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The chemistry of an insect ear: ionic composition of a liquid-filled ear and haemolymphs of Neotropical katydids

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The purpose of this study is to examine and to compare the ionic composition of the haemolymph and the ear fluid of seven species of katydids (Orthoptera: Tettigoniidae) with the aim of providing from a biochemical perspective a preliminary assessment for an insect liquid contained in the auditory organ of katydids with a hearing mechanism reminiscent of that found in vertebrates. A multi-element trace analysis by inductively coupled plasma optical-emission spectrometry was run for 16 elements for the ear liquid of seven species and the haemolymph of six of them. Based on the obtained results, it can be recognized that the ionic composition is variable among the studied insects, but sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) are the most prominent of the dissolved inorganic cations. However, the ion concentrations between the two fluids are considerably different and the absence or low concentration of Ca^{2+} is a noticeable feature in the inner ear liquid. A potential relationship between the male courtship song peak frequency and the total ion (Na^+, K^+, Mg^{2+} and Ca^{2+}) concentration of the inner ear liquid is also reported.

1. Introduction

Acoustic perception involves the mechanical transformation of sound into neural signals [1]. In vertebrates, this mechano-electrical transduction process takes place in the inner ear, where the auditory receptor cells are immersed in a specialized fluid environment that enables and sustains an adequate sensory function [2]. The inner ear is integrated by an osseous outer wall (otic capsule) and an internal membranous labyrinth [3]. The otic capsule and labyrinth are separated by a liquid medium called perilymph which is presumed to be a derivate of blood plasma [4] or cerebrospinal fluid [5]. The membranous labyrinth itself is filled with endolymph, a solution with a unique ionic composition comparable to no other body fluid [6].

Hearing systems with tympanal membranes are conditioned to match the internal space of the membrane to the acoustic impedance of the external medium in which the sound waves are being dispersed [7]. In insects, such internal space is obtained with a modified trachea or an air sac opposed to the tympanum [8–11]. The auditory sensilla of insect tympanic organs could be directly attached to the tympanic membrane as in locusts [12] and moths [13], where a middle ear component seems unnecessary, partially attached as in praying mantis [14], or separated from the tympanic membrane as in katydids, where a middle ear step is necessary for impedance conversion [15]. In all cases, the auditory neurons are surrounded by some kind of extracellular fluid medium (e.g. [16–18]). It is possible that different portions of the neuron may be surrounded by different fluids. In many katydids, these fluids are confined into an enclosed cavity known as the auditory vesicle (AV) [19] and within the smaller volume of the scolopale cells [8].

The hearing organ in katydids anatomically derives from a set of stretch receptors known as chordotonal organs [20,21]. Like other chordotonal organs,

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the auditory receptor or *crista acustica* [8,22] is composed of multicellular units called scolopidia that sit on the acoustic trachea, and the cap cells are embedded within a 'tectorial membrane' [7,20]. The scolopidia are arranged by size, with the smallest taking place at the distal end of the tibia [23–25]. As mentioned above, the *crista acustica* is not attached directly to the tympanal membranes, but instead lies directly on top of the acoustic trachea.

As in most katydids, the haemolymph channel in the forelegs runs along of the dorsal surface of the acoustic trachea [26], and previous morphological descriptions of the auditory organ considered this to be bathed by haemolymph [11]. Based on anatomical studies of the hearing system of *C. gorgonensis*, Montealegre-Z *et al*. [15] observed that the haemolymph channel in this species was not continuous as had been suggested before, but instead, they found a fluid trapped in a cavity bounded by the presence of a colloidal material at each end of the auditory organ. This cavity is the AV, and it is considered to be an important element of the auditory system as it provides a medium for the propagation of mechanical travelling waves and influences the dynamics of the tympanal membranes [19,27]. A fluid-filled cavity appears to be a required condition for the function of auditory organs in some Neotropical katydids. In other ensiferans like the tree weta *Hemideina thoracica* [28] and the field cricket *Gryllus bimaculatus* [29], the auditory sensory organ is immersed in a fluid-filled channel, in the weta composed apparently of a new class of fatty acid. *Copiphora gorgonensis*, *Panacanthus pallicornis*, *Supersonus aequoreous* and *Sphagniana sphagnorum* are so far the only species in which the presence of the AV has been reported. It is believed that other species of Neotropical katydids might possess a similar structure [19], or at least that their hearing organ requires a fluid environment that enhances the transduction of mechanical signals to neural responses [30]. The AV is separated from the scolopidial organs by the tectorial membrane [26]. The only part of the scolopidium that protrudes out of the tectorial membrane and is in contact with the AV fluid is the cap cell; below the cap cell lies the scolopale cell that contains the dendric cilia embedded in a small volume of receptor lymph. Our analysis does not relate to this receptor lymph but to the fluid contained in the much larger volume of the AV.

