

RESEARCH ARTICLE

The simple ears of noctuid moths are tuned to the calls of their sympatric bat community

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SUMMARY

Insects with bat-detecting ears are ideal animals for investigating sensory system adaptations to predator cues. Noctuid moths have two auditory receptors (A1 and A2) sensitive to the ultrasonic echolocation calls of insectivorous bats. Larger moths are detected at greater distances by bats than smaller moths. Larger moths also have lower A1 best thresholds, allowing them to detect bats at greater distances and possibly compensating for their increased conspicuousness. Interestingly, the sound frequency at the lowest threshold is lower in larger than in smaller moths, suggesting that the relationship between threshold and size might vary across frequencies used by different bat species. Here, we demonstrate that the relationships between threshold and size in moths were only significant at some frequencies, and these frequencies differed between three locations (UK, Canada and Denmark). The relationships were more likely to be significant at call frequencies used by proportionately more bat species in the moths' specific bat community, suggesting an association between the tuning of moth ears and the cues provided by sympatric predators. Additionally, we found that the best threshold and best frequency of the less sensitive A2 receptor are also related to size, and that these relationships hold when controlling for evolutionary relationships. The slopes of best threshold versus size differ, however, such that the difference in threshold between A1 and A2 is greater for larger than for smaller moths. The shorter time from A1 to A2 excitation in smaller than in larger moths could potentially compensate for shorter absolute detection distances in smaller moths.

Key words: sensory ecology, constant-frequency echolocation, horseshoe bats, predator–prey interactions, allotonic frequency hypothesis.

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INTRODUCTION

Animals obtain information about their environment *via* their sensory systems, including information about the presence of predators. To survive, animals need to detect a variety of predators, and natural selection should result in sensory systems that are adapted to encode cues of the specific predators in the sympatric community. Bats and their insect prey provide an ideal study system to investigate sensory system adaptations. Insectivorous bats are significant predators of night-flying moths (reviewed in Fullard, 1998). These bats produce ultrasonic calls during echolocation to navigate their surroundings and locate their prey, and ultrasound-sensitive ears have evolved in several groups of moths specifically to detect bats (Fullard, 1998). Depending on the family, moths have only one to four receptor cells in their ears, making these ears some of the simplest sensory structures in nature (Yack, 2004). Roeder (Roeder, 1962) observed that many moths have a bimodal escape response: they show directional flight away from quiet ultrasound, i.e. a distant bat, and erratic flight in response to loud ultrasound, i.e. a close bat. Different bat species produce echolocation calls at different frequencies, and the question originally raised by Fullard (Fullard, 1998) as to what extent can the simple ears of moths adapt to the specific frequencies used by different sympatric predators, still remains. Here we address this question by investigating moth

hearing in three different areas (UK, Canada and Denmark), which are separated geographically by ~1000 km to more than 5000 km.

Except for a single family, the Notodontidae, with only a single sensory cell per ear, moths from the superfamily Noctuoidea have two auditory receptor cells, called A1 and A2, in each of their two ears. The frequency tuning curves of these two cells are roughly the same (Fullard, 1998). Both are most sensitive to lower ultrasonic frequencies (~20–50 kHz), but A2 has thresholds ~20 dB greater than A1. Because the shape of the A1 and A2 frequency tuning curves are so similar, moths are tone-deaf; that is, they cannot discriminate between different frequencies (reviewed in Miller and Surlykke, 2001). Moth species vary greatly in size, and larger moths should be more conspicuous to bats because they provide a larger reflective surface for echolocation calls and thus a greater target strength, which will enable echolocating bats to detect them at greater distances than small moths (Surlykke et al., 1999). Surlykke et al. (Surlykke et al., 1999) found that larger moths have lower A1 thresholds for ultrasound than smaller moths, which could compensate for their increased conspicuousness to bats by enabling them to also detect bats at greater distances. Surlykke et al. (Surlykke et al., 1999) thus demonstrated that the auditory systems of individual moth species reflect their risk of predation by bats. In addition, Surlykke et al. (Surlykke et al., 1999) found size-dependent

differences in the best frequency, with larger moths being most sensitive to lower frequencies and smaller moths most sensitive to higher frequencies.

At the community level, studies have shown that the general tuning of moth ears reflects the diversity of echolocation call frequencies across the bat community in which they live (reviewed in Fullard, 1998; Ratcliffe, 2009). For example, in tropical communities with high bat species diversity and a very wide range of echolocation frequencies, moth species are sensitive to a broader range of frequencies than in temperate zones, where bat echolocation assemblages span a narrower range of frequencies (Fullard, 1982). In addition, the presence of specific bat species may also influence the hearing of sympatric moths. Fullard (Fullard, 1984) showed that the hearing of Hawaiian moths reflected the echolocation frequency of the single bat species on Hawaii. Jacobs et al. (Jacobs et al., 2008) found an increase in auditory sensitivity for two moth species at the high frequencies (above *ca.* 80 kHz) emitted by sympatric constant-frequency horseshoe bats. Horseshoe bats tend to use higher echolocation frequencies for their size than other insectivorous bats (Jones, 1999). Thus, we hypothesized that the presence of horseshoe bats, which are known to prey heavily on moths (reviewed in Jacobs et al., 2008), should influence the hearing of sympatric moths by selecting for better sensitivity at high frequencies. In tropical areas, this might be difficult to determine because of the general high diversity of bats. In temperate areas, however, most bats echolocate between 20 and 60 kHz, and thus improved sensitivity to high frequencies in moths would stand out in comparison to areas without horseshoe bats.

