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Echocardiographic values for normal conditioned and unconditioned North American whippets

Rebecca L. Stepien[1] | Heidi B. Kellihan[1] | Lance C. Visser[2] | Lisa Wenholz[1] | Virginia Luis Fuentes[3]

[1]Department of Medical Sciences, University of Wisconsin School of Veterinary Medicine, Madison, Wisconsin, USA

[2]Department of Clinical Sciences, Colorado State University College of Veterinary Medicine and Biomedical Sciences, Fort Collins, Colorado, USA

[3]Department of Clinical Science and Services, Royal Veterinary College, Hatfield, Hertfordshire, UK

Correspondence
Rebecca L. Stepien, University of Wisconsin School of Veterinary Medicine, 2015 Linden Drive, Madison, WI 53711, USA.
Email: rebecca.stepien@wisc.edu

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Abstract

Background: Echocardiographic reference intervals have not been reported for North American whippets, or for whippets that have undergone pet-level athletic training.

Objectives: To develop normal echocardiographic reference intervals for North American whippets and investigate differences in echocardiographic parameters based on athletic conditioning in pet whippets engaged in competitive sports.

Animals: One-hundred healthy North American whippets.

Methods: Dogs were examined at national shows between 2005 and 2009. Echocardiographic reference intervals were constructed and the effect of athletic conditioning on parameters of structure and function was assessed.

Results: Two dimensional, M-mode, Doppler and tissue Doppler reference ranges for healthy North American whippets are presented. Measures of left ventricular (LV) chamber diameter were larger in conditioned whippets (N = 25) and remained significantly larger than in unconditioned whippets (N = 16) when normalized for weight using allometric equations. Calculated LV mass was higher in conditioned dogs than in unconditioned dogs, and this difference persisted when LV mass was normalized by weight. Mitral E velocity was higher in conditioned dogs than in unconditioned dogs, whereas E/A and measures related to systolic function were not different.

Conclusions and Clinical Importance: Pet whippets in peak athletic condition have larger hearts than do less conditioned whippets, but measures of systolic function are similar. Whippet pet athletes may show eccentric LV hypertrophy at peak condition. Normal values for cardiac size and function in North American whippets might be

Abbreviations: a', peak late diastolic myocardial velocity determined by tissue Doppler imaging; AoLTvel, peak aortic systolic velocity, left apical view; AoSax, aortic diameter in short axis; AoSCvel, peak aortic systolic velocity, subcostal view; Avel, mitral valve peak A wave velocity; dLVvol, left ventricular diastolic volume; e', peak early diastolic myocardial velocity determined by tissue Doppler imaging; E/A, mitral valve E wave to A wave ratio; EF, ejection fraction; EPSS, E-point septal separation; Evel, mitral valve peak E wave velocity; FS, left ventricular fractional shortening; IVSd, interventricular septal thickness in diastole; IVSdN, interventricular septal thickness in diastole indexed for body weight; IVSs, interventricular septal thickness in systole; LA, AoLax: left atrial to aortic diameter ratio in long axis; LA, AoSax: left atrial to aortic diameter ratio in short axis; LADLax, left atrial diameter in long axis; LADLaxN, left atrial diameter in long axis indexed for body weight; LADSax, left atrial diameter in short axis; LV, left ventricular; LVIDd, left ventricular diameter in diastole; LVIDdN, left ventricular diameter in diastole indexed for body weight; LVIDs, left ventricular diameter in systole; LVIDsN, left ventricular diameter in systole indexed for body weight; LVmass, calculated left ventricular mass; LVWd, left ventricular wall thickness in diastole; LVWdN, left ventricular wall thickness in diastole indexed for body weight; LVWs, left ventricular wall thickness in systole; MMVD, myxomatous mitral valve disease; PVvel, peak pulmonary valve systolic velocity; s', peak systolic myocardial velocity determined by tissue Doppler imaging; sLVvol, left ventricular systolic volume; SV, stroke volume.

considered abnormal if population-specific whippet reference intervals are not used in analysis.

KEYWORDS
athletic, canine, dog, echocardiography, reference values

1 | INTRODUCTION

As a breed, whippets are typically physically active and may participate in racing, agility and other performance events as well as functioning as pet companions. The acknowledged risk of adult-onset myxomatous mitral valve disease (MMVD) in whippets[1,2] as well as recent concerns about the effect of diet on cardiac function[3,4] have increased interest in echocardiographic screening of at-risk dogs for evidence of subclinical MMVD or changes suggestive of cardiomyopathy.

Reliable reference intervals are essential when reviewing echocardiographic data for evidence of mild abnormalities. Previous studies of sighthounds including western European whippets, Australian whippets, greyhounds, Italian greyhounds and salukis[5-10] as well as large studies of various breeds of normal dogs[11] have indicated that weight or body surface area-based normal echocardiographic reference ranges for dogs in general may not be applicable to sighthounds, presumably because of sighthounds' distinctive body conformation, degree of athleticism, or both. Other methods of adjusting measurements for body size have been suggested[12,13] and some have been applied to whippets.[5]

Some studies have indicated that purposeful athletic conditioning (eg, for endurance or racing events or as treadmill training) results in cardiac hypertrophy that might be misdiagnosed as abnormal in a healthy dog,[14-16] but it is currently unclear if the degree of athletic training in casual athletes (ie, pet animals that regularly participate in organized athletic events) is sufficient to cause physiologic hypertrophy to an extent that might be misdiagnosed as abnormal.

