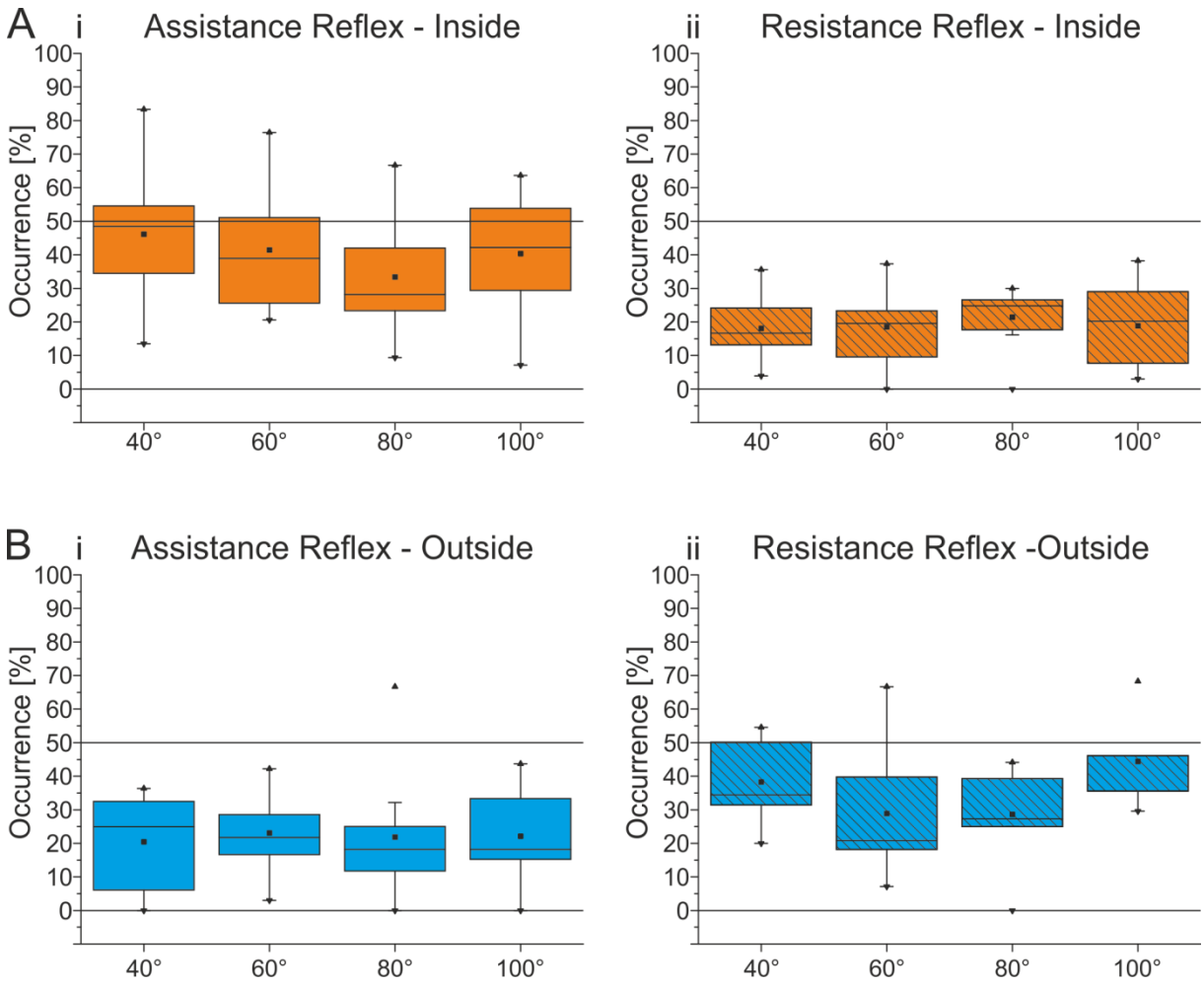



Fig. 3.7: Occurrence of AR and RR due to different stimulus amplitudes
Ai-ii: Inside middle leg: Occurrence of (i) AR and (ii) RR for different stimulus amplitudes: 40° (N = 13, n = 724), 60° (N = 13, n = 750), 80° (N = 10, n = 373), 100° (N = 10, n = 387). B i-ii: Outside middle leg: Occurrence of (i) AR and (ii) RR for different stimulus amplitudes: 40° (N = 8, n = 406), 60° (N = 9, n = 432), 80° (N = 9, n = 299), 100° (N = 5, n = 265); inside leg condition (orange); outside leg condition (blue); RR: lined box plots.

The fCO measures movement parameters of the FT joint. It can measure the position of the tibia compared to the FT joint angle, the velocity and the acceleration of tibial movement (Hofmann and Koch, 1985, Hofmann et al., 1985). In a first set of experiments, different movement parameters were tested for their likelihood of occurrence of movement reinforcement for the inside- and outside-stepping leg. The parameters were the starting angle at the beginning of the stimulation and the stimulation velocity, which represents the slope of the RS. Additionally, the stimulation amplitude, which is the difference between the starting angle and the hold-part of the RaHS, was altered. The RaHS parameters were changed every 10th stimuli, while the animal was walking. A combination of one stimulus velocity, one stimulus amplitude, and one starting angle was tested.

The parameters of the starting angle (Inside: AR: $p < 0.0012$, RR: $p < 0.003$, outside: AR: $p < 0.039$) and stimulus velocity (Inside: AR: $p < 0.0001$, RR: $p < 0.0008$, outside: AR: p

< 0.0028, RR: $p < 0.0018$) were significantly different, while the linear regression analysis (Fig. 7) shows that there is no difference between the stimulus amplitudes.

When comparing the different starting angles, I found an increase in AR occurrence with increasing starting angle from 70° to 150° in both, the inside and the outside leg (Fig. 3.5 Ai and Bi). The two smallest and largest starting angles were in both cases significantly different (inside: $p < 0.001$, outside: $p < 0.05$). The contrary was observed for the RR. The likelihood of RR occurrence was significantly decreased with increasing starting angle ($p < 0.01$) difference between 70° and 150° for the inside leg condition (Fig. 3.5 Aii). The same tendency was observed in the outside leg condition, but the changes were not statistically significant (Fig. 3.5 Bii).

The stimulus velocity also had a significant effect on the likelihood of AR and RR occurrence (Fig. 3.6). For the inside and the outside leg condition, the likelihood of AR occurrence increased with lower stimulus velocities, from 750°/s to 150°/s (inside: 750°/s to 300°/s: $p < 0.05$; 750°/s to 150°/s: $p < 0.001$; outside: 750°/s to 150°/s: $p < 0.01$; Fig. 3.6 Ai and Bi). Again, the contrary was observed in the likelihood of RR occurrence. It decreased with lower velocities and was significantly lower from 750°/s to 150°/s for both inside ($p < 0.001$; Fig. 3.6) and outside leg ($p < 0.01$; Fig. 3.6 Bii).

For the last parameter tested, the stimulus amplitude, no statistically significant differences between the amplitudes for AR and RR for each leg condition could be found (Fig. 3.7 and Fig. 3.8 iii).

As a last step, I compared the values for single parameters for inside and outside leg against each other. For all values tested, the likelihood of occurrence for AR was higher for the inside leg compared to the outside leg (Fig. 3.8). For the starting angle of 110° and 150° this was significant ($p < 0.05$), for the stimulus velocity of 300°/s ($p < 0.05$) and 150°/s ($p < 0.01$) and the stimulus amplitudes of 40° ($p < 0.01$) and 60° ($p < 0.05$) the difference between inside and outside leg was also significantly higher for the inside leg condition (Fig. 3.8 Ai-iii). The contrary was observed for the RR. The likelihood of occurrence for a RR was lower for the inside leg compared to the outside leg condition (Fig. 3.8 Bi-iii).

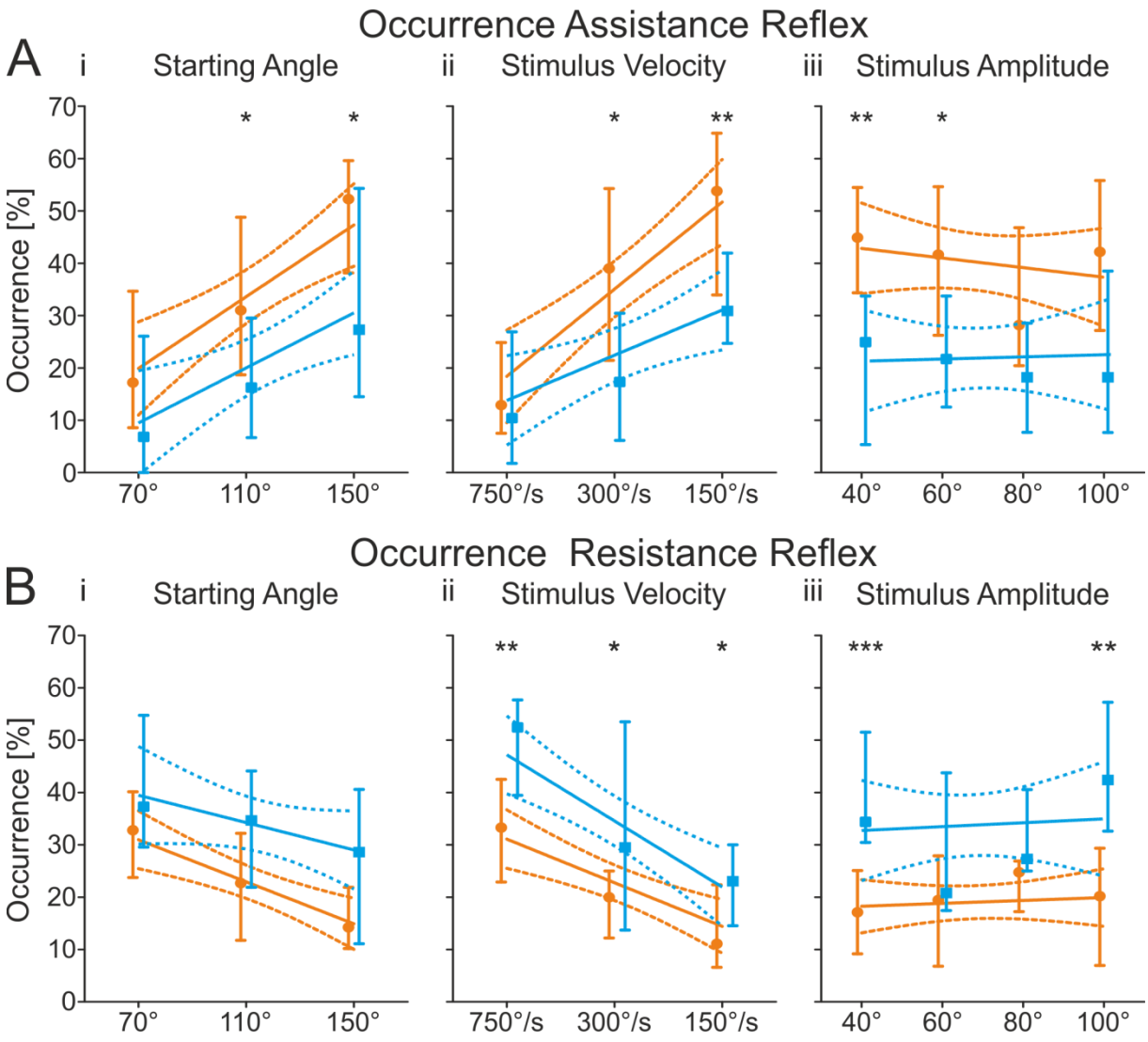



Fig. 3.8:Comparison of the occurrence of AR and RR between inside and outside leg
Regression of the likelihood of occurrence of (A) AR and (B) RR for (i) starting angles, (ii) stimulus velocities and (iii) stimulus amplitudes. Dashed lines show the 95% confidence intervals; inside leg condition (orange); outside leg condition (blue).

For all parameter combinations, the likelihood of occurrence for the third category, the undefined condition, was around 40% and was not further analyzed.

Summarizing, the larger the starting angle and the slower the velocity of the ramp of the RaHS became, the higher was the likelihood of generation of an AR. The amplitude had no major effects on the occurrence of an AR. The undefined condition was the same for all parameters, but only the likelihood of occurrence of AR and RR was changing. The overall likelihood of ARs occurrence was higher for inside-stepping condition however, the tendency of occurrence of ARs for the different parameters was the same for inside and outside condition.

As I found a significant change between the inside and outside leg in the FT joint, I also wondered if there is a task specific change in movement feedback processing in the other leg

joints. To get an insight into the influence of fCO feedback on the CTr joint, the F2 nerve, containing the ExtTi MNs, and the C1 nerve,

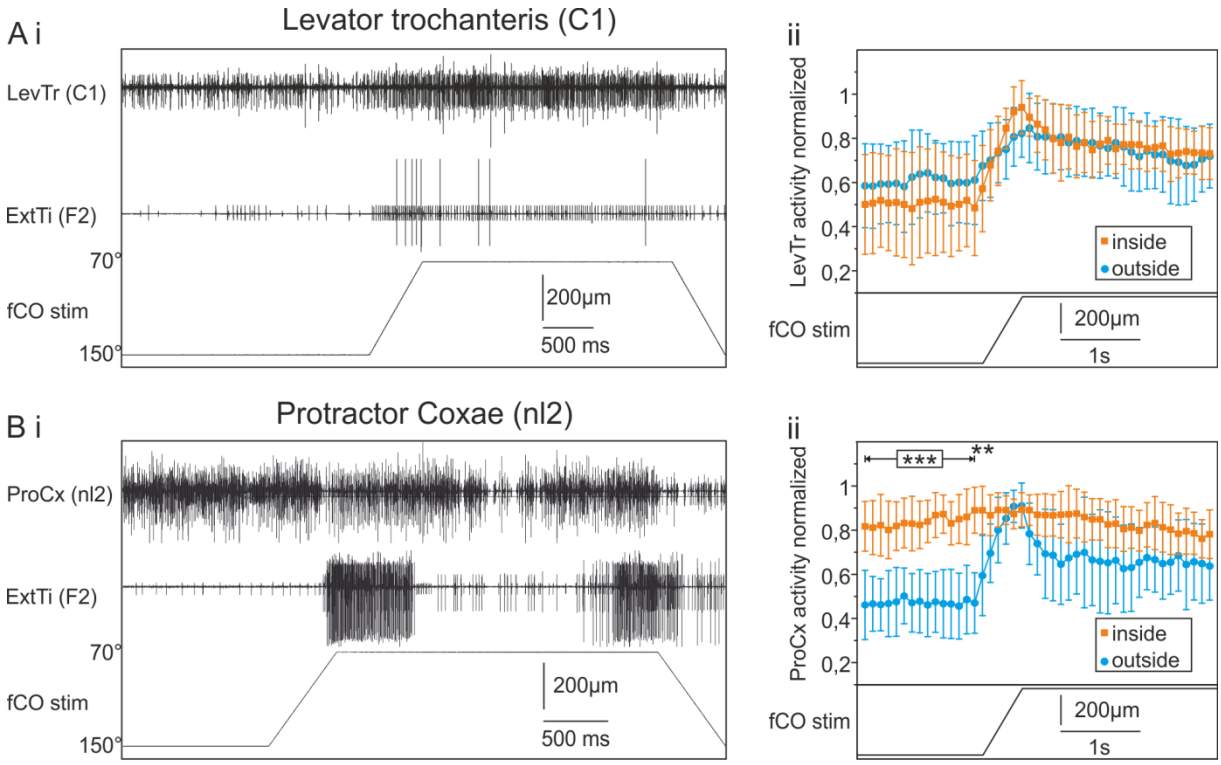



Fig. 3.9: Comparison of ThC and CTr MN activity due to fCO stimulation between inside and outside leg

A: i) Recording of the levator trochanteris nerve (C1) in the upper trace and the extensor tibiae nerve (F2) in the lower trace during fCO stimulation, when the recorded side of the animal served as inside leg; ii) Comparison of normalized *levator trochanteris* nerve activity during elongation stimuli at the fCO during inside (filled orange squares) or outside (filled blue circles) leg condition (N = 5; inside leg: n = 150, outside leg: n = 148). B: i) Recording of the *protractor coxae* nerve (nl2) in the upper trace and the *extensor tibiae* nerve (F2) in the lower trace during fCO stimulation, when the recorded side of the animal served as outside leg; ii) Comparison of normalized *protractor coxae* nerve activity during elongation stimuli at the fCO during inside (filled orange squares) or outside (filled blue circles) leg condition (N = 10; inside leg: n = 337, outside leg: n = 339).

containing LevTr MNs, were recorded with an extracellular hook electrode (N = 5; Fig. 3.9 Ai). To observe the influence of the fCO feedback on the thorax-coxa joint, the nl2-nerve, containing ProCx MNs, was recorded extracellularly with another hook electrode (N=10; Fig. 3.9Bi). Simultaneously, the fCO was stimulated with RaHS while the animals performed inside- and outside-stepping (inside: n = 337; outside: n = 339).

During these experiments LevTr MNs responses to RaHS of the inside (n = 125) and for outside leg (n = 148) were compared. In both cases the activity was increased by elongation of the fCO receptor apodeme (Fig. 3.9 Aii). During the ProCx MN recording the activity differed between inside and outside leg conditions. A higher tonic MN activity was observed during the inside leg compared to outside leg. During the inside leg, no modulation of the MN activity could be observed (Fig. 3.9Bii). However, the MN activity was very high throughout the whole time. During the outside leg, the activity was low before the ramp

stimulation started. It was significantly lower ($p < 0.001$) compared to the inside leg condition. At start of the stimulation, the activity increased during outside leg condition until a similar level of activity was reached compared to the inside leg. After the RS of the RaHS, the activity decreased slightly, however not significant to the inside leg condition.

The feedback from the fCO had an influence on the LevTr MN activity while the leg was functioning as inside or to the outside. The influence was the same in both stepping conditions. For the ProCx, the feedback seemed to have only an influence on the outside-stepping leg. During inside-stepping the ProCx was tonically active and no influence of the fCO could be measured.

## 3.4. Discussion

For the curve walking experiments, two setups could be selected. On the slippery surface, the legs of the stick insect were not mechanically coupled, however, the animal was free to move the legs in their normal trajectories (Gruhn et al., 2006, Gruhn et al., 2009 a). The second setup was a ball setup. Here, the legs are mechanically coupled (Dürr and Ebeling, 2005, Simon, 2014). The stick insect is able to walk on the ball setup, as described in previous studies, with six legs (Dürr and Ebeling, 2005) and five legs (Simon, 2014). However, during preliminary experiments it became clear that the stick insect was not able to move the ball with four or less legs in a normal rotation like it was observed with five and six legs. The legs of the animal were moved passively by the balls rotation. Also the animal was not able to move the ball away from its center of mass. Probably, with a lighter ball, or a ball without a center of gravitation, an animal with four or less legs could move the ball. Thus, it was decided to carry out the experiments on the slippery surface (Gruhn et al., 2006).

In the present study, I showed that the likelihood of occurrence of the AR is dependent on the walking direction. I additionally showed that AR are dependent on parameters measured by fCO sensory neurons. First insights into the neuronal mechanisms show a longer latency to the first AP after fCO stimulation onset during the AR during outside-stepping and a decrease in early-latency depolarizations during outside-stepping in ExtTi MNs. Intrasegmental effects of fCO feedback on the LevTr and ProCx show an increase in activity during outside-stepping in both MN groups, but only in the LevTr during inside-stepping while no modulation could be found in the ProCx.

The reflex induced by fCO feedback is state-dependent. If the animal is at rest longer than 30 seconds (Driesang and Büschges, 1996), it is considered inactive and shows the RR where ExtTi activity is increased and the FlxTi is inactivated, which helps to resist passive movement and stabilize the animal (Bässler, 1976) (Fig. 1). If the animal is in the active state this RR could be reversed. During the AR, which Bässler (1988) called the reflex reversal, the ExtTi muscle is inactivated and the FlxTi muscle is activated. This can serve several

functions. It can amplify the muscle tension of the FlxTi, generate a support of the movement, and generate propulsion in the direction movement (Bässler, 1988). Moreover, the fCO feedback has also an impact on the phase transition, and is thought to mediate the transition from stance to swing by influencing the CPG network (Bässler, 1988, Driesang and Büschges, 1996). The responses of the tibial MNs generated by fCO stimulation are divided into seven categories (Bässler, 1988). For simplification, the AR for only one muscle of the FT joint were categorized as AR, the same was true for the two categories of “no reaction” and “classification not possible”, which I categorize in the current study as undefined. Next to this, also the category of the RR was used by Bässler (1988).

In a previous study, Hellekes et al. (2011), through a comparison of inside and outside-stepping, found an increase in activity of ExtTi MNs for the outside-stepping leg. Further, during inside-stepping the likelihood of occurrence of an AR during parallel stimulation of the fCO was increased compared to straight- and outside-stepping of the front legs. Gruhn et al. (2016) revealed the influence of the direction of front leg stepping on the ProCx and RetCx in the mesothoracic leg. During inside-stepping the mesothoracic ProCx/RetCx network is coupled to the front leg movement, while the front legs do not have any influence on the ProCx/RetCx network during outside walking, where the RetCx is tonically active. The rhythm in the mesothoracic outside- and inside-stepping legs is influenced by load feedback. On the outside the RetCx activity increases with load, while, in the inside-stepping leg, the load feedback leads to flexible reactions such as an increase or decrease of ProCx activity (Gruhn et al., 2016).

