Agenda Item 8	Projects and Activities Supported by ASCOBANS
Information Document 8.6	Project report: Prediction of the Cochlear Frequency Maps of Harbour Porpoise
Action Requested	Take note
Submitted by	TiHo /ITAW

Note: Delegates are kindly reminded to bring their own document copies to the meeting, if needed.

University of Veterinary Medicine Hannover, Foundation

Prediction of the cochlear frequency maps of harbour porpoise (*Phocoena phocoena*)

(reference SSFA-ASCOBANS-2022-002_TiHo)

ASCOBANS (Agreement on the Conservation of Small Cetaceans of the Baltic, North East Atlantic, Irish and North Sea)

by

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Büsum, November 2022

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Summary

There is an increasing concern on the effects of underwater noise on hearing of cetaceans. However, cochlear frequency maps (i.e. distribution of frequencies along the cochlear spiral within the inner ear) are still lacking for marine mammal species. The aim of this study is to predict the cochlear frequency map for harbour porpoise (*Phocoena phocoena*), based on morphometrics of the sensory cells of the organ of Corti (hearing organ). In mammals, the cochlear base encodes for high frequency sounds, while low frequencies are detected in the apex but how the frequencies are distributed along the spiral is species specific. Morphometric variation occurs in cells of the organ of Corti (the hearing organ) from the apex to the base of the cochlea. These changes in cell shape and spacing are correlated to the frequencies encoded at different locations. Our study shows that this correlation can be extrapolated to other species of mammals, if they have a similar hearing range. By using geometric morphometric data from scanning electron micrographs of the inner ear from terrestrial mammals of known frequency map, it is possible to create a predicting model that can then be applied for localizing the frequencies that are encoded in each location along the cochlear spiral in mammalian species in which we only have morphologic data. We complemented previous work using Parnell's mustached bat, rat, mouse, gerbil and harbour porpoise with more data from the inner ears of two gerbils and six harbour porpoises and we tested and cross-validated several predicting models. This study represents a large step forward in predicting the cochlear frequency map for harbour porpoises. Once the map is known for a species, it will be possible to extrapolate the frequencies that are impaired if lesions are found in the inner ear of harbour porpoise, and have better understanding of the consequences for individual to have a hearing loss in a particular hearing range. In cases of noise-induced hearing loss, it will be possible to extrapolate the characteristics of potential sound sources that have triggered a damage. The current map predicted in this study will be a crucial tool for management of the effects of underwater noise on hearing in porpoises, as well as improved decision-making in conservation plans for harbour porpoises and other marine mammals.

1. Introduction

Man-made noise, at different intensity levels, can affect negatively marine mammal populations in several ways, such as masking the vital information transmitted by them, affecting their behaviour, inducing some physiological changes, or causing hearing loss. As it has been demonstrated in human and other land mammals, repeated exposure to sound at a particular frequency and intensity can cause hearing loss. This hearing loss can be temporary or permanent. In the latter case, lesions at the level of sensory hair cells of the organ of Corti (hearing organ) and its associated innervation are produced, which can be identified by electron microscopy. Hearing loss can have fatal consequences for harbour porpoises (*Phocoena phocoena*) and other species of cetaceans since hearing is essential for their survival, as it is needed for orientation, communication, and foraging.

High intensity active sonar, and other loud noise sources, like those from shipping, seismic surveys, or pile driving amongst others, can cause auditory lesions severe enough to be lethal to harbour porpoises and other cetaceans. However, scientific evidence concerning the effect of different types of noise on harbour porpoise hearing is lacking; no data are available concerning noise frequencies, intensities, and durations that can cause them permanent hearing loss. There is a need to improve our understanding on how these complex characteristics interact, which is essential to mitigate the effect of man-made noise on harbour porpoises, while preserving human progress.

To achieve this long-term goal, it is necessary to improve our knowledge on hearing capabilities and implement a routine protocol to be able to determine the presence of lesions associated to noise overexposure in harbour porpoises.

The cochlea in odontocetes and land mammals are largely comparable. They both contain the organ of Corti, or hearing organ, which is formed by the sensory cells (hair cells) and the supporting cells (Figure 1, see Lim 1986 for review). There are two types of sensory cells, inner hair cells (IHCs) and outer hair cells (OHCs), which are arranged in three parallel rows, amplify the incoming signal and are responsible for frequency sensitivity and selectivity. IHCs are aligned in one row and they transduce the mechanical sound stimulation into the release of neurotransmitter to the Type I afferent neurons that conduct the auditory information to the brain stem. Structural alterations as a consequence of noise exposure include degeneration and loss of entire hair cells, among others (Bredberg *et al.* 1972, Hu *et al.* 2000). Following cochlear hair cell death, neighboring supporting cells initiate the elimination of the hair cell, leaving a distinct scar (Lim and Dunn 1979). The presence of scars among hair cell rows is therefore an important criterion for assessing possible history of noise-induced hearing loss.

The organ of Corti is in contact with the basilar membrane that vibrates with the incoming sound and, depending of its frequency, will be encoded in one region of the cochlea or another. High frequency sounds are encoded in the base (closest to the stapes) and the low frequencies at the apex or tip of the spiral. However, the exact distribution of frequencies along the cochlear spiral is species specific.

The morphology of the organ of Corti cells changes from apex to base of the cochlea. Studies on guinea pigs (*Cavia porcellus*) and mustached bats (*Pteronotus parnellii*) related these changes in shape and morphology to the corresponding encoding frequency (Yarin *et al.*, 2014; Girdlestone *et al.*, 2020). This discovery is ground-breaking because implies that differences in shape at the level of the cuticular plate (i.e. apical portion of the cells of the organ of Corti) must have crucial biomechanical properties that are tuned according to the coding frequency. And these differences in morphology along the spiral can be measured using scanning electron micrographs. It also suggests that we can predict the encoding frequency of a particular location based on the ultrastructural morphometrics of the organ of Corti cells.

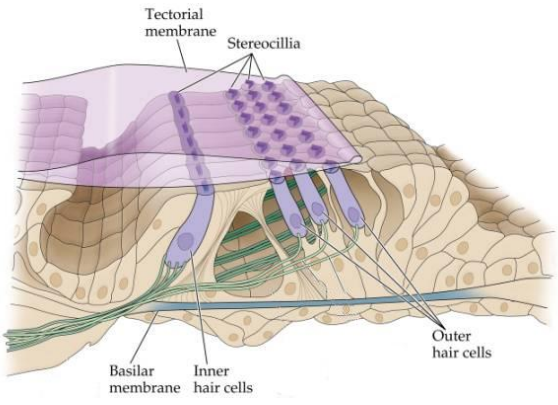

Figure 1. Schematic of the organ of Corti or hearing organ in mammals, characterized by one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs). Source: flipper.diff.org.

The cochlear frequency maps (i.e. the frequency distribution along the cochlear spiral) for harbour porpoises and other species of marine mammals are not known since they require the animals to be sacrificed. The importance of having these maps relies in the possibility to determine the frequencies that are impaired when lesions are found. In addition, in cases of noise-induced hearing loss, it will be possible to extrapolate the acoustic characteristics of a source that had trigged these lesions (Figure 2).

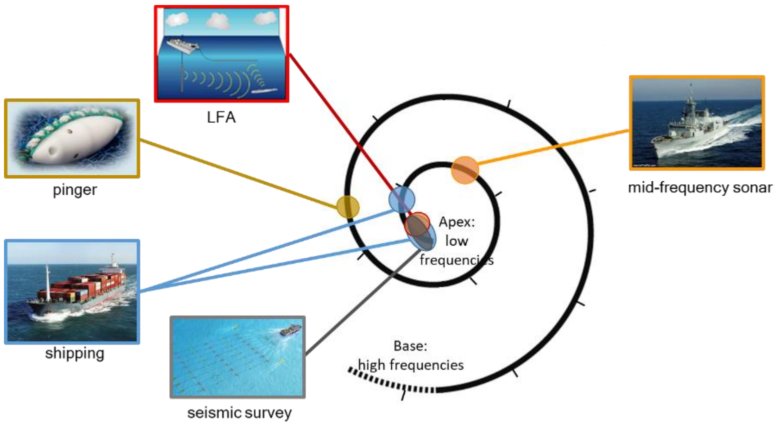

Figure 2. Schematic illustrating how sound sources can be determined from the location of a lesion once the frequency map for a species is known.

