THE VOCALIZATION MECHANISM OF THE FLORIDA MANATEE (*TRICHECHUS MANATUS LATIROSTRIS*)

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ABSTRACT

The mechanism by which Florida manatees produce vocalizations is unknown. Anatomically, the laryngeal region in manatees lacks clearly defined vocal folds. Initially we developed a method to visualize the entire manatee upper respiratory system. We then forced air through fresh necropsied manatee larynxes and generated artificial vocalizations which closely duplicated the normal vocalizations produced by live manatees, both in fundamental frequency and structure of harmonics. Here we report that sound is generated in the larynx when air vibrates bilateral strips of tissue embedded in the lateral laryngeal walls which are in close approximation anteriorly but which diverge posteriorly. We propose that these strips of tissue are the modified vocal folds containing ligaments and we support this through histological stained sections and because they are connected anteriorly to the posterior side of the thyroid cartilage and posteriorly with the arytenoidal cartilages. We also suggest that these vocalizations are then modified within the resonance cavities in the frontal area of the head and the air used to generate these vocalizations also causes a transient deformation of this region before being conserved and returned to the lungs.

1. INTRODUCTION

Vocalizations produced by the Florida manatee (*Trichechus manatus latirostris*) consist of complex acoustical phenomena which have many variable components (O'Shea and Poche, 2006) and may constitute a form of communication (Schevill and Watkins, 1965; Sonda and Takemura, 1973; Hartman, 1979; Steel, 1982; Bengtson and Fitzgerald, 1985). Our team has been studying various aspects of manatee vocalizations for the past ten years and we have recorded hundreds of individual vocalizations. These vocalizations vary widely in primary

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frequency, structure of harmonics, duration and intensity. The fundamental frequency of manatee vocalizations falls into the audible range of human hearing, but harmonics may reach well into the ultrasonic range, (up to 60,000 Hz). Individual vocalizations may also have between 3 and 24 harmonics (O'Shea and Poche, 2006).

In early anatomical studies, it was suggested that the laryngeal apparatus in manatees lacks true vocal cords, also known as vocal folds. Murie (1872) was first to describe the various laryngeal cartilages present in manatees, including the thyroid, cricoids and arytenoids. Hill (1945) clearly states that "There are no vocal cords" in manatees and goes on to describes this region as " the aryteno-epiglottal fold". In 1965 Harrison and King also concurred with the findings of Hill (1945) by reporting that the manatee larynx does not contain true vocal cords but instead suggested that the fleshy ligamentous fibro-elastic tissue situated anterior to the larynx may serve this function.

Because vocal cords, also known as vocal folds, are the common mechanism of vocalization in most mammals, these reports raised lingering questions about the true nature of the anatomical structures responsible for manatee vocalizations. Although much is now known about the characteristics of manatee vocalizations, almost nothing is known about the mechanism of their generation (O'Shea and Poche, 2006). In the mammalian respiratory system sound is generated when flowing air under pressure passes between elastic structures. These elastic structures, equivalent to vocal folds, would have to possess sharp medial edges capable of changing shape under muscle control and would have to separate under expiratory air flow, there after springing back thus generating recurring cycles of sound waves (Fink, 1975). To demonstrate this process during manatee vocalizations we studied 10 necropsied manatee larynxes in vitro by forcing puffs of air under controlled pressure through them, thus producing sound. Our results closely mimicked recorded vocalizations from living manatees, both in fundamental frequency and structure of harmonics. Our findings also suggested why during vocalizations in living animals no air is released but a shape change does take place in the head region.

2. MATERIALS AND METHODS

2.1. Silicon Modeling of the Manatee Respiratory Systems

The following is an abridged description of the method that we developed to allow visualization of the internal dimensions of both a dog and two manatee respiratory tracts. A complete description of the method and results can found in Grossman *et al*. (2011) in "On Line Journal of Biological Science, 2011. Liquid silicone in the form of MoldRite 25 (SP700501R) (Environ Molds, ArtMolds Sculpture Studio, New Jersey) mixed with 10% catalyst (by weight) was injected into the upper and lower respiratory tract of two small manatee carcasses that were made available to us at the Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, Marine Mammal Pathobiology Laboratory in St. Petersburg Florida. Manatee 1 (MSW0780, necropsy date Oct 7-8, 2008) was a female, 144 cm long; death was from natural causes. Manatee 2 (MNW0848, necropsy date Oct 7-8, 2008) was also a female, 158 cm long, with a body weight of 88.5 kg; death was from natural causes. In both animals we injected the silicone through tracheal slits and also through their nares (**Fig. 1**). In the smaller manatee calf carcass we also injected silicone into both primary bronchi as well as up into the laryngeal region. Both treated carcasses were then placed under refrigeration for 24 h to allow the silicone to cure and we then opened the respiratory structures by dissection. We then withdrew the cured models from the larynx, trachea, primary bronchi and esophagus in both animals by gently pulling it out of these structures. We soaked the silicone models in a dilute solution of Clorox and rinsed them in water. The resulting cured models have proven to be sufficiently flexible to allow for relative ease of extraction, yet robust enough to withstand repeated handling and examination.

2.1. Sound Recordings

2.1.1. On Site, Shure SM58 Air Microphone

For the recordings of sounds produced when air was forced through excised manatee larynxes *in vitro* we employed a Shure SM58 air microphone (see upper middle of **Fig. 2B**) which has an upper frequency limit of 15 kHz at 5dB. These sounds were all recorded using a Fostex Model DV 40 DVD-RAM Master Recorder (Fostex Company, a division of Foster Electric Co, Ltd) that was capable of recording two track sound files. These recordings where then transferred to our laboratory computers (one of which was running Cool Edit Pro 2.0 and the other Adobe Audition 1.5) and the duration, fundamental frequency and number of harmonics visualized. For back up and cataloguing, we also recorded these same sounds using two mini disc recorders (Tascam MD-350) which had a sensitivity range that only extended to 20 kHz. We then converted our manatee vocalizations that had been recorded as raw strength decibels (dB) in water into calibrated strength levels by adjusting the dB measurements by an additive factor of 144.80. This factor was generated from calibrated sound source measurements made in water. These numbers are reported in **Tables 1**

and 2 as Calibrated Strength Levels and are equivalent to 1 microwatt per square meter (1 μWatt/m^2). For the recordings of sounds produced when air was forced through excised manatee larynxes *in vitro* we converted these raw dB measurements into calibrated strength levels by adjusting the dB measurements by an additive factor of 118.95. This factor was generated from calibrated sound source measurements made in air. The numbers are reported in **Table 3** as Calibrated Strength Levels and are equivalent to 20 μWatt/m^2.

2.2. Off Site, Aquarian Hydrophone

For the last 3 years we have been recording manatee vocalizations and other underwater sounds at off site locations where manatees are in captivity. These locations were the Cincinnati Zoo in Cincinnati OH; the Columbus Zoo and Aquarium in Columbus, OH; The Parker Manatee Aquarium at the South Florida Museum in Bradenton FL and Homosassa Springs Wildlife State Park in Homosassa FL. At each of these locations one underwater hydrophone (Aquarian Audio H2A-XLR) was installed, with the exception of Homosassa which had two hydrophones in place. Each hydrophone was enclosed in a PVC pipe with holes drilled in it to allow water to enter the pipe and conduct sounds from the water to the hydrophone. The PVC pipes were secured to the wall of the manatee enclosures to prevent the manatees from playing with or damaging the units or hurting themselves.

Fig. 1. Injection of liquid silicon into a manatee carcasses at the florida fish and wildlife research institute, marine mammal pathobiology laboratory in St petersburg florida. The silicon is being injected into one of the nares. The tracheal region has also been opened. A modified automobile grease gun is being used to inject the silicon Grossman *et al*. (2011)
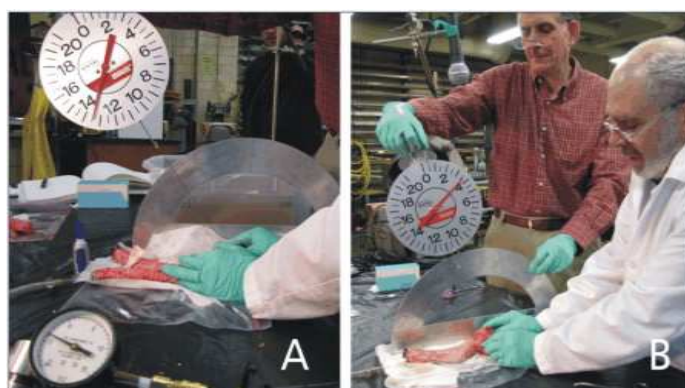
Fig. 2. A-B One experiment is presented during which sound is being generated *in vitro* when air was forced through a fresh manatee larynx. Air pressure, tension and angle had to be constantly adjusted until the experimental conditions were correct to maximize sound production from each larynx under study. **Figure 2A and B** were made at different times during the same experiment using the same larynx. In **Fig. 2A** it can be seen that this larynx has two long 26 primary bronchi, one of which is fitted with a rubber cork pierced by a glass tube attached to the air supply. The other bronchus is fitted with a solid rubber cork. The pressure of the air entering the larynx is measured with a pressure gauge seen in the lower left. Here the pressure reading is 3.8 psig (pounds/in^2 gauge pressure). The tension that is being applied to the arytenoid cartilages closing them together is measured on the tension scale at 13.5 N (newtons) and the angle applied to the sutures as measured on the steel protractor is 45° from the horizontal. Note also that the larynx is being held in place to stabilize it, while mild finger pressure is being applied. In **Fig. 2B** the tension now being applied is 3.5 N and the angle of the tensioning suture is 80° from the horizontal. The Shure SM58 air microphone used to record the experimental vocalizations can be seen at the top middle

Table 1. Normal manatee vocalization recorded data: Here we present the data for 109 manatee vocalizations recorded between 2009-2010 from living animals housed at Manatee Springs of the Cincinnati Zoo. Because various animals were housed together we do not know which animals are responsible for these individual calls, but only the date and time when the calls were recorded. For each vocalization, the fundamental frequency as well as one or more harmonic structures are presented. Sound pressure calibration strength was based on reference vocalizations and was calculated in water by adjusting the dB of the recorded vocalization by an additive factor of 144.80. The multiplier and the strength of the harmonics are also provided. See text for details

