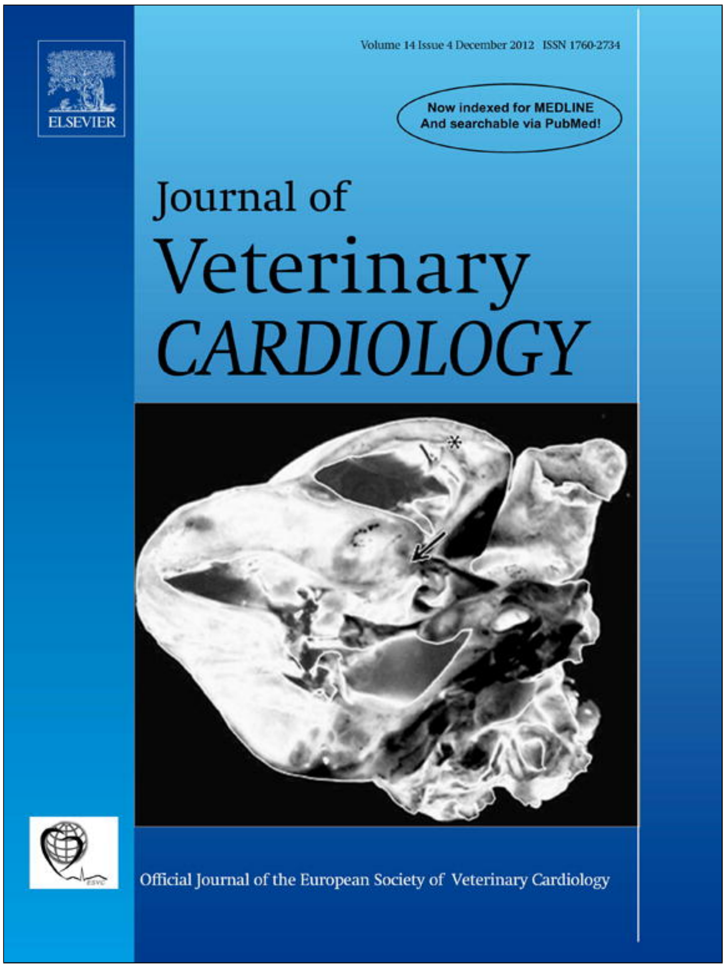
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Within-day and between-day variability of transthoracic anatomic M-mode echocardiography in the awake bottlenose dolphin (*Tursiops truncatus*)

Valérie Chetboul, DVM, PhD [a,b,*], Jonathan Lichtenberger, DVM [a], Mathieu Mellin, DVM [c], Birgitta Mercera [c], Anne-Cécile Hoffmann, DVM [a], Gwendoline Chaix, DVM [a], Emilie Trehiou-Sechi, DVM [a], Charlotte Misbach, DVM [a], Amandine Petit, DVM [a], Hervé P. Lefebvre, DVM, PhD [d], Nicolas Gaide, MSc [a], Renaud Tissier, DVM, PhD [b,e], Fabienne Delfour, PhD [c]

[a] *Université Paris-Est, Ecole Nationale Vétérinaire d'Alfort, Unité de Cardiologie d'Alfort (UCA), Centre Hospitalier Universitaire Vétérinaire d'Alfort (CHUVA), 7 avenue du Général de Gaulle, 94704 Maisons-Alfort cedex, France*
[b] *INSERM, U955, Equipe 03, 51 avenue du Maréchal de Lattre de Tassigny, 94010 Créteil cedex, France*
[c] *Delphinarium du Parc Astérix, Plailly, France*
[d] *Unité de Recherche Clinique Santé des Populations, Ecole Nationale Vétérinaire de Toulouse, 23 chemin des Capelles, 31076 Toulouse cedex 03, France*
[e] *Université Paris-Est, Ecole Nationale Vétérinaire d'Alfort, Unité de Pharmacie-Toxicologie, 7 avenue du Général de Gaulle, 94704 Maisons-Alfort cedex, France*