Based on this assumption, our main objective in this study was to determine whether the tuning of the simple ears of noctuid moths reflects the echolocation frequencies used by sympatric bat species at a community level. In their comparisons of relative detection distances for bats and moths, Surlykke et al. (Surlykke et al., 1999) only considered a bat emitting calls at the moth's best frequency, but here we take into consideration moth auditory thresholds at the different bats' peak frequencies (frequency with most energy in a bat species' search phase calls). Hence, our first goal was to determine the relationships between moth size and hearing threshold at fixed call frequencies to test whether these relationships reflect the specific call frequencies used by the moths' sympatric community of bat predators. We determined hearing thresholds of moths in Bristol, UK, a temperate area with horseshoe bats, and compared the data with those from Odense, Denmark, and Chaffey's

Locks, Ontario, Canada (Surlykke et al., 1999), two different temperate geographic locations that lack horseshoe bats. In contrast to Surlykke et al. (Surlykke et al., 1999), we did not base our analysis on the best frequency of the moths, but tested the hypothesis that the relationship between A1 sensitivity and size is significant at frequencies used by sympatric bat species. In particular, we tested whether A-cell sensitivity of moths in the UK reflects the presence of high-frequency moth specialists [*Rhinolophus ferrumequinum* calling at 82 kHz and *R. hipposideros* calling at 109 kHz (Vaughan et al., 1997)], which are not present in Denmark or Canada.

Based on a few studies (e.g. Surlykke and Miller, 1982; Fullard, 1998) it is assumed that the threshold curve of A2 follows A1 quite accurately (*ca.* 20 dB greater), but neither fine details in the relative thresholds nor the relationship across many moth species have ever been determined. A2 has been proposed to elicit close range evasive behaviour (Roeder, 1974). To analyse this and to better understand bat-moth interactions, it is important to know when A2 is activated during a bat attack. Thus, the second goal of our study was to test the hypothesis that the same relationships between best threshold, best frequency and size (surface area) exist for the A2 as for the A1 receptor and to test that these relationships remain significant after controlling for the shared evolutionary histories of the moth species considered. To this end, we determined not only A1 but also A2 thresholds for those moths tested in the bat-moth community of the UK.

MATERIALS AND METHODS

Size and auditory thresholds in UK moths

Thirteen species from 13 genera of moths were collected in Bristol, UK (Table 1). Twelve of these species belong to the family Noctuidae, and one to the family Lymantriidae. Moths in the family Lymantriidae have ears identical to those in the family Noctuidae and fall within the family Noctuidae according to a recent molecular phylogeny of moths (Mitchell et al., 2006). We collected data from both male and female moths; studies show that there is no difference in neural auditory thresholds between the sexes for most moth species in the superfamily Noctuoidea (Fullard, 2006; Fullard et al., 2007; Skals and Surlykke, 1999; Skals and Surlykke, 2000; Surlykke and Gogala, 1986; Surlykke and Treat, 1995). Moths were dissected as in Surlykke et al. (Surlykke et al., 1999), but without decapitation, to expose the auditory nerve, which was hooked with an extracellular electrode. An indifferent electrode was placed in the abdomen. The moth was placed in a Faraday cage lined with sound attenuating

Table 1. Surface area and auditory receptor characteristics for 13 UK moth species

Moth species	Surface area (mm ²)	Best frequency (kHz)		Best threshold (dB peSPL)		
		A1	A2	A1	A2	
Lymantriidae	<i>Lymantria monacha</i>	505.9±6.8 (3)	25	20	55.9±0 (2)	72.9±1.0 (2)
Noctuidae						
Acronictinae	<i>Acronicta megacephala</i>	419.9±0 (1)	30	30	44.9±1.3 (4)	63.9±1.4 (4)
Amphipyriinae	<i>Amphipyra pyramidea</i>	742.6±31.4 (7)	20	20	35.5±2.6 (6)	57.2±2.3 (6)
Bryophilinae	<i>Cryphia domestica</i>	146.6±11.2 (4)	30	30	53.2±2.4 (3)	71.2±1.3 (3)
Noctuinae	<i>Agrotis exclamationis</i>	404.7±9.3 (10)	20	20	37.2±0.8 (9)	56.1±1.3 (9)
	<i>Apamea monoglypha</i>	630.8±17.5 (10)	25	20	39.4±1.6 (9)	61.0±1.7 (9)
	<i>Noctua pronuba</i>	841.0±36.1 (11)	15	15	35.1±1.7 (10)	55.1±2.3 (10)
	<i>Ochropleura plecta</i>	242.9±5.3 (10)	25	30	47.1±2.0 (8)	64.9±0.8 (8)
	<i>Orthosia gothica</i>	358.8±33.1 (5)	30	30	42.9±1.0 (2)	64.9±3.0 (2)
	<i>Xestia c-nigrum</i>	387.4±15.7 (7)	25	25	41.1±2.5 (5)	60.7±1.4 (5)
Pantheinae	<i>Colocasia coryli</i>	340.4±21.7 (5)	30	35	48.9±3.0 (2)	64.9±7.0 (2)
Plusiinae	<i>Abrostola tripartita</i>	306.7±11.9 (7)	30	30	55.2±8.5 (3)	69.2±8.8 (3)
	<i>Autographa gamma</i>	474.9±16.8 (7)	20	20	31.5±1.6 (6)	52.5±2.7 (6)

Values are means ± s.e.m. (N), except for best frequency, which is the frequency of the best threshold for the mean threshold values of each species.

foam. A loudspeaker (ScanSpeak speaker 60102, Avisoft Bioacoustics, Berlin, Germany) was directed at the moth's ear from 30 cm away. Sound stimuli were broadcast using Avisoft Recorder software *via* a data acquisition board (USB-6251, National Instruments, Austin, TX, USA). Electrode signals were amplified (custom-built amplifier) and recorded (National Instruments USB-6251, Avisoft Recorder software) to a computer.

Sound stimuli were series of 20 ms tone-pulses (plus 2 ms ramps) with a period of 500 ms increasing in 2 dB steps from 20 to 90 dB peak equivalent SPL [peSPL r.m.s., re. 20 μ Pa of a sinusoid with the same peak-to-peak-amplitude as the pulse (Burkard, 2006)]. Pulse-series at 16 frequencies (5 to 80 kHz in 5 kHz increments) were presented once each with frequencies in random order. A-cell threshold (A1 and A2) was defined as the lowest sound pressure level to elicit A-cell spikes, with spikes elicited for each pulse of increasing amplitude thereafter, a measure that yields good estimates of what intact moths hear at threshold (ter Hofstede et al., 2011). Sound stimuli used in the UK were twice as long (20 ms) as those used in Denmark and Canada (10 ms). This was a compromise because these data were collected for multiple purposes. Noctuid moths have lower A1 thresholds for longer duration pulses than for shorter duration pulses of the same frequency (Tougaard, 1998). Tougaard (Tougaard, 1998) measured A1 thresholds for sound pulses of different durations in two species of noctuid moths and found that A1 threshold decreased by 1.7 and 2.0 dB per doubling of duration in *Spodoptera littoralis* and *Noctua pronuba*, respectively. Therefore, we first measured UK thresholds with 20 ms pulses and then added 1.85 dB, the mean value for the two species measured in Tougaard (Tougaard, 1998), to the thresholds as a correction value for comparative analyses.