Our aims were to develop normal echocardiographic reference intervals for healthy North American whippets and to investigate possible differences in echocardiographic parameters based on athletic conditioning in pet whippets engaged in competitive sports.

2 | MATERIALS AND METHODS

2.1 | Patient population

Our study was an observational cross-sectional study that was approved by the Institutional Animal Care and Use Committee at the University of Wisconsin School of Veterinary Medicine. All dog owners provided informed consent. Dogs included in the study were part of a healthy population screened at the American Whippet Club National Specialty as participants or spectators between 2005 and 2009. The National Specialty includes show, lure coursing, agility, endurance and obedience events, and subjects examined included dogs with variable degrees of athletic conditioning. Genetic background regarding racing or show breeding lines[5,7] was not investigated. Dogs were submitted for examination by their owners without regard to age or breeding status. Over the course of the events of 2005-2009, 341 dogs were examined. Dogs chosen for further analysis to develop normal reference intervals included only clinically healthy dogs with no or trivial valvular regurgitation identified by echocardiography. Only the first examination of any dog that had undergone multiple examinations over the study time frame was included for further analysis. Complete diet histories were not obtained. The time frame chosen for review was limited to years preceding recent reports of diet-related cardiac disease in dogs.

Based on previous studies suggesting that athletic conditioning may influence some cardiac parameters,[5,7,14,15,17] dogs were allocated to 4 conditioning levels based on owner assessment of their own dogs. Owners were asked to subjectively identify their dogs' athletic conditioning level as "peak" physical condition currently competing in athletic events (C1), "good" physical condition but not currently competing (C2), "pet-level" condition (daily walks and play but not training, C3) or "non-athletic" (C4).

2.2 | Examination

Heart rate was recorded by auscultation before echocardiographic examination. All echocardiograms were recorded by a single echocardiographer (VLF, a board-certified veterinary cardiologist) using an Acuson Cypress System (Siemens Medical Solutions USA, Inc., Mountain View, California) with 7V3c (2-dimensional images) and 3V2c (Doppler signals) phased array sector probes. Dogs were restrained in right and left lateral recumbency in the presence of the owner or handler on a table with a cut-out to allow placement of the ultrasound probe on the lowermost side of the thorax. A lead 2 ECG tracing was recorded simultaneously for measurement purposes. The echocardiographer was unaware of age, auscultation results, clinical history and any findings available from examinations in previous years. Echocardiographic, color- and spectral Doppler images were stored on optical discs for later off-line analysis by a single trained observer blinded to condition status (LJW). All measurements were recorded as the mean of 3 consecutive cardiac cycles.

Images were obtained in the same sequence, according to the recommendations of the Echocardiography Committee of the Specialty of Cardiology, American College of Veterinary Internal Medicine[18] for all dogs. In each view, the visible valves were screened for

TABLE 1 Echocardiographic measurements obtained from 100 North American whippets in the sequence in which they were obtained.

Parameter	Abbreviation	Units	Method
Right parasternal long axis four-chamber view (2-D)			
LV diastolic volume	dLVvol	mL	Modified SMOD, monoplane, inner edge area at end-diastole
LV systolic volume	sLVvol	mL	Modified SMOD, monoplane, inner edge area at peak systole
LA end-systolic diameter	LADLax	cm	Inflow view, diameter measured midway between MV annulus and pulmonary vein parallel to MV annulus[13]
LA end-systolic area	LAA	cm^2	Area traced using SMOD, inner edge method in basilar view excluding the pulmonary vein[13]
Ao systolic diameter	AoLax	cm	Outflow view, diameter measured at valve hinge points during systole
Right parasternal short axis view (chordal level, M-mode)			
IVS thickness in diastole	IVSd	cm	Leading edge to leading edge method at end-diastole
LV diameter in diastole	LVIDd	cm	Leading edge to leading edge method at end-diastole
LV wall thickness in diastole	LVWd	cm	Leading edge to leading edge method at end-diastole
IVS thickness in systole	IVSs	cm	Leading edge to leading edge method at end-systole
LV diameter in systole	LVIDs	cm	Leading edge to leading edge method at end-systole
LV wall thickness in systole	LVWs	cm	Leading edge to leading edge method at end-systole
E-point to septal separation	EPSS	cm	Leading edge to leading edge method at maximal E wave excursion of MV
Right parasternal short axis view (at heart base, 2-D)			
Ao diameter in short axis	AoSax	cm	Inner edge to inner edge, midpoint of right coronary cusp to opposite wall at point of left coronary cusp/noncoronary cusp convergence at early diastole[33]
LA diameter in short axis	LASax	cm	Extension of Ao diameter line from Ao wall to inner edge of opposite LA wall at early diastole[33]
PV peak systolic velocity	PVvel	m/s	Pulsed waved Doppler, sample volume placed just distal to PV leaflets
Subcostal view			
Ao peak systolic velocity	AoSCvel	m/s	Measured using continuous wave Doppler with 2-D guidance
Left apical four-chamber view (2-D)			
MV peak E wave velocity	Evel	m/s	Pulsed waved Doppler, sample volume placed at tips of open mitral valve in diastole
MV peak A wave velocity	Avel	m/s	Pulsed waved Doppler, sample volume placed at tips of open mitral valve in diastole
Left apical 3-chamber view (2-D)			
Peak Ao systolic velocity	AoLTvel	m/s	Pulsed waved Doppler, sample volume placed just distal to Ao valve leaflets/Ao sinuses
TDI e' velocity	e'	m/s	Tissue Doppler, sample volume in basal segment of LV wall at lateral MV annulus, mid-diastole[34]
TDI a' velocity	a'	m/s	Tissue Doppler, sample volume in basal segment of LV wall at lateral MV annulus, end-diastole[34]
TDI s' velocity	s'	m/s	Tissue Doppler, sample volume in basal segment of LV wall at lateral MV annulus, mid-systole[34]
Calculated parameters			
Ejection fraction	EF	%	$EF = (dLVvol - sLVvol/dLVvol) \times 100$
Fractional shortening	FS	%	$FS = (LVIDd - LVIDs/LVIDd) \times 100$
Left atrial to aortic diameter, long axis	LA:AoLax		Using LADLax and AoLax (2-D, right parasternal long axis)
Left atrial to aortic diameter short axis	LA:AoSax		Using LASax and AoSax (2-D, right parasternal short axis)