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KEYWORDS
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Abstract The use of transthoracic echocardiography in dolphins has been limited so far owing to technical and anatomical specificities. Anatomic M-mode (AMM) is a postprocessing echocardiographic technique generating M-mode studies from two-dimensional (2D) cineloops independently of the ultrasound beam orientation. The aim of the present study was to determine the within-day (repeatability) and between-day (reproducibility) variability of AMM echocardiography in awake healthy bottlenose dolphins (BN, *Tursiops truncatus*). Four adult BN trained to lie

* Corresponding author.
E-mail address: vchetboul@vet-alfort.fr (V. Chetboul).

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in left recumbency at the water surface were involved in the protocol. A total of 96 echocardiographic examinations were performed on 4 different days by a trained observer examining each BN 6 times per day. Video clips of 2D left parasternal long-axis views showing the left ventricle (LV) ventrally and the aortic root dorsally were recorded at each examination and analyzed for AMM measurements in a random order. A general linear model was used to determine the within-day and between-day coefficients of variation (CV). All examinations were interpretable allowing calculation of 10 AMM variables (i.e., end-diastolic and end-systolic ventral and dorsal LV myocardial wall thicknesses as well as LV and aortic diameters, mean aortic diameter, and LV shortening fraction). Most within- and between-day CV values (18/20) were <15%, the lowest being observed for the end-diastolic LV diameter (1.6%). In conclusion, AMM provides a simple non-invasive evaluation of heart morphology and function in the awake BN with good repeatability and reproducibility of the measurements. Further studies are required to determine the corresponding reference intervals.

Abbreviations

2D	two-dimensional
AMM	anatomic M-mode
Aomin	minimal aortic diameter
Aomax	maximal aortic diameter
BN	bottlenose dolphin
CV	coefficient of variation
LV	left ventricle/left ventricular
LVDD	left ventricular end-diastolic diameter
LVDS	left ventricular end-systolic diameter
M-mode	motion mode
SD	standard deviation
TEE	transesophageal echocardiography
TTE	transthoracic echocardiography

Introduction

Aquatic mammals may be affected by miscellaneous heart diseases including congenital cardiac defects, cardiomyopathies, parasitic heart diseases, degenerative valvular diseases, valvular endocarditis, and myocarditis.[1–4] Transthoracic echocardiography (TTE) is a well-established technique for the *antemortem* diagnosis of heart diseases both in humans and in small animals, allowing qualitative description of cardiac abnormalities and quantitative assessment of heart anatomy and function using combined two-dimensional (2D) and M-mode (motion mode). However, the use of TTE to evaluate the dolphin heart has been limited so far because of anatomic barriers (thick integument and blubber layers, large sternum, and circumferential lung) and technical challenges (difficulty for non-sedated animals to stay in a stable appropriate position for several minutes and relatively high sedation-related risks).[4,5] Dolphin heart diseases are therefore currently diagnosed by necropsy and histopathological examinations only, and cardiology knowledge of this species remains very limited.

In one study specifically dedicated to echocardiography in the bottlenose dolphin (BN, *Tursiops truncatus*), TTE yielded poor-quality images of only small portions of the heart, and the authors concluded that transesophageal echocardiography (TEE) was more effective than TTE for non-invasive examination of the dolphin heart.[4] Nevertheless, the authors reported that the TEE window was far more limited in dolphins than in primates, and that breath-holding following forced exhalation was necessary to obtain good quality TEE images (while decreasing lung interference and increasing the contact between heart and esophagus). This particular breathing behavior required 4 months of specific training of the BN involved in the study, which represents a technical limitation for the practical use of TEE in this species.

