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A

μ CT analysis of titillator insertion in the female's genitalia

Contact areas of the male titillators on the female's subgenital plate

Arthropod Structure & Development

Copulatory courtship by bushcricket genital titillators revealed by functional morphology, μ CT scanning for 3D reconstruction and female sense structures

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Running title: Copulatory courtship by bushcricket genital titillators

Abstract

Genitalia are rapidly evolving morphological structures most likely under sexual selection. Due to their internal nature they are often hidden inside the body, thus morpho-functional studies of animal genitalia are broadly lacking. Males of some bushcricket taxa bear paired genital appendices called titillators, the exact function of which is unknown since they are obscured inside the female body during pairing. To investigate titillator morphology and possible function during copulation, we studied the bushcricket *Metrioptera roeselii* (HAGENBACH, 1822) using a novel combination of independent, yet complementary, techniques. Copulating pairs were snap-frozen and scanned by X-ray micro-computed tomography (μ CT) to visualize the coupling of male and female genitalia *in situ*. Video recordings of copulating pairs also showed rhythmical insertion of male titillators into the female's genital chamber, where they percuss a softened structure on the female's subgenital plate. Movements did not induce damage to the female's structure, which lacks any sclerotized genital counterparts. Instead, scanning electron microscopy and histological sections show the female subgenital plate to be covered with two different types of sensory receptors at the contact zone between the male's titillator and the female genital chamber. We interpret the non-harmful function of the titillator processes, the lack of a genital counter-structure and the presence of sensory cells on the female's subgenital plate as indicators of a copulatory courtship function of titillators, subject to sexual selection by female choice.

Key words. Tettigoniidae, titillators, stimulatory devices, computed tomography, sexual selection, female choice

1. Introduction

Animal genitalia are highly diverse morphological structures (Eberhard, 1985) under obvious sexual selection (Eberhard, 1996, 2010a, 2010b; Hosken and Stockley, 2004; Simmons, 2014) that evolve faster than non-genitalic traits (Klaczko et al., 2015; Rowe and Arnqvist, 2012). Genitalia are not only highly diverse in form; they also develop independently of other structures of either external or internal origin (Snodgrass, 1935; Weidner, 1974). Males of various taxa bear genital structures such as spikes, hooks, barbs or claws, which they utilize during mating. Moreover, these organs have very specific functions apart from the transfer and acceptance of the male's sperm, and various explanations have been invoked for their functionality (Eberhard, 1985, 1996, 2010a, 2010b; Simmons, 2014). Male genital structures may be used as grasping organs or for internal fixation of the females during mating (Arnqvist and Rowe, 2005; Edvardsson and Canal, 2006), or to perforate the female's genitalia for a faster diffusion of potentially male-beneficial seminal fluids into the female body (Eberhard, 1998). Male genitalia may also stimulate females internally, thus triggering postcopulatory effects by female choice such as acceptance of sperm deposition, sperm transport to storage sites and delay of remating (Eberhard, 1996, 2011). Alternatively, male genitalia can also function as sperm removal devices in polygamous species (Parker, 1970; Waage, 1979) and variation in genital shape may in some cases contribute to mechanical species isolation (Shapiro and Porter, 1989; Eberhard, 2010b; Masly, 2012; Wojcieszek and Simmons, 2013). Given the enormous diversity of genital morphology and function, insect genitalia must be studied within the framework of their own sexually selected mating systems.

In bushcrickets of the family Tettigoniidae, mating can generally be classified in three phases: mate approach, copulation and spermatophore transfer (Lehmann and Lehmann, 2008). A bushcricket male attracts females with his courtship song, and the female approaches her mating partner phonotactically (Gwynne, 2001; Robinson and Hall, 2002). Once the female

has found the stridulating male, she mounts the male's back. The copulation position is achieved when the male's cerci grasp into the female's lateral sclerites at the end of the abdomen (Zippelius, 1949; Harz, 1960).

Males in several subfamilies of bushcrickets carry secondary genitalia called titillators (Vahed et al., 2011), which they insert rhythmically into the female's subgenital plate during copulation (Gerhardt, 1913, 1914; Boldyrev, 1915). At the end of mating the male transfers a large spermatophore to the female. These spermatophores comprise the ampulla, containing the ejaculate and the sperm, and the larger spermatophylax (Lehmann, 2012). The presence of titillators in many bushcrickets has been known for over a century (Gerhardt, 1913, 1914; Boldyrev, 1915) and their species specific distinctiveness has been intensively studied for taxonomic purposes.