Recording ID	FILE/RUN date	FUND FREQ	CALIBR STREN	1st PTL FREQ	MULT	CALIBR STREN	2nd PTL FREQ	3rd PTL FREQ	4th PTL FREQ	Compare with (**Table 3**)
CIN-1a	5/11/2009	2484	114.600	4921	1.981	102.48	7406	9890	12370	
CIN-1b	5/11/2009	3140	124.240	6328	2.015	102.81	9468	12600		
CIN-1c	10/16/2009	2885	102.750	5770	2.000	93.08				
CIN-1d	10/16/2009	2713	104.300	5426	2.000	90.08				
CIN-1e	10/16/2009	2670	110.370	5340	2.000	93.46				
CIN-1f	10/16/2009	2605	94.950	5211	2.000	83.46				
CIN-1g	10/16/2009	2713	104.130	5447	2.008	88.38				
CIN-1h	10/16/2009	2411	114.740	4823	2.000	85.09				LRX-1
CIN-1i	10/16/2009	2583	108.530	5167	2.000	83.51				
CIN-2a	9/30/2010	2993	100.420	5986	2.000	88.95				
CIN-2b	9/30/2010	3122	116.820	6093	1.952	78.24				
CIN-2c	9/30/2010	3036	97.550	6072	2.000	99.64				
CIN-2d	9/30/2010	2993	95.030	5986	2.000	88.85				
CIN-2e	9/30/2010	3036	98.750	6072	2.000	87.90				
CIN-2f	9/30/2010	3079	102.120	6093	1.979	90.05				
CIN-2g	9/30/2010	3100	98.020	6158	1.986	79.61				
CIN-2h	9/30/2010	3079	97.830	6136	1.993	77.73				
CIN-2i	9/30/2010	2282	87.950	4608	2.019	99.46				
CIN-2j	9/30/2010	2153	92.920	4586	2.130	100.93				
CIN-2k	9/30/2010	2131	90.510	4715	2.213	88.24				
CIN-2l	9/30/2010	2368	91.080	4500	1.900	99.18				
CIN-2m	9/30/2010	2411	102.840	4780	1.983	88.45				
CIN-2n	9/30/2010	3014	108.820	6029	2.000	87.18				
CIN-2o	9/30/2010	2347	95.220	4629	1.972	103.98				
CIN-2p	9/30/2010	2325	98.870	4694	2.019	97.26				LRX-3
CIN-2q	9/30/2010	2304	95.450	4586	1.990	100.44				
CIN-2r	9/30/2010	2239	94.730	4521	2.019	99.78				
CIN-3a	12/31/2010	3617	105.860	5447	1.506	107.73				
CIN-3b	12/31/2010	1851	106.030	3682	1.989	99.76	5534			
CIN-3c	12/31/2010	1937	101.810	3854	1.990	108.54				
CIN-3d	12/31/2010	1873	100.070	3746	2.000	108.04				
CIN-3e	12/31/2010	1916	110.300	3854	2.011	104.55				
CIN-3f	12/31/2010	1808	106.630	3617	2.001	102.59				
CIN-3g	12/31/2010	1722	97.040	3466	2.013	113.31				
CIN-3h	12/31/2010	1873	105.840	3725	1.989	107.82	5598			
CIN-3i	12/31/2010	1937	93.470	3811	1.967	111.97	5727			
CIN-3j	12/31/2010	2045	115.220	4091	2.000	109.72				
CIN-3k	12/31/2010	2002	92.730	3983	1.990	99.30	5986			
CIN-3l	12/31/2010	3596	97.440	6007	1.670	75.18				
CIN-3m	12/31/2010	1873	109.010	3746	2.000	115.75	5684			
CIN-3n	12/31/2010	1937	111.700	3897	2.012	105.48	5857			
CIN-3o	12/31/2010	2024	93.580	4005	1.979	103.87	5986			
CIN-3p	12/31/2010	1937	106.790	3875	2.001	107.84	5813			
CIN-3q	12/31/2010	1894	99.620	3789	2.001	86.86				
CIN-3r	12/31/2010	1937	108.670	3854	1.990	108.67				
CIN-3s	12/31/2010	1894	100.340	3811	2.012	107.70				
CIN-3t	12/31/2010	2002	110.550	4005	2.000	101.15				
CIN-3u	12/31/2010	1873	100.080	3725	1.989	114.06				
CIN-3v	12/31/2010	2042	106.950	4048	1.982	101.63				
CIN-3w	12/31/2010	1937	100.740	3854	1.990	103.22				
CIN-3x	12/31/2010	1959	99.210	3919	2.001	103.75				
CIN-3y	12/31/2010	1808	102.270	3617	2.001	103.00				
CIN-3z	12/31/2010	1830	99.090	3660	2.000	108.89				

Table 1. Continue

CIN-3aa	12/31/2010	1830	104.320	3660	2.000	113.190	
CIN-3ab	12/31/2010	1873	107.570	3789	2.023	106.180	
CIN-3ac	12/31/2010	2002	109.400	3983	1.990	109.890	
CIN-3ad	12/31/2010	1937	107.100	3875	2.001	104.680	
CIN-3ae	12/31/2010	2024	114.790	4048	2.000	105.030	
CIN-3af	12/31/2010	2002	112.370	4026	2.011	109.400	
CIN-3ag	12/31/2010	1894	101.740	3789	2.001	109.820	
CIN-3ah	12/31/2010	1959	108.960	3940	2.011	104.640	
CIN-3ai	12/31/2010	2002	110.470	3983	1.990	111.820	
CIN-3aj	12/31/2010	1873	105.390	3768	2.012	104.850	
CIN-3ak	12/31/2010	2045	114.970	4069	1.990	111.150	
CIN-3al	12/31/2010	2002	113.450	4026	2.011	114.410	
CIN-3am	12/31/2010	2002	104.950	4005	2.000	98.940	
CIN-3an	12/31/2010	1981	99.190	3962	2.000	94.930	
CIN-3ao	12/31/2010	1937	102.650	3854	1.990	102.020	
CIN-3ap	12/31/2010	1916	97.970	3832	2.000	95.580	
CIN-3aq	12/31/2010	1873	102.310	3725	1.989	108.240	
CIN-3ar	12/31/2010	1959	108.480	3919	2.001	120.050	
CIN-3as	12/31/2010	1959	103.620	3919	2.001	107.360	
CIN-3at	12/31/2010	2024	108.950	4026	1.989	91.526	6050
CIN-3au	12/31/2010	1851	96.770	3703	2.001	99.770	
CIN-3av	12/31/2010	1937	102.150	3897	2.012	100.750	
CIN-3aw	12/31/2010	1894	102.550	3789	2.001	100.550	
CIN-3ax	12/31/2010	1959	107.410	3919	2.001	113.250	
CIN-3ay	12/31/2010	1894	101.670	3811	2.012	102.260	
CIN-3az	12/31/2010	1894	98.550	3789	2.001	102.470	
CIN-3ba	12/31/2010	1873	107.410	3768	2.012	110.660	
CIN-3bb	12/31/2010	1916	101.170	3832	2.000	110.820	
CIN-3bc	12/31/2010	1851	92.800	3725	2.012	112.750	
CIN-3bd	12/31/2010	2045	113.800	4069	1.990	110.520	
CIN-3be	12/31/2010	2002	98.130	4005	2.000	104.360	
CIN-3bf	12/31/2010	1916	101.350	3832	2.000	103.220	
CIN-3bg	12/31/2010	1959	106.180	3919	2.001	100.750	
CIN-3bh	12/31/2010	1937	106.280	3897	2.012	107.120	
CIN-3bi	12/31/2010	2045	104.280	4091	2.000	104.280	LRX-2
CIN-3bj	12/31/2010	1873	102.380	3746	2.000	103.050	
CIN-3bk	12/31/2010	1808	101.320	3617	2.001	102.440	
CIN-3bl	12/31/2010	1808	103.090	3617	2.001	99.770	
CIN-3bm	12/31/2010	1873	94.310	3746	2.000	104.000	
CIN-3bn	12/31/2010	1679	104.490	3359	2.001	105.880	LRX-10
CIN-3bo	12/31/2010	1894	110.070	3768	1.989	112.780	
CIN-3bp	12/31/2010	1916	103.560	3832	2.000	101.450	
CIN-3bq	12/31/2010	1981	92.990	3962	2.000	97.180	
CIN-3br	12/31/2010	1916	98.980	3854	2.011	89.770	
CIN-3bs	12/31/2010	1959	95.030	3897	1.989	101.410	
CIN-3bt	12/31/2010	1916	89.320	3875	2.022	91.320	
CIN-3bu	12/31/2010	1937	95.670	3897	2.012	100.160	
CIN-3bv	12/31/2010	1808	106.760	3617	2.001	94.070	
CIN-3bw	12/31/2010	1916	101.220	3832	2.000	93.970	
CIN-3bx	12/31/2010	1894	92.860	3789	2.001	90.640	
CIN-3by	12/31/2010	1851	92.460	3531	1.908	102.660	
CIN-3bz	12/31/2010	1894	116.320	3768	1.989	109.660	
CIN-3ca	12/31/2010	1873	119.270	3768	2.012	111.350	
CIN-3cb	12/31/2010	1916	94.230	3811	1.989	106.490	
CIN-3cc	12/31/2010	1894	104.870	3897	2.058	111.350	
CIN-3cd	12/31/2010	3014	99.580	6007	1.993	86.520	
MEAN	2147.0	4267.9	1.9930				
STDEV	432.1	788.0	0.0640				
%STDEV	20.13	18.46	3.22				

Table 2. Low-frequency manatee vocalizations data: Here we present six low frequency manatee vocalizations (below the mean of 1500 Hz) recorded in 2010 from living manatees housed variously at the Cincinnati Zoo (CIN) and at the Parker Aquarium of the South Florida Museum (SFLA) in Bradenton Florida. Sound pressure calibration strength was based on reference vocalizations and was calculated in water by adjusting the dB of the recorded vocalization by an additive factor of 144.80. Once again because these animals were housed together we do not know which animal was responsible for the recorded vocalizations. See text for details

Recording ID	FILE/RUN date	FUND FREQ	CALIBR STREN	1st PTL FREQ	MULT	CALIBR STREN	2nd PTL FREQ	3rd PTL FREQ	4th PTL FREQ	Compare with (Ref. **Table 3**)
CIN-a	10/1/2010	1426.00	81.20	2877.00	2.018	61.65				
CIN-b	10/1/2010	1254.00	80.31	2414.00	1.925	72.12	4828			LRX-4, LRX-8, LRX-9
CIN-c	10/1/2010	1337.00	86.29	2112.00	1.580	68.92				
SFLA-a	2/1/2010	1270.00	116.29	2540.00	2.000	90.54				
SFLA-b	3/1/2010	947.40	109.29	1873.00	1.977	91.59	2820	4629	5534	LRX-5, LRX-6, LRX-7
SFLA-c	3/1/2010	1378.00	100.68	2756.00	2.000	110.49	4091	5512		
MEAN		1268.70		2428.70	1.917					
STDEV		170.20		382.00	0.168					
%STDEV		13.41		15.73	8.770					

Table 3. Trial vocalization data: This table contains the results of the experimental vocalization studies from our 10 necropsied larynxes collected over the period of 2009 to 2011. The fundamental frequencies generated *in vitro* by our larynx tissue varied from a high of 2449 Hz to a low of 796.8 Hz. For each of these 10 results additional clear harmonic structures with multipliers of 2 and 3X could also be identified and for 8 of these results another clear harmonic structure with a multiplier of 4X could also be identified as well (LRX #s 1, 2, 3, 4, 5, 6, 7, 8). Sound pressure calibration strength was based on reference vocalizations and was calculated in air by adjusting the dB of the recorded vocalization by an additive factor of 118.95 (see text for details). Based on the appropriate mean of the fundamental frequencies (1500 Hz), 24 the larynxes with fundamental frequencies above the mean (marked as "+"), were classified as producing Normal Frequency Experimental Vocalizations (LRX #s 1, 2, 3 and 10; 2173 Hz n = 4) and the larynxes with fundamental frequencies below the mean (marked as "-"), were classified as producing Low-Frequency Experimental Vocalizations (LRX #s 4, 5, 6, 7, 8 and 9; 1057 Hz n = 6). The data generated from LRX #3 can also be seen visually in **Fig. 8**. However, although LRX #3 generated 7 harmonics (which can be seen in **Fig. 8**), in this spread sheet we list only the first three harmonics for LRX #3 (4898 Hz, 7359 Hz and 9808 Hz.) to reduce the number of columns in the spread sheet. The additional 4 harmonics generated by LRX #3 are reported in **Fig. 8** and in the text