Anatomic M-mode (AMM) is a post-processing echocardiographic technique capable of generating M-mode studies from stored 2D cineloops independently of the ultrasound beam orientation.[6–9] This technique overcomes the main limitation of conventional M-mode echocardiography, i.e., fixed origin of the M-mode line at the sector apex, which may result in an incorrect alignment with the cardiac structures studied, and may thus lead to unreliable measurements of the latter. Using AMM, the observer has the opportunity to position the M-mode line in any spatial orientation defined from 2D views, thus improving measurements variability and increasing the ability to measure cardiac structures from various spatial directions.[6–11] We hypothesize that TTE using AMM could be suitable to assess the unsedated BN heart. To the best of our knowledge, LV morphology and function have never been quantitatively evaluated in this species.

The aims of this prospective study were therefore 1) to assess the feasibility of transthoracic AMM in healthy awake BN (optimal animal position, views, and probes), and then 2) to determine the intra-observer within-day (repeatability) and between-day (reproducibility) variability of the corresponding AMM measurements.

Animals, materials and methods

Animals

Four adult healthy BN were involved in the study, i.e., 2 females (15 and 31 years old; 180 and 250 kg, respectively) and 2 males (7 and 19 years old; 180 and 250 kg, respectively). Animals were considered healthy on the basis of visual examination before inclusion in the study (quality of interactions with trainers and other dolphins, food intake, buoyancy, observation of skin, eyes, blowhole and other body orifices).

Transthoracic echocardiographic technique and recording of 2D images

Transthoracic echocardiographic examinations were performed outdoors on the poolside of the Parc Astérix dolphinarium (Plailly, France) using a portable cardiovascular ultrasound system[f] equipped with a 3S phased-array transducer (1.5–3.5 MHz). The ultrasound unit was protected from water with a specifically manufactured waterproof case including a transparent window for the observer to see the screen and a removable top, so that an assistant could perform echo settings (transducer frequency, depth, focus location, gain, and compression) according to the observer's recommendations. The waterproof case containing the ultrasound system was placed near the edge of the pool under a black tarp when the weather was sunny (Fig. 1).

Different positions of the dolphins underwater (left and right lateral recumbency, ventral and dorsal recumbency, upright standing position) were investigated in a pilot study in an attempt to optimize image quality. For each position, the observer sat on the poolside with a trainer on her left and an aid (ACH) responsible for echocardiographic settings behind her (Fig. 1). The trainer was responsible for bringing the dolphin to an appropriate place in the water close to the observer.

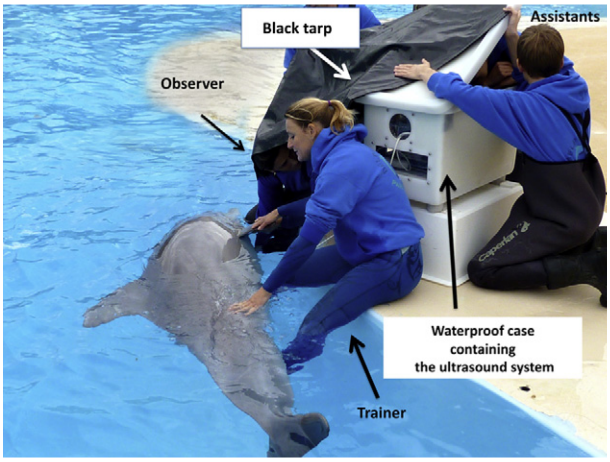

Figure 1 Photograph showing the organization of a transthoracic echocardiographic examination in a bottlenose dolphin. Left recumbency was considered the best animal position. The observer held the transducer in her right hand and the right pectoral fin in her left hand in order to help stabilize the dolphin's position and place the transducer against the dolphin's thorax.