In the current study, we used the bushcricket *Metrioptera roeselii* (HAGENBACH, 1822) from the subfamily Tettigoniinae to investigate the morphological function of bushcricket titillators. Males of *M. roeselii* possess one pair of sclerotized titillators showing moderate complexity when classified in a cross-species comparison (Vahed et al., 2011). With their well developed titillator processes bearing a number of small spines on each tip they are overall similar to a wide number of titillators in other Tettigoniidae bushcrickets (Harz, 1969). Furthermore, the mating behaviour (Zippelius, 1949; Harz, 1960) and spermatophore production (Wedell, 1993; Vahed and Gilbert, 1996; Vahed et al., 2011) of this species is well described. The morphology of the paired titillators of bushcricket males varies significantly across species in structure, shape and the number of apical spines. Two recent studies have tried to elucidate the functional aspects of the titillators for copulation. Vahed and co-authors (2011) undertook a comparative analysis of 54 bushcricket species, showing that (a) copulation duration in subfamilies possessing titillators was significantly longer than in subfamilies without titillators and (b) species with complex titillators transferred larger

spermatophores faster than species with simple titillators. Direct experimental evidence of the importance of titillators for successful matings was provided by our complementary study on the bushcricket *Metrioptera roeselii* HAGENBACH, 1822 (Wulff and Lehmann, unpublished results). Manipulative shortening of the titillators resulted in a significant number of failed spermatophore transfers and increased female resistance during matings with manipulated males. The titillators may thus play an important role in spermatophore transfer or for suppressing female rejection behaviour through stimulation. However, since the titillators are inserted into the female during copulation, their contact with the female's genitalia cannot be studied by external observation. It is therefore not surprising that morpho-functional analyses of the titillators are lacking so far.

Here we present the first morphological investigation on bushcricket titillator functionality. We apply a wide range of techniques, including digital microscopy, scanning electron microscopy and histological sections of preserved specimens of both sexes. Furthermore, the advanced technology of X-ray micro-computed tomography (μ CT) gives the possibility of visualising titillator positioning in flash-frozen pairs in copula in static 2D and rotating 3D reconstructions. Based on these results, we provide detailed descriptions of the male titillator morphology, where and in which form the titillators contact the female's genitalia during copulation, and describe the female's genital counterparts, including sensory structures on the contact sites of the female genitalia.

2. Materials and methods

2.1. Study species

M. roeselii is a common bushcricket in central Europe of moderate body size, approximately 2 cm in total length. Male *M. roeselii* possess one pair of sclerotized titillators, with a strongly projecting apical part (Harz, 1969). Individual bushcrickets were caught in June 2011 as nymphs close to Berlin, Germany (Stahnsdorf 52°23'14" N, 13°12'54" E), and reared separately to adulthood in transparent 0.5l plastic containers. Each container was covered with gauze and contained a stick for crawling. Food was provided *ad libitum* and replaced on a daily basis; it consisted of garden collected mixtures of fresh grass, dried fish food pellets (Tetramin®), oat flakes and bee pollen. Water was sprinkled on the brim of the container three to five times a day to give animals access to water. Ambient temperature in the laboratory was 22 to 25° C with a light-dark cycle of 16:8 h.

2.2. Morphology of male titillators

For morphological descriptions of internal genitalia, we used male bushcrickets fixed in 70% Ethanol. Titillators were removed from the male's abdomen with forceps and cleaned from tissues mechanically and with 70% Ethanol. The structure of the male titillators was analysed with a digital microscope (VHX-1000, zoom objective VH-Z00W, Keyence Corporation, Neu-Isenburg, Germany).