Larynx (LRX) #		Animal ID	FILE/RUN DATE	FUND FREQ	CALIBR STREN	1st PTL FREQ	MULT	CALIBR STREN	2nd PTL FREQ	3rd PTL FREQ	Compare with (Ref. **Table 1 and 2**)
1	+	LPZ102645	8/28/2009	2425.0	95.61	4839.0	1.995	77.750	7253		CIN-1h **Table 1**
2	+	SWFTM0921b	9/30/2009	2144.0	78.11	4289.0	2.000	77.810	6433	8542	CIN-3bi **Table 1**
3	+	MSW1002	1/15/2010	2449.0	63.50	4898.0	2.000	59.010	7359	9808	CIN-2p **Table 1**
4	-	MEC1003	1/15/2010	1253.0	53.17	2507.0	2.001	56.000	3761		CIN-b **Table 2**
5	-	MSTM1002	1/15/2010	1101.0	79.52	2203.0	2.001	66.010	3304	4406	SFLA-b **Table 2**
6	-	MNW1019	3/11/2010	878.9	85.97	1769.0	2.013	92.440	2648	3539	SFLA-b **Table 2**
7	-	MSE1060	3/11/2010	796.8	69.90	1582.0	1.985	73.390	2390	3175	SFLA-b **Table 2**
8	-	SWFTm1016b	5/6/2010	1183.0	87.58	2378.0	2.010	90.480	3562	4746	CIN-b **Table 2**
9	-	MSE1136	2/17/2011	1160.0	71.64	2332.0	2.010	81.260	3492	4664	CIN-b **Table 2**
10	+	MSE1146	4/4/2011	1675.0	82.50	3339.0	1.993	76.300	5015	6691	CIN-3bn **Table 1**
		MEAN		1506.6		3013.6	2.001				
		STDEV		625.2		1247.1	0.008				
			%STDEV		41.50		41.380	0.420			

Each of these hydrophones was connected with Mogami microphone cable (2354 Star Quad Mic Cable) to a small Behringer XENYX 802 mixer amplifier attached to a paired BARIX Instreamer 100 encoder that converted the analog acoustical signal into a compressed data stream set to MPEG 1 Layer 3 (MP3), 48 kHz sample rate, monaural, resulting in an approximately 64bps data rate connected to a modem with a static IP address. At the Cincinnati Zoo the provider was Time Warner Cable, at South Florida it was Brighthouse, at Homosassa it was Century Link. The data stream was then transmitted via the internet to our laboratory at Xavier University where it was then received by the other paired Barix Exstreamer decoder which reconstructed the analog signal. The data was then passed into a specialized computer where it was segmented by MediaLogger (OMT Technologies) software which saved sequential half hour files stamped by time and day for later review. These sound files were then transferred through our internal laboratory network to our editing computers. Research students and faculty in our laboratory could then

access these stored files and count manatee vocalizations in 30 min intervals which allowed them to visualize call duration, fundamental frequency, structure of harmonics and other specific characteristics. As with the On-Site Vocalization recordings we also converted these Off-Site Vocalizations made in water from raw dB into calibrated strength sound levels. The resultant calibrated strength sound levels are reported in **Table 1 and 2**.

2.3. Manatee Larynxes Utilized for this Study and the Method Employed for In Vitro Sound Generation

Manatee larynxes were obtained from manatees as listed in **Table 4** under US Fish and Wildlife permit # MA049136-3, expiration 4/14/2014. Necropsies were performed at the Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, Marine Mammal Pathobiology Laboratory in St. Petersburg Florida. Over a period of three years (2009 - 2011) we studied sound generation in 10 fresh manatee larynx specimens. When a manatee carcass arrived at the Pathobiology Laboratory in Florida, it would be necropsied for cause of death and the larynx then removed and immediately shipped in ice overnight to our laboratory in the Department of Biology at Xavier University where it was washed in ice cold saline solution and stored (but not frozen) in saline solution in a refrigerator until used in our research. Prior to their use each larynx was inspected for any sign of deterioration identified by discoloration or unpleasant odor. During the course of the three years of the study only one such larynx was found to have extensive tissue deterioration and is therefore not included in the data set. After a larynx was used for sound generation it was fixed in 10% formalin and permanently stored in the laboratory. The deteriorated larynx was also fixed and stored. Animal and larynx identification numbers, dates of animal collections, larynx run dates, animal body weights, body lengths and causes of death are all presented in **Table 4**. When larynxes arrived visual inspection clearly indicated that they were of various sizes. Unfortunately the weight of the larynx tissue could not be utilized for comparison purposes because the gross anatomy of the larynxes that arrived in our laboratory varied as a result of differences in removal methods. For example, some larynxes arrived with both bronchi attached (**Fig. 2A, B and 3A**) while others came with only part of the trachea (**Fig. 4**).

Table 4. Information on animals that provided larynxes: This table contains the following information on the manatees that provided the larynxes that were utilized in these studies: Collection dates, causes of death if known, location where the animal carcasses were collected, body weights, body length, sex, animal identification numbers and larynx identification numbers. The larynx run date is the date when the larynx was used in the vocalization study.

Larynx (Lrx) #	Animal ID	Date of collection	Larynx run date	Pertinent information
1	LPZ102745	8/26/2009	8/28/2009	Female, 255 cm long, 330 kg BW Chronic watercraft, St Marks River
2	SWFTM0921b	9/21/2009	9/30/2009	Male, 286 cm long, 317 kg BW Watercraft Injury, Marco Island, Gulf of Mexico
3	MSW1002	1/13/2010	1/15/2010	Male, 184 cm long, 115.5 kg BW Human related cause of death, other (ingestion and obstruction with plastic monofilament line), Caloosahatchee River, N. Ft Myers
4	MEC1003	1/13/2010	1/15/2010	Male, 185 cm long, 124 kg BW Cold stress, Indian River in Cocoa
5	MSTM1002	1/13/2010	1/15/2010	Female, 201 cm long, 167 Kg BW Undetermined cause of death (possible cold stress and traumatic entrapment), Indian River Port St. Lucie
6	MNW1019	3/2/2010	3/11/2010	Female, 209 cm long, 193 kg BW Cold stress, Braden River in Bradenton
7	MSE1060	3/2/2010	3/11/2010	Female, 331 cm long, 624 kg BW Inconclusive necropsy findings, middle river FT. Lauderdale
8	SWFTm1016b	5/3/2010	5/6/2010	Female, 260 cm long, 297.5 kg BW, watercraft injury Avery bayou, palm harbor
9	MSE1136	2/15/2011	2/17/2011	Male, 292 cm long, 422 kg BW Chronic watercraft, Indian River Ft. Pierce
10	MSE1146	3/28/2011	4/4/2011	Male, 210 cm long, 195.5 kg BW Chronic watercraft, tarpon basin, key largo
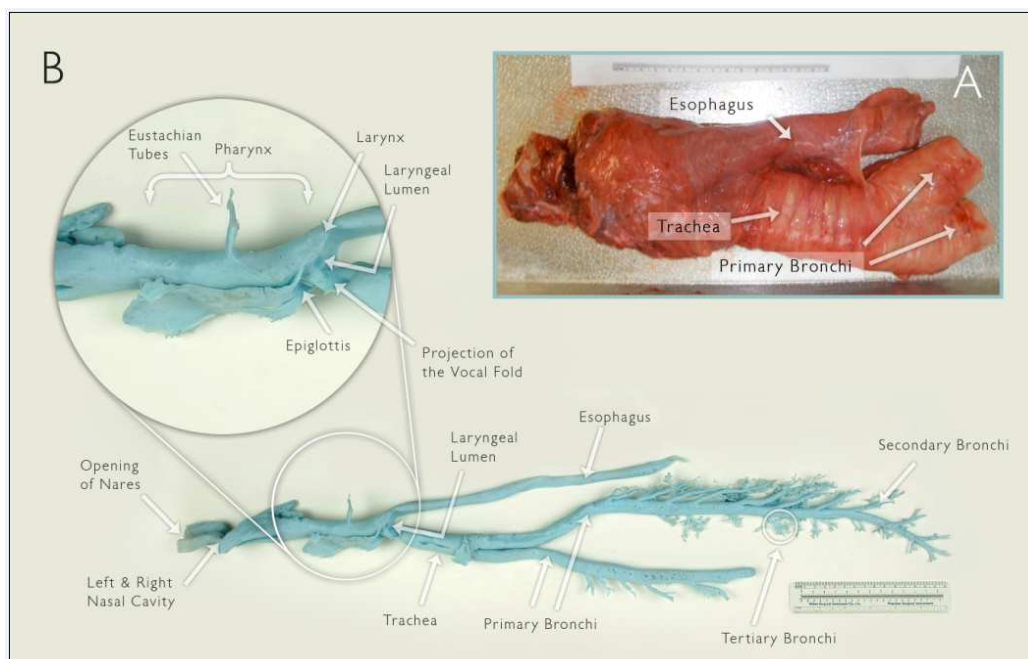
Fig. 3. And insets A and B The bottom view is of the silicon model of the internal volume of the respiratory tract from manatee MSW0780. The left and right nasal cavities can be seen, as well as the short trachea, left and right primary bronchi which are long and straight and secondary and tertiary bronchi. **Figure 3B** (inset expanded view) is of the silicon model of the glottis and pharynx. Note the presence of one of the very large Eustachian tubes (the other is on the opposite side of the model). **Figure 3A** (inset) is of a fresh manatee larynx positioned in an orientation which is similar to the silicon model. Note the short trachea which bifurcates into the left and right primary bronchi. Here the esophagus is located ventrally. The pharyngeal opening is to the left Grossman *et al.* (2011)

Fig. 4. This is a manatee larynx which had only a short piece of trachea attached. A rubber stopper has been inserted into the trachea and fixed in place with super glue. The glass tube will be attached to a compressed air source. A glass plug is blocking a second, unneeded hole in the rubber stopper

After each of the 10 larynxes were fixed in formalin they were measured for both pharyngeal circumference (at the glottal region) and tracheal circumference (at the pharyngeal attachment) and these results can be found in **Table 5A**. The purpose of these measurements will be described in the discussion section. In any case, in order for us to force air into the fresh larynx under study we fitted rubber stoppers into the cut ends of both the left and right bronchi (**Fig. 2A and 2B**) or into the short trachea (**Fig. 4**), but because the diameters of the bronchi or trachea varied with the size of the individual larynxes, we had on hand a variety of rubber stoppers that varied in size from nos. 3 to 7 which we could insert for a tight fit depending on the diameters required. For larynxes with primary bronchi the two rubber stoppers inserted were generally of the same diameter but the one placed into the right primary bronchi had a single hole drilled into it while the one placed into the left primary bronchi was solid (**Fig. 2A and B**). For the larynx with only the short trachea a single larger stopper was inserted into the tracheal opening (**Fig. 4**). All of the inserted stoppers were then fixed in place using cyanoacrylate adhesive (super glue) (**Fig. 2A and 4**).

