Physiology and mechanics of insect hearing organs

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Abbreviations

CO_2	Carbon dioxide
DPOAEs	Distortion-product otoacoustic emissions
LDV	Laser Doppler vibrometry
OAEs	Otoacoustic emissions
SPL	Sound pressure level

Zusammenfassung

Einleitung

Insekten mit Tympanalorganen produzieren deutliche Distorsions-Produkt otoakustische Emissionen (DPOAE), wie bisher an Locusten, Nachtfaltern und Laubheuschrecken gezeigt wurde. Ihre Generierung weist auf nicht-lineare mechanische Verarbeitung im Hörorgan hin. Die Emissionen zeigen weitgehend vergleichbare Charakteristiken zu denjenigen, die im Ohr von Wirbeltieren gemessen wurden, wo sie als Indikator für aktive, nicht-lineare Schall-Verstärkungsmechanismen gelten. Während der Stimulation des Hörorgans mit zwei Reintönen (f1<f2) erscheinen zusätzliche spektrale Maxima an den Frequenzen nf1-(n-1)f2 und nf2-(n-1)f1. Die 2f1-f2 Emission ist dabei die prominenteste, da sie bereits durch sehr leise Stimuluspegel evoziert wird, wie es auch bei Vertebraten der Fall ist. DPOAE-Schwellenkurven von Insekten reflektieren den Verlauf der Hörschwelle des jeweiligen Tieres und können in Frequenzbereichen deutlich über 20 kHz erfasst werden. DPOAE-Amplituden sind anfällig für Änderungen des physiologischen Zustandes des Tieres. Erste Hinweise deuteten an, dass die skolopidialen Mechanorezeptoren im Tympanalorgan von Locusten eine Rolle in der frequenz-spezifischen DPOAE-Generierung spielen könnten (Möckel et al. 2007).

Die vorliegende Arbeit besteht aus drei Einzelprojekten, die zusammen der Frage nach der Herkunft sensitiven, nicht-linearen Hörens bei hohen Frequenzen und der Generierung von otoakustischen Emissionen in Tympanalorganen von Insekten nachgingen.

Teilprojekt 1: Allgemeine Eigenschaften von DPOAE in Laubheuschrecken und deren Beeinflussung durch Pymetrozin (Möckel et al. 2011, J Comp Physiol A 197:193-202)

Das Insektizid Pymetrozin wirkt hoch-effektiv und selektiv auf Chordotonalorgane, und eine Applikation führt zum Verlust deren stimulus-spezifischen Antwortverhaltens. Es hat dagegen keinen Einfluss auf sensorische Organe, die keine Skolopidien als Rezeptortyp aufweisen, wie z.B. campaniforme Sensillen. Dies deute auf eine spezielle Wirkung der Substanz auf skolopidiale Mechanorezeptoren hin. Skolopidien sind mit der DPOAE-Generierung frequenz-spezifisch assoziiert, was durch zellgruppen-spezifische Ausschaltung mittels mechanischer Läsionen im Tympanum gezeigt wurde (Möckel et al. 2007). Im vorliegenden Teilprojekt wurde Pymetrozin appliziert, um die Mechanorezeptoren im Tympanalorgan pharmakologisch gezielt auszuschalten, ohne die mechanische Integrität des Organs zu beeinflussen.

<u>Material und Methoden</u>

Der methodische Vorteil der tropischen Laubheuschrecke *Mecopoda elongata* liegt in der anatomischen Trennung zwischen dem Hauptort der Schallaufnahme (dem Spirakel am lateralen Pronotum) und dem Ort der Schallwahrnehmung (den skolopidialen Rezeptoren in der

Vorderbeintibia). Dies erlaubt die Manipulation der Funktion des Tympanalorgans, während die zeitgleich durchgeführten akustischen Messungen nicht gestört werden.

Das methodische Ziel war, die Substanz so nah wie möglich am Organ zu applizieren, ohne dessen Mechanik und die der umliegenden Strukturen zu beeinflussen. Die Vorderbeintibia wurde dorsal und distal zum Tympanalorgan geöffnet, indem ein Stück Cuticula oberflächlich entfernt wurde. DPOAE wurden jeweils als Kontrollen im intakten Bein und nach Öffnung der Cuticula gemessen, um die Unversehrtheit des Organs zu gewährleisten. Pymetrozin wurde in den Konzentrationen 10^{-3} M (n=7), 10^{-5} M (n=7), 10^{-7} M (n=6) und 10^{-9} M (n=5) als Tropfen (jeweils 1,5µl) auf die Cuticula-Öffnung appliziert. Durch die normalen Atembewegungen des Tieres wurde die Testsubstanz in die Hämolymphe aufgenommen und erreichte somit das Tympanalorgan. Anschließend an die Applikation von Pymetrozin wurde in Abständen von 5-10 min die Auswirkung der Substanz auf die DPOAE-Amplituden bestimmt.

Ergebnisse

Tympanalorgane der Laubheuschrecke *M. elongata* produzieren DPOAE, die in ihren Eigenschaften mit denen von Locusten und Nachtfaltern vergleichbar sind. Auch hier lässt sich ein allgemein gültiges, optimales f2/f1 Frequenz-Verhältnis schwer festlegen, da es große frequenz-spezifische und individuelle Unterschiede zwischen den Tieren gibt. Jedoch evozieren auch in Laubheuschrecken generell geringe f2/f1 Frequenz-Verhältnisse zwischen 1,04 und 1,10 hohe DPOAE-Amplituden. DPOAE-Schwellenkurven geben für unterschiedliche Stimulus-Frequenzen denjenigen f2 Stimulus-Pegel an, der ausreicht, um eine Emission bestimmter Amplitude (wie -10 dB SPL als Schwellenkriterium) zu induzieren. Die Schwellenkurven zeigten deutliche Minima bei f2-Frequenzen von 17 und 18 kHz. Hier betrug der Unterschied zwischen Stimulus und Emission 44 dB. In weniger sensitiven Frequenz-Bereichen von 15 und 25 kHz (den Schwellenkurven-Maxima) beträgt diese Differenz 55-65 dB. Diese Werte liegen im Bereich der Werte für Locusten (30-50 dB) und über denjenigen für Nachtfalter (23-30 dB).

Sowohl die Cuticula-Öffnung dorsal am Vorderbein, direkt distal zum Tympanalorgan, als auch eine Applikation eines Ringer-Tropfens zeigten keine signifikanten Unterschiede zu den Emissionsamplituden im intakten Bein. Die hier etablierte Methode ermöglicht damit verlässlich die Applikation flüssiger Testsubstanzen auf das Tympanalorgan der Laubheuschrecke, ohne dessen Mechanik an sich zu stören.

Eine Applikation von Pymetrozin in den Konzentrationen zwischen 10^{-3} M und 10^{-7} M auf das Tympanalorgan führte zum deutlichen Absinken der DPOAE-Amplituden. Dieser Effekt war irreversibel innerhalb von 60 bis 120 min nach Applikation der Substanz. Der zeitliche Verlauf dieser Effekte war konzentrations-abhängig. Das Absinken der DPOAE-Amplituden nach 10^{-3} M Pymetrozin begann direkt nach Applikation, während der Effekt nach 10^{-7} M Pymetrozin erst nach 40 min begann, dann jedoch nach 60 min vergleichbare Amplituden-Veränderungen brachte wie die höhere Konzentration. Eine Konzentration von 10^{-9} M hatte dagegen keinen Einfluss.

Teilprojekt 2: Temperatur-Abhängigkeit der DPOAE in Tympanalorganen von Locusten (Möckel et al. 2012, J Exp Biol 215:3309-3316)

Vorangegangene Studien an Locusten und Laubheuschrecken ergaben Hinweise auf die frequenz-spezifische Generierung von DPOAE durch die skolopidialen Mechanorezeptoren in Tympanalorganen. Die Emissions-Erzeugung ist stark abhängig vom physiologischen Zustand des Tieres, was auf einen biologischen Hintergrund schließen lässt. Die involvierten metabolischen Prozesse sollten eine Anfälligkeit auf Veränderungen der umgebenden Temperatur aufweisen, was direkt die DPOAE-Amplituden beeinflussen sollte.

Material und Methoden

Locusten der Art *Locusta migratoria* (n=13) wurde im Abdomen dorsal unter die Cuticula ein Temperatur-Sensor eingesetzt, der die Körpertemperatur mit einer Genauigkeit von 0,1°C über die Versuchsdauer hinweg überwachte. Die Körpertemperatur wurde erhöht oder verringert, indem die umgebende Lufttemperatur innerhalb der Schallkammer durch Heiz- oder Kühlelemente verändert wurde. Die Heiz- oder Kühlelemente berührten dabei nicht das Tier auf seinem Halter, und die Position des Kopplersystems der zwei Lautsprecher und des Mikrophons für die akustischen DPOAE-Messungen wurde im gesamten Verlauf nicht verändert.

Das Absenken oder Erhöhen der Körpertemperatur im Bereich zwischen 12 und 35°C wurde jeweils durch ein Rückführen zur normalen Raumtemperatur abgewechselt. An jedem hintereinander folgendem Temperatur-Schritt wurden DPOAE gemessen, sobald die jeweilige Körpertemperatur für einige Minuten konstant war. Die Daten bei Raumtemperatur dienten dabei als Kontrolle für die Stabilität der gesamten Präparation.

Ergebnisse

Änderungen der Körpertemperatur führten zu Pegel- und Frequenz-abhängigen Modulationen der 2f1-f2 Emission. Werden f2-Frequenzen von bis zu 10 kHz als Stimuli verwendet, bewirkte eine Temperatur-Erhöhung (im Mittel um 8,5°C) einen Anstieg der DPOAE-Amplituden um ca. 10 dB, wohingegen eine abgesenkte Körpertemperatur (im Mittel um 7°C) zur Verringerung der DPOAE-Amplituden um 3-5 dB führte. Beide Effekte waren reversibel, sobald die Raumtemperatur wieder erreicht wurde, und sie betrafen nur den Bereich der sogenannten „low-level component“ der DPOAE-Wachstumsfunktion, d.h. diejenigen Emissionen, die durch geringe Stimuluspegel unter 30-40 dB SPL evoziert werden. Emissionen, die durch höhere Stimuluspegel und -frequenzen induziert wurden, blieben unbeeinflusst. Diese höhere Anfälligkeit der „low level component“ der DPOAE-Wachstumsfunktionen gegenüber physiologischen Manipulationen wurden ebenfalls in anderen Insekten und Vertebraten gezeigt.

Die Arrhenius-Aktivierungsenergie der zugrundeliegenden zellulären Komponenten wurde aus den Veränderungen der -10 dB SPL Schwellen berechnet. Diese Schwellen beschreiben die „low-level component“ der DPOAE-Wachstumsfunktionen, da sie angeben, welche Stimuluspegel gebraucht werden, um eine Emission von -10 dB SPL zu evozieren. Die Aktivierungsenergie betrug 34 und 41 $kJmol^{-1}$ (bezogen auf Wachstumsfunktionen, die mit f2

Frequenzen von 8 und 10 kHz gemessen wurden). Diese Werte geben einen Hinweis, dass ein intaktes Dynein-Tubulin-System innerhalb der Skolopidien eine tragende Rolle in der DPOAE-Generierung spielt.

Teilprojekt 3: Mechanische Basis von otoakustischen Emissionen in Tympanalorganen (Möckel et al. 2014, J Comp Physiol A 200:681-691)

Die Generierung von DPOAE in Insekten-Hörorganen beruht entscheidend auf der Intaktheit der skolopidialen Mechanorezeptoren. In Locusten setzen diese direkt innen an verschiedenen Stellen des Tympanums an. Akustisch gemessene DPOAE sollten daher während Zwei-Ton-Stimulation mechanische Korrelate innerhalb der Tympanum-Vibrationen haben.

Material und Methoden

Locusten der Art *Schistocerca gregaria* besitzen ein relativ großes, von außen frei zugängliches Tympanum. Die Versuchsdurchführung erfolgte in zwei Schritten in jeweils separaten Schallkammern: die akustische DPOAE-Messung und die Erfassung von Tympanum-Vibrationen während Zwei-Ton-Stimulation mit Hilfe von Laser Doppler Vibrometrie (n=22; 14 ♀ und 8 ♂). Durch die vorangestellten akustischen DPOAE-Messungen wurden Stimulus-Frequenz und -Pegel so optimiert, daß sie im jeweiligen Organ hohe Emissionsamplituden induzierten. Diese Optimierung war für jedes einzelne Versuchstier notwendig, da es bei Insekten allgemein große individuelle Unterschiede in der DPOAE-Erzeugung gibt, vor allem in Bezug auf das optimale f2/f1 Frequenzverhältnis. Die akustischen Versuche wurden mit Hilfe des gewohnten Kopplersystems durchgeführt, das beide Lautsprecher (für die Stimulation mit zwei Reintönen) und das Mikrophon (zur Aufnahme des vom Ohr zurückkommenden Signals) zusammenbringt. Anschließend erfolgte die Erfassung der Tympanum-Vibrationen während Zwei-Ton-Stimulation mit Hilfe von Laser Doppler Vibrometrie. Der Laserstrahl, der die Bewegungen des Tympanums erfasst, war in ein Mikroskop eingekoppelt und erforderte einen optisch freien Zugang zum Tympanum. Die beiden Lautsprecher zur Evozierung der DPOAE waren nicht direkt am Hörorgan-Eingang platziert, sondern befanden sich in 10 cm Abstand. Das Tympanalorgan wurde durch die zuvor optimierten Stimulus-Parameter beschallt und die mechanischen Korrelate der DPOAE aufgezeichnet. Der Messbereich des Lasers konzentrierte sich auf das pyriforme Vesikel (der von außen gut sichtbaren Ansatzstelle hochfrequenter Mechanorezeptoren) und umliegende Tympanum-Bereiche. Da die dort ansetzenden Rezeptoren auf hohe Frequenzen abgestimmt sind, wurden Stimuli über 15 kHz verwendet.

In zwei Fällen wurde die beschriebene Versuchsdurchführung erweitert. Nach den anfänglichen akustischen und mechanischen Messungen am intakten Tympanalorgan wurde spezifisch eine Gruppe von Mechanorezeptoren durch eine mechanische Läsion ins Tympanum funktionell ausgeschaltet. Es handelte sich um diejenigen Rezeptoren, die auf hochfrequenten Schall über ca. 12 kHz antworten und am pyriformen Vesikel am Tympanum ansetzen. Nach dieser Präparation wurden die akustischen und mechanischen Messungen wiederholt.

Ergebnisse

Mechanische DPOAE-Korrelate traten lokal begrenzt in einer bestimmten Tympanumregion auf. Sie waren in allen 22 Tieren messbar, jedoch mit Unterschieden in Bezug auf absolute Auslenkungsamplitude. Der Ort dieser zusätzlichen Tympanumvibrationen lag dabei in jeden Fall nah am pyriformen Vesikel, dem Ansatzpunkt hochfrequent abgestimmter Rezeptoren. Die Differenz der Auslenkung durch den f2-Stimulus und durch die 2f1-f2 Emission an diesen Punkt lag im Mittel zwischen 42 dB und 51 dB, was vergleichbar mit Werten aus akustischen Messreihen an Locusten ist (30-50 dB).

Die emissions-basierten Vibrationen unterschieden sich deutlich von Schwingungen, die durch externe Reintöne evoziert wurden, hinsichtlich ihrer Wellenausbreitung, Energieverteilung und der Lage der Amplitudenmaxima. Vibrationen, die durch externe Beschallung hervorgerufen wurden, zeigten Charakteristiken einer Wanderwelle, die im äußeren Bereich des Tympanums begann, Richtung Zentrum lief, frequenz-abhängig an verschiedenen Stellen ihr Amplitudenmaximum hatte und dann abebbte. Intrinsisch generierte Schwingungen waren hingegen auf die Region um die Ansatzstelle der hochfrequenten Rezeptoren begrenzt. Äußere Tympanumbereiche wurden nur sehr wenig oder gar nicht ausgelenkt.

Die selektive und funktionelle Ausschaltung der hochfrequenten Rezeptoren durch mechanische Läsionen hatte keinen Einfluss auf die allgemeinen Schwingungseigenschaften des Tympanums; die Antwort auf externe Reintöne blieb unverändert. Jedoch verschwanden nach Ausschaltung der Rezeptoren sowohl alle akustisch gemessenen DPOAE, als auch ihre durch Laser Doppler Vibrometrie erfassten mechanischen Korrelate im Schwingungsmuster des Tympanums. Ohne funktionell intakte skolopidiale Rezeptoren waren keine intern evozierten, emissions-basierten Vibrationen mehr messbar.

Diskussion

Skolopidien als Ursprung der DPOAE-Generierung in Tympanalorganen

Pymetrozin wirkt hochselektiv auf die skolopidialen Mechanorezeptoren in Tympanalorganen, indem es deren neuronale Antwort auf mechanische Stimuli blockiert. Auf zellulärer Ebene könnten sowohl die sensorischen Neurone als auch die Hilfszellen des Skolopidiums potentielle Ziele von Pymetrozin sein. Die Substanz wurde über eine Öffnung in der Cuticula gezielt auf das Organ appliziert, was zum irreversiblen Absinken der DPOAE-Amplituden führte. Dies deutet darauf hin, dass Skolopidien entscheidend an der Emissions-Generierung in Insekten beteiligt sind. Diese Erkenntnis war nicht komplett neu. Die gezielte Ausschaltung hochfrequent-abgestimmter Zellgruppen im Tympanalorgan von Locusten durch mechanische Läsionen führte zur frequenz-spezifischen Reduktion der DPOAE-Amplituden (Möckel et al. 2007). Eine günstigere Vorgehensweise könnte allerdings eine Methode sein, die das Organs an sich und die akustischen Messungen ungestört belässt, wie es während der Versuchsreihe mit Pymetrozin an Laubheuschrecken möglich war (Möckel et al. 2011). Beide Studien liefern Hinweise auf die Beteiligung der Skolopidien an der DPOAE-Generierung in Insekten, und verwenden dabei komplementäre Methoden (mechanische versus pharmakologische

Manipulationen des Organs) und unterschiedliche Tiermodelle (Locusten und Laubheuschrecken).

Das Dynein-Tubulin-System innerhalb der Skolopidien spielt eine entscheidende Rolle für potentiell aktive Mechanismen

Jeder Zelltyp (d.h. sensorisches Neuron und Hilfszellen) und zelluläre Komponenten innerhalb der skolopidialen Rezeptoren könnten potentiell die Basis für die mechanische Nicht-Linearität in Tympanalorganen sein. Mögliche metabolische Prozesse, die an der Emissionserzeugung beteiligt sind, sollten eine Anfälligkeit auf Veränderungen der umgebenden Temperatur aufweisen, was direkt die DPOAE-Amplituden beeinflussen sollte. Die Temperatur-Abhängigkeit des auditorischen Systems bei Locusten und Zikaden beruht auf intrinsischen Eigenschaften der neuronalen Rezeptoren, wohingegen die Mechanik des Tympanums über einen biologisch relevanten Bereich hinweg unverändert bleibt. Die Veränderung der Körpertemperatur der Locusten in der hier präsentierten Studie resultierte in reversiblen, pegel- und frequenz-abhängigen Modulationen der 2f1-f2 Emission. Nur Emissionen, die durch tiefe Pegel (bis 30-40 dB SPL) und Frequenzen (unter 10 kHz) evoziert wurden, zeigten Veränderungen aufgrund von Temperaturverschiebungen.

Die aus den DPOAE-Veränderungen berechnete Arrhenius-Aktivierungsenergie der zugrundeliegenden zellulären Komponenten deutet darauf hin, dass ein intaktes Dynein-Tubulin-System eine bedeutende Rolle für die Emissions-Generierung spielt. Eine Studie über die Temperatur-Abhängigkeit spontaner mechanischer Oszillationen des Johnstonschen Organs bei Mosquitos brachte dieselben Ergebnisse. Beide Gruppen von Insekten-Hörorganen (Tympanalorgan und Johnstonsches Organ) operieren zwar in sehr verschiedenen Frequenzbereichen (Ultraschall versus Frequenzbereiche unter 1 kHz), besitzen aber denselben Mechanorezeptor-Typ und könnten ähnliche nicht-lineare Mechanismen aufweisen. Für antennale Hörorgane in Mosquitos und Fruchtfliegen wurde bereits aktive Verstärkung leiser Schallereignisse nachgewiesen.

Es gibt momentan eine Debatte darüber, ob das skolopidiale Cilium beweglich ist oder nicht. Es enthält eine sogenannte „9x2+0“-Konstitution, mit neun Mikrotubuli-Paaren, die in einen konzentrischen Ring angeordnet sind, ohne ein zentrales Paar auszubilden. Da motile Cilien allgemein ein solches zentrales Mikrotubuli-Paar enthalten, wurde eine Beweglichkeit des skolopidialen Ciliums in Tympanalorganen als unwahrscheinlich angenommen. Dieser Annahme werden strukturelle Erkenntnisse gegengestellt, die zeigen, dass Cilien in skolopidialen Rezeptoren doch beweglich sein könnten. Aktive ciliäre Motilität, so wird angenommen, beruht auf der Krümmung des Ciliums. Diese wiederum basiert auf der Krafterzeugung durch Dynein-Arme, die mit den Mikrotubuli-Paaren assoziiert sind. Die Dynein-erzeugte Kraft verursacht wahrscheinlich ein Verschieben der Mikrotubuli-Paare gegeneinander und damit eine Krümmung des Ciliums, was letztendlich zur Verformung der Zellmembran, der Öffnung von Tranduktionskanälen und der Ausbildung von Rezeptorpotentialen führt. Von Centrin, einem Hauptbestandteil in als motil gezeigten Cilien, wird zusätzlich angenommen, dass es kontrollierende Wirkung auf die Dynein-basierte Krümmung des Ciliums hat.

Weitere Hinweise auf bewegliche Cilien stammen von Beobachtungen der aktiven Krafterzeugung in Johnstonschen Organen und DPOAE-Generierung in Tympanalorganen verschiedener Insektenarten (wobei DPOAE an sich noch kein Beweis für aktive Prozesse sind, sondern für nicht-lineare Mechanismen). Beide Phänomene zeigen anhand ihrer Temperatur-Abhängigkeit eine Beteiligung des Dynein-Tubulin-Systems, was die Idee der Dynein-basierten Krümmung und Beweglichkeit des Ciliums unterstützen würde.

Mechanische DPOAE-Korrelate im Vibrationsmuster des Tympanums

Otoakustische Emissionen in Hörorganen von Vertebraten sind definiert als Luftdruck-Schwankungen im äußeren Ohrkanal, die aus der mechanischen Nicht-Linearität des Innenohres resultieren und von dessen physiologischer Aktivität abhängen. Sie führen zu Trommelfellvibrationen und werden mit sensitiven Mikrophonen als leise Schallereignisse gemessen. In Insekten setzen die für die Emissions-Generierung verantwortlichen Rezeptoren direkt innen am Tympanum an. Akustisch gemessene DPOAE haben, wie in dieser Studie gezeigt, mechanische Korrelate im Vibrationsmuster des Tympanums.

Mechanische DPOAE-Korrelate traten lokal begrenzt in einer bestimmten Tympanumregion nah am pyriformen Vesikel, dem Ansatzpunkt hochfrequent abgestimmter Rezeptoren, auf. Die emissions-basierten Vibrationen unterschieden sich deutlich von Schwingungen, die durch externe Reintöne evoziert wurden, hinsichtlich ihrer Wellenausbreitung, Energieverteilung und der Lage der Amplitudenmaxima. Der mechanische Gradient des Tympanums, der für richtungs-abhängige Eigenschaften sorgt, verhindert wahrscheinlich die Ausbreitung dieser lokal evozierten Vibrationen. Diese werden dann reflektiert und erscheinen als stehende Wellen in einem sehr begrenzten Tympanumbereich.

Die selektive und funktionelle Ausschaltung der hochfrequenten Rezeptoren durch mechanische Läsionen führte zum Verschwinden der intern evozierten, emissions-basierten Vibrationen, ohne jedoch die allgemeinen Schwingungseigenschaften des Tympanums in Antwort auf Reintöne zu verändern. Diese Befunde unterstützen die Hinweise, dass tympanale Rezeptoren, ähnlich zur Situation in Säugern, die erforderlichen nicht-linearen Antwortcharakteristika besitzen, die während Zwei-Ton-Stimulation zu zusätzlichen, lokal begrenzten Auslenkungen des Tympanums führen.

Summary

Tympanal hearing organs of insects emit distortion-product otoacoustic emissions (DPOAEs) which are indicative of nonlinear mechanical sound processing. In vertebrates, otoacoustic emissions are considered by-products of metabolically highly active mechanisms that considerably improve the ear's sensitivity, selectivity and dynamic range. DPOAEs have been recorded in locusts, moths, and bushcrickets. Their general characteristics are comparable to those measured in vertebrates, despite distinct differences in external and internal ear anatomy. DPOAEs appear during simultaneous stimulation with two pure tones (f1<f2) as additional spectral peaks at frequencies of nf1-(n-1)f2 and nf2-(n-1)f1, with the 2f1-f2 emission being the most prominent one. Insect DPOAEs are highly vulnerable to manipulations that interfere with the animal's physiological state and disappear after death. First evidence from locusts suggested that scolopidial mechanoreceptors might play a role in frequency-specific DPOAE generation (Möckel et al. 2007). The overall aim of this thesis was to determine the source of sensitive, nonlinear hearing at high frequencies and of DPOAE generation in tympanal organs of insects.

The first project of the present thesis involved general characteristics of DPOAE generation in the bushcricket *Mecopoda elongata* and the selective exclusion of the scolopidial mechanoreceptors using the neuroactive insectizide pymetrozine (Möckel et al. 2011). The methodical advantages of bushcrickets for DPOAE measurements lies in the anatomical separation of the main site of sound input (spiracle at the lateral pronotum) and the site of its perception (scolopidial mechanoreceptors in the foreleg tibia). It reliably allows manipulating the function of the tympanal organ without interfering with the simultaneously performed acoustical measurements. General characteristics of DPOAEs measured in bushcrickets were comparable to those in locusts and moths. Depending on frequency, the stimulus-emission-difference of 44 to 65 dB for bushcrickets lay within or above the values reported for locusts (30–50 dB), and above that shown for moths (23–30 dB). The second part of this first project on bushcrickets examined the effects of the neuroactive insectizide pymetrozine on DPOAE generation. The compound appears to act highly effective and selectively on chordotonal organs, without affecting other sensory organs that lack scolopidial receptors. Pymetrozine solutions were applied as closely as possible to the scolopidia via a cuticle opening in the tibia, distally to the organ. Applications at concentrations between 10^{-3} and 10^{-7} M led to a pronounced and irreversible decrease of DPOAE amplitudes. Both this study on bushcrickets (Möckel et al. 2011) and an earlier one on locusts (Möckel et al. 2007) hence indicate the involvement of scolopidia in DPOAE generation in insects, by using complementary methods (pharmacological versus mechanical manipulation) and different animal models.

The second project of the present thesis investigated the temperature-dependence of DPOAEs in the locust *Locusta migratoria* (Möckel et al. 2012). The suggested biological origin of acoustic two-tone distortions in insects should involve metabolic processes, whose temperature-dependence would directly affect the DPOAE generation. Starting at a medium

temperature, the locust's body temperature was raised and lowered within the range of 12 to 35°C by changing the temperature of the surrounding air, while recording DPOAEs. Such body temperature shifts resulted in reversible, level- and frequency-dependent effects on the 2f1–f2 emission. Using low f2 frequencies of up to 10 kHz, a body temperature increase (median +8–9°C) led to an upward shift of DPOAE amplitudes of approximately +10 dB, whereas a temperature decrease (median –7°C) was followed by a reduction of DPOAE amplitudes by 3 to 5 dB. Both effects were only present in the range of the low-level component of DPOAE growth functions below f2 stimulus levels of approximately 30-40 dB SPL. Emissions induced by higher stimulus levels and frequencies (e.g. 12 and 18 kHz) remained unaffected by any temperature shifts. The higher vulnerability of the low level component in DPOAE growth functions to physiological manipulations, as observed in this study, had also been found in vertebrates and in other insects. The Arrhenius activation energy of the underlying cellular component was calculated from the –10 dB SPL thresholds (representing the low-level component) and amounted to up to 34 and 41 $kJmol^{-1}$ (for growth functions measured with 8 and 10 kHz as f2, respectively). Such activation energy values provide a hint that an intact dynein-tubulin system within the scolopidial receptors could play an essential part in the DPOAE generation in tympanal organs.

The third project of this thesis demonstrated mechanical DPOAE analogs in the tympanum's vibration pattern during two-tone stimulation in the locust *Schistocerca gregaria*, using laser Doppler vibrometry (Möckel et al. 2014). DPOAE generation crucially relies on the integrity of the scolopidial mechanoreceptors (Möckel et al. 2007, 2011), which in locusts, directly attach to the tympanal membrane. Acoustically measurable DPOAEs should therefore have mechanical analogs within the tympanum vibrations during two-tone stimulation. The experimental time line for each individual animal started with acoustical recordings of DPOAEs in order to optimize stimulation parameters that evoked largest emissions, and was then followed by laser Doppler vibrometry measurements of DPOAE analogs using the previously determined acoustic stimuli. DPOAEs were shown to mechanically emerge at the tympanum region where the auditory mechanoreceptors are attached. Those emission-coupled vibrations differed remarkably from tympanum waves evoked by external pure tones of the same frequency, in terms of wave propagation, energy distribution, and location of amplitude maxima. In contrast to traveling wave-like characteristics of externally evoked vibrations, intrinsically generated waves were locally restricted to the region around the high frequency receptors' attachment position. The mechanical gradient of the tympanal membrane that leads to direction-dependent properties probably avoids the spreading of these locally evoked waves, which are then reflected and occur only in restricted areas as standing waves. Selective inactivation of mechanoreceptors by mechanical lesions did not affect the tympanum's response to external pure tones, but abolished the emission's displacement amplitude peak. These findings provide evidence that tympanal auditory receptors, comparable to the situation in mammals, comprise the required nonlinear response characteristics, which during two-tone stimulation lead to additional, highly localized deflections of the tympanum.

1. General introduction, aims and questions

1.1. Mechanical amplification and otoacoustic emissions in vertebrate ears

To be able to evaluate solutions for sensitive hearing in insects, an overview on anatomy and function of different vertebrate hearing organs is given in this chapter. The performance of inner ears in vertebrates is considerably improved by active processes which increase their sensitivity, selectivity and dynamic range (Kemp 2008). The general terms "active process" and "cochear amplifier" for such mechanisms are hereby employed in all vertebrate ears, even if they do not possess a cochlea (Manley 2001), and actually describe more than one process.

1.1.1. Anatomy of the inner ears in vertebrates

The inner ear of frogs consists of two anatomically separated papillae. The amphibian and the basilar papilla are most sensitive to airborne sound at different frequency ranges. Their receptors are not differentiated into groups of inner and outer hair cells as it is the case in mammals, they receive both afferent and efferent innervation, and are imbedded in a relatively solid structure rather on a flexible membrane. With the upper frequency limit being species-dependent, the tonotopically organized amphibian papilla responds to frequencies between 0.1 and 1 to 1.4 kHz, whereas the basilar papilla is not tonotopically organized and primarily exited by higher stimulus frequencies above 1 kHz (Simmons et al. 2007). One exeption is the nocturnal, concave-eared torrent frog *Amolops tormotus* whose males possess ultrasonic communication capacity (Feng et al. 2006).

The turtle basilar papilla holds several hundred hair cells. They receive both afferent and efferent innervation, and are covered by a thick tectorial membrane (Sneary 1988). The electrically tuned hair cells (which is based on properties of the cell membrane ion channels, their ion-channel density and kinetics) are arranged tonotopically up to about 0.8 kHz (Crawford and Fettiplace 1980; Fettiplace 1987).

The auditory papilla of lizards is an elongated epithelium which is located over a thickened basilar membrane and holds sensory hair cells and their supporting cells. The morphology varies greatly across the lizard subgroups, such as different arrangements of hair cells in low (up to 1 kHz) and high frequency areas (3-7 kHz) along the papilla and the presence or absence of a tectorial membrane (Manley and Köppl 1998; Manley and van Dijk 2008).

The basilar papilla of birds is a relatively short, slightly curved band, with its length ranging from 2.1 mm in small songbirds up to 10 mm in the barn owl and its width decreasing from apex to base (Manley and van Dijk 2008). Hair cell bundles firmly attach to the tectorial membrane, which covers the entire papilla. The 3000 to 17000 hair cells undergo a gradual morphological and physiological transition along and across the papilla, from short, cup-shaped

hair cells at the base to relatively tall and columnar hair cells at the apex. This hair cell shape, together with bundle orientation and innervation pattern correlates with the tonotopical organization along the avian papilla. The hearing range of birds extents to frequencies of up to 12 kHz (Manley and Köppl 1998).

The mammalian cochlea is a coiled structure, consisting of three acoustically coupled scalae (scala vestibuli, scala tympani and in the middle scale media) which are separated by Reissner's membrane and basilar membrane. The basilar membrane's mechanical properties vary along the cochlea, being narrow and stiff at the base and increasing its compliance towards the apex, hence leading to a frequency-dependent positioning of the peak amplitude for a sound stimulus-induced pressure wave. The organ of Corti sits upon the basilar membrane and holds mechanosensitive hair cells and several types of specialized supporting cells, overlaid by the tectorial membrane. Hair bundles, that is, stereocilia that rise at the apical surface of hair cells, are the defining structure of that cell type. Inner hair cells (in humans arranged in one row) are the primary sensory cells and receive 90-95% of the cochlea's afferent innervation. They respond to apical fluid movement. Outer hair cells (in humans arranged in three rows) have their hair bundles directly coupled to the tectorial membrane and respond to its shearing motion against the basilar membrane, they receive many efferent contacts and fulfill an important role in amplification processes (see below). The general hearing range of mammals extents to frequencies above 100 kHz (for overview, see LeMasurier and Gillespie 2005).

Hair bundles consist of up to several hundred stereocilia per hair cell. They are arranged in rows of increasing height, are interconnected by several crosslinks, and hence are deflect during a mechanical stimulus as a cohesive unit (Crawford and Fettiplace 1985; Goodyear et al. 2005; LeMasurier and Gillespie 2007). The stereocilia contain hundreds of actin filaments along their length, with few of them anchoring as rootlet in the apical cell surface.

If the hair bundle is moved in the exitatory direction, the open probability of the involved transduction channels raises as the tension in its gating springs increases. The molecular constitution of these gating springs is still under debate. Tip links are coiled filaments that extent between the tip of a shorter stereocilium and the lateral wall of a taller neighbour, and are hence creating the bundle's directional sensitivity (Kachar et al. 2000). Cadherin 23 was suggested to be a tip link component, and essential for normal hair bundle morphology (Siemens et al. 2004). The tip links are too stiff to be acting as gating spring themselves, and are rather thought to transmit forces to an elastic gating spring (Howard and Hudpseth 1988). Compliant elements which are arranged in series with the tip links at their insertion sites are discussed to provide the required elasticity, such as components of the transduction channel itself or myosin molecules which serve as channel anchors.

Transduction channels and hence the hair cell's mechanotransduction site are thought to be located at the top of the stereocilia (Hudspeth 1982). These non-selective cation channels are, according to the prevailing hypothesis, a member of the transient receptor potential (TRP) channel family (Corey 2003).

1.1.2. Mechanotransduction and adaptation mechanisms in hair cells

Sound stimulus-induced basilar membrane vibrations elicit hair bundle deflections, which immediatley causes a direction-dependent opening or closing of non-selective transduction channels (a movement towards the tallest stereocilia opens the channels, an opposite movement closes them). The evolving receptor current is mostly due to an influx of K^+ (Corey and Hudspeth 1979) which is highly concentrated within the endolymph that bathes the hair cell's apical surface, and partly relies on inflowing Ca^{2+} ions. The hair cell's depolarization leads to voltage-dependent opening of Ca^{2+} channels near the cell's base, which stimulates neurotransmitter release and subsequent afferent signal propagation.

Sustained stimulation and hence steady hair bundle deflection bring about an important feature of the transduction channel's response: adaptation processes which reduce the response, prevent saturation, and restore the bundle's sensitivity to threshold deflections (Crawford et al. 1989). Two forms were found to exist, both depending on Ca^{2+} ions, with different time scales. Slow adaptation (tens to hundreds of milliseconds) is mediated by myosin motors (most probably myosin-1c) which lie concentrated at the insertion of the upper end of each tip link. These motors climb up and down along the actin filaments of the stereocilium, by that regulating the tension in the gating springs (Gillespie and Cyr 2004). The associated channels are not inactivated; their operation range is shifted, and they remain sensitive should a second stimulus arrive. Ca^{2+} inflow during stimulation leads to myosin's detachment from actin, the motor shifts down along the stereocilium, and the reduced gating spring tension closes the channel. As the Ca^{2+} inflow weakens, the motor climbs back up the restore the channel's resting tension. The far less understood fast adaptation (up to one millisecond) is known from amphibians, reptiles, and mammals. It is thought to be based on structural re-arrangements, mediated by Ca^{2+} entry in the cytoplasma. Suggested mechanisms would be a direct Ca^{2+} binding to the transduction channels, with the binding energy re-closing the channel, or a Ca^{2+} mediated change in the characteristics of the adaptation motors, altering its ability to regulate the gating spring's tension (Hudspeth 2008).

1.1.3. Active amplification processes in vertebrate hearing organs

Two mechanisms are discussed as the source of the "cochlear amplifier" in mammalian and non-mammalian vertebrates: active hair bundle motility and somatic electromotiliy. These metabolically highly active amplification mechanisms that greatly improve the ear's performance possess four distinct properties in all tetrapod vertebrates (e.g., Manley 2001; Hudspeth 2008; Peng and Ricci 2011):

(1) Mechanical amplification lowers hearing thresholds by 40 to 60 dB.

(2) Frequency tuning (that is, the cochlea’s capacity to separate incoming sounds into their constituent frequencies) is considerably sharpened.

(3) A compressive nonlinearity varies its gain relative to the sound input intensity (lowered gain for loud sounds, enhanced gain for low sounds) and modulates the system’s input over a wide stimulus intensity range. Thus, at low stimulus levels, the basilar membrane gets much more sensitive than a passive system.

(4) Spontaneous otoacoustic emissions (which are more a by-product than a function of the active process) are generated. In a quiet environment, sound at one or more frequencies is emitted by the ears of many species from all tetrapod classes.

Active hair bundle motility is the likely source of amplification in non-mammalian hearing organs of frogs, reptiles, and birds. Even if they do not possess somatically motile cells like mammals, non-mammalian ears show comparable hearing thresholds, frequency selectivity, and OAE generation (Manley 2001; Manley and Köppl 2008) and hence comprise the four characteristics of active hearing processes mentioned above. Spontaneous OAEs, as an argument for the existence of a mechanical amplifier, have been recorded from different non-mammalian species [e.g., Crawford and Fettiplace 1985 (turtle); Köppl and Manley 1993 (bobtail lizard); Manley et al. 1996 (gecko); Manley and Gallo 1997 (lizard); Taschenberger and Manley 1997 (barn owl); Martin and Hudspeth 1999 (bullfrog)]. The responsible bundle motility is created by an interaction of negative hair-bundle stiffness and myosin-based adaptation motors (Martin et al. 2000). Within a specific range of displacement, the bundle gives no resistance to the applied stimulus force (zero stiffness) or moves farther in response to a stimulus than the amplitude of that stimulus itself would cause (negative stiffness). This type of force generation emerges from parallel arranged transduction channels and their interactions. An external force which is applied to the hair bundle is divided equally among all the gating springs of the bundle's transduction channels, increasing the opening probabilty of each of them. The opening of one of these channels reduces the tension in its gating spring, and by that raises the tension upon all remaining gating springs, subsequently continuing until all channels are open. This coordinated gating of the transduction channels hence reduces the stiffness of the hair bundle (non-linear gating compliance) and drives it farther into displacement than the initial stimulus force, which was found to be the base of active hair bundle motility (Hudspeth 2008). Ca^{2+} dependent adaptation motors constantly adjust the transduction channel's resting position and keep the hair bundle in the range of a negative stiffness. No control parameter for active hair bundle motility have been found so far, yet there is evidence for a regulation of the Ca^{2+} inflow into the stereocilia by reducing or raising the ion's extracellular concentration, or by efferent control.

Active hair bundle motility provides amplification and filtering in non-mammalian hair cells, but has also been found to exist in the mammalian ear. Mammalian hair bundles actively move faster than initially thought, with frequency limits of about 8 kHz (Kennedy et al. 2003; Chan and Hudspeth 2005). An interaction between both mechanisms was hypothesized: Electromotility (only present in mammalian outer hair cells) would supply amplification on a cycle by cycle basis, while hair bundles could provide frequency selectivity and compressive nonlinearity (Liberman et al. 2004; Peng and Ricci 2011). In the high frequency region of the mammalian cochlea, however, the active amplification process would entirely been taken over by electromotility, whereas active hair-bundle motility woud become increasingly important toward the low-frequency apex (Hudspeth 2008).

Somatic motility (or electromotility) of mammalian outer hair cells relies on the motor protein prestin (Dallos and Fackler 2002). Prestin is a member of the SLC26 family of anion-bicarbonate transporter proteins, that lies densely packed in the lateral cell membrane (Zheng et al. 2000; Liberman et al. 2002). This form of active amplification does not need ATP or another

source of chemical energy. It solely relies on prestin's voltage-dependent conformation changes and hence the altered surface area which the molecule occupies within the cell membrane. A change in transmembrane voltage that follows acoustical stimuli leads to a contraction (during depolarisation) or elongation (during hyperpolarisation) of the cell body, with cell length changes of as much as 4 % (Brownell et al. 1985; Zenner 1986; Ashmore 1987). Outer hair cells hence generate large forces and movements that are fed back into the passive motion of basilar membrane by significantly enhancing its displacement amplitude and sharpening its wave form (the latter was suggested to be based on lateral inhibition between adjacent outer hair cells; Zhao and Santos-Sacchi 1999). That in turn directly changes the hair bundle motion and increases the inner hair cell's input (Mammano and Ashmore 1993; Robles and Ruggero 2001). Prestin is highly sensitive to intracellular anions, especially choride, whose modulation was reported to reversibly alter the outer hair cell's electromotility and cochlear amplification (Santos-Sancchi et al. 2006). Prestin-knockout animals were reported to shown severe decrease in hearing sensitivity (Liberman et al. 2002), but do generate attenuated DPOAEs at high SPLs which are due to a non-linearity based on gating compliance in hair bundle transduction and motility (Liberman et al. 2004). The forces contributed by electromotility are considerably larger than those measured from active hair bundle mechanisms so far (Peng and Ricci 2011). Outer hair cell electromotility was suggested to be controlled and modulated (among others) by efferent neurotransmitter (acetylcholine) causing hyperpolarization and decreasing axial cell stiffness, both of which being mediated by Ca^{2+} (Frolenkov 2006).

1.1.4. The generation of otoacoustic emissions

"The ear makes sound while listening to sound" (Shera 2004). This, at first glance, weird statement describes a quite remarkable feature of sensitive hearing organs in mammals and other vertebrates: Unlike any other sensory organ, they can produce physical energy comparable to that of the external stimuli they are supposed to sense. Such self-generated sound waves retrogradely leave the ear as otoacoustic emissions (OAEs).

The inner ear in general serves two roles: the frequency-selective transmission of the arriving mechanical energy and the transduction of mechanical into neural signals. OAEs, however, are generated only at the first of these steps and are a by-product of normal peripheral auditory function, that is, a non-linear and active mechanism (Kemp 2008).