2.3. Video recordings, flash freezing and X-ray μ CT-Scans

Randomly chosen males and females were paired in a mating area (30 x 30 x 20 cm; see Wulff & Lehmann, unpublished results). Matings were video recorded with a full HD digital camcorder (Samsung HMX-Q10BP/EDC, Samsung Electronics GmbH, Schwalbach/Taunus, Germany). During copulation, 31 mating pairs were submerged for 20 s in a Styrofoam

container filled with liquid nitrogen and transferred to a freezer using forceps, where couples were stored at -18°C until examination. Due to different stages of titillator insertion at the moment of flash-freezing, two couples with completely inserted titillators were selected and fixed for two weeks in 70% ethanol. They were then dehydrated for 24 h in 85 % Isopropanol and then for another 24 h in 96 % Isopropanol. Following this procedure, they were transferred for 5 days to 100 % Isopropanol and left for 7 days in an oven at 58 °C: for 24 hours in a one-by-one solution of 100 % Isopropanol and Histosec® (Merck Millipore, Darmstadt, Germany) and six more days in pure Histosec®. The couples were then fixed in paraffin. One couple was examined by high resolution μ CT using a Phoenix Nanotom® X-ray tube tomography system (GE Sensing and Inspection Technologies, Wunstorf, Germany) under the following scanning parameters: dimensions of the detector panel 2304 x 2304, 35 kV, 330 μ A, no filter, 1,5 s exposure time, 5.5 μ m voxel resolution, 1800 projections. Data reconstruction was performed in Phoenix Datos|x CT software, version 1.5.0.22 (GE Sensing and Inspection Technologies, Wunstorf, Germany), and the resulting volume was segmented and analysed in VGStudio MAX 2.1 (Volume Graphics, Heidelberg, Germany). Our main objective was to visualize the site of insertion of the titillators on the female's subgenital plate and the demarcation of the titillators from the surrounding tissue of the male. We therefore assigned different colours and opacity to the female, the titillators and the rest of the male's body in the reconstructed images. The resulting videos (Video 2 and 3) show a virtual tracking shot around the pair.

2.4. Morphology of the female subgenital plate

The dorsal surface of the female subgenital plate represents the inside of the genital chamber and the contact zone with the male's inserted titillators during copulation. This dorsal surface of the subgenital plate was therefore morphologically inspected for the occurrence of

mechanoreceptors and for injuries caused by male titillator movements during copulation. Prior to preparation, nine females were mated with two different males and fixed in 70% Ethanol. The subgenital plate of five out of the nine females was analysed by digital microscopy (VHX-1000, zoom objective VH-Z00W, Keyence Corporation, Neu-Isenburg, Germany). The subgenital plates of the remaining four females were scanned by electron microscopy (EVO LS 10 SEM, Carl Zeiss, Oberkochen, Germany). These four subgenital plates were removed from the individuals, fixed in 70% Ethanol and dried for 24 h at room temperature under a fume hood. They were coated with Au/Pd (Plano, Wetzlar, Germany) under the sputtercoater SC7640 (Polaron Manufacturing, Wales). The following scanning parameters for the SEM were used: 11.12 – 20.01 kV EHT-value, working distance of 17-18 mm, 300 V collector bias, stage at T 0.0°, 356-380 µm spot size and a magnification of 29x-5550x.

2.5. Histology of the female subgenital plate

The subgenital plates of two freshly killed virgin females were softened in a solution of 7.5 vol% formaldehyde in water (50.8 vol%) and DMSO (41.7 vol%) for three days, transferred to 50 % Ethanol for six hours and to 70% Ethanol for 30 minutes and then fixed in paraffin using an enclosed tissue processor (Hypercenter XP; Shandon Electron Corporation, Waltham). The specimens were cut with a sliding microtome (SM2000 R; Leica Instruments, Nussloch, Germany) in slices with a diameter of 10 µm and stained with hematoxylin-eosin according to standard procedures (Fischer et al., 2008), dehydrated, and mounted using an automated glass coverslipper (CV5030; Leica Instruments, Nussloch, Germany). The subgenital plate slices were examined and photographed with the Zeiss Axioskops 2 plus (Carl Zeiss Microscopy, Jena, Germany) in combination with the analysing software Axio Vision (Version 4.8.2-06-2010). Photographs were adjusted for contrast and brightness using

Photoshop CS2.