As it remains unclear, how the neuronal mechanisms change the motor output of the tibial MNs during inside- and outside-stepping. However, it would be interesting to investigate the different levels of processing with this question. Hence, various parameters of movement and position measured by different units of the fCO were compared for their influence in control of motor output in the curve walking stick insect. The evoked motor output, investigated here, was the likelihood of occurrence of AR and RR between inside- and outside-stepping legs for the FT MN. Additionally, for the ThC and CTr joint also the motor output activity changes were compared between inside- and outside-stepping leg.

The present study could easily reproduce the findings of Hellekes et al. (2011), with an increased probability of AR for inside stepping. In Fig. 3.3, the comparison of inside and outside leg in one animal showed a drastic increase in firing frequency for the outside leg (comp. Hellekes et al., 2011). However, next to the modification of the firing frequency, some modulations due to fCO stimulation were visible, also a lower number of AR was found during outside stepping. If the animal shows an AR on the outside, the firing frequency of tibial MNs was higher.

### 3.4.1. Transition angle from stance to swing phase

The phase transition from the stance to the swing phase takes place in the last third of the RS and is marked by a switch from ExtTi inactivation to activation or FlxTi activation to inactivation, respectively (Bässler, 1976, 1988, Driesang and Büschges, 1996). During AR, the ExtTi is hyperpolarized and depolarized in the transition from stance to swing phase (Bässler, 1976, 1988, Driesang and Büschges, 1996). The position of the stance to swing phase transition for *C. impigra* front legs was described as dependent on the absolute value of the stretch of the fCO (Bässler, 1986). The angle of the transition was the same independent if the fCO was moved from 140° starting angle by 500 µm or pre-stretched by 200 µm. The angle of *C. morosus* middle legs was found to be independent of the absolute value of stretch. Different starting angles, which corresponds the pre-stretch used by Bässler (1986), result in transition angles dependent on the starting angle. Furthermore, the angular difference between the starting angle and the angle of the first ExtTi AP was the same for different starting angles (Fig. 3.4), which implies the transition from stance to swing phase to happen at a certain angle after onset of the fCO stretch. This could be explained by the different functions of the front and middle leg in the stepping animal. The middle leg is mostly used for walking, while front legs could also have other functions as searching (Cruse, 1976). Also the processing of sensory feedback was found to be variable between the stick insect species (Chapter 2).

Summarizing this, the front leg of *C. impigra* and the middle leg of *C. morosus* differ in the transition from stance to swing phase. While in the case of *C. impigra* front leg the absolute position of the stretch of the fCO could elicit the transition (Bässler, 1986), in the case of the middle leg of *C. morosus* independent of the stepping direction the transition takes place after a certain angular difference from stretch onset is reached.

### 3.4.2. Influence of movement parameters is task specific

The fCO consists of sensory neurons measuring either position, velocity, acceleration or two or all of these parameters together (Hofmann and Koch, 1985, Hofmann et al., 1985). The velocity sensitive units were further investigated in how their feedback has been processed (Bässler, 1988). Bässler (1988) found the velocity information to be processed differently and reported the likelihood of occurrence of a AR to be higher the slower the velocity of movement of the FT joint. As he did not look into details about task-specificity of this response, it was interesting to remeasure his findings and compare inside- and outside-stepping leg.

The other parameters were neither tested for their likelihood of producing AR if they were changed, nor for their influence on this likelihood while the animal does step to the inside or outside.

### 3.4.3. Influence of the starting angle

In the present study, I found the more extended the leg is at the beginning of the fCO stimulation the higher the likelihood of occurrence of an AR. At the beginning of the stimulation, the animal should be at the end of the swing phase and with beginning of the stance phase, and the tarsus should be on the ground. If the leg is extended a lot, the animal needs to flex it to move it to an adequate anterior extreme position or the movement of the other joints would simply be in the air, without ground contact of the tarsus.

The other way around, if the leg is flexed a lot and in a position of i.e. 70° the leg does not need to be flexed to assist the movement of the leg. So the likelihood of occurrence for a RR is much higher here. If there would be an AR, the leg would be flexed under the body and there it could not contribute to a stable movement.

### 3.4.4. Influence of the stimulus velocity

As Bässler already showed in (1988), the stimulus velocity has an impact on the processing of the fCO signal. In the study, he showed that the slower the stimulation was, the higher was the likelihood of occurrence of an AR. This tendency was reproduced in the present study. For the inside-stepping leg as well as for the outside-stepping leg, the likelihood of occurrence for an AR was higher was slow velocities, compared to fast velocities. All the tested velocities were in the range of normal movement of the stick insect (Bässler, 1988).

For *Drosophila* it is shown that for the stabilization of the body sensory feedback from a leg is not as important for fast movements compared to slow movements (Berendes et al., 2016), where the rhythm of leg movement could be more entrained by sensory feedback. A stimulus velocity of 750°/s, a very fast movement is implicated and probably, the input from the fCO could reach the central network later during the movements and less AR could be generated.

### 3.4.5. Processing of the stimulus amplitude

The stimulation for the fCO consists of a ramp and a hold part. The stimulus amplitude, reached at the hold-part of the RaHS, did not change the likelihood of assistance of the stance movement. The four stimulus amplitudes used in the present study did not vary much in their likelihood of occurrence of AR.

### 3.4.6. Task-dependent processing of fCO feedback

Gruhn et al. (2006, 2009 a) described the leg kinematics for inside- and outside-stepping legs. For the inside-stepping leg they found that mostly the FT joint is responsible for the stance movement. Furthermore, the ThC join is moved with small, but flexible movements forwards and backwards. Therefore, the stance phase consists of large flexion and minor but more flexible forward and backward movements. For the outside-stepping leg on the other side different kinematics were described. Here, for the stance phase mostly large longitudinal movements from front to back (ThC joint), and no or only small flexion movements (FT joint) were visible. It was claimed that the inside-stepping leg turns the animal around the point of turning, which is mainly done by flexion movements while the outside leg pushes the animal forward by the large longitudinal movement of the ThC-joint (Gruhn et al., 2006, Gruhn et al., 2009 a, Gruhn et al., 2016). However, for the outside-stepping leg, the FT joint could be fixed, for example by simultaneous contraction of the FlxTi and ExtTi. Furthermore, this could be an explanation for the tonic depolarization of the ExtTi (Hellekes et al., 2011).

The AR is used to support flexion movement of the FT joint during stance phase. This is mainly used for the inside-stepping leg as the leg joint here is used to do the main movement of the leg during stance phase. On the outside, the leg joint should only be moved to rearrange the position of the tibia, if the leg is for example too extended, but no support for the main stance movement of this leg joint is needed. This could explain the difference in likelihood of occurrence of the assistance reflex between inside stepping and outside stepping.

### 3.4.7. Interjoint influences of the fCO

As described above, the other leg joints, next to the FT joint, play an important role in performing the stance phase in the inside- and outside-stepping leg. The ThC joint is mainly used in the outside-stepping leg, the CTr-joint is used in the outside- as well as in the inside-stepping leg to perform movements. The influence of the fCO on the ThC- and CTr-joint is also well described, but not compared for inside- and outside-stepping legs.

#### 3.4.7.1. Thorax-Coxa joint:

Bässler (Bässler, 1988) described in the ProCx the swing phase muscle of the ThC joint during forward walking to react with an increase in activity in 80% while the ExtTi showed an AR. The 20% of these cases, where the animals showed no increase in activity, could be a hint for the animal doing different tasks during the time of fCO stimulation. In the present study, I found the ProCx to be activated by fCO stimulation, if the animal walked to

the outside, as the kinematics, shown by the leg would suggest. For the inside stepping leg the fCO stimulation could not induce a change in motor output of the ProCx. For the inside-stepping middle leg the ProCx/RetC network seems to be influenced in timing by CS in a flexible manner, while on the outside the RetCx was activated or initiated by load stimulation (Gruhn et al. 2016). This fits to the results of fCO stimulation, which increases the activity of the ProCx during outside-stepping, but has no influence on the inside-stepping leg.

The overall activity of the ProCx was higher for the inside-stepping leg, compared to the outside-stepping leg. This could be a hint to a simultaneous contraction of the ProCx and RetCx, to stiffen the leg joint. This was also suggested by Gruhn et al. (2016). However, the feedback of the fCO during inside-stepping could be masked by the tonic activity of the ProCx, or minimized by other neuronal mechanisms, which also should be investigated.

#### 3.4.7.2. Coxa-Throchanter joint:

The activity of the CTr joint did increase independently from the task of the leg during curve walking. The LevTr, is always active to lift up the leg at the end of the stance phase to initiate the swing phase. This movement is necessary for the inside-stepping leg after the leg is flexed to extend it again, as well as the outside-stepping leg after the leg is at the posterior extreme position to move it to the anterior extreme position. The influence of the fCO on the activity of the LevTr was investigated by Hess and Büschges (1997) and the feedback was found to increase the activity of the LevTr. Also if the fCO in the inside- and outside-stepping leg is stimulated, the LevTr is always activated by this stimulation. In both cases, the tarsus does not have to have ground contact, otherwise, the animal would be moved into a different direction.

## 3.4.8. Conclusions

The position of the leg at the beginning of the stance phase, as well as the velocity of movement, measured by the fCO, seems to influence how often the movement of the femur tibia joint is supported. In the animal does change the walking direction, and a leg changes from an inside leg, to an outside leg, this also changes the probability of generating an assistance reflex. On the inside, the FT joint is used to move the animal around the point of turning, whereas on the outside the ThC joint moves the animal forward. For the FT joint, this means that on the inside the fCO feedback is needed generate AR to support the ongoing movement, whereas on the outside the feedback is not needed, because the FT joint is not used in the ongoing movement. Here, only in extreme positions of the leg, for example, if the leg is extended, the FT joint needs to be moved and an AR could occurs. The ThC joint and the CTr joint receive input from the fCO as well. For the movement of the CTr joint, it is used similarly on both tasks, the inside and the outside leg. The ThC joint used the fCO feedback

only during the outside leg. Here, the leg joint is moved the most, compared to the inside, where almost no movement of the ThC joint is visible.

First insights into neuronal mechanisms which could change the motor output of the ExtTi MNs indicate a change in synaptic transmission from the fCO afferent neurons to the ExtTi MN, as early latency depolarizations decrease during outside stepping.

# 4. Mechanisms altering the task-dependent processing of fCO feedback

## 4.1. Abstract

Afferent feedback from the fCO of the middle leg in the stick insect elicits different frequencies of occurrence of responses depending whether the leg is currently serving as the inside or outside leg during curve walking. While the leg functions as inside leg AR are generated more often, and while the leg functions as outside leg RR are generated. However, the mechanisms behind this change in occurrence remain unclear. In the present study, an increase in tonic depolarization in ExtTi MNs was observed while the leg functions as outside leg. This gain in membrane potential due to mechanical stimulation of the fCO was smaller during outside stepping, while the direct connection from the afferents to the ExtTi decreased for both inside- and outside-stepping. The membrane input resistance decreased for both inside and outside stepping legs. Recordings from two representative NSI showed also a decrease in amplitude of a monosynaptic excitatory postsynaptic potential (EPSP) during curve walking.

## 4.2. Introduction

Animals use locomotion to interact with their environment. Goal-directed locomotion is crucial for animals to reach food sources or possible mates. The neuronal mechanisms underlying goal-directed locomotion have been addressed in studies about swimming in fish and lamprey, flying in birds and insects, and terrestrial walking (Goulding, 2009, Grillner et al., 2008, Lehmann, 2004). Motor output is rhythmically generated by CPGs, and its timing and magnitude are influenced by sensory feedback (Büschges and Gruhn, 2008, Pearson, 1993). The output can be adjusted so that the legs can fulfill different tasks, such as when they function as inside or outside legs during curve-walking (Gruhn et al., 2009 a, Gruhn et al., 2016). In six-legged insects, it is known that leg trajectories and leg kinematics differ between the legs on the inside and outside of the turn (Dürr and Ebeling, 2005, Jindrich and Full, 1999, Mu and Ritzmann, 2005).

In curve-stepping stick insects, sensory feedback is processed differently between the legs inside and outside of a curve (Hellekes et al., 2011, Hellekes, 2012 and Chapter 3). In the stick insect *C. morosus*, modulation of motor activity by sensory feedback from the fCO is decreased in the middle leg when it functions as the outside leg (Hellekes et al., 2011 and Chapter 3). The fCO is a proprioceptive sense organ in the femur of each leg, measuring

acceleration, velocity, and the position of FT joint movements (Hofmann and Koch, 1985, Hofmann et al., 1985). Feedback from fCO afferents is transmitted either directly or indirectly via polysynaptic pathways, consisting of NSI and SI, to MN causing movement of the tibia via activation of the ExtTi and FlxTi muscles (c.f. Bässler, 1993, Büschges, 1990, Driesang and Büschges, 1996).

Due to the feedback of the fCO, different reactions can be generated depending on the behavioral state of the animal. In inactive animals, a RR movement is generated by extension of the tibia, which is measured by the fCO. This RR is thought to maintain the posture of the animal and to oppose passive movements by activating the ExtTi muscle if the leg is flexed (Bässler, 1977b, 1988). In the active behavioral state, animals are also capable of generating AR, which likely supports stance phase movement by activating the FlxTi if the fCO measures leg flexion (Bässler, 1977b, 1988).

During the active behavioral state, the likelihood of occurrence of AR differs depending on the function of the leg—inside stepping legs show more AR whereas outside-stepping legs generate more RR (Hellekes et al., 2011 and Chapter 3). Moreover, during outside stepping, less modulation due to the fCO feedback is visible while the ExtTi MN are tonically activated (Hellekes et al., 2011).

A mechanism influencing outside stepping is presynaptic inhibition, which decreases the AP amplitude in the afferent neurons and thereby decreases synaptic transmission (Büschges, 1995b, Sauer et al., 1997). Presynaptic inhibition is known to affect proprioceptor afferents in similar control networks in cats (Rudomin and Schmidt, 1999). In the case of stick insects, it is modulated by the CPG (Côté and Gossard, 2003, Ménard et al., 2003). A well-studied example of presynaptic inhibition in insects can be found in the auditory pathway of crickets. To prevent the animals from hearing themselves, the auditory pathway of crickets is presynaptically inhibited by an efference copy (c.f. Poulet and Hedwig, 2002, 2006), and postsynaptic inhibition of locusts is generated by afferents of the same sense organ (Burrows and Laurent, 1993).

Other leg sense organs are known to influence the magnitude of sensory feedback from the fCO. For example, Schmitz and Stein (1999) show that CS as well as HP have inhibitory synapses at fCO afferent terminals. feCS are also known to increase the frequency of AR via additional inhibitory synaptic input onto the ExtTi MNs (Akay and Büschges, 2006).

Intrinsic properties of cells, such as plateau potentials (Kiehn and Eken, 1998, review in: Rossignol et al., 2006) or increased post-synaptic excitability found in vertebrates (review in Heckman et al., 2005) and also in stick insects (Westmark et al., 2009), could also influence the responses of cells to feedback from the fCO. Furthermore, tonic depolarization of MNs during stepping varies regarding leg functioning as an inside or outside leg (Gabriel, 2005, Ludwar et al., 2005a, Ludwar et al., 2005b). Tonic depolarization and phasic

hyperpolarization and depolarization are responsible for rhythmical MN activity in the cat (Perreault, 2002), lamprey (Wallén and Grillner, 1985, Wallén et al., 1993), locust flight (Hedwig and Pearson, 1984), and the stick insect (Gabriel, 2005, Ludwar et al., 2005b). One hypothesis is that the amplitude of the tonic depolarization and the phasic synaptic input could vary for the outside stepping leg. A neurotransmitter underlying the tonic depolarization is ACh shown in MN of *Maduca sexta* (Trimmer and Weeks, 1993) and the stick insect (Westmark et al., 2009). Moreover, input resistance of MNs could be changed. In walking stick insects it is known that the membrane input resistance of MNs decreases (Büschges et al., 2004, Gabriel, 2005).

Neuromodulators, like serotonin in lamprey, are found to decrease synaptic transmission (Parker and Grillner, 1999). In locusts, the neuromodulator OA is found to increase the position feedback of the fCO, but not the response of the phasic part of the stimulation (Matheson, 1997). In stick insects, OA was found to regulate the transmission by ACh and thereby increasing the tonic response (Westmark et al., 2009).

The present study investigates mechanisms that decrease the ExtTi MN activity while modulating the fCO feedback during curve stepping, especially during outside stepping. Additionally, these mechanisms should be compared to mechanisms in inactive animals. Furthermore, the present study is supposed to show an insight into the increased activity of ExtTi MN when the middle leg functions as an outside leg. Therefore, intracellular recordings of ExtTi MN are performed, to investigate for changes in the gain induced by fCO stimulation in the ExtTi MN, monosynaptic connection from fCO afferents onto ExtTi MN, membrane input resistance and tonic depolarization, while the stick insect is stepping in a curve. Additionally, first insights into the direct afferent input from the fCO onto NSI during curve walking are investigated.

## 4.3. Materials and Methods

All experiments were carried out with adult female stick insects (*Carausius morosus* Br.), which were kept in a colony at the Biocenter of the University Cologne (Cologne, Germany). The colony was kept at 25°C and 55% humidity on a 12 h/12 h light-dark cycle. Experiments were performed at a room temperature of 20-22° under reduced light conditions. Blackberry leaves (*Rubus fructiosus*) were given ad libitum as food for the stick insects. All experimental procedures reported here comply with the German National and State Regulations for Animal Welfare and Animal Experiments.

### 4.3.1. Positioning of the stick insect for stepping experiments

The animals were glued to an animal holder (a metal rod, covered with a waxed foam platform) with dental cement (Protemp II; 3M ESPE, St. Paul, MN) dorsal side up. The animal

holder with the animal attached was then placed above a slippery surface (Gruhn et al., 2006). The animal was placed at a height that allowed the femorotibial (FT) joints of the stepping legs to be at an angle of around 90° (Epstein and Graham, 1983, Graham and Cruse, 1981, Gruhn et al., 2006). The left middle leg, however, was fixed to an extension of the animal holder in a well of wax. The joints of the left middle leg were immobilized with dental cement (Hellekes et al., 2011), and the tarsus was ablated. The middle-leg FT joint was fixed at an angle of 110°. The hind legs were amputated to prevent the animal from touching the recording electrodes.

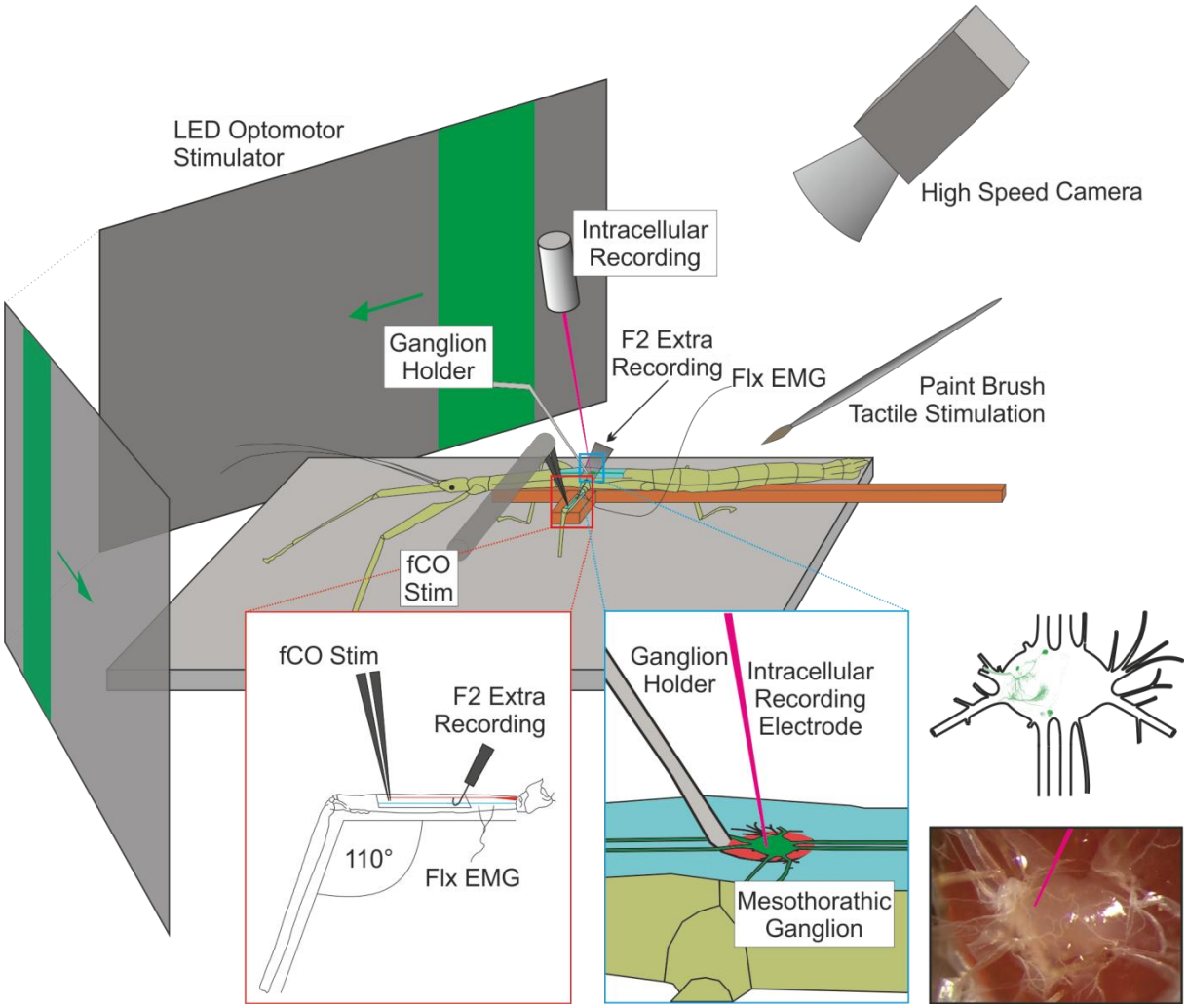



Fig. 4.1: Overview of the experimental approach with fCO stimulation, intra-, and extracellular recordings

The animal glued to an animal holder, walking on a slippery surface (Gruhn. 2006). The fCO is clamed and stimulated, while *flexor tibiae* EMG´s are recorded and the F2 nerve, containing *extensor tibiae* MN, is recorded extracellularly. The mesothoracic ganglion is placed on a ganglion holder. Intracellular recordings with a sharp microelectrode are made in the mesothoracic ganglion. The recording side of *extensor tibiae* motor neurons (green in schematic ganglion) is marked by the tip of the schematic electrode (pink line). The animal was optically and tactilely stimulated to induce curve walking, which was monitored by a high speed camera.