Several aspects of the ion composition of insect haemolymph have been previously studied from samples usually extracted from the dorsal vessel in the abdomen [31–33]. Although nothing is known of the ionic composition of the katydid inner ear fluid, the work of [31] suggests a relative stability of the Ca^{2+}/Mg ratio in the insect blood and that insects seem to have developed a greater tolerance for changes in magnesium concentration than for changes in the Ca^{2+} concentrations in the haemolymph. To the best of our knowledge, this is not just the first study reporting the ion composition for the ear liquid of Neotropical katydids in the literature but also of any insect species. Therefore, in this study, the ionic composition and ion concentration levels are measured for haemolymph and the ear liquid across seven species with the purpose of providing for the first time the chemical characterization of the ear liquid observed in Neotropical katydids. This work on the chemical composition of the ear liquid can also help in understanding which chemical elements may have a direct contribution to the hearing process in insects.

2. Results

2.1. Haemolymph versus inner ear liquid

A multi-element trace analysis by inductively coupled plasma optical-emission spectrometry (ICP-OES) was run for 16 elements (Li, Na, K, Mg, Ca, Si, Ti, Fe, Co, Ni, Cu, Zn, P, S, Sr and Cd) for the ear liquid of seven species and the haemolymphs of six of them. The ionic composition of haemolymphs is variable among insects, but sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) are the most prominent cations of the dissolved inorganic elements [31,34]. These cations were chosen as focus elements with the purpose of comparing our results with those previously published for haemolymph's chemical composition in Arthropoda [35].

Figure 1 shows that Na^+ is the cation with the highest mean concentration values in both haemolymphs and inner ear liquid, followed by K^+ and Mg^{2+}. This quantitative distribution is comparable to what has been reported in previous studies [33,36], but a notable feature is the very low concentration to non-appearance of Ca^{2+} in the inner ear liquid. The appearance of Ca^{2+} in some of the samples (figure 1*b*,*d*,*e*) can be attributed to haemolymph contamination. It is probable that during the extraction of inner ear liquid, the piercing of the tympanal plate with the glass capillary suddenly changed the inner ear's pressure, thus withdrawing haemolymph from the surrounding tissue.

2.2. Inner ear liquid ion concentration

A one-way analysis of variance (ANOVA) was performed among species to compare ion mean concentration values. There was a stastically significant difference among species regarding Na^+ ($F_{6,63} = 4.069$, $p = 0.002$), K^+ ($F_{6,63} = 3.875$, $p = 0.002$) and Mg^{2+} ($F_{6,63} = 2.658$, $p = 0.023$). There was no statistically significant difference among the groups for Ca^{2+} mean concentration values ($F_{6,63} = 1.272$, $p = 0.283$).

Post hoc comparisons to evaluate pairwise differences among species ion mean concentration were conducted with the use of Games–Howell test since there was no homogenity of variances for the dataset. Tests indicated the following results (figure 2). Na^+ concentrations are low in *S. aequoreous* (mean ± s.e. = 11.72 ± 2.18) and significantly different from *C. vigorosa* ($t = 5.698$; $p = 0.001$) and *Ne. affinis* ($t = 4.641$; $p = 0.001$). *C. gorgonensis*' values were both low in Na^+ ions (mean ± s.e. = 18.94 ± 3.98) and significantly different from *C. vigorosa* ($t = 4.71$; $p = 0.001$) and *Ne. affinis* ($t = 4.038$; $p = 0.001$). High concentration of K^+ was observed in *P. pallicornis* (mean ± s.e. = 52.24 ± 26.98), while *C. vigorosa* (mean ± s.e. = 11.48 ± 2.59) presented mean values with significant differences from *Ph. poecila* ($t = 3.916$; $p = 0.001$) and *S. aequoreous* ($t = 4.266$; $p = 0.001$). Concerning Mg^{2+}, *P. pallicornis* exhibited significant differences when compared against *C. vigorosa* ($t = 4.038$; $p = 0.004$), *Ph. poecila* ($t = 5.335$; $p = 0.003$) and *S. aequoreus* ($t = 5.336$; $p = 0.003$). Likewise, the lowest mean values were recorded in *Ph. poecila* (mean ± s.e. = 0.38 ± 0.16), *S. aequoreous* (mean ± s.e. = 0.23 ± 0.02) and *C. vigorosa* (mean ± s.e. = 2.76 ± 1.02). Finally, as was mentioned before, Ca^{2+} was not detected in most of the species and for those with some presence of Ca^{2+}, the mean values were not significantly different from those of the rest of the species ($p > 0.05$).