3. Results

3.1. Morphology of the male titillators

Males of *M. roeselii* possess three different types of paired abdominal appendages (Fig. 1a): (1) The cerci, movable appendices of the tenth tergite, act as grasping organs during copulation and are densely covered with sensilla hairs. (2) The styli at the subgenital plate are flexible and have a minor function in supporting mating. (3) The chitinized titillators are concealed inside the male's genital chamber and are only exposed during copulation. The average total length of these titillators is 3.7 mm (n=20), their diameter 193 μ m (n= 22, see also Fig. 2). The curved titillator base is attached inside the soft membrane of the genital chamber (Fig. 2a-d). The left and right arms of the paired titillators are linked at their midpoints by a bridge approximately 0.3 mm long (Fig. 2b), still inside the membrane of the genital chamber. The distance between this bridge and the apex of the titillators is 1.3 mm, of which 1.1 mm extend straight into the genital chamber (Fig. 2b, d). The titillator processes are yellow in colour, heavily chitinized, hollow and have two to six darker coloured spines at their apex. In all cases, there was one bigger spine and several smaller ones. The male in Fig. 1b bears two smaller, closely set spines of a length around 80 μ m, separated by only 60 μ m. Being 190 μ m long, the third spine is more than double in length, projects outwards and is located 100 μ m apart from the two smaller spines (Fig. 1b, 2b,d).

Figure 1: Male abdominal mating structures. a) Caudal end of the abdomen of a *M. roeselii* male, showing the styli and cerci as well as titillators and phallobasis inside the artificially exposed male genital chamber. b) Enlarged detail, showing the paired titillators.

Figure 2: Position of the paired titillators inside the male's genital chamber as revealed by μ CT. a) Titillators within the male's abdomen. The letters b,c and d represent the perspectives of the following panels. b) Titillators viewed from the ventral side. The arrow indicates the coalescence of both titillators. c) Titillators in lateral view, positioned as in figure a, d) Titillators in dorsal view.

During mating, the male's cerci grasp the base of the female's ovipositor, and thereby enclose the female's subgenital plate at both sides. The tooth on the inner side of each cercus hooks into lateral sclerites above the ovipositor. The soft styli at the proximal ends of the male subgenital plate are placed around the female ovipositor. The female subgenital plate is open and held 90° abducted (Fig. 3b). During copulation, the titillators are inserted rhythmically

into the female genital chamber and the tips contact the middle of the female subgenital plate (Fig. 3). The rhythmical insertion is repeated with a frequency of around 10 movements per minute during the mean 32 minutes of copulation (Video 1, 2 and 3).

Figure 3: Copulating *M. roeselii* pair. a) General appearance, with a rectangle indicating the area magnified in subsequent μ CT images. b) Lateral view of a flash-frozen couple. Dark blue: female, green: male, yellow: titillators. c) Enlarged detail in lateral view, see accompanying video. d) Enlarged detail as viewed from above.

Video 1: Video recording of a *M. roeselii* pair during copulation.

Bushcricket titillator morphology_R1_Video 2.ico

Video 2: Insertion of the male genital titillator into the female genital chamber during copulation. Virtual tracking of a 3D reconstruction made by μ CT scanning of a flash-frozen *M. roeselii* pair. Dark blue: female, green: male, yellow: titillators.

Bushcricket titillator morphology_R1_Video 3.ico

Video 3: Insertion of the male genital titillator into the female genital chamber during copulation. Enlarged presentation of a second virtual tracking of a 3D reconstruction made by μ CT scanning of a flash-frozen *M. roeselii* pair. Dark blue: female, green: male, yellow: titillators.

3.2. Morphology of the female's structure involved in copulation

There are no anatomical structures on the female's subgenital plate or in her genital chamber onto which the titillators could hold during copulation. In contrast to expectations of morphological counter-adaptation, the female genital chamber is internally covered by a thin and soft membrane. The female subgenital plate is approximately 2.7 mm long and at the proximal part 2.6 mm wide (Fig. 4). It has mirror-symmetry with two tips at the distal end. Those tips surround the right and left part of the ovipositor in the closed state. They are approximately 1.3 mm apart and yellow in colour (Fig. 4b).

Figure 4: Female subgenital plate. a) μ CT scan the female *M. roeselii* abdomen with opened subgenital plate. The view angle for microscopy and SEM inspection is indicated and the section plane for the histological inspection shown by a pair of scissors. b) Female subgenital plate in dorsal view, which is inside the female genital chamber.