It is crucial to have these maps for harbour porpoises to be able to identify potential damaging sound sources (Figure 2), as well as to monitor the effectiveness of the current mitigation measures.

Our first results comparing and extrapolating the morphologic variability of the sensory cells and corresponding frequency encoded along the cochlear spiral in rats (*Rattus norvegicus*), mice (*Mus musculus*), gerbils (*Meriones unguiculatus*) and Parnell's mustached bats (a species of echolocating bats) gave us the first preliminary predictions of the frequency maps for harbour porpoise using mathematical modelling (Morell *et al.,* 2018, 2019). By using geometric morphometric data from scanning electron micrographs of the inner ear from terrestrial mammals of known frequency map, it is possible to create a predicting model that can then be applied for localizing the frequencies that are encoded in each location along the cochlear spiral in mammalian species in which we only have morphologic data.

2. Objectives

The main objective of this study is **to predict the cochlear frequency map for harbour porpoise** based on morphometric characteristics of the organ of Corti.

To build up from our preliminary work and to achieve the main objective, it is necessary to accomplish the following tasks:

- **Task 1) Include more inner ears of terrestrial mammals** to make the predictive model stronger.

- **Task 2) Have more detailed information from the most apical region of the cochlea of harbour porpoise** (first 10-15% of the total length), which is the region where it is more likely to find lesions associated to noise exposure and where there is a higher morphological variability.

- **Task 3) Validate the predictions with individuals whose audiograms have been measured**

Once the predictions are validated for harbour porpoises, it will be possible to extrapolate the same predictive model to establish cochlear frequency maps (and hearing ranges) for species whose hearing capabilities been not yet been measured, such as some species of beaked whales or baleen whales.

3. Relevance to ASCOBANS

The project is highly relevant for the attainment of ASCOBANS goals in achieving and maintaining a favourable conservation status for harbour porpoises. Our project helps addressing several relevant Activities in the Agreement's current Work Plan:

7. Review new information on underwater noise, its impacts on small cetaceans and their prey species, mitigation measures, technological developments, best practices and guidelines. Make recommendations to Parties and other relevant authorities for further action.

8. Assess whether national navies' mitigation protocols for use of military sonar are effective. This requires Parties to request the mitigation protocols from the navies.

9. Review new information on ocean energy, its impacts on small cetaceans, mitigation measures, technological developments, best practices and guidelines. Make recommendations to Parties and other relevant authorities for further action.

11. Review new information on recreational sea use, impacts on small cetaceans, best practices and guidelines. Make recommendations to Parties and other relevant authorities for further action.

12. Review new information on other sources of disturbance, impacts on small cetaceans, best practices and guidelines. Make recommendations to Parties and other relevant authorities for further action.

13. Review new information on underwater munitions and, their impacts on small cetaceans and cetacean habitat. Make recommendations to Parties and other relevant authorities for further action.

41. Promote the use of joint ASCOBANS/ACCOBAMS best practice on cetacean post-mortem investigation and tissue sampling, and standardize reporting procedures of Post-Mortem Examinations statistics.

42. Continue monitoring new information on the causes of strandings and mortality of cetaceans, as well as best practice guidance on stranding responses and necropsies, and to make recommendations to Parties as appropriate.

Specifically, from Activities 7, 8, 9, 11, 12 and 13, this research:

1) helps identifying whether sound exposure for a determined source have the potential to damage the hearing of harbour porpoises.

2) monitors whether a mitigation measure needs improvement in protecting the hearing of harbour porpoises.

3) allows us to make recommendations to Parties and other relevant authorities for further actions in specific sound sources.

The project addresses Activity 41 since one of sections of the joint ASCOBANS/ACCOBAMS best practice on cetacean post-mortem investigation and tissue sampling is dedicated to ear extraction and fixation, which is critical for our study. Only very fresh ears (i.e. fixed within around 24 hours post-mortem) are suitable for analysis for noise-induced hearing loss diagnosis. If the innervation is affected, then our window of detection can be larger (up to 2-3 days possibly). However, the majority of the lesions found immediately after exposure are located at the level of the sensory cells of the organ of Corti, which are very sensitive to post-mortem decomposition. Thus, this project highlights the potential and importance of collecting the ears shortly after the death of the animal, thus enforcing the use of the joint ASCOBANS/ACCOBAMS best practises protocol.

This study also shows the need to continue monitoring the mitigation measures set in place by offshore operations, as well as contributes in establishing causes of strandings and mortality of cetaceans (Activity 42), which are highly dependent of their hearing capabilities. As mentioned above, our project promotes the use of the best practice guidance on necropsies and to make recommendations to Parties on damaging sound sources and potential improvement of mitigation measures when needed.

The project addresses the following conservation, research, and management measures from the Conservation Management Plan annexed to the Agreement text:

- 1d (“prevention of other significant disturbance, especially of an acoustic nature”) within the measures of Habitat conservation and management, and

- 3 (“full autopsies in order to collect tissues for further studies and to reveal possible causes of death”)

The prediction of the cochlear frequency map for harbour porpoises can help updating the CMS Family Guidelines on Environmental Impact Assessments for Marine Noise-generating Activities (UNEP/ASCOBANS/Res.8.11 Rev.MOP9) and/or its Technical Support Information contained in ASCOBANS/MOP9/Inf.6.2.6a. Specifically, our project will be important to address the following actions for mitigation and monitoring plans of nearly all the sources cited: “Scientific monitoring programmes, conducted during and after the activity, to assess impact”, and “Quantification of the effectiveness of proposed mitigation methods”.

In addition, this project relates to ASCOBANS Resolution 5.4: “Adverse Effects of Sound, Vessels and other Forms of Disturbance on Small Cetaceans”, Resolution 6.2: “Adverse Effects of Underwater Noise on Marine Mammals during Offshore Construction Activities for Renewable Energy Production”, and Resolution 8.10 “Small Cetacean Stranding Response”.

4. Methodology

4.1 Task 1. To include more inner ears of terrestrial mammals

To make our previous predictive model stronger, we needed to include more data from terrestrial mammals, especially those that are more sensitive to low frequencies, such gerbils or guinea pigs.

We followed the same methodology as in our preliminary work with rats, mice, gerbils, Parnell's mustached bats and harbour porpoises (Morell *et al*., 2018, 2019, Girdlestone *et al.,* 2020, Figure 3). We characterized the correlation between morphology and coding frequency using geometric morphometrics in all species from scanning electron micrographs. Geometric morphometrics analyses differences in shape taking into account all possible distance among the selected landmarks. We selected numerous landmarks (orange circles in Figure 3b) of the cuticular plate to characterize its shape in 10 locations along the spiral (Figure 3a). We used several machine learning algorithms with the morphometric data of echolocating bats and rodents (species with known frequency maps) to identify a predictive model relating morphometry with coding frequency. Then, we used this predictive model to generate predictions of the cochlear frequency map for the harbour porpoise, as previously done (Morell *et al*., 2018, 2019), but with more data to train the model with.