Some of the high variability observed for each of the ions and species cannot entirely be attributed to methodological errors or contamination, as each of the measurements (in

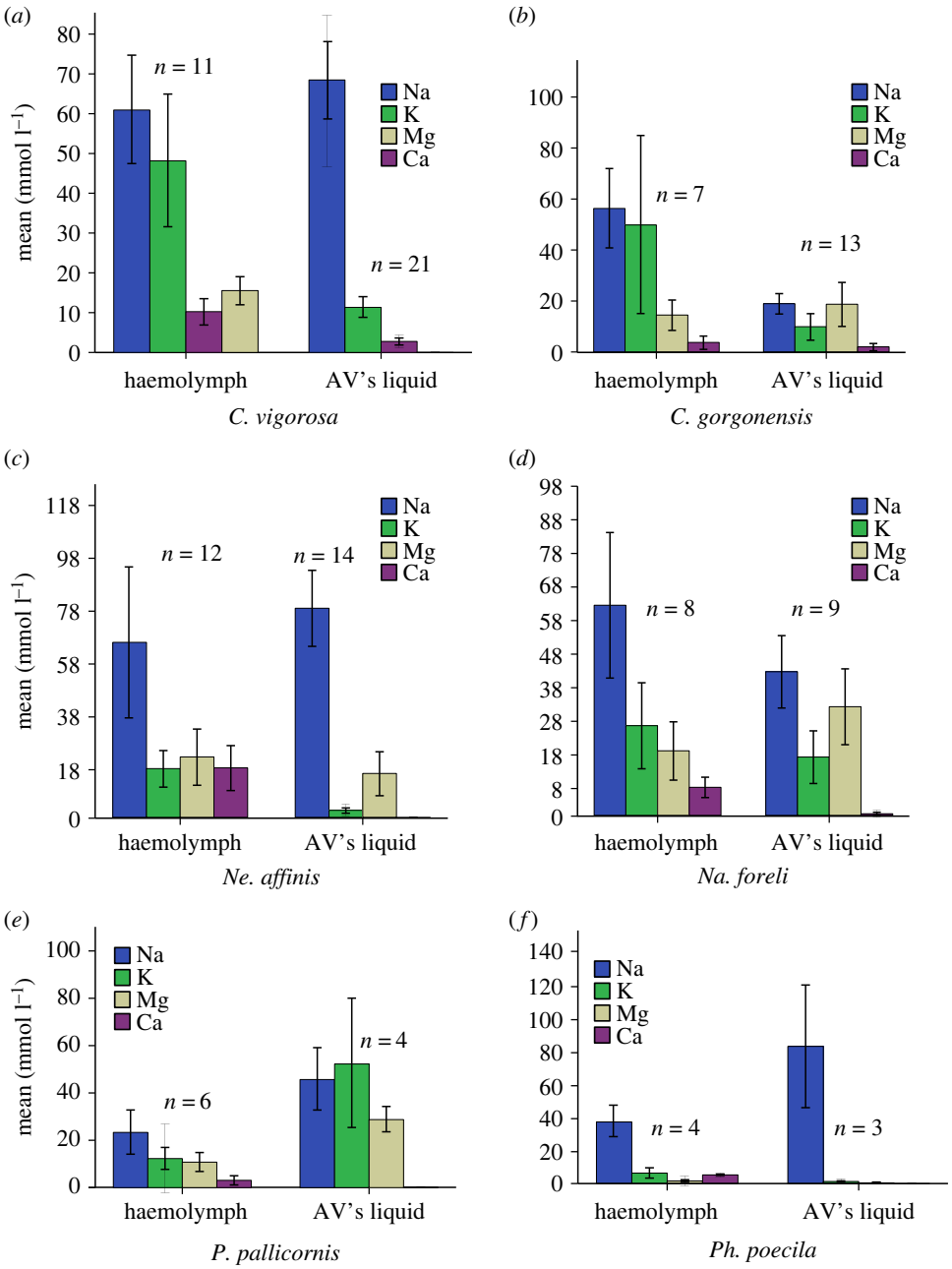

Figure 1. Comparison between haemolymphs and inner ear liquid mean concentration values in mmol l^{-1} (± s.e.) for six species, except *S. aequoreus*.

triplicate) offered a low %RSD (high precision) as ICP-OES is a very precise technique. This variability can be also attributed to the individuals' and species' natural variation in the concentration of certain ions. Given the limited number of samples in our study, individual changes in the ion concentration can produce high variability. A greater number of individuals could ameliorate this effect and offer more defined normal ranges for ionic composition of the different species.

2.3. Ca^{2+} in the inner ear liquid

Calcium is perhaps the most influential polyvalent ion found in living cells [31]. In insects, Ca^{2+} is involved in a diversity of physiological processes, e.g. it participates in intermediary metabolic processes, in locomotion and in neural signalling [34]. In view of the extremely low Ca^{2+} mean values obtained with the ICP-OES analysis for the liquid samples, average values and s.e. for the different individuals involved in this study were calculated. This allowed comparison of Ca^{2+} presence/absence and offer evidence of being a major factor in haemolymphs and inner ear liquid divergence. The results showed that in four species the inner ear liquid Ca^{2+} mean values were zero or very close to zero and very different from those obtained for haemolymph samples (table 1). However, for two species, *C. gorgonensis* and *P. pallicornis*, the averages and errors obtained did not allow us to establish a clear difference.