Several types of OAEs have been characterized and used to study the cochlea's function and dysfunction in detail. Originally discovered by Kemp (1978), OAEs have by now been demonstrated in amphibians, reptiles, birds, mammals (Peng and Ricci 2011), and insects (see chapter below). What all OAE types have in common is that their amplitudes rely on the physiological condition of the inner ear. They were initially grouped according to measurement procedures and types of stimuli to evoke them (Probst et al. 1991):

(1) Spontaneous otoacoustic emissions (SOAEs) arise in the absence of external sound stimuli. They are associated with specific tonotopic positions in the inner ear, as they appear only at certain frequencies within the range of species-specific high hearing sensitivity. They are the most striking indication of active auditory processing and may also require acoustic reflections at anatomical discontinuities in the organ. SOAEs have been found in amphibians, reptiles,

birds, and mammals (Probst et al. 1991), with the so far highest SOAE frequency of 62 kHz detected in bats (Kössl 1994).

(2) Evoked OAEs on the other hand are induced by short clicks (transient evoked OAEs, TEOAEs), by single pure tones (stimulus frequency OAEs, SFOAEs), or by two simultaneously presented pure tones (distortion-product OAEs, DPOAEs) (Kemp 2008). DPOAEs are a direct consequence of the nonlinear mechanical characteristics of the inner ear. During the processing of a signal by an amplifier with non-linear characteristics, the input signal is not only amplified, but also changed in its spectral constitution. The output signal contains wave form distortions that were not present in the input signal and that give rise to specific frequency peaks in the spectrum of the output signal. DPOAEs are primarily generated when the cochlear amplifier works at a maximal gain during the presentation of low level stimuli. During the simultaneous stimulation with two pure tones, two traveling waves propagate along the basilar membrane, with maximal amplitudes at their specific resonance places. If the frequencies of the two primaries are close together and their traveling waves partly overlap, the cochlear amplifier enhances both waves which generates so-called distortion-products. These distortion-products are manifested as basilar membrane vibrations (Rohde 2007), and as pressure waves (Ren 2004) which are transmitted retrogradely through the middle ear, cause ear drum vibrations, and are measurable with sensitive microphones as faint acoustic sounds (Kemp 2008). As DPOAE generation is mathematically related to the used stimuli, they can be used to probe the whole hearing range of an animal species and reach largest amplitudes at frequencies of high auditory sensitivity (Kössl et al. 2008).

The characterisation of OAEs has been re-defined based on their particular generation mechanism. They are hence either place-dependently generated, for example SOAEs, or wave-dependently generated, for example DPOAEs (Shera and Guinan 1999; Shera 2004).

1.2. OAEs in insects… state of research at the beginning of this project

Nonlinear sound amplification is not restricted to vertebrate ears, but might be a more general feature of all sensitive hearing organs across various animal groups. Compared to the knowledge about vertebrates, research about active processes and OAE generation in auditory systems of insects still was at its beginning. A couple of years ago, it was thought that insects would not have such nonlinear mechanical amplification and that there was only passive sound transduction. This opinion did begin to change after the discovery of OAEs in these animals.

Tympanal organs generate distinct distortion-product otoacoustic emissions (DPOAEs). When this project started, they had been recorded in locusts (Kössl and Boyan 1998a,b; Möckel et al. 2007) and moths (Coro and Kössl 1998, 2001; Kössl and Coro 2006; Kössl et al. 2007). Their presence indicated nonlinear mechanical processing in the insect's tympanal hearing organs. Insect DPOAEs showed similar characteristics compared to the ones in vertebrates, despite the fact that the sensory cells are not hair cells but primary mechanoreceptors with a ciliated neuron instead of a hair bundle composed of rows of stereocilia as in vertebrates (for anatomical details on scolopidia, see chapter 1.3.).

Characteristics of insect DPOAEs are largely comparable to those reported from vertebrate ears. They appear during simultaneous stimulation with two pure tones (f1<f2) as additional spectral peaks at frequencies of nf1-(n-1)f2 and nf2-(n-1)f1 (Fig.1.1). The 2f1-f2 emission is most prominent, as it is the case in vertebrates.

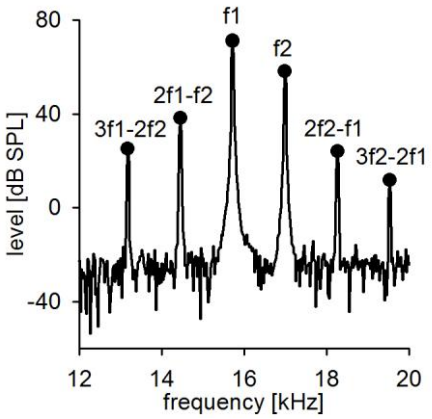

Fig.1.1 Example spectrum of a DPOAE measurement in the desert locust *Schistocerca gregaria*. Stimulus frequencies f1 15.72 kHz and f2 17 kHz, stimulus levels 70/60 dB SPL. Additional spectral peaks which had not been present in the loudspeaker signal, appear at frequencies of nf1-(n-1)f2 and nf2-(n-1)f1, with the most prominent one at 2f1-f2.

In locusts and moths, DPOAEs had been elicited already at stimuli levels near the species-specific auditory threshold, which indicates a nonlinearity of the tympanal organ at these low levels (Kössl and Boyan 1998b; Kössl et al. 2007). DPOAE threshold curves display the frequency-specific sensitivity of the tympanal organ's nonlinearity, as they reflect the course of the animal's hearing threshold. The level difference between the f2 stimulus and the emission amounts to 23-30 dB in moths (Coro and Kössl 1998), to 30-50 dB in locusts (Kössl and Boyan 1998b; see Fig.1.2a), and to 44 to 55-65 dB in bushcrickets (as shown in this thesis; Möckel et al. 2011; see Fig.1.2b), in dependence of the particular frequency. The hearing range of insects can extent into ultrasonic frequencies, as those of most mammalian species (locusts: Römer 1976; different grasshoppers: Meyer and Elsner 1996; bushcrickets: Oldfield 1985; moths: Surlykke 1984), and DPOAEs can be measured at frequencies well above 20 kHz.

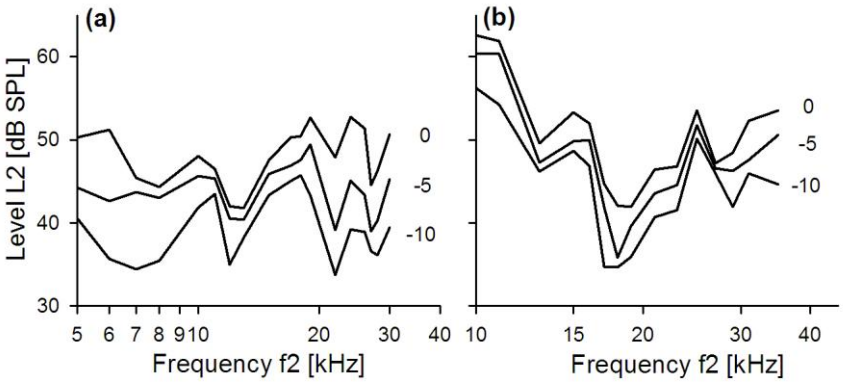

Fig.1.2 2f1-f2 DPOAE threshold curves. Two representative examples obtained for **(a)** the migratory locust *Locusta migratoria*, and **(b)** the bushcricket *Mecopoda elongata*. Levels of f2 sufficient to evoke an emission of -10, -5, and 0 dB SPL (threshold criteria) were extrapolated from DPOAE growth functions recorded at different stimulus frequencies. Level of f1 had been 10 dB above that of f2.

Tympanal organs comprise a huge variety in terms of scolopidia numbers per organ. Those of some notodontid moths possess only one scolopidium and yet produce quite large DPOAEs (Kössl et al. 2007) which allows to evaluate auditory mechanics on a more or less cellular level. On the other hand, the higher complexity of for example the locust ear permits an estimate of the interaction of several anatomical features within the organ, that is, the tympanal membrane, the adjacent tracheal system, and frequency-specific groups of scolopidial mechanoreceptors.

Insect DPOAEs are highly vulnerable to manipulations that interfere with the animal's physiological state and disappear after death. After ventilation with CO_2 which induced hypoxia in the locust *L. migratoria*, the amplitudes of DPOAEs which had been evoked by f2 frequencies below 10 kHz reversibly decreased. Those DPOAEs that were induced by higher f2 frequencies did not significantly change (Kössl and Boyan 1998a). A drop of ethyl ether topically applied onto the thorax surface of the moths *Empyreuma affinis* (two receptor cells per organ; Coro and Kössl 2001) and *Ptilodon cucullina* (one auditory receptor neuron per organ; Kössl et al. 2007) led to a strong (and partly reversible) decrease of DPOAE amplitudes within the low level component of the growth functions. First evidence originating from my diploma thesis on locusts suggested that scolopidial mechanoreceptors might play a role in frequency-specific DPOAE generation. After the selective exclusion of receptors tuned to high frequencies via mechanical lesions, DPOAE amplitudes decreased considerably in a frequency-specific manner. Current injections directly into the primary neuron’s axons led to reversible changes in DPOAE-amplitudes, which suggested that intrinsic mechanisms within the scolopidia are involved in DPOAE generation (Möckel et al. 2007). As the mechanoreceptor in its entirety had been affected by those experimental procedures, it was still unclear which of the scolopidial cell types and cell components might be crucial for nonlinear (and possibly active) mechanisms in these insect hearing organs.

Ears of different insect groups can generate high-frequency otoacoustic emissions (Kössl et al. 2008). It is to note that the presence of OAEs by itself does not necessarily imply the existence of active amplification processes within tympanal organs, as any non-linear system could produce distortions, independently of whether the non-linearity has active or passive properties. Reversible changes of OAE amplitudes during interferences with the animal’s physiological state, however, suggest an underlying active metabolism (Kössl and Boyan 1998a,b). Antennal hearing organs of flies and mosquitoes have been demonstrated to actively amplify low level sound, although at frequency ranges much lower than the working range of tympanal organs (e.g. Göpfert and Robert 2003; Göpfert et al. 2005). Although different in external structure of their hearing organs, the underlying mechanoreceptor is the same in antennal and tympanal insect hearing organs. Examinations of auditory mechanics linked with genetic manipulations in flies allowed the tracing of active amplification on a cellular and molecular level. A prestin-homologous protein was found in auditory organs of non-mammalian vertebrates and insects (Weber et al. 2003). Its involvement in active and spontaneous antennal vibrations in the mosquito (Göpfert and Robert 2001) is still unclear. Proteins within the mechanoelectrical transduction were other thinkable possibilities for active amplification in scolopidial mechanoreceptors (Göpfert et al. 2005; 2006). For a more detailed

account on amplification processes in antennal hearing organs, please see discussion section chapters 6.4 to 6.9.

Given the possibility that tympanal organs in insects were able to actively amplify incoming faint sound as it is the case in vertebrate ears, it is so far not known up to which frequency such a mechanism would maximally operate. The generation and possible mechanical constraints of OAEs in tympanal organs that are operating at high frequency ranges are subject of the present thesis.

1.3. A big world of small ears

The acoustic sense serves several intraspecific and interspecific functions in the life of an insect, such as predator detection, mate recognition, establishing and maintaining territories and groups, social interactions, and locating hosts (Yack and Dawson 2008). Insect in general are able to detect airborne sounds in the near-field as well as in the far-field, each of which requires specialized mechanoreceptive organs. In the acoustic near-field, close to the vibrating, sound-emitting source (typically within one wavelength), the velocity of displaced air particles might be capable to deflect lightweight structures such as a hair or antenna. Such movement or displacement receivers operate only at low frequencies of up to 2 kHz. Fluctuations in air pressure which propagate further away from the sound source as a pressure wave, on the other hand, are detected by pressure receivers that generally are implemented as tympanal organs (Yack 2004). The following chapters will focus on the anatomy of tympanal organs, based on the animals used in the present studies.

1.3.1. General anatomical features of tympanal organs

Tympanal organs are complex insect hearing organs that detect airborne sounds in the far-field in a broad range of frequencies (about 0.5 to >100 kHz) in different insect species. The efficiency of signal production in very small animals such as insects increases greatly with frequencies higher than a few kHz, which is why pressure detection hearing makes quite some sense in intra-specific communication (Michelsen 1992; Yager 1999). So far, tympanal organs have been found in seven insect orders, that are, in Lepidoptera, Orthoptera, Hemiptera, Diptera, Dictyoptera, Coleoptera, and Neuroptera. Their locations on the body display a high variability depending on the respective species, ranging from head to abdomen to appendages like legs and wings (Hoy and Robert 1996).

Tympanal organs in general are anatomically characterized by tympanal membranes, an adjacent tracheal system, and one or more chordotonal organs (Yack 2004):

(1) The tympanal membrane is a thinned region of exoskeleton and detects pressure changes which are transmitted through the air. Its oval or round shape is often encircled by a distinct cuticular rim, which might help to isolate the tympanum from body movements (Yager 1999). The membrane's thickness varies considerably between species, with a general tendency of thinner membranes being more sensitive to higher sound frequencies (Yack and Dawson 2008).

The tympanum's constitution ranges from rather uniform membranes, for example in noctuid moths, to more complex non-homogeneous structures where thickness changes across the membrane, as for instance found in locusts (Yager 1999). Such structural features affect the vibration pattern of the tympanum, whose general function is to focus sound-induced vibrations onto the chordotonal sensilla (French 2008).

(2) The adjacent tracheal system generally comprises an air chamber (enlarged trachea or tracheal sac) that is confined by the internal surface of the tympanum. The acoustic impedance of this chamber is comparable to the air impedance outside. Arriving pressure changes of a sound wave emitted through the air will hence be able to set the tympanum into vibration (Yager 1999). Rare exceptions are some aquatic hemipterans whose tympana are backed by fluid (Arntz 1975).

(3) Chordotonal organs in general function as proprioceptors (to detect body movements) and exteroreceptors (to detect sounds, vibrations, or gravity) throughout the insect body. The underlying sensilla are apparently not particularly adapted to their specific sensory role (Yager 1999). In tympanal organs, chordotonal organs are associated directly or indirectly with the tympanal membranes. They comprise mechanoreceptive units, called scolopidia, which range in complexity from only one scolopidium per organ in notodontid moths to up to 2000 scolopidia per organ in some cicadas and bladder grasshoppers (Yack and Dawson 2008).

1.3.2. Scolopidial mechanoreceptors

This particular class of scolopidial mechanoreceptors is unique to Insecta and Crustacea (Field and Matheson 1998). In insect chordotonal organs that detect far-field sounds, each individual scolopidial mechanoreceptor unit consists of four cell types (Fig.1.3):

(1) one or more bipolar type 1 neurons, each of which have a single dendrite with a modified cilium, and an axon which proceeds directly towards the central nervous system;

(2) a scolopale cell (the primary distinguishing feature of chordotonal organs) that forms a receptor cavity around the dendrite's cilium and a scolopale cap (an extracellular structure) at its terminal end;

(3) attachment cells that surround the distal part of the scolopale cell and the scolopale cap and that mechanically couple the receptor to the source of the applied mechanical tension, either directly or through a ligament;

and (4) glia cells which surround the sensory neuron.

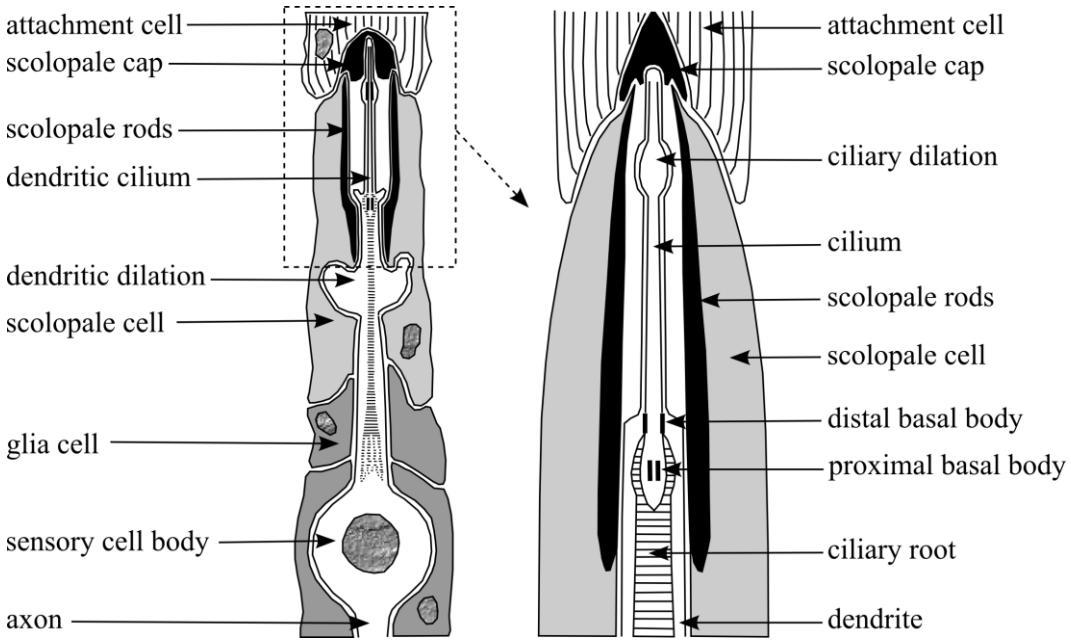

Fig.1.3 Ultrastructure of a mononematic, monodynal scolopidium from the tympanal organ of *Locusta migratoria*. Left: Schematic drawing of a scolopidium. **Right:** Detailed schematic drawing of the distal region of the scolopidium. Redrawn and modified after Gray (1960), Young (1973), and Yack (2004).

All chordotonal organs studied so far in insect tympanal organs have mononematic (they possess a scolopale cap at the cilium's terminal end), monodynal (only one dendrite associated with each scolopidium), type 1 scolopidia (ciliary segment with uniform diameter) (Fig.1.3; Gray 1960; for reviews, see Field and Matheson 1998;Yack 2004; French 2008; Yack and Dawson 2008). The functional importance of some or all of the mentioned anatomical features is not yet fully understood (see below). But their consistency in all tympanal organs points towards an essential mechanical function within the transduction process, like for instance, an especially tight coupling between vibrating structure and receptors (Field and Matheson 1998; Yack 2004; French 2008). In a few cases (geometrid moths, cicadas, mandits) and for reasons not known so far, the scolopidia posses an inverse anatomy with the scolopales pointing away from the tympanum (Yager and Hoy 1987). As a longitudinal stretching of the dendritic cilium is thought to be the adequate stimulus in scolopidia (see below), such an inverse arrangement is mechanically equivalent to the "normal" case (Yager 1999).

The dendrite's proximal inner segment holds ciliary roots and microtubules and is enlarged in form of a dendritic dilation directly proximal to the scolopale rods. The base of the scolopale rods is tied to the dendritic membrane by belt desmosomes (called dendritic collar). The distal, ciliated outer segment of the dendrite has, except for a small dilation (called ciliary dilation), a uniform diameter. The cilium stretches through a fluid-filled cylindrical cavity formed by the surrounding scolopale cell, the scolopale lumen (Yack 2004). The cilium's distal tip is coupled to the scolopale cap either by tight enclosure or insertion into the cap (Field and Matheson 1998).

Ciliary rootlets are the structural foundation for the dendritic cilium. They start out far down in the cell body at the soma or sometimes even in the axon, which might support the root's proposed function as mechanical anchor (Wolfrum 1991). The rootlets rise through the dendritic inner segment and unite into a single, cylindrical, ciliary root. More distally, it separates into nine processes that surround the proximal basal body which itself is built up by nine microtubule triplets that form a concentric ring. The nine rootlet processes then merge again into the distal basal body, which constitutes the starting point of the ciliary axoneme. The axoneme holds a ring of nine microtubule pairs without a central pair. Such a "9x2+0" assembly classifies the ciliary component within the dendritic outer segment as modified cilium. Both microtubules of a pair are hollow, with no arms, but have radial connections to the surrounding dendritic membrane at the cilium's base. Further on in the distal cilium region up to the ciliary dilation, one of the microtubules has a dense core and bears a pair of arms which are presumably the ATPase dynein (Thurm et al. 1983). Finally, distal to the ciliary dilation, the axoneme forms a clear connection (and as proposed a firm attachment) between each microtubule pair and the ciliary membrane (Field and Matheson 1998; Yack 2004).

The scolopale lumen (also called scolopale space or receptor lymph cavity) is a cylinder around the dendritic outer segment, formed by the scolopale cell. The lumen is tightly sealed, at the proximal end by the dendritic collar and at its distal end by a firm attachment via hemidesmosomes between the scolopale cell and the scolopale cap. The inner surface of the scolopale cell membrane which lies directly adjacent to the lumen is covered with scolopale rods. They are electron-dense, longitudinally oriented, intracellular structures (as opposed to the cap) and produced by the scolopale cell (Field and Matheson 1998; Yack 2004). They stretch between the scolopale cap towards the distal region of the dendritic inner segment. The rods contain mainly filamentous actin with co-localized tropomyosin and embedded longitudinal microtubules (Wolfrum 1990, 1991). The extracellular structure of the apical scolopale cap is deposited by either the scolopale cell or the attachment cell. The attachment cell couples the scolopale cell directly or indirectly (through a ligament) to the source of the applied mechanical tension.

1.3.3. Transduction in scolopidial mechanoreceptors

It is still little known about how the transduction of mechanical energy into neuronal electrical signals in tympanal organs is achieved (Yack and Dawson 2008). There are four general steps in the mechanosensory process: (1) stimulus reception (conversion of the mechanical stimuli into vibrations of the receiver), (2) coupling (transmitting these external stimuli towards the sensory neuron's transducer channels), (3) mechano-electrical transduction (mechanical deformation of a sensory cell membrane results in a variation of the receptor potential), and (4) encoding (forming specific temporal patterns of electrical impulses which are then propagated towards the central nervous system) (French 1988; Yack 2004; Albert et al. 2007a).

The functional importance of the particular anatomical features described above for each of these steps is not yet understood. A general assumption is that they play a crucial role in the tight coupling between the vibrating structure and the neuron which is essential for the detection of small fast sound vibrations. Mechanical stimuli were suggested to be transmitted by

the rigid attachment cell to the scolopale cap, resulting in lateral displacements of both cap and cilium tip, and (because all scolopidial components are mechanically linked via desmosomal structures) also of bending of the scolopale rods (Moran et al. 1977). The dendritic apex is generally proposed to be the transduction site. Stimulation of the chordotonal organ is thought to lead to a longitudinal stretching of the dendritic cilium which causes a change of the neuron's permeability at the cilium's base (French 1988). The current debate about a possible active motility of the cilium will be assessed in the discussion section 6.10. of this thesis.

A mechanical distortion of the cell membrane changes the channel opening probability (and hence the ionic conductance) between scolopale lumen and dendrite. Transduction channels in fly mechanoreceptors have been shown to open within 200 µs after the mechanical stimulus which indicated that the transduction of the stimulus occurs by direct opening of mechanically gated channels without the involvement of second messengers (Walker et al. 2000). The tightly sealed scolopale lumen holds a still unknown ionic composition that is sequestered from the hemolymph. Extrapolations from other ciliated type 1 insect mechanoreceptors, however, predict high potassium and low chloride concentrations, in analogy to receptor lymph cavities of hair sensilla. The regulated distribution of ions between scolopale lumen and cilium builds up an ionic gradient. The hence generated transepithelial potential drives the receptor current. The location of putative mechanosensitive ion channels has not yet been found (for reviews, see French 1988; Keil, 1997; Field and Matheson 1998; Eberl 1999; Yack 2004). Indirect evidence for a regulated function of the scolopale lumen came from simultaneous recordings of neuron and attachment cell in a katydid's tympanal organ (Oldfield and Hill 1986). A spike in the neuron was accompanied by the attachment cell's hyperpolarisation. This inverted reaction was suggested to be caused by an ion inflow from the scolopale lumen to the neuron upon stimulation, and the lumen being partly resupplied by a passive ion flow from the attachment cell.

Insect tympanal organs have developed different designs to guarantee high frequency hearing, while the underlying scolopidial receptor type remains the same. The tympanal organs in the insect species used for this thesis show different complexity and arrangement of mechanoreceptors, which makes each of them especially suited for specific experimental approaches. The following chapters describe the anatomical features of hearing organs in those bushcrickets and locusts which were used during this project.

1.4. The auditory system of the bushcricket *Mecopoda elongata*

Tympanal organs of bushcrickets contain structures with several morphological specifications that make them suitable for airborne sound transmission (Bailey 1990). Main site for sound input above a certain cut-off frequency is the spiracle, located posterior to the lateral pronotum (Fig.1.4a). The affiliated voluminous bullae of both sides seem to function independently, as sound transfer between them has not been demonstrated so far, but may potentially be realized in areas where they are in contact. The directly adjacent acoustical trachea within the foreleg forms an exponential horn, leading to an amplification of high-frequency sound energy above a certain cut-off frequency (e.g. Hoffmann and Jatho 1995). The subsequent trachea section has a

more or less constant diameter, and possible resonant properties are discussed (Weber 2012). The velocitiy of sound energy propagation along the trachea was found to be independent from frequency (Bangert et al. 1998). Below the knee joint, the acoustic trachea separates into two branches, with the anterior branch ending blindly. Two more or less symmetrical tympanal membranes sit at the anterior and posterior side of the foreleg tibia (Fig.1.4b). Their outer surface is subdivided into two areas, a dorsal inner plate and a surrounding membrane. Sensory response to direct sound input via the tympanal membranes, however, is restricted to frequencies below 25 kHz and requires high sound pressure levels (Hummel et al. 2011).

The peripheral scolopidial mechanoreceptor complex in the foreleg tibia consists of three parts, the subgenual organ at the proximal end, the intermediate organ, and the crista acustica at the distal end (Fig.1.4c; Yager 1999, review). The two proximal organs respond to airborne sounds and vibrations at low frequencies of up to 10 kHz. The crista acustica of bushcrickets in general is responsive to a broad sound frequency spectrum from 5 to at least 70 kHz (Oldfield 1985; Lakes and Schikorski 1990). It includes ca. 47-48 scolopidia (Weber 2004; Strauß et al. 2012) that form a linear array between the two tympanal membranes, dorsal to the anterior branch of the acoustical trachea within a hemolymph channel, and are not directly attached to the tympanal membrane. The somata of their sensory neurons are arranged close to the anterior tympanal membrane (Lakes and Schikorski 1990). The tympana themselves are partly in contact with the fluid-filled hemolymph channel and with the air-filled trachea. The receptors in the crista acustica are tonotopically organized; their best frequencies increase from proximal to distal (Oldfield 1982, 1988a; Stumpner 1996). Mechanisms for such frequency discrimination are suggested to rely on intrisic factors for the receptor's tuning (such as electrical resonance or lateral inhibition) as well as possible mechanical features of the entire organ (such as anatomical properties of distinct organ parts and the creation of sound-induced travelling waves) (Oldfield 1984, 1985; Hummel et al. 2014).

Nerve fibres of the three peripheral organ parts proximally merge into the tympanal nerve which then joins the leg nerve. Axons of the chordotonal neurons project directly from the auditory organ to the primary auditory neuropile in the prothoracic ganglion (Oldfield 1988a). Each individual receptor fiber terminates in a different area of the anterior intermediate sensory neuropile, tonotopically ordered according to its characteristic frequency (Fig.1.4d; Römer et al. 1988).

M. elongata males produce conspecific songs by moving one forewing against the other. These calling songs contain typically 10 to 14 syllables with increasing amplitude. They have a broad frequency spectrum of 2 to 80-90 kHz, with amplitude peaks around 7 kHz, 12-18 kHz, around 31 kHz, and a wide amplitude plateau in the high frequency range starting at 45 kHz (Hummel et al. 2014). The bushcricket’s hearing range extents far into the ultrasonic range of up to 70 kHz, with the highest sensitivity between 15 and 20 kHz (Hummel et al. 2011). When singing in large crowds, the males synchronize or alternate their calling songs and can maintain this condition for long periods. These song interactions establish the roles of leaders who head the chorus by a few milliseconds and their followers. Phonotaxis trials and electrophysiological measurements had the females prefering the leading males (Hartbauer et al. 2005; Fertschai et al. 2007).

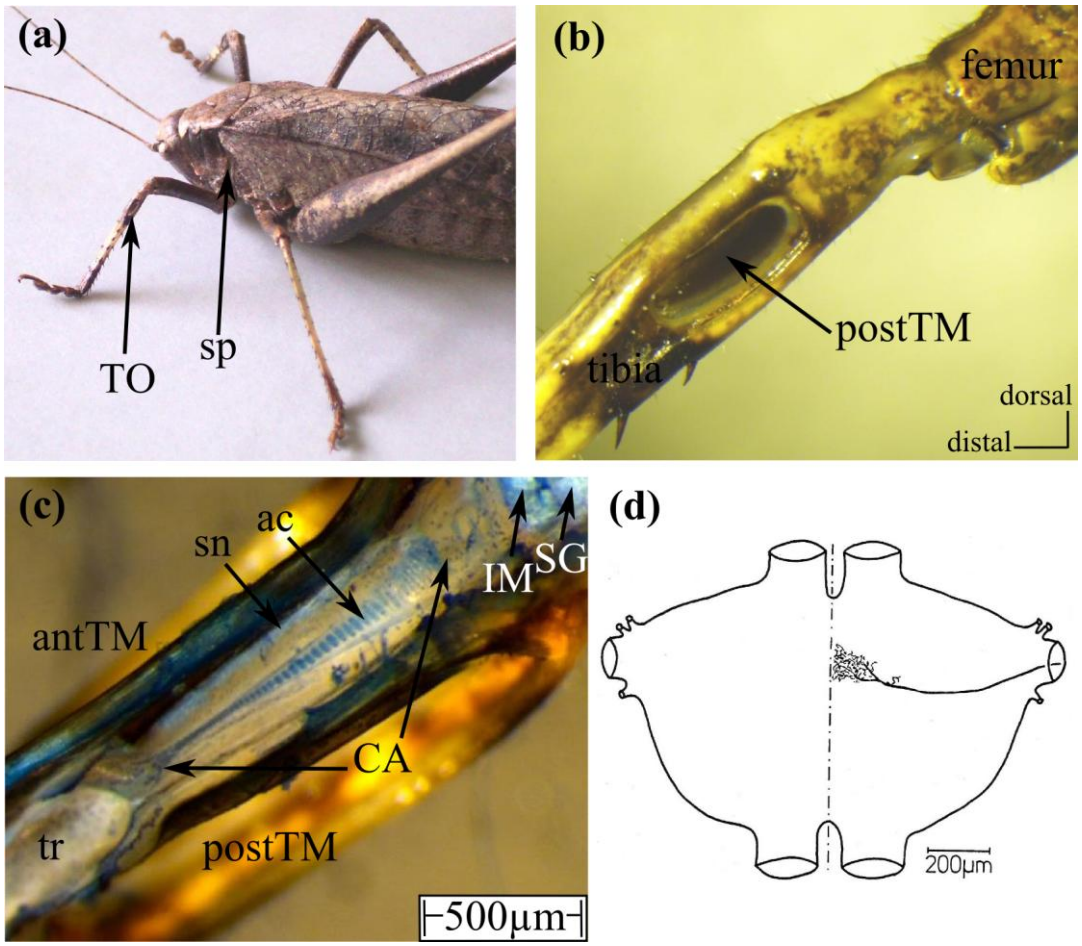

Fig.1.4 Anatomy of the auditory system in the bushcricket *M. elongata*. (a) A bushcricket with the locations of spiracle and tympanal organ indicated [sp spiracle, TO tympanal organ] **(b)** Detailed view of the foreleg tibia with one of the two tympana between which the tympanal organ is located [postTM posterior tympanum] **(c)** Exposed tympanal organ after removing the dorsal cuticle and staining with methylene blue [ac attachment cell, antTM anterior tympanum, CA Crista acustica, IM intermediate organ, post TM posterior tympanum, SG subgenual organ, sn sensory neuron, tr acoustic trachea] (changed after Kössl et al. 2008 and Weber 2012) **(d)** Terminal branching of primary auditory afferents in a bushcricket's prothoracic ganglion (from Römer et al. 1988).

1.5. The auditory system of the locusts *Locusta migratoria* and *Schistocerca gregaria*

The tympanal organ of the locust is one of the best studied hearing organs of insects. Two studies included in this thesis were performed on the migratory locust *Locusta migratoria* and the desert locust *Schistocerca gregaria*. The following chapter describes their ear anatomy. Despite of an additional small cuticle lid in front of the ear opening in *L. migratoria*, both ears are otherwise anatomically and functionally identical.

The tympanal organ of locusts is located laterally in the first abdominal segment (Fig.1.5a and b) and consists of a peripheral ganglion, called Müller's organ, a relatively large

tympanal membrane, and the adjacent tracheal system. Müller's organ comprises approximately 80 scolopidia. The tympanum is surrounded by a sclerotized cuticle ring. It is characterized by an abrupt change in thickness and stiffness between the outer thin and inner thick membrane part (Stephen and Bennet-Clark 1982). The organ is internally surrounded by an air-filled tracheal sac. Between both ears are a series of further air sacs that permit interaural sound transmission and provide the animal with a directional sense (Michelsen and Rohrseitz 1995).

Müller's organ is attached to the inside of the tympanal membrane at a spot where the two membrane regions join (Fig.1.5c). Based on the exact attachment position of the scolopidia's dendrites and their frequency tuning, four (Michelsen, 1971; Römer, 1976) or three groups of neurons (Jacobs et al., 1999) have been distinguished. Those scolopidia that are attached to the thin tympanum part at the so-called pyriform vesicle (named 'd-cells' or 'group II', respectively, by the authors mentioned above) are most sensitive to sound frequencies above 12 kHz, extending into the ultrasonic frequency range. Those coupled to the thick part of the tympanal membrane are most sensitive to lower frequencies.

Frequency analysis in the locust ear is realized primarily at the level of the tympanum. It responds to sound by travelling-wave-like vibrations whose envelopes depend on sound frequency (Windmill et al. 2005). From their initiation at the outer rim of the tympanum, waves that are induced by sound frequencies above 12 kHz are attenuated as soon as they reach the pyriform vesicle, whereas those induced by lower sound frequencies continue to travel towards the thick membrane region. Such travelling waves originate from direction-dependent properties and the mechanical gradient of the underlying vibrating structure, the tympanum (Stephen and Bennet-Clark 1982; Windmill et al. 2005). Additional to that vibration-pattern-related tuning, intrinsic properties of individual scolopidia could be partially responsible for their respective frequency tuning characteristics (Yack 2004). Sustained tracheal air pressure changes and hence tympanum displacements as it would occur during ventilation influences the vibration pattern of the tympanal membrane and the responses of auditory receptors to acoustic stimuli (Meyer and Hedwig 1995). It generally shifts the tympanum's resonance response toward higher frequencies, reducing low-frequency components of the incoming sound by about 15 dB SPL and enhancing high frequencies by about 7 dB SPL.

Axons of the scolopidial neurons of the tympanal organ project into the anterior intermediate sensory neuropile of the metathoracic ganglion (Fig.1.5d). A tonotopical organization is established within the neuropile, as projections from receptors tuned to high and low frequencies terminate in different neuropile areas (Halex et al. 1988; Römer et al. 1988).

Migratory locusts produce conspecific songs that show a broad spectrum of dominant frequencies from 6 to 16 kHz and a characteristic frequency at 7.84 ± 0.57 kHz (Meyer and Elsner 1996). Combined threshold curves of the four receptor groups within the organ cover a broad hearing range, with two minima at ca. 4-8 kHz and 12-20 kHz (Römer 1976).

In general, Orthoptera use their hearing sense primarily for conspecific communication and secondarily for predator detection (Yack and Dawson 2008). Mating behavior and aggressive interactions induced by intra-specific communication have been shown for locusts, and they display negative phonotaxis in response to batlike signals (Robert 1989; Hoy et al. 1989; Miller and Surlykke 2001). The avoidance response was provoked by stimuli above 10

kHz and 45 dB SPL, whereas lower frequencies did not evoke phontactic steering. Based on the reaction's relatively low threshold, this behavior to avoid acoustic detection of echolocating predators was suggested to be some kind of early-warning system (Robert 1989).

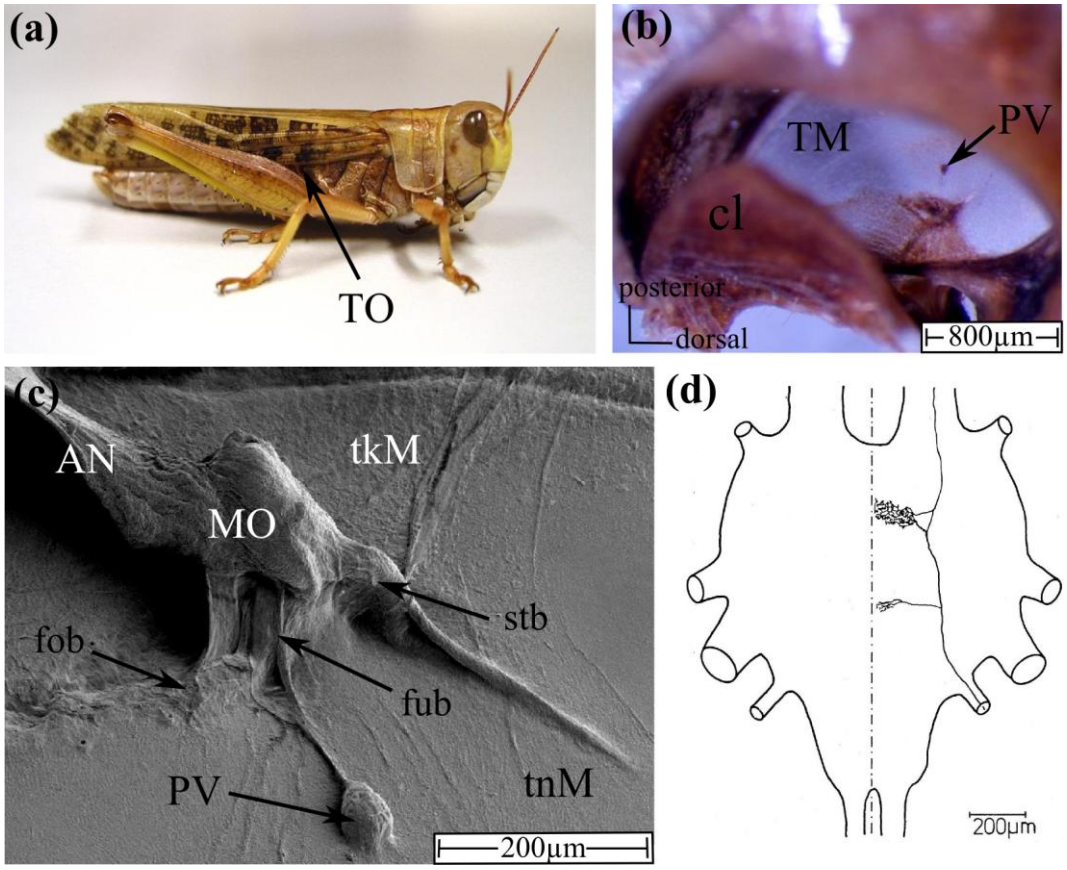

Fig.1.5 Anatomy of the auditory system in the locust *L. migratoria*. (a) A migratory locust with the location of the tympanal organ in the first abdominal segment indicated [TO tympanal organ]. **(b)** Detailed outside view of the tympanal organ [cl cuticular lid, PV pyriform vesicle, TM tympanal membrane]. **(c)** Scanning electron micrograph of the peripheral ganglion attached on the inside of the tympanum [AN auditory nerve, fob folded body, fub fusiform body, MO Müller's organ, PV pyriform vesicle, stb styliform body, tkM thick part of the membrane, tnM thin part of the membrane] **(d)** Terminal branching of primary auditory afferents in a locust's metathoracic ganglion (from Römer et al. 1988).

1.6. Aims and questions

Such diverse auditory systems as those of vertebrates and insects possess remarkable similarities, despite their differences in end organ structure and mechanisms (Peng and Ricci 2011). Tympanal hearing organs have scolopidial sensilla whose detecting organelle is embedded in an apical extracellular structure, the scolopale cap, comparable to the connection of mammalian outer hair cell bundles and tectorial membrane. Both types of hearing organs are frequency tuned and tonotopically organized. The most eminent difference of mammalian ears in comparison to those of insects are the divergence of innervation patterns and the separated function of amplification and signal transduction between hair cells. Regarding their mechanotransduction characteristics, scolopidial receptors share physiological similarities to vertebrate hair cells: an endolymph holding high K^+ concentrations which is sustained by supporting cells, a directional selectivity of the receptors, response latencies in the microsecond range, a nanometre-scale sensitivity, and voltage- and size-dependent adaptation processes (Gillespie and Walker 2001).

The overall aim of this thesis was to determine the source of sensitive, nonlinear hearing at high frequencies and of the generation of OAEs in tympanal organs of insects. Which sensory or attachment cells might be involved? Can any suggestions be made about responsible cellular components within scolopidial mechanoreceptors? Are there hints of mechanical amplification in these insect ears that operate at frequency ranges comparable to those of most mammals?

1.6.1. Evidence of a scolopidial involvement in DPOAE generation from a new animal model

Scolopidial receptor structures are associated with DPOAE generation in a frequency-specific way (Möckel et al. 2007). The procedures in this particular study included mechanical lesions which, however small of diameter (8-20 µm), disrupted both the scolopidial attachment and the tympanum itself. It could not be ruled out that the tympanum's mechanical properties had not been disturbed. I therefore sought for a new approach to specifically manipulate the scolopidia without affecting surrounding structures of the tympanal organ. Different anatomical features of the bushcricket ear compared to that of locusts allowed a controlled application of fluid substances to the organ. The method itself needed to be reliable and stable, without affecting the organ's integrity and ability to generate DPOAEs. The pharmacological substances which were applied had known effects on sensory cells, attachment cells and/or their cell components, in order to correlate possible changes in DPOAE generation to receptor mechanisms. The tested substance in this respective study was the neuroactive insecticide pymetrozine which acts highly effective and selectively on chordotonal organs, without affecting other sensory organs that lack scolopidial receptors (Ausborn et al. 2005).

The questions concerned in this part of my thesis were therefore: How can scolopidial receptors be manipulated and possibly disabled without interfering with the organ’s mechanical integrity? Does the neuroactive insecticide Pymetrozine that acts selectively on those auditory receptors have any effect on DPOAE generation?

1.6.2. Possible cell components involved in DPOAE generation in insects

Evidence of the involvement of scolopidia in DPOAE generation was brought about by studies on locusts (Möckel et al. 2007) and bushcrickets (Möckel et al. 2011). Both suggested a biological origin of acoustic two-tone distortions. Such mechanisms should involve metabolic processes, whose temperature-dependence would directly affect the DPOAE generation. A relationship between body temperature shifts, the rate of potential DPOAE changes, and the hence calculated activation energy would provide conclusions on possible mechanisms involved in non-linear hearing in tympanal organs.

The respective study therefore addressed the following questions: Which components of the intracellular machinery could contribute to mechanical non-linearity? Is non-linear hearing in tympanal organs of locusts based on mechanical principles similar to those found in Johnston's organs of mosquitoes and flies that operate at much lower frequency ranges?