In order to force air into the larynx the following laboratory set up was utilized: Electric air pump,

regulatory valve, pressure gauge, rubber tube, glass tube through the rubber stopper with the hole. Air pressure during a research run was maintained at approximately 6 psig (lbs/in^2 gauge pressure) (**Fig. 2A**) as measured on the pressure gauge. Immediately prior to the beginning of a research run each larynx was inspected at the glottal opening to locate the two visibly thickened structures which were oriented in an anterior-to-posterior direction (**Fig. 5A to C and 6**). We propose that these structures are the source of the manatee vocalizations and we will discuss this in our results and conclusions section of this study.
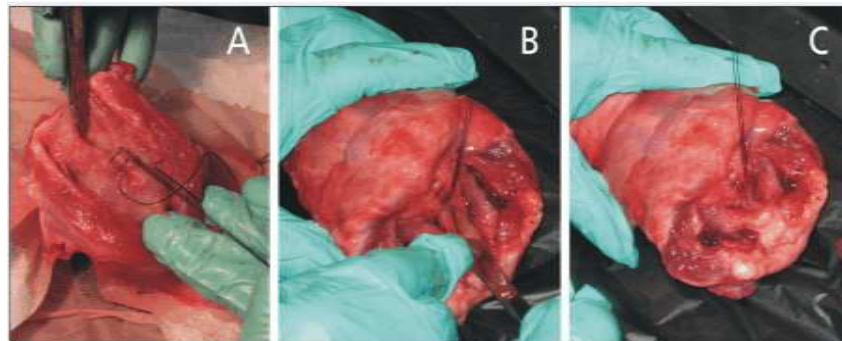
Fig. 5. A, B and C This figure is to be viewed as a progression. We placed a 2-0 braided black silk suture on a cutting needle through the posterior end of the strips of tissue in the area of the arytenoid cartilages. The purpose was to cause midline approximation of these tissues. The 27 suture was cut to leave about 10 inches of length on each side. However, when tension was placed on the suture, a defect remained posteriorly, so, in order to seal this opening, a continuous 2-0 braided silk suture was used to approximate the margins and close the defect. This suture was also left long so as to allow manual tension to be applied to maintain complete closure during the run. See text for further details
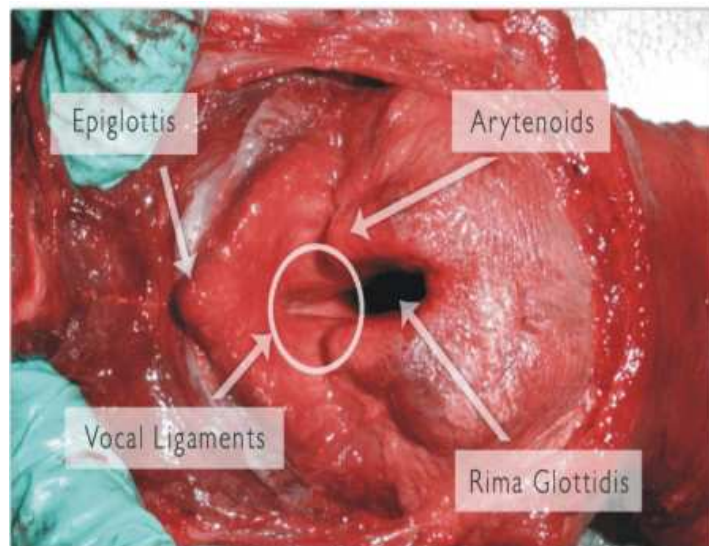
Fig. 6. This is a front view of a manatee larynx showing the glottal opening (Rima Glottidis) surrounded by the two protruding arytenoid cartilages. The leaf shaped epiglottis can be seen as a projecting structure at the middle left. The circled region marked as "vocal ligaments" indicates the location of two bilateral strips of cartilaginous tissue embedded in the lateral laryngeal walls along the margins of the glottis and which are in close approximation anteriorly but which diverge posteriorly. Our hypothesis is that these represent modified vocal ligaments which run along the margins of the rudimentary vocal folds. We suggest that these vocal ligaments are connected anteriorly to the posterior side of the thyroid cartilage and posteriorly with arytenoid cartilages. See text for details

Table 5A. Experimental larynx parameters: Here we present the fundamental frequencies and harmonic structures (1st PTL-3rd PTL) generated experimentally by our necropsied manatee Larynxes (LRX 1-10) along with the following anatomical information for the animal who supplied the larynx: Sex, body weights and body lengths. Additionally, we also provide the formalin fixed glottal circumference and formalin fixed tracheal circumference of each of these experimental larynxes

LRX no	Fund freq (Hz)	1st PTL freq	2nd PTL freq	3rd PTL freq	Sex	Body length (cm)	Body weight (kg)	Glot circ (cm)	Trac circ (cm)
LRX-1	2425.0	4839	7253		F	255	330.00	14.75	12.0
LRX-2	2144.0	4289	6433	8542	M	286	317.00	24.50	18.0
LRX-3	2449.0	4898	7359	9808	M	184	115.50	16.00	11.5
LRX-4	1253.0	2507	3761		M	185	124.00	15.50	11.5
LRX-5	1101.0	2203	3304	4406	F	201	167.00	21.00	15.0
LRX-6	878.9	1769	2648	3539	F	209	193.00	18.00	12.5
LRX-7	796.8	1582	2390	3175	F	331	624.00	20.50	15.5
LRX-8	1183.0	2378	3562	4746	F	260	297.50	17.00	14.0
LRX-9	1160.0	2332	3492	4664	M	292	422.00	20.00	16.5
LRX-10	1675.0	3339	5015	6691	M	210	195.50	18.50	14.0

Table 5B. Experimental larynx correlation coefficients: The anatomical information provided in **Table 5A** was used to calculate the correlation coefficients presented here. Although there was no significant correlation between fundamental frequencies and any of these anatomical parameters, there was a significant correlation between larynx circumference and tracheal circumference, as well as body length and body weight. See text for details

Regression analysis	Slope	I-CEPT	CORR COEF
Fundamental frequency and:			
Body length	0.0	264.7	-0.19
Body weight	-0.1	389.4	-0.29
Larynx circumference	0.0	20.0	-0.20
Tracheal circumference	0.0	15.1	-0.19
Larynx circumference and:			
Body length	8.4	85.8	0.49
Body weight	19.3	-80.3	0.37
Tracheal circumference	0.7	1.3	0.93
Tracheal circumference and:			
Body length	15.9	17.4	0.70
Body weight	39.3	-273.8	0.56
Larynx circumference	1.2	1.0	0.93
Body length and:			
Body weight	3.0	-436.9	0.96

Because our *in vitro* laryngeal studies employed non-living tissue, the absence of muscle tone was an inevitable hurdle which we had to overcome. Normally during sound generation contraction provided by the various laryngeal muscle groups (Fink, 1975) would alter the tension on the vibrating surfaces. In our case we attempted to mimic these muscular contractions by applying tension to the arytenoid cartilages (**Fig. 6**) to control their position and to cause midline approximation of the vibrating surfaces. We accomplished this with a 2-0 braided black silk suture on a cutting needle which was placed through the posterior end of the strips of tissue in the area of the arytenoid cartilages (**Fig. 5A to C**), the rotation of which would *in vivo* cause midline approximation of these tissues. The suture was cut to leave approximately

10 inches of length on each side (**Fig. 2A, B and 5A to C**). However, when tension was placed on the suture, a defect remained posteriorly, which *in vivo* would have been sealed by muscular contraction (Fink, 1975). Therefore, in order to seal this opening, a continuous 2-0 braided silk suture was used to approximate the margins and close the defect (**Fig. 5C**). This suture was also left long so as to allow manual tension to be applied to maintain complete closure during the run. The ends of the initial single suture were tied and this loop was attached to a tension scale. As tension was applied to the scale (**Fig. 2A and B**), the tension was transmitted into the arytenoid cartilages closing them together. This allowed us to obtain a very rough estimate of the force that had to be applied to the cartilages to close the glottal opening. Using a

protractor we were also able to obtain a rough estimate of the angle of the tensioning sutures (as force was being applied between the arytenoid cartilages and the tension scale) that would maximize sound generation (**Fig. 2A, B**). However, during sound generation it was still necessary to apply finger pressure on the anterior and posterior sides of the arytenoid cartilages to prevent the larynx from lifting off the laboratory bench as force was applied to the tensioning sutures and also to maintain a constant closure of the glottal opening (**Fig. 2A and B**).

The typical experimental conditions which were used to standardize our laryngeal studies were as follows: Air pressure range was maintained between 2.5 and 10 psig with 6 psig as the typical pressure used for most of the experiments. (This pressure is similar to the subglottal pressure measured in experimental human subjects where the range was from about 8 psig to about 26 psig; see (Fink, 1975.) Suture angle was between 30 and 70°C with 70°C used for most of the studies. Force range measurements using a spring balance were between 12 and 19 N (newtons) with 14 N used for most of the studies. Finger pressure applied to the larynxes was in the range of 23 N as measured with a spring balance. To maximize sound production for each larynx, air pressure, tension and suture angle had to be constantly adjusted until the experimental conditions were met. The vocalization data generated during these studies was then recorded by our Fostex on DVD discs, after which it was transferred to computers in our laboratory where we reviewed the recordings obtained from each larynx under study.

2.4. Histological Staining of Manatee Larynx Tissue Employing Masson's Trichrome Stain

In collaboration with Dr. Gregory Boivin (DVM, MS, Professor and Director, Laboratory Animal Resources, Wright State University Histology Laboratory), manatee larynx tissue was cut into 5 micron sections and stained using Collagen-Masson's Trichrome Stain (Bacnroft and Stevens, 1990). Blocks of tissue were cut from two manatee larynxes (#6: MNW1019, 2 Mar 2010; #3: MSW1002, 13 Jan 2010) that had previously been used for our sound generation studies and which were fixed in formalin. The blocks of tissue were sectioned through the region of the lateral walls surrounding the glottal opening midway between where the tissue is in close approximation anteriorly and where it diverges posteriorly (**Fig. 11**). This region which we hypothesize contained modified vocal

ligaments was previously outlined in **Fig. 6**. The stained sections were then viewed and areas of epithelium, skeletal muscle and connective tissue were identified and photographed.

3. RESULTS

3.1. Silicon Modeling of the Manatee Upper and Lower Respiratory Tract and Larynx

In **Fig. 3** the silicon model produced from the hollow spaces present in the upper and lower respiratory tract of the immature female manatee MSW0780 is shown. As can be seen for manatee MSW0780, the shapes of the interior spaces of the nasal cavity, oral cavity, pharynx, larynx and short trachea are clearly defined. In this model the location of the laryngeal lumen is apparent as well as the impression of the epiglottis. However, what we did not expect to observe in our model was the impression made by a projecting structure located rostral to the laryngeal lumen and caudal to the trachea which could be anatomically equivalent to rudimentary vocal folds (**Fig. 3,** Inset B). The location of a nasal septum can be inferred from the separation between left and right nasal cavities. Additionally, our silicone modeling technique allowed us to visualize both primary bronchi as well as the secondary and tertiary bronchi. These long, straight primary bronchi were expected because manatees possess unusual elongated dorsal lungs, as well as hemi-diaphragms (Rommel and Reynolds, 2000; Reynolds *et al.*, 2002; Rommel and Lowenstein, 2001). It is also interesting to note the presence of unusually large Eustachian tubes in our model, attached to the pharyngeal region, as observed first by SA Rommel (personal communication). The presence of such large Eustachian tubes thus lends further credence to our modeling technique.