2.4. Ion concentration and calling song carrier frequency

A Pearson product-moment correlation coefficient was calculated to assess the relationship between ion concentration (inner ear liquid and haemolymph samples) and male song carrier frequency. There was a significant inverse relationship between inner ear liquid total sum of the ion mean concentration and carrier frequency ($r = -0.801$, $n = 71$, $p = 0.007$; figure 3*a*). By contrast, there was not a significant relationship

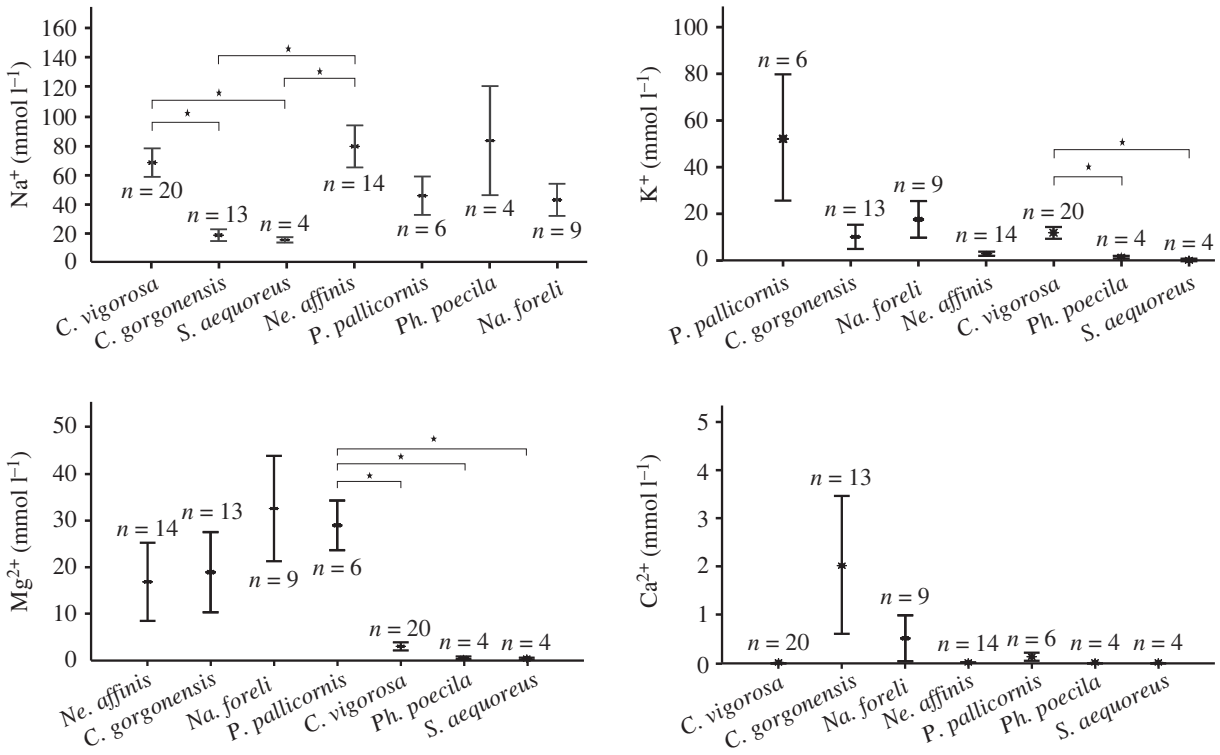

Figure 2. Inner ear liquid mean ion concentration values in mmol l^{-1} (± s.e.) for the seven studied species. Asterisk (*) indicates significant differences with a 95% confidence level ($p > 0.05$).

Table 1. Ca^{2+} mean concentration values in haemolymphs and inner ear liquid for the different species under study.

species	liquid mean ± s.e.	haemolymph mean ± s.e.
C. vigorosa	0	15.55 ± 3.52
C. gorgonensis	2.01 ± 1.43	3.74 ± 2.59
Na. foreli	0.48 ± 0.48	8.37 ± 3.03
Ne. affinis	0	18.73 ± 8.49
P. pallicornis	0.12 ± 0.08	3.07 ± 1.93
Ph. Poecila	0	5.35 ± 0.33

between the haemolymph ion concentration and the male calling song peak frequency ($r = -0.187$, $n = 47$, $p = 0.209$; figure 3*b*).

3. Discussion

3.1. Ion concentration

Taking into account that the most recent study for haemolymph ion concentration in Orthoptera was provided approximately 70 years ago [31–33], large differences in ionic concentration values from a diversity of species have been reported and there is great variability in results obtained even for a single species [37]. Therefore, the concentration of inorganic cations in the haemolymph fluctuates strongly within taxa and it is also dependent of the developmental stage [38], making it difficult to establish a reference range from the available literature. The observed variability could be related to differences in techniques and not necessarily to differences across species. Although there is not an established reference range for inorganic ion concentration values, it has been considered that for this taxonomic group (Orthoptera), there is a tendency for a relatively high Na^+:K^+ ratio (greater than 9) while the Mg^{2+}:Ca^{2+} ratio is about 1 [33,34], and this condition is believed to represent an early evolutionary stage [35]. The haemolymph's mean values for Na^+, K^+, Mg^{2+} and Ca^{2+} obtained for the six species used in this analysis do not follow this tendency suggested for the order Orthoptera. As seen in figure 1, a trend showing greater concentration of ions of Na^+ and K^+ is observed, while mean values for Mg^{2+} and Ca^{2+} ions are close to the data range observed in the literature [34,38,39].