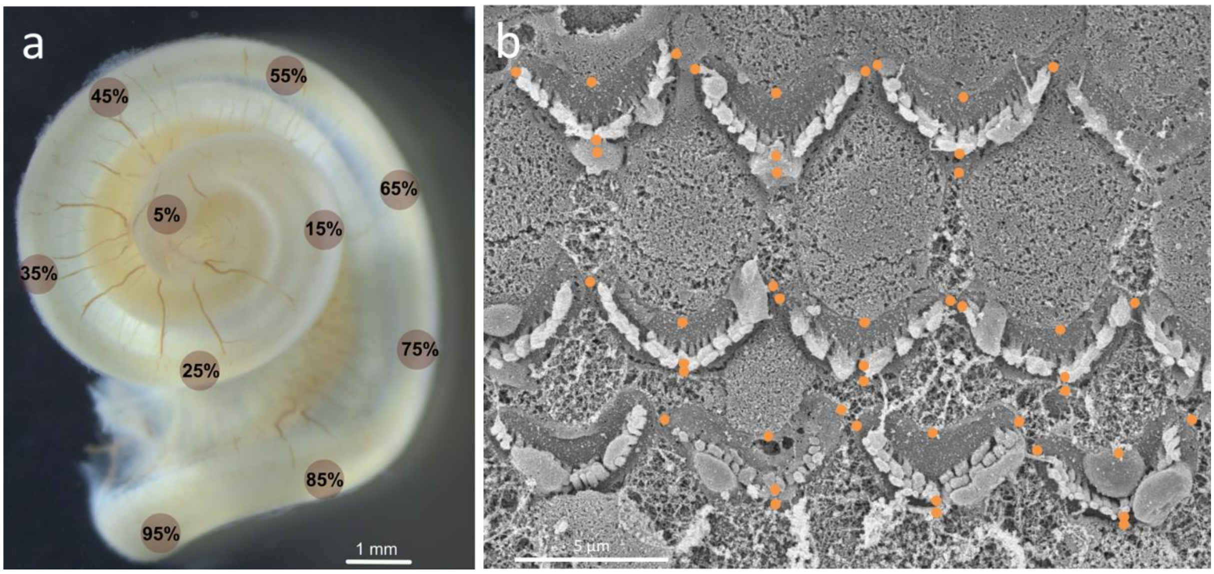

Figure 3. a) Cochlea of a harbour porpoise, indicating the 10 locations where the measurements have been taken from. b) Scanning electron micrograph of the organ of Corti of a harbour porpoise. The positions of the landmarks in the outer hair cells for the geometric morphometric analysis are highlighted in orange.

We used the software tpsDig and MorphoJ to digitize and process geometric morphometric data, and r for coding the predictive models. The dissection of the inner ears, processing for scanning electron microscopy (SEM), imaging and digitizing (placing the landmarks) was done by Dr Maria Morell and Laura Rosas at ITAW. The data treatment and coding was performed by Dr. Adrien Caplot at INM, who has expertise in this field and have worked already with the preliminary data. The Department of Osteology and Biomechanics of UKE Hamburg has a first-rate SEM that was used for this project.

Inner ears from two gerbils were provided by Dr. Artëm Djuba at INM and seven inner ears from guinea pigs already dissected and coated that were used for previous projects, were provided by Dr. Jing Wang, INM. Table 1 shows all the data that was integrated in this study, from both previous and the current research.

Table 1. Summary of the species, origin and number of ears processed in this study, or used from our own previous work. Information on hearing ranges according to their cochlear frequency maps are also provided. The hearing range from harbour porpoise can be found at Kastelein *et al.,* 2002.

Species	Scientific name	Origin	Number of ears	Cochlear frequency map	Hearing range	Current/ previous work
Mustached bat	*Pteronotus parnellii*	Germany	7	Kössl and Vater, 1985	23-111 kHz	previous
Rat	*Rattus norvegicus*	France and Canada	4	Müller, 1991	0.5-54 kHz	previous
Mouse	*Mus musculus*	France	5	Müller and Smolders, 2005	6-68 kHz	previous
Mongolian gerbil	*Meriones unguiculatus*	France	7	Müller, 1996	0.25-30 kHz	previous and current
Guinea pig	*Cavia porcellus*	France	7	Tsuji and Liberman, 1997	0.15-55 kHz	current
Harbour porpoise	*Phocoena phocoena*	The Netherlands and Canada	11	_________	0.25-180 kHz (Kastelein *et al.,* 2002)	previous and current

Several predicting algorithms, such as Random Forest, Support Vector Machine (lineal, radial, sigmoidal and polynomial), K-Nearest Neighbor and Decision Tree were compared and validated, using the Leave-one-out cross-validation method (i.e. one row is left so the model can be trained on the other rows, then the model can predict the row left aside). The error of prediction was first calculated for each species (the model was trained with a species and the prediction tested within the same species). The predictive algorithms that had less errors in all the species were selected to then compare data between species, and finally used to predict the cochlea frequency map for harbour porpoises.

4.2. Task 2. To have more detailed information from the most apical region of the cochlea in harbour porpoises

The apical region of the cochlea is the area where the lowest frequencies are encoded. It is also the region where it is more likely to find lesions associated to noise exposure and where there is a higher morphological variability.

In our first preliminary work, we observed that in the first 10-15% of the cochlea, there was a large frequency distribution (from 125 Hz up to 32 kHz). To be able to fulfil the ultimate objective to be able to determine which frequencies are impaired and which is the potential source/s that have trigged this damage, we need to have more precise information of which frequencies are encoded in each percentage. We used the ears of some of the harbour porpoises that we have already prepared that had intact apex, and took additional images at (2%, 4%, 6%, up to 15%). We added the ears of another 6 porpoises into this analysis to have a better overview of the inter-individual variability, and took detailed images of the apex each percentage, as well as in each 10% of the rest of the spiral, as performed before (15%, 25%, 35%, etc). We followed the same methodology as described above, and the ears were processed according to previously optimized protocols for cetacean species (Morell *et al.,* 2015, 2017, 2021, 2022).

The new inner ears, as well as some of the previous ones, were provided by Dr. Lonneke IJsseldijk (Utrecht University, Table 2).

Table 2. Details of the harbour porpoises from this study, tissue and type of analysis, stranding date and location.

Id	ear	sex	length (cm)	weight (kg)	age class	necropsy date	pres. code	origin
cet 401A_Seamarco Pp02	right	M	147	44.4	A	04.02.2015	1	The Netherlands
cet 404A_UT1495 Pp	right	M	105	14.8	J	17.02.2016	1	The Netherlands
cet 405A_UT1509 Pp	right	F	113	26	J	11.03.2016	1	The Netherlands
cet 410B_UT1528 Pp	left	M	75	6.5	N	30.06.2016	1	The Netherlands
cet 412A_UT1532 Pp	right	M	82	8.5	N	18.07.2016	1	The Netherlands
cet 413B_UT1535 Pp	left	F	146	46	A	28.07.2016	1	The Netherlands
cet 415B_Jack Pp	left	M	156		A	12.08.2016	1	Canada
cet 427B_UT15697 Pp	left	M	105	24.5	J	24.02.2017	1	The Netherlands
cet 430A_UT15697 Pp	right	F	133	53	A	25.04.2017	1	The Netherlands
cet 432A_UT1602 Pp	right	M	147	41	A	04.07.2017	1	The Netherlands
cet 433A_UT15611 Pp	right	F	167	44.5	A	01.08.2017	1	The Netherlands

Id: identification code, F: female, M: male, N: neonate, J: juvenile, SA: subadult, A: adult, pres.: preservation (1 fresh and not frozen, up to 5 mummified).

4.3 Task 3. To validate the predictions with individuals whose audiograms have been measured

We validated our frequency maps by using the data from individual cet 401A_Seamarco Pp02, whose behaviour audiograms have been measured regularly by Dr. Ron Kastelein from SEAMARCO, The Netherlands (Kastelein *et al.,* 2010). The cochlear frequency map was generated for this individual alone, to be able to correlate the results of the prediction with the measured frequency hearing range of the individual.

5. Results

5.1 Comparison among several predicting algorithms

To select the best predicting algorithms, the error of prediction was calculated per each species for several machine learning techniques that can perform well for small datasets. Previous research was performed using more algorithms such as neural networks, but were already discarded for this study since the error was too large.