For each moth, we determined the best frequency (BF; frequency of lowest threshold across all frequencies), best threshold (BT; threshold at BF) and surface area (SA). We used the means across individuals within a species of BT, BF and SA in regression analyses, and we transformed SA to \log_{10} SA to achieve linearity. We used ImageJ software (National Institutes of Health, Bethesda, MD, USA) to measure moth SA from digital photographs of moth specimens with the wings completely spread (Table 1). Moth sample sizes for neural data ranged from two to 10 individuals per species (Table 1), representing typical sample sizes for these data in the literature. In addition, studies have found that five to six individuals per species provided sufficient statistical power to detect significant differences in thresholds between moth species (Fullard et al., 1997; Fullard, 2001; Muma and Fullard, 2004; Jacobs et al., 2008), most likely because of low variation in thresholds within each species (Table 1).

Linear regressions were run on both species-level data and phylogenetically independent contrasts (PICs), i.e. values for each variable (BT, BF and \log_{10} SA) that statistically control for the putative effects of evolutionary relationships between species. Although it is important to establish whether a relationship exists with the species-level data, it is also important to remember that species are not independent data points; some species are more closely related to each other than are others (Felsenstein, 1985). This violates an assumption of parametric statistical tests and could result in spurious correlations of traits based on shared ancestry. Therefore, it is important to validate species-level relationships by using a measure that removes the potentially confounding effect of shared evolutionary history among species (Felsenstein, 1985). Calculating and running statistical tests on PICs is a commonly used method for this, and detailed descriptions of this method are given in Harvey and Pagel (Harvey and Pagel, 1991) and Garland et al. (Garland et al., 1992). Sample sizes were 13 species for species-

level data and 11 species for the PICs (two genera were not in the phylogeny).

To calculate PICs, we first pruned a molecular phylogeny of noctuid moths (Mitchell et al., 2006) using Mesquite (Maddison and Maddison, 2010) to produce a new tree containing only our UK species while preserving branch lengths. We used this new phylogeny and COMPARE (Martins, 2004) to calculate PIC values for each variable for each species. Linear regressions on PICs were forced through the origin, a requirement explained in Garland et al. (Garland et al., 1992). Briefly, PICs are calculated by subtracting the values for the variables between sister taxa or nodes, but the direction of subtraction is arbitrary because equivalent phylogenies can be drawn in multiple ways. PICs for the independent variable are calculated first and this dictates the direction of subtraction for the dependent variable. However, for the particular cases in which the difference between the independent variable values for two species is zero, there is no information regarding the direction of subtraction. In these cases, the difference between the dependent variable values could be either the positive or negative value, and thus the expected value for the dependent variable difference is also zero.

Comparing the slopes of size and auditory threshold relationships across communities

Different bat species produce search-phase echolocation calls with different, species-characteristic peak frequencies (frequency with the most energy), and different locations vary in the species that comprise the local bat community. Therefore, we obtained audiograms of moths from the UK, Denmark and Canada and analysed the relationship between moth hearing threshold and size at fixed frequencies (in contrast to best frequency) and determined whether it differed between locations with different bat communities. Methods for measuring A1 thresholds and SA for 17 moth species in Denmark and 26 moth species in Canada (Surlykke et al., 1999) were similar to those described above for the UK moth species [for a detailed description of methods, specific species and sample sizes, see Surlykke et al. (Surlykke et al., 1999)]. We determined the relationship between moth size and threshold at 14 frequencies relevant to bat echolocation (15–80 kHz in 5 kHz increments) by running linear regressions of A1 threshold *versus* \log_{10} SA for moths at each location (UK, Denmark and Canada). We then compared the significance and slope of these relationships at each frequency with the number of bat species that produce calls with the greatest energy at that frequency in each community. The peak frequency of the echolocation calls produced when bat individuals are flying over open field in search of prey or are in transit between roosts were obtained from the literature for all the bat species in each location (Table 2).

RESULTS

Size and auditory thresholds in UK moths

BF decreased with increasing moth size for both the A1 and A2 receptor cells, and the slopes of both relationships were significant for species-level data (A1: $F_{1,11}=9.3$, $P=0.011$; A2: $F_{1,11}=14.2$, $P=0.003$; Fig. 1A), but only for A2 for the PICs (A1: $F_{1,9}=3.5$, $P=0.092$; A2: $F_{1,9}=23.6$, $P=0.001$). For both receptors, BT decreased significantly with increasing moth size, for both species-level data (A1: $F_{1,11}=6.8$, $P=0.024$; A2: $F_{1,11}=6.2$, $P=0.030$; Fig. 1B) and PICs (A1: $F_{1,9}=8.7$, $P=0.016$; A2: $F_{1,9}=5.6$, $P=0.042$). The slopes of these two regressions, however, were significantly different (two-tailed test for difference between two population regression coefficients, $t=3.638$, $P<0.01$). Specifically, A2 BT significantly increased with

Table 2. Mean peak frequencies from the literature for all the bat species in each location