TABLE 1 (Continued)

Parameter	Abbreviation	Units	Method
E/A	E/A		Ratio of peak E wave velocity to peak A wave velocity (mitral inflow)
LV mass (calculated)	LVmass	g	LVmass = 1.04 [LVIDd + LVWd + IVSd^3] − 13.6 g, using M-mode measurements obtained from right parasternal short axis view at level of chordae tendineae[19]
LV mass/kg	LVmass/kg	g/kg	Calculated LVmass/kg body weight
LV diastolic volume/kg (mL/kg)	dLVvol/kg	mL/kg	LV diastolic volume by monoplane SMOD (right parasternal long axis view) per kg body weight
LV systolic volume/kg (mL/kg)	sLVvol/kg	mL/kg	LV systolic volume by monoplane SMOD (right parasternal long axis view) per kg body weight
Stroke volume	SV	mL	SV = dLVvol − sLVvol (2-D, right parasternal long axis)
Stroke volume/kg	SV/kg	mL/kg	Calculated SV/kg body weight

Note: References are provided when applicable.
Abbreviations: Ao, aortic valve; IVS, interventricular septum; LA, left atrium; LV, left ventricle; MV, mitral valve; PV, pulmonic valve; SMOD, Simpson's method of discs; TDI, tissue Doppler imaging; 2-D, two-dimensional imaging.

anatomic abnormalities or regurgitation jets. Dogs with any valvular regurgitation considered more than trivial were excluded from further analysis.

Measurements were obtained from both 2-dimensional (2-D) and M-mode images. Details of measurement abbreviations, the order of and methods by which the values were obtained, calculated values and formulae used are presented in Table 1. Left ventricular M-mode measurements were obtained at the level of the chordae tendineae by the leading-edge to leading-edge method. End-diastolic measurements were obtained using image frames at the onset of the QRS complex, and end-systolic measurements were obtained at the point of maximal septal systolic excursion. Volumetric measurements were obtained using a monoplane Simpson's method of discs by tracing the endocardial border of the chamber in question using the right parasternal long-axis 2-D images optimized for LV inflow.[7] Left ventricular mass and LV mass/kg were estimated using M-mode measures of interventricular septum in diastole (IVSd), left ventricular wall in diastole (LVWd) and left ventricular internal diameter in diastole (LVIDd) according to previously described methods.[19]

2.3 | Statistical methods

Commercial software programs (Prism 9, GraphPad Software LLC, San Diego, California and MedCalc Statistical Software, MedCalc Software bvba, Ostend, Belgium) were used for data analysis. Descriptive statistics were generated for population characteristics and normal dataset distribution was assessed using a Shapiro-Wilk test. Multiple regression was used to explore the effects of conditioning (C1 vs "not C1" categorization), age, body weight and sex (without regard to neuter status) on selected echocardiographic measures of cardiac structure and function. Ninety-five percent reference intervals were generated using the "robust method," as recommended by Clinical Laboratory Standards Institute guidelines when the reference sample does not exceed 120 subjects.[20] Ninety percent confidence intervals were generated for reference limits using a bootstrapping technique.

Selected linear measurements (left atrial diameter in long axis [LADLax], LVIDd, left ventricular internal diameter in systole [LVIDs], IVSd, LVWd) were normalized (indexed) to body weight by dividing by body weight (kg)^b. The constant b was specific to each measurement and was generated by allometric scaling or nonlinear regression using the power equation, $Y = ax^b$, where a represents the proportionality constant, b represents the scaling exponent, Y is the linear echocardiographic measurement, and x is body weight (kg).[12]

To further investigate the effects of high-level athletic conditioning, dogs were separated into 2 subgroups based on condition score; "conditioned" dogs included dogs categorized by their owners as C1 (n = 27), and "unconditioned" dogs (n = 16) included dogs categorized as C3 or C4. Selected parameters were compared between these groups using an unpaired t test or Mann-Whitney U test, as appropriate. No adjustment was made for multiple comparisons. Significance for all statistical tests was set at $P < .05$.