### 4.3.2. Mechanical stimulation of the fCO with extracellular and intracellular recording of motor activity

The preparation was performed as in Chapter 3. Additionally, the animal was opened dorsally between the prothoracic and the metathoracic segments (Fig. 4.1). A well was formed with petroleum jelly and filled with saline to prevent the ganglia from drying out. The gut was moved aside and the mesothoracic ganglion was placed on a wax-covered ganglion holder. The ganglion was fixed on the ganglion holder using cactus thorns, and the sheath around the ganglion was digested with a protolytic enzyme powder (Pronase E; Merck, Germany) for around 30 s. Intracellular recordings were done with sharp glass microelectrodes with a resistance of 15-25 MΩ. The electrodes were pulled using a Sutter Micropuller (P-1000; Sutter Instrument Company, Novato, CA, USA) and then filled with 3M potassium acetate/0.1M potassium chloride. The intracellular signals were recorded in bridge mode and amplified with an intracellular amplifier (SEC-10L, NPI electronic, Tamm, Germany).

### 4.3.3. Electrical stimulation of the fCO afferents with extracellular and intracellular recording of motor activity

The preparation was performed as described in Chapter 4.3.1. The additional preparation was performed as in Sauer and colleagues (1995). The trochanter, coxa, and the proximal part of the femur were opened dorsally to access the F1 nerve, the fCO afferent nerve. A two hooked stimulation electrode was placed at the F1 nerve to electrically stimulate the fCO afferents (Fig. 4.2). Extracellular hook electrodes (modified from Schmitz et al., 1988) were placed on the *nervus cruris* (NCr) to record the fCO afferents and on the nl3, containing all ExtTi MNs axons, to record the ExtTi MNs activity. Additionally, intracellular recordings were performed from ExtTi MNs as described in Chapter 4.3.2.

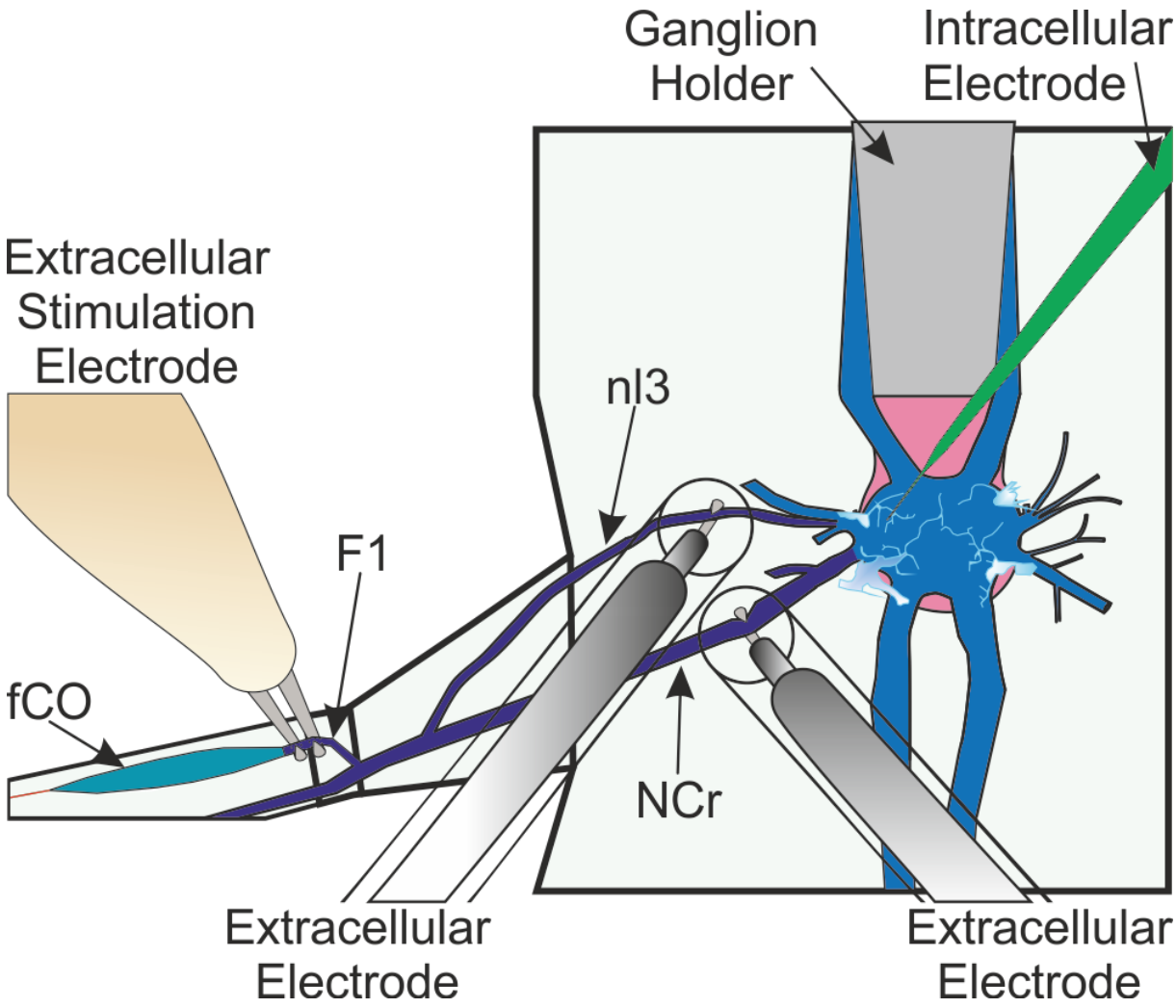



Fig. 4.2: Schematic drawing of the electric stimulation approach
The mesothoracic ganglion is placed on a ganglion holder. The F1 nerve, containing the fCO afferents is placed on a two hooked stimulation electrode. The NCr, containing the fCO afferents, and the nl3, containing extensor tibiae MN, are recorded extracellularly. Additionally, extensor tibiae and nonspiking interneurons are recorded intracellularly. Tracheae on the ganglion are used as landmarks.

### 4.3.4. Curve walking.

The preparation described in 4.3.2 and 4.3.3 was used. Additionally, both hind legs were amputated to prevent the animal from touching the electrodes, whereas the other legs were free to move. To induce walking the animal was stimulated on the abdomen with a paintbrush (Graham and Epstein 1985). To induce curve walking a visual stimulation with two LED screens (8x8 green light LED panels, Electronics Workshop, University of Cologne, Cologne, Germany), showing a striped pattern (one stripe per LED screen) with vertical movement, was used. Directional curve walking was elicited by moving the pattern to the left or to the right (comp. Gruhn et al., 2009 a, Hellekes et al., 2011). This curve walking setup was used in all experiments described here. Walking activity was monitored by video recordings, with an high speed camera (AOS S-PRI, AOS Technology AG, Baden Daettwil,

Switzerland, resolution: 400 × 1,024 pixel, frame rate: 7 fps, shutter speed: 2000 µs) from the dorsal side of the animal.

### 4.3.5. Data Acquisition

All physiological signals, which were recorded, were amplified and filtered (e.g. von Uckermann and Büschges, 2009). To record the signals, the analogue signal was converted with a Micro 1401 A/D converter (sampling rate, 12.5 kHz) in digital signals and then recorded with Spike2 data acquisition/ analysis software (version 5.04; Cambridge Electronic Design).

### 4.3.6. Statistical analysis

All physiological signals, which were recorded, were amplified and filtered (e.g. von Uckermann and Büschges, 2009). To record the signals, the analogue signal was converted with a Micro 1401 A/D converter (sampling rate, 12.5 kHz) in digital signals and then recorded with Spike2 data acquisition/analysis software (version 5.04; Cambridge Electronic Design).

## 4.4. Results

The experiments described above led to a significant increase in the occurrence of RR during outside stepping. In the present study, I aimed to investigate the mechanisms responsible for the differences between inside and outside stepping legs. To that end, I performed intracellular recordings of ExtTi MNs to compare the membrane potential, depolarization during mechanical stimulation of the fCO, EPSPs elicited by electrical stimulation of the fCO afferents, and membrane resistance.

The aim of the first experiment was to receive an overview of changes in ExtTi MNs during curve walking compared to the resting animal. Therefore, I tested the change in depolarization, which represents the gain of the stimulation during fCO stimulation, with a linear motor. Here, the inactive leg, which was producing the RR, was compared to the outside- as well as the inside-stepping leg.

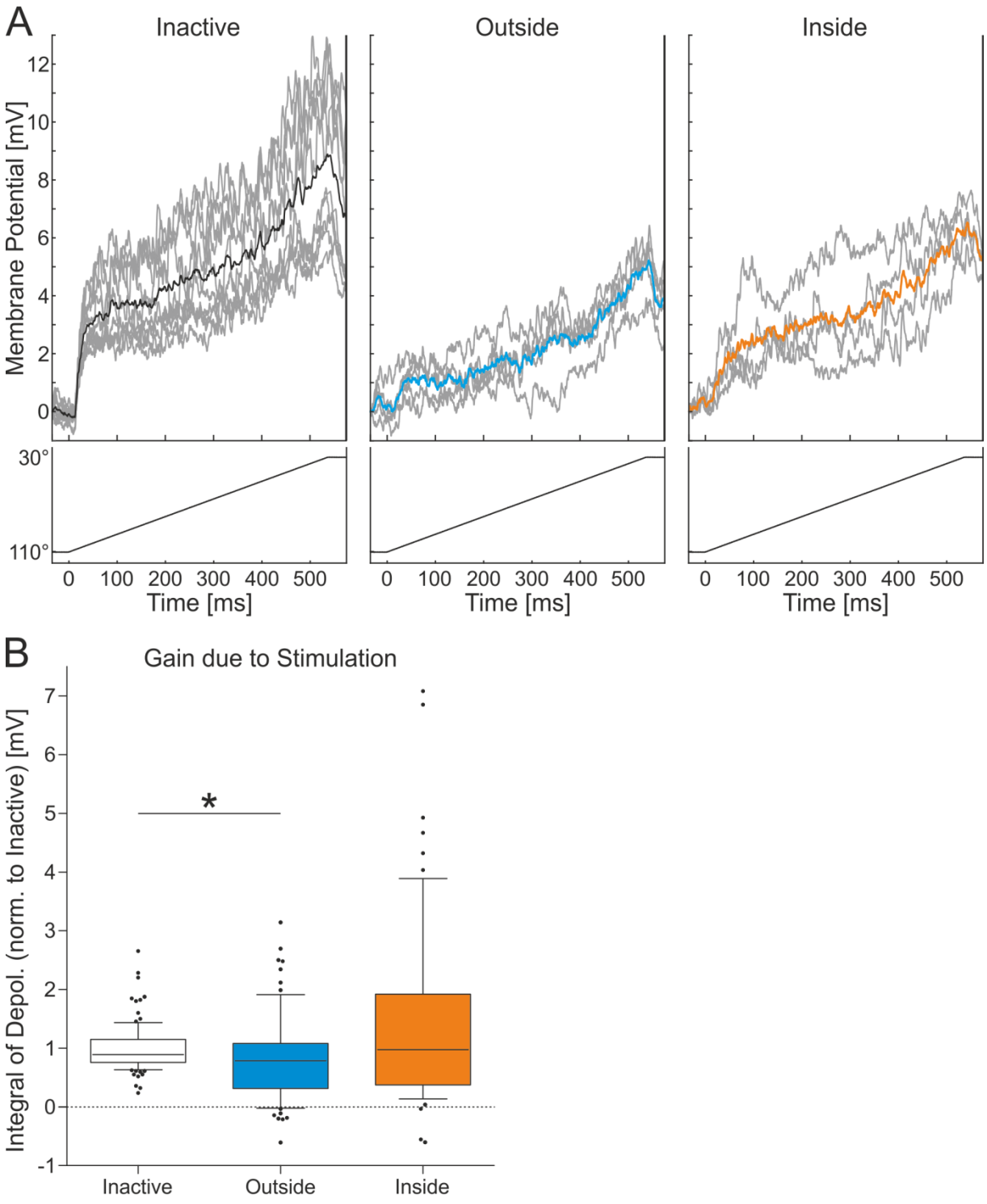



Fig. 4.3: Gain in ExtTi MNs membrane potential due to mechanical fCO stimulation
(A) One example animal: Overlays of membrane potential waveforms, with zero at the onset of the mechanical fCO stimulation. (B) Integral of the membrane potential overlays during mechanical fCO stimulation. Inactive (N = 8, n = 103); outside (N = 8, n = 74); inside (N = 8, n = 65); membrane potential overlay parts in grey, mean of the overlays: inactive in black, outside in blue, inside in red; box plots: inactive leg condition (white); outside leg condition (blue); inside leg condition (orange); significance value: $P < 0.05$ = *.

In inactive animals, ramp stimulation of the fCO led to a sharp depolarization of the membrane potential of the ExtTi MN. This increase lasted up to 25 ms and started 3-5 ms after stimulation onset. Afterwards, the membrane potential increased further, but more

slowly, to a maximum value at the end of the ramp stimulation. Data of one example animal are shown in Fig. 4.3. Here, data of several membrane potential waveforms are overlaid at the onset of the stimulation of the fCO with the mean membrane potential waveform. For better comparison, the membrane potential waveforms had similar membrane potentials at the onset of the stimulation. Within this representative animal, the membrane potential of the outside stepping leg was shaped similarly to that of the inactive leg, with a decreased amplitude for the first depolarization and the overall depolarization. The initial depolarization and the maximal depolarization of the inside stepping leg varied more than those of the outside stepping leg.

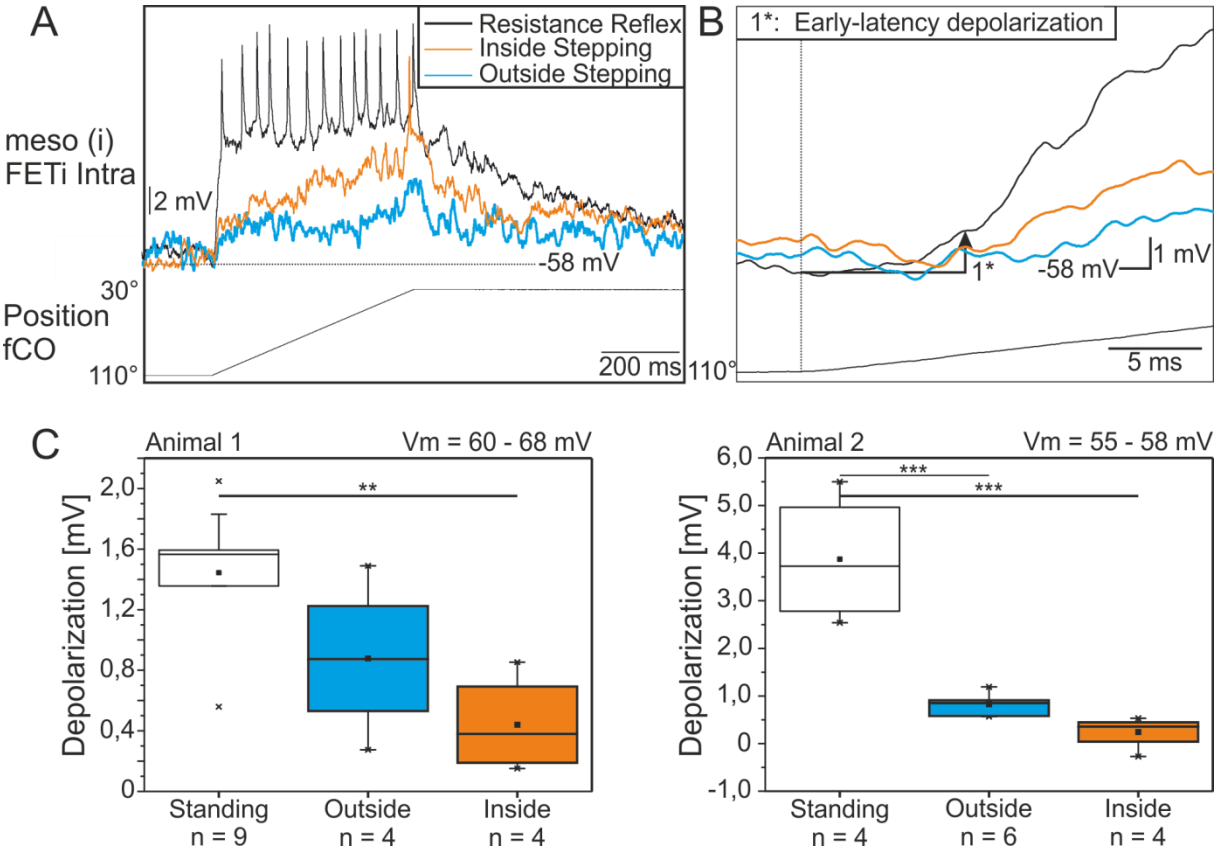



Fig. 4.4: Early latency depolarization during curve walking in ExtTi MN
(A) Overlay of membrane potential during mechanical fCO stimulation for inactive, outside and inside leg condition. (B) Overlay of membrane potential at the onset of mechanical fCO stimulation with early latency depolarization for inactive, outside and inside leg condition. (C) Amplitude of the early latency depolarization in two exemplary animals. Inactive leg condition (black line or white box); outside leg condition (blue); inside leg condition (orange); significance values: P < 0.01 = **; P < 0.001 = ***.

To analyze the gain during the mechanical stimulation of the fCO, the integrals of multiple membrane potential waveforms during fCO stimulation were compared. In 7 of 10 similar starting membrane potential experiments measured in eight animals, the integrals were smaller in the outside leg compared to the inactive leg; in 2 of 3, the integral was larger; and in 1 of 10 there was no difference. For the inside stepping leg, 60% of the integral of the

experiments were smaller, and 20% were larger or the same. By pooling the different experiments and normalizing the integrals to the mean of the inactive leg, the values of the outside-stepping legs (N = 8, n = 74, median: 0.79 mV/s, IQR: 0.77 mV/s) were significantly smaller compared to the values of the inactive leg (N = 8, n = 103, median: 0.89 mV/s, IQR: 0.39 mV/s). The inside-stepping leg (N = 8, n = 65, median: 0.98 mV/s, IQR: 1.55 mV/s), however, was not significantly different from the inactive and the outside stepping leg. The gain of the membrane potential depolarization due to mechanical stimulation of the fCO was significantly smaller ($P < 0.05$) during outside stepping compared to the inactive animal showing a RR. While the leg was used as an inside leg, the gain of the membrane depolarization was more variable, but not smaller, compared to the inactive animal showing a RR.