About 1.2 mm proximally from the edge of the subgenital plate begins a paired structure on the internal part, which is contacted by the titillators during copulation. This structure is folded outward and softer than the surrounding tissues (Fig. 4b). Using SEM, the surface of the female subgenital plate is seen to be covered with numerous campaniform receptors of about 4 μm length, regularly interspersed from each other by 6 μm (Fig. 5b). Secondary to these ubiquitously distributed sensilla, a number of trichoform sensilla, with lengths a magnitude larger (50 μm), are irregularly distributed on the subgenital plate, especially at the lateral sides (Fig. 4b, 5a). In the histological cross-section and from staining with hematoxylin and eosin, these trichoform sensilla (Fig. 6a: SH) are clearly visible. The sensory hairs are innervated by an axon leading to the inside of the membrane (Fig. 6b: Ax), where axons of multiple sensory hairs are connected to form a single cell layer of 10 μm thickness (Fig. 6b). However, the cross-section revealed not only these sensory hairs, but also hill-shaped sensory cells (Fig. 6a: SC). These hill-shaped sensory cells span the 60-70 μm thick genital-chamber-membrane, without being detected on the surface by SEM examination (Fig. 5). After remating with a second male, the macroscopic and microscopic inspection of the subgenital plate revealed no scars or any form of injury (n=9).

Figure 5: Female sense cells on the dorsal = inside of the subgenital plate (SEM). I) Entire subgenital plate with the styli oriented towards the top. Marked areas are enlarged in subsequent images. a) Sensory hairs on the edge of the subgenital plate. b), b+), b++), b+++)) Median area of the subgenital plate with sensilla, shown in increasing magnification.

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301 **Figure 6:** Histological sections of the female subgenital plate stained with hematoxylin and
302 eosin. a) Two different sensory cells at the edge of the subgenital plate, SC: hill-shaped
303 sensory cell, SH: sensory hair. b) Trichoform sensory hair with the axon (Ax) connected to
304 other nervous cells, forming a single cell layer on the inner side.

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4. Discussion

Male *M. roeselii* possess three different types of paired abdominal appendages, which serve alternative functions. From direct observation including video recording we confirm that the cerci act as grasping organs during copulation (Zippelius, 1949; Gerhardt, 1913; Boldyrev, 1915). This mate securing function of the male cerci is generally recognized for bushcrickets, used in some species to enforce matings (Vahed, 2002; Vahed and Carron, 2008) and mechanical incompatibility between closely related species adds to reproductive isolation (Rentz, 1972; Lehmann, 1998; Lehmann and Heller, 1998). The soft and unspecialized styli at the tip of the subgenital plate support matings by being placed around the female's ovipositor (Zippelius, 1949; Gerhardt, 1913; Boldyrev, 1915). The bushcricket titillators fulfil several criteria supporting the hypothesis that sexual stimulation by genital copulatory courtship is the main function, leading to sexual selection by female choice.

In corroboration with the function of the bushcricket titillators to stimulate the female during copulation, we observed and video recorded a rhythmical insertion and retraction of males' titillators. This is in direct agreement with observations of copulatory movements in a number of titillator-bearing bushcricket species (Gerhardt, 1913, 1914; Boldyrev, 1915). However, previous studies were limited to direct observations, while cryo-fixation in conjunction with μ CT scanning provides new insights into the otherwise hidden placement of the male titillator. The μ CT reconstruction together with the mating videos suggest tapping of the titillator processes in the middle of the female's subgenital plate. This tapping together with the rhythmical movement during copulation and the non-sharp tips are not in favour of titillators acting as anchors. However, bushcricket titillators could have a direct function in mating; since in *M. roeselii* they are fused in the middle, they can only be moved together and could therefore be used to hold or pull open the subgenital plate of the females mechanically (compare Video 2). Such a stabilizing function is also suggested for titillators in

Ephemeroptera during copulation (Gaino et al., 2009). As the complete opening of the female's subgenital plate is required for the correct attachment of the spermatophore, this hypothesis could also explain why some males with ablated titillators had fatal problems in correct spermatophore transfer (Wulff and Lehmann, unpublished results).