3 | RESULTS

Results from examinations of 100 whippets (56 female or spayed female, 44 male or castrated male) were assessed. Population characteristics are presented in Table 2. At the time of examination, dogs were assessed by their owners as being in Condition 1 (C1, n = 27), Condition 2 (C2, n = 55), Condition 3 (C3, n = 14) or Condition 4 (C4, n = 2). Two dogs did not have a condition score recorded. Echocardiographic results (2-D, M-mode, Doppler, calculated values) are presented in Table 3, including number of measurements available, mean, *SD*, median, range and calculated 95% reference limits with 90% confidence intervals. Measurements obtained from sub-optimal images were excluded from analysis; not all values were available for all dogs.

TABLE 2 Population characteristics of 100 normal North American whippets.

Clinical data	N	Mean	*SD*	Median	Min-Max	Other
Age (years)[a]	100	3.3	2.2	2.7	.4-9.0	IQR = 1.75-4.40
Body weight (kg)[a]	100	15.2	2.4	14.7	9.8-22.2	IQR = 13.3-16.6
Heart rate (bpm)[a]	100	99.0	21.9	90.0	60.0-160.0	IQR = 80.0-110.0
Sex (female/male)	100					56 female, 44 male

Abbreviation: IQR, interquartile range.
[a]Reject normality (Shapiro-Wilk test).

Assessment of the influence of age, sex, weight, and C1 status is presented in Table 4. Sex had no apparent effect on any measured or calculated echocardiographic values. Age was not associated with any linear or volumetric echocardiographic measurements but was associated with several indirect measures of function. A weak but significant negative association was found between age and E wave velocity (Evel; $P = .0005$; $r = -0.35$), a weak but positive association of age with A wave velocity (Avel; $P = .009$; $r = 0.27$) and a moderate negative association between age and E wave to A wave ratio (E/A; $P < .0001$; $r = 0.42$). Weight was positively associated with measures of left atrial, aortic and left ventricular dimensions and volumes; these associations were negated by indexing to body weight by use of allometric equations (Table 5) or by normalizing the values by comparison to aortic size (left atrial to aorta in the long axis [LA:AoLax], left atrial to aorta in the short axis [LA:AoSax]) or on a per kilogram (kg) basis. The categorization of C1 (yes/no) showed a weak but positive association with calculated LVmass ($P = .03$; $r = 0.18$) and this association was retained when LVmass was normalized on a per kg basis ($P = .04$; $r = 0.21$).

Results of comparisons between the "conditioned" and "unconditioned" groups are presented in Table 6. "Conditioned" dogs were younger and had lower heart rate on examination than did "unconditioned" dogs, but weight did not differ between the groups. No difference was found in measures of left atrial diameter (LADLax, LA:AoSax, LA:AoLax) between the groups. Measures of left ventricular chamber diameter (LVIDd, LVIDs, E-point septal separation [EPSS]) were significantly larger in "conditioned" dogs, and left ventricular diameters normalized for weight using the allometric equations (LVIDdN and LVIDsN) remained significantly larger than those of "unconditioned" dogs. Regardless of conditioning status, LVIDd and LVIDs were frequently above a published reference range for dogs of unspecified breed and similar weight[21] (Figure 1). The IVSd was significantly higher ($P = .04$) in "conditioned" dogs but this difference was not significant for IVSdN. Left ventricular volume in systole and diastole tended to be higher in "conditioned" dogs, but the differences were not significant. Calculated LVmass was higher in "conditioned" dogs vs "unconditioned" dogs ($P = .007$; Figure 2) and this difference persisted when LVmass was normalized by weight ($P = .002$). Among the functional measurements examined, Evel was higher in "conditioned" dogs ($P = .005$), whereas E/A and measures related to systolic function (ejection fraction [EF], fractional shortening [FS], tissue Doppler imaging of peak systolic velocity [s']) did not differ.

4 | DISCUSSION

Multiple studies have established breed-related differences in normal echocardiographic values in dogs, particularly sighthounds.[5-9,11,22-24] Previous studies of whippets have involved populations in Europe[5,7,11,23] and Australia[6] and have rendered population-specific reference ranges and indexed values. Indexing of echocardiographic variables, whether on a per kg basis, by body surface area or by use of allometric equations derived for application to all breeds[12,13] has allowed development of reference intervals applicable over a wide range of body weights. Breed-specific reference intervals and indexed values have been advocated for echocardiographic measurements and calculated values. This recommendation is especially important when comparing echocardiographic measurements of chamber size in a breed where adult body weight may vary by 100% in a healthy population or in single breed populations that may differ because of local genetic breeding pools. The population of healthy whippets in our study was drawn from whippets bred and living in North America and ranged in weight from 9.8 to 22.2 kg. The mean weight of these dogs was significantly higher than that of dogs reported in previous studies drawn from European whippet populations,[5,7,23] emphasizing the need for population-specific indexed reference intervals when weight is used in the calculations. As in previous studies, the dogs in our study had left ventricular chamber and wall dimensions generally larger than those published for dogs of nonspecified breeds.[11,21] The coefficients of allometric equations developed for these North American whippets (Table 5) differ from those developed in European whippets[5] and from coefficients developed for application over a range of breeds.[11-13] Our results provide reference intervals for echocardiographic parameters of cardiac size and function in North American whippets and add indexed values for selected commonly used parameters as well as additional information on measurements not previously available in whippets.