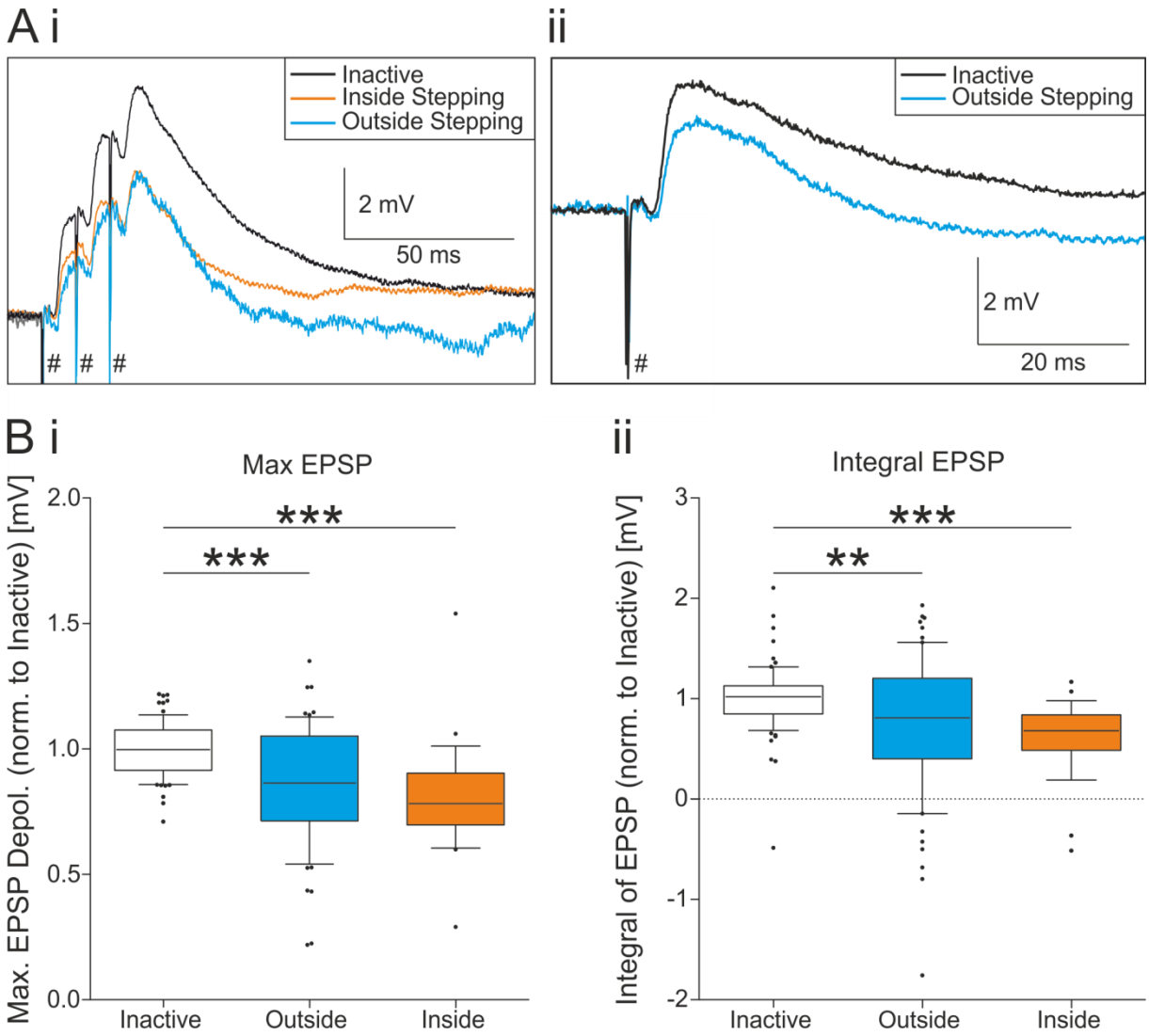



Fig. 4.5: Electrical stimulation of fCO afferents and intracellular ExtTi recording
(A) EPSPs in *extensor tibiae* MN elicited by electrical fCO stimulation (i) with three and (ii) one stimulation. (B) Comparison of the maximal amplitude (i) and the integral (ii) of the EPSP elicited in ExtTi MN. Inactive (N = 8, n = 75); outside (N = 8, n = 69); inside (N = 5, n = 26); inactive leg condition (black line or white box); outside leg condition (blue); inside leg condition (orange); significance values: $P < 0.01$ = **; $P < 0.001$ = ***; electrical stimulation marked with rhombus.

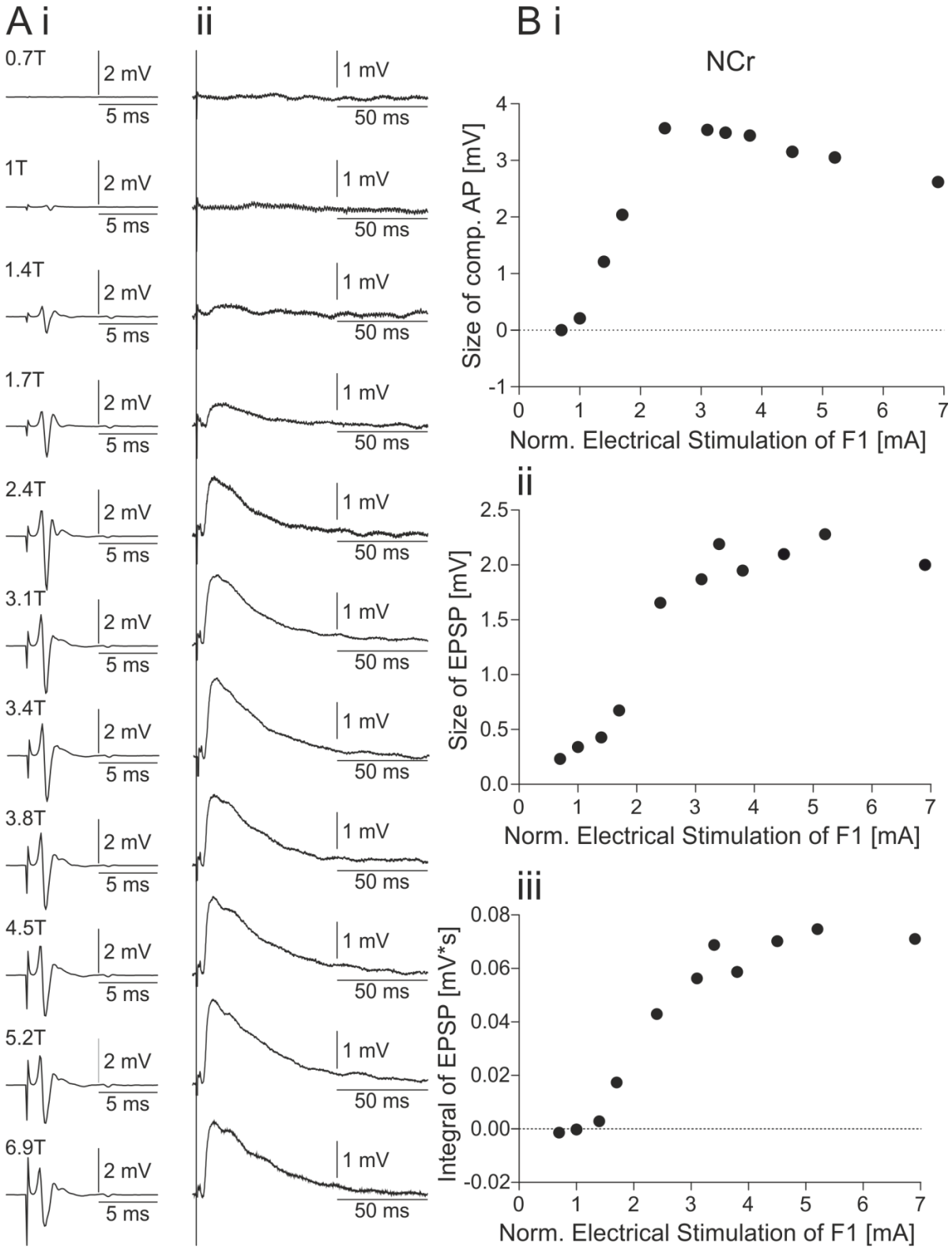


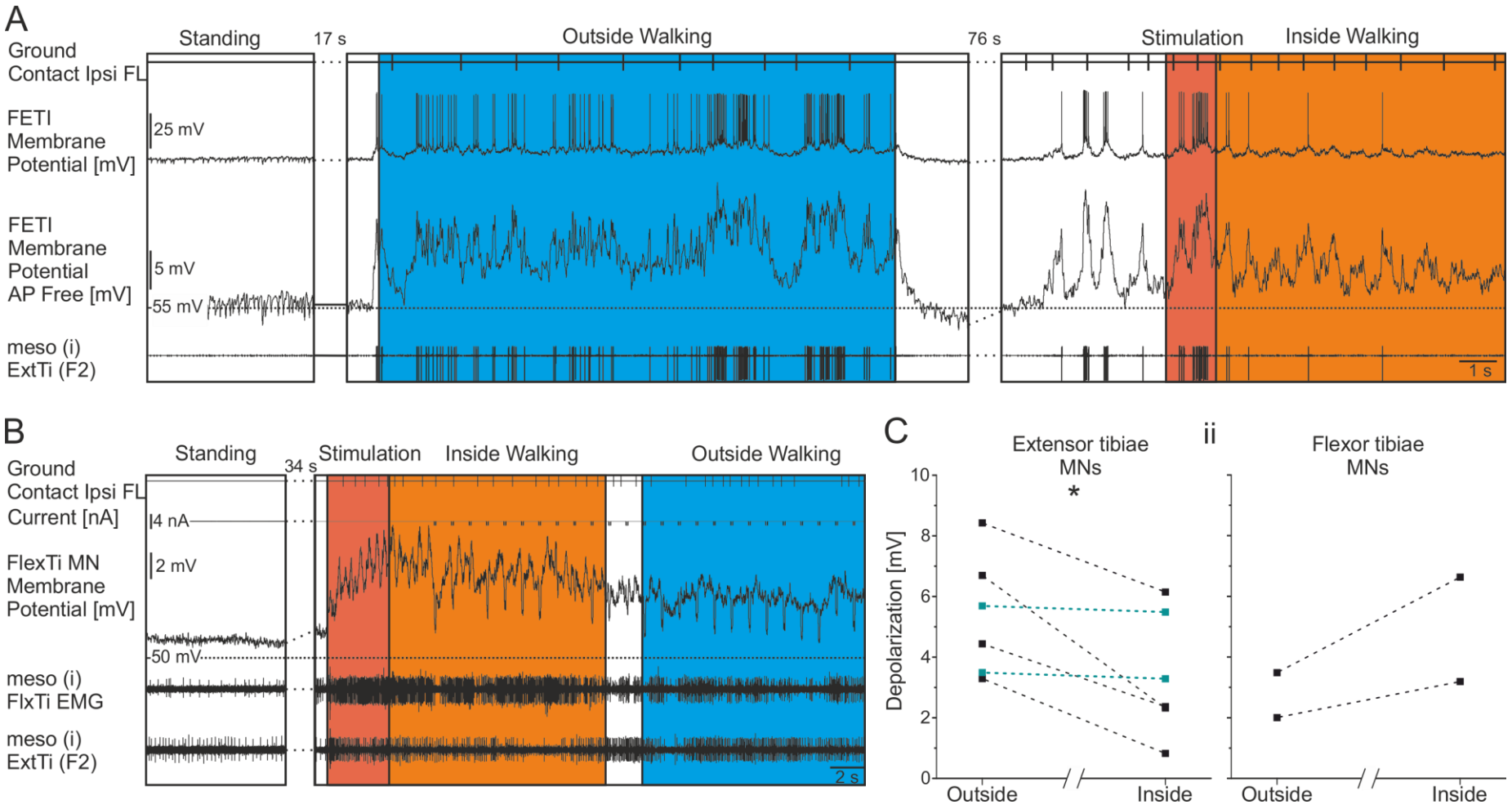

Fig. 4.6: Stimulus response ExtTi MN to electrical fCO stimulation

(Ai) Compound action potential recorded in the NCr, and (ii) the intracellular recorded EPSP in ExtTi MN after electrical stimulation of the F1 nerve, containing all fCO afferents. (B) Stimulus response curves to electrical stimulation of the F1 nerve from (i) the amplitude of the compound action potential in the NCr, (ii) the amplitude, and (iii) the integral of the EPSP in ExtTi MN.

The mechanical stimulation lasted up to 500 ms. During this time, the ExtTi MN could be affected by a number of different influences like SI and NSI (Büschges, 1995a). Due to the high number of influences, a conclusion on the mechanisms underlying changes between inactive, outside and inside stepping leg cannot be elucidated.

Table 4.1: Values for mean, standard deviation (SD), median, and interquartile range (IQR) for the early latency depolarization in specific membrane potential ranges

| | Membrane Potential [mV] | | Mean [mV] | SD [mV] | Median [mV] | IQR [mV] |
|---|---|---|---|---|---|---|
| Animal 1 | -60 : -68 | Inactive | 1.52 | 0.47 | 1.65 | 0.25 |
| | | Outside | 0.93 | 0.53 | 0.92 | 0.73 |
| | | Inside | 0.47 | 0.34 | 0.40 | 0.53 |
| Animal 2 | -55 : -58 | Inactive | 3.87 | 1.35 | 3.73 | 2.19 |
| | | Outside | 0.83 | 0.23 | 0.85 | 0.33 |
| | | Inside | 0.24 | 0.35 | 0.36 | 0.41 |
| Animal 3 | -52 : -55 | Inactive | 0.87 | 0.16 | 0.79 | 0.29 |
| | | Outside | 0.12 | 0.17 | 0.10 | 0.28 |
| Animal 4 | -61 : -65 | Inactive | 1.04 | 0.30 | 0.89 | 0.45 |
| | | Outside | 0.98 | 0.65 | 0.84 | 0.83 |
| | -65 : -68 | Inactive | 2.17 | 0.52 | 2.13 | 0.55 |
| | | Outside | 1.67 | 1.04 | 1.77 | 1.62 |
| Animal 5 | -52 : -55 | Inactive | 0.49 | 0.31 | 0.44 | 0.27 |
| | | Outside | 0.78 | 0.27 | 0.81 | 0.39 |
| Animal 6 | -56 :-63 | Inactive | 0.98 | 0.26 | 0.92 | 0.26 |
| | | Outside | 0.69 | 0.61 | 0.39 | 1.12 |
| Animal 7 | -76 : -80 | Inactive | 0.71 | 1.88 | 1.29 | 2.58 |
| | | Outside | 2.84 | 1.85 | 1.89 | 2.47 |
| Animal 8 | -56 : -60 | Inactive | 0.67 | 0.31 | 0.65 | 0.25 |
| | | Outside | 0.99 | 0.48 | 1.03 | 0.97 |


A
Standing
17 s
Outside Walking
76 s
Stimulation
Inside Walking
Ground Contact Ipsi FL
FETI Membrane Potential [mV]
25 mV
FETI Membrane Potential AP Free [mV]
5 mV
-55 mV
meso (i) ExtTi (F2)
1 s
B
Standing
34 s
Stimulation
Inside Walking
Outside Walking
Ground Contact Ipsi FL Current [nA]
4 nA
FlexTi MN Membrane Potential [mV]
2 mV
-50 mV
meso (i) FlxTi EMG
meso (i) ExtTi (F2)
2 s
C
Extensor tibiae MNs
*
ii
Flexor tibiae MNs
Depolarization [mV]
10
8
6
4
2
0
Outside
Inside
Outside
Inside

However, there is a monosynaptic connection from the fCO afferents to the ExtTi MN (Driesang and Büschges, 1996). The early latency depolarization (ELD) is a monosynaptic influence leading to a EPSP in the ExtTi MN in the first 5-12 ms after the onset of the mechanical stimulation (Driesang and Büschges, 1996) and as I could find changes in the sharp increase of the membrane potential at the onset of the mechanical stimulation, I had a closer look on the ELD in several animals.

We compared the first peak ELD between the inactive animal, which is uninfluenced by other feedback than the fCO stimulation, and the curve walking animal (Fig. S. 3) . In two example animals shown in Fig. 4.4, the ELD measured in the inactive leg was significantly larger compared to the ELD of the inside-stepping leg (animal 1: inside (n = 4): mean: 0.47 mV, SD: 0.34 mV, inactive (n = 9): mean: 1.52 mV SD: 4.47 mV, $P < 0.01$; animal 2: inside (n = 4): mean: 0.24 mV, SD: 0.35 mV, inactive (n = 4): mean: 3.87 mV SD: 1,35 mV, $P < 0.001$). Furthermore, the EDL of the outside-stepping leg was smaller in 7 out of 10 measurements (N = 8, n = 10). In two of these seven measurements, this was significantly smaller (animal 2: $P < 0.001$ (see Fig. S. 3), animal 3: $P < 0.5$). In 2 of 10 measurements, the maximum value of the ELD had a higher value, which did not significantly differ from the EDL in the inactive animal, and, in one measurement, the ELD stayed the same (see Table 4.1). Taken together, the ELD decreased in most of the cases in the outside-stepping leg and significantly in the two inside-stepping leg measurements. As I found a change in the maximum amplitude of the ELD during curve walking, I used another approach to gain a deeper insight into the monosynaptic connection. I electrically stimulated the afferent fCO neurons with a double hook electrode, to elicit EPSPs in the postsynaptic ExtTi MN to compare the amplitude as well as the area under the EPSP between the inactive and the curve walking animal.

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Fig. 4.7: Tonic depolarization in ExtTi and FlxTi MN during outside and inside leg stepping (A) A fast FETi shows a decrease in tonic depolarization during outside leg stepping. The touchdown of the ipsilateral front leg is shown in the upper trace, FETi MN recording is shown with APs (2nd trace) and with APs eliminated (3rd trace), a nerve recording of the F2 nerve with the ExtTi MN is shown in the last trace. (B) FlxTi MN recording (3rd trace) shows increase in tonic depolarization during inside leg stepping. Ground contact of the ipsilateral front leg in the top trace, current injection in the 2nd trace, one FlxTi EMG (4th trace) and extracellular F2 nerve recording (bottom trace). (C) Comparison of the tonic depolarization in (i) ExtTi MN and (ii) FlxTi MN. Outside leg condition (blue); inside leg condition (orange); tactile stimulation (red); (C) black lines show experiments decrease and blue lines with no change; significance value: $P < 0.05$ = *.

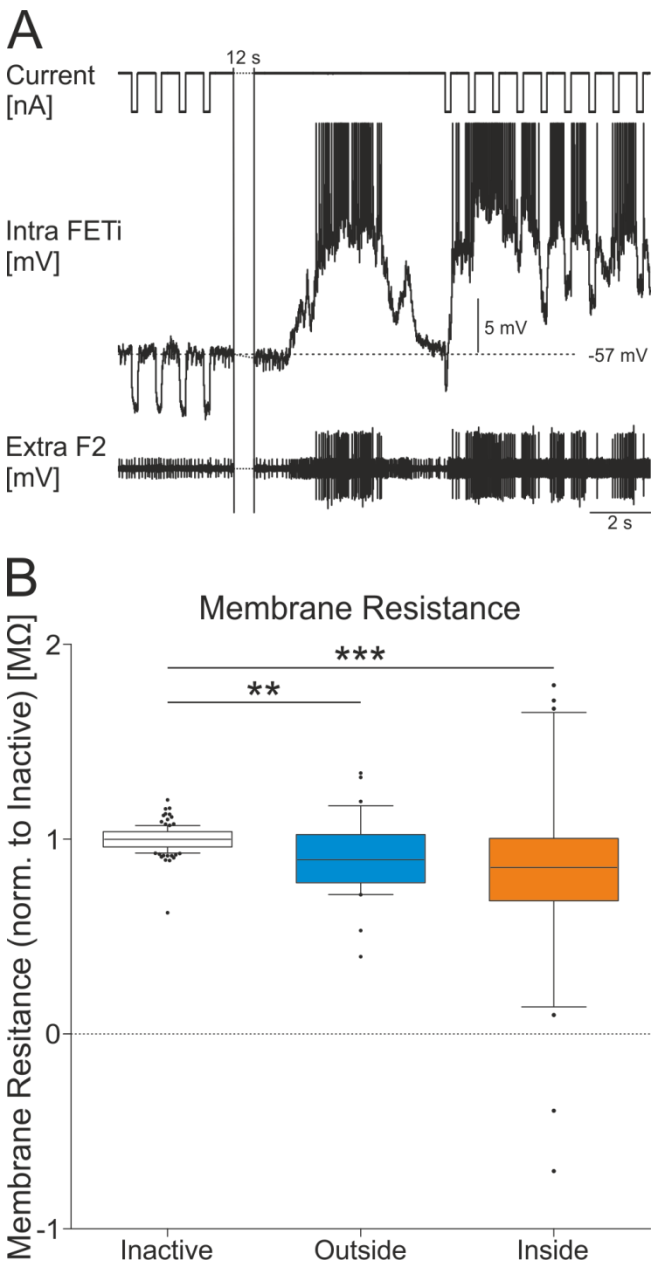



Fig. 4.8: Comparison of membrane input resistance between inactive, inside and outside leg condition in ExtTi MN

(A) Recording of FETi MN. Upper trace shows current, with negative injections of 1nA, second trace shows an intracellular recording of FETi MN with resting membrane potential at -57mN and a walking sequence, third trace shows an extracellular recording of the F2 nerve with ExtTi MN. (B) Comparison of the membrane input resistance between inactive, inside and outside leg condition. Inactive (N = 5, n = 105); outside (N = 5, n = 33); inside (N = 4, n = 31); inactive leg condition (white); outside leg condition (blue); inside leg condition (orange); significance values: P < 0.05 = *; P < 0.01 = **; P < 0.001 = ***.