The obtained analytical results confirm that the ion compositions of the inner ear liquid and haemolymph samples were mainly constituted by Na^+, K^+, Mg^{2+} and Ca^{2+} (figure 1); however, their concentration varies between the two solutions. A factor contributing to the variation between haemolymph and inner ear liquid concentration is the possible binding of these ions to other dissolved molecules such as proteins, carbohydrates or nucleic acids [40]. It was observed in *Periplaneta americana* that haemolymphs sampled from different body parts of the same specimen showed an unequal distribution of Na^+, K^+ and Ca^{2+} [37]. Since inorganic ions circulate rather without restrictions in an aqueous medium, it is likely that the binding of slowly diffusing macromolecules in the haemolymphs to inorganic ions enhances their distribution [40]. In the case of katydid ear with a fluid-filled cavity or AV, the contained liquid is separated from the haemolymphs circulating through the tibia [15]. This physical isolation potentially filters out particles and determines the presence of certain molecules in the inner ear liquid and consequently the local concentrations of ions. In the haemolymph, Ca^{2+} is associated with proteins, derived proteins from metabolic processes, and other organic

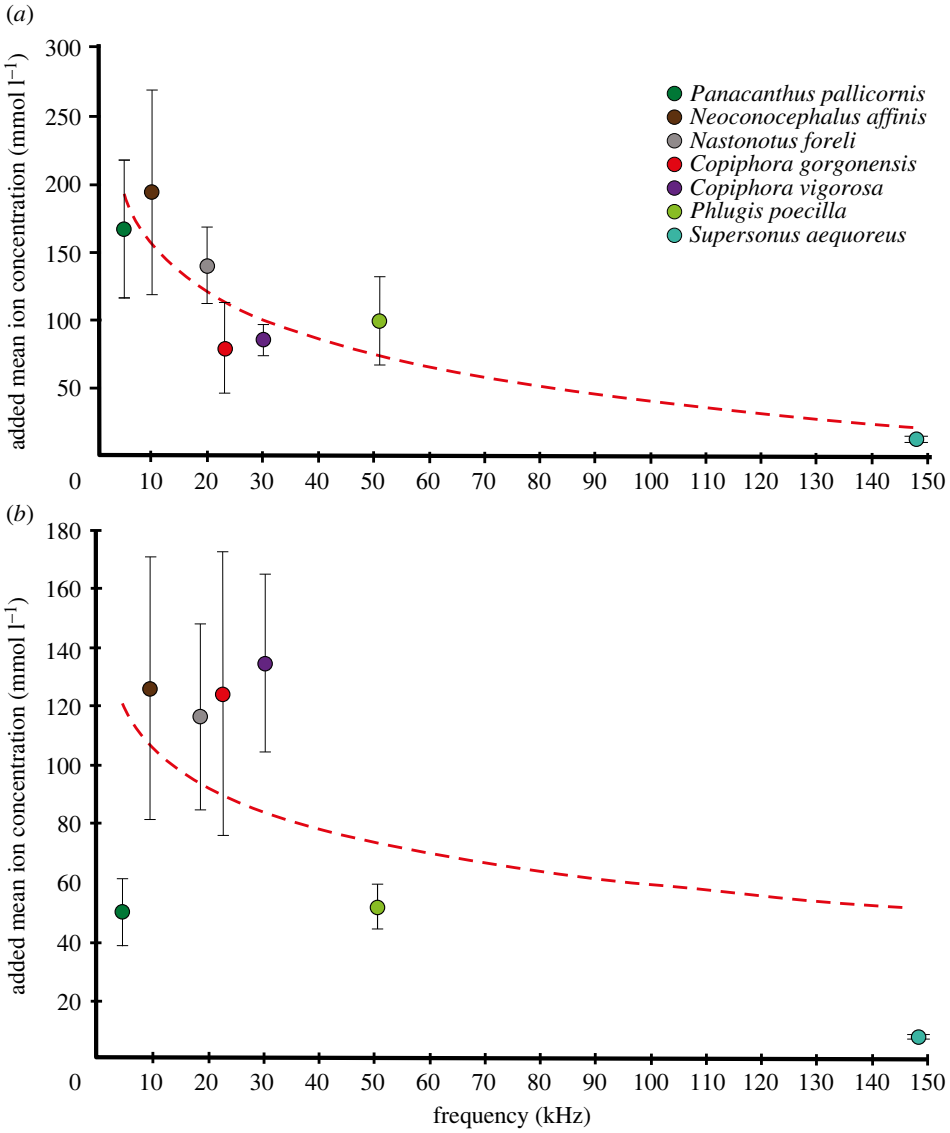

Figure 3. Relationship between added ion mean concentration and carrier frequency of male song. (*a*) Inner ear liquid combined ion mean values (Na^+, K^+, Mg^{2+} and Ca^{2+}). (*b*) Haemolymph combined ion mean values (Na^+, K^+, Mg^{2+} and Ca^{2+}).

compounds [31], while its low concentration in most of the inner ear liquid samples (table 1) could be explained by the unavailability of those organic compounds in the inner ear. Low concentration of Ca^{2+} is also a common feature in the mammalian cochlear fluids, with Ca^{2+} concentration values in the endolymph of 0.02 mM, and 0.2 mM for the perilymph [3,6], whereas in the serum on blood plasma Ca^{2+} concentration is around 1.16 mM [41].