Although our study mainly focuses on the structure of the male titillators, a greater focus on the female's genitalia structure is also needed (Simmons, 2014) for a more comprehensive understanding of genital function and evolution. Unfortunately, females are systematically underrepresented in such investigations (Ah-King et al., 2014). One identified reason for this is the inappropriate adherence to the single hypothesis of genitalia as a lock-and-key system (Shapiro and Porter, 1989). This precludes any alternative hypotheses and focuses on male genital function (Eberhard, 2010a, 2010b, 2011; Simmons, 2014). In one of the few studies of orthopteran female genitalia (Alexander and Otte, 1967; reviewed in Sakai and Kumashiro, 2004), it was revealed that the posterior margin of the subgenital plate of *Gryllus bimaculatus* crickets serves as an anchoring site for the hook shaped male epiphallus and that inside the female genital chamber, small sclerotised structures occur (Sakai and Kumashiro, 2004).

Whereas females of the bushcricket *M. roeselii* share with crickets the soft walls of the genital chamber, which forms an inner space protected by the subgenital plate when not mating, they completely lack any internal sclerotized structures (Wulff and Lehmann, 2014). Moreover, μ CT reconstruction of male and female contact during titillator insertion (Fig. 3) with complementary visual inspection reveals a contact area covered by a thin elastic membrane without any rigid structures (Fig. 4). This membrane is folded outward and is softer than the surrounding tissues (Fig. 4). Despite the growing evidence for genital evolution in concert between the sexes (Brennan et al., 2007; Evans et al., 2011; Rönn et al., 2011; Simmons and Garcia-Gonzalez, 2011; Yassin and Orgogozo, 2013; Matsumura et al., 2014; Simmons, 2014), *M. roeselii* lacks a morphological correspondence between male and female genitalia.

Therefore the lock-and-key hypothesis can be refuted as a driving force in the evolution of bushcricket titillators, similar to the rejection for the majority of animals (Eberhard, 1985, 2010a, 2010b).

Species recognition and the prevention of hybridization between closely related species can be an important selective force in the evolution of genitalia (Eberhard, 2010b; Masly, 2012). Therefore, many species have male genitalia acting as clasping and grasping organs (Polak and Rashed, 2010; Grieshop and Polak, 2012; Khila et al., 2012). Species recognition is however unlikely to select for the rapid and divergent evolution of bushcricket titillators as the female genitalia do not have a counter structure to the titillators. Without locking it is less possible for bushcricket males to secure their mates (Eberhard, 1985; Simmons, 2001; Hosken and Stockley, 2004). Males of *M. roeselii* are also not able to fix their genitalia directly into the soft walls of the female genital chamber, as we found no signs of perforation caused by the titillators on the inner side of a female subgenital plate which might be indicative of fixation. After remating with a second male, the macroscopic and microscopic inspection of the subgenital plate revealed no scars or any form of injury. This lack of wounds furthermore excludes the hypothesis (Eberhard, 1998) that titillator processes allow a faster transmission of male-derived seminal fluid substances into the female by rupturing or perforating the female's body walls, which has been shown for genital spines in seed beetles (Hotzy and Arnqvist, 2009; Hotzy et al., 2012).

We can also reject the hypothesis that titillators act as devices for sperm removal. In *M. roeselii*, the titillators are about 560 μm broad, whereas the diameter of the duct, which leads to the female's spermatheca where sperm is stored reaches only about 200 μm (Wulff and Lehmann, 2014). Hence the titillators are too broad to enter the spermatheca and consequently cannot directly interfere with female sperm storage. In some species males can force females to eject sperm of previous mates (Cordoba-Aguilar, 1999; Simmons, 2001). This possibility

can also be excluded here, as no sperm ejection in remated females was detected (Wulff and Lehmann, unpublished results).

Our morphological analysis shows that bushcricket titillators percuss a softened structure on the female's subgenital plate, the surface of which is covered with two different sensory cells (Fig. 5). In contrast to the many studies covering genital morphology (reviewed in Eberhard, 1985; Simmons, 2014), few data exist on sensory structures linked to genitalia (e.g. Kumashiro and Sakai, 2001a, 2001b ; Acebes et al., 2003; Gaino et al., 2009) and the single study in bushcrickets is restricted to the sensilla on the external male postabdomen (Faucheux, 2012). Limited data exist for sensilla on genitalia of both sexes (Rossignol and McIver, 1977; Kim and Teiji 2004; Dtingelhof and Schmitt, 2010; Córdoba-Aguilar et al., 2015). The many campaniform sensilla on the inner side of the female subgenital plate are comparable to mechanoreceptors found on other insect body structures (Altner, 1977; Keil, 1997; Keil and Steinbrecht, 1984), however without further analysis of the sensilla a contact chemoreception can not be excluded. Detection of the titillator movements by the female is hence possible. Since the sensory receptors are not clustered but evenly distributed over the subgenital plate, it is likely that the rhythm of the titillator movement and not the exact location gives the relevant stimulus. Furthermore, bushcricket females could have evolved preferences for certain copulatory movements through processing of sensory impressions deeper in the nervous system. The copulatory courtship would also explain our experimental results, in which surgical ablation of the titillator processes had no effect on the copula duration prior to sperm transfer but resulted in female resistance during copulations (Wulff and Lehmann, unpublished results).