To have a measure for the efficiency of the stimulation, the NCr, which contains the axons of the fCO afferents, was recorded and a compound AP could be elicited with a electrical stimulation of the F1 nerve. Further, to identify the necessary stimulation strength, for each recording a stimulus response curve was made. Stimulation strength, which elicited the first measurable compound AP, was called 1T. With increasing stimulation strength the compound AP also increased until a maximum size was reached, at which point all neurons in the F1 nerve should be firing an AP. The same was true for the intracellularly recorded EPSP, elicited by the compound AP. For the following experiments a stimulation strength was chosen, which elicited a compound AP and an EPSP, which were both a multiple of the threshold 1T. In **Fig. 4.6** an representative stimulus response curve of an ExtTi MN for the maximal amplitude of the EPSP, and the integral under the EPSP is shown against the stimulus strength, with the first compound AP elicited at 1T (0.06 mA) stimulation strength. The saturation was reached at a stimulation strength of 3.4 T (0.2 mA), while the compound AP reached its maximum value at 2.4 T (0.14 mA).

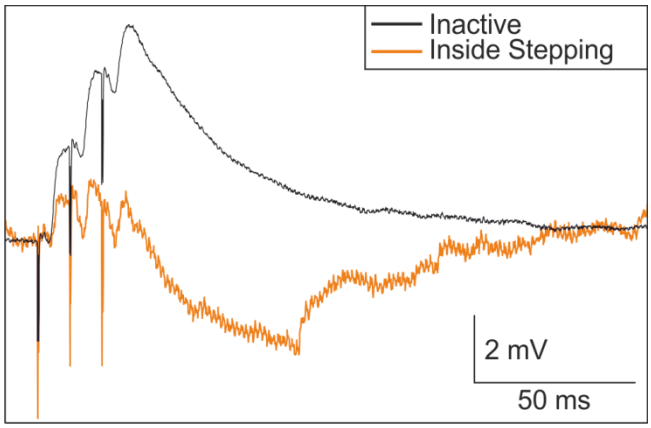



Fig. 4.9: Intracellular recording of an ExtTi MN showing EPSP and IPSP

Comparison of an intracellular ExtTi MN recording with three electrical stimulations of the F1 nerve, for inactive leg condition (black) and inside leg condition (orange). Inside leg condition first shows small EPSP and then a large IPSP.

As I already knew, the ELD, elicited by mechanical stimulation of the fCO, was smaller while the animal was walking in a curve. Hence, to have a more accurate measure, I used the electrical stimulation to elicit APs in the fCO afferents, and additionally, let the animal walk in a curve induced by an optical stimulation with the LED screen in front of the animal. The EPSPs evoked during curve walking were compared to EPSPs elicited by the inactive animal. EPSPs in the inactive animal are not influenced by the mechanisms active in the walking animal and are therefore supposed to be very stereotypic.

In 4 out of 6 animals in the curve walking situation the EPSP measured in the ExtTi MN was smaller compared to the EPSP in the inactive animal. In the example recording, in black an EPSP of a inactive leg is shown, which is compared to the EPSP of the outside-stepping leg in blue for one stimulation, and as a sum of three stimulations. Additionally, in orange the sum of the EPSPs after three stimulations for the inside-stepping leg are displayed. In both cases, the outside-stepping and inside-stepping leg with three stimulations is smaller compared to the inactive leg (**Fig. 4.5**).

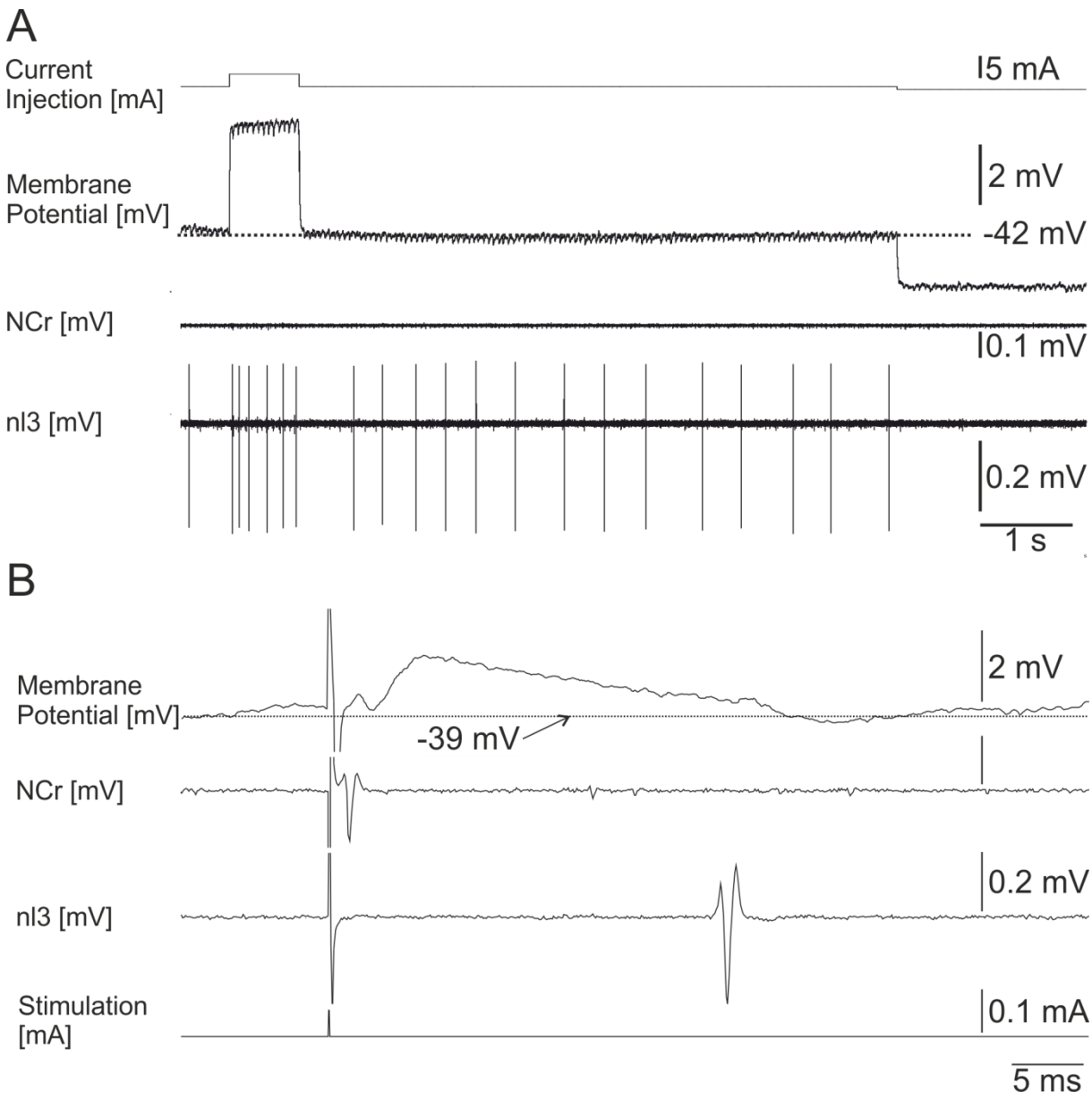



Fig. 4.10: Recording of a potential NSI, with excitatory and inhibitory connection onto ExtTi MN

(A) Intercellular recording of a potential NSI, eliciting an increase in spiking in the extracellular recorded nl3 nerve, with depolarizing and a decrease with hyperpolarizing current injection. Current in the top trace, intracellular recording of the potential NSI in the second trace, extracellular recordings of NCr in the third trace and nl3 in the bottom trace. (B) Intracellular recording of the potential NSI, showing a large EPSP due to electrical F1 nerve stimulation, extracellular recordings of the NCr, showing compound AP after electrical stimulation of fCO afferents and nl3, showing ExtTi AP. Intracellular recording of the potential NSI in the top trace, extracellular recordings of NCr in the second trace and nl3 in the third trace, electrical stimulation of the F1 nerve in the bottom trace.

For quantification the maximum amplitude and the integral beneath the EPSP from six animals was taken, and normalized to the mean of the inactive leg. In all cases if the animals are pooled, the outside-stepping leg (N = 6, n = 69, median: 0.86 mV, IQR: 0.34 mV) and the inside-stepping leg (N = 6, n = 26, median: 0.78 mV, IQR: 0.20 mV) had significantly reduced maximal EPSP amplitudes ($P < 0.001$) compared to the inactive leg (N = 6, n = 75, median:

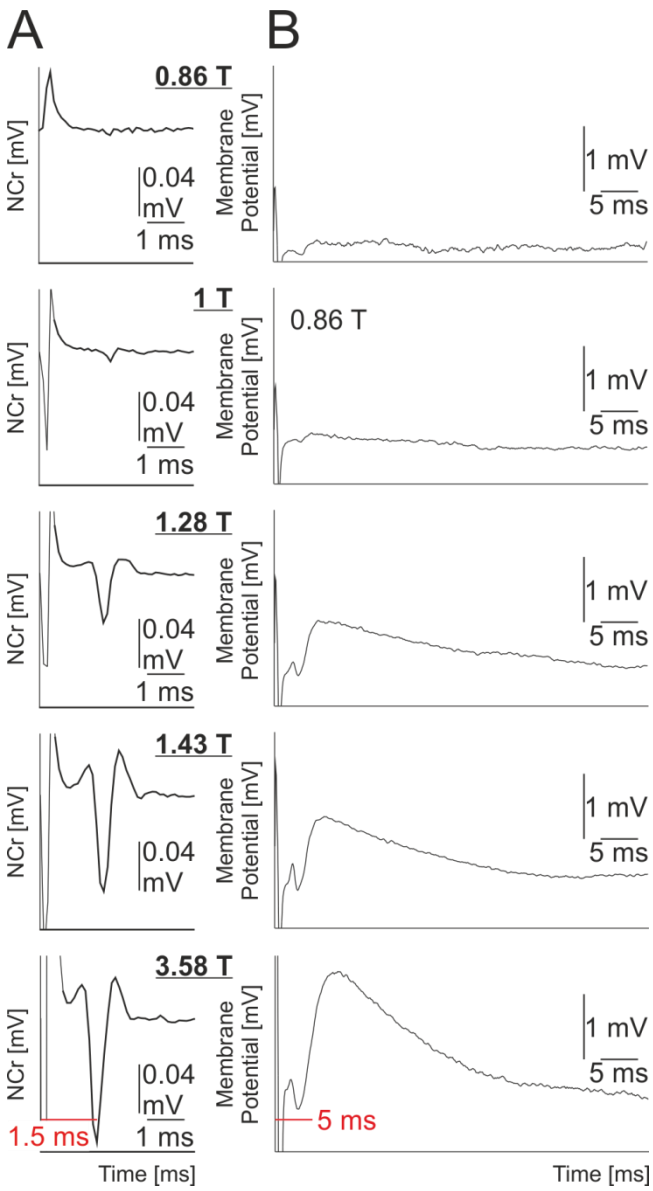



Fig. 4.11: Stimulus response in potential NSI (Fig. 4.10) to electrical fCO stimulation
(A) The compound action potential recorded in the NCr and (B) the intracellular recorded EPSP in the potential NSI after electrical stimulation of the fCO.

1.00 mV, IQR: 0.17 mV). The same was true for the integral of the EPSP. The integral for the outside-stepping (N = 6, n = 69, median: 0.81 mV/s, IQR: 0.80 mV/s, P < 0.01) and the inside-stepping leg (N = 6, n = 26, median: 0.68 mV/s, IQR: 0.35 mV/s, P < 0.001) were significantly smaller compared to the EPSP measured in the inactive leg (N = 6, n = 75, median: 0.85 mV/s, IQR: 0.28 mV/s).

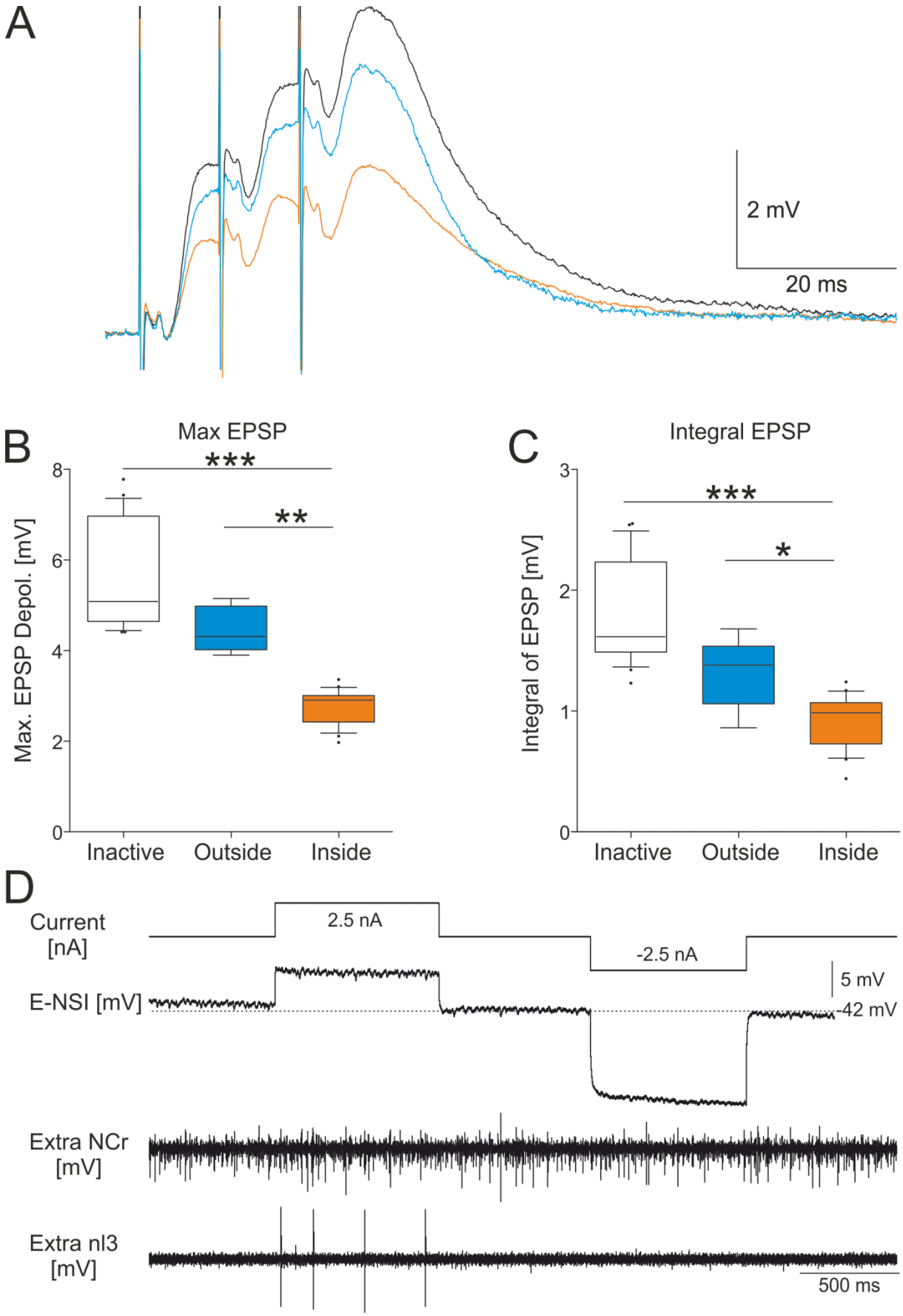
In the next experiment the underlying tonic depolarization was evaluated. For six animals the membrane potential difference between outside- or inside-stepping leg to the inactive leg of the ExtTi during multiple sequences was compared (Fig. 4.7). During curve walking the membrane potential was more depolarized compared to the inactive leg. Furthermore, in 4 out of 6 animals the outside-stepping leg displayed a more depolarized membrane potential compared to the inside stepping leg. In 2 out of 6 animals, the membrane potential showed no difference between the outside- and inside-stepping leg. Pooled together, the depolarization of the outside stepping leg was increased compared to the inside-stepping leg (P < 0.05).

For the FlxTi, the muscle antagonist, the opposite was true. For 2 out of 2 animals tested in the present study and for six more animals of previous study by Katja Hellekes (personal communication), the membrane potential was more depolarized during inside- compared to outside-stepping. Summarizing, the curve walking led to an increase in membrane potential during the stepping sequences. For the ExtTi the membrane potential was more depolarized during outside stepping, the opposite was true for the FlxTi.

A presynaptic inhibition has been shown for forward walking by Sauer and Büschges (1997), but needs to be investigated further for curve walking as it is a likely mechanism to modify the input to the ExtTi MN. However, another possibility could be postsynaptic mechanisms changing the membrane input resistance of the cell and therefore leading to a decrease in the EPSP size.

The easiest way to check for changes in membrane input resistance is to use short current pulses of 200 ms with an amplitude of -1 nA, as shown in Fig. 4.8, and measure the change in membrane potential due to the current flow. Using Ohms law the membrane resistance was calculated. During both outside- ($N = 5$, $n = 33$, median: 0.89 MΩ, IQR: 0.25 MΩ) as well as the inside-stepping ($N = 4$, $n = 31$, median: 0.85 MΩ, IQR: 0.32 MΩ), a significant decrease in the membrane resistance was measured in five animals (outside: $P < 0.01$; inside: $P < 0.001$), compared to the inactive animal ($N = 5$, $n = 125$, median: 1.00 MΩ, IQR: 0.08 MΩ). In both cases also the variance was increase compared to the inactive animal.

As described above, presynaptic inhibition could not be ruled out as a mechanism in the ExtTi MNs leading to the measured decrease of the ELD, as well as the EPSP, elicited by a monosynaptic transmission from fCO afferent neurons after electrical stimulation of these. However, membrane input resistance was decreased during curve walking, which should lead to smaller changes evoked by these connections. Additionally, membrane potential was more depolarized during curve walking, which also leads to smaller changes in membrane potential due to the afferent input.


A
2 mV
20 ms
B
Max EPSP
***
**
Max. EPSP Depol. [mV]
0
2
4
6
8
Inactive
Outside
Inside
C
Integral EPSP
***
*
Integral of EPSP [mV]
0
1
2
3
Inactive
Outside
Inside
D
Current [nA]
2.5 nA
-2.5 nA
E-NSI [mV]
5 mV
-42 mV
Extra NCr [mV]
Extra nl3 [mV]
500 ms

Another interesting effect was visible during curve walking. With an electrical stimulation of the fCO afferents, which was at the saturation level of stimulation, next to the EPSPs, which were always elicited, a large long lasting hyperpolarization could be evoked. First, due to the stimulation a small EPSP could be seen, and after around 50 ms a large inhibitory postsynaptic potential (IPSP) was observed (Fig. 4.9). In the present study, the IPSP was measured only three times, two of which were during inside-stepping and also one time during outside-stepping.

Next to the ExtTi MNs, also NSI, which had an effect on the ExtTi, were investigated for their changes during curve walking in regard to the monosynaptic connection of fCO afferents on the NSI. The NSI were divided into two groups: the excitatory NSI, and the inhibitory NSI. As an example, one excitatory NSI is shown, which had a membrane potential of -42 mV. The frequency of ExtTi APs measured in the nl3 was increased while a depolarizing current was injected into the cell. Further, the nl3 was silent while a hyperpolarizing current was injected, which led to the conclusion that this cell had an excitatory effect on the ExtTi MN. The ENSI also showed a large long lasting EPSP elicited by the electrical stimulation of the fCO afferents. This is shown in Fig. 4.10, together with the stimulus response (Fig. 4.11). If the cell was slightly depolarized to 39 mV, each EPSP in the ENSI elicited also one AP in the nl3.