3.2. Inner ear liquid compartmentalization and biomechanical effects

In insects, the haemolymph ion concentration varies significantly among individuals and also among the different body parts of the same specimen [37]. Ion concentration is affected by diet, water content and the heterogeneous flow of the haemolymph through the body [35,40]. If the inner ear liquid is considered as being a continuation of the haemolymph channel, its ionic concentration could be susceptible to fluctuations, just as occurs in other body regions. If this is the case, sudden variations in ion content might have negative effects on the auditory response by increasing or reducing its sensitivity. Therefore, an interesting hypothesis to test in the future is that a constant ion concentration in katydids can be reached by means of physical separation of the inner ear liquid from the haemolymph.

From the biomechanical perspective, the containment of the inner ear liquid effectively makes it an incompressible fluid. Thus, this feature has an effect on the movement of the auditory sensilla embedded in the liquid. A similar condition takes place in the inner ear of mammals, where the cochlear fluid is restricted by the cochlea's osseous walls [42]. In addition, endolymph and perilymph pressures are maintained equally [43,44] by highly compliant membranes bounding the endolymphatic space [45]. It is believed that the incompressibility of the perilymph is crucial for the mechanical movement of the basilar membrane and the opening of ion channels of the outer hair cells [46]. The fluid mass affects the dynamics of the basilar membrane, loading its different parts by amounts that depend upon the local wavelength [47,48]. Although poorly understood, katydid ears with reported AV might be endowed with a softer enclosure than the vertebrate cochlea. This enclosure is provided by the dorsal cuticle and surrounding structure such as the tympanal membranes, acoustic trachea and the colloidal material at each end of the inner ear cavity [15,27].

A stable and specific viscosity represents another advantage of the ear liquid isolation. However, in this study, we did not measure the viscosity. This physical property is greatly determined by the chemical and ionic composition of substances dissolved in a solution [49], and it has effects on physiological processes. For instance, studies on cochlear models show that an increase in endolymph viscosity causes a decrease in basilar membrane motion [50], and the hearing sensitivity is thus reduced [51]. In another model, the generation of standing waves was dependent of the fluid viscosity [52]. In the same manner, a specific viscosity of the ear liquid in katydids could be an adding factor for the optimal response of the hearing organ, and this could be regulated by the ionic composition and in particular by the concentration of Ca^{2+} in the ear liquid. As has been observed in cells, low amounts of calcium in the protoplasm reduce its viscosity, and high quantities of Ca^{2+} cause a gelling reaction [53]. Whether the observed low concentration of Ca^{2+} in the inner ear liquid is an adaptation for the auditory response in katydids remains to be investigated. Fluid dynamics studies should help to understand better this phenomenon.

3.3. Male calling song peak frequency and ion concentration

As mentioned above, ion concentration has an effect on propagation of the travelling wave if the medium density and viscosity are considered [54]. As can be seen in figure 3*a*, there is a relationship between the male calling song peak frequency and the total ion (Na^+, K^+, Mg^{2+} and Ca^{2+}) concentration of the inner ear liquid. However, there is a lack of experimental data to elucidate how a specific concentration of ions is related to the propagation of travelling waves carrying information from acoustic energy.

4. Conclusion

Based on the obtained analytical chemistry results, it can be assumed that the inner ear liquid is mainly composed of the same ions that have been reported for the haemolymph. However, the ion concentrations between the two fluids are different and the absence of Ca^{2+} is a noticeable feature in the inner ear liquid. Although the analytical technique chosen for the analysis provided information regarding the elemental composition, a more detailed chemical characterization is still required for the identification of other compounds or macromolecules such as proteins, carbohydrates or lipids. A more comprehensive description of the ear liquid could shed light on the lipidic nature suggested by Montealegre-Z *et al*. [15], taking into account that a lipid-filled cavity has been reported for the ear of the tree weta *Hemideina* sp. [28]. Also, in this work evidence that there is a relationship between the male courtship song peak frequency and the total ion (Na^+, K^+, Mg^{2+} and Ca^{2+}) concentration of the inner ear liquid has been shown. Interestingly, while the Ensifera species reported to have AV exhibit tympanal flaps or pinnae, those reported to show a continuous haemolymph channel exhibit naked or exposed tympana. This deserves more investigation, and the next step should involve non-invasive methods to measure fluid dynamics.