After 4 sessions of trials, as already described by Miedler,[g] the most suitable animal position for obtaining correct to good quality 2D images was judged to be left lateral recumbency, with the probe placed under the water on the left side of the thorax, ventrally just near the sternum, at the level of the caudal portion of the left pectoral fin (Figs. 1 and 2). This probe placement provided a 2D parasternal long-axis view of the heart showing the LV ventrally and the globular aortic root dorsally (Figs. 3 and 4). After only a few sessions, the 4 dolphins were easily conditioned by the training staff to stay in this position for several minutes.

Protocol: assessment of intra-observer within-day and between-day variability of AMM variables

A total of 96 echocardiographic examinations were performed on 4 different days over a 2-week period by a single trained observer (VC), Diplomate of the European College of Veterinary Internal Medicine (Cardiology). Each dolphin was examined 6 times

[f] Vivid i, GE Healthcare, 9900 Innovation Drive, Wauwatosa, WI 53226, USA.

[g] Miedler S. Sonographic approach of the heart of *Tursiops truncatus* [abstract]. In: Proceedings of the 31th Annual Symposium of the European Association of Aquatic Mammals; 2003 Mar 14–17; Tenerife, Spain.

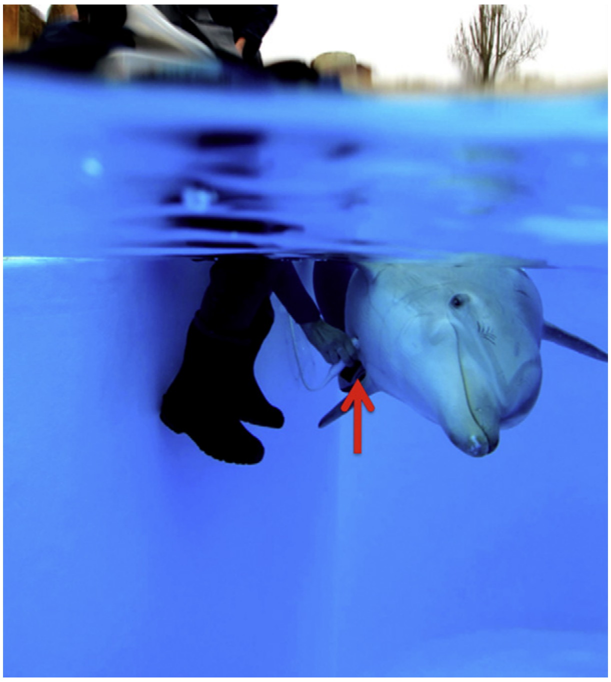

Figure 2 Photograph showing one of the bottlenose dolphins involved in the study, lying in the water on its left side near the poolside for recording of transthoracic two-dimensional echocardiographic images. The probe was first placed under the water just near the sternum approximately at the level of the caudal portion of the left pectoral fin (arrow). It was then slightly and slowly displaced on the left side of the thorax until an optimal left parasternal long-axis view was obtained of both the left ventricle and the aortic root. With the courtesy of Vincent Capman.

per day. The time between echocardiographic examinations was highly variable (from several minutes to 2 hours) owing to fixed feeding hours and daily rest periods, and depending also on the time for BN to stay in the appropriate position. Video clips of the previously described left parasternal long axis view, including at least 3 cardiac cycles, were recorded at each examination and stored in the system's hard drive. Second harmonic tissue imaging was used to obtain optimal 2D images. These video clips were then randomly analyzed for AMM measurements by the same observer and on the same day of the recordings. No specific randomization criteria was used except that the same animal could not be used twice consecutively. Anatomic M-mode images, showing motions of the LV at the top and of the aorta below (Fig. 5), were generated from digital 2D cineloops using a specific software.[h] The electronic AMM cursor was carefully placed across the LV and the aortic root, perpendicular to the aortic and dorsal LV myocardial walls (Fig. 4). Eight AMM variables were measured from inner edge to inner edge, i.e., ventral LV free wall thickness at end-diastole and end-systole, LV end-diastolic (LVDD) and end-systolic (LVDS) diameters, dorsal LV free wall thickness at end-diastole and end-systole, minimal (Aomin) and maximal (Aomax) aortic diameters. The mean aortic diameter, (Aomax + Aomin)/2, and the LV shortening fraction (%), defined as (LVDD − LVSD)/LVDD, were also calculated. Each echocardiographic variable was assessed once during the cardiac cycle for which the endocardial borders were considered as best defined. Heart rate was also assessed from each AMM view by calculating the time interval between 2 maximal thickenings of the dorsal LV free wall.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using computer software.[i] Briefly, and as already described in our previous ultrasound imaging validation studies performed on small animals,[12–18] the following linear model was used to analyze the within-day and between-day variability of the AMM variables:

$$Y_{ijk} = \mu + \text{day}_i + \text{dolphin}_j + (\text{day} \times \text{dolphin})_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} is the kth value measured for dolphin j on day i, μ is the general mean, day_i is the differential effect of day i, dolphin_j is the differential effect of dolphin j, $(\text{day} \times \text{dolphin})_{ij}$ is the interaction term between day and dolphin, and ε_{ijk} is the model error. The SD of repeatability was estimated as the residual SD of the model and the SD of reproducibility as the SD of the differential effect of day. The corresponding coefficients of variation (CV) were determined by dividing each SD by the mean.

Results

All echocardiographic examinations were interpretable and all 10 AMM variables could be calculated for each time. The mean heart rate ± SD during AMM examination was 54 ± 14 bpm (34–91), the lowest heart rates (<40 bpm) being recorded after sustained breath holding.

Table 1 gives the values for the 960 repeated AMM measurements. Within-day and between-day

[h] Vivid i BT 10 SW appl R. 10.3.0., GE Healthcare, 9900 Innovation Drive, Wauwatosa, WI 53226, USA.

[i] Systat version 10.0, SPSS Inc., Chicago, IL, USA.

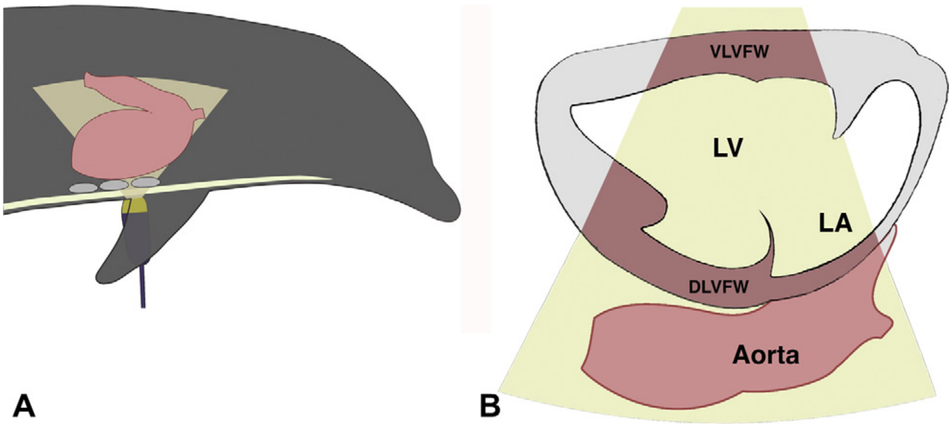

Figure 3 (A) illustrates the spatial orientation of the ultrasound plane within the dolphin heart to obtain a left parasternal long-axis view of the left ventricle ventrally and of the aortic root dorsally, as shown in (B). The transducer is placed ventrally near the sternum on the left side of the thorax and the plane traverses the heart from the left ventral to left dorsal side of the thorax. DLVFW, dorsal left ventricular free wall; LA, left atrium; LV, left ventricle; VLVFW, ventral left ventricular free wall.