Figure 4 shows the results of error of prediction in octaves per species and algorithm (or regression model). Since the scale was different, a one octave error was highlighted with a discontinued line, to better compare among graphs. In general, the algorithms Random Forest and Lineal Support Vector Machine displayed less errors in all the species tested. All predicting algorithms performed badly for the guinea pigs, with close to one octave of error. This usually indicates that the number of samples is not enough to have strong predictive models and more data is needed to have reliable predictors.

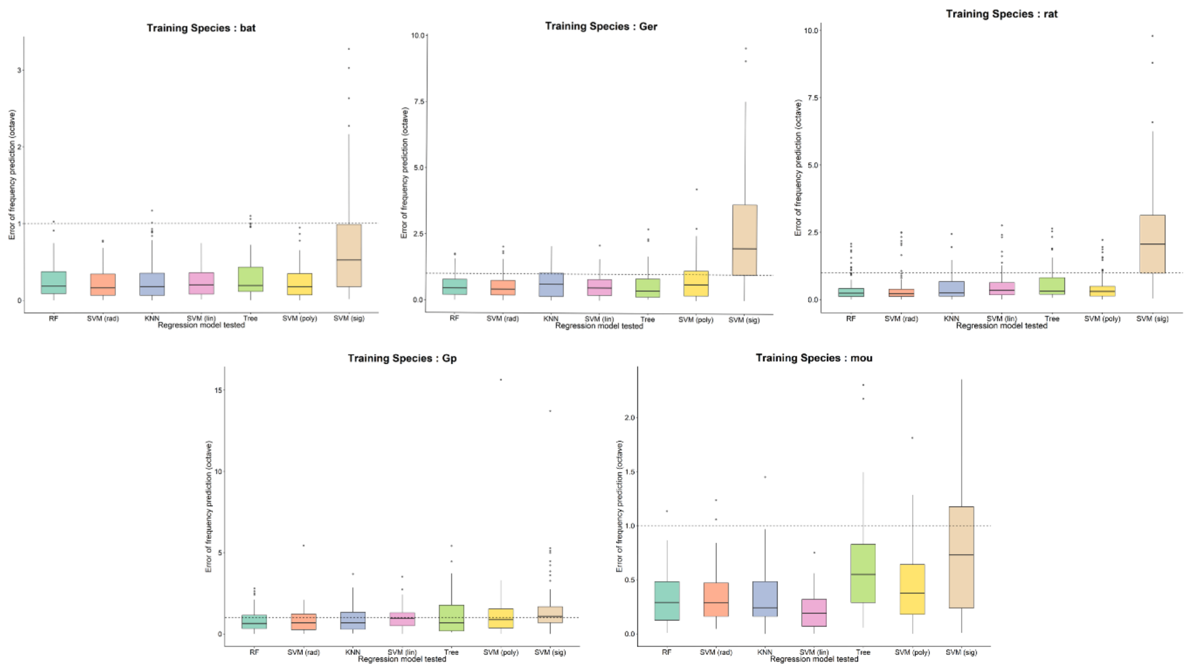

Figure 4. Error of prediction in octaves per each species for several predicting algorithms, using the Leave one out - cross validation method. The dotted line marks the location of one octave. RF: Random Forest, SVM: Support Vector Machine, rad: radial, lin: lineal, sig: sigmoidal, pol: polynomial, KNN: K-Nearest Neighbor, Tree: Decision Tree, bat: mustached bat, Ger: gerbil, Gp: guinea pig, mou: mouse.

5.2 Predictive model 1: Lineal Support Vector Machine

5.2.1 Cross-validation among terrestrial species

After selecting the best predictive models, we cross-validated each of them first within the same species. For example, the model was trained with data from mice (correlation morphometrics-frequency) and subsequently asked to predict the frequencies encoded along the spiral in mice (with only morphometrics data). The comparison between the real and predicted frequencies allows validating the performance of the model, as well as to calculate the coefficient of correlation.

The correlation between the predicted frequency (blue dots in Figure 5) and the real frequency, according to published frequency maps (red lines in Figure 5), was calculated for the five species of terrestrial mammals using the Linear Support Vector Machine model. While the coefficient of correlation was above 0.9 for rat and mouse, and around 0.8 for mustached bat and gerbil, it was only 0.63 for guinea pigs. Again, this indicates that more data from guinea pigs is needed to have reliable predictions for this species.

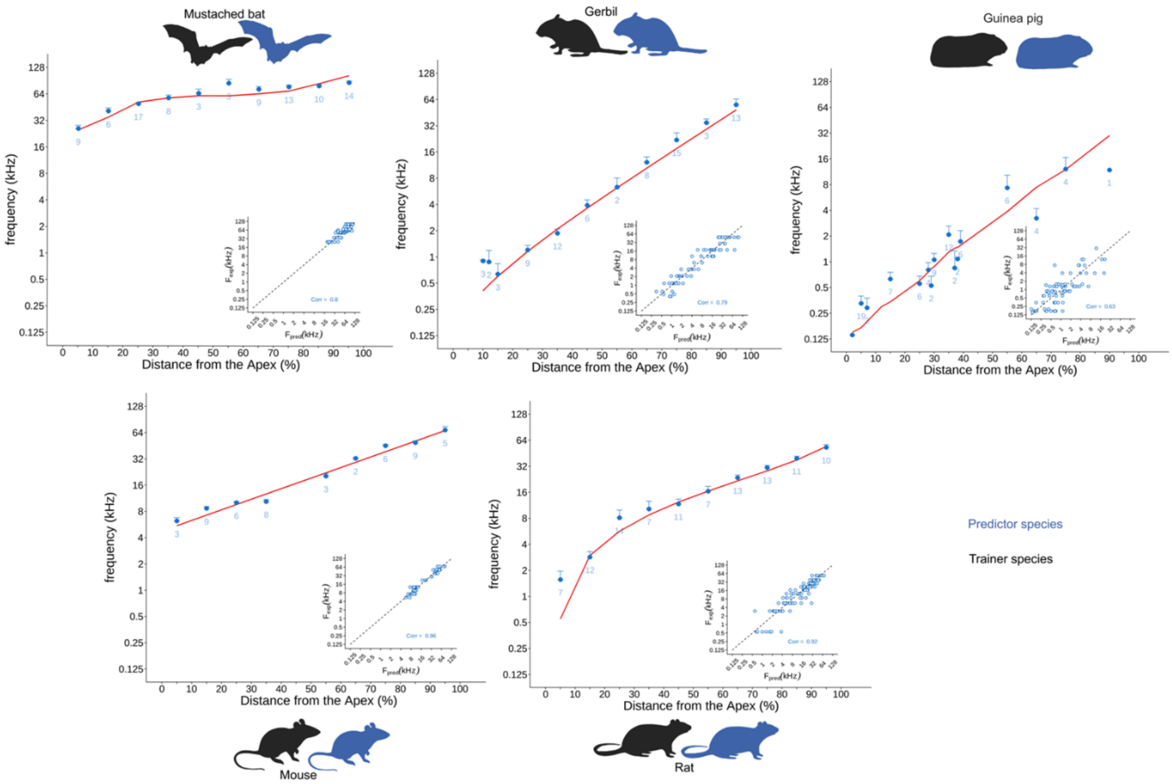

Figure 5. Correlation between predicted frequency (blue dots) and published frequency map (red line) for mustached bat (Kössl and Vater, 1985), rat (Müller, 1991), mouse (Müller and Smolders, 2005), gerbil (Müller, 1996) and guinea pig (Tsuji and Liberman, 1997) using the Linear Support Vector Machine model. The dataset of a particular species was tested for the same species.

Furthermore, a cross-validation between pairs of species was done, i.e., one species was used to train the model with, and then the model was used to predict the frequencies for another species along the cochlear spiral with only morphometric information. With this, we could test whether the predictive model and the correlation morphometrics-coding frequency could be extrapolated to different species.