Table 2. Studied species and their corresponding mating song peak frequency.

species	*n*	song peak frequency (kHz)
Copiphora vigorosa	21	30
Copiphora gorgonensis	13	23
Nastonotus foreli	9	19
Neoconocephalus affinis	14	10
Panacanthus pallicornis	6	5
Phlugis poecila	4	51
Supersonus aequoreous	4	148

5. Methods

5.1. Inner ear liquid extraction

For the chemical analysis of the liquid contained in the inner ear, seven species of katydid were sampled (table 2). Inner ear fluid was extracted from live specimens maintained in colonies as described in [55]. Specimens were placed on a platform made out of cork (5 cm × 2 cm) and gently restrained with staple clamps over the legs and the abdomen, while the front legs were held to the front by a brass wire (figure 4). Once the insect was immobilized, the external cuticle of the tympanal slit was excised to gain visual and mechanical access to the tympanal membranes (figure 4*b*), with the exception of *Phlugis poecila* in which the intact tympanal slits allowed adequate access. The excisions were done using small pieces of stainless-steel double-edge razor blades placed in a blade holder. In order to avoid any contamination from the bleeding to surrounding areas, small pieces of tissue paper were used to dry the ablated area until the bleeding stopped by coagulation.

The liquid was extracted by capillarity action using glass microcapillaries, which were pulled from borosilicate glass tubing (external diameter: 1.0 mm, internal diameter: 0.8 mm; B120-69-8, Linton Instruments, Norfolk, UK) using a micropipette puller (P30; Sutter Instruments, Novato, CA, USA) to produce tips with a width of approximately 10 µm. The microcapillary was mounted in a micromanipulator for precise movement of the tip through the tympanal plate into the fluid-filled area beneath at the proposed position of the inner ear (figure 4). With this method, it was possible to extract samples of approximately 0.20–0.50 µl of liquid per ear. This technique also allowed the extraction of fluid without affecting the integrity of the auditory organ and reducing (not completely eliminating) contamination from other body fluids. Extracted liquid volume was measured by injecting example samples into oil and measuring the diameter of the resultant spherical drop.

As a control, haemolymph samples were also extracted from the coxa of the hind leg of the same specimen employing the same procedure as with the ears. The coxa of the hind leg was chosen in order to ensure that the haemolymph sample was not associated with the ear liquid. The ear and haemolymph samples were deposited in a 0.5 ml polypropylene tube (Brand®, Sigma-Aldrich, Dorset, UK) containing 20 µl of ethyl acetate (Sigma-Aldrich, Dorset, UK). Ethyl acetate was used as a preservative solvent, since it does not react with the water contained in the samples. All the samples were kept in a freezer at approximately −20°C.

5.2. Inductively coupled plasma optical-emission spectrometry

ICP-OES was used as the analytical method for the study of the elemental composition of the inner ear liquid. This analytical