1.6.3. Mechanical DPOAE analogues within the tympanum motion

Otoacoustic emissions in vertebrates are defined as air pressure fluctuations recorded in the outer ear canal (Kemp 1978). They are caused by vibrations of the eardrum driven by the cochlea and recordable with sensitive microphones. Relating that to tympanal organs, the question was whether DPOAEs occur within the tympanal membrane motion during stimulation with two pure tones. Such additional tympanum displacements at the emission frequency would be expected around the attachment position of the scolopidial receptors. Acoustical DPOAE recordings were combined with mechanical measurements of DPOAE analogues within the tympanum's movement, using laser Doppler vibrometry. The desert locust was chosen for its relatively large, freely accessible tympanum which acts as a pressure receiver (like the mammalian ear) and the directly attached scolopidial mechanoreceptors (which is in evident contrast to the situation found in mammals where any direct or indirect interference from the interposed middle ear are subject for discussions; for overview, see Janssen and Müller 2008). Provided that scolopidia are the source of OAE generation, their operations should directly act onto the tympanum.

The questions addressed in this respective study were therefore: Are mechanical correlates of DPOAEs measurable within the tympanum movement during stimulation with two pure tones? Are there any distinctive features in the vibration pattern of such internally evoked displacements which contrast them to externally evoked tympanum movements? Which consequence has the selective exclusion of high-frequency receptors on the mechanical DPOAE analogues as well as the wave forms induced by the two stimuli?

2. Otoacoustic emissions in bushcricket ears: general characteristics and the influence of the neuroactive insecticide pymetrozine

Doreen Möckel, Ernst-August Seyfarth, and Manfred Kössl

Journal of Comparative Physiology A (2011) 197:193-202

Author contributions Experiment design by all authors; preliminary test, controls and all experiments performed by D. Möckel; data collection and presentation by D. Möckel; data analysis by D. Möckel with consultation from E.-A. Seyfarth and M. Kössl; D. Möckel wrote the manuscript with contributions from all authors.

Abstract The tympanal organ of the bushcricket *Mecopoda elongata* emits pronounced distortion-product Otoacoustic emissions (DPOAEs). Their characteristics are comparable to those measured in other insects, such as locusts and moths, with the 2f1–f2 emission being the most prominent one. Yet the site of their generation is still unclear. The spatial separation between the sound receiving spiracle and the hearing organ in this species allows manipulations of the sensory cells without interfering with the acoustical measurements. We tried to interfere with the DPOAE generation by pharmacologically influencing the tympanal organ using the insecticide pymetrozine. The compound appears to act selectively on scolopidia, i.e., the mechanosensor type characteristically constituting tympanal organs. Pymetrozine solutions were applied as closely as possible to the scolopidia via a cuticle opening in the tibia, distally to the organ. Applications of pymetrozine at concentrations between 10^{-3} and 10^{-7} M to the tympanal organ led to a pronounced and irreversible decrease of the DPOAE amplitudes.

Anlage 1

Erklärung über Anteile der Autoren/Autorinnen an den einzelnen Kapiteln der Promotionsarbeit

Titel der Publikation/ des Manuskripts:	**Möckel D, Seyfarth E-A, Kössl M (2011) Otoacoustic emissions in bushcricket ears: general characteristics and the influence of the neuroactive insecticide pymetrozine. J Comp Physiol A 197:193-202**	
	Was hat der/die Promovierende bzw. was haben die Co-Autoren/Autorinnen beigetragen[#]	Name des/der jeweiligen Autors/Autoren/Autorin*
(1) Entwicklung und Planung	Möckel D 25%, Seyfarth E-A 50%, Kössl M 25%	Möckel D, Seyfarth EA, Kössl M
(2) Durchführung der einzelnen Untersuchungen/ Experimente	vorbereitende Tests und Verhaltensversuche mit der Substanz an Laub- und Feldheuschrecken; Vorversuche an Laubheuschrecken zur optimalen Präparation; Kontrollversuche, um die Stabilität der Präparation zu testen; Durchführung aller Experimente	Möckel D
(3) Erstellung der Daten-sammlung und Abbildungen	Auslesen der Rohdaten; Erstellung aller Abbildungen der Publikation	Möckel D
(4) Analyse/Interpretation der Daten	Analyse der Rohdaten; statistische Zusammenfassung und Auswertung der Daten	Möckel D
	Diskussion der Daten	Seyfarth E-A, Kössl M
(5) übergeordnete Einleitung/ Ergebnisse/Diskussion	Möckel D 80%, Seyfarth E-A 10%, Kössl M 10%	Möckel D, Seyfarth EA, Kössl M
[#]Bei 2, 3 und 4 bitte kurze inhaltliche Angaben der jeweiligen Anteile, bei 1 und 5 reichen prozentuale Angaben		*Mehrfacheintragungen möglich

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ORIGINAL PAPER

Otoacoustic emissions in bushcricket ears: general characteristics and the influence of the neuroactive insecticide pymetrozine

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Keywords DPOAE · Tympanal organ · Chordotonal organ · Scolopidium

Abbreviations

DPOAE	Distortion-product otoacoustic emission
SPL	Sound pressure level

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Introduction

The tympanal hearing organs of insects such as locusts, bushcrickets and moths produce distortion-product otoacoustic emissions (DPOAEs; Kössl and Boyan 1998a, b; Coro and Kössl 1998, 2001; Kössl and Coro 2006; Möckel et al. 2007; Kössl et al. 2007). DPOAEs are evoked by simultaneous stimulation of the hearing organ with two pure tones (f1 < f2). They appear as spectral peaks of nf1–(n−1)f2 and nf2–(n−1)f1, with the 2f1–f2 emission being the most prominent one (see, e.g., Fig. 1a). They reach their largest amplitudes at frequencies of species-specific high auditory sensitivity, and can, in locusts and moths, be evoked by stimulus levels near the auditory threshold (Kössl et al. 2008).

Similar to the situation found in vertebrate inner ears, insect DPOAEs are a by-product of non-linear sound processing in the hearing organs. They are vulnerable to manipulations that affect the physiological state of the animal (Kössl and Boyan 1998a, b; Coro and Kössl 2001; Kössl et al. 2007) and are influenced by changes of the membrane potential caused by electrical stimulation of the auditory afferents (Möckel et al. 2007). Local lesions that affect a particular group of auditory neurons (i.e., the d-cells) tuned to high frequencies in the locust ear delete only those DPOAEs that are evoked by stimulus tones above 15 kHz (Möckel et al. 2007). This suggests that scolopidia, i.e., a particular type of mechanosensor characteristic of all chordotonal organs (including tympanal organs), are directly involved in the frequency-specific DPOAE generation in insect ears.

DPOAEs in insect ears resemble those recorded from vertebrates. But the mechanism of their generation is still unclear, while two possibilities exist in vertebrates. In the case of reptiles and birds, active sensory hair-bundle

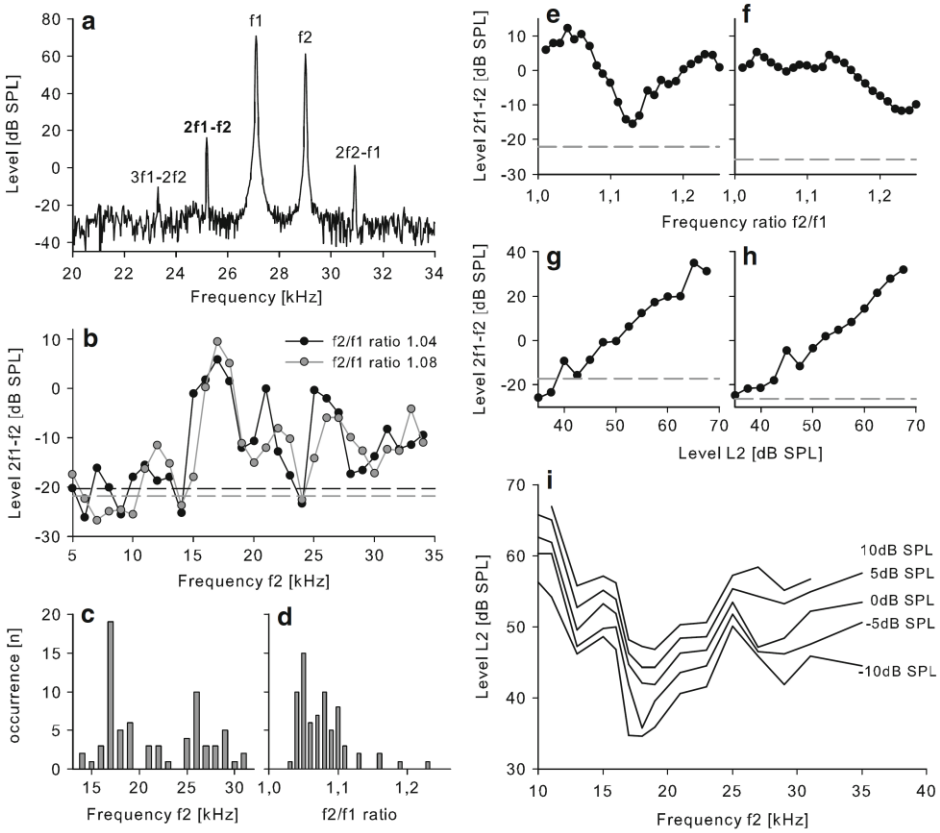

Fig. 1 General characteristics of DPOAEs in the bushcricket *Mecopoda elongata*. **a** Example spectrum of a DPOAE measurement (f1 27.1 kHz; f2 29 kHz; L1/L2 70/60 dB SPL). **b** DPOAE audiograms for f2 frequencies between 5 and 34 kHz, both measured in the same animal (L1/L2 60/50 dB SPL; *black line* f2/f1 ratio 1.04; *gray line* f2/f1 ratio 1.08). Dashed lines show the respective noise level for each DPOAE audiogram expressed as the average + 1 SD. **c** Summary of the f2 frequencies that evoked high emission levels in DPOAE audiograms and were chosen for further investigations. **d** Summary of the best f2/f1 ratios that were determined for the f2 frequencies used in the presented experiments. **e**, **f** Level of DPOAEs plotted against the f2/f1 ratio (e: f2 17 kHz; f: f2 27 kHz; L1/L2 60/50 dB SPL). *Dashed lines* show the noise level expressed as the average + 1 SD. **g**, **h** DPOAE growth functions (g: f1 16.33 kHz, f2 17 kHz; h: f1 23.90 kHz, f2 27 kHz). The level of f1 was always 10 dB above that of f2. *Dashed lines* show the noise level expressed as the average + 1 SD. The data in e–h were all obtained in the same preparation. **i** 2f1–f2 DPOAE threshold curves measured in one individual animal. The levels of f2 that were sufficient to evoke a DPOAE of −10, −5, 0, 5, and 10 dB SPL (threshold criteria) were extrapolated from DPOAE growth functions. The f1 level was 10 dB above that of f2

motility seems to be the main source of non-linear mechanical amplification (Manley 2001). In mammals, cochlear amplification is based on the electro-motility of outer hair cells produced by the motor protein prestin (see, e.g., Dallos and Fakler 2002), and the possible involvement of active hair-bundle motility is still discussed (e.g., Liberman et al. 2004).

Tympanal organs are complex insect hearing organs which are anatomically characterized by tympanal membranes, an adjacent tracheal system, and one or more chordotonal organs (for an overview, see Yack 2004). In bushcrickets, structures suitable for airborne sound transmission include the spiracle (main site for sound input above a certain cut-off frequency, posterior to the lateral pronotum), the acoustical trachea (running towards the foreleg tibia), and two tympanal membranes (on both sides of the foreleg tibia). Chordotonal organs in general function as proprioceptors and exteroreceptors throughout the insect body. In tympanal organs, they are associated directly or indirectly with the tympanal membranes. They comprise one or more mechanoreceptive units, called scolopidia, each of which consists of one to four bipolar sensory neurons and accessory cells. The part of the tympanal organ of *Mecopoda elongata* which perceives airborne sound from 5 to 50 kHz is called crista acustica, and includes ca. 47 scolopidia (Weber 2004) that form a linear array between the two tympanal membranes, dorsal to the acoustical trachea, with the somata of the sensory neurons arranged close to the anterior tympanal membrane (for an overview, see Lakes and Schikorski 1990). The receptors in

the crista acustica are tonotopically organized; their best frequencies increase from proximal to distal (Oldfield 1982, 1988; Stumpner 1996).

Bushcrickets as a model to study DPOAEs in insects hold great experimental advantages, as it is possible to manipulate the function of the tympanal organ without disturbing the simultaneously performed acoustical measurements because the main site of sound input and the site of its perception are anatomically separated. This methodical approach is, for anatomical reasons, not possible in other animal models such as locusts or moths.

The aim of the present study was to test if the insecticide pymetrozine affects mechanical sound processing in tympanal organs. Pymetrozine was developed primarily against plant-sucking insects. Contact with the compound does not cause general neuronal intoxication, but rather a specific impairment of feeding which leads to starvation (Harrewijn and Kayser 1997). Migratory locusts, in addition, showed intoxication symptoms by fully extending their hindlegs, indicating a direct impact on the neurosensory or motor systems (Kaufmann et al. 2004). Studies on the femur–tibia joint control loop in locusts revealed pymetrozine's high potency and selectivity for chordotonal organs, including the femoral chordotonal organ that monitors the femur–tibia joint, as well as other chordotonal organs that are associated with the wing hinge stretch receptor and the tegula (Ausborn et al. 2005). These organs ceased responding after direct application of pymetrozine. Pymetrozine had no influence, however, on other examined sensory organs which do not possess scolopidia, such as campaniform sensilla or hair sensilla. These findings point to specific effects of pymetrozine on receptors of the scolopidial type. Our present study shows that application of pymetrozine to the tympanal organ is followed by a pronounced decrease of DPOAE amplitudes. The results indicate that scolopidial structures are critically involved in the generation of DPOAEs in insect ears.

Materials and methods

Animals and preparations

Tropical bushcrickets (*M. elongata*) were raised from a breeding colony in our laboratory. They were kept at room temperature (ca. 25°C) and relative humidity of 50–60%. All experiments reported here were carried out with adult animals. We used both males and females, as previous studies with other tettigoniids used both sexes and did not report any obvious differences in the functional anatomy of their ears (Oldfield 1982; Stumpner 1996). After anesthetizing the animals with CO_2, the 2nd and 3rd leg-pairs and the wings were removed. The animals were then tethered with their thorax onto a metal holder using rosin-beeswax. The forelegs were also fixed with beeswax to two supports to maintain both legs in an extended position. For better access to the spiracle entrance, the surrounding cuticle flap was partly removed under visual control. Recovery from anesthesia was verified by observing normal breathing movements and normal reflexes upon touching the antennae. During the DPOAE measurements, CO_2 was not reapplied, and the animals remained awake. After the experiments, they were re-anesthetized and sacrificed by decapitation.

Measurement of distortion-product otoacoustic emissions

The experimental setup and measuring procedures followed previous reports, as described by Möckel et al. (2007) and Kössl et al. (2008). The generation of the two acoustic stimuli and the analysis of the measured microphone signal were accomplished by custom software which controlled a Microstar DAP 840 DSPcard (sampling rate 200 kHz). The two output channels were connected to two attenuators (TDT System3 Tucker Davis Technologies), an amplifier, and two 1 in. microphones (Microtech Gefell, Germany, Type 103.1) serving as loudspeakers. The acoustic signals were measured by an additional 1/2 in. microphone (Brüel & Kjaer 4133) connected to the input channel of the DSP-card by a preamplifier (Brüel & Kjaer 2660) and an amplifier (Brüel & Kjaer 2610). The two adjacent channels for stimulation with f1 and f2 and the recording of the microphone signal were brought together in an acoustic coupler. The tip diameter of the acoustic coupler was adapted to the size of the spiracle of the bushcrickets. Tethered animals were placed in a soundproof chamber whose interior was kept at room temperature (maximum fluctuations ± 1°C). The sound system was calibrated in situ, with the coupler in measuring position at the entrance of the spiracle. During acoustical stimulation with two pure tones of different frequencies ($f1 < f2$), the level of the f1 stimulus was chosen to be 10 dB above that of the f2 stimulus, as this induces maximal DPOAE levels (Kössl and Boyan 1998a, b). The microphone signal was averaged 100–300 times before fast Fourier transform (FFT) analysis. Each presentation of a pair of pure-tone stimuli added up to 4.2 s for 100 averages, making the measurement of a DPOAE growth function about 1.5–2 min long, depending on the number of level steps.

Recordings of DPOAEs included the following procedures: DPOAEs were elicited over a wide frequency range (5–34 kHz) with constant frequency separation and level of the two stimuli (f2/f1 ratio 1.08; 60/50 dB SPL) to obtain so-called DPOAE audiograms. From these audiograms, the

f2-stimulus frequencies evoking large 2f1–f2 emissions were chosen for further measurements. The respective f1 frequency was adjusted according to the optimum f2/f1 ratio that evoked the highest 2f1–f2 emission. To obtain DPOAE growth functions, both stimulus frequencies were kept constant and their levels were increased in 2-dB steps, starting from 30/20 or 40/30 dB SPL and reaching up to 74/64 to 80/70 dB SPL, with the maximum stimulus levels fixed for each respective experiment to avoid any setup-generated distortions. DPOAE frequency threshold curves were interpolated from such growth functions for threshold criteria of −10, −5, 0, 5, and 10 dB SPL DPOAE levels at different f2 frequencies. The background noise level was measured 100 Hz below the DPOAE frequency.

The measurement of DPOAE audiograms and the determination of the best f2/f1 ratio for the chosen f2 frequency were only performed once, at the beginning of each respective experiment. Once both stimulus frequencies, f1 and f2, had been determined, they were kept constant for all consecutive growth functions.

Application the test substances

Pymetrozine was dissolved in saline according to Hoyle (1953): NaCl 8,2 g/l; $CaCl_2$ 0,22 g/l; $MgCl_2$ 0,19 g/l; $KHCO_3$ 0,4 g/l; KH_2PO_4 0,82 g/l. Contrary to previous studies (Harrewijn and Kayser 1997; Kaufmann et al. 2004; Ausborn et al. 2005), in which the authors first dissolved pymetrozine in dimethylsulfoxide (DMSO) before dilution with saline, we dissolved the compound merely in saline, as preliminary experiments had shown that DMSO affects DPOAE amplitudes, even at low concentrations. Pymetrozine was tested at concentrations of 10^{-3} M ($n = 7$ preparations), 10^{-5} M ($n = 7$), 10^{-7} M ($n = 6$), and 10^{-9} M ($n = 5$). A total of 33 preparations were tested for effects after pymetrozine application, of which 8 had to be rejected. The rejection was caused either by disturbed measurements, as the respective animals moved too much, or when the substance drop was not taken in by the hemolymph, which was controlled throughout the duration of the experiments.

Our aim was to apply the test substance as closely as possible to the sense organ without disturbing the mechanics of the tympanal organ and its surrounding structures. The foreleg tibia was opened under visual control by removing a piece of cuticle with the help of a micro-scalpel. The location of the opening was chosen to lie on the dorsal side of the leg distally of the tympanal membranes (see Fig. 2a). The tissue directly underneath the cuticle was kept intact, as a lesion in the trachea leads to the loss of all DPOAEs. The test substance was applied once per organ on top of the opening in amounts of ca. 1.5 µl per trial using a syringe. The test substance was taken up by hemolymph and thus reached the crista acustica. Preliminary tests with tracer solutions confirmed that the applied substance was transported across the entire organ (methylene blue in less than 10 min; Indian ink in less then 40 min). In order to open the tibia cuticle, the acoustic coupler had to be retracted, and the animal was taken out of the sound proof chamber. Afterwards, the animal (on its holder) was placed in the sound proof chamber again, and the acoustic coupler was re-positioned. The integrity of the tympanal organ was controlled by DPOAE measurements after the tibia had been opened. The spatial separation of the acoustic coupler from the actual site of sound perception, i.e., the tympanal organ in the tibia, allowed an application of the test substance without removing the acoustic coupler from the spiracle entrance. DPOAEs were recorded immediately after the drop was applied, and then at intervals of 5–10 min for at least 60 min. The optimum ratio of the stimulus frequencies f1 and f2 was determined only pre-operatively and was not checked again after opening the tibia cuticle and applying the substance. Once determined, f1 and f2 were kept the same for all following growth function measurements in that respective animal, which are, (1) with an intact organ, (2) after opening the tibia cuticle, and (3) at certain time points after the application of the test substance, to provide comparability between measurements throughout the course of the experiments. During a different set of experiments with the same agent (data not shown), the best f1/f2 ratio was defined separately at each time point and the growth functions were measured with the respective frequency pair. The hereby determined best ratios at each time point differed only to a small amount compared to the ones found at the beginning of the procedure, with the absolute DPOAE amplitudes continuously descending during the time course after pymetrozine application.

Control measurements

Control measurements were performed in order to rule out that the application itself might influence the DPOAEs. The following control conditions were assessed: (1) the stability of the DPOAE amplitudes in an intact animal without any further manipulation ("intact"; $n = 6$); (2) controlling the integrity of the DPOAEs after opening the cuticle distally of the tympanal organ ("opening"; $n = 7$); and (3) estimating the possible influence of applying saline drops of the same volume as used in the actual pymetrozine tests ("saline"; $n = 11$).

Data analysis

Each experiment started with the measurement of a DPOAE growth function (given as "0 min"). It was then

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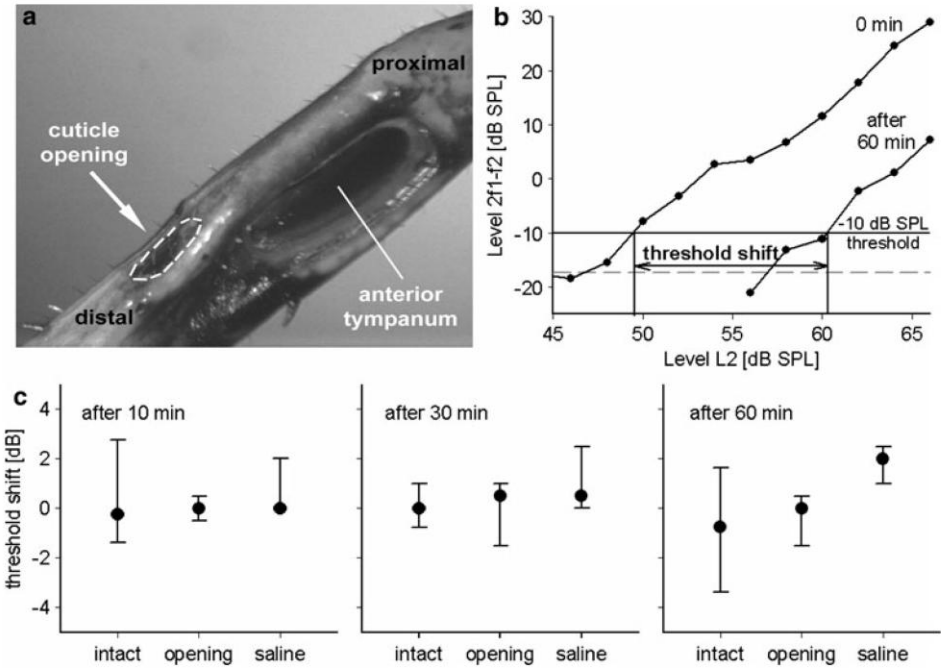

Fig. 2 **a** Dorso-lateral view of the right foreleg tibia of *M. elongata*. Indicated are the location and size of the cuticle opening. **b** Two examples of DPOAE growth functions measured before ("0 min") and 60 min after application of 10^{-7} M pymetrozine ("60 min"). From each growth function, the −10 dB SPL threshold was determined as the f2 stimulus level that was sufficient to evoke a 2f1–f2 DPOAE of −10 dB SPL. The threshold shift between these two growth functions was used to quantify DPOAE changes due to manipulations at the organ. f1 28.98 kHz; f2 31 kHz. *Dashed line* shows noise level expressed as the average + 1 SD. **c** Control measurements showing the DPOAE threshold shift within 10, 30, and 60 min in intact animals ("intact", $n = 6$ organs), after opening the tibia cuticle ("opening", $n = 7$ organs), and after application of saline to the organ ("saline", $n = 11$ organs). Values are given as median with the 25th and 75th percentiles

followed either by control recordings ("intact", "opening", and "saline") or by the application of pymetrozine at different concentrations. The experimental paradigm included the measurement of several DPOAE growth functions, one immediately before each respective manipulation on the organ, and later repetition at certain time intervals. The −10 dB SPL thresholds (i.e., the L2 level which is sufficient to evoke an emission of −10 dB SPL) were obtained from both the "0 min" growth function (i.e., the one obtained pre-operatively) and any subsequently measured growth function. The shifts that occurred between the threshold of the "0 min" growth function and the subsequent ones were calculated, and are hence called threshold shifts. For example, the "10 min threshold shift" represents a shift in the −10 dB SPL threshold that occurred between the pre-operative growth function and the one obtained 10 min after a certain manipulation. These threshold shifts were determined for all control recordings as well as for the pymetrozine experiments at each tested concentration.

Figure 2b shows a representative example of such calculations for two growth functions (0 and 60 min after 10^{-7} M pymetrozine). The −10 dB SPL threshold is marked, as well as the corresponding L2 levels at which the threshold is reached in both growth functions. The shift between these values, hence called "threshold shift", is marked with an arrow. This threshold shift between growth functions served as the basis for a statistical evaluation of the observed effects. In the present example, the growth function measured before application of pymetrozine reached the threshold at an L2 level of 49.4 dB SPL. 60 min after application, DPOAE amplitudes strongly decreased, and the threshold was at 60.4 dB SPL. Hence the threshold shift between 0 and 60 min amounted to 11 dB.

The threshold shifts that occurred in each experimental condition were averaged as the medians and the 25th and 75th percentiles (i.e., their range contained 50% of all threshold shift values of each particular group). The values of each experimental group were compared with those obtained for the other treatment groups and tested for statistically significant differences (Wilcoxon rank sum test for unpaired samples, performed with the Statistics Toolbox of Matlab R2007b). The null hypothesis "no difference"

was rejected at p values <0.05 (indicated with *), <0.01 (**), and <0.001 (***).

Results

Basic properties of DPOAEs in the bushcricket *M. elongata*

The 2f1–f2 emission is the most prominent in the bushcricket ear, as is the case in tympanal organs of other insects, such as locusts and moths. All results presented in this study focus on the 2f1–f2 emission. Additional emissions such as the 2f2–f1 and 3f1–2f2 emission appear successively with increasing levels of the primary tones (see the example in Fig. 1a).

DPOAE audiograms were measured at the beginning of each experiment and showed 1–6 emission level maxima (for f2/f1 ratio 1.04: median 2 maxima ± 1.1; for f2/f1 ratio 1.08: median 3 maxima ± 1.1). Their exact frequencies depended on the individual animal and on the used f2/f1 ratio. Two such measurements, recorded with an f2/f1 ratio of 1.04 and of 1.08 in the same animal, are provided in Fig. 1b. The highest emission level was reached at f2 frequencies of 17, 21, and 25 kHz for a ratio of 1.04, and at 17, 27, and 33 kHz for a ratio of 1.08.

One or two f2 frequencies that evoked high emission levels in DPOAE audiograms were chosen for each organ for further investigations. Figure 1c summarizes those chosen f2 frequencies, and shows a clear prevalence of auditory maxima around 17 and 26 kHz. For f2 frequencies between 5 and 20 kHz, the median f2 frequency was 17 ± 1.23 kHz. For f2 frequencies between 21 and 34 kHz, the median f2 frequency was 26 ± 2.77 kHz.

2f1–f2 levels greatly depended on the ratio of the two stimulus frequencies f1 and f2. They showed two distinct maxima, usually in the range of small and of large f2/f1 ratios. Figure 1e shows such an example at an f2 frequency of 17 kHz, with 2f1–f2 level maxima at a frequency ratio of ca. 1.04 and 1.23. In other cases, the 2f1–f2 level remained on a high level over a wide range of f2/f1 ratios and then decreased with growing f2/f1 ratios (Fig. 1f; f2 frequency of 27 kHz). Figure 1d summarizes the determined best f2/f1 ratios during the experiments presented here, showing a clear prevalence of small ratios between 1.04 and 1.10 (median ratio 1.07 ± 0.036).

To measure DPOAE growth functions for a given f2 frequency (Fig. 1g, h), the respective f1 frequency was adjusted according to the f2/f1 ratio that evoked maximum 2f1–f2 levels. The 2f1–f2 level grew with increasing stimulus levels and reached values well above noise level. In a few cases, the growth functions displayed a small notch at intermediate stimulus levels.

Frequency threshold curves were generated by extrapolating the level of f2 that was sufficient to evoke a DPOAE of −10, −5, 0, 5, and 10 dB SPL (threshold criteria) from DPOAE growth functions obtained for different f2 frequencies (Fig. 1i). The frequency threshold curves showed a pronounced minimum at f2 frequencies of 17 and 18 kHz, where stimulus levels of about 45/35 dB SPL were sufficient to evoke an emission of −10 dB SPL. Additional, but less distinct minima appeared at f2 frequencies of 13 and 29 kHz. Local threshold maxima occurred at f2 frequencies of 15 and 25 kHz, where stimulus levels of ca. 60/50 dB SPL were required to evoke an emission of −10 dB SPL.

Control measurements

Threshold shifts which occurred under the three control conditions, which were, “intact”, “opening” and “saline”, are presented in Fig. 2c. No significant differences were found between these control groups when tested against each other ($p > 0.05$).

Pymetrozine reduces DPOAE amplitudes in *M. elongata*

Figure 3 provides representative examples of growth functions that were measured before and after the application of pymetrozine at two different concentrations. After application of 10^{-3} M pymetrozine (Fig. 3a), the DPOAE amplitudes decreased by 5–15 dB within the first 10 min, changed only slightly until 30 min after application, and were further reduced by 5–10 dB after 60 min. For 10^{-7} M pymetrozine (Fig. 3b), the DPOAE amplitudes measured 10 min after application varied within a range of 5 dB compared to the measurement before applying the substance, decreased by 5–10 dB after 30 min, and dropped by another 8–15 dB after 60 min. Application of 10^{-9} M pymetrozine, the lowest concentration used in this study, did not have any obvious effect on the DPOAE levels (data not shown in detail).

As observed for half the applications (10^{-3} M: 5 out of 7; 10^{-5} M: 2 out of 7; 10^{-7} M: 3 out of 6), pymetrozine caused at the effective concentrations a different decreasing behavior of the growth functions, in a way that those DPOAE amplitudes evoked by lower stimulus levels were more affected than the ones evoked by higher stimulus levels. In the other cases, the growth functions showed an equal down-shift of the DPOAE amplitudes across all stimulation levels. The reasons for this unequal effect on the growth function behavior depending on the stimulus levels must remain unclear.

Figure 4 summarizes the threshold shifts that were observed in growth functions measured after the

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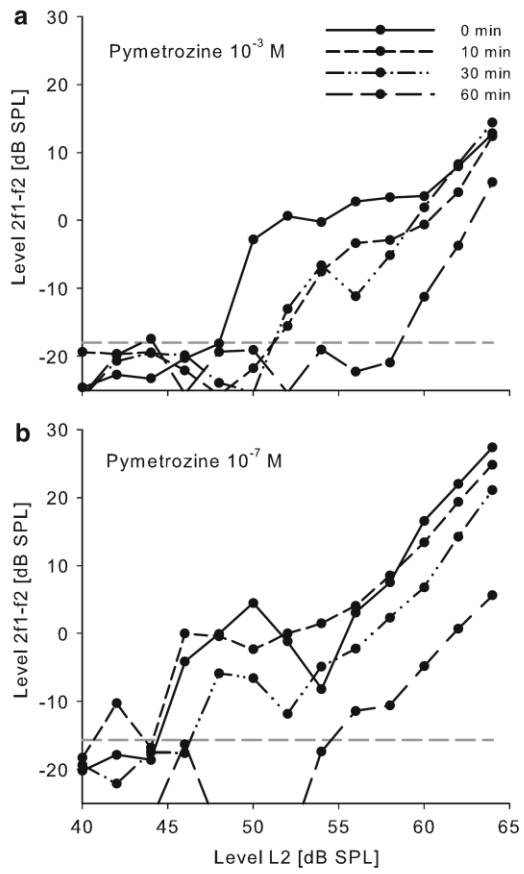

Fig. 3 Examples of the effect of two different pymetrozine concentrations on DPOAE growth functions. Measurements before (0 min) and 10, 30, and 60 min after application are indicated by the 4 different line patterns. **a** Pymetrozine concentration 10^{-3} M; f1 24.048 kHz; f2 25 kHz. **b** Pymetrozine concentration 10^{-7} M; f1 24.755 kHz; f2 26 kHz. *Dashed line* shows noise level expressed as the average + 1 SD

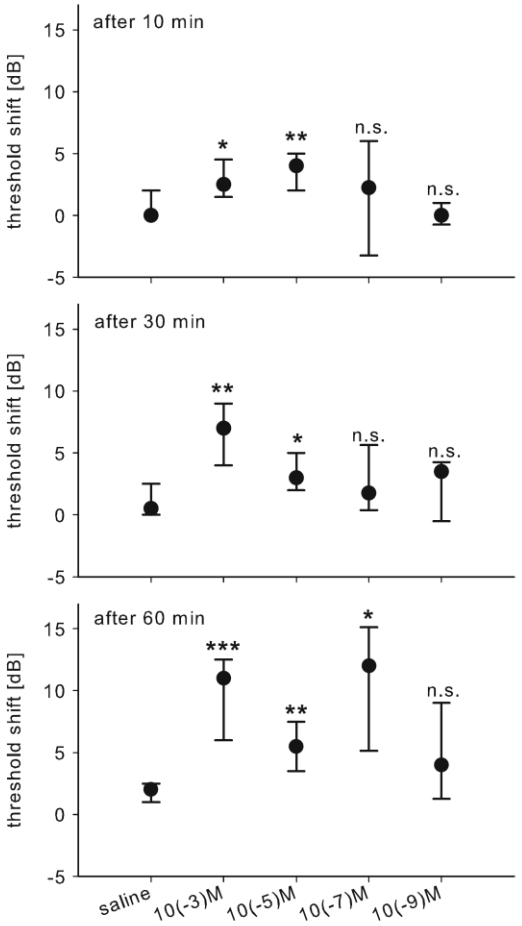

Fig. 4 Threshold shifts between DPOAE measurements before and at certain time points after the application of saline and of 4 pymetrozine concentrations (saline $n = 11$ organs, 10^{-3} M $n = 7$ organs, 10^{-5} M $n = 7$ organs, 10^{-7} M $n = 6$ organs, and 10^{-9} M $n = 5$ organs). Values are given as median with the 25th and 75th percentiles. Possible differences between threshold shifts observed after pymetrozine application ("10^{-3} M", "10^{-5} M", "10^{-7} M", "10^{-9} M") and the ones obtained for the saline control measurements ("saline") were tested statistically, for each time point, respectively (10, 30, and 60 min after application). *Asterisks* indicate statistically significant differences (Wilcoxon test for unpaired samples; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; *n.s.* not significant)

application of pymetrozine at different concentrations. Possible differences between these threshold shifts and the ones obtained for the saline control measurements were tested statistically. Threshold shifts induced by the highest pymetrozine concentrations, 10^{-3} and 10^{-5} M, were statistically different from the saline controls at all time points (10, 30, and 60 min after application; $p < 0.05$). Pymetrozine at a concentration of 10^{-7} M led to statistically significant differences in the threshold shifts only after 60 min ($p < 0.05$). The lowest pymetrozine concentration tested, 10^{-9} M, had no statistically significant influence on DPOAE thresholds compared to the saline controls ($p > 0.05$).

The time-dependency of the effects is displayed in Fig. 5, with data for saline, and pymetrozine concentrations of 10^{-3} and 10^{-7} M, obtained for all time points. Threshold shifts determined after saline application (triangles) kept a low level over the entire experimental procedure, pointing to only small DPOAE amplitude changes. The decrease of the DPOAE amplitudes observed after 10^{-3} M pymetrozine (circles) began directly after application and continued to the last measured time point at 60 min after application, indicated by increasing thresholds shifts. The lower pymetrozine concentration of 10^{-7} M (squares) was followed by a delayed effect onset which started 40 min after application, but reached about the same threshold shift values after 60 min.

The observed threshold shifts were not reversible within our examination period of 60 min after application of pymetrozine. In some cases, the experimental procedure

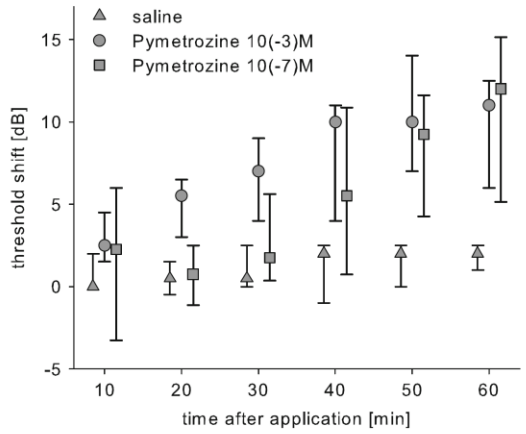

Fig. 5 Threshold shifts between DPOAE measurements before and at all time points after the application of saline ($n = 11$ organs) and of pymetrozine at concentrations of 10^{-3} M ($n = 7$ organs) and of 10^{-7} M ($n = 6$ organs). Values are given as median with the 25th and 75th percentiles. The medians and percentiles are offset around each respective time point to provide better visibility

was extended to more than 60 min, but still revealed no recovery of the effects. This is comparable to the results of Ausborn et al. (2005) who reported no restoration of stimulus-related responses in the femoral chordotonal organ after pymetrozine application within the observation period of 2 h.

Discussion

DPOAEs in bushcricket ears

The characteristics of the DPOAEs measured in the tympanal organs of *M. elongata* are comparable to those previously recorded from the hearing organs of locusts and moths (Kössl and Boyan 1998a, b; Coro and Kössl 1998, 2001; Kössl and Coro 2006; Möckel et al. 2007; Kössl et al. 2007). The 2f1–f2 emission is most prominent, as it is evoked at the lowest stimulus levels. An optimum f2/f1 ratio is difficult to define; generally small ratios between 1.04 and 1.10 evoke the largest 2f1–f2 emissions. Both findings also apply to locusts and moths. This implies that the 2f1–f2 emission shows maximum levels if the emission frequency is close to the stimulation frequencies, which is typical of non-linear resonators. At the most sensitive frequencies, which lie around 17 and 18 kHz in *M. elongata*, the level of the emission is about 44 dB lower than the stimulus levels; for less sensitive frequencies, the emission level is 55–65 dB lower than the stimulus levels. These values are within or above the range reported for locusts (30–50 dB; Kössl and Boyan 1998a, b), and above that reported for moths (23–30 dB; Coro and Kössl 1998). The lower sensitivity, especially when compared to moths, may be caused by the measurement procedures which were different for both animal groups. In moths and locusts, the devices for stimulation and recording were placed directly in front of the tympanal membrane, to which the scolopidia are attached on the inside. In our present experiments with bushcrickets, we placed the stimulation and recording channels at the entrance of the spiracle. Consequently, both the stimulation signals and the much lower emission signals have to travel over a much longer distance, extending between spiracle and tympanal organ. Nonetheless, the situation found in bushcrickets is of great experimental advantage, as it allows direct manipulation of the organ while the acoustical measurements at the spiracle remain undisturbed.

Pymetrozine and its effects on DPOAEs

Pymetrozine appears to act selectively on scolopidia, i.e., the mechanosensor type constituting all chordotonal organs. Selective action was reported in a study on the locust femoral chordotonal organ, a proprioceptor detecting limb movements. In this preparation, pymetrozine application caused the loss of stimulus-related responses, revealed either by temporary tonic discharges or by altogether abolished spike activity (Ausborn et al. 2005). The crista acustica is also comprised of scolopidial receptors. Together with their accessory structures such as tympanal membranes and the tracheal system, they function as exteroreceptors to detect acoustic far-field stimuli. In an earlier study (Möckel et al. 2007), we reported the frequency-specific deletion of DPOAEs following mechanical ablation of a certain group of scolopidia. With the scolopidia identified as targets of pymetrozine, it has now been possible to influence the DPOAE-activity pharmacologically, without mechanical interference. The exact site of pymetrozine action at the molecular level, however, remains unclear (see below).

Studies on locust thoracic ganglia in situ (Kaufmann et al. 2004) and the femoral chordotonal organ (Ausborn et al. 2005) determined the threshold concentration for pymetrozine effects to be 10^{-8} M. The results in our present study correspond well to these findings. But we cannot exclude the possibility that the applied substance concentrations were reduced by the surrounding hemolymph before reaching the auditory organ in our experiments. We observed changes in DPOAE amplitudes after application of pymetrozine concentrations as low as 10^{-7} M, while our lowest concentration tested, 10^{-9} M, did not produce significant differences compared to the control and/or start measurements.

Ausborn et al. (2005) reported an all-or-none mode of pymetrozine action, with the effect being either absent or

fully present. Dose-dependency was expressed merely by the increase in the number of unaffected animals in tests with relatively low concentrations. In our study, 60 min after application, the median values for threshold shifts using concentrations of 10^{-3} and 10^{-5} M pymetrozine differed from each other (11.1 and 5.6 dB, respectively; see Fig. 4), but both proved to be significantly different from their controls ($p < 0.01$). These divergences are probably due to the differences of the initial DPOAE amplitudes and the slope of the DPOAE growth functions in individual tympanal organs.

Furthermore, it seems important to consider that the substance was applied distally from the organ itself, was then taken up by the hemolymph, and thus reached the crista acustica. Preliminary tests with dyes confirmed transport across the entire organ within minutes. It still is possible that differences in diffusion time of the pymetrozine concentrations applied here played a role in the observed effects. With our lowest effective concentration, 10^{-7} M, it took 50 min to induce threshold shifts that were significantly different from those of the saline controls (see Fig. 5). At higher concentrations, 10^{-3} and 10^{-5} M, however, such statistically significant difference in threshold shifts occurred already 10 min after application. These variations in the observed onset of the threshold shifts for the different concentrations could be caused by slightly unequal diffusion times, as all effective pymetrozine concentrations induced the same threshold shift values 60 min after application.

Pymetrozine acts highly selectively on a distinct class of sensor cells, the scolopidia. Yet the compound's action remains unknown at the molecular level. The substance does not necessarily affect an amplifier; it could also be possible that only passive cellular structures were affected, which caused properties of the organs to change and thereby influenced the DPOAE amplitudes. As shown in moths and grasshoppers, similar to vertebrates, DPOAE growth functions are comprised of two components, a low-level and a high-level component, which possess different physiological vulnerability (Coro and Kössl 2001). Such an effect separation into two components was observed in half of the pymetrozine applications, as the sensitive low-level component was more vulnerable to the influence of the substance than the high-level component, which would not support the possibility that only passive structures were affected. Each scolopidium in the tympanal organ of *M. elongata* consists of one bipolar sensory neuron and its accessory cells. Potentially, the substance could affect all these cell types. Future experiments should, therefore, include intracellular recordings from identified sensory neurons and their accessory cells upon application of pymetrozine. Among insect mechanosensory organs, the chordotonal organs are the only ones with a fully developed ciliary apparatus (ultrastructure reviewed by Field and Matheson 1998; Yack 2004; Field 2005). This distinct feature might help reveal the molecular mechanism of DPOAE generation in tympanal organs and hence of non-linear auditory processing in insect ears.