3.2. Sound Generation from In vitro Necropsied Manatee Larynxes

In **Table 3** (Trial Vocalization Data) we present the results of the sound frequency profiles generated experimentally from 10 necropsied manatee larynxes which were provided to us over the period of 2009 to 2011 by the Marine Mammal Pathobiology Laboratory in St. Petersburg Florida. In **Table 1** (Normal Manatee Vocalization Data) we present a comparison of 109 actual manatee vocalizations (with a mean of approximately 2100 Hz), recorded from living animals housed at the Manatee Springs Exhibit of Cincinnati

Zoo and Botanical Garden (CIN) from 2009 to 2011. Over this time period the following manatees were housed together: Little Joe and Slip, Aug 2005-Oct 2009; CC Baby and Turner, Mar 2010 to Nov 2, 2010; and Betsy, Illusion and Wooten, Nov 8, 2010 to Nov 8, 2011 when Illusion was transferred to Florida's Lowry Park Zoo leaving Betsy and Wooten in Cincinnati. Clearly because these different animals were housed together we do not know which specific animals are responsible for the individual calls presented in **Table 1**, but only the date and time when the calls were recorded and thus the animal grouping. In addition, the appearance of one of these manatee vocalizations can be found in **Fig. 7** (Normal Manatee Vocalization). Note the fundamental frequency here is 3200 Hz with three clear harmonics present at 6400, 9600 and 12800 Hz.

In **Table 2** (Low-Frequency Manatee Vocalization Data) we present six low frequency manatee vocalizations (with a mean of approximately 1500 Hz), recorded in 2010 from living manatees housed variously at the Cincinnati Zoo (CIN) and the Parker Manatee Aquarium of the South Florida Museum (SFLA) in Bradenton Florida. Animals at the Parker Aquarium that were 11 housed together during 2010 were: Bolee, Jan 14, 2010-May 17, 2010; Teco 2, Jan 14, 2010-May 19, 2010; Cayman 4B, Jan 14, 2010-May 24, 2010; as well as Snooty who is a permanent resident at South Florida. Once again because these animals were housed as a group we do not know which animal was responsible for the recorded vocalizations.

In **Table 1 to 3** particular entries are highlighted in orange. This highlighting is designed to allow specific comparison of the data between tables. For example in **Table 3**, LRX #1 is to be compared with CIN 1h which can be found in **Table 1**; LRX #2 in **Table 3** is to be compared with CIN-3bi in **Table 1**; while LRX #7 in **Table 3** is to be compared with SFLA-b in **Table 2**.

One other observation requiring clarification in **Table 1 to 3** relates to the number of partials displayed. For the data in **Table 3** the experimental results include one and sometimes two partials. However in **Table 1** while all of the 109 vocalizations contain the 1st partial, only 11 demonstrate a 2nd partial and of these CIN-1a and 1b possess a 3rd partial, while only CIN-1a also possesses a 4th partial. Similarly for **Table 2**, while all 6 Low Frequency Manatee Vocalizations demonstrate a 1st partial, three of these also possess a 2nd partial, two possess a 3rd partial and only SFLA-c possesses a 4th partial. The reason for this is related to the fact that these vocalization streams are being transmitted through the internet from our off site

locations at the Cincinnati Zoo and at the South Florida Museum. The highest partial frequency that can be transmitted is affected by (i) the fundamental frequency of the particular vocalization and (ii) the lossy nature of the compression algorithm imposed by the encoder/decoder pair (as described in "Materials and Methods" above) which in this case is MP3. Because data collected during our experimental larynx vocalization studies utilized a Shure microphone directly connected to our Fostex recorder, higher frequency content is present since this data is not limited by encoder sampling rate and data compression.

In our experimental results presented in **Table 3** (Trial Vocalization Data), the fundamental frequencies generated *in vitro* by our larynx tissue varied from a high of 2449 Hz to a low of 796.8 Hz. For each of these 10 results an a clear partial with a multiplier of 2X could be identified and for 8 of these results another clear partial with a multiplier of 3X could be identified as well (LRX #s 1, 2, 4, 5, 6, 7, 8). Statistically the results generated from these 10 larynx studies possessed a mean fundamental frequency of 1506 +/- 625 Hz (one standard deviation) which is low when compared with typical vocalizations produced by living animals, where the fundamental frequency was 2147+/-432 Hz (one std. dev.) n = 109 (**Table 1**; Normal Manatee Vocalization Data).

To make valid comparisons between our experimental vocalization results and normal manatee vocalizations we subdivided our experimental results in **Table 3** into two experimental larynx groups based on the fundamental frequencies produced. Those fundamental frequencies that fell above the mean of 1506 Hz were classified as Normal Frequency Experimental Vocalizations marked with a "+" sign (in **Table 3** see; LRX #s 1, 2, 3 and 10; mean 2173 Hz n = 4), while 351 those fundamental frequencies that fell below this mean were classified as Low-Frequency Experimental Vocalizations marked with a "-" sign (in **Table 3** see; LRX #s 4, 5, 6, 7, 8 and 9; mean 1062 Hz n=6). In **Fig. 8** the appearance of such a "Normal Frequency Experimental Vocalization" generated by LRX #3 (as reported in **Table 3**) is presented. Here the fundamental frequency is 2449 Hz with 7 clear partials at 4898, 7359, 9808, 13000, 15900, 18399, 20900 Hz and possibly 23500 Hz. In **Fig. 9** we present an example of a "Low Frequency Experimental Vocalization" generated by LRX #4 (as reported in **Table 3**). Here the fundamental frequency is 1253 Hz with three clear partials present at 2507, 3761 and 4406 Hz.
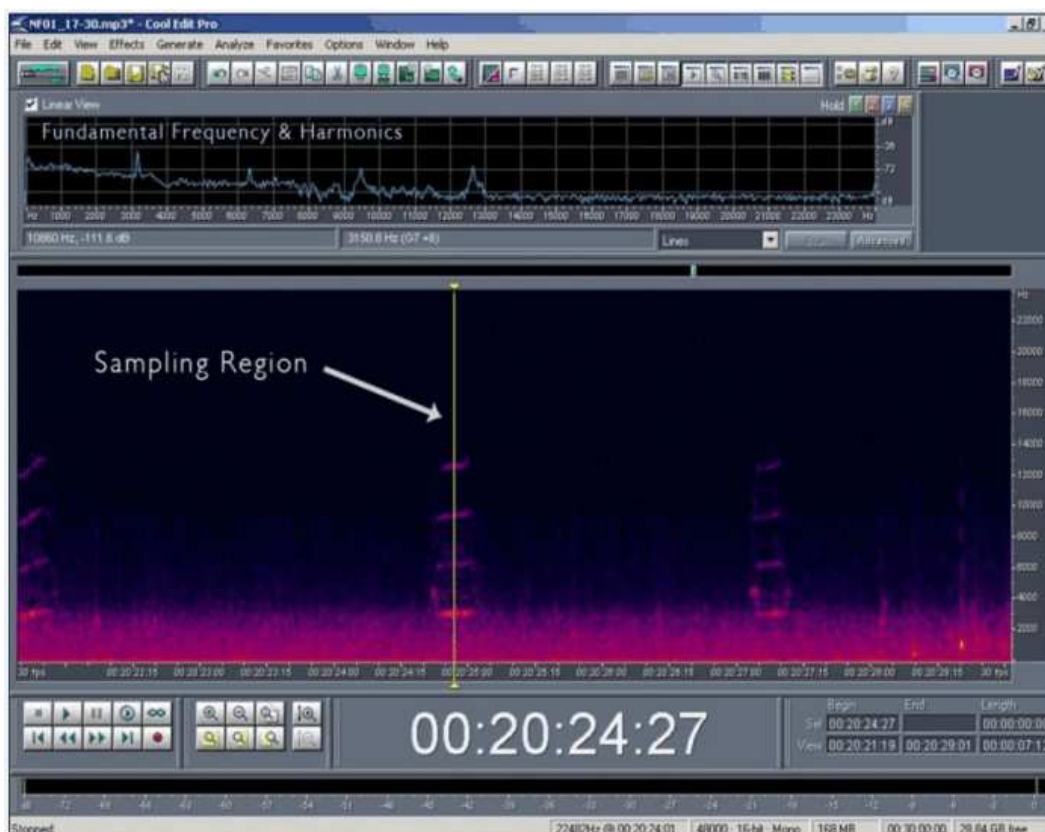
Fig. 7. Appearance of a normal manatee vocalization produced by a living manatee. The vocalization patterns were analyzed using Cool Edit Pro. The horizontal scale is in "SMPTE 30 fps" format, which is one of the standards used in the music and video industry. The time stamp is properly read as HH:MM:SS:FF, where HH is hours, MM is minutes, SS is seconds and FF is frames from the file, not clock time. There are 30 frames per second (fps) in this format, making each frame 3 1/3 hundredths of a second long. To convert from frames to seconds, divide the number of frames by 30. The vertical scale is frequency in Hertz (Hz). Three vocalizations are shown here which were all probably produced by the male manatee, Slip, who was housed at the Cincinnati Zoo some years ago. In the center vocalization the yellow dotted line represents the sampling region. The duration of this single vocalization is 0.33 sec. The brightest band is the fundamental frequency at 3158 Hz. There are also three strong harmonic peaks at 6328, 9486 and 12,650 Hz. This underwater sound recording was made on site at Manatee Springs of the Cincinnati Zoo. Because this was made on site we did not employ our small in situ tank hydrophone but instead 28 utilized an F33 Reciprocal Navy Transducer rented from the Naval Undersea Warfare Center, Underwater Reference Division, Newport Rhode Island, for which the frequency response was known. Furthermore, since these vocalizations were not transmitted through the internet the patterns seen here contain additional harmonic structures which are usually eliminated as has been described in detail in the text. Therefore with less compression noise we can visualize the harmonic structures up to 12,650 Hz. These on site vocalizations were recorded on the same Fostex recorder that was used in our studies when we generated experimental vocalizations by 836 forcing air through our fresh necropsied manatee larynxes (see the section on Sound Recordings)

We then statistically compared the experimental data from our "Normal Frequency Experimental Vocalization" group of **Table 3** with our large data set of 109 Normal Manatee Vocalizations (**Table 1**) recorded from manatees at the Cincinnati Zoo.

We also statistically compared the experimental data from the "Low Frequency Experimental Vocalization" group of **Table 3** with the Low-Frequency Manatee Vocalizations (**Table 2**) which we recorded from living manatees at both the Cincinnati

Zoo and the South Florida Parker Aquarium. Such low frequency vocalizations, while not typical, do occur and we were able to identify six from our database with lower fundamental frequencies (**Table 2**; mean 1267 +/- 170 Hz one std.dev., n = 6). Our results (found in **Table 6**) utilizing the Student's T-Test at the 0.05 significance level indicate that there is no significance difference for high or low frequency experimental vocalization groups compared with normal manatee vocalizations for the Fundamental Frequencies and the 1st partial. However, if we more appropriately utilize the non-parametric Wilcoxon Rank-Sum Test for small samples, we find that there is significance at the Alpha 0.05 level (test statistic critical value of 52) and Alpha 0.10 level (test statistic critical value of 50) for Experimental Vocalizations compared with Low-Frequency Manatee Vocalizations for fundamental frequencies. Note that the Wilcoxon value for our Fundamental Frequency had an observed test statistic of 53, which is only one integer greater than then 52, where 0.05 is the critical Alpha value. However, using the Wilcoxon test to measure significance for both High and Low Experimental Vocalizations compared with Normal Manatee Vocalizations, we see no significance for the 1st partial (observed rank-sum test statistic = 48) or for the multiplier (observed rank-sum test statistic = 30). We define the multiplier as the ratio between the first partial and the fundamental frequency. From physical theory this ratio should be precisely two.