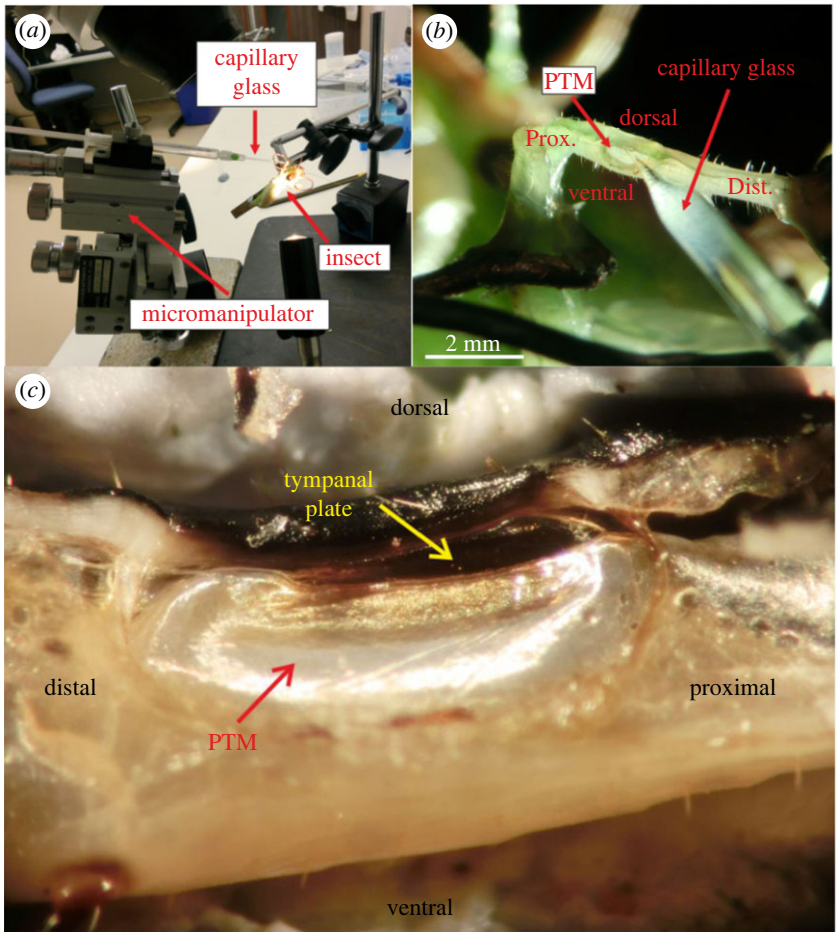

Figure 4. Mounting of specimen and close-up of the insect's ear. (*a*) Specimen mounted for inner ear liquid extraction. (*b*) Lateral view of the right leg. Once the posterior tympanal membrane (PTM) is exposed and the inner ear identified, the tip of the capillary glass is inserted through the tympanal plate. Proximal (Prox.) and distal (Dist.). (*c*) Close-up of the posterior tympanal membrane and tympanal plate of *Nastonotus foreli*.

technique involves emission spectroscopy that uses an inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at wavelengths characteristic of a particular element [56]. This is accomplished by ionizing the sample with inductively coupled plasma and then using the spectral emission of the different elements to identify and quantify the ions [57].

For the analysis, an ICP-OES Thermo Scientific™ iCAP™ 7000 ICP-OES Analyser (Thermo Scientific, UK) was used for quantitative/qualitative elemental analysis of the ear cavity samples. The ICP-OES was connected to a CETAC® ASX260 auto-sampler with nine standard racks and two sets of sample trays each with 60 sample racks. All analyses were performed using axial mode as it offers a greater sensitivity at low concentrations. The following elements were selected for analysis using the specified wavelength in brackets [nm]: lithium [670.784], sodium Na [589.592], potassium K [766.490], magnesium Mg [279.553], calcium Ca [393.366], titanium Ti [334.941], iron Fe [259.940], phosphorus P [177.495] and sulfur S [180.731]. Elements like titanium were selected as a marker to assess any potential external contamination as it is present in soil dust and other surfaces but unlikely to be found in biological samples. None of the samples analysed showed any traces of titanium, confirming the clean nature of the sampling method. Samples were first diluted in 1 ml of deionized water, then vortexed for one minute to ensure obtaining a homogeneous sample. Following this procedure, the vortexed sample was added to a test tube with 3 ml of deionized water and loaded onto an auto-sampler tray. As a control, a reagent blank (deionized water) was used in between samples and a multi-element standard solution, a periodic table mix certified reference material of elemental standards (Fluka Analytical,Sigma-Aldrich GmbH, Switzerland) was used to calibrate the concentration of ions present in the samples. Liquid flow rates were controlled with a peristaltic pump, with sample flow at 1 ml min^{-1}. A standard data acquisition run was implemented for the analysis of 110 samples, with three replicates per sample. The plasma used was generated using a flow of argon gas. Data were acquired using the software supplied by the manufacturer.

5.3. Data analysis

After all the raw data were collected, a preliminary data treatment was necessary for calculating the concentration in mmol l^{-1} of the elements present in the samples. For this, the data from the only distilled water samples (blanks) were subtracted from those of the samples because they represented values already present in the solvent and those added by contamination. Sample contamination is imposed by different sources in the laboratory, among them, the need of diluting sample solutions prior to the analysis and by the equipment itself. Element concentrations were calculated using a calibration curve from standard solutions. Those elements found at very low concentrations (close to zero) and unlikely to contribute to the chemical profile were discarded.

Graphical representations of the means and s.e. were generated for ion concentration of haemolymph and inner ear liquid samples. An independent *t*-test was applied to test the difference between haemolymph and inner ear liquid Ca^{2+} mean concentration values. To compare the mean ion concentration among species, an ANOVA was performed. A *post hoc* comparison using Games–Howell test was applied to establish which pairs of groups were significantly different. The relationship

between male courtship song peak frequency and inner ear liquid ion concentration was tested with a Pearson correlation. The statistical analyses were performed by using SPSS Version 23 (IBM Corp., Armonk, NY), and all *p*-values were two-sided. Values of *p* of less than 0.05 were considered to indicate statistical significance.

Ethics. College of Science Research Ethics Committee (COSREC), University of Lincoln granted permission to conduct this research under number COSREC-2014-02 and authorized the participation of all researchers involved in this project. The Colombian Ministry of Environment granted a permit for fieldwork at Gorgona National Park (decree DTS0-G-31 11/2007 and decree DTS0-G-090 14/08/2014). All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Data accessibility. Curated data are available in figshare. The data are stored as an Excel file at http://dx.doi.org/10.6084/m9.figshare.23589651. The access pathway is https://figshare.com/s/c703d99bf73018a27200.

Authors' contributions. F.A.S.: conceptualization, data curation, formal analysis, investigation, methodology, software, validation, visualization and writing—original draft; F.M.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization and writing—review and editing; J.G.-R.: conceptualization, data curation, formal analysis, investigation, methodology, resources, supervision, validation, visualization and writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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