Bat species	Location	Peak frequency (kHz)	Literature source
<i>Barbastella barbastellus</i>	UK	35	1,2,3
<i>Nyctalus leisleri</i>	UK	28	1,2,3,4
<i>Plecotus austriacus</i>	UK	28	2
<i>Rhinolophus ferrumequinum</i>	UK	81	1,2,3
<i>Rhinolophus hipposideros</i>	UK	109	1,2,3,5
<i>Eptesicus serotinus</i>	UK, D	30	1,2,3,4
<i>Myotis bechsteinii</i>	UK, D	57	1,2,3
<i>Myotis brandtii</i>	UK, D	53	1,2,3,5
<i>Myotis daubentonii</i>	UK, D	49	1,2,3,6
<i>Myotis mystacinus</i>	UK, D	51	1,2,3
<i>Myotis nattereri</i>	UK, D	56	1,2,3,5
<i>Nyctalus noctula</i>	UK, D	22	1,2,3,4
<i>Pipistrellus nathusii</i>	UK, D	40	1,2,4
<i>Pipistrellus pipistrellus</i>	UK, D	49	1,2,3,5
<i>Pipistrellus pygmaeus</i>	UK, D	54	1,2,3
<i>Plecotus auritus</i>	UK, D	45	1,2,3,5
<i>Myotis dasycneme</i>	D	42	6
<i>Vespertilio murinus</i>	D	25	2,4
<i>Eptesicus fuscus</i>	C	30	4
<i>Lasionycteris noctivagans</i>	C	28	4
<i>Lasiurus borealis</i>	C	38	4
<i>Lasiurus cinereus</i>	C	20	4
<i>Myotis leibii</i>	C	44	7
<i>Myotis lucifugus</i>	C	45	4
<i>Myotis septentrionalis</i>	C	59	7
<i>Pipistrellus (Perimyotis) subflavus</i>	C	44	8

UK, United Kingdom; D, Denmark; C, Canada.

Literature sources: [1] Vaughan et al., 1997; [2] Obrist et al., 2004; [3]

Parsons and Jones, 2000; [4] Bogdanowicz et al., 1999; [5] Waters and Jones, 1995; [6] Siemers et al., 2001; [7] Fenton and Bell, 1981; [8] MacDonald et al., 1994.

A1 BT for both species-level and PIC data, but with a slope of <1 (species-level data: $F_{1,11}=187.7$, $P<0.001$; PICs: $F_{1,9}=107.9$, $P<0.001$; Fig. 1C). This means that moth species with low A1 BTs, i.e. sensitive ears, have relatively large differences between the A1 and A2 cell thresholds ($\sim\Delta 20$ dB) and moth species with high A1 BTs, i.e. less sensitive ears, have relatively small differences between the A1 and A2 cell thresholds ($\sim\Delta 15$ dB). Because low A1 BTs are found in larger moths and high A1 BTs in smaller moths, this difference between A1 BT and A2 BT (Δ BT) should also increase with moth size, which was supported by a positive slope between moth size and Δ BT (species-level data: $F_{1,11}=3.5$, $P=0.089$; PICs: $F_{1,9}=7.5$, $P=0.023$; Fig. 1D). Thus, across all moth species, the largest moths had large differences between A1 and A2 BTs of ca. 20 dB, but this difference decreased to only ca. 15 dB in the smallest moths (Fig. 1D).

Next, we tested the relationships between moth size and auditory threshold across the entire experimental frequency range, i.e. irrespective of the moths' BF. We determined the relationships at individual frequencies (from 5 to 80 kHz) for both A1 and A2 from our UK moth sample. Fig. 2 shows the scatterplots and regression results for these relationships. At 5 kHz there was neither a clear difference between A1 and A2 threshold nor a strong relationship with size. This might be due to the steeply increasing sensitivity to higher frequencies for all moths, making it likely that the sensory cells are responding to even weak higher harmonics of the stimulus. Thus, we make no further reference to the results at 5 kHz. At low frequencies (10–20 kHz) there were significant and steep relationships between size and threshold, which follows from the

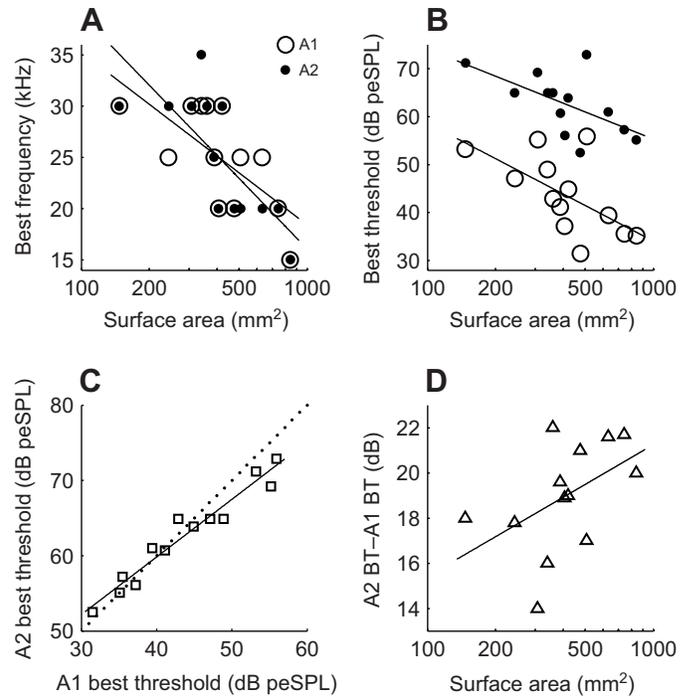


Fig. 1. Auditory characteristics of the A1 and A2 receptors in 13 species of noctuid moths from the UK: relationships between (A) best frequency and surface area, (B) best threshold and surface area, (C) A2 and A1 best threshold, and (D) the difference between A1 best threshold and A2 best threshold and surface area. \log_{10} (surface area) values were used to calculate linear regressions (solid lines). Large circles containing black dots in A are overlapping A1 and A2 data points. The dotted line in C provides an example of a line with a slope of 1 for comparison with the regression line.

general shape of the moth threshold curve; larger moths not only have lower thresholds in general, but also have their BF at these low frequencies. There were few significant relationships at intermediate frequencies (25 to 50–55 kHz), which again follows from the shape of typical threshold curves; the smaller the moth, the higher the BF (Fig. 1), and therefore this mid-frequency range compares thresholds above BF for large moths with thresholds at BF for smaller moths (Fig. 2A). Finally, at the highest frequencies (above 60–65 kHz) there was again a significant relationship between size and threshold, reflecting that these frequencies are above the BF for both large and small moths, where thresholds vary in smaller increments with frequency. In other words, at the low and high ends of our frequency spectrum, larger moths were more sensitive to sound than were smaller moths, but this relationship did not exist at intermediate frequencies. Both the A1 and A2 cell exhibit significant relationships between threshold and moth size at lower and higher frequencies, but the slopes for the A2 cell are always shallower and were significant at fewer frequencies than those of the A1 cell because, as shown by Fig. 2, the difference between A1 and A2 threshold is greater for larger moths than for smaller moths at all frequencies.