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References

Ausborn J, Wolf H, Mader W, Kayser H (2005) The insecticide pymetrozine selectively affects chordotonal mechanoreceptors. J Exp Biol 208:4451–4466

Coro F, Kössl M (1998) Distortion-product otoacoustic emissions from the tympanic organ in two noctuoid moths. J Comp Physiol A 183:525–531

Coro F, Kössl M (2001) Components of the 2f1–f2 distortion-product otoacoustic emission in a moth. Hear Res 162:126–133

Dallos P, Fakler B (2002) Prestin, a new type of motor protein. Nat Rev Mol Cell Biol 3:104–111

Field LH (2005) The chordotonal organ: a uniquely invertebrate mechanoreceptor. In: Christensen TA (ed) Methods in insect sensory neuroscience. CRC Press, Boca Raton, pp 61–105

Field LH, Matheson T (1998) Chordotonal organs of insects. Adv Insect Physiol 27:1–228

Harrewijn P, Kayser H (1997) Pymetrozine, a fast-acting and selective inhibitor of aphid feeding. In situ studies with electronic monitoring of feeding behaviour. Pestic Sci 49:130–140

Hoyle G (1953) Potassium ions and insect nerve muscle. J Exp Biol 30:121–135

Kaufmann L, Schürmann F, Yiallouros M, Harrewijn P, Kayser H (2004) The serotonergic system is involved in feeding inhibition by pymetrozine. Comparative studies on a locust (*Locusta migratoria*) and an aphid (*Myzus persicae*). Comp Biochem Physiol 138C:469–483

Kössl M, Boyan GS (1998a) Otoacoustic emissions from a non-vertebrate ear. Naturwissenschaften 85:124–127

Kössl M, Boyan GS (1998b) Acoustic distortion products from the ear of a grasshopper. J Acoust Soc Am 104:326–335

Kössl M, Coro F (2006) L1, L2 maps of distortion-product otoacoustic emissions from a moth ear with only two auditory receptor neurons. J Acoust Soc Am 120:3822–3831

Kössl M, Coro F, Seyfarth E-A, Nässig WA (2007) Otoacoustic emissions from insect ears having just one auditory neuron. J Comp Physiol A 193:909–915

Kössl M, Möckel D, Weber M, Seyfarth E-A (2008) Otoacoustic emissions from insect ears: evidence of active hearing? J Comp Physiol A 194:597–609

Lakes R, Schikorski T (1990) Neuroanatomy of tettigoniids. In: Bailey WJ, Rentz DCF (eds) The Tettigoniidae: biology,

systematics and evolution. Crawford House Press, Bathurst, pp 166–190

Liberman MC, Zuo J, Guinan JJ Jr (2004) Otoacoustic emissions without somatic motility: Can stereocilia mechanics drive the mammalian cochlea? J Acoust Soc Am 116:1649–1655

Manley GA (2001) Evidence for an active process and a cochlear amplifier in nonmammals. J Neurophysiol 86:541–549

Möckel D, Seyfarth E-A, Kössl M (2007) The generation of DPOAEs in the locust ear is contingent upon the sensory neurons. J Comp Physiol A 193:871–879

Oldfield BP (1988) Tonotopic organization of the insect auditory pathway. Trends Neurosci 11:267–270

Oldfield BP (1982) Tonotopic organisation of auditory receptors in Tettigoniidae (Orthoptera: Ensifera). J Comp Physiol 147:461–469

Stumpner A (1996) Tonotopic organization of the hearing organ in a bushcricket. Naturwissenschaften 83:81–84

Weber M (2004) Untersuchungen über neuroaktive Substanzen in tympanalen Hörorganen der Laubheuschrecke *Mecopoda elongata.* Diplomarbeit, Fachbereich Biologie, Universität Frankfurt am Main

Yack JE (2004) The structure and function of auditory chordotonal organs in insects. Microsc Res Tech 63:315–337

2.9. Appendix to Möckel et al. 2011

2.9.1. Additional remarks on the experimental procedures

2.9.1.1. Foreleg position and its influence on DPOAE amplitudes

The forelegs for the animals were fastened to two supports that held them in an extended, but not fully stretched position. In first tests (n=2; 1♀ and 1♂), the tibia-femur angle was varied between 15° (leg fully bent) and 180° (leg fully stretched out) to estimate a possible influence of the leg angle on the mechanics of the hearing organ. No significant change in DPOAE amplitudes was found in the tested frequency range (10-34 kHz; Fig. 2.6).

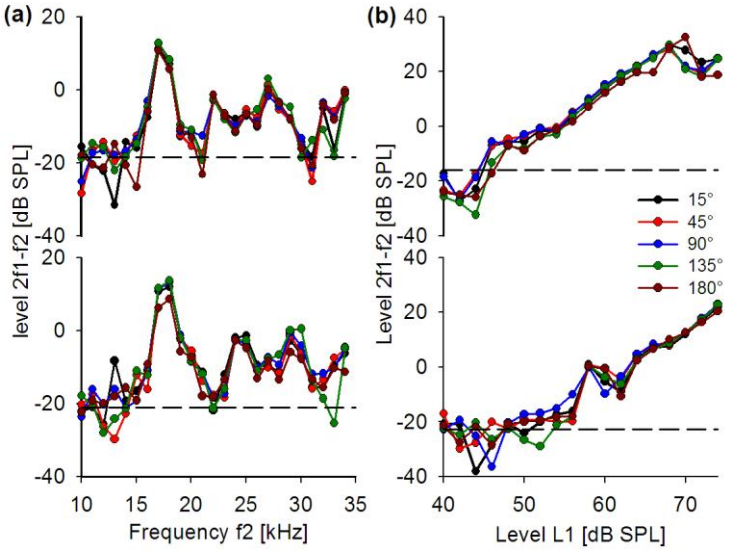

Fig.2.6 The tibia-femur angle has no influence on 2f1-f2 ampitudes during DPOAE measurements in *M. elongata.* The angle between foreleg tibia and femur amounted to 15°(leg fully bent), 45°, 90°, 135° and 180° (leg fully stretched). All shown data was obtained in the same animal. **(a)** DPOAE audiograms for f2. Stimulus levels 60/50 dB SPL. f2/f1 ratio 1.08 (top) and 1.12 (bottom). **(b)** DPOAE growth functions. Stimulus frequencies 15.72/17 kHz (top) and 23.80/25 kHz (bottom). Horizontal dashed lines indicate the noise level expressed as means + 1SD.

2.9.1.2. The acoustic coupler for microphone and loudspeakers

DPOAE measurements were conducted using an acoustical coupler whose tip fitted the entrance of the spiracle. The coupler was specifically made according to the spiracle's size and measurement arrangements. It consisted of pipette tips (size medium or small) which had been acuminated and clued together in a certain angle. The final coupler tip diameter amounted to about 1.3 mm. The study about the influence of pymetrozine was done at the beginning of this thesis. The two loudspeaker channels were brought together within the connecting piece that held them, with their signal reaching the ear in a combined channel (shown in Fig.2.7a). At a later stage of my thesis, this arrangement was slightly changed (see chapter 3.8.).

The spiracle entrance in the bushcricket *M. elongata* is partly covered by the pronotum. In order to insert the coupler slightly, the spiracle entrance had to be expanded by removing small cuticle pieces from the pronotum. That preparation had to done quite carefully, as removing too much of the pronotum cuticle caused severed bleeding and hemolymph flowing

into the tracheal system, which could only be stopped in the fewest cases. Once that preparation was completed, the tip of the coupler was inserted into the spiracle opening and bulla, without pointing the tip onto tracheal tissue within.

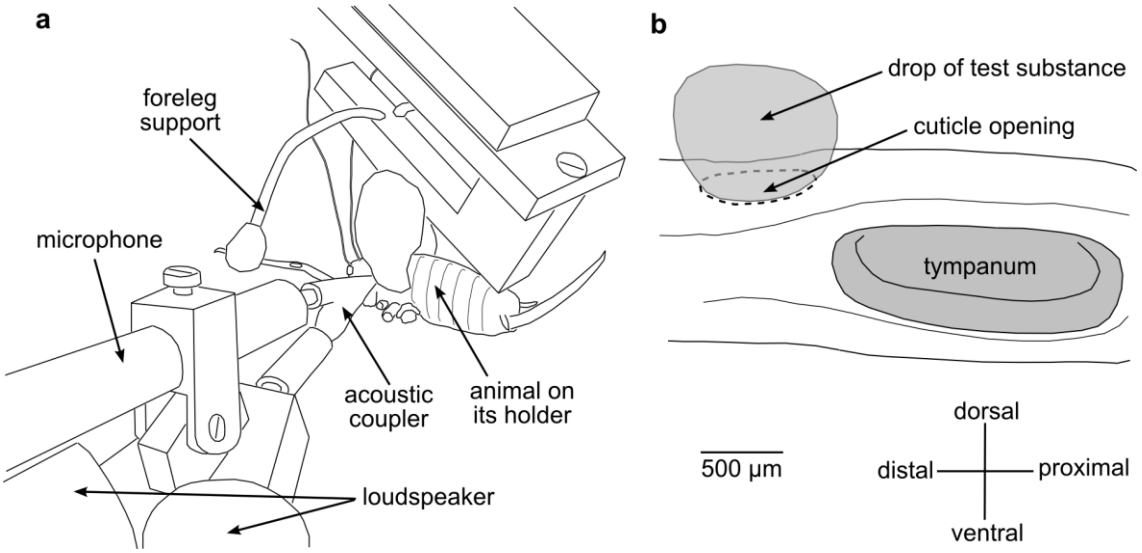

Fig.2.7 Experimental set up for DPOAE recordings during the application of a test substance via an opening in the foreleg tibia of *M. elongata*. (a) Arrangement for acoustical DPOAE recordings. **(b)** Posterior view on the foreleg tibia, showing the location and size of the cuticle opening and the approximate size of a test substance drop. Please see main text for details on the experimental procedure.

2.9.1.3. Measurements without anesthesia

The animals were awake during all measurements. This was confirmed by normal breathing behaviour and fast reflexes upon touching the antennae. In general, it proved a good pre-experimental treatment to deprive the animals of food for one day before the experiments, and of water during the night before. This prevented heavy bleeding during the preparations and allowed a faster wound closure. During longer experiments, the animals were offered small wet tissue pieces to take up water between measurements. The light source within the sound-proof chamber was turned off, which usually calmed the animals down throughout the experiment.

2.9.1.4. Test fluid application to the tympanal organ

In preceding experiments, several sizes and locations for the cuticle opening within the foreleg tibia were tested, with DPOAEs and their possible cuticle opening-related changes being recorded for at least 60 min. The cut to remove the cuticle had to be as shallow as possible, because too deep incisions led to wounds in the acoustical trachea, with hemolymph entering the system. Whereas even smallest cuticle openings directly above the organ caused irreversible reductions of DPOAE amplitudes (Fig.2.8 left and middle column), a small opening about half the length of the tympanal membranes, located at the dorsal side of the leg just distally from the organ, proved to have no effect (Fig.2.8 right column).

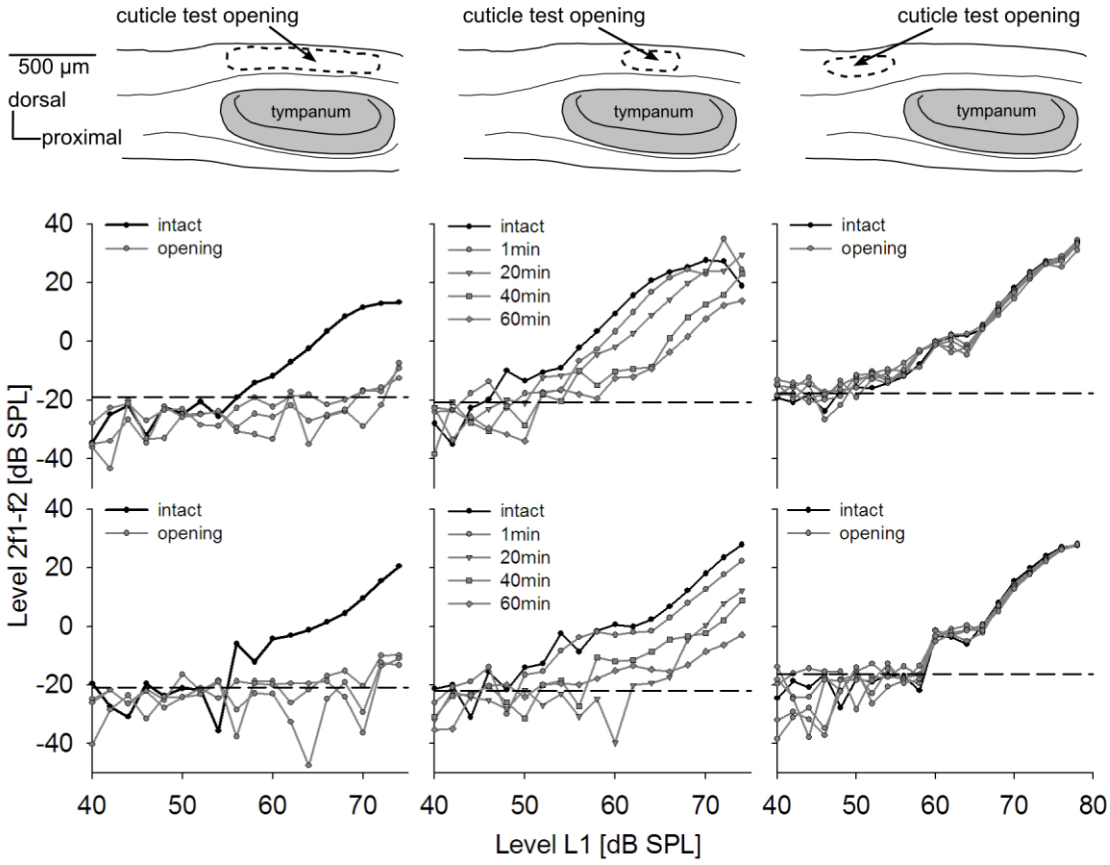

Fig.2.8 DPOAE growth functions measured before and after opening the tibia cuticle at various locations. Posterior views on the foreleg tibia display the corresponding cuticle openings above each column. **Left column:** cuticle opening approximately the length of the tympanal membranes, directly above the tympanal organ. Stimulus frequencies: 16.80/21 kHz (top) and 25/27 kHz (bottom); growth functions measured before and three times after opening, respectively. **Middle column:** Only small cuticle opening, directly above the tympanal organ. Stimulus frequencies: 15.72/17 kHz (top) and 25/27 kHz (bottom); growth functions measured before and at several time points after opening, respectively. **Right column:** small cuticle opening, about half the length of the tympana, directly distally to the organ. Stimulus frequencies: 18.09/19 kHz (top) and 18.26/19 kHz (bottom); growth functions measured before and five times after opening, respectively.

Probably caused by breathing movements of the animal, the test substance drop was taken up by the hemolymph. The uptake of the drop was visually controlled throughout the experimental course. Legs in Orthoptera possess pulsatile organs that create a hemolymph flow, ventrally towards the tip and dorsally back to the thorax (Hustert 1999; Pass 2000). Substances applied via an opening located distal to the tympanal organ on the dorsal side of the leg should therefore reach the organ quickly. To estimate the path of the substances after they were applied and taken up by the hemolymph system, I tested the same approach using diluted ink and found their particles being spread across the entire organ and further proximal towards the knee joint and femur.

2.9.2. Effects of pymetrozine on DPOAE amplitudes in the Greater wax moth *Galleria mellonella*

As a short side project, the compound's influence was first tested on the Greater wax moth, *Galleria mellonella*, together with Prof. Frank Coro (then University of Havana, Cuba) during his stay in Frankfurt in 2006. The animals possess two tympanal organs on the ventral side of their thorax. The attachment position of the respective scolopidia is clearly visible through the intact tympanum. Drop application directly onto the tympanum led to an irreversible reduction of DPOAE amplitudes (data not shown). This approach, however, left quite some room for methodical discussions, such as a possible disturbance of the tympanum's mechanical properties or whether the substance had actually reached the scolopidia within the organ, and was therefore not further pursued, but rather replaced by the methodically much more controlled application in bushcrickets.

2.9.3. DPOAE measurements in the field cricket *Gryllus bimaculatus*

The ear anatomy of field crickets, another member of the Ensifera, is also well studied, and those species are used in a variety of auditory research projects. As these animals show acoustical communication with great behavioral relevance in a broad frequency range, I did a series of test measurements to record sensitive DPOAEs. Although DPOAEs were recordable, eventually the size of the hearing organ proved to be too small to position the acoustical measuring devices in a reliable way across a line of experiments.

3. Temperature dependence of distortion-product otoacoustic emissions in tympanal organs of locusts

Doreen Möckel, Manfred Kössl, Julian Lang, and Manuela Nowotny

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Author contributions Experiment design by D. Möckel and M. Nowotny; preliminary tests performed by D. Möckel; experiments performed by D. Möckel and J. Lang; data collection and presentation by D. Möckel; data analysis by D. Möckel with consultation from M. Kössl and M. Nowotny; D. Möckel wrote the manuscript with contributions from M. Kössl and M. Nowotny

Abstract Distortion-product otoacoustic emissions (DPOAEs) in tympanal organs of insects are vulnerable to manipulations that interfere with the animal's physiological state. Starting at a medium temperature, we raised and lowered the locust's body temperature within the range of 12 to 35°C by changing the temperature of the surrounding air, while recording DPOAEs. These experimental manipulations resulted in reversible amplitude changes of the 2f1–f2 emission, which were dependent on stimulus frequency and level. Using low f2 frequencies of up to 10 kHz, a temperature increase (median +8–9°C) led to an upward shift of DPOAE amplitudes of approximately +10 dB, whereas a temperature decrease (median –7°C) was followed by a reduction of DPOAE amplitudes by 3 to 5 dB. Both effects were only present in the range of the low-level component of DPOAE growth functions below L2 levels (levels of the f2 stimulus) of approximately 30 dB SPL. DPOAEs evoked by higher stimulus levels as well as measurements using higher stimulation frequencies above 10 kHz remained unaffected by any temperature shifts. The Arrhenius activation energy was calculated from the –10 dB SPL thresholds (representing the low-level component) of growth functions, which had been measured with 8 and 10 kHz as f2 frequencies and amounted to up to ~34 and 41 $kJmol^{-1}$, respectively. Such activation energy values provide a hint that the dynein-tubulin system within the scolopidial receptors could play an essential part in the DPOAE generation in tympanal organs.

Anlage 1

Erklärung über Anteile der Autoren/Autorinnen an den einzelnen Kapiteln der Promotionsarbeit

Titel der Publikation/ des Manuskripts:	**Möckel D, Kössl M, Lang J, Nowotny M (2012) Temperature dependence of distortion-product otoacoustic emissions in tympanal organs of locusts. J Exp Biol 215:3309-3316**	
	Was hat der/die Promovierende bzw. was haben die Co-Autoren/Autorinnen beigetragen[#]	Name des/der jeweiligen Autors/Autoren/Autorin*
(1) Entwicklung und Planung	Möckel D 50%, Nowotny M 50%	Möckel D, Nowotny M
(2) Durchführung der einzelnen Untersuchungen/ Experimente	vorbereitende Tests; Kontrollversuche, um die Stabilität der Präparation zu testen; Durchführung der Experimente	Möckel D
	Durchführung der Experimente	Lang J
(3) Erstellung der Daten-sammlung und Abbildungen	Auslesen der Rohdaten; Erstellung aller Abbildungen der Publikation	Möckel D
(4) Analyse/Interpretation der Daten	Analyse der Rohdaten; statistische Zusammenfassung und Auswertung der Daten	Möckel D
	Diskussion der Daten	Kössl M, Nowotny M
(5) übergeordnete Einleitung/ Ergebnisse/Diskussion	Möckel D 70%, Kössl M 15%, Nowotny M 15%	Möckel D, Kössl M, Nowotny M
[#]Bei 2, 3 und 4 bitte kurze inhaltliche Angaben der jeweiligen Anteile, bei 1 und 5 reichen prozentuale Angaben		*Mehrfacheintragungen möglich

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RESEARCH ARTICLE

Temperature dependence of distortion-product otoacoustic emissions in tympanal organs of locusts

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SUMMARY

Distortion-product otoacoustic emissions (DPOAEs) in tympanal organs of insects are vulnerable to manipulations that interfere with the animal's physiological state. Starting at a medium temperature, we raised and lowered the locust's body temperature within the range of 12 to 35°C by changing the temperature of the surrounding air, while recording DPOAEs. These experimental manipulations resulted in reversible amplitude changes of the $2f_1–f_2$ emission, which were dependent on stimulus frequency and level. Using low f_2 frequencies of up to 10 kHz, a temperature increase (median +8–9°C) led to an upward shift of DPOAE amplitudes of approximately +10 dB, whereas a temperature decrease (median –7°C) was followed by a reduction of DPOAE amplitudes by 3 to 5 dB. Both effects were only present in the range of the low-level component of DPOAE growth functions below L2 levels (levels of the f_2 stimulus) of approximately 30 dB SPL. DPOAEs evoked by higher stimulus levels as well as measurements using higher stimulation frequencies above 10 kHz remained unaffected by any temperature shifts. The Arrhenius activation energy was calculated from the –10 dB SPL thresholds (representing the low-level component) of growth functions, which had been measured with 8 and 10 kHz as f_2 frequencies and amounted to up to ~34 and 41 kJ mol^{-1}, respectively. Such activation energy values provide a hint that the dynein-tubulin system within the scolopidial receptors could play an essential part in the DPOAE generation in tympanal organs.

Key words: Arrhenius plot, dynein, tubulin, *Locusta migratoria*.

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INTRODUCTION

Upon simultaneous stimulation with two pure tones f_1 and f_2 ($f_1<f_2$), tympanal organs of insects such as locusts (Kössl and Boyan, 1998; Möckel et al., 2007), moths (e.g. Kössl and Coro, 2006; Kössl et al., 2007) and bushcrickets (Möckel et al., 2011) emit pronounced distortion-product otoacoustic emissions (DPOAEs) that resemble those measured in the ears of vertebrates (Kössl et al., 2008). They appear as additional spectral peaks at frequencies of $nf_1–(n–1)f_2$ and $nf_2–(n–1)f_1$. In insects as well as in vertebrates, the $2f_1–f_2$ emission is the most prominent one, as it is evoked by the lowest stimulus levels. The largest $2f_1–f_2$ amplitudes are reached at frequencies of high auditory sensitivity, and in moths and locusts, the $2f_1–f_2$ DPOAEs are elicited with stimuli levels near the species-specific auditory threshold (Kössl et al., 2008).

The $2f_1–f_2$ emission often shows non-monotonic growth behavior with increasing stimulus levels, and the slope of the corresponding growth function changes at intermediate stimulation levels. The two parts of the growth function are referred to as the low-level component and the high-level component. The low-level component is more strongly affected by changes in the animal's physiology induced by hypoxia (Kössl and Boyan, 1998) or the application of ethyl ether (Coro and Kössl, 2001; Kössl et al., 2007), suggesting a metabolically sensitive biological origin of the acoustic two-tone distortions. Evidence that the scolopidia, the auditory mechanoreceptor cells in tympanal organs, are involved in DPOAE generation comes from two types of experiments. In one, mechanical lesions in the locust ear, which affected a specific group of scolopidia tuned to high sound frequencies above 12 kHz, deleted only those DPOAEs that were evoked by stimulus frequencies above 15 kHz (Möckel et al., 2007). In the other, the anatomical separation of the main site of sound input (the spiracle at the prothorax) and the site of its perception (the tympanal organ in the foreleg tibia) in bushcrickets permitted the local application of the insecticide pymetrozine. The compound appears to act selectively on scolopidia (Ausborn et al., 2005), causing a pronounced and irreversible decrease of DPOAE amplitudes (Möckel et al., 2011). The underlying cellular components, however, which may be involved in DPOAE generation in insects, have not been identified so far.

The tympanal organ of locusts consists of a peripheral ganglion, called Müller's organ, a large tympanal membrane, and an adjacent tracheal system. Müller's organ comprises approximately 80 scolopidia, each of them containing a bipolar sensory neuron that is coupled to the inside of the tympanal membrane *via* its accessory cells (Gray, 1960). Based on the exact attachment position of their dendrites and their frequency tuning, four (Michelsen, 1971; Römer, 1976) or three groups of neurons (Jacobs et al., 1999) have been distinguished. Those scolopidia that are attached to the thin part of the tympanal membrane at the so-called pyriform vesicle (named 'd-cells' or 'group II', respectively, by the authors mentioned above) are most sensitive to sound frequencies above 12 kHz. Those coupled to the thick part of the tympanal membrane are most sensitive to lower frequencies. Frequency analysis is determined by

the travelling-wave-like vibration pattern of the tympanal membrane. From their initiation at the outer rim of the tympanum, waves that are induced by sound frequencies above 12 kHz are attenuated as soon as they reach the pyriform vesicle, whereas those induced by lower sound frequencies continue to travel towards the thick membrane region (Windmill et al., 2005).

Hearing involves mechano-electrical transduction mechanisms. For locusts (Oldfield, 1988) and cicadas (Fonseca and Correia, 2007), sensory transduction has been shown to be sensitive to changes in body temperature. The sensitivity and the characteristic frequency to which the receptor is tuned both increase with increasing temperature. In both of these previous studies, these effects exceeded those expected from merely mechanical property changes of the auditory organ. Laser Doppler vibrometry measurements of the tympanal vibrations in cicadas in response to acoustical stimulation were independent of temperature within a biologically relevant range (18–35°C). The changes in the neuronal response characteristics owing to shifts in body temperature were therefore suggested to be caused by intrinsic properties of the receptor neurons.

After initially being considered to be purely linear mechanical systems, insect ears were shown, as mentioned above, to comprise non-linear sound processing. Such non-linearity and generation of sensitive DPOAEs were found to depend on intact scolopidia, to be vulnerable to changes concerning their physiology, and could potentially rely on each of the scolopidial cell types. The present study concerns two open questions: (1) which components of the intracellular machinery could contribute to mechanical non-linearity; and (2) is non-linear hearing in tympanal organs of locusts based on mechanical principles similar to those found in Johnston's organs of mosquitoes and flies? The suggested biological origin of acoustic two-tone distortions should involve metabolic processes, whose temperature-dependence would directly affect the DPOAE generation. A relationship between body temperature shifts and the rate of potential DPOAE changes, plotted into an Arrhenius graph and converted into the activation energy, would provide conclusions on possible mechanisms involved in non-linear hearing in tympanal organs. Such assumptions would allow a comparison with non-tympanic insect hearing organs such as those found in mosquitoes and flies, which have a much lower frequency range of operation.

MATERIALS AND METHODS

Animals and preparations

Adult locusts [*Locusta migratoria* (Linnaeus 1758), Insecta, Caelifera, Acrididae] were acquired from a commercial supplier (Terra Tropica, Friedrichsdorf, Germany) and kept in the laboratory at room temperature. Previous studies reported no obvious gender differences in the functional anatomy or in DPOAE generation (Kössl and Boyan, 1998; Möckel et al., 2007). We therefore used males and females (N=13) for the present study. After slight anesthesia with CO_2, legs and wings were removed, and the animals were laterally fixed with their thorax onto a metal holder using rosin-beeswax. The cuticular lid that partly covers the ear opening was removed under visual control. The temperature sensor probe of a digital thermometer (Voltcraft Digital Thermometer K101, Conrad Electronic, Hirschau, Germany) was placed under the dorsal abdomen cuticle *via* a small cuticle opening and was also fixed onto the metal holder. CO_2 was not re-applied after these preparations, and the animals remained awake during all measurements. Normal breathing movements and fast reflexes upon touching the antennae indicated recovery from anaesthesia. After the experiments, the animals were re-anesthetized and killed by decapitation.

Measurement of DPOAEs

Experimental setup and measuring procedures followed a previous report on DPOAEs in locusts (Möckel et al., 2007). A Microstar DAP 840 DSP card (Microstar Laboratories, Bellevue, WA, USA; sampling rate 200 kHz) that was controlled by custom software accomplished the generation of the two acoustic stimuli and the analysis of the incoming microphone signal. The two output channels were connected to two attenuators (TDT System3, Tucker Davis Technologies, Alachua, FL, USA), an amplifier, and two 1 inch microphones (Microtech Gefell, Gefell, Germany, Type 103.1) that served as loudspeakers. The acoustic signals were measured by an additional ½ inch microphone (Brüel & Kjær, Bremen, Germany, Type 4133) connected to the input channel of the DSP card by a preamplifier (Brüel and Kjær, Type 2660) and an amplifier (Brüel & Kjær, Type 2610). The adjacent channels for stimulation and recording were brought together in an acoustic coupler whose tip diameter fitted the size of the locust's ear opening. The animals, fixed on the metal holder, were placed in a soundproof chamber. The coupler was positioned rectangular to the tympanum at a distance of 0.2–1 mm. The remaining space between the coupler and the cuticle rim that surrounds the ear was sealed using Vaseline or toothpaste. The sound system was calibrated *in situ*, with the coupler placed in measuring position. The arrangement of animal and coupler was not affected by the manipulations during the course of the experiment, as only the surrounding air temperature was changed in order to shift the animal's body temperature.

During acoustical stimulation with two pure tones of different frequencies (f_1<f_2), the level of the f_1 stimulus (L1) was chosen to be 10 dB above that of f_2 (L2), as this has been found to induce maximal DPOAE levels (Kössl and Boyan, 1998). The measured microphone signal was averaged 100 times before fast Fourier transform (FFT) analysis. Each presentation of a pair of pure-tone stimuli took 4.2 s for 100 averages. Accordingly, the measurement of a DPOAE audiogram and growth function took approximately 1.5–2 min, depending on the respective number of frequency/level steps.

Recordings of DPOAEs included the following procedures: DPOAE audiograms were obtained by evoking DPOAEs over a wide frequency range (1–30 kHz) while keeping a constant frequency separation and level of the two stimuli [f_2/f_1 ratio of 1.04, 1.08 and 1.15; L1/L2 60/50 dB sound pressure level (SPL)]. From these DPOAE audiograms, f_2 frequencies that elicited large $2f_1–f_2$ emissions were chosen for further measurements. The respective f_1 frequency was adjusted for each f_2 frequency according to the optimum f_2/f_1 ratio that evoked the largest $2f_1–f_2$ emissions. DPOAE growth functions were obtained by keeping both stimulus frequencies constant and increasing their levels in 2 dB steps, starting from 10/0 or 20/10 dB SPL and reaching up to 64/54 dB SPL. At the used stimulus levels, no setup-generated distortions were visible above the noise level. Background noise level was measured 100 Hz below the DPOAE frequency.

Measurements of DPOAE audiograms and growth functions were repeated at each consecutive body temperature step. The f_2/f_1 ratio for the chosen f_2 frequency was determined only once for each respective animal, and both stimulus frequencies, f_1 and f_2, were kept constant for all consecutive growth functions. Frequencies for f_2 were chosen to be 8 kHz (N=11), 10 kHz (N=5), 12 kHz (N=5) and 18 kHz (N=7).

Shifting the body temperature of the animals

The animal's body temperature was shifted by changing the temperature of the surrounding air within the soundproof chamber.

A Peltier element (PKE 36H 021, Petron, Fürth, Germany) and additional heating and cooling pads were placed around the metal holder that carried the animal. The temperature sensor that had been placed under the abdomen cuticle recorded the resulting body temperature shift with a resolution of 0.1°C.

The procedure involved measurements with the animal's body temperature at medium temperatures (which correlated with the surrounding room temperature), and at body temperatures that were shifted up and down, compared with the preceding medium temperature, in the range between 12 and 35°C. DPOAEs were recorded at each consecutive body temperature step. The return to the medium temperature in between served as a control condition to the temperature shifts and made it possible to assess the stability of the preparation during the course of the experiment. DPOAE measurements were started as soon as the respective body temperature was stable for several minutes. As the return to the medium temperature was achieved by merely removing the heating and cooling devices, breaks between these respective temperature steps could take up to 30 min.

Data analysis

To evaluate temperature-induced changes in DPOAE amplitude and sensitivity, two approaches were chosen. First, after the measurement of $2f_1–f_2$ growth functions at different temperatures, the changes in DPOAE amplitudes at given L2 stimulus levels (Fig. 1A) were assessed by subtraction of the respective growth functions and displayed as DPOAE level shift (Fig. 1B). The background noise level in all measured growth functions had a median value of –23.8±6.1 dB SPL, and to keep a safe distance to the noise level, –15 dB SPL was used as a reference. DPOAE levels below –15 dB SPL were therefore rejected from further analyses. Second, from the two growth functions obtained at different temperatures, the L2 level that induced a DPOAE threshold level of e.g. –10 dB SPL was interpolated (Fig. 1C) and the two threshold values were subtracted (Fig. 1D).

In the DPOAE level shift data, positive difference values indicate increased DPOAE amplitudes at the respective body temperature in comparison to the medium temperature condition (Fig. 1B). The used growth functions were measured at f_2 stimulus frequencies of 8 and 18 kHz, and the level shifts were separately averaged and displayed as medians and the 25th and 75th percentiles (their range hence containing 50% of the values). For each 2 dB step of the L2 level, the values of both f_2 frequency groups were compared and tested for statistically significant differences, using a Wilcoxon rank sum test for unpaired samples (MATLAB R2007b, Statistics Toolbox, The MathWorks, Natick, MA, USA). The null hypothesis 'no difference' was rejected at P-values <0.05.

For the DPOAE threshold shifts, positive values indicate higher L2 levels, and negative values lower L2 levels, that were sufficient to evoke the emission threshold level. The temperature shift indicated the change of the animal's body temperature in relation to the initial medium temperature. For each DPOAE growth function measured at lowered or raised temperatures, a value of DPOAE threshold shift and temperature shift was obtained (Fig. 1D). All resulting threshold shifts for the respective f_2 stimulus frequencies of 8, 10, 12 and 18 kHz were plotted using linear regression curves (Regression Wizard, Sigma Plot 10.0, Systat Software, San Jose, CA, USA).

Using Arrhenius coordinates, the determined values for DPOAE thresholds were plotted against the body temperatures (converted into Kelvin) that had been measured during the experiments. DPOAE thresholds were chosen as opposed to the actual DPOAE amplitudes to achieve better comparability across all measured animals. By connecting data points for each individual animal, the slope m and hence the activation energy (E_A) were determined, using the following equation:

$$|E_A| = m\,(-R), \qquad (1)$$

where R is the gas constant (8.314 J mol^{-1} K^{-1}). We used the absolute value of the calculated activation energy, as the inclusion of DPOAE thresholds implicated an inverted relationship, that is, an increase of DPOAE amplitudes in the measured growth functions caused decreased DPOAE thresholds. The values were separately calculated for every animal and averaged. Three data sets (two for 8 kHz and one for 10 kHz) that were included in the threshold shifts displayed in Fig. 4 were excluded from the Arrhenius plot because they consisted of two measured temperature steps without intermediate temperature values.

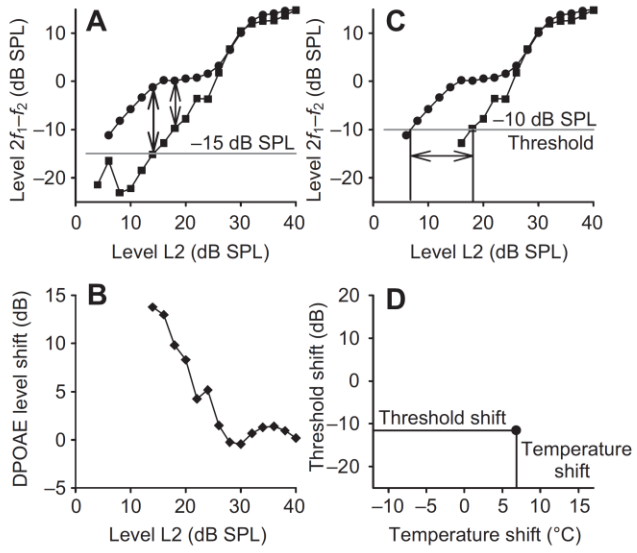

Fig. 1. Illustration of analysis paradigms regarding temperature-dependent effects on distortion-product otoacoustic emission (DPOAE) growth functions in *Locusta migratoria*. (A,B) Calculation of DPOAE level shifts. (A) Growth functions measured at the initial medium temperature (squares) were subtracted from those obtained at a shifted body temperature (circles). Only DPOAE data points ≥–15 dB SPL (grey line), well above the noise level, were used for subtraction. (B) The subtraction curve represents the amplitude shift between these two DPOAE growth functions (indicated by arrows in A) plotted against the respective L2 stimulation level. (C,D) DPOAE threshold shifts in dependence on the temperature shift. (C) DPOAE thresholds (i.e. the L2 level that is sufficient to evoke an emission of a certain level, for example –10 dB SPL) were determined for both growth functions. The L2 level shift between these values ('threshold shift') is marked by an arrow. (D) The resulting data point is defined by the DPOAE threshold shift (y-axis) and the corresponding temperature shift (x-axis). Used stimuli f_1/f_2: 6.909/8 kHz. Body temperatures: 18.4°C (medium temperature; squares) and 25.2°C (temperature shift up; circles).

RESULTS

The temperature dependency of DPOAE amplitudes in the locust was investigated by measuring DPOAE audiograms and growth functions at different body temperatures (representative examples in Fig. 2). It is noteworthy that DPOAE audiograms obtained using very low stimulus levels of 30/20 dB SPL did not evoke emissions above the noise level (Fig. 2A, grey circles). Only when the body temperature of the animal was raised by ~9°C were DPOAEs evoked by low f_2 frequencies of up to ~9 kHz, and they reached levels of >20 dB above noise level for 7 and 8 kHz (Fig. 2A, black circles). When DPOAE audiograms were recorded at higher stimulus levels, such as 60/50 dB SPL, there were no obvious effects induced by different body temperatures (Fig. 2B).

DPOAE growth functions were measured at different body temperatures using f_2 frequencies of 8, 10, 12 and 18 kHz (representative examples in Fig. 2C–F). DPOAE amplitudes during control measurements at medium temperatures (grey lines) displayed only slight variations within a range of 3 dB, demonstrating a reversibility of any temperature-dependent effects, once the animal's body temperature had returned to its original value. DPOAEs emerged from the noise level at an L2 level of 10 to 20 dB SPL, depending on the respective f_2 frequency, and reached ~40 dB SPL at the highest L2 levels of 54 dB SPL. If the body temperature of the animal was decreased or increased, DPOAE amplitude modulations occurred only for those emissions that had been recorded with low f_2 frequencies up to 10 kHz. Also, such modulations concerned only the so-called low-level component of these growth functions, that is, those DPOAEs that were evoked by low L2 stimulus levels of up to 25–30 dB SPL (Fig. 2C,D). When the body temperature was lowered by ~6°C, the emissions were slightly reduced in amplitude by ~3–10 dB (Fig. 2C,D, black triangles). Raising the animal's body temperature by ~7°C caused an increase of DPOAE amplitudes by ~10–15 dB (Fig. 2C,D, black circles). No such effects were observed for DPOAEs that were evoked by higher L2 levels (above 25–30 dB SPL). At higher stimulus levels, the emission levels remained the same over the entire applied temperature range. Such temperature- and L2-level-dependent effects, however, appeared only for emissions evoked by f_2 frequencies up to ~10 kHz. Amplitudes of DPOAEs evoked by higher f_2 frequencies did not differ when measured at the medium temperature or at shifted body temperatures (Fig. 2E,F).

DPOAE level shifts represent amplitude changes between DPOAE growth functions measured at different temperatures (see Materials and methods; Fig. 1A,B). Data obtained for f_2 frequencies of 8 kHz (black circles) and 18 kHz (grey circles) were separately averaged for decreased and increased body temperatures (Fig. 3A and 3B, respectively). The lack of data points for the lowest L2 levels comes from the fact that here often the DPOAE amplitude of one of the two measurements was below –15 dB SPL and too close to the noise level to allow a reliable subtraction of DPOAE levels obtained at two temperature conditions. Using f_2 frequencies of 8 kHz, a reduction of body temperature (temperature decreased by ~7°C in comparison to the medium temperature at the beginning; Fig. 3A) induced a small DPOAE amplitude shift of approximately –3 to –5 dB at L2 levels between 24 and 32 dB SPL. If 18 kHz was used as the f_2 frequency, no such shift in DPOAE amplitudes within a range of ±2 dB was recorded; this shift would represent the usual fluctuations between growth function measurements. An increase of body temperature (temperature increased by ~8–9°C; Fig. 3B) led to a strong increase of DPOAE amplitudes measured with 8 kHz as the f_2 frequency. This amplitude enhancement was found within the low-level component of the growth functions, for L2 levels between ~14 and 40 dB SPL. The largest average DPOAE level shift of ~9–12 dB is evident at L2 levels of 18 to 24 dB SPL. Level shifts of DPOAEs evoked by f_2 frequencies of 18 kHz decreased by only –2 to –3 dB at L2 levels of 36–40 dB SPL. When comparing one

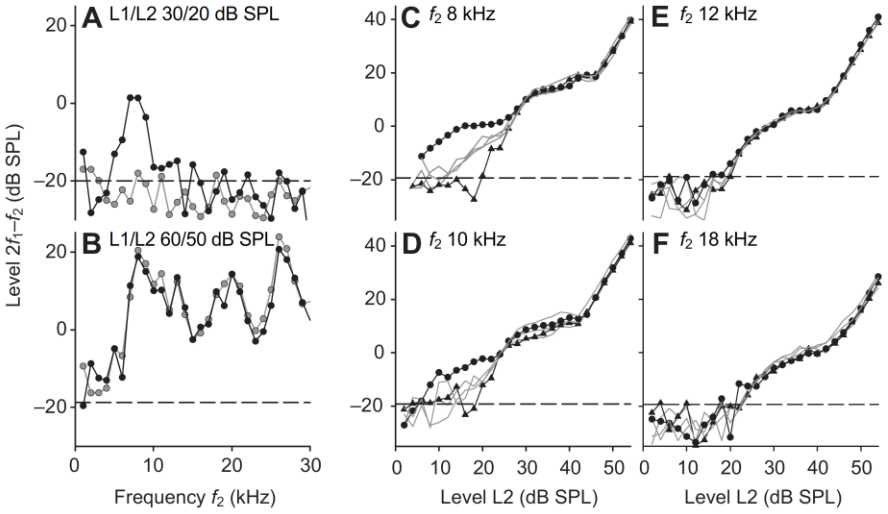

Fig. 2. Effects of a shifted body temperature on DPOAEs in *L. migratoria*. (A,B) DPOAE audiograms for f_2 frequencies between 1 and 30 kHz (f_2/f_1 ratio=1.15), measured in the same animal. Body temperatures: 16.6–16.8°C (grey circles) and 25.3–26.2°C (black circles). Stimulus levels: (A) 30/20 dB SPL; (B) 60/50 dB SPL. (C–F) DPOAE growth functions for different stimulus frequencies, measured in the same animal. Each series of recordings consisted of five growth functions, measured at: (1) initial medium temperature, (2) downward temperature shift, (3) medium temperature, (4) upward temperature shift and (5) medium temperature. Body temperatures: 24.3–25.2°C (temperature shift up, black circles), 12.1–12.5°C (temperature shift down, black triangles) and 18.3–18.7°C (medium temperature, grey lines). Stimuli: (C) 6.909/8 kHz, (D) 8.008/10 kHz, (E) 11.23/12 kHz and (F) 16.211/18 kHz. Horizontal dashed lines in all panels indicate the noise level expressed as means + 1 s.d.