Figure 6 shows the results of the cross-validation of the Linear Support Vector Machine model across all the pairs of species. It showed that the predictive model can be applied among species of comparable hearing range. For example, since mustached bats can hear sounds between 25 and 111 kHz, when training the model with data from bat and asked to predict rat, it over predicted those frequencies between 0.5 and 25 kHz. That is logic since there was no input in the model to determine the frequencies below 25 kHz. However, the model predicted correctly in the majority of the cases when the trained and predicted species overlapped in frequency range. The current data from guinea pig did not predict well the other species, but this was expected since the previous methods already indicated that our dataset is not ready yet to be used in the predictions and more data should be added.

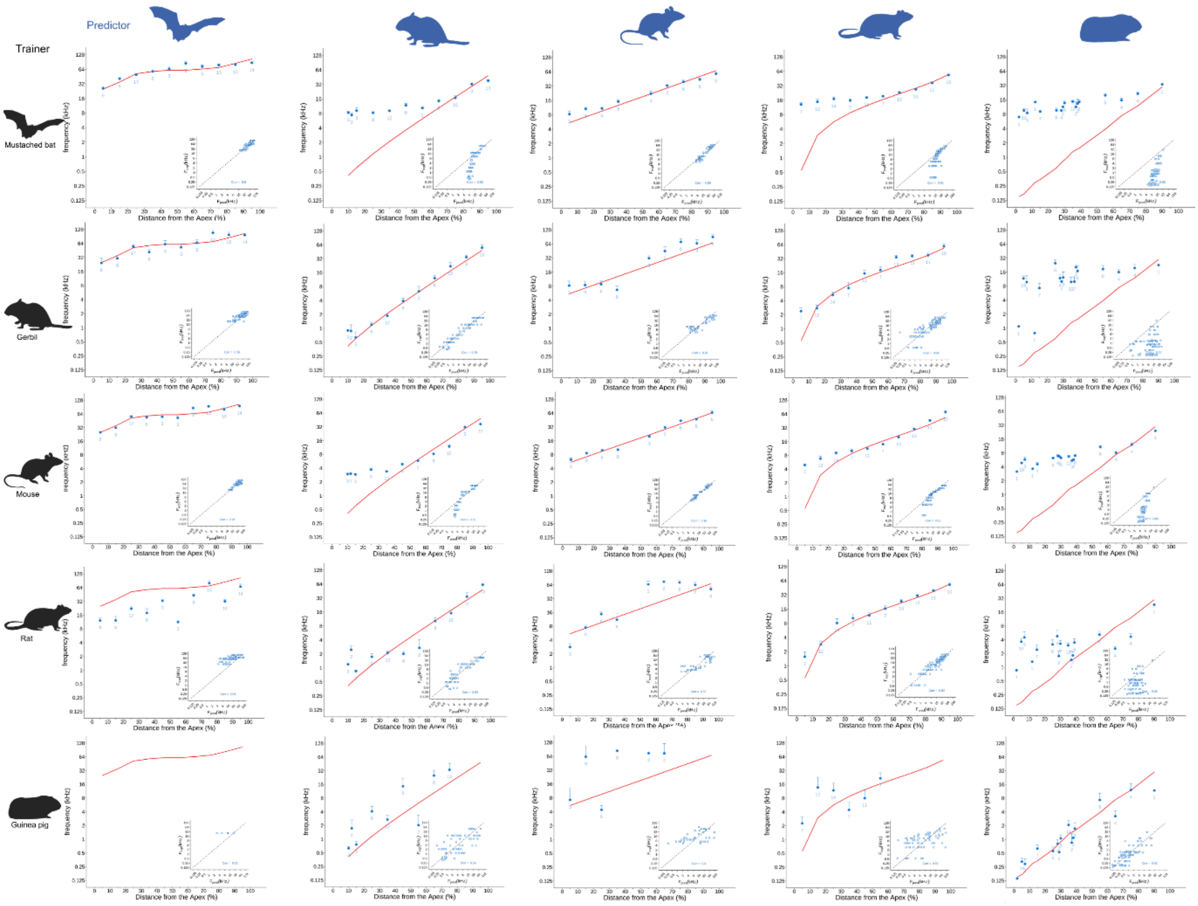

Figure 6. Cross-validation using the Linear Support Vector Machine model to test its performance and correlation among pairs of species. The model was trained using the species in black, to predict the frequencies (blue dots) of the species in blue. The published cochlear frequency map for each species is shown in red.

5.2.2 Prediction of cochlear frequency map for harbour porpoise

Once the predictive model was tested and validated among different species, the predictive algorithm created with data (morphometrics and coding frequencies) from mustached bat, rat, mouse and gerbil was used to predict the frequency map of for harbour porpoises based on morphometrics of the organ of Corti (Figure 7). The morphometrics from the five previous harbour porpoises, as well as the new six porpoises were combined for this prediction. Figure 7 shows the predicted distribution of frequencies along each 10% of the spiral, except for the first 15% of the apex where the frequencies where predicted every 2%, to have a higher detail of the frequency distribution of the apex.

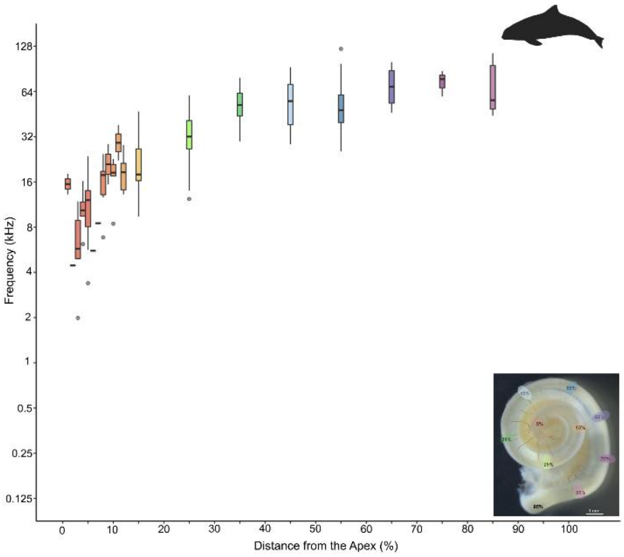

Figure 7. Prediction of the cochlear frequency map for harbour porpoise using the Linear Support Vector Machine predicting model, using the following training species: mustached bat, rat, mouse and gerbil.

Since the data from guinea pigs that are the species with a lower hearing range (from 125 Hz onwards, like porpoises) could not be used, the current prediction at the apex of the cochlea was likely above the real frequency. There were no data to train the model for the highest frequencies heard by harbour porpoises in the extreme base (up to 180 kHz). The mustached bats hear 105 kHz at 95% distance from the apex. Consequently, the current predictive model could not be trained for frequencies above 105 kHz. Thus, the frequency estimation of the most basal region of the cochlea of the harbour porpoise will likely be under-predicted.

5.2.3 Validation of the prediction for harbour porpoise

The morphometric data from individual cet 401A_Seamarco Pp02, a porpoise whose audiogram has been routinely measured using behavioural methods, was used separately to generate a prediction of its cochlear frequency map (Figure 8a). While this individual showed a hearing range between 125 Hz to 180 kHz, the prediction of its audiogram did not fully displayed the totality of the frequency range, likely due to the fact that the guinea pigs could not be incorporated and that there is no data above 105 kHz to train the model with.