We did not find independent associations between sex and any of the variables assessed. This finding contrasts with previous studies,[5,7] in which males tended to have some values that were higher than observed in females. In those studies, the sex association did not persist when values were indexed, again supporting use of indexed normal values when assessing cardiac size in this breed. Age distribution in the dogs of our study showed a predominance of younger animals, as would be expected in a healthy population traveling to national specialty events that include both show and performance trials. Nonetheless, increasing age was found to be significantly associated with measures reflecting decreased LV early filling velocities

TABLE 3 Reference intervals (95% RI, generated using "robust" method [n = 100] reporting lower limit and upper limit, each with 90% confidence intervals) for measured and calculated echocardiographic variables in normal North American whippets.

						95% reference intervals
Echo measurement	**N**	**Mean**	***SD***	**Median**	**Min-Max**	**Lower limit (90% CI) – upper limit (90% CI)**
2-D/M-MODE						
LADLax (cm)	99	3.47	.33	3.46	2.64-4.34	2.81 (2.71-2.90), 4.14 (4.03-4.23)
LASax (cm)	96	2.62	.35	2.60	1.71-3.56	1.89 (1.79-1.98), 3.30 (3.20-3.41)
AoLax (cm)	99	1.70	.14	1.70	1.34-2.04	1.43 (1.40-1.47), 1.98 (1.93-2.01)
AoSax (cm)	96	2.04	.20	2.02	1.52-2.57	1.64 (1.59-1.71), 2.42 (2.35-2.47)
LAA (cm^2)	99	9.82	1.82	9.61	5.82-14.26	6.12 (5.69-6.60), 13.42 (12.85-13.92)
LA:AoLax	98	2.05	.19	2.04	1.53-2.55	1.67 (1.61-1.72), 2.43 (2.38-2.48)
LA:AoSax	96	1.28	.17	1.27	.82-1.62	.95 (.90-1.00), 1.62 (1.57-1.67)
IVSd (cm)[a]	97	1.02	.14	1.00	.75-1.39	.72 (.69-.77), 1.28 (1.23-1.32)
LVIDd (cm)	97	3.73	.36	3.75	2.69-4.66	3.02 (2.90-3.12), 4.44 (4.34-4.55)
LVWd (cm)	97	.88	.13	.87	.63-1.22	.61 (.57-.64), 1.13 (1.09-1.17)
IVSs (cm)[a]	97	1.23	.17	1.20	.84-1.94	.88 (.81-.94), 1.56 (1.49-1.62)
LVIDs (cm)	97	2.88	.38	2.84	1.93-3.89	2.10 (2.00-2.21), 3.63 (3.51-3.75)
LVWs (cm)	97	1.13	.17	1.11	.73-1.6	.77 (.73-.82), 1.47 (1.42-1.52)
LVmass (g)	97	121.3	31.9	119.4	60.5-207.5	56.0 (47.7-64.4), 183.7 (173.9-193.0)
EPSS (cm)[a]	90	.43	.15	.42	.22-.89	.12 (.08-.16), .72 (.66-.77)
DOPPLER						
PVvel (m/s)[a]	88	.97	.21	.93	.56-1.91	.51 (.42-.61), 1.35 (1.25-1.45)
AoSCvel (m/s)	81	1.74	.27	1.73	1.03-2.41	1.19 (1.11-1.29), 2.29 (2.20-2.38)
AoLTvel (m/s)	97	1.50	.26	1.47	.99-2.19	.96 (.89-1.03), 1.99 (1.90-2.08)
Evel(m/s)	97	.79	.14	.80	.40-1.29	.52 (.47-.57), 1.06 (1.02-1.11)
Avel (m/s)[a]	97	.39	.12	.38	.16-.72	.14 (.11-.17), .62 (.58-.66)
E/A[a]	97	2.22	.74	2.16	1.05-4.78	.67 (.42-.87), 3.62 (3.36-3.88)
e′ (m/s)[a]	99	.19	.05	.18	.07-.37	.07 (.05-.09), .29 (.27-.30)
a′ (m/s)[a]	99	.12	.11	.09	.05-.89	.05, .53[b]
s′ (m/s)[a]	99	.14	.04	.14	.08-.25	.07 (.05-.08), .21 (.2-.22)
Volume/function						
FS (%)[a]	96	23.2	5.7	23.0	10.0-43.0	11.1 (9.2-13.2), 34.0 (32.1-35.9)
EF (%)[a]	99	59.0	7.9	59.9	33.8-82.2	43.9 (41.2-46.8), 75.5 (73.0-78.1)
dLVvol (mL)[a]	99	52.0	11.4	50.9	30.0-80.8	27.9 (24.3-30.9), 73.5 (69.6-77.2)
sLVvol (mL)[a]	99	21.7	8.0	20.1	7.8-53.5	3.9 (1.1-6.9), 36.3 (32.9-39.3)
SV (mL)	99	30.3	6.0	29.7	16.4-53.5	18.0 (16.0-20.1), 42.2 (40.1-44.1)
Indexed parameters						
LADLaxN ($cm/kg^{.391}$)	99	1.21	.09	1.20	.86-1.41	1.03 (1.00-1.06), 1.39 (1.36-1.42)
IVSDdN ($cm/kg^{.222}$)[a]	97	.56	.07	.55	.43-.75	.40 (.38-.42), .70 (.67-.72)
LVWdN ($cm/kg^{.335}$)[a]	97	.35	.05	.35	.26-.47	.25 (.24-.26), .45 (.43-.47)
LVIDdN ($cm/kg^{.332}$)	97	1.52	.12	1.52	1.17-1.81	1.28 (1.24-1.32), 1.77 (1.73-1.80)
LVIDsN ($cm/kg^{.433}$)	97	.89	.10	.90	.61-1.15	.69 (.66-.72), 1.09 (1.06-1.12)
LVmass/kg (g/kg)[a]	97	8.00	1.73	7.69	4.75-11.73	4.4 (3.96-4.86), 11.41 (10.85-11.92)
dLVvol/kg (mL/kg)	99	3.42	.49	3.40	2.39-4.65	2.42 (2.29-2.57), 4.38 (4.23-4.52)
sLVvol/kg (mL/kg)[a]	99	1.42	.42	1.33	.48-3.07	.47 (.33-.64), 2.19 (2.02-2.35)
SV/kg (mL/kg)	99	2.00	.30	2.00	1.25-2.81	1.4 (1.32-1.50), 2.6 (2.51-2.68)