Comparing the slopes of size and auditory threshold relationships across communities

A1 and A2 audiograms of UK moths had the typical shape for noctuid moths and were similar in shape to those measured by Surlykke et al. (Surlykke et al., 1999) for moths in Canada and Denmark (Fig. 3A–C). Also, except for *Apamea monoglypha* showing some

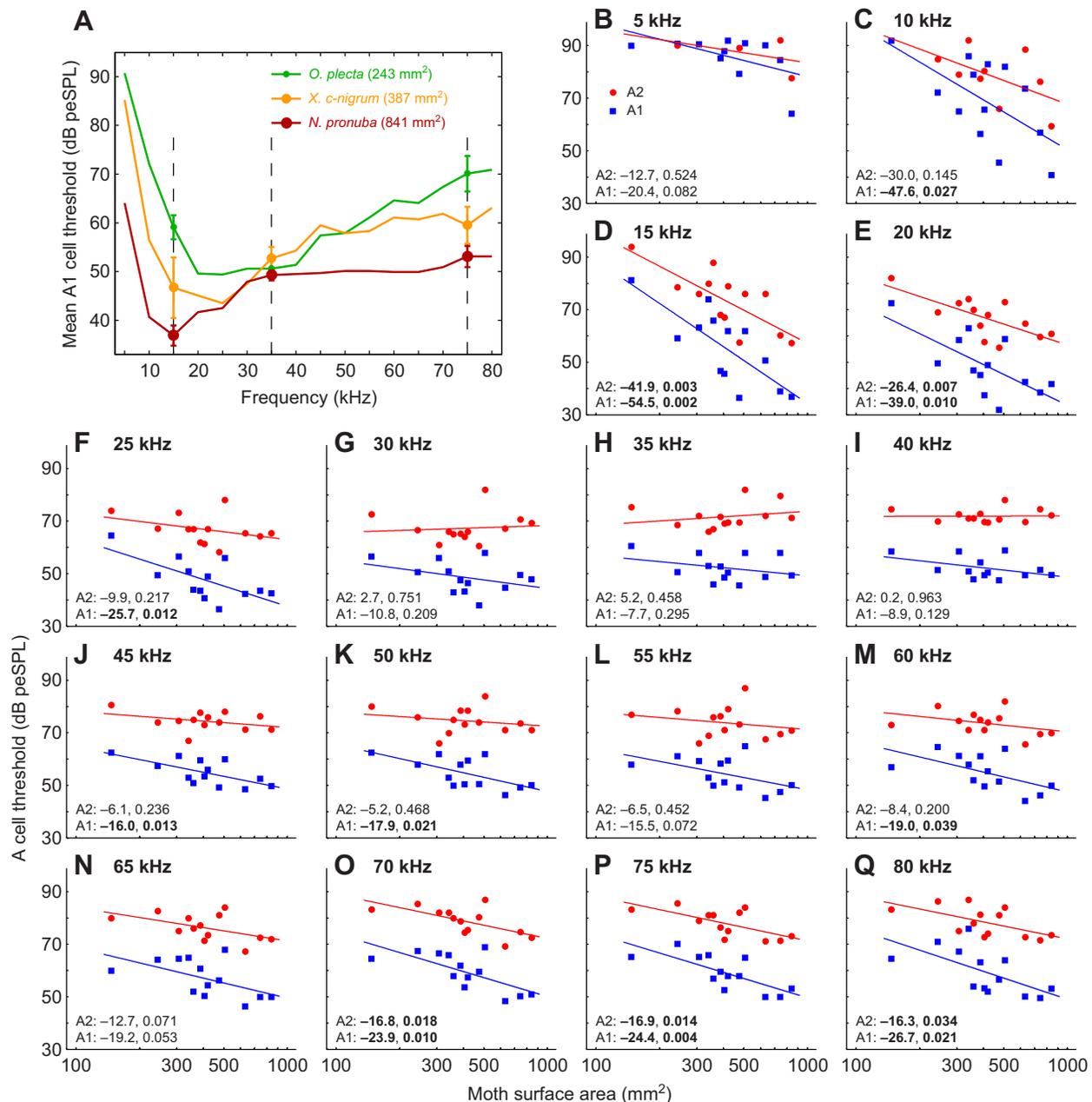


Fig. 2. Relationship between moth size and hearing threshold. (A) Example audiograms of three differently sized UK moths. Vertical dashed lines at fixed frequencies show that larger moths have lower hearing thresholds at low and high frequencies, but not at intermediate frequencies (error bars are \pm s.e.m.). (B–Q) Scatterplots and linear regression lines of A1 and A2 thresholds of 13 UK moth species as functions of surface area at 16 sound frequencies. \log_{10} (surface area) values were used to calculate linear regressions (solid lines). The slope of the regression line and its P -value (two-tailed t -test) are given in each panel and significant slopes are highlighted in bold.

differences (Fig. 3D), the shapes of the audiograms for the moth species found in both the UK and Denmark or Canada were quite similar within each species (Fig. 3E–H), suggesting that the data collected using different equipment and in different locations are comparable. The absolute A1 thresholds were generally higher in the UK than the Denmark or Canada populations for each of these species, but the size of the moths in the UK populations of these species were also smaller than the moths in the other populations, possibly explaining this difference in threshold. However, we compared the slopes of the size–threshold relationships and not the absolute thresholds for three areas. As examples of these relationships, Fig. 4

shows that at low frequencies (15 kHz) there is a strong correlation between size and auditory threshold in all three geographical areas, with all locations having steep negative slopes. In the mid-frequency range, there is almost no change in sensitivity with size, as the slopes approach zero at 40 kHz. Thus, in the low- and mid-frequency range, the results from Denmark and Canada support those from the UK (Fig. 2). However, at high frequencies, the results vary between the areas, with a strong correlation between sensitivity and size in UK moths (e.g. a steep negative slope at 80 kHz), but no correlation between sensitivity and size and slopes a little below or above zero for the other two areas (Canada and Denmark).