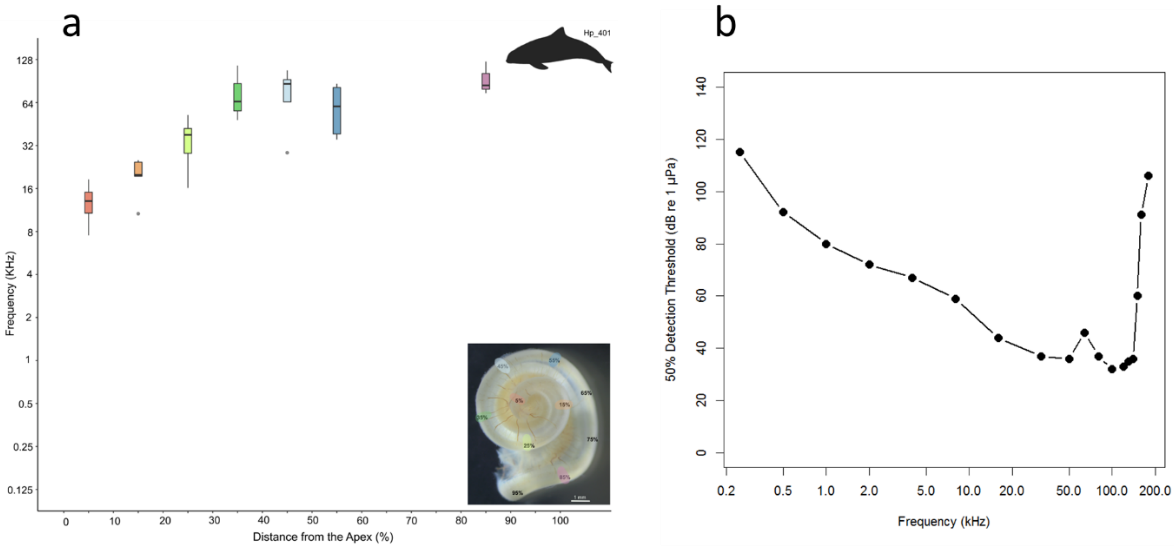

Figure 8. a) Prediction of the cochlear frequency map for cet 401A_Seamarco Pp02 using the Linear Support Vector Machine predicting model, b) audiogram from cet 401A_Seamarco Pp02 (Kastelein *et al*., 2010)

5.3 Predictive model 2: Random Forest

5.3.1 Cross-validation among terrestrial species

The same procedure that was followed for the Linear Support Vector Machine (section 5.2) model was adopted by the Random Forest method. Figure 9 shows the correlation between the predicted frequency (blue dots) and the published frequency maps (red lines) when the model was trained with data from the same species. The coefficient of correlation was at or above 0.95 for rat, mouse, and gerbil, at 0.81 for mustached bat, and 0.71 for guinea pigs. Although the correlation coefficients were slightly higher that with the Linear Support Vector Machine method, more data from guinea pigs is needed to have reliable predictions for this species.

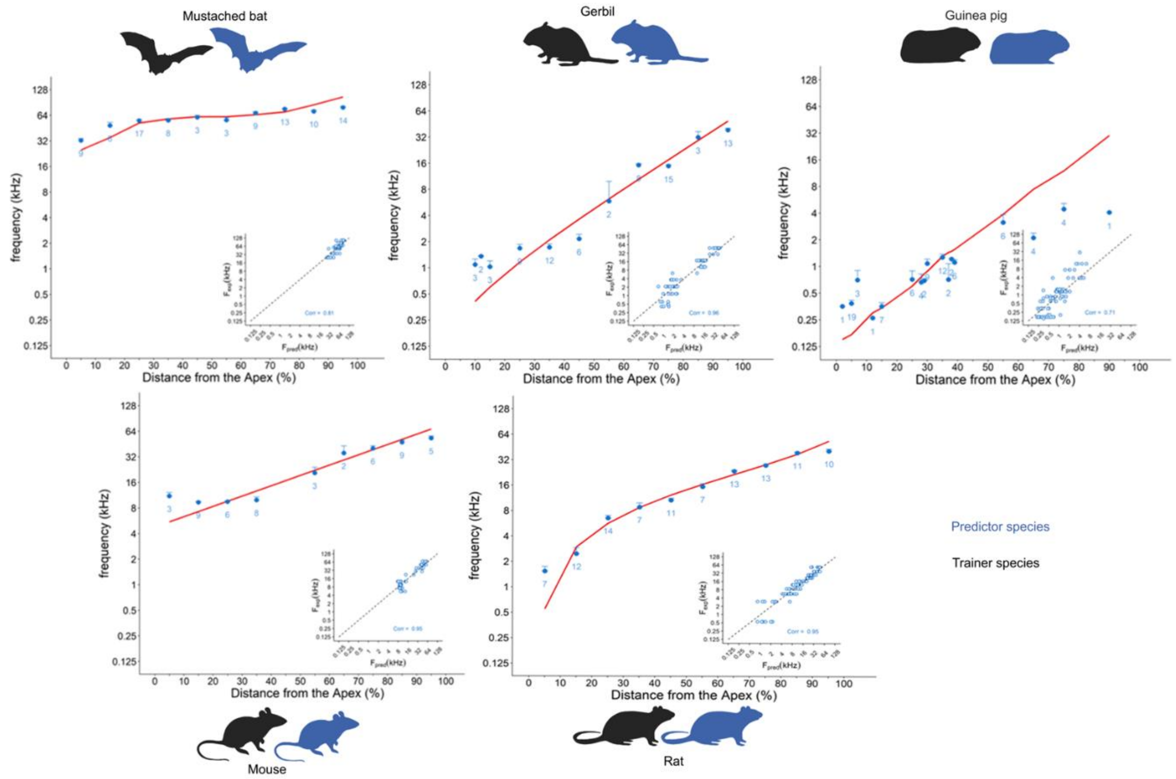

Figure 9. Correlation between predicted frequency (blue dots) and published frequency map (red line) for mustached bat (Kössl and Vater, 1985), rat (Müller, 1991), mouse (Müller and Smolders, 2005), gerbil (Müller, 1996) and guinea pig (Tsuji and Liberman, 1997) using the Random Forest model. The dataset of a particular species was tested for the same species.

The cross-validation of the Random Forest model across all the pairs of species (Figure 10) also showed that the predictive model can be applied among species of comparable hearing range. The predictions of guinea pig with other species were better than for the Linear Support Vector Machine algorithm. However, more morphometric data from the organ of Corti from guinea pigs should be gathered to be able to include them to the predictive model, also to further compare which predictive method performs better.