Based on the information presented in **Table 5A**, we calculated the correlation coefficients for various parameters related to the 10 larynxes used in our experiments (**Table 5B**). There were no significant correlations of fundamental frequencies generated by these larynxes when compared with manatee body weights, manatee body length, tracheal circumference, or glottal circumference. Furthermore, glottal circumference was not significantly correlated with either body weight or body length. But it was correlated (r = 0.93) with tracheal circumference. Also as expected there was a strong correlation (r = 0.96) between body weight and body length.

Table 6. Statistical analyses: Here we statistically compare the results from our Normal Frequency Trial Vocalizations (from **Table 3,** indicated by "+") against Normal Manatee Vocalizations (**Table 1**). We also statistically compare the results from the Low-Frequency Trial Vocalizations (from **Table 3**, indicated by "-") against Low-Frequency Manatee Vocalizations (**Table 2**). The Students T-test at the 0.05 significance level demonstrates no significance difference for Normal Freauency Trial Vocalizatios (**Table 3**) or Low Frequency Trial Vocalizations (**Table 2**) compared with Normal Manatee Vocalizations for the Fundamental Frequency, the 1st harmonic structure and the ratio. However, if we utilize the Wilcoxon Rank-Sum test for small samples we find that there is significance at the Alpha 0.05 level (critical value of 52) and Alpha 0.10 level (critical value of 50) for Low Frequency Experimental Vocalizations compared with Low Frequency Manatee Vocalizations, but only for Fundamental Frequencies. Note that the Wilcoxon value for our Fundamental Frequency was 53, which is only one integer greater than then 52 where the critical Alpha value is 0.05. However, using the Wilcoxon test to measure significance for Low Frequency Experimental Vocalizations compared with Normal Manatee Vocalizations, we see no significance for the 1st harmonic structure, the multiplier or the ratio

|	FREQ		FREQ	
	N	FUND	1ST PTL	MULT
T-TEST FOR:	6	0.07	0.196	0.262
LOW-FREQ GROUP				
(Data from Table 3, Marked "-")				
T-TEST FOR:	4	0.895	0.854	0.555
HIGH-FREQ GROUP				
(Data from Table 3, Marked "+")				
WILCOXON R-S:				
RANK-SUM		53	48	30
CRITICAL VALUES:				
ALPHA = 0.05	(26, 52)	(26, 52)	(26, 52)	Only fundamental frequency
ALPHA = 0.10	(28, 50)	(28, 50)	(28, 50)	Is significant here
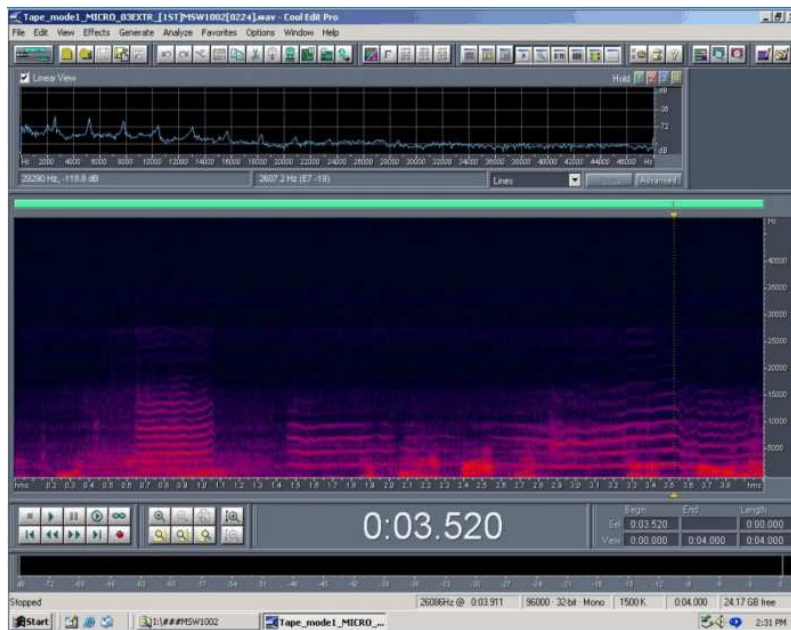
Fig. 8. This is the appearance of a Normal Frequency Experimental Vocalization generated when air was forced through the fresh manatee larynx LRX #3 (MSW1002). The pattern was analyzed using Cool Edit Pro. The sampling region is indicated by the yellow dotted line. Here the fundamental frequency is 2449 Hz with 7 clear harmonic structures at 4898, 7359, 9808, 13000, 15900, 18399, 20900 Hz and possibly 23500 Hz. The data generated by this larynx is also included in **Table 3** (LRX #3) but only the first three harmonic structures are listed in this table to limit the number of columns in the spreadsheet

Fig. 9. This is the appearance of a Low Frequency Experimental Vocalization generated when air was forced through the fresh manatee larynx LRX #4 (MEC1003). The pattern was analyzed using Cool Edit Pro. The sampling region is indicated by the yellow dotted line. Here the fundamental frequency is 1253 Hz with three clear harmonic structures present at 2507 Hz, 3761 Hz and 4406 Hz

3.3. Histological Results for Trichrome Staining of Manatee Larynx Tissue

According to the report provided by Gregory Boivin, the Trichrome stained manatee laryngeal tissue sections appear to be anatomically consistent with human laryngeal tissues:

*"Examined were 4 sections of tissue from the larynxes of 2 manatees. The sections were examined to determine the presence of vocal cords in the manatee (**Fig. 12-15**). A characteristic feature of the vocal cord in humans is the presence of a stratified squamous epithelium. In the deep layers of the true vocal cord there is a muscular layer. In the false vocal cord there is a large glandular component. All samples demonstrated moderate autolysis. Epithelium loss was evident in all sections. The dorsal (rostral) sections from both manatees had abundant glandular tissue underlying a stratified squamous epithelium. These sections are histologically consistent with the false vocal cords of humans. The ventral sections from both manatees have a large muscle in the deep layer of the larynx with fibrous tissue overlying the muscle. The epithelium is stratified squamous. The histologic appearance of the ventral sections is consistent with the true vocal cord in humans.*
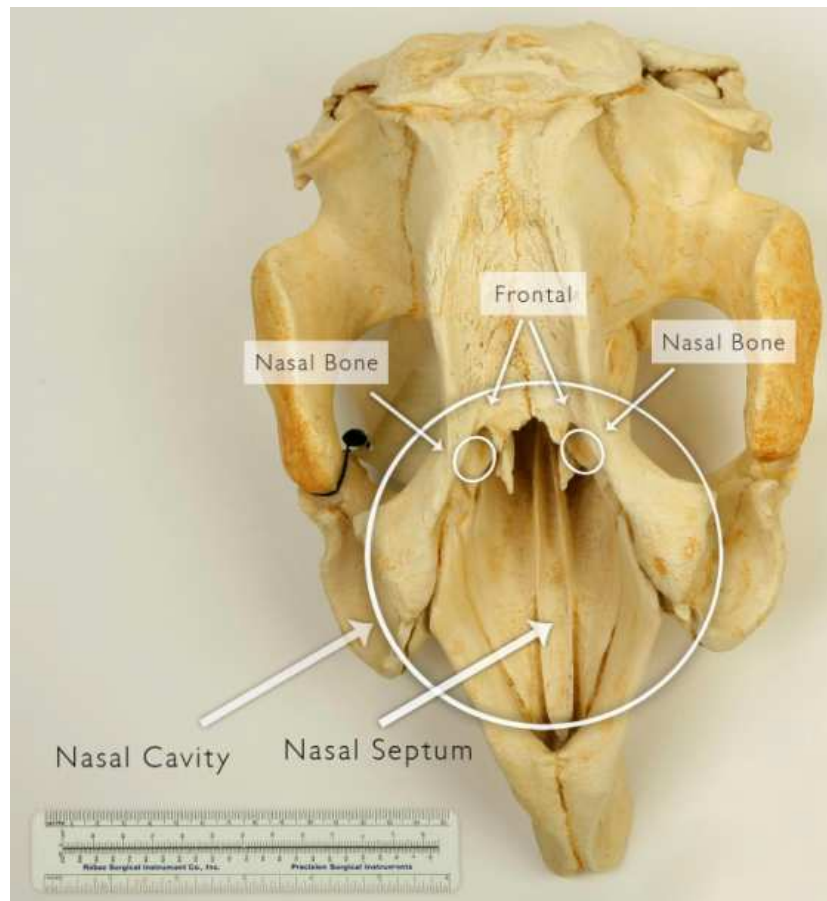
Fig. 10. This is the model of a manatee skull (*Trichechus manatus latirostris*). Note the location of the nasal septum within the open nasal cavity. In manatees the two almond shaped nasal bones are reduced and appear as part of the frontal bones (Rommel *et al.*, 2009)
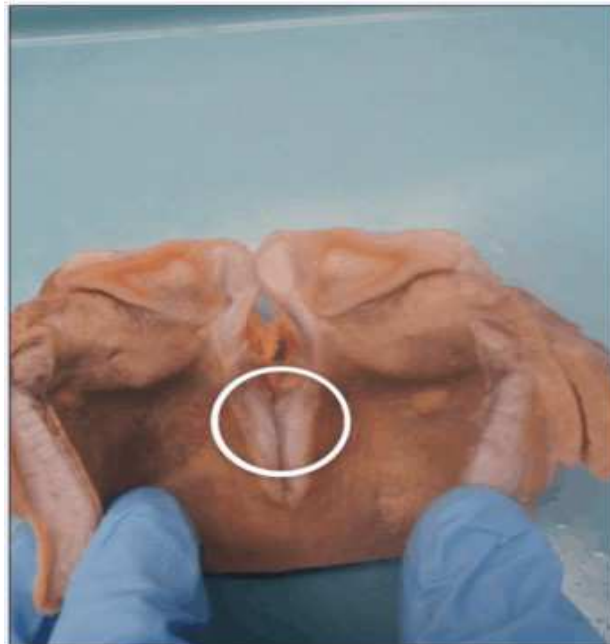
Fig. 11. Blocks of tissue were cut from two manatee larynxes (MNW 1019 (#6) 2 Mar 2010; MSW 1002 (#3) 13 Jan 2010 that had previous been used for our sound generation studies and 29 which were fixed in formalin. The blocks of tissue were cut through the region of the lateral walls surrounding the glottal opening midway between where the tissue is in close approximation anteriorly and where it diverges posteriorly. This photograph is of larynx MNW 1019. The circled area in this figure indicates where the tissue sections that were stained by the Trichome method were excised. Slide provided by Dr. Boivin
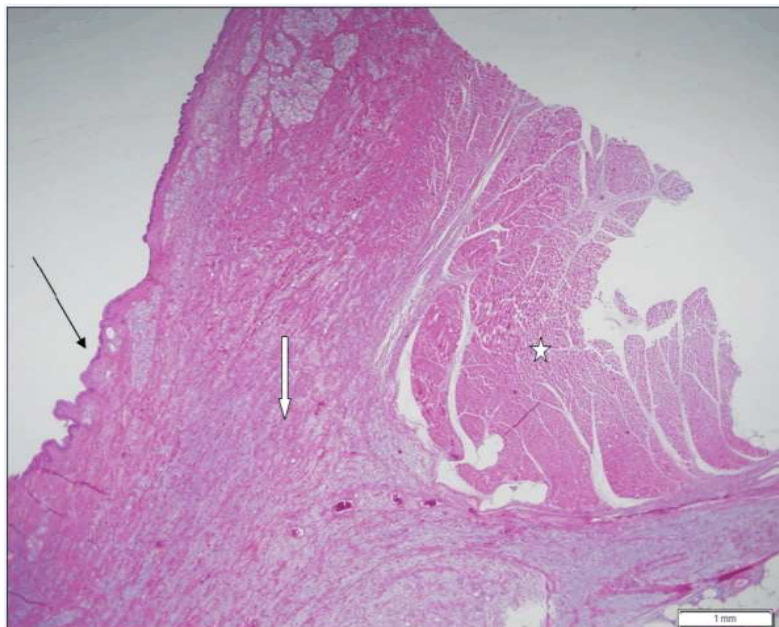
Fig. 12. Low magnification of the region where we propose the manatee vocal cord is located. This view is of the skeletal muscle (star), squamous epithelium (black arrow) and fibrous tissue (white arrow). This region was excised from the muscle shown in the circled area of **Fig. 11**. (Scale 1 mm). Slide provided by Dr. Boivin
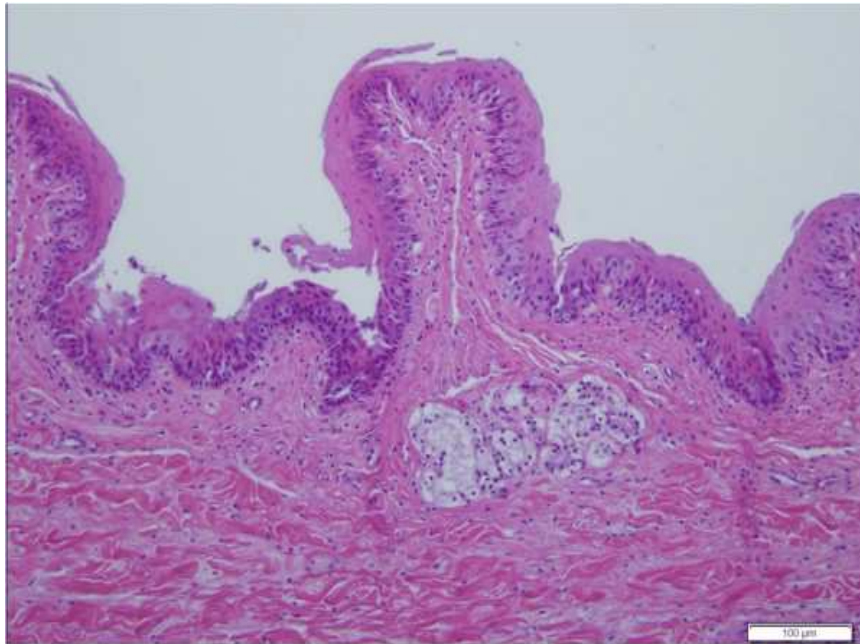
Fig. 13. Surface epithelium of what we propose to be the true manatee vocal cord. This region was excised from the muscle shown in the circled area of **Fig. 11**. (Scale 100 um) Slide provided by Dr. Boivin
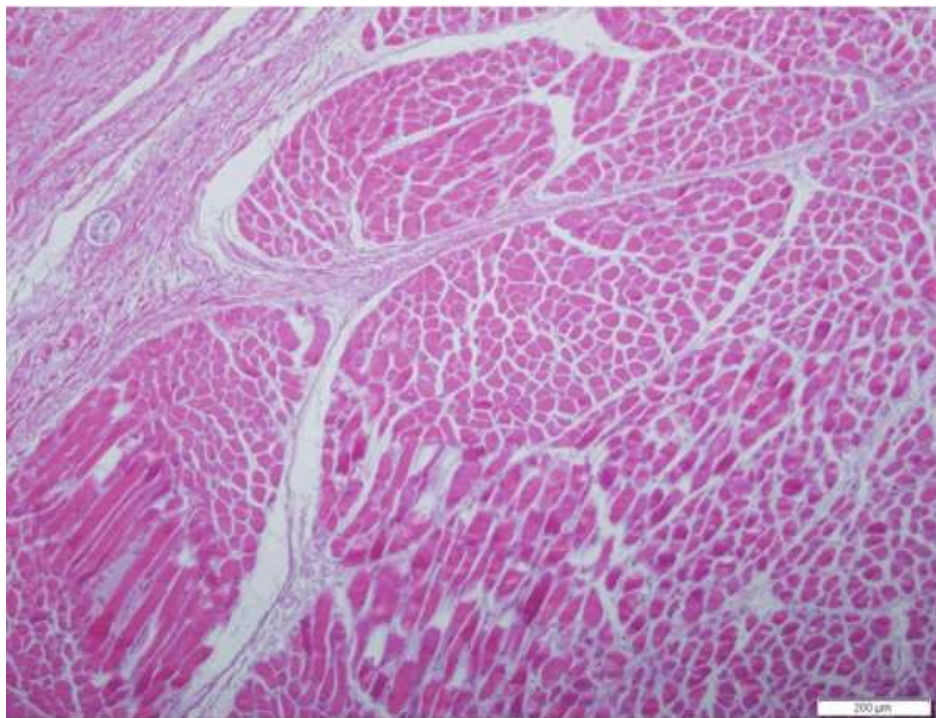
Fig. 14. Skeletal muscle in the body of the vocal cord. This region was excised from the muscle shown in the circled area of **Fig. 11**. (Scale 200 um) Slide provided by Dr. Boivin
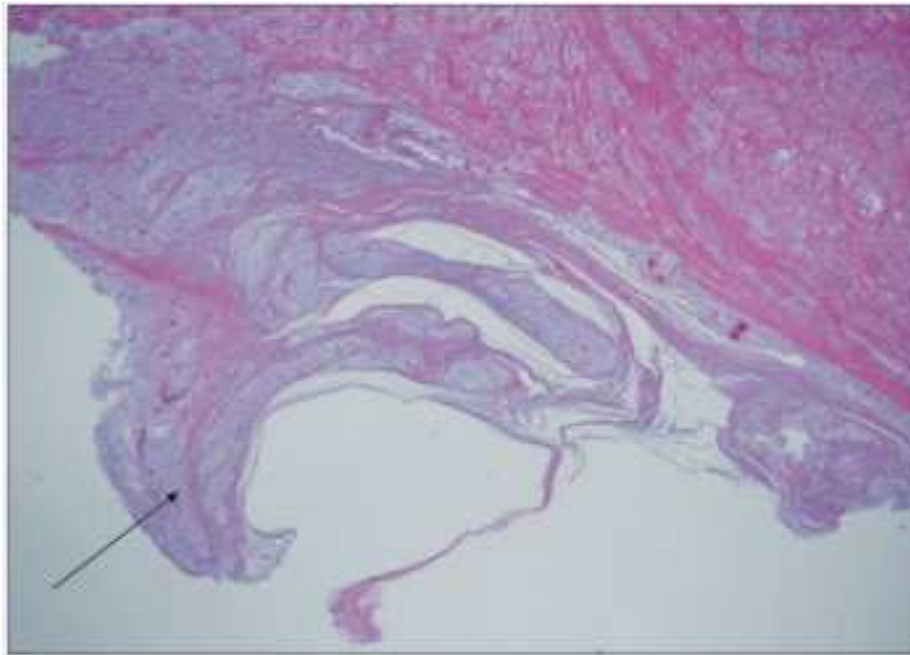
Fig. 15. Potential vocal fold consisting of fibrous tissue (arrow) extending from the surface of the vocal cord. This region was excised from the muscle shown in the circled area of **Fig. 11**. 880 (Scale 1 mm) Slide provided by Dr. Boivin

4. DISCUSSION

Sound generation by manatees, as in other mammals, occurs in the larynx and is accomplished when expiratory air is forced across the medial edge of strips of tissue, which we propose are modified vocal ligaments which are analogous to true vocal folds present in other mammals. These modified vocal ligaments (which are orientated in an anterior-posterior direction), are connected anteriorly to the posterior side of the thyroid cartilage and posteriorly to the arytenoid cartilage. Thus, as expired air is forced between these structures, the air current forces them open 415 after which they spring back cyclically creating sound waves (Fink, 1975). By visual inspection, these modified vocal ligaments appear as two bilateral strips of cartilaginous tissue embedded in the lateral laryngeal walls which are in close approximation anteriorly, but diverge posteriorly (**Fig. 5A to C and 6**) and may also be analogous to the fleshy ligamentous fibro-elastic tissue of Harrison and King (1965).

In the living manatee, during sound generation by the larynx, we suggest that paired laryngeal muscle groups medially rotate the arytenoid cartilages causing opposition or adduction thus creating midline approximation of these modified vocal ligaments. In our model we mimicked this midline approximation by providing force through tensioning sutures and also utilized mild finger pressure. Thus, when we forced air through this region we were able to generate sound vibrations from each larynx with primary (fundamental) frequencies and harmonic (partial) structures comparable to vocalizations made by living manatees. While only four larynxes in our study generated fundamental frequencies that fell in the typically normal range (**Table 3**; mean 2173 Hz n = 4) and six larynxes generated fundamental frequencies that fell in the low range (**Table 3**; mean 1062 Hz n = 6), statistically there were no significance differences between these experimental results and normal or low frequency vocalizations produced by living manatees (**Table 1, 2 and 6**) as measured with the Student's T-Test. Use of the non-parametric Wilcoxon Rank-Sum test for small samples indicated slight significance at the 0.05 alpha level for the fundamental frequency but not for the first harmonic or other parameters with respect to the low frequency vocalizations. These statistical results support the validity of our *in vitro* model.

Why did the fundamental frequencies vary in our experimental studies? We suggest three possible causes. Firstly, larynx tissue was in the early stages of decomposition with associated anatomical changes; secondly, larynx muscle flaccidity was responsible; or

thirdly, variations in larynx mass were responsible. Since, as described in the results section, we were unable to correlate larynx weight with the primary vocalization frequency produced by each of the larynxes, we instead used body weight, body length, larynx circumference (at the region of the epiglottis) and tracheal circumference (at the pharyngeal attachment) (**Table 5B**). We found no significant correlation between frequency vs. body weight (r = -0.29), body length (r = -0.19), laryngeal circumference (r = -0.20) or tracheal circumference (r = -0.19). We did find significant correlations between body weight vs. length (r = -0.96) and laryngeal Vs. tracheal circumferences (r = -0.93). However, because we had not planned at the inception of our experiments to make such circumference measurements, the larynxes had already been fixed in formalin. We reasoned that although formalin fixed larynxes would lose their pliable consistency and might also experience slight tissue shrinkage, the circumferential ratio would still be related that of the fresh tissue (**Table 5B**). While this may be an erroneous conclusion, tracheal circumference does appear to be at least moderately related to body length (r=-0.70) and tracheal and laryngeal circumferences are also more highly correlated (r = -0.93). Thus at least as far as we can ascertain, variations in fundamental frequencies between the larynxes under study did not correlate with larynx size as measured by circumference.