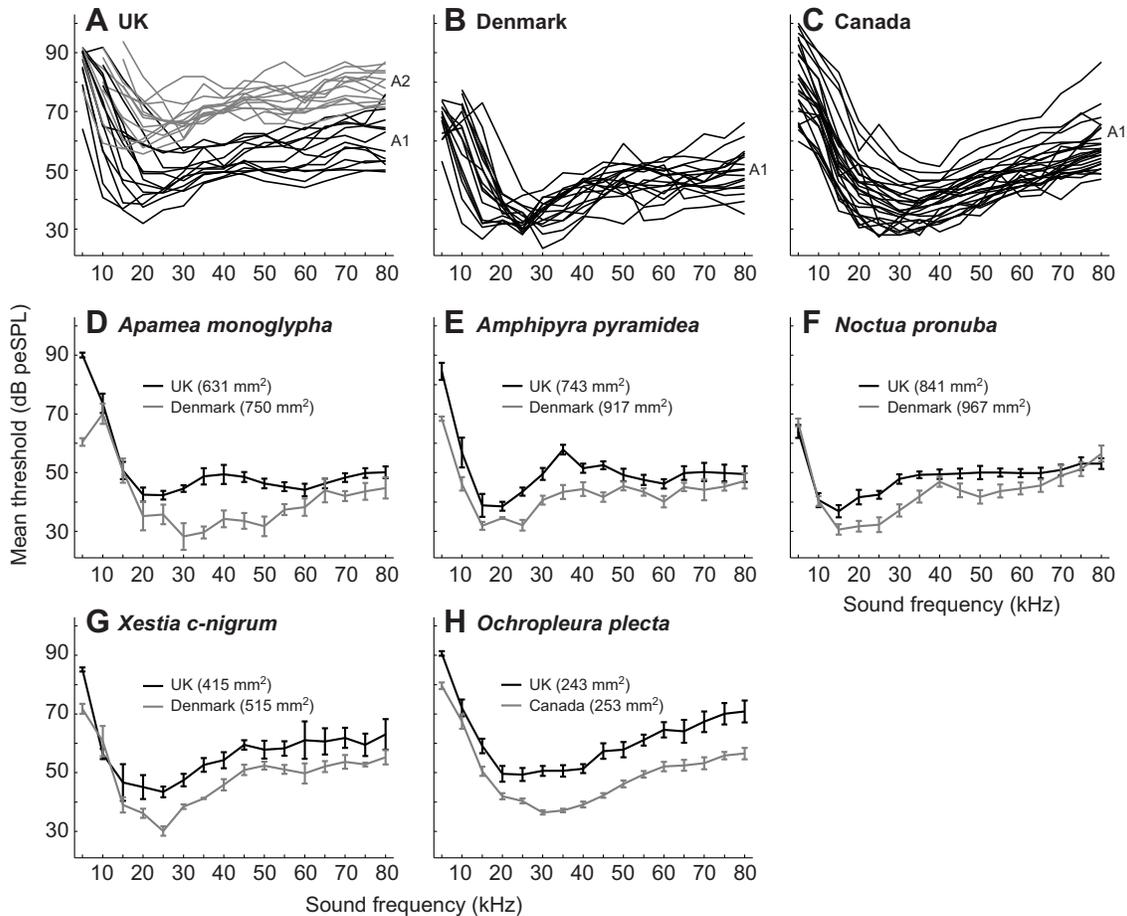


Fig. 3. (A–C) Audiograms for all moth species from the three locations. (D–H) Audiograms of five moth species with two locations in common. The shapes of the tuning curves were generally similar. UK thresholds were generally higher than those from Denmark and one species from Canada, but these moths also had smaller mean surface areas.

Analyzing this relationship between size and sensitivity for each of 14 tested frequencies (Fig. 5) confirms the strong correlation between size and sensitivity at low frequencies for all three areas. The mid-frequencies have more shallow slopes, which are significant for Canada, but not for Denmark or the UK. At the high-frequency end, the UK moths stand out by exhibiting a strong correlation between size and sensitivity for both A cells, whereas neither Canada nor Denmark moths show significant relationships. Further, there is an interesting frequency range around 50–60 kHz in which Denmark moths and also A1 cells of UK moths show significant relationships between sensitivity and moth size.

Next we considered each location with respect to the number of bat species that produce calls with the greatest energy at the tested frequencies. In Canada, all of the bat species have echolocation call peak frequencies below 65 kHz, and the threshold–size relationship for moths was only significant at 65 kHz and below (Fig. 5A). In Denmark (Fig. 5B), the frequency range of 50–60 kHz (i.e. the range for which there were significant relationships between moth size and sensitivity), corresponded with a greater number of bat species calling in this range than at other frequencies and also relative to the lower number of bat species calling in this range in Canada (Table 2, Fig. 5). The UK community is the only one of the three locations that has bat species calling with the most energy at very high frequencies (the horseshoe bats *R. ferrumequinum* and *R. hipposideros* at 80+ kHz), which is of particular interest in light of

the significant and steep correlation between moth size and sensitivity at these very high frequencies (Fig. 5C).

DISCUSSION

In addition to confirming the results of Surlykke et al. (Surlykke et al., 1999) that larger moths have lower A1 best thresholds than smaller moths, the main results of our study (1) verify the relationship between size and auditory sensitivity for the less sensitive A2 receptor and identify a previously unknown size-dependent relationship with A1 sensitivity, and (2) show that these size–sensitivity relationships vary with sound frequency and geographic location. Specifically, we first found that the size–BT relationship holds for A2, but the sensitivity difference between A1 and A2 BT is related to moth size and is smaller in smaller moths. In theory, this relationship between size and the difference between A1 and A2 BT could be advantageous to moths, and we discuss this possibility below. These relationships between size and sensitivity were identified at the species level and held when the putative effects of phylogenetic relationships were controlled for statistically in the analyses. Second, we found that the relationship between size and A1 sensitivity does not hold in the mid-frequency range if the data are analysed in a bat-specific way (by call frequency) instead of a moth-specific way (the moth's best frequency). In addition, the moth size–auditory sensitivity relationships at each frequency differed between three geographic