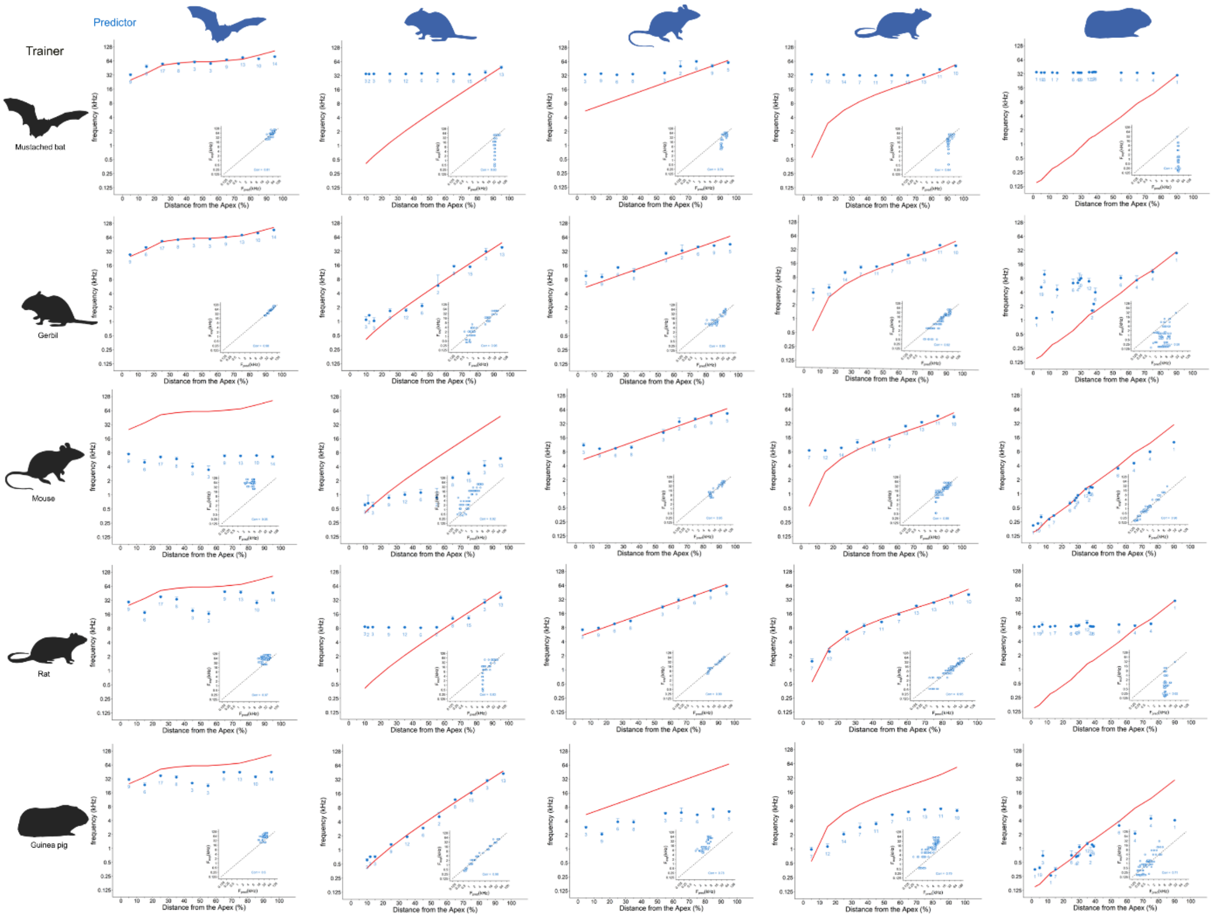

Figure 10. Cross-validation using the Random Forest model to test its performance and correlation among pairs of species. The predictive model was trained with the species in black and was used for the species in blue. The blue dots are the results of the prediction while the red line corresponds to the published cochlear frequency map for the species

5.3.2 Prediction of cochlear frequency map for harbour porpoise

Predictive model created for data from mustached bats, rats, mice and gerbils using Random Forest was used to predict the cochlear frequency map for harbour porpoise. The results obtained (Figure 11) are quite similar than with the previous model. While the frequencies predicted are in the frequency range from harbour porpoise hearing, and it is likely that the mid-frequency range is well predicted, more low frequency predictors should be added to have a better estimate of the frequency distribution of the apex.

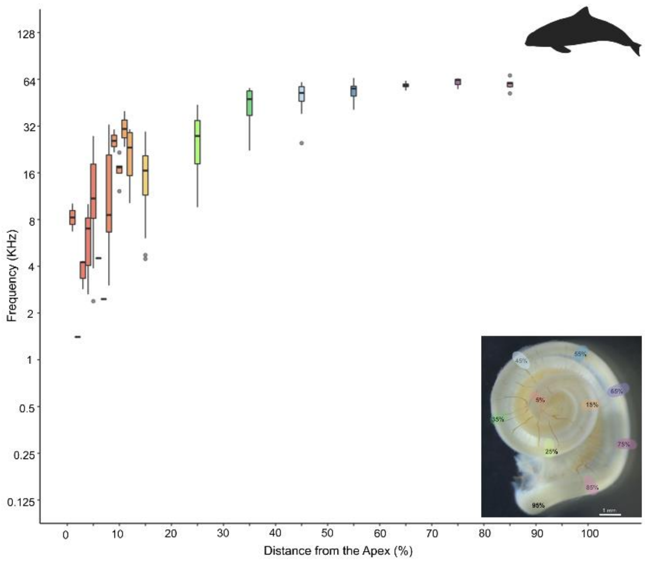

Figure 11. Prediction of the cochlear frequency map for harbour porpoise using the Random Forest predicting model, using the following training species: mustached bat, rat, mouse and gerbil.

5.3.3 Validation of the prediction for harbour porpoise

Prediction of the cochlear frequency map for individual cet 401A_Seamarco Pp02 (Figure 12) using the Random Forest model was possibly worse for the lowest frequency end than Linear Supporting Vector Machine. This validation will also help comparing among different mathematical models.

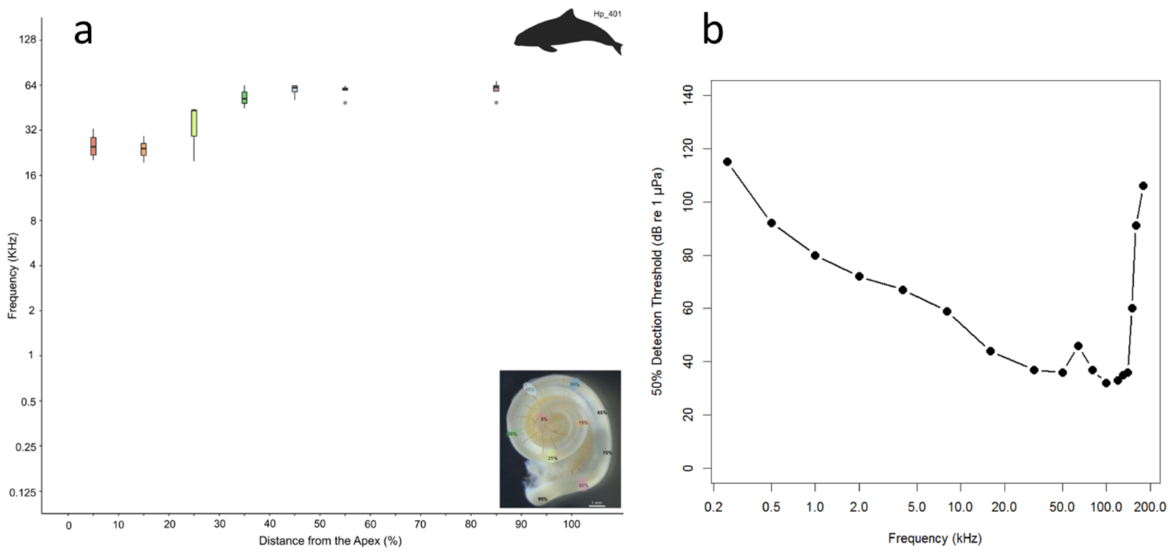

Figure 12. a) Prediction of the cochlear frequency map for cet 401A_Seamarco Pp02 using the Random Forest predicting model, b) audiogram from cet 401A_Seamarco Pp02 (Kastelein *et al.,* 2010)

6. Discussion

This study allowed including morphometric information of the sensory cells of the organ of Corti of two gerbils, seven guinea pigs and six harbour porpoises. The data from terrestrial mammals was used to make our predictive model stronger, especially for the low frequency ranges. While the data from gerbils was enough to complement our previous work for the species, the validation of our predictions with guinea pigs showed that there was not enough data from guinea pigs to make reliable predictions in any of the models. A priori, the use of the model Random Forest showed less error than Lineal Support Vector Machine. A higher number of inner ears from guinea pigs should be included in the analysis to have reliable predictive models. Thus, for the moment, we excluded the data from guinea pigs for the current predictions of the cochlear frequency maps for harbour porpoises. Once more data is added, several predictive models will be compared again using the leave-one-out cross-validation method and a final predictive algorithm will be chosen to be used for harbour porpoises and other marine mammal species.

We could add a finer resolution information for the apex of the cochlea of harbour porpoises, the location within the spiral that encodes for lower frequencies, since it is the region where it is expected to find the majority of the lesions, if they are associated to underwater anthropogenic sound exposure. New scanning electron micrographs from the five porpoises we had information from were taken every 2% of the cochlea from 0 to 12% of the apex. We processed the inner ears from six more porpoises and took detailed images from the apex, as well as from the rest of the spiral (every 10% from 15% onwards) to include these new data into the predictive model. By enlarging the dataset of harbour porpoise using individuals of different age, we can now better characterize the inter-individual variability.

We predicted the cochlear frequency map for harbour porpoise using two models (Random Forest and Linear Support Vector Machine) that were trained using the data from mustached bat, rat, mouse and gerbil. Since there were not enough low frequency data from terrestrial mammals, the model could not yet predict well the low frequency hearing end for harbour porpoise, according to published audiograms. However, it is expected that just by adding a few more cochleas from inner ears from guinea pig, it will be possible to adjust the low frequency end of the prediction. And the same for the extreme base, since there are no other animals of known frequency map that can hear up to 180 kHz, the extreme base was likely under predicted.