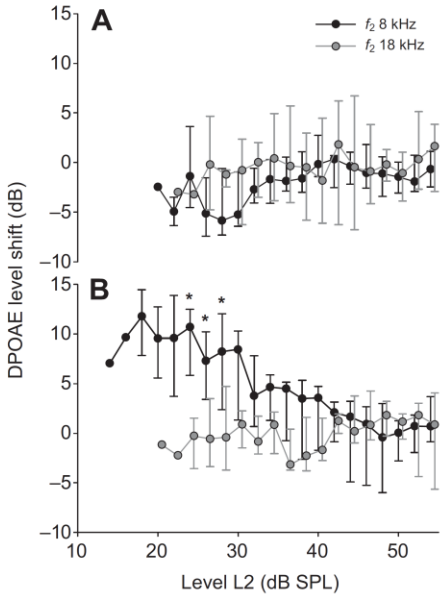

Fig. 3. Average DPOAE level shifts for f_2 frequencies of 8 kHz (black circles) and 18 kHz (grey circles). Given are DPOAE level changes at a shifted body temperature compared with those measured at the initial medium temperature, plotted against the respective L2 level (see also Fig. 1A,B). (A) Downward temperature shifts for f_2=8 kHz (N=11, –6.5±1.6°C) and f_2=18 kHz (N=7, –6.1±2.0°C). (B) Upward temperature shifts for f_2=8 kHz (N=11, 8.8±2.7°C) and f_2=18 kHz (N=7, 7.5±2.2°C). Data are expressed as medians and 25th and 75th percentiles. Asterisks indicate statistically significant differences between the two frequency groups (Wilcoxon test for unpaired samples, *P<0.05). The symbols for the 18 kHz data sets are graphically offset in the horizontal direction by 0.5 dB to provide better visibility compared with the 8 kHz values.

frequency group with the other, increased body temperatures caused statistically significant differences between DPOAE level shifts in an L2 level range between 24 and 28 dB SPL (P<0.05).

DPOAE threshold shifts represent the difference in threshold value (that is, L2 levels sufficient to evoke an emission of a certain level) obtained from two DPOAE growth functions. The shifts were assessed at two different threshold criteria to represent the low-level component (–10 dB SPL threshold; Fig. 4A,B) and the high-level component (20 dB SPL threshold; Fig. 4C,D) of the DPOAE growth functions. Such threshold shifts were set into relation to the shift in temperature at which the compared growth function had been measured (see Materials and methods; Fig. 1C,D). These temperature shifts spanned from approximately –11 to +16°C, based on the value of the original medium temperature during the first measurements. Emissions evoked by low f_2 frequencies such as 8 and 10 kHz and low L2 stimulus levels were highly temperature dependent (Fig. 4A). The –10 dB SPL thresholds increased with lowered body temperatures, and decreased with raised body temperatures. Threshold shifts amounted up to approximately +8–9 dB and –12 dB if the body temperature had been shifted by –10°C and +10°C, respectively, resulting in a threshold temperature shift of –1.10 dB °C^{-1} for 8 kHz and –1.07 dB °C^{-1} for 10 kHz (linear regression plots in Fig. 4A, black line for 8 kHz and grey line for 10 kHz, with R^2 values of 0.82 and 0.77, respectively). In contrast, emissions also evoked by low L2 stimulus levels, but with higher f_2 frequencies such as 12 and 18 kHz, did not depend on body temperature (Fig. 4B). A temperature dependence of DPOAE thresholds was also absent when higher L2 stimulus levels were used, in recordings with 8 and 10 kHz (Fig. 4C) as well as with 12 and 18 kHz (Fig. 4D). DPOAE threshold shifts were small and scattered close to 0 dB across the entire temperature shift range, with threshold shift values of –0.16 to 0.02 dB °C^{-1} for all f_2 frequencies (linear regression plots, Fig. 4B–D). Apart from outliers, most data points (87.2–100%) in all of the eight data groups lay within a ±5 dB interval centred close to the corresponding linear regression plots.

As reported above, only emissions evoked by low L2 stimulus levels and low f_2 frequencies of 8 and 10 kHz showed a dependence on temperature. Based on these data sets, the logarithms of the L2 values for –10 dB SPL DPOAE thresholds, representing the low-level component of the DPOAE growth functions, were plotted against the inverse body temperatures (T, converted into Kelvin), using Arrhenius coordinates (Fig. 5). Data points for each individual animal were connected and are shown as grey lines. The slopes of the resulting curves were not linear, but displayed a break point at medium temperatures. For measurements using an f_2 frequency of 8 kHz (Fig. 5A, N=9 animals), median values for these break points amounted to 20.3±1.7°C, ranging from 18.4 to 23.1°C. For measurements using an f_2 frequency of 10 kHz (Fig. 5B; N=4 animals), median values amounted to 18.9±0.7°C, ranging from 18.4 to 20.1°C. The level-dependent variation between the individual animals occurred as consequence of different sensitivities in each measured tympanal organ. The individual graphs, however, had comparable slopes for the temperature range both below and above the break point. Based on these slopes, the activation energy was calculated for each temperature range and animal. For measurements using f_2 frequencies of 8 and 10 kHz, the average activation energy values in the warm temperature range (above 20.3 and 18.9°C; 1000/T below ~3.40 and 3.42) amounted to 34.01±12.24 and 41.08±13.58 kJ mol^{-1}, respectively. The corresponding values for the cold temperature range amounted to lower activation energy values of 12.74±5.03 and 2.64±4.78 kJ mol^{-1}, respectively.

DISCUSSION

DPOAEs are vulnerable to manipulations that interfere with the animal's physiology, such as changing its body temperature. In the present study, a change of body temperature resulted in level- and frequency-dependent effects on the $2f_1$–f_2 emission, which were reversible, once the animal's body temperature was back to its original medium value. Increasing the locust's body temperatures led to enhanced amplitudes of DPOAEs, which had been measured with low stimulation levels (below ~30–40 dB SPL L2 level) and using low f_2 frequencies of up to 10 kHz. The high-level component remained unchanged, as well as those emissions that were evoked by higher stimulation frequencies (e.g. 12 and 18 kHz).

The low-level component of the DPOAE growth function in vertebrates (e.g. Lukashkin et al., 2002) and in insects (e.g. Kössl et al., 2007) was found to be physiologically much more vulnerable than the high-level component, which could lead to the suggestion of two L2-level-dependent mechanisms for DPOAE generation. For mammals, Lukashkin et al. (Lukashkin et al., 2002), however, provided evidence that both components are evoked by a single source, a non-linear amplifier with saturating input/output characteristics. Mammalian cochlear amplification of basilar membrane vibration is largest at low levels (Dallos and Fakler, 2002), and the impact of active amplification on the basilar membrane vibration reaches saturation if the input levels are increased beyond ~40–60 dB SPL. If the gain of the cochlear amplifier is changed *via* physiological manipulations, this would

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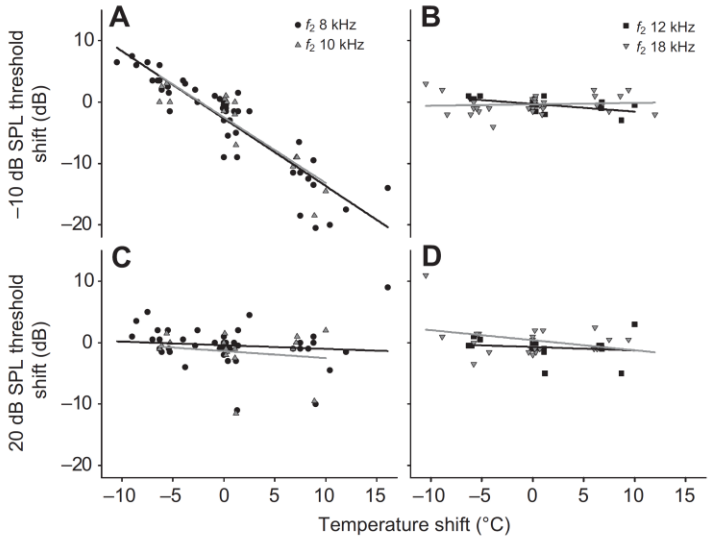

Fig. 4. Temperature-dependent shift of DPOAE threshold (see also Fig. 1C,D). Threshold levels were chosen to represent the low-level component (–10 dB SPL threshold; A,B) and the high-level component (20 dB SPL threshold; C,D) of the DPOAE growth functions. (A,C) f_2 frequencies of 8 kHz (47 measurements from 11 animals; black circles) and 10 kHz (17 measurements from five animals; grey triangles up). (B,D) f_2 frequencies of 12 kHz (17 measurements from five animals; black squares) and 18 kHz (30 measurements from seven animals; grey triangles down). Linear regression lines are fitted to each set of data. R^2 values are as follows: –10 dB SPL threshold: 8 kHz, 0.817 (A, black line); 10 kHz, 0.771 (A, grey line); 12 kHz, 0.301 (B, black line) and 18 kHz, 0.008 (B, grey line); 20 dB SPL threshold: 8 kHz, 0.012 (C, black line); 10 kHz, 0.027 (C, grey line); 12 kHz, 0.022 (D, black line) and 18 kHz, 0.096 (D, grey line).

influence the low-level component much more strongly than the high-level component, even if there is a single source of non-linearity (see Lukashkin et al., 2002). This explanation is supported by findings in a notodontid moth that has tympanal organs with only one auditory receptor neuron (Kössl et al., 2007). DPOAE growth functions from such hearing organs are also characterized by low- and high-level components that have a different emission phase and are separated by pronounced notches. In addition, the moth's DPOAE growth functions are strongly vulnerable to physiological manipulation, all of this apparently being contributed by only one scolopidium. As shown in the present study, changing the physiological state of the locust by enhancing its body temperature led to increased DPOAE amplitudes for low-level stimulation, whereas high-level DPOAEs were temperature insensitive. Level-dependent temperature effects have also been reported for vertebrates. In the amphibian papilla of leopard frogs (Meenderink and van Dijk, 2006) and in rats [for temperatures below 33°C (Khvoles et al., 1998)], lowered body temperatures led to decreased DPOAE amplitudes induced by low stimulus levels.

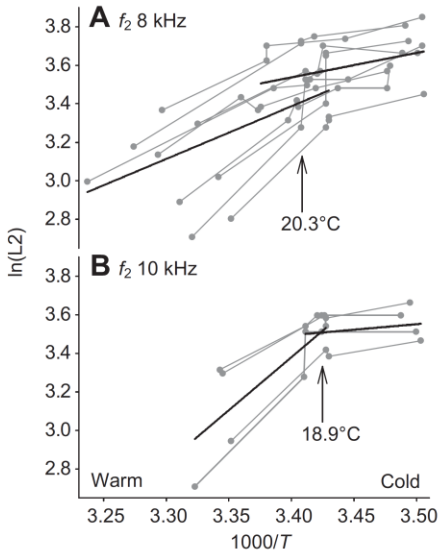

Fig. 5. Arrhenius plot of DPOAE thresholds for a threshold criterion of –10 dB SPL. This criterion was chosen to represent the low-level component of the growth functions. Plotted are the logarithms of these L2 threshold values against the inverted temperature (T, in Kelvin). Data points of each individual animal are connected (grey lines). Linear regression plots were fitted to the warm and the cold temperature ranges, and are shown in black. (A) DPOAE thresholds determined from growth functions measured for f_2=8 kHz (N=9 animals). R^2 values for the linear regression plots: warm temperature range, 0.332; cold temperature range, 0.151. (B) DPOAE thresholds determined from growth functions measured for f_2=10 kHz (N=4 animals). R^2 values for the linear regression plots: warm temperature range, 0.669; cold temperature range, 0.055.

Temperature dependence within the auditory pathway of insects has been reported for locusts (Oldfield, 1988) and cicadas (Fonseca and Correia, 2007). Both of these studies indicated that the magnitude of the effects regarding frequency tuning and sensitivity exceeded those one would expect from changes in the passive mechanical properties of the tympanum. Laser vibrometry measurements of tympanum vibrations in the cicada revealed that temperature changes between 18 and 35°C had no impact on the tympanal response to acoustic stimulation (Fonseca and Correia, 2007). The temperature shifts in the present study ranged between 12 and 35°C and were comparable to those used by Fonseca and Correia. The temperature effects on DPOAEs in locusts are therefore most probably not caused by a mere temperature-dependent change in the mechanical properties of the tympanum. Also, one would expect that any shifts in the vibration amplitude of the tympanum would have caused DPOAE amplitude changes at all L2 levels, and therefore would not only affect the low-level component of the DPOAE growth function, as was the case in our study. Our findings are more likely caused by frequency-selective transduction mechanisms based upon intrinsic properties within the scolopidia.

A change in the ambient temperature brought about frequency-selective effects within the auditory pathway for both locusts and cicadas. Oldfield (Oldfield, 1988) reported that the temperature-dependent shift in characteristic frequency was larger in receptors tuned to frequencies below 10 kHz than in receptors tuned to higher frequencies. In cicadas, intracellular recordings from interneurons having two sensitivity maxima (3–8 and 14–24 kHz, respectively)

displayed temperature-dependent effects only at the low frequency band (Fonseca and Correia, 2007). An increase in temperature was followed by upward shifts of the characteristic frequency and by enhanced sensitivity. The same frequency-dependent results were found in auditory nerve recordings, where there is a shift of tuning and an increase in sensitivity during a temperature rise only at low frequencies. The authors of both studies suggested that a temperature dependence of possible electrical tuning properties of individual scolopidial receptors could be responsible for the effect, comparable to findings on electrical tuning in non-mammalian hair cells (Fettiplace, 1987). In hair cells, electrical tuning is based on membrane potential resonances produced by the density and kinetics of ion channels, such as that of large conductance potassium channels (e.g. Bai et al., 2011), and is limited to low frequencies. The temperature sensitivity of such ion channels (Crawford et al., 1989) might explain the frequency selectivity of our observations, assuming that: (1) electrical resonances were present in insect sensory neurons and (2) would act at frequencies up to 10 kHz. So far, however, there is no experimental evidence for either of these assumptions.

Warren et al. (Warren et al., 2010) recently reported a temperature dependence concerning spontaneous mechanical oscillations of the Johnston's organ in mosquitoes. Spontaneous oscillations are caused by active mechanisms, selectively amplifying the antennal hearing organ's response to low-level stimuli (Göpfert and Robert, 2001). Scolopidia are the mechanoreceptors of both the non-tympanic Johnston's organ of mosquitoes and the locust tympanal organ. However, the external anatomy of the two organs is quite different because the former is specialized to detect the particle velocity, and the latter the sound pressure component, of incoming sound. Particle velocity attenuates rapidly with increasing frequency, which leads to very different frequency ranges of operation, i.e. several hundred Hz for mosquitoes [although certain species have been found to detect frequencies of up to 2 kHz (Cator et al., 2009)] *versus* frequencies in the ultrasonic range in locusts, which is comparable to the frequency range of the sound-pressure-sensitive ears of vertebrates [for an overview, see Yack (Yack, 2004)]. After transferring their data on temperature dependency into an Arrhenius plot, Warren et al. (Warren et al., 2010) also found a break point in their function, at 17°C, which is comparable to our findings of break points of ~20°C (for growth functions using 8 kHz for f_2) and ~19°C (for growth functions using 10 kHz for f_2). Based on the slope of these curves, the activation energy was determined to be 30 and 43 kJ mol^{-1} for the warm and cold temperature range, respectively. For the warm temperature range, this activation energy is comparable to values of ~34 and ~41 kJ mol^{-1} for f_2 frequencies of 8 and 10 kHz, respectively. The corresponding activation energies for the cold temperature range, however, were ~13 and ~3 kJ mol^{-1}, respectively, and are significantly lower than those calculated for mosquitoes. The reasons for this difference remain unclear. The locust's activation energy concerning the warm temperature range, however, is in good accordance to that found for mosquitoes by Warren et al. (Warren et al., 2010). Taking into account the activation energy values for dynein and myosin given in the literature (median values of 31 and 66 kJ mol^{-1}, respectively), Warren et al. concluded that the generation of spontaneous mechanical oscillations of the Johnston's organ relies on an intact dynein-tubulin system within the sensory cilium, which is also emphasized by its vulnerability to colchicine applications. Despite the differences in external anatomy and frequency range described above, the temperature dependence of the kinetics of the non-linear mechanical active processes (sound processing) in these non-tympanic and tympanic insect hearing organs are surprisingly similar. The activation energy values for the underlying mechanisms in both species are comparable, with the implication that the mechanical non-linearity is linked to the mechanoreceptor type. Furthermore, as now found in locusts, the underlying mechanism is not restricted to low frequency ranges of several hundred Hz, which is typical of mosquito hearing, but seems to be capable of operating at much higher frequencies of up to 10 kHz.

Studies on *Drosophila* mutants regarding the power gain provided by motile mechanosensory neurons (which are amplifying the mechanical input of the ear by adding mechanical energy) determined that proteins within the mechanoelectrical transduction apparatus could possibly carry out such processes (Göpfert et al., 2005). Dynein motors could play such an amplifying role during mechanoelectrical transduction. The dendrite of scolopidial sensory neurons elongates distally into a modified cilium which is surrounded by the scolopale space, whose cavity holds a receptor lymph with high K^+ concentration, comparable to the vertebrate scala media [for an overview, see Yack (Yack, 2004)]. Within the cilium, nine microtubule doublets form a circular arrangement. The links between adjacent microtubule doublets may well be dynein, which is distributed along the length of the cilium, except in the ciliary dilation region. One has to keep in mind, though, that the activation energy calculations based on Arrhenius plots could be problematic if the examined processes occurred in complicated systems such as hearing organs (James, 1959). The data that led to the calculated activation energy were derived from the output of an intact and complex organ. The kinetics as well as the distribution of dynein within the cilium, however, hint that molecules of this particular protein family could be the basis for the observed non-linear processes – spontaneous flagellum oscillations of the Johnston's organ and DPOAE generation in tympanal organs. As the present study aimed to compare the mechanical properties and activation energy values for both types of hearing organs, we followed, as close as possible, the paradigm for the Arrhenius plot derived activation energy used by Warren et al. (Warren et al., 2010). An application of colchicine, as performed for the mosquito, and its potential effects on DPOAEs could corroborate this assumption. Yet in the locust, such an experimental treatment is problematic for anatomical reasons. Müller's organ (which comprises the scolopidia) sits on the inner surface of the tympanal membrane and is surrounded by air-filled chambers. The substance would have to be applied either directly onto the tympanal membrane or topically to the surface of the thorax. Besides the uncertainty about whether the substance reached the organ, both approaches would affect the adjacent structures and their mechanical properties to such an extent that would make the interpretation of the outcome of the experiments ambiguous.

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REFERENCES

Ausborn, J., Wolf, H., Mader, W. and Kayser, H. (2005). The insecticide pymetrozine selectively affects chordotonal mechanoreceptors. *J. Exp. Biol.* **208**, 4451-4466.

Bai, J.-P., Surguchev, A. and Navaratnam, D. (2011). β_4-Subunit increases Slo responsiveness to physiological Ca^{2+} concentrations and together with β_1 reduces surface expression of Slo in hair cells. *Am. J. Physiol.* **300**, C435-C446.

Cator, L. J., Arthur, B. J., Harrington, L. C. and Hoy, R. R. (2009). Harmonic convergence in the love songs of the dengue vector mosquito. *Science* **323**, 1077-1079.
Coro, F. and Kössl, M. (2001). Components of the $2f_1$-f_2 distortion-product otoacoustic emission in a moth. *Hear. Res.* **162**, 126-133.
Crawford, A. C., Evans, M. G. and Fettiplace, R. (1989). Activation and adaptation of transducer currents in turtle hair cells. *J. Physiol.* **419**, 405-434.
Dallos, P. and Fakler, B. (2002). Prestin, a new type of motor protein. *Nat. Rev. Mol. Cell Biol.* **3**, 104-111.
Fettiplace, R. (1987). Electrical tuning of hair cells in the inner ear. *Trends Neurosci.* **10**, 421-425.
Fonseca, P. J. and Correia, T. (2007). Effects of temperature on tuning of the auditory pathway in the cicada *Tettigetta josei* (Hemiptera, Tibicinidae). *J. Exp. Biol.* **210**, 1834-1845.
Göpfert, M. C. and Robert, D. (2001). Active auditory mechanics in mosquitoes. *Proc. Biol. Sci.* **268**, 333-339.
Göpfert, M. C., Humphris, A. D. L., Albert, J. T., Robert, D. and Hendrich, O. (2005). Power gain exhibited by motile mechanosensory neurons in *Drosophila* ears. *Proc. Natl. Acad. Sci. USA* **102**, 325-330.
Gray, E. G. (1960). The fine structure of the insect ear. *Philos. Trans. R. Soc. Lond. B* **243**, 75-94.
Jacobs, K., Otte, B. and Lakes-Harlan, R. (1999). Tympanal receptor cells in *Schistocerca gregaria*: correlation of soma positions and dendrite attachment sites, central projections and physiologies. *J. Exp. Zool.* **283**, 270-285.
James, T. W. (1959). Synchronization of cell division in Amoebae. *Ann. N. Y. Acad. Sci.* **78**, 501-514.
Khvoles, R., Freeman, S. and Sohmer, H. (1998). Effect of temperature on the transient evoked and distortion product otoacoustic emissions in rats. *Audiol. Neurootol.* **3**, 349-360.
Kössl, M. and Boyan, G. S. (1998). Acoustic distortion products from the ear of a grasshopper. *J. Acoust. Soc. Am.* **104**, 326-335.
Kössl, M. and Coro, F. (2006). L1,L2 maps of distortion-product otoacoustic emissions from a moth ear with only two auditory receptor neurons. *J. Acoust. Soc. Am.* **120**, 3822-3831.
Kössl, M., Coro, F., Seyfarth, E.-A. and Nässig, W. A. (2007). Otoacoustic emissions from insect ears having just one auditory neuron. *J. Comp. Physiol. A* **193**, 909-915.
Kössl, M., Möckel, D., Weber, M. and Seyfarth, E. A. (2008). Otoacoustic emissions from insect ears: evidence of active hearing? *J. Comp. Physiol. A* **194**, 597-609.
Lukashkin, A. N., Lukashkina, V. A. and Russell, I. J. (2002). One source for distortion product otoacoustic emissions generated by low- and high-level primaries. *J. Acoust. Soc. Am.* **111**, 2740-2748.
Meenderink, S. W. F. and van Dijk, P. (2006). Temperature dependence of anuran distortion product otoacoustic emissions. *J. Assoc. Res. Otolaryngol.* **7**, 246-252.
Michelsen, A. (1971). The physiology of the locust ear. I. Frequency sensitivity of single cells in the isolated ear. *Z. Vgl. Physiol.* **71**, 49-62.
Möckel, D., Seyfarth, E.-A. and Kössl, M. (2007). The generation of DPOAEs in the locust ear is contingent upon the sensory neurons. *J. Comp. Physiol. A* **193**, 871-879.
Möckel, D., Seyfarth, E.-A. and Kössl, M. (2011). Otoacoustic emissions in bushcricket ears: general characteristics and the influence of the neuroactive insecticide pymetrozine. *J. Comp. Physiol. A* **197**, 193-202.
Oldfield, B. P. (1988). The effect of temperature on the tuning and physiology of insect auditory receptors. *Hear. Res.* **35**, 151-158.
Römer, H. (1976). Die Informationsverarbeitung tympanaler Rezeptorelemente von *Locusta migratoria* (Acrididae, Orthoptera). *J. Comp. Physiol. A* **109**, 101-122.
Warren, B., Lukashkin, A. N. and Russell, I. J. (2010). The dynein-tubulin motor powers active oscillations and amplification in the hearing organ of the mosquito. *Proc. Biol. Sci.* **277**, 1761-1769.
Windmill, J. F. C., Göpfert, M. C. and Robert, D. (2005). Tympanal travelling waves in migratory locusts. *J. Exp. Biol.* **208**, 157-168.
Yack, J. E. (2004). The structure and function of auditory chordotonal organs in insects. *Microsc. Res. Tech.* **63**, 315-337.

3.8. Appendix to Möckel et al. 2012

3.8.1. Additional remarks on the experimental procedures

3.8.1.1. The acoustic coupler for microphone and loudspeakers

The acoustical coupler with which experiments on locusts were conducted was, like in bushcrickets, specifically made to fit the size of the ear opening, its tip diameter amounted to about 2.5 mm. In contrast to earlier experiments, the arrangement of the coupler was changed at a later stage of my thesis. As a way of reducing possible set-up generated artefacts which would mask quadratic distortion-products (f2-f1), a different coupler was used in which the three channels for acoustical stimulation and microphone signal remained separated all the way to the tip of the coupler (Fig.3.6 and Fig. 4.6a).

3.8.1.2. The advantage of locusts over bushcrickets for this particular study

Initial experiments had shown a frequency-dependency of DPOAE amplitude shifts induced by a change in body temperature, with low frequency DPOAEs being particularily affected. The locust's ear reliably generates DPOAEs using low stimulus frequencies around 7-8 kHz. Such low stimulus frequencies do not lead to significant OAE emission in the ears of the bushcricket *M. elongata* where DPOAE generation starts at stimulus frequencies of about 12 kHz upwards (Möckel et al. 2011). The locust's tympanal organ possesses a relatively large tympanum, with the mechanoreceptors directly coupled to its inside. The recorded DPOAEs are larger in amplitude compared to those in bushcrickets, with a stimulus-emission-difference amounting to about 30-50 dB (Kössl and Boyan 1998b). Another factor to not use the bushcrickets for this study was that a change in body temperature during preliminary experiments highly disturbed them, resulting in fast breathing and other movements which in turn caused problems with the acoustical recordings. The locust was therefore chosen for this study, as they all in all remained calmer (n=13).

3.8.1.3. Shifting and monitoring the animal's body temperatue

The experimental course involved DPOAE recordings (1) at the initial medium temperature, (2) after a downward temperature shift, (3) after the return to medium temperature, (4) after an upward temperature shift and (5) after the final return to medium temperature.

Cooling the animals did not affect the animals much in terms of changes in their motor activity. But an increase of body temperature above ca. 30-35°C made them very nervous and uncomfortable, with a lot of fast breathing activity and movement of the extremities which very much disturbed the acoustical recordings. Once started, it took the animals a long time to stop that behaviour, so care was taken to not rise above this temperature threshold.

The body temperature of the animals was permanently monitored throughout the course of the experiments via a temperature sensor (resolution of 0.1°C) which had been placed under

the abdomen cuticle (Fig.3.6). Its position was chosen to provide some distance from the tracheal air sacs surrounding the ear, in order to prevent any effect which a wound in the tracheal structures and inflowing hemolymph might have, and should give a good measure of the overall body temperature.

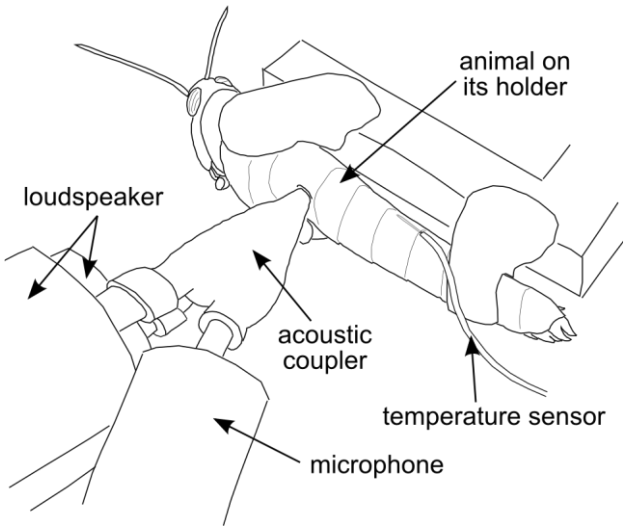

Fig.3.6 Experimental set up for DPOAE recordings during a shift of body temperature in *L. migratoria*. Arrangement for acoustical DPOAE recordings. A temperature sensor to control the body temperature was placed under the dorsal abdomen cuticle. Please see main text for details on the experimental procedure.

3.8.1.4. Arguments against an experimental application of colchicine onto the ear of locusts

Besides the mentioned advantages, the locust's ear and its particular anatomy also holds certain limitations. These constraints mainly concern the accessibility of the scolopidial mechanoreceptors for manipulations without infering with the organ's overall mechanical function. Applications of fluid test substances as done in the bushcrickets are not possible or at least quite problematic, due to anatomical reasons. Müller's organ which comprises the scolopidia sits on the inside surface of the tympanal membrane, surrounded by air-filled chambers. There are three ways to apply a substance to the tympanal organ in a locust. The first would be to apply the drop onto the surface of the tympanum itself. After some time of exposure, the drop would have to be removed in order to restart the acoustical emission measurements. The difficulty with such an approach would be the uncertainty about whether the substance reached the organ und how it affected the tympanum. To avoid that, another way would be to topically apply the substance to the surface of the thorax. But in this case, it would somehow have to enter the air-filled chambers that surround the organ. The third possibility would be to directly inject a substance into the cavity behind the tympanum which would also cause rather severe changes in the organ's mechanical properties. All these approaches would affect the adjacent structures and their mechanical properties in such an extent that would make an interpretation of the outcome of acoustical experiments more than ambitious. Out of those reasons, and apart from some initial tests, I refrained from experiments that involved applying fluid test substances to the locust's ear. Controls in terms of applying colchicine in order to confirm the suggested involvement of the dynein-tubulin system within the scolopidia in DPOAE generation were therefore not performed.

3.8.1.5. Colchicine applications to the tympanal organ of bushcrickets

Bushcrickets of the species *M. elongata* are not applicable for experiments about body temperature shifts and their effects on DPOAE generation, as hardly any emissions are evoked at the temperature-affected frequency range. A colchicine application, however, could be performed much more reliable then in locusts, via a cuticle opening close to the tympanal organ in the foreleg tibia, similar to that used during the pymetrozine study.

Colchicin has disruptive effects on microtubule turnover (that is, their normal balance between free and bound tubulin molecules) and prevents repolymerisation of microtubules after mechanical / acoustical stimulation (Höger and Seyfarth 2001). The application of a drop of colchicine (25 mM) was followed by an irreversible decrease in DPOAE amplitudes across all stimulus levels, starting in most animals within 20-30 min. The amplitude decrease hereby occurred stepwise and reached values of up to 30 dB (n=32 organs).

Such DPOAE experiments were immediately followed by histological stainings to provide evidence of the involved molecules (n=15 organs). These α-tubulin antibody stainings were performed together with Dr. Melanie Weber. It stains bound tubulin which should not be present anymore after mechanical stimulation during colchicine application. The histology results, however, were very inconsistent, and reasons for that could unfortunately not be found.

4. Measurement of sensitive distortion-product otoacoustic emissions in insect tympanal organs

Manfred Kössl, and Doreen Möckel

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Author contributions M. Kössl and D. Möckel wrote the manuscript

Comment to Moir, Jackson, Windmill (2011) No evidence for DPOAEs in the mechanical motion of the locust tympanum. J Exp Biol 214:3165-3172.

Anlage 1

Erklärung über Anteile der Autoren/Autorinnen an den einzelnen Kapiteln der Promotionsarbeit

Titel der Publikation/ des Manuskripts:	**Kössl M, Möckel D (2012) Measurement of sensitive distortion-product otoacoustic emissions in insect tympanal organs. J Exp Biol 215:566-567. Comment to Moir et al. (2011) J Exp Biol 214:3165-3172**	
	Was hat der/die Promovierende bzw. was haben die Co-Autoren/Autorinnen beigetragen[#]	Name des/der jeweiligen Autors/Autoren/Autorin*
(1) Entwicklung und Planung	Kössl M 50%, Möckel D 50%	Kössl M, Möckel D
(2) Durchführung der einzelnen Untersuchungen/ Experimente		
(3) Erstellung der Daten-sammlung und Abbildungen		
(4) Analyse/Interpretation der Daten		
(5) übergeordnete Einleitung/ Ergebnisse/Diskussion	Kössl M 50%, Möckel D 50%	Kössl M, Möckel D
[#]Bei 2, 3 und 4 bitte kurze inhaltliche Angaben der jeweiligen Anteile, bei 1 und 5 reichen prozentuale Angaben		*Mehrfacheintragungen möglich

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Datum/Ort: 05.12.2014 Frankfurt am Main	*Datum:* 05.12.2014
	zustimmende Bestätigung der vorgenannten Angaben
Unterschrift Promovend/Promovendin	*Unterschrift Betreuer/Betreuerin*

Measurement of sensitive distortion-product otoacoustic emissions in insect tympanal organs

In a recent paper in *The Journal of Experimental Biology*, Moir and colleagues did not find evidence for DPOAEs in the motion of the locust typanum (Moir et al., 2011).

Insects with tympanal organs produce sensitive DPOAEs (distortion-product otoacoustic emissions), as shown in locusts, several moth species and bushcrickets (for a review, see Kössl et al., 2008). Their characteristics are largely comparable to those reported from vertebrate ears. Insect DPOAEs are highly vulnerable to manipulations that affect the animal's physiology: hypoxia (Kössl and Boyan, 1998), ethyl ether (Kössl et al., 2007), electrical auditory nerve stimulation (Möckel et al., 2007), a neuroactive insecticide (Möckel et al., 2011) and acoustic suppression by third tones (Kössl and Coro, 2006). These findings strongly indicate a biological origin of acoustical two-tone distortions. Mechanical data from Michelsen (Michelsen, 1971) and Windmill et al. (Windmill et al., 2005) showed for locusts that the tympanum's thin part is maximally deflected by high frequency stimuli, and therefore scolopidia attached at the pyriform vesicle (PV) were most sensitive to that frequency range. Exclusion of these scolopidia *via* mechanical ablation specifically reduced the levels of high frequency DPOAEs evoked by stimuli above 15 kHz. Scolopidia were therefore suggested to be directly involved in frequency-specific DPOAE generation in insects (Möckel et al., 2007) [see also fig. 6 of Kössl et al. (Kössl et al., 2008)].

Moir et al. (Moir et al., 2011) aimed to record mechanical two-tone distortions in the movement of the tympanum and to relate these mechanical responses to the $2f1–f2$ DPOAE frequency defined by the used acoustical stimuli. Several times, the authors termed their measurements 'DPOAEs', which in association with their chosen method (laser Doppler vibrometry) is not quite correct. DPOAEs are defined as sounds that can be recorded in the outer ear canal, resulting from cochlear mechanical non-linearity and depending on physiological activity (for a review, see Kemp, 2008). Likewise, in the case of locusts, they are acoustically recorded close to the tympanum, to which the auditory receptors are directly attached. The authors' second aim was to visualize the effect that hypoxia has on tympanum vibration. Within the limits of their experimental setup, as stated in the last paragraph of Moir et al. (Moir et al., 2011), both parts of their work failed to show positive results. From our extensive experience with insect DPOAEs, we feel we should comment on the present study.

Possible reasons for difficulties in measuring mechanical two-tone distortions

One important factor for measuring sensitive DPOAEs is the acoustical coupler and its tip. The coupler consists of separate tubes for two speakers for stimulation and a microphone to record the emission. To record sensitive DPOAEs in locusts, it is crucial that the microphone is aimed perpendicular to the tympanal membrane, and is positioned as close to it as possible without making direct contact. Additionally, to measure sensitive DPOAEs especially in the low frequency range, it is essential to seal the ear with the coupler in position and hence produce a closed system (see also Kemp, 2008). This creates a situation where a large portion of the sound detected by the microphone comes from the tympanum. The study of Moir et al. (Moir et al., 2011), however, worked with an open system, as required for laser Doppler vibrometry. The authors used the stimulation parameters given in Kössl and Boyan (Kössl and Boyan, 1998), which in that study had produced an emission of 10 dB SPL in a closed system, but did not measure DPOAEs in their preparation. They also calculated the expected mechanical tympanum vibration associated with a 10 dB SPL tone based on a planar source model in an open system. However, the actual DPOAE recording situation (see above) and the complex mechanics of the locust tympanum might not be sufficiently reflected in this simple simulation. In this respect, it would be advisable, if possible, to record DPOAEs and mechanical tympanum vibration with the same closed coupler system, as Dalhoff et al. (Dalhoff et al., 2007) did for the human tympanum. This coupler included an optical fiber for the laser. Of course, the locust tympanum with its directly attached sensory cells and the strong membrane inhomogeneities cannot easily be compared with the human tympanum, and parallel acoustic and mechanical measurements in the locust are needed.

The second part of Moir et al.'s study involved the influence of hypoxia, which reduces DPOAE amplitude (Kössl and Boyan, 1998). As we interpret their data, the authors did not measure mechanical ($2f1–f2$) distortions or their modulation during hypoxia, but found that mechanical vibrations evoked by $f1$ or $f2$ were not affected by CO_2. Of course, during DPOAE recordings, we would not expect that hypoxia influences $f1$ or $f2$ responses but only the much smaller $2f1–f2$ response, whose mechanical analogue Moir et al. could not measure.

Artefacts

In their Results section, Moir and colleagues report the presence of setup artefacts at the DPOAE frequency of about 32 dB SPL (0.8 mPa). The acoustical stimuli used may amount to ~72 dB SPL for $f1$ (82 mPa) and to ~63 dB SPL for $f2$ (28 mPa) and not the other way round as stated in their fig. 4 legend (which is obviously a typing error). This very high distortion level within their sound-producing system might have been caused by the use of a single loudspeaker to deliver both pure-tones. Using two loudspeakers would cause much lower setup distortion during DPOAE measurements and also could be beneficial for investigating their mechanical analogue on the tympanal membrane. It is a common praxis in our lab to use two loudspeakers for delivering the $f1$ and the $f2$ stimulus and also to keep the stimulation and DPOAE recording channels separated as far as the tip of the acoustical coupler, positioned close to the tympanum. In the original report on locust DPOAEs, setup-produced distortions appeared just above the background noise level (–15 to –25 dB SPL) at high stimulation levels of 83 to 91 dB SPL (Kössl and Boyan, 1998). Sensitive locust preparations allow DPOAE measurements at stimulation levels as low as 10–20 dB SPL, giving a safe distance of more than 60 dB to any setup-generated distortion. DPOAEs from tympanal organs reach their highest threshold sensitivity at frequencies defined by the hearing range of the investigated species (cf. Kössl and Boyan, 1998; Coro and Kössl, 1998; Kössl et al., 2008). The pronounced differences in DPOAE threshold characteristics between diverse animal groups (moths, locusts, bushcrickets and several mammalian species) rules out setup distortions whose characteristics would depend on the recording system and not the animal species.

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References

Coro, F. and Kössl, M. (1998). Distortion-product otoacoustic emissions from the tympanic organ in two noctuoid moths. *J. Comp. Physiol. A* **183**, 525-531.

Dalhoff, E., Turcanu, D., Zenner, H. P. and Gummer, A. W. (2007). Distortion product otoacoustic emissions measured as vibration on the eardrum of human subjects. *Proc. Natl. Acad. Sci. USA* **104**, 1546-1551.

Kemp, D. T. (2008). Otoacoustic emissions: concepts and origins. In *Active Processes and Otoacoustic Emissions* (ed. G. A. Manley, R. R. Fay and A. N. Popper), pp. 1-38. Springer, New York: Springer Handbook of Auditory Research.

Kössl, M. and Boyan, G. S. (1998). Acoustic distortion-products from the ear of a grasshopper. *J. Acoust. Soc. Am.* **104**, 326-335.

Kössl, M. and Coro, F. (2006). L1,L2 maps of distortion-product otoacoustic emissions from a moth ear with only two auditory receptor neurons. *J. Acoust. Soc. Am.* **120**, 3822-3831.

Kössl, M., Coro, F., Seyfarth, E.-A. and Nässig, W. A. (2007). Otoacoustic emissions from insect ears having just one auditory neuron. *J. Comp. Physiol. A* **193**, 909-915.
Kössl, M., Möckel, D., Weber, M. and Seyfarth, E.-A. (2008). Otoacoustic emissions from insect ears: evidence of active hearing? *J. Comp. Physiol. A* **194**, 597-609.
Michelsen, A. (1971). The physiology of the locust ear, I. Frequency sensitivity of single cells in the isolated ear, II. Frequency discrimination based upon resonances in the tympanum, III. Acoustical properties of the intact ear. *Z. Vergl. Physiol.* **71**, 49-128.
Möckel, D., Seyfarth, E.-A. and Kössl, M. (2007). The generation of DPOAEs in the locust ear is contingent upon the sensory neurons. *J. Comp. Physiol. A* **193**, 871-879.
Möckel, D., Seyfarth, E.-A. and Kössl, M. (2011). Otoacoustic emissions in bushcricket ears: general characteristics and the influence of the neuroactive insecticide pymetrozine. *J. Comp. Physiol. A* **197**, 193-202.
Moir, H. M., Jackson, J. C. and Windmill, J. F. C. (2011). No evidence for DPOAEs in the mechanical motion of the locust tympanum. *J. Exp. Biol.* **214**, 3165-3172.
Windmill, J. F. C., Göpfert, M. C. and Robert, D. (2005). Tympanal travelling waves in migratory locusts. *J. Exp. Biol.* **208**, 157-168.

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Response to ‘Measurement of sensitive distortion-product otoacoustic emissions in insect tympanal organs’

We thank Kössl and Möckel for their correspondence regarding our article (Moir et al., 2011) and the Editor for the opportunity to reply.

In general, the occurrence of distortion products (also called ‘intermodulation’ or ‘intermodulation distortion’) indicates that the system in question is non-linear. This non-linearity can really be anything: from active feedback to passive structural non-linearities. A single-frequency input into a non-linear system will result in a distorted signal that, spectrally, will contain a series of harmonics of the fundamental. Similarly, if two frequencies are input, then intermodulation will occur and sidebands at various arithmetic combinations of the two input frequencies will exist in the frequency spectrum.

A distortion-product otoacoustic emission (DPOAE) is thus a result of such an intermodulation, recorded as a sound emission from an ear. For sound to be emitted from any system, a structure within that system must be vibrating (as in a loudspeaker). In the case of locust ears, the DPOAE sounds are understood to originate from the tympanal organ. As a result, there should be a mechanical signature on the motion of the tympanal organ – something we aimed to measure. Over a large number of animals, at both ethologically relevant sound pressures and louder, we were unable to measure the largest distortion product (at $2f1–f2$) in the mechanical motion of the tympanum using laser Doppler vibrometry. As pointed out by Kössl and Möckel, sensitivity of this effect to the physiology of the insect indicates a biological origin, and the mechanosensitive neurones (scolopidia), which attach to the tympanum, were the most likely candidate for the production of DPOAEs in insects (Kössl et al., 2008).

If the locust ear produces DPOAEs as part of its everyday sound transduction behaviour, then we should be able to measure the structure vibrating to generate them (within the limits of our measurement system), regardless of whether the system is closed or not. Therefore, if the tympanal membrane is that structure, and is receiving the correct two-tone sounds [as is clearly evident from measurements showing that the membrane vibrates at those tone frequencies; see fig. 2, Moir et al. (Moir et al., 2011)], such that DPOAEs are being produced by the same membrane (or the scolopidia attached to the membrane) then that tympanal membrane must also be vibrating at the $2f1–f2$ distortion-product frequency. Such vibrations were not recorded (down to the laser vibrometer’s 3 pm noise floor).

Kössl and Möckel suggest that the use of an acoustic coupler and its tip are important for measuring DPOAEs. The use of an acoustic coupler is simply a method by which faint sounds from a small source can be recorded by a microphone. While this is clearly beneficial for measuring very quiet sounds, it is irrelevant for our report. The existence of a DPOAE at a known frequency and sound pressure must correlate with the motion of a structure emitting the sound. Our investigation could not find evidence of the tympanal membrane vibrating in relation to known DPOAEs.

The reasonable concern of artefacts in the current experiments was also raised. When studying an effect that is derived from any non-linearity, artefacts must be carefully noted and avoided. However, we were very careful not to overdrive our single loudspeaker so as not to generate artefactual two-tone distortion; this was monitored using the microphone. It was only possible to produce distortion-products by overdriving the loudspeaker and not the animal, and we took this into account throughout our experiments [see fig. 4, Moir et al. (Moir et al., 2011)]. In any case, the presence or not of some acoustic artefacts in the setup used does not in any way relate to the absence of any evidence for measured vibrations at any distortion-product frequencies.