However, O'Shea and Poche (2006) have reported that vocalization frequency is negatively correlated with body weight, where larger females produce vocalizations with lower primary (fundamental) frequencies. Since our studies did not demonstrate such a correlation with body weight we suggest that our *in vitro* model was responsible. We attribute this to the flaccidity of the laryngeal muscle tissue coupled with the absence of the resonance cavities of the upper respiratory tract and head. However, while our model is not a perfect imitation of the vocal apparatus found in living manatees, we are convinced that the larynxes we studied were not damaged anatomically. This is supported because all ten of the experimental larynxes we studied were able to generate fundamental frequencies and associated partial frequencies with the correct multipliers. Furthermore, these frequency patterns were not statistically different from vocalizations produced by living animals. Additionally, all the larynxes we studied were pink and had only minimal odor when they were utilized. We attempted to use each larynx as quickly as possible after its arrival in our laboratory and until use we stored the tissue under refrigeration in ice cold saline which was changed twice daily. However, review of **Table 1** will show that while many of these larynxes were used within 2 days of their necropsy (LRX #1, 3, 4,

5, 9) some were used days later (LRX #2, 6, 7, 8, 10). Review of the primary (fundamental) frequencies generated (**Table 3**) suggests that the length of storage time did not affect primary frequency. For example in **Table 1**, LRX #1 and 3 which produced high frequencies (2425 and 2449 Hz) were used 2 days after they were necropsied, while LRX #2 which was used 9 days after it was necropsied also produced a high frequency (2144 Hz). Additionally LRX #4, 5 and 9 were used 2 days after necropsy but produced low frequencies (1253, 1101, 1160 Hz). Also, although we knew when and where the animals were collected, we did not know how long the manatee carcasses may have been floating before collection, nor did we know the water temperature. Thus all of these unknowns may have played a role in the state of the larynx tissue that we received. These factors are constraints on any future research involving manatee tissues.

Given these facts we suggest that the non-living and flaccid muscle tissue was primarily responsible for the variation in the frequencies we observed. During our studies we attempted to provide measurable and repeatable outside constrictive force to the arytenoidal cartilages such that they would provide midline approximation to the vibratory surfaces of these larynxes. Nonetheless this was only a weak substitute for the controlled muscle contractions produced by living manatees during vocalizations.

One of the serendipitous spinoffs of our research was the observation that manatees possess very large Eustachian tubes which we visualized in our silicon modeling studies (**Fig. 3**) and which are far larger than those found in dogs (Grossman *et al*., 2011). The presence of large Eustachian tubes in manatees was first reported in the anatomical studies by S.A. Rommel (personal communication). Hill (1945) does mention that "The thin sheet of fibers of the levator palate cover the Eustachian enlargement…" but does not otherwise provide any further details. Although the function provided by these exceptionally large Eustachian tubes in manatees remains unknown we propose two possible hypotheses.

4.1. One Hypothesis: Directionality of Sounds Received

Christensen-Dalsgaard (2005); and Christensen-Dalsgaard and Manley (2005) have suggested that "Any ear in which the two tympana are coupled through Eustachian tubes or interaural canals is potentially a pressure difference receiver. However, for the ear to exhibit any significant directionality, the sound from the contralateral ear must reach the ipsilateral ear with little excess attenuation." Furthermore, ears with such interaural difference are "inherently directional simply because their response varies as an arbitrary function

of azimuth or elevation (Christensen-Dalsgaard, 2005; Christensen-Dalsgaard and Manley, 2005; Popper and Fay, 2005). Perhaps in manatees, the large diameter of the Eustachian tubes coupled to each other through the larynx may allow sound pressure to reach both sides of the tympanic membranes and provide manatees with increased ability to differentiate the directionality of a sound source under water.

## 4.2. A Second Hypothesis: Pressure Equalization During Diving

It is also conceivable that the presence of large Eustachian tubes may allow the manatee middle ear to rapidly equalize pressure across the tympanic membrane when diving or surfacing. This is supported by Wartzok and Ketten (1999) who state that "All marine mammals have special adaptations…" including "broad Eustachian tubes……consistent with deep rapid diving and long-term submersion…". Such pressure equalization across the tympanic membrane would increase manatee hearing sensitivity because it would limit inward or outward tympanic membrane deformation during external pressure changes.

4.3. Regulation of Airflow During Vocalization

One question that remains to be answered is why there is no air released from living manatees during a vocalization. In order to answer this question one must first consider the structure of the nasal cavity in the manatee skull. As can be seen in **Fig. 10** the anterior frontal region of the manatee skull is not closed by nasal bones but remains open (Rommel and McLellan, 2002; Rommel *et al.*, 2009), as compared to the skull of many other animals. In the living manatee this open area is covered by skin, fat, muscle and some cartilage (SA Rommel personal communication and direct observations by the authors). This point is important when we consider the mechanism by which manatee vocalizations are transmitted from this anterior frontal region.

For a vocalization to occur, first air is forced from the animal's lungs through the trachea, larynx, pharynx and into the nasal cavity. This air flow results in sound vibration produced by the vocal folds/ligaments within the manatee larynx. As the vocal folds/ligaments vibrate short bursts of sound are produced (Fink, 1975). Second, because the nares remain closed, the forced air does not exit the nasal cavity during a vocalization. From observations first reported by Gerstein (1955) and confirmed recently by both the manatee keepers at the Cincinnati Zoo & Botanical Garden and members of our own research team, we also believe that this displaced air collected in the nasal cavity causes an observable, momentary swelling of the external central nasal area (**Fig. 16**).
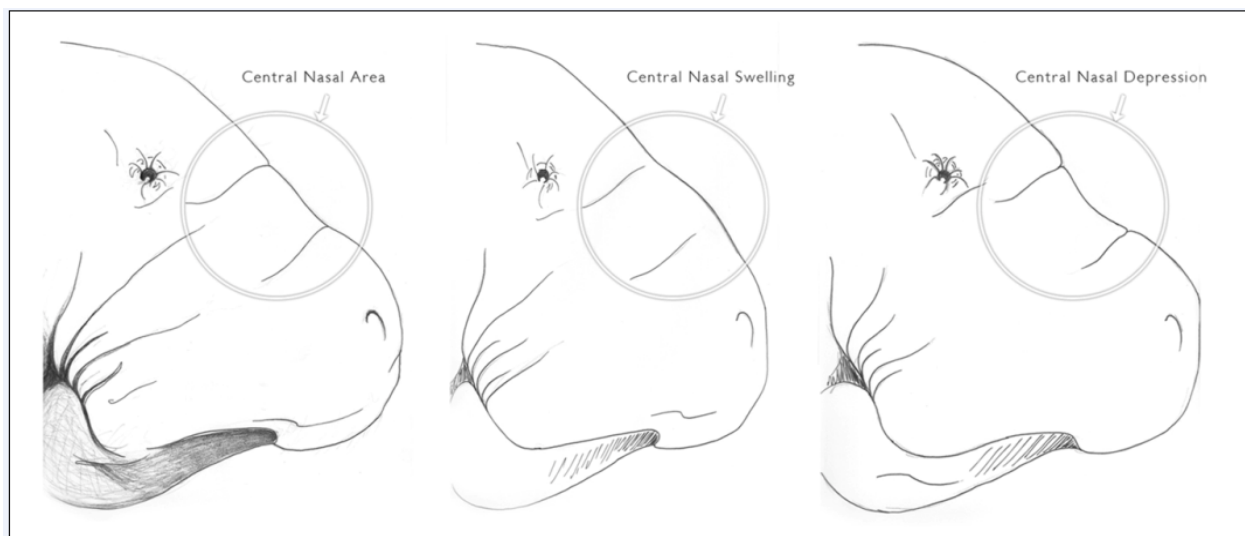
Fig. 16. Depiction of the manatee nasal region shape change during a vocalization (images should be viewed in sequence). The depiction is based on repeated observations, by both the manatee keepers and our research staff, of vocalizations made by various manatees (Slip and Little Joe and later by Illusion) at the Cincinnati Zoo and Botanical Garden. Note that the central nasal region swells and then rebounds during an active vocalization. Because the nasal cavity in manatees is not closed by nasal bones this region exhibits what appears to be elastic recoil due to contained air pressure. See text for details

The associated deformation of the nasal cavity is facilitated because of the absence of bone in this region, along with the presence of muscle, connective tissue and some cartilage. We have also recorded video of this frontal head movement made by Illusion, one of the three manatees housed at Manatee Springs at the Cincinnati Zoo, while she was vocalizing and we believe that this lends support to our hypothesis (see Supplemental Video Material). Finally, the air used to generate these vocalizations is conserved within the respiratory tract and returned to the lungs, so that the buoyancy of the animal is not compromised. This then accounts for our observation that at the termination of a vocalization, the central nasal region that was swollen due to air retention, rapidly deflates and then indents (**Fig. 16**). Additionally, we suggest that the deformable tissue in this region also acts like a drum head allowing the vocalization energy to be more efficiently dissipated into the surrounding water where it is perceived as an audible or ultrasonic squeak. Without this, the sound energy would have to travel through the massive manatee flesh and solid bone where it would be attenuated. This observation also supports the suggestion by Anderson and Barclay (1995) that sounds made by sirenians may originate in their frontal area.

5. CONCLUSION

Our results suggest that vocalizations produced by the Florida manatee are generated in the larynx when air vibrates a pair of visibly thickened vocal structures orientated in an anterior to posterior direction surrounding the Rima Glottidis and which are connected anteriorly to the posterior side of the thyroid cartilage and posteriorly with arytenoidal cartilages. Based on their histology and their location we propose that these structures are modification vocal ligaments. We further suggest that the vocalizations produced by these structures are modified within the resonance cavities in the frontal area and the air used to generate these vocalizations also causes a transient deformation of this region before being conserved and returned to the lungs.

6. ACKNOWLEDGEMENT

6.1. Supplementary Material

The following supplementary material is available for this article online:

S1: Audio File; Normal Manatee Vocalizations produced by a living manatee at the Cincinnati Zoo http://www.xavier.edu/manateeresearch/documents/1S1AudioFileCincinnati.mp3

S1A: Appearance of three vocalizations in audio file S1 http://www.xavier.edu/manateeresearch/images/S1AAppearanceofthreevocalizationsinaudiofileS1.bmp

S2: Audio File squeak chirps from animal at Homosassa http://www.xavier.edu/manateeresearch/documents/1S2AudioFileHomosassasqueakchirps.mp3

S2A: Appearance of squeak chirps in audio file S2 http://www.xavier.edu/manateeresearch/images/S2AAppearandeofthreevocalizationsinaudiofileS2.bmp

S3: Audio File of artificial vocalization made when air was forced through larynx MXTM1002 http://www.xavier.edu/manateeresearch/documents/S3ArtificialvocalizationbylarynxMXTM1002.wav

S3A: Appearance of artificial vocalization from larynx MXTM1002 from audio file S3 23 http://www.xavier.edu/manateeresearch/images/1S3AAppearanceofS3vocalizationbyLarynxMXTM1002.bmp

S4: Video File of Illusion's head movement during her vocalizations videotaped at the Cincinnati Zoo 8 http://www.xavier.edu/manateeresearch/documents/S4 manatee-all.mp4

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