As observed before in our first preliminary predictions, we found a large region of the cochlea that codifies for the same frequency range, in both methods (see Figure 13 for comparison among predicting methods). The region extends from 45% to 95% from the apex, although we would expect that at 95% the frequencies encoded would be closer to 180 kHz. This area of enhanced frequency selectivity is usually called an auditory or acoustic fovea. An acoustic fovea is also found in cochlear frequency maps of echolocating bats (Kössl and Vater, 1985), and correspond to the frequencies of maximum hearing sensitivity for the species. In the current prediction of the cochlear frequency map for harbour porpoise, the frequencies associated to the acoustic fovea are slightly lower than the frequencies of maximum hearing sensitivity of harbour porpoises (Kastelein *et al.,* 2002). We will include a few more cochleas from mustached bats to the analysis to expand the dataset for the high frequencies.

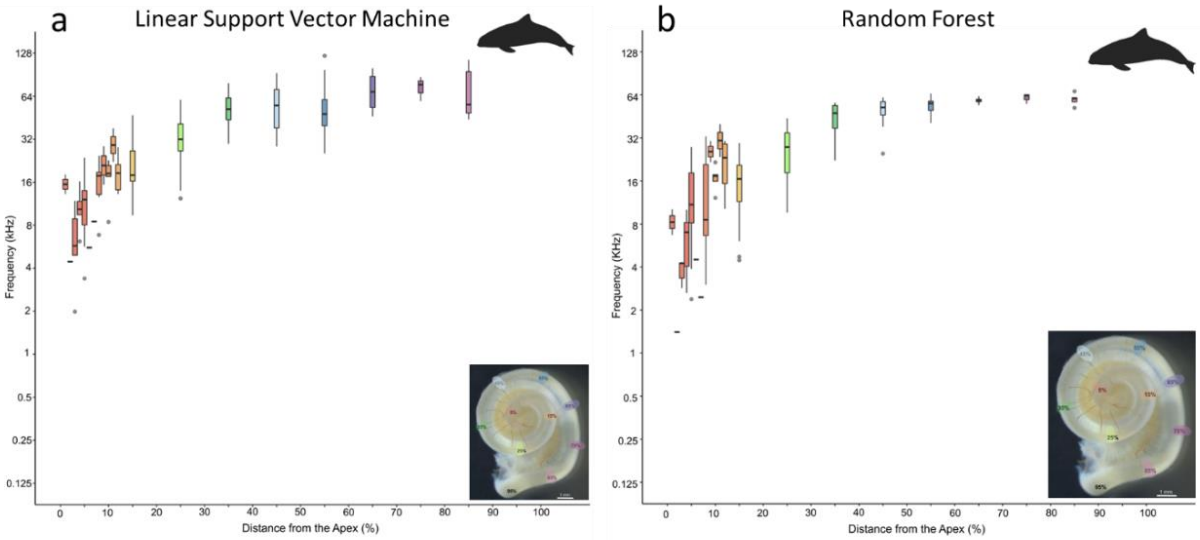

Figure 13. Prediction of the cochlear frequency map for harbour porpoise using the a) Linear Support Vector Machine (Figure 7), and b) Random Forest (Figure 11) predicting models, shown together for comparison. The training species were the mustached bat, rat, mouse and gerbil.

7. Conclusions and Implications for Conservation and Management of Small Cetaceans

This study shows that it is possible to extrapolate the correlation between morphometrics of the sensory cells of the organ of Corti and the associated frequency. This correlation can be extrapolated to other species of mammals, if they have a similar hearing range. By using morphometric data of the inner ear from terrestrial mammals of known frequency map, it is possible to create a predicting model that can then be applied for localizing the frequencies that are encoded in each location along the cochlear spiral in mammalian species in which we only have morphologic data. Although the results of the cross-validation of our predicting models show that we should include more data from guinea pigs, we expect that we can have the final prediction of the cochlear frequency map for harbour porpoises in the upcoming months, especially for the lower frequency hearing range of the species.

This research highlights the importance of **collecting the ears** during post-mortem examinations (see joint ASCOBANS/ACCOBAMS best practises protocol for more detailed information on the recommended protocols for ear extraction and fixation, IJsseldijk *et al.,* 2019). Information on inner ear analysis can be used for diagnosis purposes, such as to determine if the individual suffered a hearing impairment, and its more likely aetiologies.

This study represents a large step forward in predicting the cochlear frequency map for harbour porpoises. Once the map is finalized, it will be possible to extrapolate the frequencies that are impaired once lesions are found in the inner ear of harbour porpoises, and have better understanding of the consequences for individual to have a hearing loss in a particular hearing range. In cases of noise-induced hearing loss, it will be possible to extrapolate the frequency characteristics of potential sound sources that have triggered a damage. This map will be a crucial tool for management of the effects of underwater noise on hearing in porpoises, as well as improved decision-making in conservation plans for harbour porpoises and other marine mammals.

The implications of the including the study of the ears of harbour porpoises (and other marine mammals) in the post-mortem examinations**, combined with the prediction of the cochlear frequency maps** are:

1. Monitor the effects of mitigation measures of sound sources: If a mitigation measure has been implemented but animals strand after an operation, it is possible to detect newly formed lesions (Morell *et al.,* 2020) that can be related with the stranding, with the analysis of the inner ears. In cases of noise-induced hearing loss, the most likely sources can be identified once the cochlear frequency map is known. Thus, by including the analysis of the inner ear in the full post-mortem protocol, it will be possible to evaluate the efficacy of several mitigation measures that are currently being used.
2. Monitor the "acoustic health" of populations: The routine analysis of the inner ears allows detecting porpoise populations that might be at risk of over exposure or are more vulnerable and that need prioritizing for setting up specific conservation and management plans. The combination of ear analysis and frequency mapping allows detecting which are the potential sources that might have more influence in inducing permanent hearing loss and might need alternative mitigation measures.
3. Predict hearing ranges of marine mammals whose audiograms are not known yet: Once the cochlear frequency map is fully created and predicted for one species, such as for harbour porpoise, it can be then applied for other species in which we still have no information on their hearing capabilities, such as for example some species of beaked whales or baleen whales. This has large implications for underwater noise management, because it is challenging to predict effects without knowing what some species may be able to hear.
4. Bring information for the development of indicators within the descriptor 11 of the Marine Strategy Framework Directive (MSFD): Descriptor 11 emphasizes on the severity of noise pollution in the marine environment and the need to understand and mitigate its impact on the ecosystem. The cochlear frequency maps can be a tool used by regulators to better understand the consequences of hearing loss at certain frequencies, which is important for determining or predicting ecological impacts in their feeding behaviour, detection of threats (nets, predators, or beaches), communication or navigation. At the same time, cochlear frequency maps can allow identifying potential sources that trigger a damage in cases of noise-induced hearing loss, which is essential for monitoring the effectiveness of mitigation measures (as highlighted in points 1 and 2) as well as detecting sources that can affect certain populations that are more vulnerable and might need more immediate action.

Acknowledgements

We would like to thank Lonneke IJsseldijk (Utrecht University), Jing Wang (INM) and Artëm Djuba (INM) for providing the samples from harbour porpoises, guinea pigs, and gerbils, respectively that were used for this research. The inner ears from mustached bats were kindly provided by Manfred Kössl (Goethe University), from rats by Marc Lenoir (INM) and Wayne Vogl (University of British Columbia), from one of the harbour porpoises by Martin Haulena (Vancouver Aquarium) and Stephen Raverty (Animal Health Center), and from another harbour porpoise by Ron Kastelein (Seamarco). We also thank Javier Latorre Sánchez who draw the silhouettes of the mammals for the figures used in this report. This study was funded by ASCOBANS (SSFA-ASCOBANS-2022-002_TiHo).

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