Finally, we are not questioning the existence of DPOAEs in locusts, or their physiological dependence, the evidence for which is strong. However, it is concerning that we were not able to measure a mechanical two-tone vibration on the locust tympanum with an experimental setup that is capable of doing so. Rather than put these results in the ‘file drawer’, we believe it is better that the scientific community is made aware of these results so that the question of where DPOAEs are generated can be resolved more quickly. We very sincerely hope that our work will be followed by that of others in order to continue to examine, and seek to explain, all the intricate workings of the locust (and other) insect ears.

10.1242/jeb.068098

References

Kössl, M., Möckel, D., Weber, M. and Seyfarth, E.-A. (2008). Otoacoustic emissions from insect ears: evidence of active hearing? *J. Comp. Physiol. A* **194**, 597-609.
Moir, H. M., Jackson, J. C. and Windmill, J. F. C. (2011). No evidence for DPOAEs in the mechanical motion of the locust tympanum. *J. Exp. Biol.* **214**, 3165-3172.

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5. Mechanical basis of otoacoustic emissions in tympanal hearing organs

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Author contributions Experiment design by D. Möckel and M. Nowotny; preliminary tests and all experiments performed by D. Möckel; data collection and presentation by D. Möckel; data analysis by D. Möckel with consultation from M. Kössl and M. Nowotny; D. Möckel wrote the manuscript with contributions from M. Kössl and M. Nowotny

Abstract Tympanal hearing organs of insects emit distortion-product otoacoustic emissions (DPOAEs), which in mammals are used as indicator for nonlinear cochlear amplification, and which are highly vulnerable to manipulations interfering with the animal's physiological state. Although in previous studies, evidence was provided for the involvement of auditory mechanoreceptors, the source of DPOAE generation and possible active mechanisms in tympanal organs remained unknown. Using laser Doppler vibrometry in the locust ear, we show that DPOAEs mechanically emerge at the tympanum region where the auditory mechanoreceptors are attached. Those emission-coupled vibrations differed remarkably from tympanum waves evoked by external pure tones of the same frequency, in terms of wave propagation, energy distribution, and location of amplitude maxima. Selective inactivation of the auditory receptor cells by mechanical lesions did not affect the tympanum's response to external pure tones, but abolished the emission's displacement amplitude peak. These findings provide evidence that tympanal auditory receptors, comparable to the situation in mammals, comprise the required nonlinear response characteristics, which during two-tone stimulation lead to additional, highly localized deflections of the tympanum.

Anlage 1

Erklärung über Anteile der Autoren/Autorinnen an den einzelnen Kapiteln der Promotionsarbeit

Titel der Publikation/ des Manuskripts:	**Möckel D, Nowotny M, Kössl M (2014) Mechanical basis of otoacoustic emissions in tympanal hearing organs. J Comp Physiol A 200:681-691**	
	Was hat der/die Promovierende bzw. was haben die Co-Autoren/Autorinnen beigetragen[#]	Name des/der jeweiligen Autors/Autoren/Autorin*
(1) Entwicklung und Planung	Möckel D 50%, Nowotny M 50%	Möckel D, Nowotny M
(2) Durchführung der einzelnen Untersuchungen/ Experimente	vorbereitende Tests; Kontrollversuche, um die Stabilität der Präparation zu testen; Durchführung der Experimente	Möckel D
(3) Erstellung der Datensammlung und Abbildungen	Auslesen der Rohdaten; Erstellung aller Abbildungen der Publikation	Möckel D
(4) Analyse/Interpretation der Daten	Analyse der Rohdaten; statistische Zusammenfassung und Auswertung der Daten	Möckel D
	Diskussion der Daten	Nowotny M, Kössl M
(5) übergeordnete Einleitung/ Ergebnisse/Diskussion	Möckel D 70%, Nowotny M 15%, Kössl M 15%	Möckel D, Nowotny M, Kössl M
[#]Bei 2, 3 und 4 bitte kurze inhaltliche Angaben der jeweiligen Anteile, bei 1 und 5 reichen prozentuale Angaben		*Mehrfacheintragungen möglich

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ORIGINAL PAPER

Mechanical basis of otoacoustic emissions in tympanal hearing organs

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Abstract Tympanal hearing organs of insects emit distortion–product otoacoustic emissions (DPOAEs), which in mammals are used as indicator for nonlinear cochlear amplification, and which are highly vulnerable to manipulations interfering with the animal's physiological state. Although in previous studies, evidence was provided for the involvement of auditory mechanoreceptors, the source of DPOAE generation and possible active mechanisms in tympanal organs remained unknown. Using laser Doppler vibrometry in the locust ear, we show that DPOAEs mechanically emerge at the tympanum region where the auditory mechanoreceptors are attached. Those emission-coupled vibrations differed remarkably from tympanum waves evoked by external pure tones of the same frequency, in terms of wave propagation, energy distribution, and location of amplitude maxima. Selective inactivation of the auditory receptor cells by mechanical lesions did not affect the tympanum's response to external pure tones, but abolished the emission's displacement amplitude peak. These findings provide evidence that tympanal auditory receptors, comparable to the situation in mammals, comprise the required nonlinear response characteristics, which during two-tone stimulation lead to additional, highly localized deflections of the tympanum.

Keywords DPOAE · Laser Doppler vibrometry · Locust · Tympanum · Insect

Abbreviations

DPOAE	Distortion–product otoacoustic emission
LDV	Laser Doppler vibrometry
SPL	Sound pressure level

Introduction

Insects with tympanal organs produce sensitive distortion–product otoacoustic emissions (DPOAEs), as shown in locusts (Kössl and Boyan 1998; Möckel et al. 2007, 2012), moths (Kössl and Coro 2006; Kössl et al. 2007) and bushcrickets (Möckel et al. 2011), that in mammals are used as indicator for nonlinear cochlear amplification. Characteristics of insect DPOAEs are largely comparable to those reported from vertebrate ears. They appear during simultaneous stimulation with two pure tones ($f_1 < f_2$) as additional spectral peaks at frequencies of $nf_1 - (n-1)f_2$ and $nf_2 - (n-1)f_1$. The $2f_1 - f_2$ emission is most prominent, as it is evoked by lowest stimulus levels, with the largest amplitude reached at frequencies of high auditory sensitivity. In moths and locusts, the $2f_1 - f_2$ DPOAEs are elicited already at stimuli levels near the species-specific auditory threshold (Kössl et al. 2008). Insect DPOAEs are highly vulnerable to manipulations that interfere with the animal's physiological state, such as hypoxia (Kössl and Boyan 1998) or ethyl ether (Kössl et al. 2007). First evidence that the auditory mechanoreceptors and specifically the dynein–tubulin system within could be relevant for DPOAE generation came from selective exclusion of mechanoreceptors via mechanical lesions (on locusts, Möckel et al. 2007), the effects of a neuroactive insecticide (on bushcrickets,

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Möckel et al. 2011), and the temperature dependence of mechanical organ vibrations and DPOAE amplitudes (on mosquitoes, Warren et al. 2010; on locusts, Möckel et al. 2012).

Otoacoustic emissions in vertebrates are air pressure fluctuations recorded in the outer ear canal (Kemp 1978, 2008). They result from cochlear mechanical nonlinearity, depend on the cochlea's physiological state, are transmitted retrogradely through the middle ear and cause ear drum vibrations (Dalhoff et al. 2007). These vibrations are emitted as sounds and therefore recordable with sensitive microphones. The first and so far only study to simultaneously record DPOAEs and mechanical tympanum vibrations in humans used a coupler that included the acoustical channels and an optical laser beam fiber, and reported tympanum displacements at the $2f_1 - f_2$ frequency of 1–8 pm (Dalhoff et al. 2007).

Our study combines acoustical DPOAE recordings with mechanical measurements of DPOAE analogs within the tympanum's movement of the desert locust *Schistocerca gregaria*, using laser Doppler vibrometry (LDV). In contrast to the indirect coupling of the tympanic membrane of mammals to the sensory epithelium, the locust's tympanal membrane is directly coupled with about 80 scolopidial mechanoreceptors (Gray 1960; Michelsen 1971; Römer 1976; Jacobs et al. 1999), which are the potential source of DPOAE generation. Up to now, however, studies failed to show that DPOAEs are generated mechanically on the tympanum (Moir et al. 2011; comment by Kössl and Möckel 2012; see also the discussion). The locust tympanum comprises an abrupt change in thickness and stiffness between the outer thin and inner thick membrane part (Stephen and Bennet-Clark 1982), and it responds to sound by traveling wave-like vibrations whose envelopes depend on sound frequency (Windmill et al. 2005). Low sound frequencies induce waves that start at the outer rim of the tympanum and continue to travel towards the thick membrane region. For high frequencies ≥12 kHz, there is a distinct maximum of tympanum vibration close to the location of the pyriform vesicle, the attachment zone for high-frequency receptor cells. High-frequency DPOAEs also depend on the integrity of receptor cells attached at the pyriform vesicle (Möckel et al. 2007), a structure clearly visible through the intact tympanal membrane. In the present study, we therefore focused on this tympanal region and assessed mechanical DPOAE analogs evoked by high-frequency stimuli above 15 kHz.

We demonstrate mechanical correlates of DPOAEs within the tympanum movement during stimulation with two pure tones. DPOAEs are acoustic events that can be recorded with sensitive microphones, as done in the first part of the experimental time line. The second part of our approach dealt with the underlying vibrating structure, the tympanal membrane that gave rise to these additional sound events. Its motion pattern showed additional mechanical events that were not evoked by external stimulation, but were correlated with the acoustical DPOAE events by frequency. The displacement pattern of such internally evoked DPOAE analogs were locally restricted around the attachment position of the high-frequency receptors and by that differed profoundly from vibrations which had been evoked by external stimuli.

Materials and methods

Animals and preparations

Both male and female adult desert locusts (*Schistocerca gregaria*; $n = 22$; 14 ♀ and 8 ♂; commercial supplier, Frankfurt) were used for the present study, as no obvious gender differences in functional anatomy, tympanum vibration pattern and DPOAE generation have been reported (Kössl and Boyan 1998; Windmill et al. 2005; Möckel et al. 2007). After anesthesia using CO_2, legs and wings were removed to provide free access to the tympanum. The acoustic tracheal system of the locust ear itself was not further altered or harmed. The animals were fixed laterally with their thorax onto a metal holder using rosin-beeswax. Once these preparations were finished, CO_2 was not re-applied, and the animals remained awake during the course of all measurements, confirmed by normal breathing movements and fast reflexes upon touching the antennae. After the experiments, the animals were re-anesthetized with CO_2 and killed by decapitation.

Experimental outline

The measurements were conducted in two different experimental set-ups in separate soundproof chambers. The interior of these chambers was kept at room temperature (maximum fluctuations during experiments ±1 °C). Each experiment started with acoustical DPOAE measurements, during which the optimal stimulation frequencies f_1 and f_2 to evoke large $2f_1 - f_2$ emissions were determined (Fig. 1a). Only animals that had produced sufficient DPOAEs ($2f_1 - f_2$ level at least 10 dB above noise level for medium stimulation levels) during the acoustical measurements were used for further investigations. There were individuals without recordable DPOAEs, possibly caused by age or ecdysis defects of the tympanal organ and surrounding structures. Using young and medium aged adult animals, our criterion included all but one locust (95.6 %), the data of which were not used. The animal on its holder was then moved to the laser Doppler vibrometry (LDV) set-up to record the tympanum's mechanical responses upon

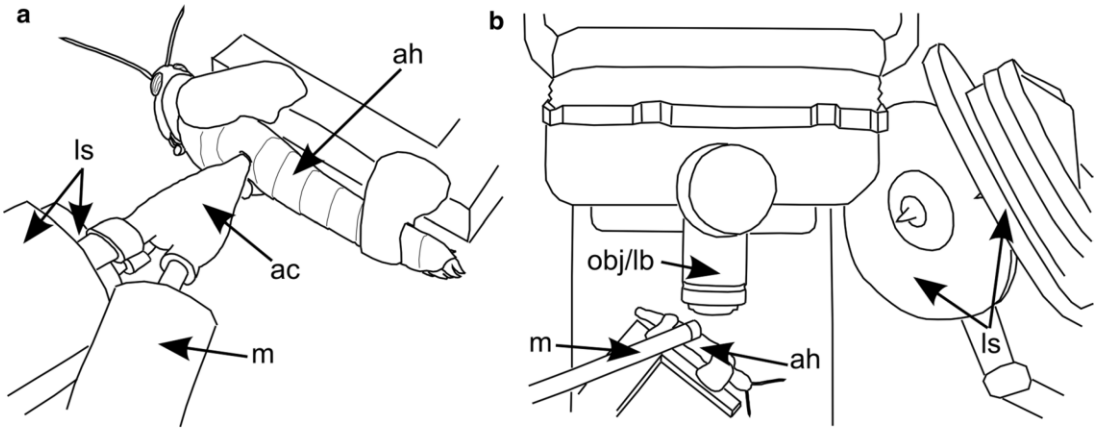

Fig. 1 Experimental set-ups. **a** Arrangement for acoustical DPOAE recordings and **b** for mechanical laser Doppler vibrometry (LDV) measurements. (*ac* acoustic coupler, *ah* animal on its holder, *ls* loudspeakers, *m* microphone, *obj/lb* objective lens of the microscope leading the laser beam)

stimulation with the two pure tones f_1 and f_2 which had been defined during the previous acoustical experiments (Fig. 1b). Control measurements included the presentation of the calculated $2f_1 - f_2$ emission frequency as pure tone with stimulation levels of 5–20 dB SPL. The use of these low levels hereby induced small displacement amplitudes as it is the case with the emerging emission frequency evoked during stimulation with f_1 and f_2.

In two cases, the experimental paradigm was expanded. After the initial acoustical and mechanical measurements on the intact tympanal organ, a specific cell-group of high-frequency receptors (d-cells) was selectively disabled. A mechanical lesion was made in the connection between the attachment point of the d-cells at the pyriform vesicle and the peripheral ganglion using a thin insect needle held by a micromanipulator (experimental details, Möckel et al. 2007). Both the acoustical and the mechanical measurements were then repeated.

The acoustical DPOAE recordings and the mechanical LDV measurements were both done using f_1 and f_2 stimulus levels of up to 70 dB sound pressure level (SPL). Based on the threshold curves for the different receptor groups in the locust's ear (Römer 1976), an involvement of receptors other than those tuned to high frequencies (d-cells) might only set in at the very highest of our used stimulus levels at 70 dB SPL. The main part of the stimulus-level range lies within the operating range of only the d-cells.

Acoustical measurements of DPOAEs

The procedures followed previously reported DPOAE experiments on locusts (Möckel et al. 2007, 2012). The generation of the two acoustic stimuli and the analysis of the incoming microphone signal was accomplished with a Microstar DAP 840 DSP card (Microstar Laboratories; sampling rate 200 kHz) that was controlled by custom software. The two output channels were connected to two attenuators (TDT System3, Tucker Davis Technologies), an amplifier, and two 1 in. microphones (Microtech Gefell, Type 103.1) that served as loudspeakers. An additional 0.5 in. microphone (Brüel & Kjær, Type 4133) measured the incoming acoustical signals and was connected by a preamplifier (Brüel and Kjær, Type 2660) and an amplifier (Brüel & Kjær, Type 2610) to the input channel of the DSP card. The channels for stimulation with f_1 and f_2 and the recording of the microphone signal came together in an acoustic coupler whose tip diameter fitted the size of the locust's ear opening. The small remaining space between the coupler and the cuticle rim that surrounds the tympanal organ was not sealed to prevent a contamination of the organ by the sealing medium during the transfer from one set-up to the other. Yet as the tip of the acoustic coupler was fitted for the size of the ear opening, the positioning of the coupler already created a nearly closed acoustic system which is essential for sensitive DPOAE measurements especially in the low frequency range. The orientation of the acoustic coupler was hereby optimal in relation to the tympanum and highly reproducible from one tested animal to the next. The sound system was calibrated in situ, with the coupler placed in measuring position, in the same plane as the tympanum and as close as possible at a distance of 0.2–1 mm.

The tympanal organ was stimulated with two pure tones of different frequencies ($f_1 < f_2$). The level of the f_1 stimulus (L1) was chosen to be 10 dB above that of f_2 (L2), as this has been found to induce maximal DPOAE levels (Kössl and Boyan 1998). The measured microphone signal was averaged 100 times before fast Fourier transform (FFT)

analysis. Each presentation of a pair of pure tone stimuli took 4.2 s for 100 averages. Accordingly, each measurement took approximately 1.5–2 min, depending on the respective number of frequency/ratio/level steps.

The following procedures for DPOAE recordings were used: DPOAE audiograms were obtained by evoking DPOAEs over a wide frequency range (1–30 kHz) with constant frequency ratio and stimulus levels (f_2/f_1 ratio of 1.04, 1.08 and 1.15; L1/L2 60/50 dB SPL). Based on these DPOAE audiograms, f_2 frequencies that elicited large $2f_1 - f_2$ emissions were chosen for further measurements. The main focus hereby lay on frequencies above about 15 kHz. The respective f_1 frequency was adjusted for each f_2 frequency according to the optimum f_2/f_1 ratio that evoked the largest $2f_1 - f_2$ emissions. DPOAE growth functions were recorded by keeping both stimulus frequencies constant and increasing their levels in 2 dB steps, from 20/10 to 70/60 dB SPL. At these stimulus levels, no set-up-generated distortions were detectable above the noise level of ≤ -20 dB SPL. Background noise level was measured 100 Hz below the DPOAE frequency.

Mechanical measurements of tympanum vibrations

The vibration pattern induced by stimulating the ear with two pure tones f_1 and f_2 was measured using a microscanning laser Doppler vibrometer (LDV) scanning system (MSV-Z-040, Polytec). The sensor head (OFV-534, Polytec) was coupled via a microscope adapter (OFV-072, Polytec) to a microscope (Axio ExaminerA1, Zeiss) with a 4× objective (N-Acroplan, Zeiss).

Under video control (VCT-101, Polytec) and using an external light source (Schott KL1500), the animal on its holder was positioned under the microscope that was coupled to the LDV system. The inward slope of the locust's tympanal membrane and the half overlapping cuticle rim surrounding it prevented the scanning of the entire tympanum. With numbers slightly varying for respective animals, 118–125 laser measurement points were therefore positioned at the pyriform vesicle (the attachment point of the high-frequency receptors) and the adjacent parts of the thin and thick membrane that were clearly visible through the microscope. Distances between single measurement points amounted to about 57.33 µm, and a total tympanum area of about 0.345 mm^2 was covered.

The nature of optical measurements using an LDV required free access of the laser beam to the tympanum. Two loudspeakers (R2904/700000 and R2904/700005, ScanSpeak) were positioned in 10 cm distance to the tympanal organ. A 1/4 in. microphone (MK301, Microtech Gefell), placed 1 cm next to the animal and aiming towards the loudspeakers was used for sound system calibration and as a control of any set-up-generated distortions. As opposed to the nearly closed system during acoustical DPOAE measurements, this created (referring to the methodical approach) an open acoustic measuring system. The acoustical measurement of DPOAEs simultaneously to LDV recordings of the tympanum vibrations, however, was not possible in this experimental set-up. Acoustic stimulus generation and microphone signal analysis were accomplished by an external soundcard (RME fireface 400, RME Audio; sampling rate 48 kHz) that was controlled by custom software (MATLAB R2007b). The two output channels were connected to an amplifier (Rotel RB 850 Stereo Power Amplifier) and then passed on to the loudspeakers. The microphone signal fed into a preamplifier (Brüel & Kjær, Type 0350) and an amplifier (Brüel & Kjær, Type 2610) and from there to the input channel of the external soundcard as well as to the Scan Controller of the LDV system.

Applying a Hanning window FFT analysis, the vibration responses of the tympanal membrane were recorded during the continuous acoustical stimulation as velocity of the motion with a resolution of 31.25 Hz. Using a sampling rate of 128 kHz, the recording of each measurement point took 32 ms for 30 averages, amounting to a total of about 2:20 min per recording, depending on the number of measurement points.

Frequencies which had been optimized to generate large DPOAEs during previous acoustical DPOAE measurements were used as stimuli for the LDV recordings. Their levels ranged from 20/20 to 70/70 dB SPL. Both f_1 and f_2 were presented at the same level. The use of two loudspeakers in order to avoid system generated distortions caused a certain angle between both. Only one of the loudspeakers was oriented optimally and perpendicular to the tympanum. This, in turn resulted in a 10 dB difference in the measured relative displacement amplitudes, similar to the situation in acoustical DPOAE recordings (Fig. 2).

Data analysis was performed using the PSV 8.7 software (Polytec) and Sigma Plot 10.0 (Systat Software). Data were not smoothed prior to analysis. An exception was made when evaluating the distribution of wave forms across the entire measured area. The threshold for minimal displacement amplitudes was set at 0.01 nm, with lower values being rejected.

Results

Additional spectral peaks at the emission frequency

We were able to demonstrate mechanical distortions at the $2f_1 - f_2$ emission frequency on the locust tympanum during stimulation with two pure tones. In all 22 tested animals, maximum amplitudes of such additional tympanum vibrations lay close to the pyriform vesicle. In terms of their absolute displacement amplitudes, tympanum vibrations of

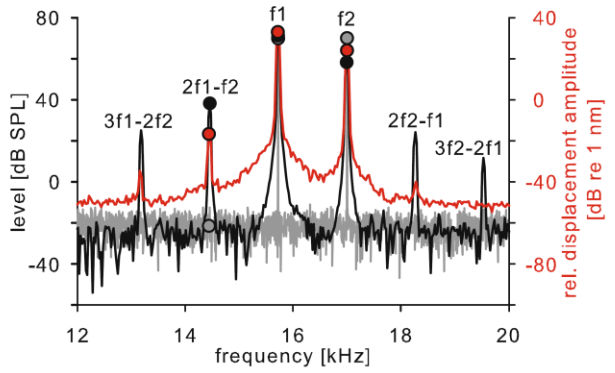

Fig. 2 Acoustically and mechanically measured amplitude spectra as well as controls against system artifacts during high level stimulation. Stimulus frequencies f_1 15.72 kHz and f_2 17 kHz. *Black* Acoustically recorded DPOAEs (stimulus levels 70/60 dB SPL), with additional spectral peaks appearing at frequencies of $nf_1 - (n-1)f_2$ and $nf_2 - (n-1)f_1$. *Red* LDV measured tympanal displacement amplitude, given as median of 15 laser points centered at and around the pyriform vesicle (stimulus levels 70/70 dB SPL). Using two loudspeakers to avoid set-up generated distortions, with hence only one oriented optimal and perpendicular to the tympanum, resulted in a 10 dB difference in relative displacement amplitudes, similar to acoustical DPOAE recordings. *Gray* The loudspeaker signal which had been recorded simultaneously during each LDV measurement via a microphone placed next to the animal served as control for any system generated distortions and displayed even at the highest used stimulus levels of 70/70 dB SPL only the two stimulus frequencies at f_1 and f_2, but no further peaks at the emission frequencies. This finding of no distortions within the loudspeaker signal is also backed by the low coherence between the loudspeaker signal and the velocity of the tympanum vibrations at the $2f_1 - f_2$ emission frequency (data not shown). Their median values averaged across all measured stimulation levels amounted to 0.0390 ± 0.0241, demonstrating no correlation between loudspeaker signal and the $2f_1 - f_2$ emission frequency in the vibration pattern (f_1: 0.9397 ± 0.073; f_2: 0.9083 ± 0.1426)

the two pure tone stimuli as well as of the $2f_1 - f_2$ emission varied across the tested animals. Amplitudes of stimulus and emission which have been obtained at the pyriform vesicle using the same f_2 stimuli, that is, 17 and 18 kHz with several L2 levels, were grouped together, to visualize their distributions over animals (Table 1; values given as mean ± standard deviation). Note that more than one frequency-level combination had been used in respective animals. For a better comparability with values reported in the literature for acoustical DPOAE measurements, the corresponding differences in displacement amplitudes between the f_2 stimulus tone and the $2f_1 - f_2$ emission were calculated (Table 1, lower row). Using 17 kHz as f_2 stimulus, such differences amounted to mean values of 42.4, 51.4, 45.4, and 50.9 dB for L2 levels of 70, 65, 60, and 55 dB SPL, respectively. In case of f_2 being 18 kHz, these mean differences amounted to 46.6, 46.9, 45.5, and 46.4 dB for the mentioned stimulus levels. Such values are comparable to acoustical DPOAE measurements reported for locusts (30–50 dB stimulus–emission difference, Kössl and Boyan 1998).

We determined the averaged contribution of the $2f_1 - f_2$ emission's displacement amplitude along a transect line across the tympanum (Fig. 3; $n = 15$; f_2 17 kHz, L2 70 dB SPL). The arrangement of laser measurement points varied between animals. Their location is therefore displayed as distance from the pyriform vesicle. Negative distance values hereby indicate the direction towards the thick tympanal membrane region and positive distance values the direction towards the thin membrane region (as indicated by arrows below the graph). The mean emission displacement amplitudes reached their largest values of about −20 dB re 1 nm at and around the pyriform vesicle and decreased to values around −45 dB re 1 nm towards the thick membrane region and to about −35 dB re 1 nm towards the thin membrane region.

We chose to show two representative examples, one with large vibration amplitudes at the emission frequency (Figs. 4, 5), and a second example comparing LDV measurements before and after selectively excluding the high-frequency receptors (Fig. 6). The arrangement of laser measurement points (Fig. 4a) and stimulus frequencies (f_1 15.72, f_2 17 kHz) were kept the same in both animals.

During stimulation with f_1 and f_2 (marked blue and green; Fig. 4b), the mechanical distortion appeared as additional spectral peak at the $2f_1 - f_2$ emission frequency (marked red), with largest displacement amplitudes at the pyriform vesicle, the mechanoreceptor's attachment position (laser point 61). For spectra obtained at and around the pyriform vesicle, stimulation levels of 70/70 dB SPL evoked emission displacement amplitudes of −19 to −12 dB re 1 nm, with differences between f_2 and $2f_1 - f_2$ amounting to 39.7 dB. Depending on location, further emission peaks at $3f_1 - 2f_2$ or $2f_2 - f_1$ were clearly distinguishable from the background noise floor (marked gray; −35 to −28 dB re 1 nm).

Differences between internally and externally evoked tympanum vibrations

Quite striking was the distinct difference between waveforms of internally and externally evoked vibrations. Internally evoked vibrations emerged during two-tone stimulation with f_1 and f_2, with the emission frequency not being present in the loudspeaker signal. Externally evoked vibrations were induced by using the calculated emission frequency as pure tone stimulus, at low stimulus levels. Both waves vibrated at the same frequency, yet they differed profoundly, in terms of wave propagation, energy distribution and location of amplitude maxima.

Instantaneous waveforms of internally evoked tympanum vibrations plotted along a transect line across the central tympanum region (from the thick membrane part, along the course of the scolopidial dendrites, across the pyriform

Table 1 Tympanum displacement amplitudes measured in different animals are given for the f_2 stimulus tone and corresponding $2f_1 - f_2$ emission as well as the stimulus-to-emission difference

	L2 70 dB SPL		L2 65 dB SPL		L2 60 dB SPL		L2 55 dB SPL	
	17 kHz $n = 15$	18 kHz $n = 9$	17 kHz $n = 6$	18 kHz $n = 6$	17 kHz $n = 9$	18 kHz $n = 8$	17 kHz $n = 6$	18 kHz $n = 6$
Rel. displacement amplitude of f_2 stimulus (dB re 1 nm)	23.74 ± 5.26	25.51 ± 4.63	21.64 ± 4.35	22.17 ± 2.52	13.68 ± 6.83	12.95 ± 7.62	11.99 ± 2.71	10.29 ± 3.58
Rel. displacement amplitude of $2f_1 - f_2$ emission (dB re 1 nm)	−18.69 ± 11.10	−21.06 ± 11.12	−29.77 ± 12.93	−24.72 ± 6.55	−31.66 ± 10.80	−32.53 ± 10.54	−38.92 ± 9.12	−36.08 ± 4.94
f_2-stimulus-to-$2f_1 - f_2$-emission-difference (dB)	42.42 ± 10.07	46.57 ± 13.78	51.41 ± 10.53	46.89 ± 8.29	45.33 ± 11.27	45.48 ± 13.41	50.90 ± 9.36	46.37 ± 5.09

Data that had been obtained using 17 and 18 kHz as f_2 stimulus with several L2 stimulus levels were grouped together. Values for each frequency-level combination are given as mean ± standard deviation

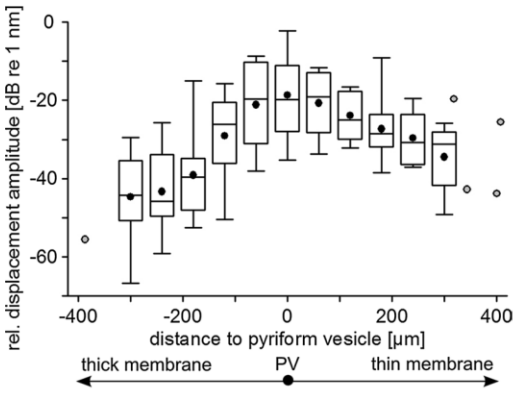

Fig. 3 Contribution of displacement amplitudes for the $2f_1 - f_2$ emission frequency along a transect line across the tympanum. The location of the laser measurement points is displayed as distance from the pyriform vesicle, indicated by *arrows* below the graph. All data sets ($n = 15$ measurements) were obtained using 17 kHz and 70 dB SPL as f_2 stimulus frequency and level. Displacement amplitude values are grouped in intervals of 60 µm around the pyriform vesicle and given as mean (*black circles within boxes*), median (*horizontal line within boxes*), 75/25th percentiles (*boxes*) and 5/95th percentiles (*error bars*). *Gray circles* are single data points outside the distance intervals

vesicle, towards the thin membrane area; see Fig. 4a) displayed locally restricted amplitude maxima of 0.25 nm at the pyriform vesicle (Fig. 4c, left column). Displacements were slightly smaller at adjacent areas and minimal at the thick membrane region as well as towards the tympanum's outside rim. Corresponding phase data for the emission (red; Fig. 4c, lower left panel) also showed a maximum at the pyriform vesicle, with decreasing values (phase lag) towards both ends of the transect line. In contrast to that, the phase of both stimuli steadily decreased in the direction of propagation from the thin towards the thick part of the membrane (f_1 blue and f_2 green; Fig. 4c, lower left panel). The decrease in phase values signifies that the motion of the tympanum lags the phase of the stimulus signal. Instantaneous waveforms of externally evoked tympanum vibrations at the emission frequency, however, differed profoundly from those of the intrinsically generated $2f_1 - f_2$ wave event (Fig. 4c, right column), in terms of energy distribution and location of amplitude maxima. Its phase data corresponded with findings for pure tone stimuli during DPOAE generation (Fig. 4c, lower right panel). The stimulus levels of these externally evoked vibrations (20 dB SPL) hereby were chosen to induce even smaller displacement amplitudes than that of the internally evoked emission, 0.074 versus 0.25 nm, respectively (note that *y*-axes in Fig. 4c differ to still provide visibility of the different instantaneous waveforms).

Tympanum responses and their spatial spread across the measured area (Fig. 5a) further highlighted differences between internally (Fig. 5b) and externally evoked displacements (Fig. 5c). Internally evoked DPOAE analogs in the

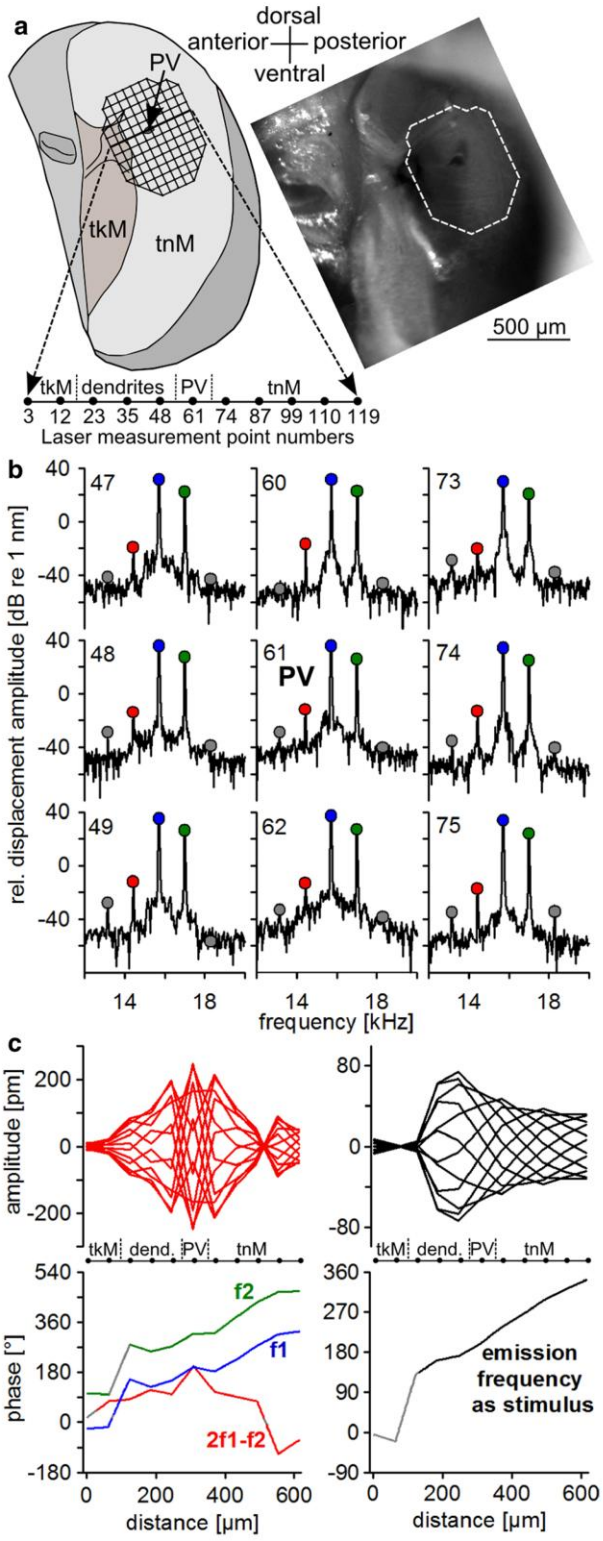

Fig. 4 Amplitude spectra of single laser points and internally versus externally evoked tympanum displacements. **a** Schematic exterior view of the locust's tympanal organ, with the arrangement of laser points at the pyriform vesicle and adjacent parts of the thin and thick membrane (*left*), and under video control during LDV recordings (*right*). Distances between laser points 57.33 µm, covered area about 0.345 mm^2. A transect line across the central tympanum region (from the thick membrane part, along the course of the scolopidial dendrites, across the pyriform vesicle, towards the thin membrane area) was used for further analysis. (*PV* pyriform vesicle, *tkM* thick and *tnM* thin part of tympanum). **b** Amplitude spectra of the nine laser points at and around the pyriform vesicle during stimulation with two pure tones (*blue* f_1 15.72 kHz; *green* f_2 17 kHz; 70/70 dB SPL; *red* $2f_1 - f_2$ emission; *gray* further emission peaks). **c** Tympanum displacement along a transect line (given in **a**), during stimulation with two pure tones (*left panel*; f_1 15.72 kHz; f_2 17 kHz; 70/70 dB SPL) and using the emission frequency as pure tone stimulus (*right panel*; 14.44 kHz; 20 dB SPL). *Upper graphs* instantaneous waveforms of tympanum vibration at the $2f_1 - f_2$ emission frequency, with the amplitude response given in 30° phase intervals for one cycle motion of 360°. *Lower graphs* phase data (*gray* amplitude responses below 0.1 nm)

tympanum's motion were locally restricted and centered around the attachment position of the high-frequency receptors, with their wave nodes and troughs being reminiscent of standing waves (Fig. 5b). The displacement of surrounding tympanum regions within the dorsal, ventral and posterior edges of our measurement area was smaller or absent. Externally evoked vibrations, however, caused larger tympanum displacements at all remaining parts of the measured area, propagating from posterior locations towards the pyriform vesicle (Fig. 5c). Note that, as expected during high-frequency stimulation, the tympanum's thick part (anterior edge of measured area) was not or only slightly displaced in both cases (dark colors in the scan views). Animations of the full oscillation cycle from which these scan views were taken are available as Electronic supplementary material (waveform animation of internally evoked tympanum displacements, ESM 1; waveform animation of externally evoked tympanum displacements, ESM 2).

To describe the displacement's distribution across the observed tympanum area, we compared displacement amplitudes relative to the f_2 level which had been measured at and around the pyriform vesicle (PV) with those at an area posterior to it (averaged for nine measurement points in each case). For L2 70 dB SPL, the mean stimulus-to-emission differences amounted to 39.9 dB (PV) and 46.6 dB (posterior), for 65 dB SPL to 47.0 dB (PV) and 51.5 dB (posterior), for 60 dB SPL to 37.8 dB (PV) and 46.0 dB (posterior), and for 55 dB SPL to 49.5 dB (PV) and 51.0 dB (posterior).

Vibration pattern after the selective exclusion of high-frequency receptors

In two animals (of which we show one representative example), the receptive function of the high-frequency receptors was selectively excluded via a mechanical lesion into the connection between their attachment site and the peripheral ganglion. Acoustically recorded DPOAEs in the

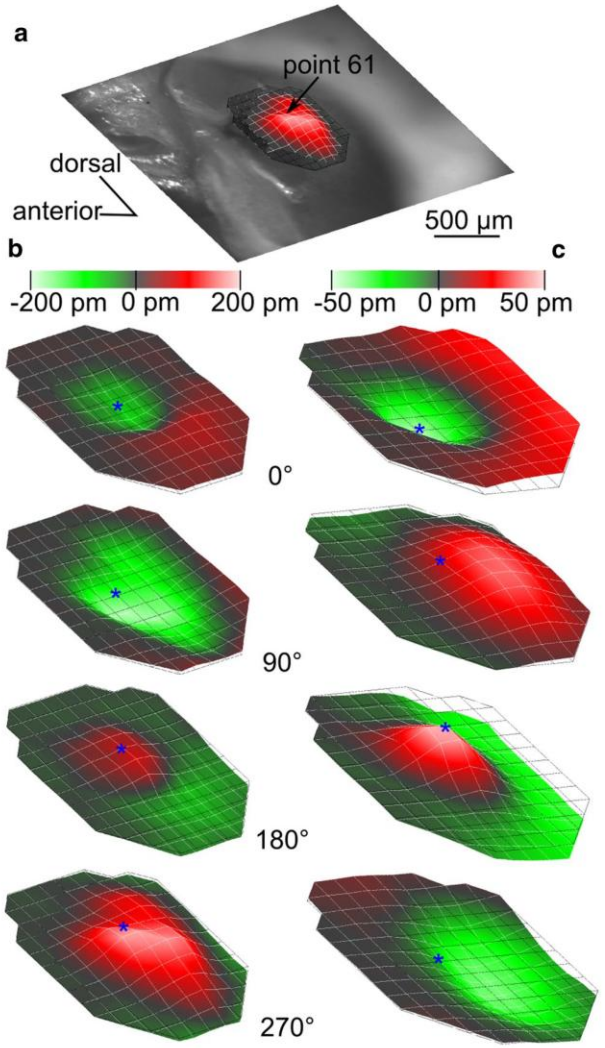

Fig. 5 Waveforms of internally and externally evoked tympanum displacements. **a** Laser measurement area on the tympanum. **b** Tympanum's displacement pattern for the $2f_1 - f_2$ emission (14.44 kHz) evoked by f_1 15.72 kHz and f_2 17 kHz (70/70 dB SPL). **c** Tympanum's displacement pattern for the emission frequency used as pure tone stimulus (14.44 kHz, 20 dB SPL). Four phases of one oscillation cycle, 0°, 90°, 180° and 270°, are given. *Scale bars* indicate the displacement amplitude (*red* outward displacement, *green* inward displacement). Zero displacement is marked by the *black grid*. *Arrow* and *asterisks* designate laser point 61 at the pyriform vesicle. Data smoothed prior to analysis

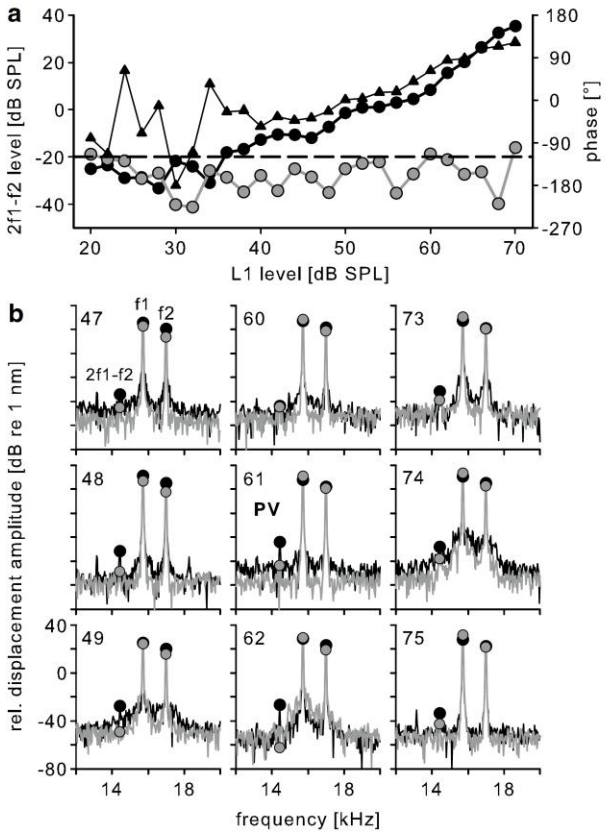

Fig. 6 Selective exclusion of high-frequency receptors via mechanical lesion. Recordings in the intact organ (DPOAE *black circles*; corresponding phase data *black triangles*) and after setting the lesion into the connection between the receptor's attachment position at the pyriform vesicle and the peripheral ganglion (*gray circles*). **a** Acoustical DPOAE growth functions. f_1 15.72 kHz, f_2 17 kHz. *Dashed line* displays noise level expressed as means +1 SD. **b** Amplitude spectra of the laser measurement point positioned at the pyriform vesicle (number 61) and of the 8 adjacent points during stimulation with two pure tones (f_1 15.72 kHz; f_2 17 kHz; 70/70 dB SPL). Note that the $2f_1 - f_2$ emission is strongly reduced at almost all measuring points after the lesion. Distances between measurement points 57.33 µm

intact organ (DPOAE growth function, Fig. 6a; black circles) steadily increased with increasing stimulus levels and amounted to 35 dB SPL at the highest stimulus levels of 70/60 dB SPL. The emission growth had a slope of 1.10 and thereby followed findings of an earlier study on locusts (Kössl and Boyan 1998), according to which $2f_1 - f_2$ distortion amplitudes obtained with high f_2 stimulus frequencies above 10 kHz grew with a steeper slope (1.10–1.48) than those recorded with lower stimulus frequencies (slope between 0.46 and 0.78). As soon as the emission had been above background noise level up to about 50 dB SPL L1 level, the phase remained almost constant and then started to increase steadily with increasing L1 levels, without displaying any abrupt phase shifts (Fig. 6a, black triangles). Pronounced notches as found for example in moths, associated with phase changes of up to 180° (Kössl et al. 2007), are much less common in DPOAE recordings from locust ears. The combination of the slight compression at low levels and quasi constant phase is reminiscent of sensitive DPOAE growth in mammals with an active cochlear amplifier. The acoustically recorded DPOAEs then decreased profoundly in the lesioned organ, with the

emission level close or below average noise level (Fig. 6a, gray circles).