SD and CV of AMM variables are shown in Table 2. The majority of within- and between-day CV values (18/20, 90%) were <15%. All within-day CV values [1.60–7.32%] were <10%, the lowest being observed for LVDD. More than half of the between-day CV values (6/10) were <10% [3.67–8.52%], the lowest also being recorded for LVDD. The two highest CV values (17.31% and 20.54%) were obtained for the two systolic LV myocardial walls (dorsal and ventral, respectively).

Discussion

Transthoracic echocardiography could represent a valuable tool for the non-invasive *antemortem* diagnosis of dolphin heart diseases. It could also be a useful imaging technique for non-invasive routine follow-ups or for performing cardiovascular physiological studies in this species. The present study provides for the first time quantitative data on LV morphology and function in awake

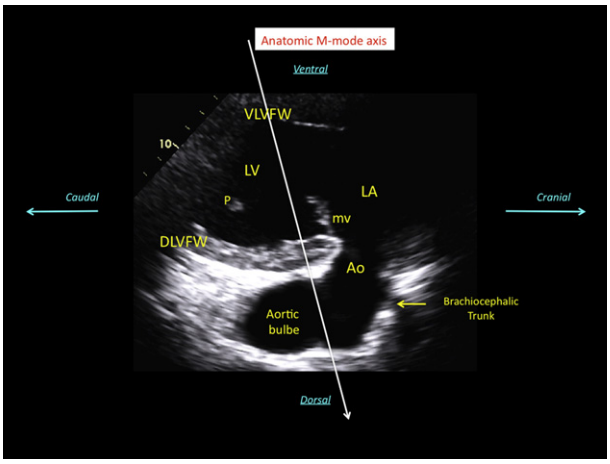

Figure 4 Two-dimensional left parasternal long-axis view obtained in one of the bottlenose dolphins involved in the study, showing the left ventricle (LV) ventrally and the aortic root (Ao) dorsally. Part of the left atrium (LA) is also seen as well as one of the 2 mitral valve leaflets (mv). The anatomic M-mode cursor is placed over the LV and the Ao, perpendicular to the dorsal left ventricular free wall (DLVFW) and aortic walls. VLVFW, ventral left ventricular free wall.

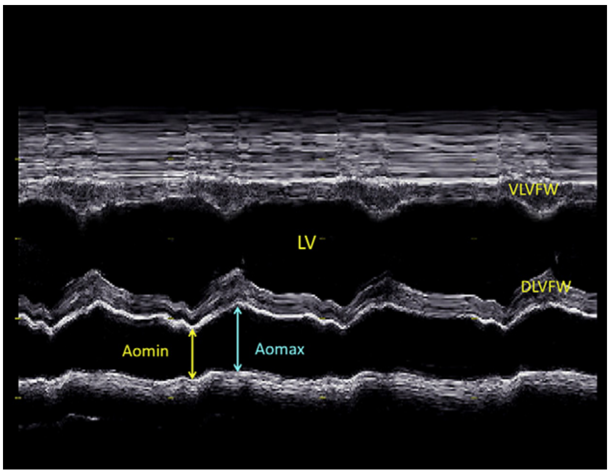

Figure 5 M-mode echocardiogram obtained in one of the bottlenose dolphins involved in the study and generated from a two-dimensional left parasternal long-axis view (see Figs. 3 and 4). This M-mode view shows from top to bottom, the ventral left ventricular free wall (VLVFW), the left ventricular cavity (LV), the dorsal left ventricular free wall (DLVFW), and the aortic root (Ao). The aorta moves upwards and increases in diameter during systole (Aomax). Conversely, it moves backwards and decreases in diameter during diastole (Aomin).

Table 1 Mean ± SD, minimum and maximum values of repeated measurements of echocardiographic variables obtained by a trained observer in 4 bottlenose dolphins (*Tursiops truncatus*) from 96 transthoracic examinations using anatomic M-mode.