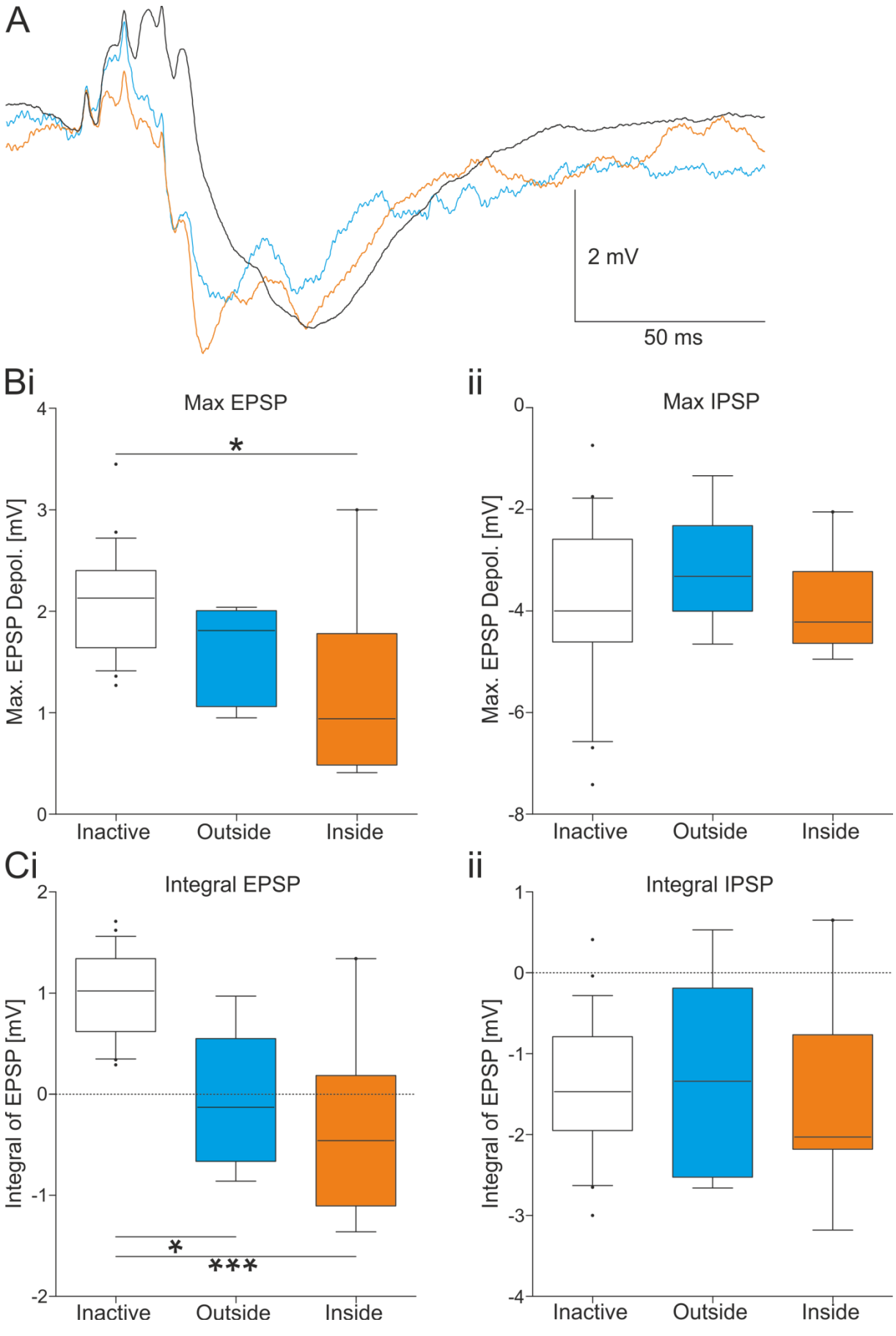
The next NSI was, when a depolarizing current was injected, exciting the ExtTi MN while no effect during hyperpolarization was found (Fig. 4.12). The neuron showed large EPSPs due to stimulation of the F1 nerve with maximal amplitudes around 3 mV. During stimulations with an interval of 10 ms the maximal amplitude increased up a maximum value of 7.78 mV. However, during curve walking this amplitude decreased compared to the median 4.71 mV (IQR: 2.22 mV) of the inactive leg (n = 29) during outside-stepping of the leg (n = 9) to amplitudes around median of 4.31 mV (IQR: 0.81 mV) with three stimulations and during inside-stepping of the leg (n = 22) even to a median of 2.71 mV (IQR: 0.55 mV), which was significantly smaller ($P < 0.001$). The inside-stepping leg showed also significantly smaller EPSPs compared to the outside-stepping leg ($P < 0.01$). Moreover, the integral of the

Fig. 4.12: Excitatory NSI recorded intracellularly with electrical stimulation of fCO afferents

(A) EPSPs in the NSI elicited by three electrical fCO stimulations. Comparison of the maximal amplitude (B), and the integral (C) of the EPSP elicited in NSI. (D) Depolarizing current injection (top trace) into the intracellular recorded NSI (second trace) elicited APs in ExtTi MN recorded extracellular in the nl3 (bottom trace), but not in the NCr (third trace). Inactive (n = 22); outside (n = 9); inside (n = 22); inactive leg condition (black line or white box); outside leg condition (blue); inside leg condition (orange); significance values: $P < 0.05$ = *; $P < 0.01$ = **; $P < 0.001$ = ***.

EPSP had the same tendency, the values were smaller during curve walking (outside: median: 1.38 mV/s ,IQR: 0.41 mV/s; inside: median: 0.99 mV/s, IQR: 0.31 mV/s) compared to the inactive leg (median: 1.51 mV/s, IQR: 0.72 mV/s). The integral of the inside-stepping leg was smaller than the one of the inactive leg ($P < 0.001$) as well as of the outside-stepping leg ($P < 0.05$).

Another NSI was recorded, which was also exciting the SETi while a depolarizing current was injected. Additionally, the NSI elicited a slight decrease the SETi AP frequency as well while the cell was hyperpolarized (Fig. 4.13). Interestingly, this cell received EPSPs in the first 30 ms after three stimulation of the F1-nerve. After 30 ms the cell also received long lasting IPSPs, lasting for up to 100ms (Fig. 4.14). To analyze the behavior of the cell to electrical stimulation of the fCO nerve, the reaction was divided into the EPSP and the IPSP, which were analyzed separately. During curve walking, the EPSP was decreasing around 10 ms earlier to the second phase, the IPSP phase, compared to the IPSP in the inactive leg (inactive: n = 23; outside: n= 5; inside: n = 9). The maximal amplitude of the EPSP was also decreased ($P < 0.05$) for the inside-stepping leg (inactive: median: 2.13 mV, IQR: 0.72 mV; outside: median: 1.81 mV, IQR: 0.80 mV; inside: median: 0.94 mV, IQR: 0.84 mV). The integral measured during 30 ms after stimulus begin was smaller for outside- (median: -0.13 mV, IQR: 0.60 mV, $P < 0.05$) and inside-stepping leg (median: -2.03 mV, IQR: 0.97 mV, $P > 0.001$) compared to the integral of the inactive leg (median: 1,02 mV, IQR: 0.66 mV). However, for the second phase, the IPSP, no difference between the inactive leg and the curve stepping leg was be found. The maximal amplitude of the IPSP was only slightly smaller for the outside stepping (inactive: median: 4.00 mV, IQR: 1.71 mV; outside: median: -3.32 mV ,IQR: 0.06 mV; inside: median: -4.22 mV, IQR: 1.29 mV). Also the integral of the IPSP was not different (inactive: median: 1.47 mV/s, IQR: 1.07 mV/s; outside: median: -1.34 mV/s ,IQR: 1.48 mV/s; inside: median: -2.03 mV/s, IQR: 0.97 mV/s).


A
2 mV
50 ms
Bi
Max EPSP
Max. EPSP Depol. [mV]
*
Inactive
Outside
Inside
ii
Max IPSP
Max. EPSP Depol. [mV]
Inactive
Outside
Inside
Ci
Integral EPSP
Integral of EPSP [mV]
*
***
Inactive
Outside
Inside
ii
Integral IPSP
Integral of EPSP [mV]
Inactive
Outside
Inside

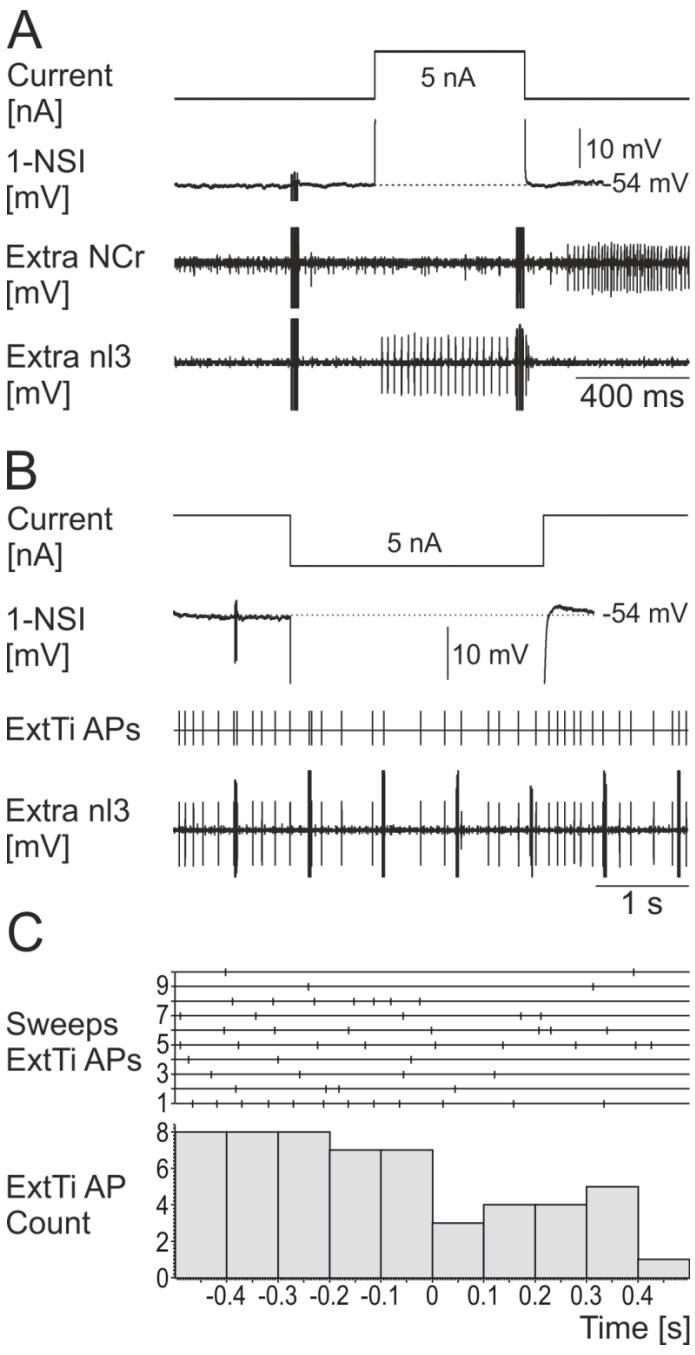



Fig. 4.13: Response of ExtTi MN to current injection in the excitatory and inhibitory NSI

(A) Depolarizing current injection into the NSI elicited APs of ExtTi MN in the extracellular nl3 recording; (B) Example of hyperpolarizing current injection into the NSI eliciting decrease in AP frequency of ExtTi MN in the extracellular nl3 recording; (C) Peristimulus time histogram for quantification of effect of ExtTi MN due to hyperpolarizing current injection into the NSI. (A and B) Current injection (top trace), NSI intracellularly recorded (2nd trace), extracellular recordings of nl3 (bottom trace) and NCr (3rd trace in B), memory channel with APs of ExtTi MN (3rd trace in A).

Summarizing, the excitatory NSI received EPSPs from fCO afferents while being stimulated electrically at the F1 nerve. The EPSP was decreased during curve stepping especially in the inside leg compared to the inactive leg. One of the analyzed cells also received an IPSP after the EPSP, which was not altered by curve stepping.

Fig. 4.14: Excitatory and inhibitory NSI recorded intracellularly with electrical stimulation of fCO afferents

(A) Overlays of inactive, outside and inside leg condition membrane potential parts from the intracellularly recorded NSI showing an EPSPs followed by IPSPs elicited by three electrical fCO stimulations . (B) Comparison of the maximal amplitude of the EPSP (i) and the IPSP (ii). (C) Comparison of the integral of the EPSP (i) and the IPSP (ii). Inactive (n = 23); outside (n = 5); inside (n = 9); inactive leg condition (black line or white box); outside leg condition (blue); inside leg condition (orange); significance values: $P < 0.05$ = *; $P < 0.001$ = ***.

## 4.5. Discussion

The task-dependency of the frequency of AR and RR was discussed in second Chapter. The task of the left middle leg was to function as the outside or inside leg of a curve. Therefore, stimulated by an optical stimulator, the stick insect had to step in curves on a slippery surface (Gruhn et al., 2006). The frequency of AR was higher for the inside-stepping leg compared to an outside-stepping leg. On the other hand, more RR and less modulation were found while the leg functioned as an outside leg (Chapter 3 and Hellekes et al., 2011). Several previous studies have investigated the generation of AR (cf. Bässler, 1988, Büschges and Bässler, 1998). However, little is known about the mechanisms generating RR and decreasing modulation during stepping when a leg functions as an outside leg. Hence, the goal of this Chapter is to gain more insight into the mechanisms responsible for the motor output while no AR is generated.

First, to get an overview of the changes in ExtTi MNs during the mechanical stimulation of the fCO while the leg was used as a inside- or outside-stepping leg, and compared to the inactive leg. Therefore, the MNs were recorded intracellularly and the animal was stimulated with an optical stimulator to induce curve walking. The integral of the membrane potentials measured in ExtTi MNs during stimulation of the fCO showed that the gain due to stimulation was lower for the outside leg compared to the inactive leg, where the animal showed a typical RR. While the leg functioned as inside-stepping leg, no significant change was found. This is probably due to the high variability of measured values. Here, while the animal was at rest, curve walking did not influence the membrane potential, which means all changes were elicited by fCO stimulation. However, as the mechanical stimulation lasted about 500 ms, the gain of outside- as well as inside-stepping legs could be reduced by multiple influences. The first depolarization, a monosynaptic connection from the afferent neurons to the ExtTi MN, however, could only be influenced by few ongoing effects, as no polysynaptic connection should be fast enough to reduce the ELD. Moreover, to have a more accurate view on the monosynaptic connections of the fCO afferents to the ExtTi MNs, I used an experimental approach where the F1 nerve, the nerve from the fCO to the NCr, was electrically stimulated with a two-hooked electrode, while an ExtTi MN was recorded intracellularly. The results show that, during curve walking, the size of the EPSPs in the ExtTi MNs elicited by the electrical stimulation of the F1 nerve were smaller for both the inside- and outside-stepping leg. Thus, the direct input from fCO afferents was decreased during curve stepping on a slippery surface. The mechanisms leading to the decrease in input from the fCO afferents could be a presynaptic inhibition of the afferents itself, which are known to have inhibitory effects during the RR, which leads to decreased AP size and, thereby, to decreased EPSPs in the postsynaptic MN (Büschges, 1995b, Sauer et al., 1997). So far, this presynaptic

inhibition has only been known as a polysynaptic effect, generated by afferents innervating an inhibitory neuron, leading to postsynaptic inhibition. However, it is possible that permanent presynaptic inhibition is present during walking as well, which needs to be investigated.

A second mechanism, investigated in the present study, decreasing the input of the fCO afferents onto MNs could result from changes in membrane resistance of the ExtTi MNs themselves. For five recordings from ExtTi MNs, I found a decrease in input resistance, which could account for at least part of the decrease in EPSP size. Next to the decrease in input resistance in the ExtTi MNs, an increase in tonic depolarization during front leg stepping was found (Ludwar et al., 2005b). Especially for the outside-stepping leg, a significant increase in ExtTi MN tonic depolarization was found compared to the inside-stepping leg (Hammel et al., in prep). However, this was not a mechanism leading to the decrease observed in present study, as the membrane potentials for the inactive, outside-, and inside-stepping legs were kept at the same potential with only minor difference. Nevertheless, interleg as well as central influences could change the excitability of the MN as shown by Westmark et al. (2009) for OA. Interleg and central influences, for example influences of neuromodulators (Stolz et al., in prep.) or front leg stepping could change the excitability not only in MNs, but also in the polysynaptic pathways via SI and NSI (Ludwar et al., 2005b). Also other leg sense organs have shown to influence the sensory feedback from the fCO, like the CS and hairplates on the same leg (Stein and Schmitz, 1999).

In addition to the EPSPs elicited by electrical stimulation of the F1 nerve, some stimulations were found where the EPSP was followed by a strong IPSP, which could be polysynaptic and be part of the AR. It was only elicited with strong electrical stimulations and more often in the inside stepping leg.

### 4.5.1. The femoro-tibial joint control network

One of the key sensory organs contributing to the FT joint control network is the fCO, measuring movement and position of the joint. These signals are processed in a neuronal network that then generates the RR, AR (Bässler, 1993), or other motor outputs, which are not one a RR or an AR, but rather between those motor outputs (Bässler, 1988). Three levels of fCO information processing could be found in this neuronal network, starting first with the fCO afferents in the ventral fCO, which respond to either the position, velocity, acceleration, or combinations of these parameters (Büschges, 1994, Hofmann and Koch, 1985, Hofmann et al., 1985) in addition to vibration-sensitive neurons in the dorsal fCO (Stein and Sauer, 1999). The fCO afferents measuring velocity and position are known to receive presynaptic inhibition initiated by neurons of the same type, caused by excitation of an inhibitory interneuron (Büschges, 1995b, Sauer et al., 1997). Second, feedback from the fCO to the

ExtTi MN is processed by direct monosynaptic connections as well as indirect polysynaptic connections (Büschges et al., 2000). The fCO feedback is processed by NSI and SI. Third, the ExtTi MNs are either excited or inhibited by some of the NSIs. In previous studies, NSIs have been identified that either excite or inhibit the ExtTi MNs and are additionally involved in the processing of fCO feedback (Akay et al., 2001, Büschges, 1990, Stein and Sauer, 1998). NSIs that excite or inhibit the ExtTi MNs are called excitatory (ENSIs) or inhibitory (INSIs), respectively (Büschges, 1990). Further, the next level of processing would be the activation of the different ExtTi MNs and movement itself. First, ExtTi force is produced by simultaneous activation of the common inhibitor, which inactivates the ExtTi muscle and the two excitatory MNs (SETi and FETi) (Bässler and Storrer, 1980). The last level would be movement of the tibia, produced by the ExtTi muscle and the antagonistic FlxTi muscle (Bässler and Stein, 1996). As this neuronal network is capable of not only producing the RR analyzed in the present study, but also the AR, Bässler (1993) described the distributed processing of this FT joint control network as parliamentary principle consisting of these five levels acting in parallel and also antagonistically (cf. Kristan, 2000, Morton and Chiel, 1994).

### 4.5.2. Investigation of a monosynaptic connection from fCO to ExtTi MN

In the following paragraph, I am going to discuss the different influences, which could decrease the gain of the monosynaptic and polysynaptic pathways from the fCO afferents onto the ExtTi MNs.

The gain during stimulation of the fCO on the ExtTi MNs was decreased during curve stepping, significant during outside stepping (Fig. 4.3). However, the influences of the second and third level, which was measured mainly here, are, as described in the last paragraph, the polysynaptic pathways, which might be very complex. Hence, it is obvious to first have a look at a less complex connection. In the study of Driesang and Büschges (1996) a connection was described, leading to a membrane depolarization in the ExtTi MN 3-12 ms after the onset of fCO stimulation. This connection is defined as a direct, monosynaptic connection from the fCO afferents onto the MN (Driesang and Büschges, 1996).

This depolarization was visible even if the leg generated an AR and more interestingly for the present study, the ELD was of the same amplitude (Driesang and Büschges, 1996). During curve stepping the ELD was decreased in its amplitude and integral. This led to the assumption that the direct connection from the fCO afferents onto the ExtTi MNs was altered by influences occurring during curve stepping. Therefore, to have a closer look on the direct connection from the fCO onto the ExtTi another preparation was used (Fig. 4.2). The F1 nerve was stimulated electrically to induce an compound AP of the fCO afferents. The ExtTi MNs and also NSIs were recorded intracellularly to compare changes in the postsynaptic

potential, elicited by the compound AP of the fCO afferents. During curve stepping, the EPSP, visible in ExtTi MNs, was reduced both in the amplitude as well as in the integral.

### 4.5.3. Presynaptic inhibition

The first level in the FT control network is the presynaptic inhibition of fCO afferents. The afferents of the ventral part of the fCO measure three parameters of tibial movement, the acceleration, the position, and the velocity, either alone or in a combination (Büschges, 1994, Hofmann and Koch, 1985, Hofmann et al., 1985). The excitatory afferents, measuring one set of fCO movement, are modulated by inhibitory interneurons, which are activated by a set of afferents measuring the same movement parameters (Sauer et al., 1997). Thereby, the action potential size in the fCO afferents is decreased (Sauer et al., 1997), and with it the amount of synaptic transmission, which could be measured in the size of the postsynaptic potential. This effect was shown by blocking the presynaptic inhibition with the application of picrotoxin (PTX), a noncompetitive γ-Aminobutyric acid (GABA) agonist, which led to an increase in response to fCO stimuli in most of the tested NSI (Sauer et al., 1997). Additionally to the increase in response in the NSIs, also the ExtTi MNs responded stronger to the fCO stimulation compared to the stimulations before the application of PTX (Sauer et al., 1997). However, this was tested with inactive animals showing RR. Büschges (1995b) demonstrated the presynaptic inhibition to be stronger during the RR and thereby decreasing the fCO afferent feedback to be transmitted to its full extent. On the other side, during the AR the presynaptic inhibition was strongly decreased, which would then increase the amplitude if the transmitted feedback from the fCO afferents. The source of the change in presynaptic inhibition between RR and AR could not be shown in these studies. However, the presynaptic inhibition might be a mechanism used to interact with the self generated feedback from the fCO, as an efference copy. This mechanism is used in the auditory pathway of crickets to inhibit the processing of auditory information while sound production (cf. Poulet and Hedwig, 2002, 2006). As the goal of the present study was to find mechanisms responsible for less modulation during the outside leg stepping, the presynaptic inhibition of the fCO afferents, perhaps as an corollary discharge, could play a significant role in decreasing of the fCO input on the ExtTi MNs, as well as on NSIs.