Our novel approach, using a combination of X-ray micro-computed tomography, scanning electron microscopy, video recording and *in situ* histology, revealed new insights into bushcricket genitalia that would have otherwise been unattainable. The combined evidence

suggests that the main function for the bushcricket titillator is stimulating the female during copulation, and therefore acting as copulatory courtship devices. Their evolution may be selected by female choice to accept matings only from males with appropriate stimulating structures and movements. Further application of these independent yet complementary methods to other species will undoubtedly provide new insights into genital form, function and evolutionary significance, thus expanding our understanding of insect reproductive systems.

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Figure legends

Figure 1: Male abdominal mating structures. a) Caudal end of the abdomen of a *M. roeselii* male, showing the styli and cerci as well as the titillators and phallobasis inside the artificially exposed male genital chamber. b) Enlarged detail, showing the paired titillators.

Figure 2: Position of the paired titillators inside the male's genital chamber as revealed by μ CT images. a) Titillators within the male's abdomen. The letters b,c and d represent the perspectives of the following panels. b) Titillators viewed from the ventral side. The arrow indicates the coalescence of both titillators. c) Titillators in ventral view, positioned as in figure a, d) Titillators in dorsal view.

Figure 3: Copulating *M. roeselii* pair. a) General appearance, with a rectangle indicating the area magnified in subsequent μ CT images. b) Lateral view of a flash-frozen couple. Dark blue: female, green: male, yellow: titillators. c) Enlarged detail in lateral view, see accompanying video. d) Enlarged detail as viewed from above.

Figure 4: Female subgenital plate. a) μ CT scan the female *M. roeselii* abdomen with opened subgenital plate. The view angle for microscopy and SEM inspection is indicated and the section plane for the histological inspection shown by a pair of scissors. b) Female subgenital plate from dorsal, which is inside the female genital chamber.

Figure 5: Female sense cells on the dorsal=inside of the subgenital plate (SEM). I) Entire subgenital plate with the styli oriented towards the top. Marked areas are enlarged in subsequent images. a) Sensory hairs on the edge of the subgenital plate. b), b+), b++), b+++)

Median area of the subgenital plate with sensillae, shown in increasing magnification

Figure 6: Histological sections of the female's subgenital plate stained with hematoxylin and eosin. a) Two different sensory cells at the edge of the subgenital plate, SC: hill-shaped sensory cell, SH: sensory hair. b) Trichoform sensory hair with the axon (Ax) connected to other nervous cells, forming a single cell layer on the inner side.

612 **Video 1:** Video recording of a *M. roeselii* pair during copulation.

613

614 **Video 2:** Insertion of the male genital titillator into the female genital chamber during
615 copulation. Virtual tracking of a 3D reconstruction made by μ CT scanning of a flash-frozen
616 *M. roeselii* pair. Dark blue: female, green: male, yellow: titillators.

617

618 **Video 3:** Insertion of the male genital titillator into the female genital chamber during
619 copulation. Enlarged presentation of a second virtual tracking of a 3D reconstruction made by
620 μ CT scanning of a flash-frozen *M. roeselii* pair. Dark blue: female, green: male, yellow:
621 titillators.

622

Highlights

- Tettigoniidae bushcricket males bear paired genital appendixes called titillators.
- Copulating pairs were video recorded, cryo-fixated and scanned with μ CT.
- Video recording showed rhythmical titillator movements without anchoring.
- Titillators touch sensilla on the inner side of a female's subgenital plate.
- Titillators seem to function as stimulatory devices during copulatory courtship.

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