In the intact organ, the traveling wave-like tympanum displacements evoked amplitudes of 25–31 dB re 1 nm and 20–25 dB re 1 nm for the f_1 and f_2 stimuli, respectively. The internally evoked emission displacement amplitude amounted to −44 to −24 dB re 1 nm (Fig. 6b, black). In the lesioned organ, after the high-frequency receptors had been disabled, externally presented stimuli f_1 and f_2 (as well as the emission frequency used as pure tone) still evoked traveling wave-like responses within the tympanum's vibration pattern, with displacement amplitudes for f_1 and f_2 of 23–33 dB re 1 nm and 14–23 dB re 1 nm (Fig. 6b, gray). For the stimuli f_1 and f_2, the mean amplitude difference between intact and lesioned condition amounted therefore only to 1.0 ± 2.6 dB and -3.0 ± 2.4 dB, respectively. The lesioned tympanum, however, lacked any additional, internally evoked emission displacements that had been present with intact high-frequency mechanoreceptors. Displacement amplitudes at the $2f_1 - f_2$ frequency dropped to the noise level, to values of −63 to −38 dB re 1 nm, and were on an average -11.0 ± 10.3 dB lower than before the receptor's exclusion (Fig. 6b, gray).

Discussion

Distortion–product otoacoustic emissions (DPOAEs) in insects are emitted from tympanal organs and depend on the physiological state of the animal (Möckel et al. 2012). We could demonstrate mechanical correlates of DPOAEs within the locust's tympanum movement during stimulation with two pure tones, using laser Doppler vibrometry (LDV). The differences in terms of absolute displacement amplitudes, for stimuli as well as for the emission frequency, in individual animals might originate from the methodical situation required for LDV measurements (for details, please see the "Methods" section). The exact arrangement of the locust below the laser beam and the orientation of loudspeakers and microphone in relation to the ear opening might slightly vary between individuals. That together with intraspecific differences between individual animals probably account for the observed variation in displacement amplitudes (Fig. 3; Table 1). Apart from these variations, mechanical distortions at the $2f_1 - f_2$ emission frequency were recorded in all tested 22 locusts, and we chose two representative example animals to discuss our results.

A recently published work showed mechanical correlates of DPOAEs within the tympanum's motion pattern in moth hearing organs (Mora et al. 2013). Possessing only one to four sensory neurons, these tympanal organs produce large DPOAEs, whose mechanical analogs, measured at the stigma (that is, the attachment position of the sensory receptors to the tympanum), were coherent to acoustically obtained DPOAEs. The overall shape of tuning curves obtained using the two noninvasive approaches (acoustical DPOAE recordings and LDV measurements) reflect those from earlier, much more invasive neurophysiological methods, by that underlining their validity.

We focused on $2f_1 - f_2$ emissions that crucially rely on the high-frequency receptors' integrity (Möckel et al. 2007), and found largest emission displacement amplitudes at the receptor's attachment position to the tympanum at the pyriform vesicle. Such internally evoked vibrations differed profoundly from externally evoked waves of the same frequency, as shown by analyzing their contribution along a transect line covering the relevant tympanal structures (Fig. 4) as well as the spatial spread of waves across the measured tympanum area (Fig. 5). This cannot merely be explained by the absolute amplitude of the respective vibration, because the level of the emission frequency used as pure tone was chosen to evoke displacement amplitudes that were even lower than the ones of the $2f_1 - f_2$ emission evoked by two pure tones. Traveling wave-like characteristics of externally evoked vibrations originate from direction-dependent properties of the vibrating structure, the tympanum (Stephen and Bennet-Clark 1982; Windmill et al. 2005), and make it rather unlikely that any internally evoked vibration would travel in the reverse direction. Such intrinsically generated waves were locally restricted to the region around the attachment position of the high-frequency receptors. Their wave nodes and troughs are reminiscent of standing waves close to the position of their origin. The mechanical gradient of the tympanal membrane probably avoids the spreading of the locally evoked waves, which are then reflected and occur only in restricted areas as standing waves.

These findings are consistent with the assumption of the scolopidial mechanoreceptors to be the source of otoacoustic emissions. Antennal hearing organs of mosquitoes and flies, that comprise the same mechanoreceptor type as tympanal organs, but operate as auditory particle velocity sensors at much lower frequencies, have been demonstrated to actively amplify low level sound (Göpfert et al. 2005; Nadrowski et al. 2008), and those species use their own ear distortions for mating song recognition (Gibson and Russell 2006; Cator et al. 2009; Warren et al. 2009). DPOAEs are, in the mammalian cochlea, a by-product of active amplification processes (Kemp 2008). For the active cochlear amplifier, critical oscillators have been shown to be the key element, providing the hearing organ with such properties as frequency selectivity, high sensitivity, and a wide dynamic range, as well as nonlinear response characteristics, the generation of distortion products, and the existence of spontaneous otoacoustic emissions (Duke and

Jülicher 2008). Distortion–product otoacoustic emissions recorded from tympanal organs in several insect species gave a hint that these hearing organs might also comprise active mechanisms which provide them with their sensitive perception abilities and which are physiologically highly vulnerable (e.g., Kössl and Boyan 1998; Kössl et al. 2007, 2008; Möckel et al. 2011). The presence of a critical oscillator in tympanal organs was suggested by the observance of occasionally present spontaneous oscillations of the tympanum of an Indian tree cricket at low frequencies (Mhatre and Robert 2013). Up to which sound frequencies such an active process in tympanal organs of insects is capable to operate, however, is still open.

Moir et al. (2011) recently published a study using a similar approach to record mechanical correlates of DPOAEs, but failed to obtain proof of ear generated distortions. The authors did not acoustically measure DPOAEs prior to LDV recordings and hence did not optimize stimulation parameters for each experimental animal individually, e.g. in terms of optimal frequency ratio f_2/f_1, as it is advisable to account for distinct inter-individual variations regarding DPOAEs in insects (Kössl and Möckel 2012). Moir et al. (2011) used stimulus frequencies and levels as given in a previous DPOAE study by Kössl and Boyan (1998) which then had been conducted under quite different conditions (with a tightly closed acoustic system as required for sensitive distortion measurements especially at frequencies below 10 kHz). After not being able to obtain any of the expected coherent DPOAE analogs within the tympanum's vibration pattern, the authors increased both stimulus levels (which in the previous report of Kössl and Boyan, 1998, had evoked even larger emission amplitudes above 10 dB SPL). These increased stimulus application in turn produced quite pronounced system artifacts that would occlude distortions produced by nonlinearity of the tympanal organ, most probably caused by the use of only one loudspeaker (Moir et al. 2011). We used two loudspeakers for f_1 and f_2 generation, and confirmed the absence of system artifacts in the combined loudspeaker signal by simultaneous microphone recordings (Fig. 2, gray) and by low coherence values for the tympanum's mechanical $2f_1$–f_2 response. In the second part of their work, Moir et al. (2011) then moved on to test whether their previously not found DPOAE analogs within the tympanum motion would be affected during hypoxia. CO_2-induced hypoxia reversibly decreases distortion amplitudes (Kössl and Boyan 1998). Again not able to find DPOAE correlates within the locust's tympanum motion, the authors only showed that the membrane's velocity at f_1, f_2 and $2f_1 - f_2$ did not alter significantly.

In general, traveling waves result from mechanical properties of the substrate of wave propagation, such as its mass, stiffness and damping, rather then from any physiological mechanism (von Békésy 1960; Robles and Ruggero 2001). We refer to Windmill et al. (2005) when addressing the waves that propagate across the locust tympanum as traveling waves. The data, however, were also consistent with multimodal tympanum vibrations with a frequency dependency that arises from nonhomogenous properties of the tympanal membrane and boundary effects of the attachment of the tympanal membrane at its edges (Michelsen 1971). We interfered with the high-frequency receptor's functional integrity by selectively excluding them via mechanical lesion, while leaving basic mechanical properties of the tympanum intact. Without intact high-frequency mechanoreceptors, the tympanum's response to externally presented stimuli still displayed traveling wave-like characteristics, but lacked any additional emission displacements (Fig. 6). The selective exclusion of a specific group of receptors tuned to high frequencies was subject of an earlier publication of our group (Möckel et al. 2007). Lesions were set into different regions of the tympanum using the same technical approach as in the present study. Only those into the connection between the attachment position of the high-frequency receptors and the peripheral ganglion led to irreversible amplitude decreases of DPOAEs evoked by high stimulus frequencies. This disconnection was verified by SEM examinations following the acoustical measurements. Several controls accompanied that earlier study. Lesions next to the pyriform vesicle into the tympanum which did not disable the receptors had no effect on the examined DPOAEs. Lesions directly into the peripheral ganglion led to a decrease of DPOAEs evoked by all used stimulus frequencies. No controls have been performed in the present study, as it repeated an already established technique. This approach has been a part in a much more difficult experimental procedure that included the acoustical and mechanical measurements of DPOAEs and their analogs, as well as their directly subsequent elimination via delicate mechanical lesions. The lesion experiments of the present study therefore represent yet another control for the already published findings. We conclude that the lesions did not impair mechanical properties and vibration patterns of the tympanum itself, but affected the connection between auditory mechanoreceptors and the tympanum. This emphasizes that insect DPOAEs are of physiological origin and depend on the mechanoreceptors integrity.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Cator LJ, Arthur BJ, Harrington LC, Hoy RR (2009) Harmonic convergence in the love songs of the dengue vector mosquito. Science 323:1077–1079

Dalhoff E, Turcanu D, Zenner HP, Gummer AW (2007) Distortion product otoacoustic emissions measured as vibration on the eardrum of human subjects. Proc Natl Acad Sci USA 104:1546–1551

Duke TAJ, Jülicher F (2008) Critical oscillators as active elements in hearing. In: Manley GA, Fay RR, Popper AN (eds) Active processes and otoacoustic emissions. Springer, New York, pp 63–92

Gibson G, Russell I (2006) Flying in tune: sexual recognition in mosquitoes. Curr Biol 16:1311–1316

Göpfert MC, Humphris ADL, Albert JT, Robert D, Hendrich O (2005) Power gain exhibited by motile mechanosensory neurons in *Drosophila* ears. Proc Natl Acad Sci USA 102:325–330

Gray EG (1960) The fine structure of the insect ear. Philos Trans R Soc Lond B 243:75–94

Jacobs K, Otte B, Lakes-Harlan R (1999) Tympanal receptor cells of *Schistocerca gregaria*: correlation of soma positions and dendrite attachment sites, central projections and physiologies. J Exp Zool 283:270–285

Kemp DT (1978) Stimulated acoustic emissions from within the human auditory system. J Acoust Soc Am 64:1386–1391

Kemp DT (2008) Otoacoustic emissions: concepts and origins. In: Manley GA, Fay RR, Popper AN (eds) Active processes and otoacoustic emissions. Springer, New York, pp 1–38

Kössl M, Boyan GS (1998) Acoustic distortion products from the ear of a grasshopper. J Acoust Soc Am 104:326–335

Kössl M, Coro F (2006) L1, L2 maps of distortion–product otoacoustic emissions from a moth ear with only two auditory receptor neurons. J Acoust Soc Am 120:3822–3831

Kössl M, Möckel D (2012) Measurement of sensitive distortion–product otoacoustic emissions in insect tympanal organs. J Exp Biol 215:566–567 Correspondence to Moir et al. 2011, JEB 214

Kössl M, Coro F, Seyfarth E-A, Nässig WA (2007) Otoacoustic emissions from insect ears having just one auditory neuron. J Comp Physiol A 193:909–915

Kössl M, Möckel D, Weber M, Seyfarth E-A (2008) Otoacoustic emissions from insect ears: evidence of active hearing? J Comp Physiol A 194:597–609

Mhatre N, Robert D (2013) A tympanal insect ear exploits a critical oscillator for active amplification and tuning. Curr Biol 23:1952–1957

Michelsen A (1971) The physiology of the locust ear I. Frequency sensitivity of single cells in the isolated ear II. Frequency discrimination based upon resonances in the tympanum III. Acoustical properties of the intact ear. Z Vergl Physiol 71:49–128

Möckel D, Seyfarth E-A, Kössl M (2007) The generation of DPOAEs in the locust ear is contingent upon the sensory neurons. J Comp Physiol A 193:871–879

Möckel D, Seyfarth E-A, Kössl M (2011) Otoacoustic emissions in bushcricket ears: general characteristics and the influence of the neuroactive insecticide pymetrozine. J Comp Physiol A 197:193–202

Möckel D, Kössl M, Lang J, Nowotny M (2012) Temperature dependence of distortion–product otoacoustic emissions in tympanal organs of locusts. J Exp Biol 215:3309–3316

Moir HM, Jackson JC, Windmill JFC (2011) No evidence for DPOAEs in the mechanical motion of the locust tympanum. J Exp Biol 214:3165–3172

Mora EC, Cobo-Cuan A, Macías-Escriva F, Pérez M, Nowotny M, Kössl M (2013) Mechanical tuning of the moth ear: distortion–product otoacoustis emissions and tympanal vibrations. J Exp Biol 216:3863–3872

Nadrowski B, Albert JT, Göpfert MC (2008) Transducer-based force generation explains active process in *Drosophila* hearing. Curr Biol 18:1365–1372

Robles L, Ruggero MA (2001) Mechanics of the mammalian cochlea. Physiol Rev 8:1305–1352

Römer H (1976) Die Informationsverarbeitung tympanaler Rezeptorelemente von *Locusta migratoria* (*Acrididae*, *Orthoptera*). [Processing of information by tympanal receptors of *Locusta migratoria* (*Acrididae*, *Orthoptera*).]. J Comp Physiol A 109:101–122

Stephen RO, Bennet-Clark HC (1982) The anatomical and mechanical basis of stimulation and frequency analysis in the locust ear. J Exp Biol 99:279–314

von Békésy G (1960) In: Wever ED (ed) Experiments in hearing. McGraw-Hill, New York

Warren B, Gibson G, Russell IJ (2009) Sex recognition through midflight mating duets in *Culex* mosquitoes is mediated by acoustic distortion. Curr Biol 19:485–491

Warren B, Lukashkin AN, Russell IJ (2010) The dynein-tubulin motor powers active oscillations and amplification in the hearing organ of the mosquito. Proc R Soc B 277:1761–1769

Windmill JFC, Göpfert MC, Robert D (2005) Tympanal travelling waves in migratory locusts. J Exp Biol 208:157–168

5.9. Appendix to Möckel et al. 2014

5.9.1. Electronic supplementary material

The online version of this article contains electronic supplementary material. It consists of two waveform animations of internally and externally evoked tympanum displacements. Scan views from these oscillation cycles, at 0°, 90°, 180°, and 270°, are included within the print version of the article (Fig.5). Both animations can be found on the included CD.

ESM_1_Emission-displacement (.mpg) Waveform animation of internally evoked tympanum displacements. Tympanum's displacement pattern for 2f1-f2 (14.44 kHz) evoked by f1 15.72 kHz and f2 17 kHz (70/70 dB SPL).

ESM_2_Pure-tone-displacement (.mpg) Waveform animation of externally evoked tympanum displacements. Tympanum's displacement pattern for the emission frequency used as pure-tone stimulus (14.44 kHz, 20 dB SPL).

Scale bars indicate the displacement amplitude (red outward, green inward displacement). Blue mark designates laser point 61 at the pyriform vesicle. Data smoothed prior to analysis.

5.9.2. Additional remarks on the experimental procedures

The locust's tympanum is slightly tilted which held certain problems during the positioning of the animal and the arrangement of the 118 to 125 laser measurement points. The animal was positioned in such a way that the tympanum was arranged as flat as possible (that means, in a 90° angle) below the laser beam. The cuticle rim that surrounds the membrane overlaps with a part of the tympanum and hence prevented the scanning of the entire membrane. An area centered around the pyriform vesicle (the attachment position of the high frequency receptors) and the adjacent membrane regions was chosen for examination. This area amounted to about 0.345 mm^2. The area of an entire tympanum was determined for *L. migratoria* to be 2.32 ± 0.24 mm^2 (Meyer and Elsner 1996). Under the assumption of a more or less similar size in *S. gregaria*, the measurement area in the present study covered about 14.9 % of the whole tympanum. This measurement area included those membrane regions which are of special importance for the detection and processing of high frequency sounds as used in the study. Membrane displacements induced by such frequencies travel across the thin membrane region and pyriform vesicle and are then attenuated (Windmill et al. 2005). I am therefore confident that the chosen tympanum area properly reflects the events during stimulation with two pure tones and the generation of DPOAE analogs.

6. General discussion

Distortion-product otoacoustic emissions in insects had been recorded in locusts (Kössl and Boyan 1998a,b; Möckel et al. 2007), moths (Coro and Kössl 1998; Kössl and Coro 2006; Kössl et al. 2007), and bushcrickets (Möckel et al. 2011). Their general characteristics are comparable to those measured in vertebrates, despite distinct differences in external and internal ear anatomy. The following chapters 6.1. to 6.3. summarize and discuss the findings of this PhD project and the current state of research on insect DPOAEs. In parallel to research on DPOAE generation in tympanal organs, active mechanical processing in antennal hearing organs was subject of many studies. As these ears possess the same type of mechanoreceptor as tympanal organs, the development in this area will be outlined in chapters 6.4. to 6.9. Concluding remarks will bring together suggestions about possible mechanisms, their location within the scolopidial receptors, and potential further steps necessary to fully understand the functioning of these sensory organs (chapter 6.10.).

6.1. Scolopidia are the source for DPOAE generation in tympanal organs

Tympanal organs of locusts and moths produce large DPOAE amplitudes which are measured by placing the acoustic coupler directly in front of the tympanal membrane to whose inside surface the scolopidial mechanoreceptors are attached. The mechanoreceptors had been so far manipulated by drop applications of ethyl ether directly onto the thorax surface (Coro and Kössl 2001), by hypoxia induced by CO_2 ventilation (Kössl and Boyan 1998a), and via mechanical lesions into the tympanal membrane (Möckel et al. 2007). These procedures, however, created uncertainties whether the substance actually reached the organ, if the membrane's mechanical properties changed, or how shifting the coupler in order to provide experimental access to the membrane might affect sensitive measurements. The methodical advantages of bushcrickets for DPOAE measurements lies in the anatomical separation of the main site of sound input (spiracle at the lateral pronotum) and the site of its perception (scolopidial mechanoreceptors in the foreleg tibia). It reliably allows to manipulate the function of the tympanal organ while the simultaneously performed acoustical measurements (with the coupler positioned at the spiracle entrance) are not disturbed. General characteristics of DPOAEs measured in bushcrickets are comparable to those in locusts and moths. Depending on frequency, the stimulus-emission-difference of 44 to 55-65 dB (Möckel et al. 2011, Fig.1) proved to be within or above the values reported for locusts (30–50 dB; Kössl and Boyan 1998a, b) and above that shown for moths (23–30 dB; Coro and Kössl 1998). This lower sensitivity might be explained by the distance between stimulation and recording channels at the entrance of the spiracle and the generation site of the DPOAEs at the tympanal organ in the tibia (see discussion section, Möckel et al. 2011). Nonetheless, the insect group of the Tettigoniidae family with its well-studied hearing

organ anatomy holds great experimental advantages, for example to apply fluid substances very close to the hearing organ to test their effects on DPOAE generation.

One of these tested substances, the insectizide pymetrozine, was originally developed against plant-sucking insects, and contact with the compound leads to feeding impairment and death through starvation (Harrewijn and Kaiser 1997). After the suggestion of an impact on neurosensory or motor system (Kaufmann et al. 2004), a study on the femur–tibia joint control loop in locusts revealed its selective action on mechanoreceptors of the scolopidial type, with the loss of stimulus-related responses (Ausborn et al. 2005). Other examined sensory organs which do not possess scolopidia such as campaniform sensilla or hair sensilla were not affected. An application of pymetrozine via an opening distally to the tympanal organ caused irreversibly reduced DPOAE amplitudes (Möckel et al. 2011, Fig.4 and 5), which indicated that scolopidial structures are critically involved in DPOAE generation in insects. Shifted emission amplitudes, however, do not necessarily mean that a possibly active amplifer was affected by the compound. A conceivable action solely against passive cellular structures which would change the organ's mechanical properties and hence DPOAE amplitudes, however, was not supported, as the low and high-level component of DPOAE growth functions had a different physiological vulnerability (Möckel et al. 2011, Fig.3). On a cellular level, both sensory neurons and/or the accessory cells within scolopidia might potentially be targets of pymetrozine. At present, its exact site of action on a molecular level is still unknown. An ongoing study on the stick insect *Carausius morosus* at the University of Cologne (Förster et al. 2013) aims to determine how and at which target sides the femoral chordotonal organ is affected. Whether Pymetrozine acts on the mechanoelectrical transduction process or directly influences the receptor potential is not yet understood.

The crucial involvement of scolopidial mechanoreceptors in DPOAE generation was not a completely new finding. Selective exclusion of specific cell groups in the tympanal organ of locusts via mechanical lesions caused frequency-specific DPOAE amplitude reductions (Möckel et al. 2007). A more favourable approach, however, might be a method that leaves the organ itself and the acoustical measurement devices undisturbed, as was done by pymetrozine application to the bushcricket's tympanal organ (Möckel et al. 2011). Both of these two studies provided evidence for the involvement of scolopidia in the DPOAE generation in insects, by using complementary methods (mechanical versus pharmacological treatment of the organ) and different animal models (locusts and bushcrickets).

6.2. The scolopidia's dynein-tubulin system plays a crucial role for potentially active mechanisms

Each cell type (sensory neuron and accessory cells) and cellular components within the scolopidial receptors could potentially be the basis for the mechanical non-linearity which is exhibited in tympanal organs. The emission's suggested biological origin should involve metabolic processes whose temperature dependence would directly affect the DPOAE generation. For locusts (Oldfield 1988b) and cicadas (Fonseca and Correia 2007), a temperature dependence within the auditory pathway of insects had been suggested to result from intrinsic

properties of the neuronal receptors, while the tympanum's mechanical properties should remain unchanged across a biologically relevant temperature range. Shifting the locust's body temperature (between 12 and 35°C) resulted in reversible, level- and frequency-dependent effects on the 2f1–f2 emission (Möckel et al. 2012, Fig.2, 3 and 4). A body temperature increase led to enhanced amplitudes of those DPOAEs which had been evoked using low stimulation levels below ~30-40 dB SPL (the low-level component of the DPOAE growth function) and f2 frequencies of up to 10 kHz. Emissions induced by higher stimulus levels and frequencies (e.g. 12 and 18 kHz) remained unchanged. The higher vulnerability of the low level component in DPOAE growth functions to physiological manipulations as it was observed in this study had also been found in vertebrates (e.g. Lukashkin et al. 2002) and in other insects (e.g. notodontid moth, Kössl et al. 2007; bushcricket, Möckel et al. 2011). Despite this separation of effects, however, there is evidence that both components are evoked by a single source, a non-linear amplifier with saturating input/output characteristics (Lukashkin et al. 2002). Amplification in the mammalian cochlea is largest at low level stimuli (Dallos and Fakler 2002) until it saturates at higher input levels beyond ~40-60 dB SPL. If the gain of the cochlear amplifier is changed via physiological manipulations, this would influence the low-level component much more strongly than the high-level component (Lukashkin et al. 2002). That tympanal organs in moths which have only one scolopidium also exhibit an unequal vulnerablility of low versus high level component of their DPOAE growth functions (Kössl et al. 2007) further supports this suggestion.

The Arrhenius activation energy of the underlying cellular component, derived from the amount of DPOAE changes during body temperature shifts, was calculated from the -10 dB SPL thresholds (representing the growth function's low-level component) and amounted to up to ~34 and 41 $kJmol^{-1}$ (for growth functions measured with 8 and 10 kHz as f2, respectively; Möckel et al. 2012, Fig.5). Such values are in accordance to those derived from temperature-dependent changes of spontaneous mechanical oscillations of the Johnston's organ in mosquitoes (Warren et al. 2010). The kinetics and the distribution of dynein within the distal cilium region (Thurm et al. 1983) provided a hint that an intact dynein-tubulin system could hereby play an essential role. Please see also the discussion section 6.10.2. about the debate on ciliary motility and its molecular basis.

While sharing the same mechanoreceptors, both types of ears operate at very different frequency ranges, yet the mechanical principles of these non-linear processes (spontaneous flagellum oscillations of the non-tympanic Johnston's organ and DPOAE generation in tympanal organs) might be similar (see also next chapter). And as shown for locusts, the mechanism seems to be capable of operating at frequencies of up to 10 kHz.

An application of colchine into the Johnston's organ of the mosquitoes as done by Warren et al. (2010) leads to a disruption of microtubules in the dendritic cilia of mechanoreceptors in arthropods (Moran and Varela 1971). Such a treatment would have corroborated the involvement of the dynein-tubulin system, but proved methodically problematic in our study on locusts. The anatomical situation (the scolopidia sitting on the membrane's inner surface, surrounded by air-filled chambers) would only allow an application directly onto the tympanal membrane, an injection through the membrane, or an application topically to the surface of the thorax, with uncertainties about how the adjacent structures and

their mechanical properties were affected and whether the substance actually reached the organ. In addition, a second series of experiments on bushcrickets that involved shifting the body temperature and colchicine applications also met quite pronounced methodical problems (see chapter 3.8.1.). We therefore decided to not include these additional experiments in the study.

6.3. Mechanical DPOAE analogs in the tympanum's vibration pattern during two-tone stimulation

Otoacoustic emissions in the ears of vertebrates are air pressure fluctuations in the outer ear canal, resulting from mechanical non-linearity of the cochlea and depending on its physiological activity (Kemp 2008). Transmitted retrogradely through the middle ear, they cause ear drum vibrations and are recordable with sensitive microphones as faint acoustical sounds. A study in humans to simultaneously record DPOAEs and mechanical tympanum vibrations used a coupler that included the acoustical channels and an optical laser beam fiber, and reported tympanum displacements at the 2f1-f2 frequency of 1–8 pm (Dalhoff et al. 2007). Scolopidial mechanoreceptors have been found to be the potential source of DPOAE generation in insects (Möckel et al. 2007, 2011). In locusts and moths, these mechanoreceptors are directly coupled to the inside of a large tympanal membrane. Acoustically measurable DPOAEs should therefore have mechanical analogs within the tympanum vibrations during two-tone stimulation.

A first attempt by Moir et al. (2011) failed to obtain proof of mechanical correlates of DPOAEs in the ears of two locust species, *Schistocerca gregaria* and *Locusta migratoria*. The study, however, held several methodical deficits. The authors did not acoustically measure DPOAEs prior to the LDV recordings, and stimulation parameters were hence not optimized for each experimental animal; something that had proven crucial to account for distinct inter-individual variations in earlier insect studies. They rather used stimulus parameters which in an earlier study (Kössl and Boyan 1998b) under quite different conditions had been sufficient to evoke pronounced DPOAEs. Using only one loudspeaker to present both stimuli led to quite pronounced system artifacts that would occlude distortions produced by non-linearity of the tympanal organ (Moir et al. 2011). For a more detailed examination, please also see our comment, Kössl and Möckel (2012) and the discussion section of Möckel et al. (2014).

Mechanical correlates of DPOAEs within the tympanum’s motion pattern were demonstrated for the moth *Empyreuma pugione* (Mora et al. 2013). The animal's two sensory receptors per ear attach at the stigma to a relatively large tympanum (Coro and Perez 1993). Combining and comparing two noninvasive approaches (acoustical DPOAE recordings and LDV measurements) led to tuning curves whose overall shape reflected those from earlier, much more invasive neurophysiological methods, by that underlining their validity. LDV recordings were done in a single-point aquisition mode, measuring the tympanum's vibration pattern at the stigma, the attachment position of the mechanoreceptors (Mora et al. 2013).

The existence of mechanical correlates of DPOAEs within the tympanum's vibration pattern during two-tones stimulation was demonstrated for locusts in our own study (Möckel et al. 2014). Scolopidia in the tympanal organ of the locust *Schistocerca gregaria* attach at several

positions to the tympanal membrane (e.g. Römer 1976) whose vibrational characteristics enable the primary frequency analysis within this hearing organ (Windmill et al. 2005). High-frequency sound above ~12 kHz induces tympanum vibrations with a distinct maximum close to the pyriform vesicle, the attachment zone for high-frequency receptor cells. As these receptors' integrity is crucial for high-frequency DPOAE generation (Möckel et al. 2007), LDV measurements covered this tympanal region and assessed mechanical DPOAE analogs evoked by high-frequency stimuli above 15 kHz. To account for distinct inter-individual variations regarding DPOAEs generation, the experimental time line for each individual animal started with acoustically measured DPOAEs in order to optimize stimulation parameters that evoked largest emissions, and was followed by LDV measurements of DPOAE analogs using previously determined stimuli (Möckel et al. 2014, Fig.1). Mechanical distortions appeared as additional spectral peak at the 2f1-f2 emission frequency, with largest displacement amplitudes at the pyriform vesicle (Möckel et al. 2014, Fig.4). The variability in absolute displacement amplitudes across animals were probably due to methodical reasons and individual differences (Möckel et al. 2014, Fig.3 and Table 1; please also refer to Methods section), but f2-stimulus-to-2f1-f2 emission-differences compared well with values reported for acoustical DPOAE measurements in locusts (Kössl and Boyan 1998b).

Emission-based, internally evoked vibrations differed profoundly from stimulus-based, externally evoked waves of the same frequency, in terms of wave propagation, energy distribution, and location of amplitude maxima (Möckel et al. 2014, Fig.4 and 5). In contrast to traveling wave-like characteristics of externally evoked vibrations, intrinsically generated waves were locally restricted to the region around the high frequency receptors' attachment position. The mechanical gradient of the tympanal membrane that leads to direction-dependent properties (Stephen and Bennet-Clark 1982; Windmill et al. 2005) probably avoids the spreading of these locally evoked waves which are then reflected and occur only in restricted areas as standing waves. After functionally eliminating the high-frequency receptors via mechanical lesion, the tympanum's response lacked any additional emission displacements while leaving basic mechanical properties of the tympanum intact (Möckel et al. 2014, Fig.6). These findings provide evidence that tympanal auditory receptors, comparable to the situation in mammals, comprise the required nonlinear response characteristics which during two-tone stimulation lead to additional, highly localized displacements of the tympanum.

6.4. First hints for active amplification in tympanal hearing organs

The findings reported above substantiate the assumption that scolopidial mechanoreceptors were the source of otoacoustic emissions in tympanal organs. In mammals, DPOAEs arise as by-product of active amplification processes (Kemp 2008). DPOAEs recorded from tympanal organs in several insect species indicated that the observed sensitive perception abilities of those hearing organs and the DPOAE's physiological vulnerablility might also comprise active mechanisms (e.g., Kössl and Boyan 1998a,b; Kössl et al. 2007, 2008; Möckel et al. 2011). The mere presence of emissions, however, does not neccessarily confirm the existence of active

amplification processes at low sound pressure levels, as those distortions could also be based on purely passive mechanisms.

Vertebrate ears reach their high sensitivity and frequency slelectivity by actively amplifying sound-induced vibrations inside the ear. The cochlear amplifier thereby leads to (i) amplification which lowers the hearing threshold, (ii) a sharpened frequency tuning, (iii) a compressive non-linearity which leads to a specific enhancement of small vibrations induced by faint sounds, and (iv) the occurrence of self-sustained oscillations (spontaneous OAEs) when sound is absent (Hudspeth 2008). Mechanically based on outer hair cell motility which converts electrical into mechanical energy with the help of the motor protein prestin, these physiologically vulnerable phenomena were suggested to involve critical oscillators as key element (Duke and Jülicher 2008). The term "critical oscillator" is used for a dynamical system which is balanced right at the edge of an oscillatory instability, the so-called critical value. Below the critical value, the system holds a quiescent state, but can be disturbed by mechanical forces just above thermal noise. Once the critical value is exceeded, the system becomes unstable and oscillatory and generates spontaneous oscillations at specific frequencies (Geurten et al. 2013).

The presence of a critical oscillator in tympanal organs was suggested by the observation of occasionally present spontaneous oscillations of the tympanum of the Indian tree cricket *Oecanthus henryi*, using Doppler vibrometry (Mhatre and Robert 2013). These tiny self-sustained oscillations were sharply tuned to kilohertz-range frequencies of the animal's conspecific song. The authors further described a switch between an "on" and "off" state, when tympana which had previously been quiescent were found to abruptly produce spontaneous oscillations. Such "on" state tympana displayed nonlinear behavior: At low sound pressure levels, the clearly tuned tympanum showed a sensitivity peak at the song's frequency range of up to 4 kHz, whereas at higher stimulus levels, the tuning proved less sharp, with a broader sensitivity. During the "off" state, however, the tympanum comprised no specific tuning and resembled the "on" state's high level response (Mhatre and Robert 2013). Up to which sound frequencies the suggested active amplification and adaptive tuning in tympanal organs of insects is capable to operate, however, is still open.

6.5. Johnston’s organs and tympanal organs – same mechanoreceptor, similar mechanism?

The generation of sensitive DPOAEs in tympanal organs documents the presence of non-linear auditory processing. Proofing the existence of active amplification of low-level sound within that type of ears has been difficult so far. Antennal hearing organs of mosquitoes and flies, called Johnston’s organs, however, were shown to comprise active force generation, exhibited as spontaneous antennal vibrations in the absence of sound, induced by pharmacological and genetic manipulations. Apart from anatomical differences of the involved classes of hearing organs, autonomous oscillations of antennal hearing organs and DPOAE generation in tympanal organs might share similar underlying cellular mechanisms.

Tympanal and Johnston's organs process different modalities of sound that reaches the ear. The former detects the sound pressure component and operates at frequencies that extent into the ultrasound range, similar to most mammals. The latter is sensitive to the particle velocity component of incoming sound and has its working range at much lower frequencies of generally below 1 kHz (Yack and Dawson 2008, review). The underlying mechanoreceptor type, namely scolopidia, is the same in both hearing organs, yet their external anatomy, that is, of the structures which channel sound energy towards the mechanoreceptors, differs profoundly. Tympanal organs possess tympanal membranes and an adjacent tracheal system. In Johnston's organs, flagellum and arista (in mosquitoes and flies, respectively) function as sound receiver.

Johnston`s organs realize both hearing and graviception by using mechanosensory neurons that detect movements of the antennae. In mosquitoes, the actual receiver is an elongated flagellum which generally exhibits sexual dimorphism as those of males bear a large number of long hairs (Göpfert et al. 1999). It is connected at its base to the second antennal segment, the pedicel, which contains the Johnston`s organ. Between the pedicel and the scape (the first antennal segment) insert the most distal antennal muscles (Göpfert and Robert 2001). Each Johnston`s organ of male mosquitoes contains about 15000 sensory neurons, that of females about 7500 sensory neurons (Boo and Richards 1975a,b). In fruit flies, each antenna is composed of three segments (scape, pedicel, and funiculus) and a feather-like arista, with the third segment and the arista forming the sound receiver as they rotate together about the longitudinal axis of the funiculus upon acoustic stimulation (Göpfert and Robert 2002). As in mosquitoes, the second antennal segment contains the sensory neurons, but in much smaller numbers. *Drosophila´*s about 480 sensory neurons per organ are anatomically divided into five subgroups (Kamikouchi et al. 2009; subgroup D not examined): Subgroups A and B (together about 150-250 neurons) respond to high-frequency and low-frequency sound-induced receiver vibrations, respectively; and subgroups C and E (together about 200 neurons) answer maximally to static receiver reflections as occurring for example when the animal walks up or down a wall. In addition to a discrimination via their response characteristics, the subgroups also differ in their central projections, and in the expression of the TRPN channel NompC which is specific for sound-sensitive receptors (Kamikouchi et al. 2009).

6.6. Active force generation in Johnston's organs of mosquitoes and flies

Laser vibrometry measurements of deflections of the mosquito's antennal receiver during sound simulation display non-linear responses (Göpfert and Robert 2001). They are sharply tuned around the frequency of highest sensitivity (female ca. 250 Hz, male ca. 430 Hz), with largest displacements occurring during lowest stimulus intensities. Such a reverse intensity-dependence disappears after death. As the most direct evidence for active force generation, the receiver also shows autonomous vibrations at restricted frequencies in the absence of sound, occasionally observed in untreated animals, and reliably induced via DMSO or ethanol application. Electrical activity within the Johnston's organ along with spontaneous vibrations and the absence of direct muscle attachments to the flagellum indicates mechanoreceptor motility as the base of these active auditory mechanics (Göpfert and Robert 2001). The *Drosophila* auditory system also

exhibits non-linear response characteristics, which result in an intensity-dependent frequency tuning (Göpfert and Robert 2002). Only at low intensities, the antenna is tuned to the species-specific songs (dominant song frequency 160-210 Hz). Increased stimulus intensities which would occur during close-range song communication between male and female shift the antenna's response frequency towards higher frequencies (and slightly decreases its sensitivity). These effects were hypothesized to be caused by non-linear stiffness changes of the antenna. They provide a dynamic range compression in the *Drosophila* ear, with improved sensitivity at low intensities and reduced sensitivity at high intensities. These modulations of the receiver's mechanical response are physiologically vulnerable. They reversibly linearize during hypoxia and disappear after death (Göpfert and Robert 2003). The origin of non-linearity and spontaneous oscillations was analysed in several *Drosophila* mutants which exhibited defects in different regions of the scolopidia: the linker proteins between scolopale cap and antennal receiver (nompA2), a mechanosensory transduction channel (nompC2), the ciliary dilation (btv5P1), or the dendritic cilium (tilB). The results suggest that the neuron's motion generation depends on functioning mechanosensory transduction channels and that the receiver becomes linear and passive if it is disconnected from the neurons (Göpfert and Robert 2003).

In the mammalian cochlea, a member of the anion transporter family SLC26, Prestin, accounts for the active mechanical amplification (Dallos and Fakler 2002). Auditory organs of non-mammalian vertebrates (teleost fish) and insects (mosquitoes and flies) were also shown to exhibit Prestin-related SLC26 proteins (Weber et al. 2003). Whether these proteins serve a similar function during active amplification in insect ears, however, remains unclear.

Motile mechanosensory cells in the fly's Johnston's organ amplify the ears input by adding mechanical energy into the receiver's fluctuations, demonstrating a pronounced power gain (Göpfert et al. 2005). The animal hereby seems to adjust the energy contributions of its auditory neurons. Such a control of the system's amplification gain keeps it near an oscillatory instability which makes the hearing organ itself more sensitive to sound. During mosquito mating behavior, the male's antenna responds in three distinct phases, depending on varying stimuli levels arriving from by-passing females (Jackson and Robert 2006): an amplification with the antennal response being larger than expected from these faint sounds when the female is still far away, a compression (negative gain) to soften her sound when she is nearby and dampen the antennal oscillations in order to avoid overstimulation, and a final amplification induced by decreasing stimulus levels at an increasing distance between both animals.

6.7. Wing beat matching and the behavioral relevance of distortion-product generation

Flying mosquitoes were found to conduct an interactive auditory behaviour which plays a crucial role in sexual recognition (Gibson and Russell 2006; Warren et al. 2009). As a response to the flight tone of the other sex, both male and female modulate their wing-beat frequencies to reach a frequency match, hereby displaying a feedback control between sensory input at the antennae and the flight muscles' motor output. In *Toxorhynchites brevipalpis*, such frequency synchronization is performed as long as the two original wing-beat frequencies of male and

female had been within 60 Hz and at a similar level, whereas same-sex-pairs react with diverging flight tones (Gibson and Russell 2006). *Culex* mosquitoes (Warren et al. 2009) and the malaria vector *Anopheles gambiae* (Pennetier et al. 2010) match their wing beat frequencies by converging the nearest shared harmonic within the flight tone signal. Such high sound frequencies are above the sensory-neural response range of Johnston`s organs, but within the flagellum's mechanical response range. The flight tone matching is realized with the help of the difference tone f2-f1, a distortion product which is generated by the interaction of the wing-beat harmonics of male and female. These two high-frequency "primaries" do not elicit any receptor potentials, whereas the low-frequency difference tone evokes large electrical responses of the sensory organ. Wing-beat frequencies were adjusted until the difference tone dropped in frequency which meant the flight tones were closely matched. These were the first studies to report an acoustic distortion to not just be a by-product of auditory processing, but to serve as a sensory reference with behavioural relevance (Warren et al. 2009; Pennetier et al. 2010).

It should be mentioned that in the case of the noctuid moth *Noctua pronuba* (which has tympanal organs instead of antennal hearing organs), increasing stimulus levels as would be emitted by an approaching bat, also lead to a shift of the resonant frequency of the moth's auditory system, from 20-40 kHz to 70-80 kHz, probably caused by a change in stiffness of the system (Windmill et al. 2006).

6.8. Gating springs and adaptation motors, but no efferent control within the antennal receiver

Mechanosensation in general consists of stimulus reception, coupling, mechano-electrical transduction, and encoding (French 1988; Yack 2004; Albert et al. 2007a; also see introduction chapter 1.3). The step of mechano-electrical transduction is thought to involve a direct gating of the mechanotransducer ion channels by the applied stimulus force, leading to a change of their opening probability (Gillespie and Walker 2001). The involved sensory structures which route the stimulus forces towards those channels should hence be mechanically coupled. Mechanotransducers of vertebrate hair cells are mechanically accessed by elastic "gating springs" which connect to the channel's gates. Upon mechanical stimulation, the hair bundles are deflected and rotate about their base, creating a shearing motion between adjacent stereocilia. The actual transducer sits at the tips of the stereocilia, linked via fine filaments, called tip links (Howard and Hudspeth 1988; Hudspeth and Gillespie 1994; Beurg et al. 2009). A stretching of these gating springs opens the connected ion channels, followed by an inflow of K^+ and Ca^{2+} ions into the cell body which then leads to a depolarization of the cell membrane, synaptic vesicle release and the formation of action potentials in spiral ganglion neurons.

The antennal receiver in *Drosophila* was also suggested to contain similar functioning elements (Albert et al. 2007b). During a maintained stimulation, the transducers adapt and hereby re-gain their sensitivity, by re-adjusting the gating-spring tension, hence shifting their operating ranges and restoring the channel opening probability. This re-adjusting of the gating-spring tension is achieved via adaptation motors which - comparable to the situation found in vertebrate hair cells (Fettiplace and Hackney 2006) - might also be involved in active

amplification processes within the fly's ear (Albert et al. 2007b). Nadrowski et al. (2008) composed their model of the *Drosophila* hearing organ by using a harmonic oscillator (representing the antennal receiver) and transducer units which each consisted of a force-gated ion channel, a gating spring and adaptation motors. The only non-linearity within this model was a sigmoid function of the gating-spring tension to characterize the opening probability of the ion channels. That and the co-operation of channels, gating springs and adaptation motors were sufficient to account for findings of active, transducer-based amplification processes in the fly´s ear (Nadrowski et al. 2008, Bechstedt and Howard 2008).

If faint sound reaches the inner ears of vertebrates, sound-induced vibrations are amplified by somatic motility of hair cells and/or hair bundle motility, to enhance the mechanical input within the ear (Robles and Ruggero 2001). This mechanical feedback-function, the cochlear amplifier, is centrally regulated and altered by efferent fibres via synaptic contacts with the outer hair cells. These efferent modulations change both the electrical and mechanical properties of the hair cells, e.g. by affecting the cells hyperpolarization and axial stiffness of hair cells, directly regulating prestin, or by changing the intracellular pressure or chloride concentration (for review, see Frolenkov 2006). A comparable efferent control mechanism is generally thought to be absent in auditory chordotonal organs of insects, such as tympanal organs and Johnston`s organs (Field and Matheson 1998). Kamikouchi et al. (2010) showed that auditory neurons in *Drosophila* lack peripheral synapses and efferent innervations, and suggested that the fly's mechanical feedback amplification is solely controlled within the organ itself, e.g. by an ionic regulation of the scolopale lumen. This tightly sealed cavity holds a Ka^{+}-rich endolymph that encloses the presumable mechanotransduction site around the dendritic outer segment of the scolopidial neuron (Field and Matheson 1998; Yack 2004). It represents a functional analogy to the cochlear endolymphatic space which is a crucial factor for normal hair cell function (Wangemann 2006).