Echocardiographic parameter	Mean ± SD	Minimum–maximum
Ventral left ventricular free wall thickness in diastole (mm)	12.7 ± 0.44	11.5–13.9
Ventral left ventricular free wall thickness in systole (mm)	21.2 ± 1.24	18.5–23.8
Left ventricular end-diastolic diameter (mm)	66.2 ± 3.80	61.3–75.6
Left ventricular end-systolic diameter (mm)	40.3 ± 2.00	35.0–43.9
Dorsal left ventricular free wall thickness in diastole (mm)	12.7 ± 0.50	11.3–14.4
Dorsal left ventricular free wall thickness in systole (mm)	21.0 ± 1.13	18.6–23.0
Shortening fraction (%)	39.0 ± 4.07	32.0–52.0
Minimal aortic diameter (mm)	40.0 ± 1.28	36.3–42.9
Maximal aortic diameter (mm)	46.8 ± 1.72	43.0–50.2
Mean aortic diameter (mm)	43.4 ± 1.27	40.2–46.4

BN using a safe, effective ultrasound technique, i.e., AMM TTE. This non-invasive imaging approach represents an initial step in the development of functional cardiology in cetaceans.

Few data are available regarding echocardiographic evaluation of the dolphin heart. Fetal echocardiography was recently described in BN by Sklansky et al.[19] The authors reported that a detailed assessment of fetal BN cardiovascular status, including 2D imaging and color flow mapping of the heart and great arteries as well as pulsed Doppler evaluation of the umbilical artery and vein, could be obtained between 8 and 9 months of gestation.[19] Another study, performed by the same authors on 4 adult BN trained to hold their breath following forced exhalation, showed that the TEE technique yielded high-quality images of the entire heart (atrioventricular and arterial valves, interatrial and interventricular septa, left and right atrial cavities, left and right ventricles, ascending aorta and main pulmonary artery).[4] The latter report also demonstrated mild tricuspid regurgitation in all BN, and mild aortic regurgitation in one BN using color flow Doppler mode.[4] Nevertheless, despite breath holding, reliable quantitative TEE measurements of ventricular size and function could not be obtained because of inconsistent animal positioning, and only maximal valve diameters could be measured.[4]

Advances in conventional ultrasound imaging techniques, including AMM echocardiography, have afforded new opportunities for non-invasive cardiac analysis in humans and various animal species.[6–11] The standard M-mode provides monodimensional echocardiograms and is commonly used for linear cardiac measurements, including ventricular diameters and myocardial wall thicknesses. This conventional echocardiographic technique is characterized by a high temporal resolution, and is therefore suitable for studying mobile structures.[8] However the major limitation of the standard M-mode is that the analysis line can only rotate on a fixed point, i.e., the sector apex.[8] The AMM technique overcomes this fixity drawback, as it provides M-mode images by orienting the analysis line in any direction according to the observer's desire.[8] Any cardiac structure in every angle shot

Table 2 Within-day and between-day variability, expressed as standard deviations (SD) and coefficients of variation (CV), of anatomic M-mode variables obtained by a trained observer on 4 bottlenose dolphins (*Tursiops truncatus*) from 96 transthoracic echocardiographic examinations.

Echocardiographic parameter	Within-day		Between-day	
	SD	CV (%)	SD	CV (%)
Ventral left ventricular free wall thickness in diastole (mm)	0.40	3.16	0.85	6.69
Ventral left ventricular free wall thickness in systole (mm)	1.00	4.72	4.35	20.54
Left ventricular end-diastolic diameter (mm)	1.06	1.60	2.43	3.67
Left ventricular end-systolic diameter (mm)	1.92	4.76	3.44	8.52
Dorsal left ventricular free wall thickness in diastole (mm)	0.46	3.65	1.03	8.13
Dorsal left ventricular free wall thickness in systole (mm)	0.94	4.47	3.64	17.31
Shortening fraction (%)	2.86	7.32	3.84	9.83
Minimal aortic diameter (mm)	0.96	2.39	4.41	11.04
Maximal aortic diameter (mm)	1.11	2.36	2.93	6.25
Mean aortic diameter (mm)	0.81	1.87	4.91	11.31