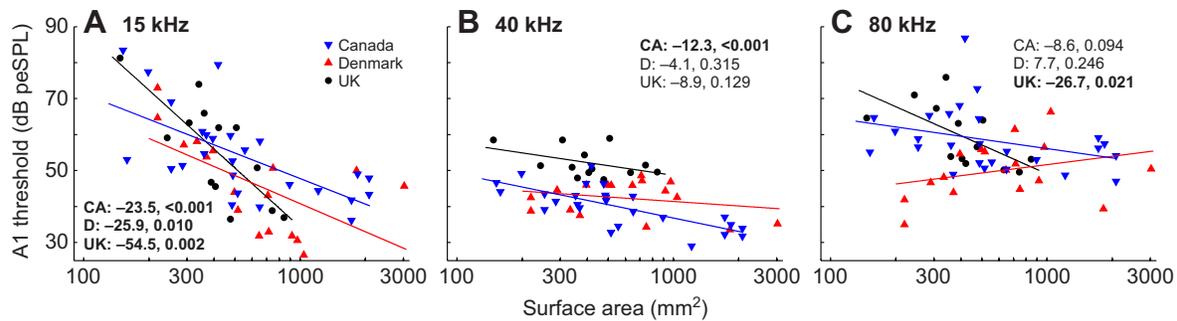


Fig. 4. Example scatterplots and linear regression lines of A1 threshold as a function of surface area for three locations at three sound frequencies. \log_{10} (surface area) values were used to calculate linear regressions. The slope of the regression line and its P -value (two-tailed t -test) are given in each panel and significant slopes are highlighted in bold.

areas, with a match to the distribution of echolocation frequencies of sympatric bat species, suggesting that perhaps it is the relationship between size and sensitivity of moth ears, more than the absolute threshold *per se*, that reflects the impact of bat predation.

A-cell best frequency

Our data confirmed the negative relationship between moth size and best frequency of the A1 cell shown by Surlykke et al. (Surlykke et al., 1999), and established the same relationship for the A2 cell. This was an important starting point for our study as it suggests that the relationships between size and sensitivity could differ amongst the different call frequencies used by bats. Surlykke et al. (Surlykke et al., 1999) concluded that the relationship between the BF of moth ears and moth size is an epiphenomenon of size, as larger tympana will have lower resonant frequencies. Even so, this relationship might still be advantageous for the moths, but it is unclear whether this is the case. Larger bats produce lower frequency echolocation calls than smaller bats (Bogdanowicz et al., 1999; Jones, 1999). Higher frequency echolocation calls improve the detection ability for smaller objects (Safi and Siemers, 2010), but acoustic calculations and empirical evidence show that even large bats with low echolocation call frequencies are capable of detecting prey that are smaller than the smallest moths in this study (Waters et al., 1995; Houston et al., 2004; Jakobsen et al., 2013). Thus, it does not seem likely that the relationship between BF and size in moths is related to their conspicuousness to different bat species using different call frequencies. However, large bats might actively

select larger prey whereas smaller bats might be physically restricted to the capture and handling of smaller prey (Aldridge and Rautenbach, 1987; Jones, 1990), which could render size-related differences in moth BF advantageous.

Function of A1 and A2 cells

The thresholds of A1 cells show that they most likely start firing at sound intensities at which the bat has not yet detected the moth, giving the moth a chance to escape undetected (Roeder, 1967; Roeder, 1998; Surlykke et al., 1999). However, the function of the A2 cell remains controversial. This cell might trigger erratic evasive flight, as suggested by Roeder (Roeder, 1974), or it might simply increase the dynamic range of the ear, as data from closely related moth families suggest (Surlykke, 1984; Ratcliffe et al., 2009). Our results show that A1 thresholds decrease with increasing moth size (i.e. smaller moths have less sensitive ears) and with a steeper slope than A2 thresholds for all frequencies (Figs 1, 2). As a result, the difference in threshold between A1 and A2 is greater in larger than smaller moths; these size-dependent threshold differences could perhaps have an adaptive function. Surlykke et al. (Surlykke et al., 1999) estimated that across all sizes and A1 thresholds for moths, moths detect bats at ~ 10 times the distance that bats detect the echo from the moths. This corresponds with the approximate 20 dB (10 times) difference between the thresholds of the A1 and A2 cells in moths, meaning that the A2 cell will start to fire at approximately the same time as the bat detects the echo from the moth. Given that, in general, bats fly much faster than moths, directional flight by the moth might no longer be effective once the bat has detected the

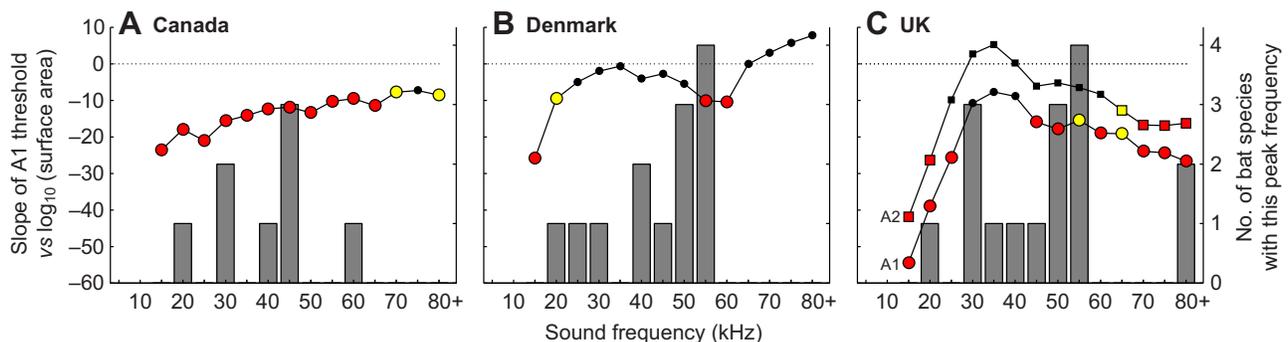


Fig. 5. Comparison between the relationship of auditory threshold and moth size (left y-axes) and the bat assemblages (right y-axes) at different sound frequencies and for three locations. Line plot: slope of the regression between A cell threshold (circles, A1; squares, A2) and \log_{10} (surface area) for all frequencies (cf. Fig. 3), with P -value indicated by symbol colour (red, $P < 0.05$; yellow, $0.05 \leq P < 0.1$; black, $P \geq 0.1$). Bar plot: number of bat species calling (peak frequency) at that frequency.

moth, and thus more drastic, unpredictable evasive flight could be a more effective means of avoiding capture by the bat.