6.9. The function of transient receptor potential (TRP) channels for amplification mechanisms

Several types of transient receptor potential (TRP) channels exist in mechanosensory cells that are used for hearing (Walker et al. 2000), and they seem to play a crucial role, as shown using several *Drosophila* mutants (Göpfert et al. 2006). Amplification mechanisms and hence the system’s gain as well as its ability to exhibit spontaneous oscillations depend on the integrity of the NompC ("no mechanoreceptor potential C", also called TRPN1) channel. Two independent TRP vanilloid (TRPV) channels (Nan and Iav) seem to be necessary to regulate NompC’s amplificatory gain via a negative control function, and their disruption leads to the occurrence of spontaneous antennal oscillations (Göpfert et al. 2006).

The proposal of the ciliary dilation to be the candidate site for transduction in chordotonal organs was supported by the localization of microtubule-associated proteins whose absence in mutant flies led to a loss in mechanosensory transduction and amplification (Bechstedt et al. 2010). Within the distal cilium region up to the ciliary dilation, the ring of nine microtubule pairs that builds up the axoneme forms (presumably) dynein-arms (Thurm et al.

1983). Such ciliary dynein-arms are missing in TilB (touch insensitive larva B) *Drosophila* mutations, causing a dysfunction in sperm flagella and in ciliated dendrites of chordotonal organs (Kavlie et al. 2010). The importance of an intact dynein-tubulin system within the sensory cilium was also emphasized by findings that spontaneous oscillations of the antenna in *Culex* mosquitoes are dependent on the ambient temperature and vulnerable to colchicine applications to the organ (Warren et al. 2010; see also Möckel et al. 2012, discussion section).

The TRPN1 channel NompC was reported to be essential for the sound receptor function of those mechanosensory neurons which mediate hearing within the antennae of fruit flies. The other half of the organ's about 500 neurons has a gravity/ wind sensor function and operates NompC-independent (Effertz et al. 2011). The TRPN1 channel's integrity had been found to be crucial for amplification mechanisms in Johnston's organs, with TRPV channels implementing a regulatory function (Göpfert et al. 2006). Using immunostaining, TRPN1 was shown to be localized at the cilium's distal tip, with the ciliary dilation being its proximal limit, whereas ciliary TRPV channels were found to be restricted to a more proximal region (Lee et al. 2010). The authors suggested that this spatial distribution proximal and distal to the ciliary dilation might separate the cilium into two zones with different functions, as also axonemal dynein-arms are only found within the proximal (TRPV channel) zone. A further study on the functional distribution between TRP channels reported somewhat other findings, probably caused by methodical differences between calcium imaging (Effertz et al. 2011) and electrophysiological recordings of sub-threshold signals (Lehnert et al. 2013): The authors of the latter study recorded from giant fiber neurons whose dendrites were coupled via electrical synapses to axons of defined populations of Johnston's organ neurons within the central nervous system. TRPN1 (NompC) channels were hereby found to not be required for transduction itself, as transduction still persisted in their absence (Lehnert et al. 2013). The authors rather suggested a role of TRPN1 in establishing a normal sensitivity of the transduction complex to antennal movements, analogous to prestin in the mammalian cochlea which is not absolutely required for transduction. TRPV channels Nanchung and Inactive on the other hand were found to be necessary for responses to sound-induced antennal vibrations and were therefore proposed as components of the transduction complex.

Additional to forming a mechanically gated ion channel, TRPN1 (NompC) channels also feature ultrastructural specializations like compliant membrane-microtubule connectors, as it was found for campaniform mechanoreceptors in *Drosophila* (Liang et al. 2013). Mechanical stimulation is generally accomplished if an external mechanical stimulus acts onto an extracellular structure which is then shifted relative to the cytoskeleton (Gillespie and Walker 2001). Being elastically tethered between them, the membrane and membrane-bound channels are deformed by such a shift. The compliant membrane-microtubule connector filaments are such an intracellular link between the ion channel within the membrane and cytoskeletal microtubules, and might therefore be candidates for gating springs (Liang et al. 2013).

6.10. Possible active mechanisms, their location and frequency range of operation

6.10.1. Ionic composition of the receptorlymph within the scolopale space

Mechanosensitive receptors in vertebrate ears are bathed in a K^+-rich endolymph (Corey and Hudspeth 1979; Bechstedt and Howard 2008). For chordotonal organs of insects, the scolopale lumen that surrounds the dendritic outer segment (and presumable mechanotransduction sites) holds a still unknown ionic composition. It is tightly sealed from the hemolymph by intercellular junctions between supporting cells which may be electrically connected by gap junctions. The scolopale lumen was therefore suggested to serve the same function as its vertebrate analog, the scala media. Neurexin is a component of chordotonal septate junctions in *Drosophila* and was proposed to form this seal (Baumgartner et al. 1996). Other ciliated type 1 insect mechanoreceptors in campaniform and bristle organs were shown to contain a receptor lymph which is K^+-rich and Cl^--poor (Eberl 1999), and a similar composition was predicted for chordotonal organs.

6.10.2. Possible active motility of the scolopidial cilium

There is a current debate on whether the scoloidial cilium is able to perform an active beat or contraction, or not. The cilium of type 1 scolopidia constitutes a 9x2+0 configuration, with nine microtubule pairs arranged in a concentric ring and a central pair missing (Yack 2004). Motile cilia characteristically possess such central microtubules, and active ciliary movement was therefore thought unlikely. Recent observations, however, started to weaken the prevailing view of non-motile cilia. Arguments both for or against a motility are based on several ultrastructural features of the cilium (French 1988; Field and Matheson 1998), with the answer to this question still being open.

Scolopale rods of the receptors contain actin filaments which in general could fulfil either a contraction mechanism or serve as stabilizing structures. In systems where they possess a motile function, their activity depends on an interaction with the motorprotein myosin. Myosin, however, has not been found so far within scolopale rods (Wolfrum 1990, 1992). The co-localization of tropomyosin with these actin filaments rather suggests a stabilization function. Tropomyosin supports the assembly of actin filaments, prevents their disassembly, and increases the rigidity of the scolopale rods (Wolfrum 1997).

As opposed to the prevailing view of non-motile cilia based on the absence of the central microtubules, cilia were nonetheless reported to be potentially motile (Eberl 1999). Ciliary rootlets are the structural foundation for the dendritic cilium and function as mechanical anchor (Wolfrum 1991). Starting far down in the cell body, they rise through the dendritic inner segment and unite into a single, cylindrical, ciliary root. The rootlets contain the Ca^{2+}-binding protein centrin (Wolfrum 1997). Centrin is a contractile protein and the major component of ciliary and flagellar rootlets of motile green algae. In the presence of raised Ca^{2+} concentrations, it is thought to induce contractions of green algal ciliary rootlets. Wolfrum (1997) therefore suggested that normal function of insects sensilla might include Ca^{2+}-modulated contractions of

their ciliary rootlets. Such contractions are thought to be involved in sensory transduction and adaptation. One of the effects of active ciliary motility is the bending of the cilium which is based on force generated by dynein arms associated with the nine microtubule pairs that constitute the ciliary axoneme (Moran et al. 1977; Thurm et al. 1983). The dynein-generated force was suggested to cause a sliding between the microtubule pairs (Warner and Satir 1974), locally restricted at the site of mechanical stimulation. The bend is then propagated to the distal basal body at the base of the cilium which by that is shifted in its position. This bend in the region of the ciliary base causes a distortion of the cell membrane that could lead to an opening of transduction channels and the production of a receptor current (Moran et al. 1977). Ciliary rootlet contractions based on centrin could serve to affect the magnitude of the displacement of the distal basal body or re-locate it to its original position after stimulation (Wolfrum 1997).

More findings supporting the idea of motile cilia came from observations of active force generation in Johnston's organs (e.g. Göpfert and Robert 2003; Göpfert et al 2005) and DPOAE generation in tympanal organs of several insect species (although DPOAEs by themselves are no proof for active processes; Kössl et al. 2008). Both spontaneous oscillations in Johnston's organs of mosquitoes (Warren et al. 2010) and DPOAE generation in locusts (Möckel et al. 2012) displayed a temperature-dependence. The calculated Arrhenius energy of the underlying cellular component pointed towards the dynein-tubulin system which would support the idea of a dynein-based bending within the cilium as described above.

6.10.3. The frequency range of a potentially active process within tympanal organs

The occurrence of DPOAEs by itself does not nesessarily mean that active amplification processes are involved. Their generation in general could as well be based on purely passive mechanisms. There are, however, possible hints that amplification processes in the hearing organs of insects might operate at the lower frequency range and that the system switches into a passive nonlinear mode at higher frequencies. There are several notions that such a shift might occur around 10 kHz, with the system working differently below and above that range.

DPOAE growth functions recorded in locusts showed different slopes, depending on stimulus frequencies (Kössl and Boyan 1998b). Emission amplitudes evoked by f2 frequencies below 10 kHz slowly increased for stimulus levels up to about 50-60 dB SPL (slope 0.46-0.78), with a steeper increase to follow at higher stimulus levels. Those evoked by f2 frequencies above 10 kHz showed a steeper initial growth (slope 1.1-1.48). The authors suggested the mechanics of the thick part of the tympanum and/or interactions of its thick and thin part to give rise to the sensitive low-frequency distortions, and the high-frequency distortions being caused by vibrations of the thin membrane part of the tympanum alone. But the separation between the frequency dependence of the growth function slopes might as well be caused by a shift from potentially active to purely passive mechanisms. To verify the hypothesis, one would have to record DPOAEs from insect species which do not possess an anatomical separation between low and high frequency receptors such as locusts, but rather have a continuous array of receptors, such as bushcrickets, and reliably generate DPOAEs in frequency ranges well below 10 kHz.

Another hint for an active amplification process in the frequency range below 10 kHz comes from whole nerve recordings in the locust (Römer 1976; Yack and Dawson 2008). The threshold tuning curves display a striking drop of sensitivity of up to 20 dB for stimulus frequencies above 10 kHz. This could simply reflect different numbers of involved receptors for each frequency group. A small group of the approximately 80 receptors in Müller's organ respond best to high frequencies above 12 kHz, the rest to frequencies below that (Jacobs et al. 1999). Or maybe the higher sensitivity in the treshold tuning curves for frequencies below 10 kHz is caused by amplification of incoming sound.

DPOAE generation displayes different vulnerability for changes in the animal's physiological state below and above 10 kHz. Hypoxia during the application of CO_2 leads to decreased DPOAE amplitudes evoked by stimuli below 10 kHz, whereas those evoked by higher frequencies are not affected (Kössl and Boyan 1998a,b). The temperature-dependence of DPOAEs in locusts brought about a sharp separation between DPOAEs evoked by stimuli at or below 10 kHz and by higher frequencies. Those induced by stimuli below 10 kHz showed level-dependent effects upon shifting the animal's body temperature. Those induced by higher frequencies remained unaffected (Möckel et al. 2012). The suggested active involvement of the dynein-tubulin system might work up to 10 kHz, and the passive system above that does not rely on the molecules' operation.

All these suggestions still need experimental verifing. Active hair bundle motility and adaptation mechanisms operate at much lower frequencies than somatic motility, and in non-mammals are restricted to frequencies below about 3 kHz (Manley et al. 2001; LeMasurier and Gillespie 2005). The hearing ranges of non-mammalian vertebrates are confined to low frequencies, with the upper limit being about 11 kHz in the barn owl (spontaneous OAEs measured at 9 to 11 kHz; Taschenberger and Manley 1997). In the case of mammalian outer hair cells, the kinetics of fast adaptation are fast enough that active hair bundle motility would be able to drive amplification at relevant auditory frequencies (Kennedy et al. 2003). A frequency limit for active hair bundle mechanisms of about 8 kHz might be calculated from the adaptation time constant of 120 µs in mammalian outer hair cells. Assuming that there really is an active amplification process working at frequencies up to 10 kHz in insect ears, then insect mechanoreceptors, despite all anatomical differences, operate in a quite similar way compared to vertebrates.

7. References

Albert JT, Nadrowski B, Göpfert MC (2007a) *Drosophila* mechanotransduction: Linking proteins and function. Fly 1:4:238-241

Albert JT, Nadrowski B, Göpfert MC (2007b) Mechanical signatures of transducer gating in the *Drosophila* ear. Curr Biol 17:1000-1006

Arntz B (1975) Das Hörvermögen von *Nepa cinerea L.*: Zur Funktionsweise der thorakalen Seolopalorgane [Hearing in *Nepa cinerea L.*: Functioning of the Pterothoracic Scolopal Organs] J Comp Physiol 96:53-72

Ashmore JF (1987) A fast motile response in guinea-pig outer hair cells: the cellular basis of the cochlear amplifier. J Physiol (Lond) 388: 323-347

Ausborn J, Wolf H, Mader W, Kayser H (2005) The insecticide pymetrozine selectively affects chordotonal mechanoreceptors. J Exp Biol 208:4451–4466

Bai J-P, Surguchev A, Navaratnam D (2011) β_4-Subunit increases Slo responsiveness to physiological Ca^{2+} concentrations and together with β_1 reduces surface expression of Slo in hair cells. Am J Physiol Cell 300:C435-C446

Bailey WJ (1990) The ear of the bushcricket. In: Bailey WJ, Rentz DCF (eds) The tettigoniidae: Biology, systematics and evolution. Springer. pp 217-247

Bangert M, Kalmring K, Sickmann T, Stephen R, Jatho M, Lakes-Harlan R (1998) Stimulus transmission in the auditory receptor organs of the foreleg of bushcrickets (Tettigoniidae) I. The role of the tympana. Hear Res 115:27-38

Baumgartner S, Littleton JT, Broadie K, Bhat MA, Harbecke R, Lengyel JA, Chiquet-Ehrismann R, Prokop A, Bellen HJ (1996) A *Drosophila* neurexin is required for septate junction and blood-nerve barrier formation and function. Cell 87:1059-1068

Bechstedt S, Howard J (2008) Hearing mechanics: A fly in your ear. Curr Biol 18:869-870

Bechstedt S, Albert JT, Kreil DP, Müller-Reichert T, Göpfert MC, Howard J (2010) A doublecortin containing microtubule-associated protein is implicated in mechanotransduction in *Drosophila* sensory cilia. Nature Comm 1:11 doi: 10.1038 / ncomms1007

Beurg M, Fettiplace R, Nam J-H, Ricci AJ (2009) Localization of inner hair cell mechanotransducer channels using high-speed calcium imaging. Nature Neurosc 12:553-558

Boo KS, Richards AG (1975a) Fine structure of the scolopidia in the Johnston`s organ of male *Aedes aegypti* (L.) (Diptera, Culicidae). Int J Insect Morphol & Embryol 4:549-566

Boo KS, Richards AG (1975b) Fine structure of scolopidia in Johnston`s organ of female *Aedes aegypti* compared with that of the male. J Insect Physiol 21:1129-1139

Brownell WE, Bader CR, Bertrand D, Ribaupierre YD (1985) Evoked mechanical responses of isolated cochlear outer hair cells. Science 227:194-196

Cator LJ, Arthur BJ, Harrington LC, Hoy RR (2009) Harmonic convergence in the love songs of the dengue vector mosquito. Science 323:1077-1079

Chan DK, Hudspeth AJ (2005) Ca^{2+} current-driven nonlinear amplification by the mammalian cochlea *in vitro*. Nature Neurosci 8:149-155

Corey DP, Hudspeth AJ (1979) Ionic basis of the receptor potential in a vertebrate hair cell. Nature 281:675–677

Corey DP (2003) New TRP channels in hearing and mechanosensation. Neuron 39:585–588

Coro F, Perez M (1993) Threshold and suprathreshold responses of the auditory receptors in an arctiid moth. Experientia (Birkhäuser Verlag) 49:285-290

Coro F, Kössl M (1998) Distortion-product otoacoustic emissions from the tympanic organ in two noctuoid moths. J Comp Physiol A 183:525–531

Coro F, Kössl M (2001) Components of the 2f1-f2 distortion-product otoacoustic emission in a moth. Hear Res 162:126-133

Crawford AC, Fettiplace R (1980) The frequency selectivity of auditory nerve fibres and hair cells in the cochlea of the turtle. J Physiol 306:79-l25

Crawford AC, Fettiplace R (1985) The mechanical properties of ciliary bundles of turtle cochlear hair cells. J Physiol 364:359-379

Crawford AC, Evans MG, Fettiplace R (1989) Activation and adaptation of transducer currents in turtle hair cells. J Physiol 419:405–434

Dalhoff E, Turcanu D, Zenner HP, Gummer AW (2007) Distortion product otoacoustic emissions measured as vibration on the eardrum of human subjects. Proc Natl Acad Sci USA 104:1546–1551

Dallos P, Fakler B (2002) Prestin, a new type of motor protein. Nat Rev Mol Cell Biol 3:104-111

Duke TAJ, Jülicher F (2008) Critical oscillators as active elements in hearing. In: Manley GA, Fay RR, Popper AN (eds) Active processes and otoacoustic emissions. Springer, New York, pp 63–92

Eberl DF (1999) Feeling the vibes: Chordotonal mechanisms in insect hearing. Curr Opinion Neurobiol 9:389-393

Effertz T, Wiek R, Göpfert MC (2011) NompC TRP channel is essential for *Drosophila* sound receptor function. Curr Biol 21:592–597

Feng AS, Narins PM, Xu CH, Lin WY, Yu ZL, Qiu Q, Xu ZM, Shen JX (2006) Ultrasonic communication in frogs. Nature 440:333-336

Fertschai I, Stradner J, Römer H (2007) Neuroethology of female preference in the synchronously singing bushcricket *Mecopoda elongata* (Tettigoniidae; Orthoptera): why do followers call at all? J Exp Biol 210:465–476

Fettiplace R (1987) Electrical tuning of hair cells in the inner ear. Trends Neurosci 10:421-425

Fettiplace R, Hackney CM (2006) The sensory and motor roles of auditory hair cells. Nature Rev Neurosci 7:19-29

Field LH, Matheson T (1998) Chordotonal organs of insects. Adv Insect Physiol 27:1–228

Field LH (2005) The chordotonal organ: a uniquely invertebrate mechanoreceptor. In: Christensen TA (ed) Methods in insect sensory neuroscience. CRC Press, Boca Raton, pp 61–105

Fonseca PJ, Correia T (2007) Effects of temperature on tuning of the auditory pathway in the cicada *Tettigetta josei* (Hemiptera, Tibicinidae). J Exp Biol 210:1834-1845

Förster J, Ebbingshaus-Kintscher U, Büschges A (2013) Determining mode of action of pymetrozine - From single cell to system level. 10th Göttingen Meeting of German Neuroscience Society T21-2A

French AS (1988) Transduction mechanisms of mechanosensilla. Ann Rev Entomol 33:39-58

French AS (2008) Biophysics of chordotonal organs. In: Dallos P, Oertel D (eds) The Senses: A Comprehensive Reference, Vol.3, Audition, Elsevier, pp 211-216

Frolenkov GI (2006) Regulation of electromotility in the cochlear outer hair cell. J Physiol 576:43–48

Geurten B, Spalthoff C, Göpfert MC (2013) Insect hearing: Active amplification in tympanal ears. Curr Biol 23:950-952

Gibson G, Russell I (2006) Flying in tune: sexual recognition in mosquitoes. Curr Biol 16:1311–1316

Gillespie PG, Walker RG (2001) Molecular basis of mechanosensory transduction. Nature 413:194-202

Gillespie PG, Cyr JL (2004) Myosin-1c, the hair cell's adaptation motor. Annu Rev Physiol 66:521–545

Goodyear RJ, Marcotti W, Kros CJ, Richardson GP (2005) Development and properties of stereociliary link types in hair cells of the mouse cochlea. J Comp Neurol 485:75–85

Göpfert MC, Briegel H, Robert D (1999) Mosquito hearing: Sound-induced antennal vibrations in male and female *Aedes aegypti*. J Exp Biol 202:2727–2738

Göpfert MC, Robert D (2001) Active auditory mechanics in mosquitoes. Proc R Soc Lond B 268:333-339

Göpfert MC, Robert D (2002) The mechanical basis of *Drosophila* audition. J Exp Biol 205:1199–1208

Göpfert MC, Robert D (2003) Motion generation by *Drosophila* mechanosensory neurons. Proc Natl Acad Sci 100:5514-5519

Göpfert MC, Humphris ADL, Albert JT, Robert D, Hendrich O (2005) Power gain exhibited by motile mechanosensory neurons in Drosophila ears. Proc Natl Acad Sci USA 102:325-330

Göpfert MC, Albert JT, Nadrowski B, Kamikouchi A (2006) Specification of auditory sensitivity by *Drosophila* TRP channels. Nature Neurosci 9:999-1000

Gray EG (1960) The fine structure of the insect ear. Philos Trans R Soc Lond B 243:75-94

Halex H, Kaiser W, Kalmring K (1988) Projection areas and branching patterns of the tympanal receptor cells in migratory locusts, *Locusta migratoria* and *Schistocerca gregaria*. Cell Tiss Res 253:517-528

Harrewijn P, Kayser H (1997) Pymetrozine, a fast-acting and selective inhibitor of aphid feeding. *In situ* studies with electronic monitoring of feeding behaviour. Pestic Sci 49:130–140

Hartbauer M, Kratzer S, Steiner K, Römer H (2005) Mechanisms for synchrony and alternation in song interactions of the bushcricket *Mecopoda elongata* (Tettigoniidae: Orthoptera). J Comp Physiol A 191:175–188

Hoffmann E, Jatho M (1995) The acoustic trachea of Tettigoniids as an exponential horn: Theoretical calculations and bioacoustical measurements. J Acoust Soc Am 98:1845-1851

Höger U, Seyfarth E-A (2001) Structural correlates of mechanosensory transduction and adaptation in identified neurons of spider slit sensilla. J Comp Physiol A 187:727-736

Howard J, Hudspeth AJ (1988) Compliance of the hair bundle associated with gating of mechanoelectrical transduction channels in the bullfrog's saccular hair cell. Neuron 1:189–199

Hoy R, Nolen T, Brodfuehrer P (1989) The neuroethology of acoustic startle and escape in flying insects. J Exp Biol 146:287-306

Hoy RR, Robert D (1996) Tympanal hearing in insects. Annu Rev Entomol 41:433-450

Hoyle G (1953) Potassium ions and insect nerve muscle. J Exp Biol 30:121–135

Hudspeth AJ (1982) Extracellular current flow and the site of transduction by vertebrate hair cells. J Neurosci 2:1–10

Hudspeth AJ, Gillespie PG (1994) Pulling springs to tune transduction: Adaptation by hair cells. Neuron 12:1-9

Hudspeth AJ (2008) Making an effort to listen: mechanical amplification in the ear. Neuron 59:530–545

Hummel J, Kössl M, Nowotny M (2011) Sound-induced tympanal membrane motion in bushcrickets and its relationship to sensory output. J Exp Biol 214:3596-3604

Hummel J, Wolf K, Kössl M, Nowotny M (2014) Processing of simple and complex acoustic signals in a tonotopically organized ear. Proc R Soc B 281:20141872

Hustert R (1999) Accessory hemolymph pump in the mesothoracic legs of locusts, (*Schistocerca gregaria* forskal) (Orthoptera, Acrididae). Internat J Insect Morphol 28:91-96

Jackson JC, Robert D (2006) Nonlinear auditory mechanism enhances female sounds for male mosquitoes. Proc Natl Acad Sci 103:16734–16739

Jacobs K, Otte B, Lakes-Harlan R (1999) Tympanal receptor cells of *Schistocerca gregaria*: correlation of soma positions and dendrite attachment sites, central projections and physiologies. J Exp Zool 283:270–285

James TW (1959) Synchronization of cell division in Amoebae. Ann NY Acad Sci 78:501-514

Janssen T, Müller J (2008) Otoacoustic emissions as a diagnostic tool in a clinical context. In: Manley GA, Fay RR, Popper AN (eds) Active processes and otoacoustic emissions. Springer, New York, pp 421-460

Kachar B, Parakkal M, Kurc M, Zhao Y, Gillespie PG (2000) High-resolution structure of hair-cell tip links. Proc Natl Acad Sci USA 97:13336–13341

Kavlie RG, Kernan MJ, Eberl DF (2010) Hearing in *Drosophila* requires TilB, a conserved protein associated with ciliary motility. Genetics 185:177–188

Kamikouchi A, Inagaki HK, Effertz T, Hendrich O, Fiala A, Göpfert MC, Ito K (2009) The neural basis of *Drosophila* gravity-sensing and hearing. Nature 458:165-172

Kamikouchi A, Albert JT, Göpfert MC (2010) Mechanical feedback amplification in *Drosophila* hearing is independent of synaptic transmission. Europ J Neurosci 31:697–703

Kaufmann L, Schürmann F, Yiallouros M, Harrewijn P, Kayser H (2004) The serotonergic system is involved in feeding inhibition by pymetrozine. Comparative studies on a locust (*Locusta migratoria*) and an aphid (*Myzus persicae*). Comp Biochem Physiol 138C:469–483

Keil TA (1997) Functional morphology of insect mechanoreceptors. Microsc Res Tech 39:506-531

Kemp DT (1978) Stimulated acoustic emissions from within the human auditory system. J Acoust Soc Am 64:1386–1391

Kemp DT (2008) Otoacoustic emissions: concepts and origins. In: Manley GA, Fay RR, Popper AN (eds) Active processes and otoacoustic emissions. Springer, New York, pp 1–38

Kennedy HJ, Evans MG, Crawford AC, Fettiplace R (2003) Fast adaptation of mechanoelectrical transducer channels in mammalian cochlear hair cells. Nature Neurosc 6:832-836

Khvoles R, Freeman S, Sohmer H (1998) Effect of temperature on the transient evoked and distortion product otoacoustic emissions in rats. Audiol Neurootol 3:349-360

Köppl C, Manley GA (1993) Spontaneous otoacoustic emissions in the bobtail lizard. I: General characteristics. Hear Res 71:157–169

Kössl M (1994) Otoacoustic emissions from the cochlea of the 'constant frequency' bats, *Pteronotus parnellii* and *Rhinolophus rouxi.* Hear Res 72:50-72

Kössl M, Boyan GS (1998a) Otoacoustic emissions from a nonvertebrate ear. Naturwissenschaften 85:124–127

Kössl M, Boyan GS (1998b) Acoustic distortion products from the ear of a grasshopper. J Acoust Soc Am 104:326–335

Kössl M, Coro F (2006) L1,L2 maps of distortion-product otoacoustic emissions from a moth ear with only two auditory receptor neurons. J Acoust Soc Am 120:3822-3831

Kössl M, Coro F, Seyfarth E-A, Nässig WA (2007) Otoacoustic emissions from insect ears having just one auditory neuron. J Comp Physiol A 193:909–915

Kössl M, Möckel D, Weber M, Seyfarth E-A (2008) Otoacoustic emissions from insect ears: evidence of active hearing? J Comp Physiol A 194:597–609

Kössl M, Möckel D (2012) Measurement of sensitive distortion–product otoacoustic emissions in insect tympanal organs. J Exp Biol 215:566–567. Correspondence to Moir et al. (2011) J Exp Biol 214

Lakes R, Schikorski T (1990) Neuroanatomy of tettigoniids. In: Bailey WJ, Rentz DCF (eds) The Tettigoniidae: biology, systematics and evolution. Crawford House Press, Bathurst, pp 166–190

Lee J, Moon S, Cha Y, Chung YD (2010) *Drosophila* TRPN(= NOMPC) channel localizes to the distal end of mechanosensory cilia. PLoS ONE 5(6): e11012. doi:10.1371/journal.pone.0011012

Lehnert BP, Baker AE, Gaudry Q, Chiang A-S, Wilson RI (2013) Distinct roles of TRP channels in auditory transduction and amplification in *Drosophila*. Neuron 77:115–128

LeMasurier M, Gillespie PG (2005) Hair-cell mechanotransduction and cochlear amplification. Neuron 48:403–415

LeMasurier M, Gillespie PG (2007) Hair bundles: keeping it together. Nat Neurosci 10:11-12

Liang X, Madrid J, Gärtner R, Verbavatz J-M, Schiklenk C, Wilsch-Bräuninger M, Bogdanova A, Stenger F, Voigt A, Howard J (2013) A NOMPC-dependent membrane-microtubule connector is a candidate for the gating spring in fly mechanoreceptors. Curr Biol 23:755-763

Liberman MC, Gao J, He DZZ, Wu X, Jia S, Zou J (2002) Prestin is required for electromotility of the outer hair cell and for the cochlear amplifier. Nature 419: 300-304

Liberman MC, Zuo J, Guinan JJ Jr (2004) Otoacoustic emissions without somatic motility: Can stereocilia mechanics drive the mammalian cochlea? J Acoust Soc Am 116:1649–1655

Lukashkin AN, Lukashkina VA, Russell IJ (2002) One source for distortion product otoacoustic emissions generated by low- and high-level primaries. J Acoust Soc Am 111:2740-2748

Mammano F, Ashmore JF (1993) Reverse transduction measured in the isolated cochlea by laser Michelson interferometry. Nature 365:838-841

Manley GA, Köppl C (1998) Phylogenetic development of the cochlea and its innervation. Curr Opinion Neurobiol 8:468-474

Manley GA, Gallo L, Köppl C (1996) Spontaneous otoacoustic emissions in two gecko species, *Gekko gecko* and *Eublepharis macularius*. J Acoust Soc Am 99:1588-1603

Manley GA, Gallo L (1997) Otoacoustic emissions, hair cells, and myosin motors. J Acoust Soc Am 102:1049-1055

Manley GA (2001) Evidence for an active process and a cochlear amplifier in nonmammals. J Neurophysiol 86:541–549

Manley GA, Kirk DL, Köppl C, Yates GK (2001) *In vivo* evidence for a cochlear amplifier in the hair-cell bundle of lizards. Proc Natl Acad Sci USA 98:2826-2831

Manley GA, Köppl C (2008) What have lizard ears taught us about auditory physiology? Hear Res 238:3-11

Manley GA, van Dijk P (2008) Otoacoustic emissions in amphibians, lepidosaurs, and archosaurs. In: Manley GA, Fay RR, Popper AN (eds) Active processes and otoacoustic emissions. Springer, New York, pp 211-260

Martin P, Hudspeth AJ (1999) Active hair-bundle movements can amplify a hair cell's response to oscillatory mechanical stimuli. Proc Natl Acad Sci USA 96:14306-14311

Martin P, Mehta AD, Hudspeth AJ (2000) Negative hair-bundle stiffness betrays a mechanism for mechanical amplification by the hair cell. Proc Natl Acad Sci USA 97:12026-12031

Meenderink SWF, van Dijk P (2006) Temperature dependence of anuran distortion product otoacoustic emissions. J Assoc Res Otolaryngol 7:246-252

Meyer J, Elsner N (1996) How well are frequency sensitivities of grasshopper ears tuned to species-specific song spectra? J Exp Biol 199:1631-1642

Meyer J, Hedwig B (1995) The influence of tracheal pressure changes on the responses of the tympanal membrane and auditory receptors in the locust *Locusta migratoria* L. J Exp Biol 198:1327-1339

Mhatre N, Robert D (2013) A tympanal insect ear exploits a critical oscillator for active amplification and tuning. Curr Biol 23:1952–1957

Michelsen A (1971) The physiology of the locust ear I. Frequency sensitivity of single cells in the isolated ear II. Frequency discrimination based upon resonances in the tympanum III. Acoustical properties of the intact ear. Z Vergl Physiol 71:49–128

Michelsen A (1992) Hearing and sound communication in small animals: evolutionary adaptations to the laws of physics. In: Webster DM, Fay RR, Popper AN (eds) The evolutionary biology of hearing. Heidelberg and New York: Springer-Verlag. pp 61–78

Michelsen A, Rohrseitz K (1995) Directional sound processing and interaural sound transmission in a small and a large grasshopper. J Exp Biol 198:1817-1827

Miller LA, Surlykke A (2001) How some insects detect and avoid being eaten by bats: Tactics and countertactics of prey and predator. BioScience 51:570-581

Möckel D, Seyfarth E-A, Kössl M (2007) The generation of DPOAEs in the locust ear is contingent upon the sensory neurons. J Comp Physiol A 193:871-879

Möckel D, Seyfarth E-A, Kössl M (2011) Otoacoustic emissions in bushcricket ears: general characteristics and the influence of the neuroactive insecticide pymetrozine. J Comp Physiol A 197:193-202

Möckel D, Kössl M, Lang J, Nowotny M (2012) Temperature dependence of distortion–product otoacoustic emissions in tympanal organs of locusts. J Exp Biol 215:3309–3316

Möckel D, Nowotny M, Kössl M (2014) Mechanical basis of otoacoustic emissions in tympanal hearing organs. J Comp Physio, A 200:681-691

Moir HM, Jackson JC, Windmill JFC (2011) No evidence for DPOAEs in the mechanical motion of the locust tympanum. J Exp Biol 214:3165–3172

Mora EC, Cobo-Cuan A, Macías-Escrivá F, Pérez M, Nowotny M, Kössl M (2013) Mechanical tuning of the moth ear: distortion-product otoacoustic emissions and tympanal vibrations. J Exp Biol 216:3863–3872

Moran DT, Varela FG (1971) Microtubules and sensory transduction. Proc Natl Acad Sci USA 68:757-760

Moran DT, Varela FJ, Rowley JC (1977) Evidence for active role of cilia in sensory transduction. Proc Natl Acad Sci USA 74:793-797

Nadrowski B, Albert JT, Göpfert MC (2008) Transducer-based force generation explains active process in *Drosophila* hearing. Curr Biol 18:1365–1372

Oldfield BP (1982) Tonotopic organisation of auditory receptors in Tettigoniidae (Orthoptera: Ensifera). J Comp Physiol 147:461–469

Oldfield BP (1984) Physiology of auditory receptors in two species of Tettigoniidae (Orthoptera: Ensifera). J Comp Physiol A 155:689-696

Oldfield BP (1985) The tuning of auditory receptors in bushcrickets. Hear Res 17:27-35

Oldfield BP, Hill KG (1986) Functional organization of insect auditory sensilla. J Comp Physiol A 158:27-34

Oldfield BP (1988a) Tonotopic organization of the insect auditory pathway. Trends Neurosci 11:267–270

Oldfield BP (1988b) The effect of temperature on the tuning and physiology of insect auditory receptors. Hear Res 35:151-158

Pass G (2000) Accessory pulsatile organs: Evolutionary innovations in insects. Annu Rev Entomol 45:495–518

Peng AW, Ricci AJ (2011) Somatic motility and hair bundle mechanics, are both necessary for cochlear amplification? Hear Res 273:109-122

Pennetier C, Warren B, Dabiré KR, Russell IJ, Gibson G (2010) "Singing on the Wing" as a mechanism for species recognition in the malarial mosquito *Anopheles gambiae*. Curr Biol 20:131–136

Probst R, Lonsbury-Martin BL, Martin GK (1991) A review of otoacoustic emissions. J Acoust Soc Am 89:2027-2067

Ren T (2004) Reverse propagation of sound in the gerbil cochlea. Nature Neurosc 7:333-334

Robert D (1989) The auditory behaviour of flying locusts. J Exp Biol 147:279-301

Robles L, Ruggero MA (2001) Mechanics of the mammalian cochlea. Physiol Rev 8:1305–1352

Rohde WS (2007) Distortion product otoacoustic emissions and basilar membrane vibration in the 6-9 kHz region of sensitive chinchilla cochleae. J Acoust Soc Am 122:2725-2737

Römer H (1976) Die Informationsverarbeitung tympanaler Rezeptorelemente von *Locusta migratoria* (*Acrididae, Orthoptera*). [Processing of information by tympanal receptors of *Locusta migratoria* (*Acrididae, Orthoptera*).]. J Comp Physiol A 109:101–122

Römer H, Marquart V, Hardt M (1988) Organization of a sensory neuropile in the auditory pathway of two groups of Orthoptera. J Comp Neurology 275:201-215

Santos-Sacchi J, Song L, Zheng J, Nuttall AL (2006) Control of mammalian cochlear amplification by chloride anions. J Neurosci 26:3992–3998

Shera CA, Guinan JJ (1999) Evoked otoacoustic emissions arise by two fundamentally different mechanisms: A taxonomy for mammalian OAEs. J Acoust Soc Am 105:782-798

Shera CA (2004) Mechanisms of mammalian otoacoustic emission and their implications for the clinical utility of otoacoustic emissions. Ear & Hearing 25:86-97

Siemens J, Lillo C, Dumont RA, Reynolds A, Williams DS, Gillespie PG, Müller U (2004) Cadherin 23 is a component of the tip link in hair-cell stereocilia. Nature 428:950–955

Simmons DD, Meenderink SWF, Vassilakis PN (2007) Anatomy, physiology, and function of auditory end organs in the frog inner ear. In: Narins PM, Feng AS, Fay RR, Popper AN (eds) Hearing and sound communication in amphibians. Springer, New York, pp 184-220

Sneary MG (1988) Auditory receptor of the red-eared turtle: I. General ultrastructure. II. Afferent and efferent synapses and innervation patterns. J Comp Neurology 276:573-606

Stephen RO, Bennet-Clark HC (1982) The anatomical and mechanical basis of stimulation and frequency analysis in the locust ear. J Exp Biol 99:279–314

Strauß J, Lakes-Harlan R (2003) Development of the auditory system of *Mecopoda elongata* (Orthoptera). In: Elsner N, Zimmermann H (eds) The neurosciences from basic research to therapy. Proceeding of the 5th Meeting of the German Neuroscience Society 2003 (29th Göttingen neurobiology conference), pp 406-407

Stumpner A (1996) Tonotopic organization of the hearing organ in a bushcricket. Naturwissenschaften 83:81–84

Surlykke A (1984) Hearing in notodontid moths: a tympanic organ with a single auditory neurone. J Exp Biol 113:323-335

Taschenberger G, Manley GA (1997) Spontaneous otoacoustic emissions in the barn owl. Hear Res 110:61-76

Thurm U, Erler G, Gödde J, Kastrup H, Keil TH, Völker W, Vohwinkel B (1983) Cilia specialized for mechanoreception. J Submicrosc Cytol 15:151-155

von Békésy G (1960) In: Wever ED (ed) Experiments in hearing. McGraw-Hill, New York

Walker RG, Willingham AT, Zuker CS (2000) A *Drosophila* mechanosensory transduction channel. Science 287:2229–2234

Wangemann P (2006) Supporting sensory transduction: cochlear fluid homeostasis and the endocochlear potential. J Physiol 576.1:11–21

Warner FD, Satir P (1974) The structural basis of ciliary bend formation. Radial spoke positional changes accompanying microtubule sliding. J Cell Biol 63:35-63

Warren B, Gibson G, Russell IJ (2009) Sex recognition through midflight mating duets in *Culex* mosquitoes is mediated by acoustic distortion. Curr Biol 19:485-491

Warren B, Lukashkin AN, Russell IJ (2010) The dynein-tubulin motor powers active oscillations and amplification in the hearing organ of the mosquito. Proc R Soc B 277:1761-1769

Weber T, Göpfert MC, Winter H, Zimmermann U, Kohler H, Meier A, Hendrich O, Rohbock K, Robert D, Knipper M (2003) Expression of prestin-homologous solute carrier (SLC26) in auditory organs of nonmammalian vertebrates and insects. Proc Natl Acad Sci USA 100:7690–7695

Weber M (2004) Untersuchungen über neuroaktive Substanzen in tympanalen Hörorganen der Laubheuschrecke *Mecopoda elongata*. Diplomarbeit, FB Biologie, Universität Frankfurt/Main

Weber M (2012) Zelluläre Komponenten des tympanalen Hörorgans von Laubheuschrecken und deren mögliche Einflüsse auf die Schallrezeption. Dissertation, FB Biologie, Universität Frankfurt/Main

Windmill JFC, Göpfert MC, Robert D (2005) Tympanal travelling waves in migratory locusts. J Exp Biol 208:157-168

Windmill JFC, Jackson JC, Tuck EJ, Robert D (2006) Keeping up with bats: Dynamic auditory tuning in a moth. Curr Biol 16:2418–2423

Wolfrum U (1990) Actin filaments: the main components of the scolopale in insect sensilla. Cell Tissue Res (1990) 261:85-96

Wolfrum U (1991) Tropomyosin is co-localized with the actin filaments of the scolopale in insect sensilla. Cell Tissue Res 265:11-17

Wolfrum U (1992) Cytoskeletal elements in arthropod sensilla and mammalian photoreceptors. Biol Cell 76:373-381

Wolfrum U (1997) Cytoskeletal elements in insect sensilla. Int J Insect Morphol & Embryol 26:191-203

Yack JE (2004) The structure and function of auditory chordotonal organs in insects. Microsc Res Tech 63:315-337

Yack JE, Dawson JW (2008) Insect ears. In: Dallos P, Oertel D (eds) The Senses: A Comprehensive Reference, Vol.3, Audition, Elsevier, pp 35-53

Yager DD, Hoy RR (1987) The midline metathoracic ear of the praying mantis, *Mantis religiosa*. Cell Tissue Res 250:531–541

Yager DD (1999) Structure, development, and evolution insect auditory systems. Microsc Res Tech 47:380-400

Young D (1973) Fine structure of the sensory cilium of an insect auditory receptor. J Neurocytol 2:47-52

Zenner HP (1986) Motile responses in outer hair cells. Hear Res 22:83-90

Zhao HB, Santo-Sancchi J (1999) Auditory collusion and a coupled couple of outer hair cells. Nature 399:359-362

Zheng J, Shen W, He DZZ, Long KB, Madison LD, Dallos P (2000) Prestin is the motor protein of cochlear outer hair cells. Nature 405:149-155

8. Appendix

8.1. Scientific classification

Phyllum	Arthropoda
Class	Insecta
Subclass	Orthoptera

Order	Ensifera	Caelifera	Caelifera
Family	Tettigoniidae	Acrididae	Acrididae
Genus	Mecopoda	Locusta	Schistocerca
Species	*Mecopoda elongata*	*Locusta migratoria*	*Schistocerca gregaria*

8.2. Keeping of animals / Source of supply

The tropical bushcricket species *Mecopoda elongata* has its native main range in south-east Asia. The animals used for this thesis came from our own breeding colony at the zoological institute Frankfurt/Main. Animals to start this colony had been provided by Prof. Heiner Römer, University Graz, Austria and were originally caught in Malaysia. At room temperatures of 25-28°C and relative humidity of 50-60%, the animals were immediately after eclosion brought up in plastic containers of different sizes, providing them with lettuce, oat flakes, fish food and fresh water. The animals were held according to age, but without any gender separation and mating control. Oviposition boxes were placed in a warming cabinet that assured constant temperatures. The time span from oviposition to eclosion amounts to about 6 to 8 weeks, and further 10 to 12 weeks until the last ecdysis, with considerable variation in duration, but also in the size of individuals during these seven larval stages (Strauss and Lakes-Harlan 2003).

The migratory locust *Locusta migratoria* natively ranges in Africa and parts of Asia, the desert locust *Schistocerca gregaria* in Africa, the Middle East and Asia. Animals for this thesis were obtained from commercial suppliers in Friedrichsdorf and Frankfurt/Main, Germany. They were kept at room temperature with an additional heating lamp and normal air humidity. Lettuce, wheat sprouts, oat flakes, fish food and fresh water were provided.

Selbstständigkeitserklärung

Ich erkläre hiermit, dass ich die Doktorarbeit selbstständig verfasst und keine anderen als die angegebene Quellen und Hilfsmittel benutzt habe.

Frankfurt am Main, den 08. Dezember 2014