can therefore be analyzed. Additionally, AMM is a post-processing technique that can be performed on 2D stored images in the absence of the animal, which represents a real advantage for its practical use in aquatic mammals. Lastly, as AMM allows free orientation of the M-mode line, a perfect alignment (i.e., perpendicular to the heart axis) can therefore be consistently obtained in spite of morphovolumetrical variability,[8] which may help in improving the repeatability and reproducibility of the measurements. Several studies have demonstrated that AMM can increase the reproducibility and accuracy of standard M-mode LV measurements in humans, thereby limiting the risk of overestimating LV dimensions related to scan line misalignments.[6,9] Similarly, AMM has been shown to quantify LV and left atrial dimensions with a greater accuracy and less variability than conventional M-mode in healthy dogs.[11] The present study also demonstrates that AMM TTE provides a rapid and safe quantitative assessment of the unsedated BN heart.

One of the major prerequisites before a new technique can be proposed for *in vivo* investigation is to assess its repeatability and reproducibility. Our results show that the repeatability and reproducibility of the AMM technique in the awake BN are adequate for both research and routine clinical use: 90% and 80% of the within- and between day CV values were $<$15% and 10% respectively, which indicates a good to excellent variability of most of the AMM measurements.[20,21] These CV values are similar to those obtained in unsedated small animals using standard M-mode TTE.[20,21]

Although AMM may be affected by 2D echocardiography limitations, i.e., lower frame rate as compared to standard M-mode, recent advances in digital imaging technology have led to increased 2D frame rates of up to 250 frames/s, thus providing reconstruction of good quality M-mode images, similar to those obtained in BN in the present study.[8,11]

Sklansky et al. demonstrated that the TTE image quality in adult BN was not improved by breath holding. Similarly in the present study, no breath holding conditioning was necessary to obtain good quality AMM views.[4] Animals were positioned in left lateral recumbency with minimal restraint. Additionally, this position was easily and quickly obtained after a few training sessions, which represents a major advantage of this technique compared to TEE.

In the present study, the heart rate recorded during AMM examination was between 34 and 91 bpm, the lowest values being measured after prolonged breath holding. Similar findings in awake adult BN were reported by Sklansky et al.[4] In their study, heart rates ranged from 60 to 70 bpm, and rapidly decreased to between 25 and 35 bpm during sustained breath holding following forced exhalation.[4] Such an acute decrease in heart rate (up to more than 50% from basal values) is a well described physiological adaptation to apnea in aquatic mammals including dolphins, both at rest beneath the water surface and during dives.[22,23]

This preliminary work on TTE in BN presents several limitations. Quantitative measurements were limited to the left heart and aorta. The interventricular and interatrial septa, as well as the right heart, were not evaluated. These cardiac structures were relatively difficult to analyze using TTE, owing to the relatively small window size. Additionally, our previous studies in dogs and cats showed that echocardiography is a highly observer-dependent examination.[20,21] The results presented here are thus only valid for the observer involved, and the authors encourage marine mammal veterinarians to determine their own AAM TTE variability, before undertaking further echocardiographic studies in BN.

Conclusion

In conclusion, AMM TTE provides a simple, rapid and safe quantitative assessment of LV size and function in the awake BN, with good repeatability and reproducibility as well as minimal animal restraint and conditioning. This imaging technique could be used for both clinical and research assessment of the normal and failing BN heart. Further studies are now required to determine the corresponding reference intervals in large healthy BN populations. Further investigations are also warranted to analyze potential gender and body weight effects on AMM variables, as described in small animals. The accuracy of the described technique for the *antemortem* diagnosis of dolphin heart diseases should also be assessed.

Conflict of interest

There are no conflicts of interest.

Acknowledgments

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