A simplified theoretical example illustrates how smaller differences between A1 and A2 thresholds could be adaptive for smaller moths, regardless of which hypothesis for A2 function is correct. To illustrate this point, consider a situation where large and small moths had the same 20 dB difference between A1 and A2 thresholds. If such were the case, the absolute distance between bat and moth at the time the bat detects the echo from the moth and the A2 cell starts to fire would be greater for larger than smaller moths. For example, a large moth detecting a bat 30 m away with its A1 cell will be detected by this bat, and the moth's A2 cell will start to fire, at 3 m; a small moth detecting a bat at 10 m with its A1 cell will be detected by this bat, and this smaller moth's A2 cell will start to fire, at 1 m. In the case of the smaller moth, these shorter distances would decrease the time available for the small moth to initiate erratic evasive flight before capture. If, however, the difference between A1 and A2 thresholds is 14 dB for the small moth, the A2 cell would start to fire when the bat is 2 m instead of 1 m away, providing the smaller moth with more time to effect an appropriate behavioural reaction and thus improve the moth's chances of survival.

Comparison between absolute thresholds and size–threshold relationships

The absolute thresholds for the A1 cell were somewhat different between the three locations, and this could be due to slight differences in the recording methods, discrepancies in threshold criteria between locations, or both. They could also represent real differences between locations. There are many potential ecological and behavioural factors that could select for differences in these absolute thresholds between locations, such as the habitats used by the bats and moths, the intensity of the echolocation calls of the bats in each location, and possible tradeoffs between defence and mating for moths in different environments depending on the duration of the warmer season or density of bats.

However, the main focus of our study was not to compare absolute thresholds, but to compare the slopes of the relationships between A1 threshold and moth size at different frequencies. These relationships, i.e. correlation between threshold and size, are not affected by consistent differences in absolute thresholds between locations, but only relative differences in thresholds between moth species within a specific community. There is a strong relationship between BT and size in moths (Fig. 1B), but because the frequency of BT (i.e. BF) varies for different moth species (Fig. 1A), and because many bats produce calls with most of the energy at a restricted range of frequencies, it was unclear whether this relationship held true at specific frequencies, and hence whether it applied to many of the predators in the moths' given community. When the data were analyzed based on specific frequencies rather than based solely on the moths' best frequency, a picture emerges distinct from that presented by Surlykke et al. (Surlykke et al., 1999), as the relationships in our study were not significant at each frequency, but were more likely to be significant for frequencies at which there were more bat species calling in the moth's community.

Local tuning of moth ears

One of the important outcomes of our study was that the relationship between auditory threshold and size in moths reflected the specific sympatric bat community in all three locations. As argued above, we propose here to use the slopes of threshold *versus* size, not absolute threshold, as a measure of bat selection pressure on moth hearing,

which reflects the relative differences in thresholds between moth species within a specific community. Therefore, we tested for linear regressions between A1 threshold and size at specific frequencies typical for the echolocation calls of different bat species and compared these with the number of bat species with the most energy in their calls at these frequencies. We found that threshold *versus* size relationships in moths were only significant at specific call frequencies in each area and that those frequencies were different between the three areas (UK, Canada and Denmark). Our results revealed that the significant relationships were predominantly found at frequencies that are used by most bat species in the moths' community, indicating local tuning of moth ears to the calls of their sympatric predators.

The number of bat species calling at each frequency is only a rough estimate of predation and selection pressure by bats on moths, which also depends on the abundance, habitat use, preferred prey and foraging style, amongst other factors, of each bat species. Unfortunately, no survey method is unbiased (acoustic methods are biased towards species with low-frequency, high-intensity calls, and trapping methods are biased towards low-flying species). Therefore, including estimates of abundance at this stage might add noise instead of meaningful data to the comparisons. For this reason, we refrained from statistical analyses of the data and simply show the general pattern. However, it is important to consider that only sympatric bats can have a potential impact on moth hearing, and this is why we used the number of species calling in different frequency bands as a first proxy for predation pressure for each location, a measure that is imperfect, but provides the most straightforward comparison between locations.

For example, horseshoe bats, which only exist in one of our three locations, are significant predators of moths (Vaughan, 1997), and our results suggest that they have an effect on the hearing system of UK moths at high frequencies, as only UK moths in our study show a correlation between auditory threshold and size at high frequencies, whereas there is no correlation at high frequencies in communities that lack these species (e.g. Canada and Denmark). The allotonic frequency hypothesis suggests that some bat species have evolved echolocation calls composed of peak frequencies outside the best hearing ranges of sympatric eared moths and, as a result, consume proportionately more eared moths than do bats calling at frequencies to which moths are most sensitive (Fullard, 1998). Several studies have confirmed a relationship between higher echolocation call frequency and increasing number of moths in the diets of different bat species (Pavey and Burwell, 1998; Schoeman and Jacobs, 2003; Schoeman and Jacobs, 2011; Pavey et al., 2006). In our study, a more detailed measure of the adaptation of moth species to their predator community proves informative. Our finding of a significant relationship between A-cell threshold and size at high frequencies in the UK, taken together with the results of Jacobs et al. (Jacobs et al., 2008) and Fullard et al. (Fullard et al., 2008), suggest that moths have adapted to this pressure and that high-frequency calling horseshoe bats have a significant and underappreciated impact on the auditory systems of eared moths. Therefore, despite the extreme simplicity of moth ears, we find evidence of auditory threshold differences matching the specific cues provided by the predators in their community, possibly reflecting sensory adaptations for survival.

LIST OF ABBREVIATIONS

BF	best frequency
BT	best threshold
PICs	phylogenetically independent contrasts
SA	surface area

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AUTHOR CONTRIBUTIONS

All authors contributed to the design, execution and writing of this study. H.M.t.H. and H.G. are co-first authors, M.W.H. and A.S. are co-last authors.

COMPETING INTERESTS

No competing interests declared.

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