### 4.5.4. Influence of other leg sense organs

In addition to the fCO afferents of the same type (Sauer et al., 1997), the feedback from the fCO is also altered by other sense organs of the same leg, like CS or HP in form of presynaptic inhibition (Stein and Schmitz, 1999). Moreover, the CS do influence the ExtTi MNs directly during stance phase when the leg is loaded (Akay et al., 2001, Schmitz et al.,

2015, Chapter 2). If the fCO and the feCS are stimulated simultaneously the likelihood of occurrence of an AR is increased (Akay and Büschges, 2006). During stimulation of the feCS the FETi and SETi MN does receive hyperpolarizing synaptic input (Akay et al., 2001, Akay and Büschges, 2006). Furthermore, during front leg stepping middle leg *Levator* (LevTr) and *depressor trochanteris* (DepTr) MNs have been entrained by stimulation of CS on the middle leg (Borgmann et al., 2011)

However, the middle leg investigated in the present study was fixed to the animal holder, and therefore, only passive cuticle strains by muscle forces might activate CS phasically (Zill et al., 2017), while other leg sense organs then the fCO might have a tonic activity. Nevertheless, this probably would not have an effect on changes between the tasks, the animal had to fulfill in the present study.

### 4.5.5. Interleg and central influences

In the experiment, the front and all contra lateral middle legs were stepping on a slippery surface. Intersegmental influences from the front and the contralateral legs could play a role in changing the tonic depolarization as well as the entrainment of the explored middle leg. Ludwar (2005a) demonstrated that stepping in the front legs is entraining the MN in the mesothoracic ganglion. This entrainment is not caused by the CPGs of the front leg, as a rhythm induced by pilocarpine, a muscarinic acetylcholine (ACh) agonist, in the prothoracic ganglion has no influence on the activity in the mesothoracic ganglion. However, stimulation of the fCO in the front leg could entrain the ipsilateral FlxTi and ExtTi in the mesothoracic ganglion (Ludwar et al., 2005a). Moreover, mesothoracic MN, as well as interneurons (E4 and I2) are tonically depolarized during front leg stepping, and the membrane potential changes phasically in correlation with front leg step phase (Ludwar et al., 2005b). During the tonic depolarization also the input resistance of nine investigated MNs has decreased (Ludwar et al., 2005b).

The underlying depolarization, found by Ludwar et al. (2005b) could be induced by the neuromodulator OA, which was demonstrated to elicit the switch to the active state in stick insects by injection in the hemolymph (Büschges et al., 1993), and also can induce flight and running in locust by injection on the neurophil (Sombati and Hoyle, 1984). Here, DUM neurons localized in the same ganglion were made out to be responsible for OA release in the thoracic ganglion. Westmark et al. (2009) demonstrated ACh to induce the tonic depolarization and OA to increase it, probably via interneurons. Next to this, OA is also shown to decrease the depolarizing effect of ACh in mesothoracic MN (Westmark et al., 2009). However, ACh is also thought to be responsible for a presynaptic inhibition of sensory terminals in *manduca sexta* (Trimmer and Weeks, 1989) and the locust (Judge and Leitch, 1999).

In a present study by Stolz et al (in prep.) DUM neurons in the suboesophageal ganglion were also hypothesized to release OA in the thoracic ganglia. By stimulation of these OAergic DUM neurons, a state change in response to fCO stimulation from a strong RR to a weaker RR or even AR could be induced (Stolz et al. in prep.). Although, OA could be one of the sources for the change in modulation of fCO feedback during turning, the DUM neurons are not influencing one hemi segment alone, as their axons bifurcate in symmetrically onto both body sides (Bräunig and Burrows, 2004, Goldammer et al., 2012). The release of OA from DUM neurons could only contribute to the task-dependent changes described here, if one side would be presynaptically inhibited.

During walking, the ExtTi MNs also receive a tonic depolarization (Ludwar et al., 2005b). While the leg functions as an outside-stepping leg, this depolarization is larger compared to the inside-stepping leg. The increase in the tonic depolarization, found for the leg function as outside-stepping leg, could be due to an increase in OA. Furthermore, Stolz et al. (in prep.) could show a decrease in gain due to stimulation of the fCO while OAergic DUM neurons were activated.

However, this was not the reason for the decrease in gain while the fCO was mechanically stimulated, as well as in EPSP size, measured as ELD and with electrical stimulation, because the analyzed membrane potentials were in the same range at the onset of stimulation. The overall tonic depolarization could still be one reason for the decrease in the gain of the fCO stimulation.

### 4.5.6. Membrane input resistance

In addition, postsynaptic mechanisms could play a role in the task dependent change, which would then lead to changes in the membrane input resistance of the ExtTi MNs. The membrane input resistance is known to change during walking in stick insects ExtTi MN (Büschges et al., 2004, Gabriel, 2005). To measure for potential changes in input resistance small current pulses were injected into the ExtTi MN either while the animal was inactive or curve stepping. This experiment showed that indeed the membrane input resistance of ExtTi cells was decreased during outside-stepping as well as inside-stepping. Thus, the decreased membrane input resistance, which is elicited by opening of channels in the membrane, might be one mechanism used during curve stepping, to reduce the depolarization elicited by the fCO afferents into the ExtTi MNs. However, the influences, changing the membrane input resistance in the ExtTi MN, and with it an opening of ion channels in the membrane, are still unknown. One potential source, which does decrease the membrane input resistance is the tonic depolarization, which was found during curve walking. Even though, the membrane potential was kept same potential, while the animal was inactive or walking in a curve, the membrane input resistance remains altered, as the channels remain open.

### 4.5.7. Influence of nonspiking and spiking interneurons

One level of parallel processing of input from the fCO afferents happens in NSI. During the generation of the AR the relative weighting of the excitatory and inhibitory NSI is changed (Bässler and Büschges, 1990, Driesang and Büschges, 1996). The NSI have specific membrane changes during RR or AR. Some of the NSI are found to influence one or both of the reactions. The NSIs E1 and E4 support only the RR, E7 and I2 oppose the RR, I2 supports the AR, E1 and 4 oppose RR. The NSI E2, 3 ,5, 6 and I1 support the motor output during both reactions (Driesang and Büschges, 1996).

### 4.5.8. Task dependent changes in NSI

Hellekes (2012) could show that some NSI also change the weighting between inside- and outside-stepping leg. During outside-stepping the NSI E2/3 showed reduction of a strong inhibition evoked by fCO stimulation, which was strongly visible during inside-stepping. For another NSI, E5/6, the membrane potential changed from a slight hyperpolarization for the inside-stepping leg to a slight depolarization while the leg stepped to the outside (Hellekes, 2012). The NIS E8, did show a hyperpolarization for the outside-stepping leg with fCO stimulation, which was not found for the inside-stepping leg, however, for this neuron only a qualitative analysis was possible (Hellekes, 2012). Hence, for other NSI (E4, E9/10, I2 and I) no differences were found between the outside- and inside-stepping leg (Hellekes, 2012). The NSI E4 shows three different reactions during generation of the AR, where in two out of three possibilities also APs of the SETi were shown during the inactive phase (Driesang and Büschges, 1996). As described above, some NSI in the mesothoracic ganglion change their activity due to curve stepping in the middle leg (Hellekes, 2012). However, the mechanisms behind these changes remained unclear. In the present study, two interesting NSIs were recorded, while the fCO nerve was stimulated electrically. The PSPs of these NSIs did change due to curve walking. The first NSI, increasing the firing frequency of ExtTi MNs, did receive an EPSP due to the stimulation. During inside-stepping, the monosynaptic input decreased, strongly, and only slightly during outside stepping. As this NSI is increasing the activity of ExtTi MNs, this neuron might play a role, on the decrease in firing for the inside stepping leg compared to the outside, where a tonic activity was often visible (Hellekes et al., 2011).

In another NSI, an EPSP, shortly after the electrical stimulation was followed by a delayed IPSP. The NSI, by injecting a depolarizing current, was increasing the firing of the SETi while it was decreasing the firing frequency of the SETi slightly by injecting hyperpolarizing current. The EPSP was reduced in size for both inside- and outside-stepping while the ISPS did not change due to curve stepping. Interestingly, the EPSP, which was

evoked by three stimulations of the fCO nerve, did end 5 ms earlier when the animal did step into a curve compared to the inactive animal (Fig. 4.14). This neuron also could play a role in decreasing the gain of the fCO stimulation, as the excitatory connection of this neuron is decreased during curve stepping.

Unfortunately, the morphology of these NSI was not investigated and also no mechanical stimulation of the fCO was made. Therefore, it was not possible to classify the cells into the system introduced by Büschges (1990).

## 4.6. Conclusions

To summarize, in the present study, I investigated changes in the membrane potential gain due to fCO feedback during curve walking and the mechanisms altering this gain. I found the overall gain during mechanical fCO stimulation, as well as the direct connection from the fCO afferents into ExtTi MN to be reduced during curve stepping, especially, while the leg functions as an outside-stepping leg. The tonic depolarization, occurring during walking in the mesothoracic ExtTi MN, was increased in the outside-stepping leg compared to the inside-stepping leg. The membrane input resistance was reduced during curve stepping. A mechanism decreasing the gain could be an increased OA release during outside-stepping, increasing the tonic depolarization, also a presynaptic inhibition could contribute to a decrease in the synaptic transmission from fCO afferents to the ExtTi MNs.

# 5. General Discussion

My dissertation introduces various approaches to investigate the role of sensory input on the control of motor activity of a stepping insect leg in different behavioral contexts. This resulted in three main questions. First, how does the influences of feedback from leg sense organs control the magnitude and timing of stance and swing phase muscles? Second, how is this sensory feedback from leg sense organs processed task-dependently and -independently to contribute to changes in movement behavior? Third, which are the potential mechanisms that could elicit such changes in sensorimotor processing?

The discussion follows this main questions and places the results of my studies in the current scientific discourse.

## 5.1. Sensory influence on magnitude and timing of muscle activation

The topic is addressed in the first and second chapter of the thesis. In the first chapter, the influence of TD and ground contact on the magnitude and timing of muscle activity and the sensory modalities involved in the control were investigated. In the second chapter, the influence of movement and position of the FT joint on the timing of muscle activity was analyzed. Sensory influence on the magnitude of stance phase muscle activity

Feedback about load during stepping affects the magnitude of all stance phase muscles activities. These results parallel the results for the stance phase muscles of the cat, where experiments demonstrated that magnitude of the stance muscles *gastrocnemius lateralis* and *vastus lateralis* are heavily influenced by ground contact (Gorassini et al., 1994). In the stick insect, load feedback, particularly from the trCS (Akay et al., 2007), is known to increase the activity of RetCx, and, during backward walking, also the ProCx activity as well as that of the DepTr during the stance phase (Bässler, 1967, Bässler, 1972b, Schmitz, 1986a). Even though, the maximal value of RetCx activity did not change when the leg was missing ground contact, the overall activity decreased. This confirms the results of Akay et al. (2007).

The stance phase muscle of the FT joint, the FlxTi is controlled in its activity by load and movement feedback. Various load sensors on the leg of the stick insect are well known to influence the magnitude of FlxTi activation (Akay et al., 2001, Akay et al., 2004, Zill et al., 2014). My work confirms, the importance of load sensing CS on the leg of the stick insect for FlxTi activity magnitude. Interestingly, the activity of the FlxTi at the beginning of stance phase is also enhanced by the fCO signaling of FT joint position (review in Büschges, 2005).

DepTr activity is reinforced by increasing load through changing the body height above ground during regular steps on solid ground (Rosenbaum et al., 2010). The RetUng activity is influenced by force feedback evoked by tarsal flexion with a latency of 35 ms (Zill et al., 2010, Zill et al., 2014). The trapdoor approach demonstrates that the magnitude of all stance phase muscles but the DepTr is reduced when the load information is missing, which matches the findings from the cat (Gorassini et al., 1994). The only notable difference is the increasing effect on DepTr muscle activity. This increase as shown in Fig. 2.5 and Fig. 2.8 appears contradictory to the result in the other stance phase muscles. However, it can be explained by the influence of another sensory modality. The Input from the trHP, as missing feedback from these HP suppresses depression of the leg and therefore could account for the observed increase in DepTr activity (Schmitz, 1986a).

### 5.1.1. Sensory influence on the timing of muscle activity

The timing of motor activity is controlled by different leg sense organs. What are the potential sensory organs involved in this timing? Most of the stance phase muscles are activated around the TD or shortly before (Rosenbaum et al., 2010). Rosenbaum and colleagues (2010) demonstrated that the RetCx and ProCx, which can both be used as stance phase muscle depending on the walking direction, are activated before TD, which leads to the conclusion that TD signaling should not influence their activation. In the resting animal, activation of the vcxHP elicits RetCx and ProCx muscle activation (Bässler, 1977b, Büschges and Schmitz, 1991, Cruse et al., 1984). Coxal hair rows are also known to influence the activation of the ProCx muscle activity, probably also during backward walking (Cruse et al., 1984). And finally, both, RetCx and ProCx are known to be activated by the different groups of trCS (Akay et al., 2004, Akay et al., 2007). However, because of the described activation of both muscles before TD, this latter influence is not likely to be causal for the activation. Rosenbaum and colleagues (2010) also reported a time shift in the activation of the RetCx and ProCx during experiments with a two-legged preparation, which I found as well. This points towards intersegmental timing influences that seem to account for the discrepancy between muscle activity onset and time of leg TD. It has been described before that front leg stepping has an influence on the activation of the RetCx (Borgmann et al., 2007, Borgmann et al., 2009). One sense organ responsible for this feedback could be the front leg fCO (Ludwar et al., 2005a).

The two following muscles are activated long before TD. The DepTr is activated by extension of the tibia, which is measured by the fCO (Bässler, 1967, Bucher et al., 2003, Graham and Bässler, 1981, Hess and Büschges, 1999) around 93 ms before the TD (Rosenbaum et al., 2010). If the leg is at a certain position, a stimulation of trochanteral hair plates (trHPs) activates DepTr MN as well (Schmitz, 1986a).

The RetUng, the tarsal retractor, is activated around the time of lift off, around 130 ms before TD (Fischer et al., 2001 and Chapter 2). In the Canterbury tree weta (*Hemideina femorata*), an RR in the RetUng could be evoked by stimulation of the fCO through extending the tibia (Field and Rind, 1981). However, as the RetUng is activated early in swing phase, local load detection during TD could hardly play a role in this. Interestingly, cutting the RetUng apodeme did cause a great increase in variability of FlxTi activation latency, with a maximum up to 200 ms. This result is an indicator for the existence of an as yet not described sense organ, which may be connected to the RetUng apodeme. The RetUng is, on the one side connected to the tarsus and on the other to the proximal femur, where one of its three muscle parts is located (Bässler, 1983). Such an apodeme receptor, connected to the RetUng apodeme could have a very short latency to the ganglion in the ventral nerve cord from its origin in the leg of the stick insect, probably in the proximal femur. In my study all the muscles above are activated before the TD, which excludes load feedback from TD as sensory stimulus for their activation (Rosenbaum et al., 2010 and Chapter 2).

The FlxTi is the only stance phase muscle activated after TD (compare Fig. 2.8). Earlier work had already demonstrated that the FlxTi is activated 8 ms after TD, independent of the height of the animal above ground, the leg is touching, or the direction the animal turns to during walking (Gruhn et al.2006; Rosenbaum et al. 2010; Berendes et al. 2013). Trapdoor experiments revealed that, when no ground contact occurs, the FlxTi is still activated in most of the cases, but with a delay of up to 100 ms (Berendes et al., 2013). This leads to the conclusion that no position signal is controlling the activation of the FlxTi and the source of the missing sensory information remains unknown. Here, I could prove that without the feCS the latency to FlxTi activation is prolonged. However, with other load sensors still intact the FlxTi was still activated earlier than during SIH. Without the other main load sensing organs, the trCS, the feCS, and the tiCS together with an amputation of the tarsus, the activation to FlxTi occurs even later. This leads to the conclusion that not only the feCS, but also the other leg sense organs measuring load, contribute to the timing of FlxTi activation.

The FlxTi is still activated in more than 80% of all steps into the hole, albeit with a longer latency. In this context, the results by Berg et al. (2015) could offer an indication for a potential mechanism. A NSI I4, changes its activity and gating of the motor activity of the FT joint MNs, when the leg is lacking ground contact, and initiates searching movements. On the other hand I4 does not activate the FlxTi while the leg has ground contact. It is possible that, if the leg is missing the TD signal at one point in time, I4 is gating the activation of the FlxTi. This would mean that the FlxTi is not only activated by ground contact. If information about the ground contact is missing, central drive may elicit FlxTi muscle activity to initiate searching. In addition, FlxTi activation could also be weakly controlled by central mechanisms. Using the muscarinic agonist pilocarpine, rhythmical activity in FT MN pools

can be elicited in a deafferented animal (Büschges, 1995a, c). As the FlxTi activation fails in 19% of all steps into the hole, however, the central influences appear to be rather weak.

One other possibility of initiation a FlxTi movement is that a passive flexion of the FT joint is initiated at the end of a leg extension (Hooper et al., 2009), which in return could activate the fCO feedback. The latter is known to enhance the FlxTi during stance phase (Bässler, 1973, Bässler, 1977b). Another mechanism, which could be responsible for FlxTi activation during SIH, is the production of cuticular strain through muscle contractions (Zill et al., 2012, Zill et al., 2014), which could also initiate FlxTi activation. As DepTr and RetUng are active long before the TD, these muscles could produce a cuticular strain and thereby activate the FlxTi. However, so far, no such activation in unresisted movements has been recorded.

Not only the timing of stance muscle but also swing muscle activity is controlled by sensory feedback. For instance, the transition from the RetCx to the ProCx during forward walking is evoked by stimulation of a hair plate that measures the position of the coxa with respect to the thorax (Bässler, 1977b). The activation of the LevTr can be induced by unloading signals from the same leg, reported by CS (Akay et al., 2001, Akay et al., 2004, Akay et al., 2007) or by position signals from the coxa (Cruse, 1985). Finally, the transition from FlxTi to the ExtTi is mediated the fCO as well as unloading signals from feCS and trCS (Akay et al., 2001 and Chapter 2, Bässler, 1986).

### 5.1.2. Task-dependent processing of the magnitude and timing of muscle activity

The processing of load feedback of the ThC joint is confirmed to be processed in a task-specific manner during curve walking. While load feedback leads to RetCx activation in the outside leg, it can activate either RetCx or ProCx or even fail to activate one of these muscles (Gruhn et al., 2016). The feedback from the fCO, is also not processed in the same way during all movements, as it was described by Bässler working on RR and AR (Bässler, 1977a, 1986, 1988). Its processing differs not only between the standing and the walking animal, but also between different behavioral contexts. Again for curve walking animals, Hellekes and colleagues (2011) reported that the occurrence of the AR depends on the function of the leg.

During locomotion, animals adjust their movements to navigate in their environment. Therefore, they move task-dependently for example during climbing, backward movements, or turning. If an animal climbs, the front legs can be used as feelers (Cruse, 1976) to find ground on the other side of a gap (Bläsing and Cruse, 2004a, b, Pick and Strauss, 2005), and also as normal walking legs, supporting the animal and pushing it towards the movement direction (Cruse, 1976). During turning the legs of a six legged animal have to fulfill different

tasks. The legs inside of a turn pull the animal into the turn, while the legs outside of the turn push the animal forward and also into the turn. The trajectories of legs inside and outside of a turn differ from each other as a result of kinematics changes of the leg (Dürr and Ebeling, 2005, Strauss, 1990). While the leg inside of a turn is flexed during stance phase, the ThC joints only show minor movements. On the other hand, the leg outside of the turn is retracted during stance phase with long movements along the body axis, while the FT joint is stiff (Dürr and Ebeling, 2005, Gruhn et al., 2009 a).

In this context, I investigated the effects of sensory feedback from the fCO on swing phase muscles during inside- and outside-stepping. The motor activity of the ProCx was unaffected while the leg functions as inside-stepping leg, but was strongly activated by fCO stimulation in the outside leg. The LevTr, on the other hand is activated by fCO stimulation in both, inside and outside leg. LevTr is thus not influenced in a task-dependent way, at least not in this behavioral context, which fits the results of Hammel and colleagues (in prep.), who describe the deafferented motor output of this joint to be task-independent. For the FT joint, I investigated the different movement and position parameters measured by the fCO and their influence on the motor output during inside- or outside-stepping. For the FTi joint, I could confirm the results by Hellekes and colleagues (2011) with an increased occurrence of AR during inside-stepping, also for all parameters measured, and increased tonic ExtTi activity during outside-stepping. Interestingly, the tonic activity was reduced in experiments with a large starting angle. This result could be explained by the feedback of position sensitive cells in the fCO. These could be generating less excitatory feedback at an extended FT joint angle, and thus excite the ExtTi MN less than at a more flexed starting angle (compare Büschges, 1994, Matheson, 1990).