Note: Not all values were available for all dogs.

Abbreviations: Ao, aortic valve; IVS, interventricular septum; LA, left atrium; LV, left ventricle; MV, mitral valve; PV, pulmonic valve; SMOD, Simpson's method of discs; TDI, tissue Doppler imaging; 2-D, 2-dimensional imaging.

[a]Reject normality (Shapiro-Wilk test).

[b]Confidence intervals could not be calculated.

TABLE 4 Association of peak athletic conditioning (Condition 1 status, n = 27), body weight, age and sex with selected echocardiographic measures of structure, volume and function in normal North American whippets.

	N	Condition 1 status (yes/no)	Body weight (kg)	Age (mo)	Sex (M/F)
Structural measures					
LADLax (cm)	99	NS	$r = .40$, $P < .0001$	NS	NS
LASax (cm)	96	NS	$r = .28$, $P = .03$	NS	NS
AoLax (cm)	99	NS	$r = .37$, $P < .0001$	NS	NS
AoSax (cm)	96	NS	$r = .41$, $P < .0001$	NS	NS
LA:AoLax	98	NS	NS	NS	NS
LA:AoSax	96	NS	NS	NS	NS
IVSd (cm)[a]	97	NS	NS	NS	NS
LVIDd (cm)	97	NS	$r = .42$, $P < .0001$	NS	NS
LVWd (cm)	97	NS	NS	NS	NS
IVSs (cm)[a]	97	NS	NS	NS	NS
LVIDs (cm)	97	NS	$r = .28$, $P = .002$	NS	NS
LVWs (cm)	97	NS	NS	NS	NS
LVIDdN (cm/kg^{332})	97	NS	NS	NS	NS
LVIDsN (cm/kg^{433})	97	NS	NS	NS	NS
LVmass (g)	97	$r = .18$, $P = .03$	$r = .34$, $P < .0001$	NS	NS
LVmass/kg[a] (g/kg)	97	$r = .21$, $P = .04$	NS	NS	NS
EPSS (cm)[a]	90	NS	NS	NS	NS
Volume measures					
dLVvol (mL)[a]	99	NS	$r = .50$, $P < .0001$	NS	NS
sLVvol (mL)[a]	99	NS	$r = .36$, $P = .0001$	NS	NS
dLVvol/kg (mL/kg)	99	NS	NS	NS	NS
sLVvol/kg (mL/kg)	99	NS	NS	NS	NS
SV (mL)	99	NS	$r = .46$, $P < .0001$	NS	NS
SV/kg	99	NS	NS	NS	NS
Functional measures					
EF (%)[a]	99	NS	NS	NS	NS
FS (%)[a]	96	NS	NS	NS	NS
Evel (m/s)	97	NS	NS	$r = -.35$, $P = .0005$	NS
Avel (m/s)[a]	97	NS	NS	$r = .27$, $P = .009$	NS
E/A	97	NS	NS	$r = -.42$, $P < .0001$	NS

Note: The *r* value is presented to estimate the strength of the association when significant ($P < .05$, bold).
Abbreviations: Ao, aortic valve; IVS, interventricular septum; LA, left atrium; LV, left ventricle; MV, mitral valve; NS, association was not significant; PV, pulmonic valve; SMOD, Simpson's method of discs; TDI, tissue Doppler imaging; 2-D, 2-dimensional imaging.
[a]Reject normality (Shapiro-Wilk test).