What are the mechanisms behind the observed differences in the occurrence of AR and RR between the two body sides? At the beginning of the AR the FlxTi is excited and the ExtTi inhibited. Now, one possibility could be that the angle at which the transition occurs is different in the outside- and inside-stepping leg.

Bässler found that the transition angle in the front leg of *C. impigra* remained the same independent of the starting angle (Bässler, 1986), which based on observed kinematics, is likely to be different between the inside- and outside-stepping leg (Gruhn et al., 2009 a). This FT joint angle at the transition from FlxTi to ExtTi activity was reported to be between 85° and 75°. In contrast to Bässler (1986), I found the transition angle to change depending on the starting angle, but the resulting angular difference to be the same for all starting angles. This suggests that the transition from the FlxTi to the ExtTi occurs at a specific angular difference after the onset of the stimulation and is completely independent of the role of the leg functioning as inside- or outside-stepping leg.

The difference to the results of Bässler (1986) could be explained by species specificity and the function of the different legs. The front legs can either be used as feelers or as legs, providing support and propulsion, while the middle legs are only used for support and propulsion (Cruse, 1976). In addition, the described species-specific differences in FlxTi activation in the trap door experiments (Chapter 2) suggest that also species-specific differences may exist in the influence of processing of fCO feedback between the two phasmid species.

## 5.2. Mechanisms for changing the task-dependent sensorimotor processing

The question about mechanisms behind the task-dependent processing remains so far unanswered. To approach these mechanisms intracellular recordings of ExtTi MN were performed while the leg functions as either an inside- or outside-stepping leg. The processing of fCO feedback onto ExtTi MN is altered such that the amplitude of membrane potential gain was decreased during outside-stepping. The amplitude of direct connections from the fCO afferents onto the ExtTi MN was also reduced during curve stepping.

Gruhn and colleagues (2016) postulated two possible mechanisms for task-dependent changes in processing of CS feedback during curve walking. First, a presynaptic inhibition of CS afferents, and secondly, a different weighting of the parallel pathways of processing (Gruhn et al., 2016). Furthermore, as a third mechanism, the MN excitability, could be altered by neuromodulators. These mechanisms could not only be influencing the task-dependent processing of the CS during curve walking but also the fCO feedback during the same task. In my work, I focused on task-dependent postsynaptic changes probably induced by the third mechanism.

A tonic depolarization, which is occurring during stepping of a leg (Büschges et al., 1994, Büschges, 1998, Ludwar et al., 2005a, Ludwar et al., 2005b), could evoke a reduction of membrane input resistance as a result of channel opening, shown in the present work during curve walking. In ExtTi MN this tonic depolarization was increased and in FlxTi MN decreased during outside-stepping (Hammel et al. in prep. and Chapter 4). A similar tonic depolarization in the hawk moth *Manduca sexta* (Trimmer and Weeks, 1993), and stick insects (Westmark et al., 2009), is dependent on the presence of the neurotransmitter ACh. A neuromodulator, which is found to increase the tonic depolarization and regulating the ACh input onto MN (Westmark et al., 2009), is the biogenic amine OA. In a present study of Stolz and colleagues (in prep.) a modulatory effect of OAergic DUM neurons onto the processing of fCO feedback was found.

OA is also increasing the activity of the position sensitive portion of the fCO feedback (Ramirez et al., 1993), which could in return contribute to a more depolarized MN membrane

potential, while the leg functions as an outside leg. Additionally, OA is found to mimic a state dependent change in fCO feedback processing (Büschges et al., 1993, Stolz et al., in prep). Although, OA could be one of the sources for the change in modulation of fCO feedback during turning, DUM neurons are not influencing one hemi segment alone, as their axons bifurcate symmetrically onto both body sides (Bräunig and Burrows, 2004, Goldammer et al., 2012). A release of OA from DUM neurons could only be assumed to play a differential role between the two body sides, if one of the two projections were presynaptically inhibited. This however remains to be demonstrated.

The mechanism of presynaptic inhibition could also decrease the afferent input onto the ExtTi MN, but has not been investigated so far during curve walking. A presynaptic inhibition was found to decrease the afferent synaptic input onto the ExtTi MN (Sauer et al., 1997). The synaptic terminals of the fCO afferents are inhibited by fCO afferents of the same type (Sauer et al., 1997), and by sensory influences of the trHP and CS (Stein and Schmitz, 1999).

The last mechanism suggested by Gruhn and colleagues (2016) was a task-dependent weighting of parallel pathways via interneurons. Several NSI showed task-dependent changes in processing of fCO feedback (Hellekes, 2012 and Chapter 4). However, their direct influence onto the motor activity during curve walking remains to be shown.

## 5.3. Conclusion

In my thesis I described the contributions of sensory influences onto the timing and magnitude control of the motor activity and how these sensory influences are processed in a task-dependent manner. With a trapdoor setup approach I proved the influence of load feedback from ground contact on the timing of the FlxTi muscle and the magnitude of all stance phase muscles. I could also confirm the timing of the ExtTi muscle to be dependent on fCO information. The motor activity evoked by sensory feedback from this sense organ is dependent on the task of the leg. Intracellular recordings of ExtTi MN revealed a reduction of synaptic transmission from fCO afferents, especially, in outside-stepping legs. In my first chapter, I proved the magnitude of FlxTi activity to be strongly increased by sensory feedback of CS. In the second and third chapter of my work, I could confirm that the magnitude of FT MN activity and feedback of the fCO to the MN is strongly dependent on the task of the leg. This task-dependent processing of sensory feedback onto the FT MN could be present for the CS as well, as shown by Gruhn et al (2016) for the ThC MN.

Different weighting of parallel pathways could influence the motor activity task-dependently, not only in curve walking but also between walking and searching (Berg et al., 2015). This mechanism could be also responsible for the FlxTi activation when the leg steps into the trapdoor and lacks ground contact.

The importance of presynaptic inhibition found for the fCO afferents (Sauer et al., 1997) could also be relevant for the processing of load feedback from CS afferents. Kasemir (2018) recently showed a CS influence in MN of the contralateral leg after the application of picrotoxin, a noncompetitive GABA agonist. Akay and colleagues (2007) could find task-dependent processing of CS as well. During forward and backward walking, the influence of CS from the front leg was either activating the RetCx or the ProCx. Both studies imply that the pathways, responsible for processing of feCS information are existing, however, they could be presynaptically inhibited.

In my dissertation I could prove that motor activity is influenced by various sense organs. This influence can be processed in a way to evoke task-specific changes in motor activity. Whereby so far, I could confirm one mechanism leading to changes, even if indications for other mechanisms contributing exist. The influences of different weighting of parallel pathways via NSIs, presynaptic inhibition, or influences of neuromodulators on changes in task-dependent motor output remain unclear.

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# Abbreviation

| | | |
|---|---|---|
| AR | - | Reflex reversal |
| ACh | - | Acetylcholine |
| CS | - | Campaniform sensilla |
| CPG | - | Central pattern generator |
| CTr | - | Coxa-trochanter |
| DepTr | - | Depressor trochanteris |
| EMG | - | electromyogram |
| EPSP | - | Excitatory postsynaptic potential |
| ExtTi | - | Extensor tibiae |
| fCO | - | Femoral chordotonal organ |
| feCS | - | Femoral campaniform sensilla |
| FETi | - | Fast extensor tibiae motor neuron |
| FlxTi | - | Flexor tibiae |
| FT | - | Femoro-tibial |
| GABA | - | γ-Aminobutyric acid |
| HP | - | Hair plate |
| IPSP | - | Inhibitory postsynaptic potential |
| LevTr | - | Levator trochanteris |
| MN | - | Motor neuron |
| NCr | - | Nervus cruris |
| NSI | - | Nonspiking interneuron |
| OA | - | Octopamine |
| ProCx | - | Protractor coxae |
| RaHS | - | Ramp-and-hold-stimulus |
| RetCx | - | Retractor coxae |
| RetUng - | - | Retractor unguis |
| RR | - | Resistance Reflex |
| RS | - | Ramp stimulus |
| SD | - | Standard deviation |
| SETi | - | Slow extensor tibiae motor neuron |
| SG | - | Step on the ground |
| SI | - | Spiking interneuron |
| SIH | - | Step in the hole |
| Sm | - | Smoothed |
| Rect | - | rectified |
| tiCS | - | tibial campaniform sensilla |
| trCS | - | trochanteral campaniform sensilla |
| trHP | - | trochanteral hair plate |
| taCS | - | Tarsal campaniform sensilla |
| TD | - | Touchdown |
| ThC | - | Thoracocoxal |
| tiCS | - | Tibial campaniform sensilla |
| trCS | - | Trochanteral campaniform sensilla |
| trHP | - | Trochanteral hair plate |
| vcxHP | - | Ventral coxal hair plate |
| vTD | - | Virtual touchdown |

# Appendix

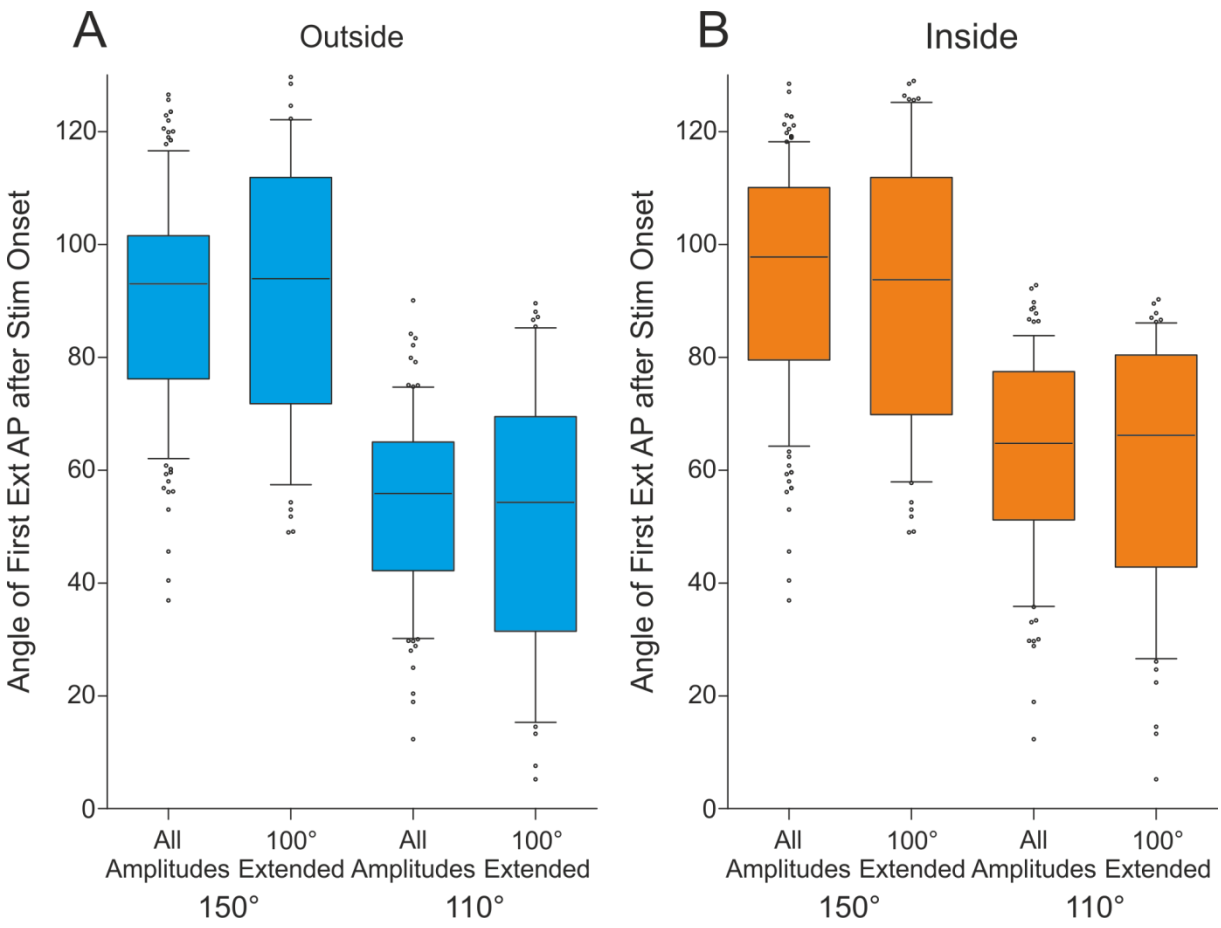


Fig. S. 1: Transition angle and angular difference for 100° and all amplitudes

Comparison of all amplitudes (40°, 60°, 80° and 100°) and an extended 100° (with earlier start of *extensor tibiae* activity) amplitude for the (A) angle of the first *extensor tibiae* action potential (AP) after stimulation onset for reflex reversals, (B) Angular Difference between the stimulus onset and the first *extensor tibiae* action potential (AP) for reflex reversals; outside: 150° (all amplitudes: N = 8, n = 47; 100° extended: N = 4, n = 36); 110° (all amplitudes: N = 6, n = 41, 100° extended: N = 4, n = 33); inside: 150° (all amplitudes: N = 7, n = 119; 100° extended: N = 6, n = 63); 110° (all amplitudes: N = 7, n = 93, 100° extended: N = 6, n = 61); inside leg condition (orange); outside leg condition (blue).

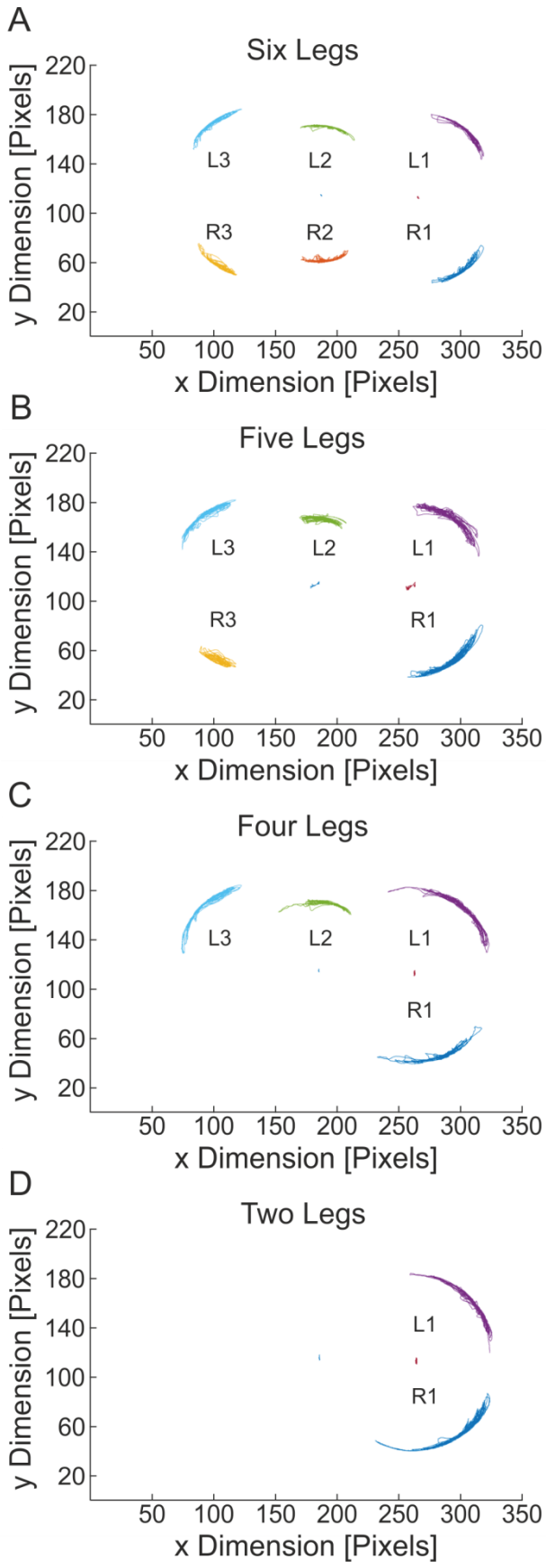


Fig. S. 2: Carausius morosus stepping trajectories on a ball tracked at the femorotibial joint

Trajectories of the position of the FT joint in x and y dimension in a walking sequence of 30 seconds. The animal was fixed at an animal holder over a rohacell- ball, floating on pressurized air. The animal was walking with six (A), five (B), four (C) and two (D) legs. The right middle leg was removed first (B), then the right hind leg (C) and the contralateral middle and hind leg (D). Trajectories are shown for the left front leg (L1) in purple, left middle leg (L2) in green, left hind leg (L3) in light blue, right front leg (R1) in dark blue, right middle leg (R2) in red and right hind leg (R3) in orange.

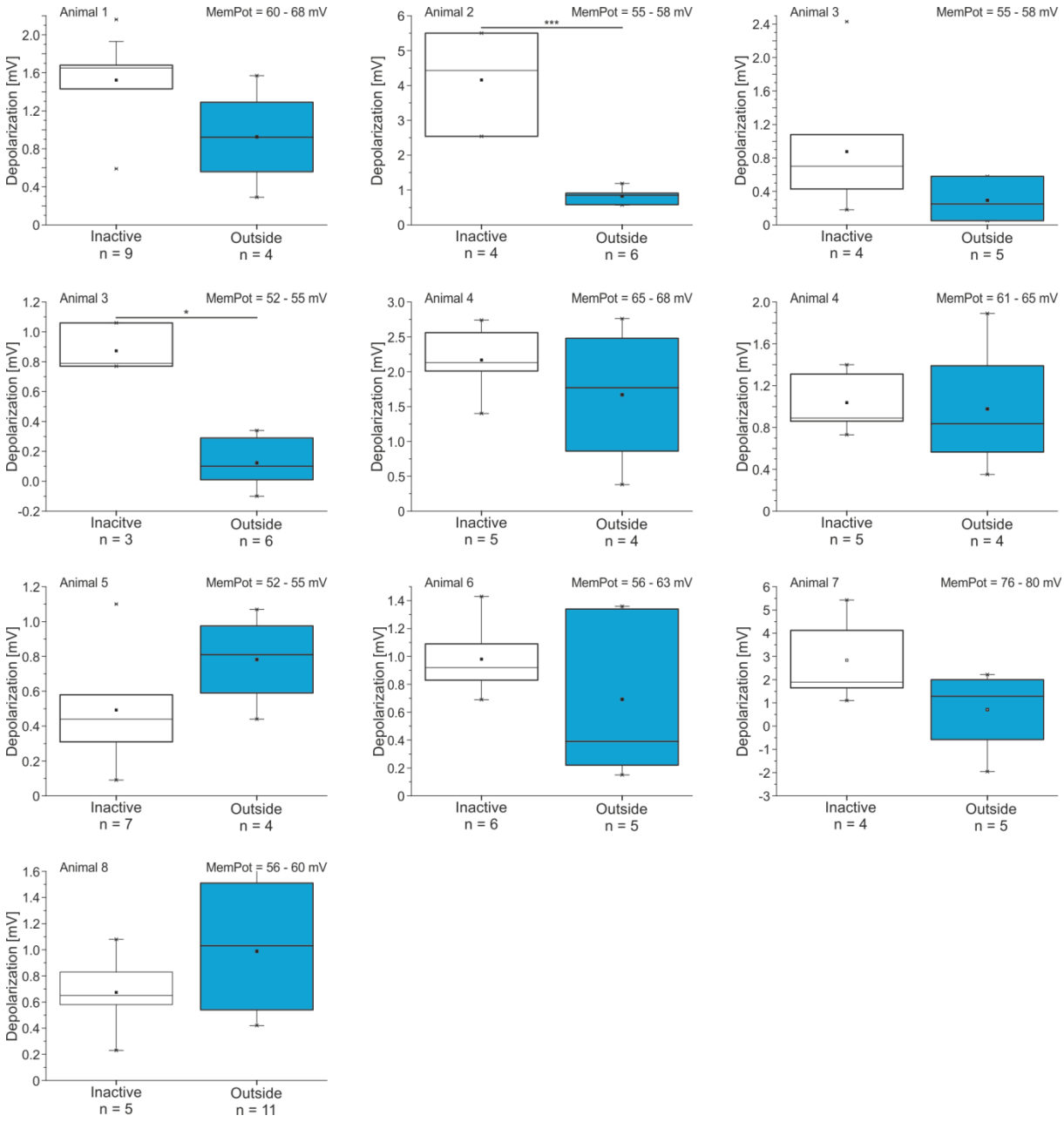


Fig. S. 3: Early latency depolarization changes in *extensor tibiae* MN in eight animals.

Amplitude of the early latency depolarization in eight animals for inactive and outside leg condition. Inactive leg condition (white); outside leg condition (blue); significance values: $P < 0.05$ = *; $P < 0.001$ = ***.

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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzen Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen – , die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von oben angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss der Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Ansgar Büschges betreut worden.

Köln, den 03.07.2018