(decreased mitral Evel, increased mitral Avel, and decreased E/A in the absence of changes in preload). The negative association between age and Evel, and age and E/A and the positive association of age and Avel are similar to the associations reported previously in 2 different studies of dogs of various breeds[25,26] and consistent with previous reports in people where age was the most important determinant of Doppler-assessed LV early filling in healthy subjects, so much so that decade-specific reference ranges for these values may be used in people.[27,28] Although parameters associated with LV early filling decreased with age, no dog, regardless of age, had an E/A ratio < 1.0, a value considered to indicate impaired myocardial relaxation.[25,29] In contrast to other studies of dogs of multiple breeds,[25] many whippets in our study had E/A ≥ 2.0 (53/97, 55%) and E/A values ≥2.0 were more common in the younger dogs (38/56 [68%] dogs ≤3 years vs 14/41 [34%] dogs >3 years of age). Increased E/A ratio because of a predominance of early diastolic filling may reflect so-called "supernormal" diastolic function in these healthy dogs. Similar findings are seen in athletic people at rest, wherein an E/A ratio > 2 is expected, with predominance of the early diastolic phase of filling.[30]

Previous studies have confirmed that whippets as a breed have increased LV dimensions and volumes on a body weight basis but found little[5] or no[7] difference between dogs purpose-bred for racing

TABLE 5 Results of the linear regression analyses describing how log_{10} of selected linear chamber measurements relate to log_{10} body weight in 100 healthy North American whippets.

Echo measurement	*a*	SE of Y estimate	Scaling exponent *b*	SE of *b*	R^2
IVSd (n = 97)	.55	.100	.222	.085	.07
LVIDd (n = 97)	1.51	.064	.332	.054	.28
LVWd (n = 97)	.35	.109	.335	.092	.12
LVIDs (n = 97)	.89	.089	.433	.076	.26
LADLax (n = 99)	1.20	.061	.391	.052	.37

Note: The constants derived permit the calculation normalized echocardiographic linear measurements for any body weight (in kg) using the equation: measured linear dimension (in cm)/body weight (in kg)^b, where *b* is the scaling exponent respective to each index. All correlations were statistically significant ($P < .05$).
Abbreviations: Ao, aortic valve; IVS, interventricular septum; LA, left atrium; LV, left ventricle; MV, mitral valve; NS, association was not significant; PV, pulmonic valve; SMOD, Simpson's method of discs; TDI, tissue Doppler imaging; 2-D, 2-dimensional imaging.

TABLE 6 Comparison of selected echocardiographic values between "conditioned" dogs and "unconditioned" dogs.

Parameter	Conditioned dogs		Unconditioned dogs		*P* value[a]
	Median [range] N	Mean (*SD*)	Median [range] N	Mean (*SD*)	
Age (mo)	30 [11-70] 27	33 (14)	62 [24-104] 16	64 (28)	**<.0001**
Weight (kg)	15.2 [11.6-20.4] 27	15.4 (2.5)	14.7 [12.0-19.0] 16	15.0 (2.2)	NS
HR (bpm)[b]	90 [60-160] 27	97 (22)	110 [70-150] 16	110 (20)	**.013**
2-D/M-mode measurements					
LADLax[b]	3.62 [2.78-4.14] 27	3.58 (.37)	3.33 [2.64-4.34] 16	3.41 (.37)	NS
LA:AoSax	1.30 [1.12-1.57] 26	1.32 (.12)	1.22 [.98-1.44] 15	1.24 (.13)	NS
LA:AoLax	2.07 [1.58-2.46] 27	2.08 (.20)	2.00 [1.53-2.55] 16	2.04 (.23)	NS
IVSd	1.02 [.79-1.39] 25	1.05 (.14)	.92 [.80-1.27] 16	.96 (.13)	**.036**
LVWd	.90 [.67-1.19) 25	.90 (.12)	.88 [.65-1.19] 16	.87 (.16)	NS
LVIDd	3.85 [3.14-4.57] 25	3.83 (.35)	3.52 [3.12-4.27] 16	3.54 (.29)	**.01**
LVIDs	2.98 [2.33-3.71] 25	2.99 (.39)	2.68 [2.12-3.26] 16	2.66 (.31)	**.007**
EPSS	.44 [.23-.74] 25	.47 (.15)	.33 [.22-.57] 13	.34 (.10)	**.007**
dLVvol	50.9 [34.4-78.5] 27	53.4 (12.5)	42.9 [35.4-73.0] 16	47 (10)	NS
sLVvol[b]	20.0 [14.9-40.2] 27	22.2 (7.4)	17.3 [11.5-37.7] 16	19.2 (7)	NS
LVmass[b]	132.6 [80.8-207.5] 25	132.7 (33.2)	97.1 [69.5-176.1] 16	104.4 (31.1)	**.007**
Indexed values					
LADLaxN[b]	1.25 [1.01-1.34] 27	1.23 (.08)	1.19 [.86-1.38] 16	1.19 (.12)	NS
IVSdN[b]	.55 [.44-.73] 25	.57 (.08)	.53 [.45-.68] 16	.53 (.07)	NS
LVIDdN[b]	1.53 [1.31-1.81] 25	1.55 (.11)	1.44 [1.28-1.62] 16	1.45 (.10)	**.004**
LVWdN[b]	.36 [.29-.45] 25	.36 (.04)	.35 [.27-.47] 16	.35 (.06)	NS
LVIDsN[b]	.94 [.74-1.10] 25	.91 (.10)	.82 [.61-1.07] 16	.83 (.10)	**.016**
LVmass/kg	8.62 [5.91-11.28] 25	8.58 (1.52)	6.68 [4.75-10.03] 16	6.94 (1.56)	**.002**
Functional measures					
Evel	.84 [.50-1.11] 26	.83 (.14)	.72 [.59-.83] 16	.72 (.08)	**.005**
E/A	2.2 [1.2-3.6] 26	2.2 (.6)	1.9 [1.1-3.8] 16	2.1 (.8)	NS
EF[b]	60.7 [38.8-68.2] 27	58.8 (7.3)	60.3 [48.4-69.7] 16	60.0 (6.9)	NS
FS[b]	21.0 [10.0-39.0] 25	22.1 (6.0)	24.5 [14.0-43.0] 16	24.9 (6.0)	NS
s′	.13 [.08-.20] 27	.13 (.03)	.14 [.09-.20] 16	.14 (.03)	NS

Note: Abbreviations and LV mass calculations appear in Table 1. Significant *P* values (<.05) appear in bold.
Abbreviation: NS, no significance.
[a]Compares mean (*SD*) if normal distribution or median [range] if non-normal distribution.
[b]Reject normality (Shapiro-Wilk).

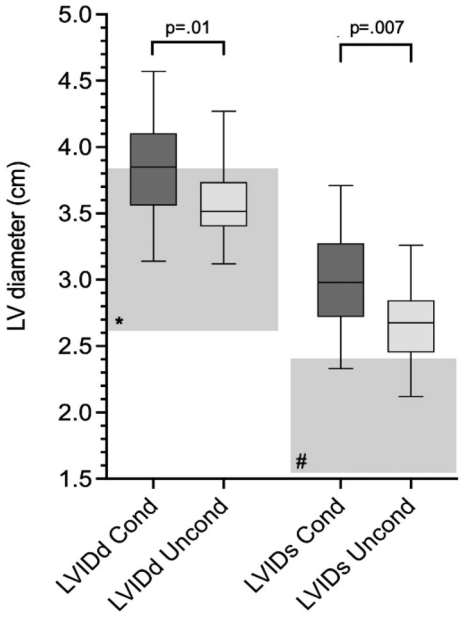

FIGURE 1 Box and whiskers plot comparing left ventricular diameter in diastole and systole between "conditioned" (n = 25) and "unconditioned" (n = 16) dogs (Mann-Whitney *U* test, $P < .05$). Line: median value, box: 25th and 75th percentile, whiskers: range. The shaded boxes indicate the reference interval for dogs of nonspecified breed within a similar weight range (11.8-22.7 kg) in *diastole and #systole.[21] LV, left ventricular; LVIDd, left ventricular diameter in diastole; LVIDs, left ventricular diameter in systole; Cond, "conditioned" dogs; Uncond, "unconditioned" dogs.

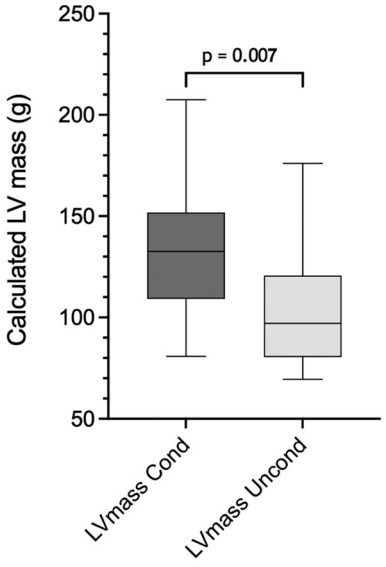

FIGURE 2 Box and whiskers plot comparing calculated left ventricular mass between "conditioned" (Cond, n = 25) and "unconditioned" (Uncond, n = 16) dogs (Students *t* test, $P < .05$). Line: median value, box: 25th and 75th percentile, whiskers: range.

vs dogs bred for show events. Those studies did not investigate the effect of degree of athletic conditioning at the time of examination. The effect of conditioning was examined in 2 ways in our study. The categorization of C1 (yes/no) showed a weak but positive association with calculated LVmass ($P = .03$; $r = 0.18$) and this association was retained when LVmass was normalized on a per kg basis ($P = .04$; $r = 0.21$), but C1 status was not associated with other measures of structure or function examined when compared to all other (C2, C3, and C4) dogs as a group. The calculation of LV mass is an approximation based on studies of people[19] and, although calculated LVmass may not be precisely accurate, it provides a basis by which the effect of body weight in conditioned animals can be examined. This method utilizes familiar and easily attained echocardiographic measurements and allows comparison to other canine athletes. Both conditioned and unconditioned dogs had higher LVmass/kg values than untrained sled dogs in a previous study, but similar measurements to those obtained from sled dogs after endurance training.[14] These findings may reflect a genetic component to left ventricular mass in whippets regardless of training compared to non-purebred sled dogs, a population that reflects more genetic heterogeneity. The calculation of LVmass includes measures of LV diameter, IVS thickness and LVW thickness in diastole. The influence of each of these component parameters individually did not reach significance in our analysis, but taken together, the calculated values indicated a difference. This first analysis of association used dogs of all conditioning categories, and the inclusion of C2 dogs (dogs in "good but not peak" condition) may have obscured true differences and weakened associations. Direct comparison of groups that were more different in degree of conditioning (the second analysis, "conditioned" vs "unconditioned" dogs) eliminated the mixed conditioning of group C2 and allowed for better differentiation of echocardiographic findings. This analysis investigated differences in structure and function between dogs considered to be in "peak" athletic condition and dogs considered to be "unconditioned."

As might be expected, currently competing "conditioned" dogs were younger as a group than were "unconditioned" dogs. Resting heart rate was lower in "conditioned" dogs, and lower resting heart rates in conditioned human and canine athletes have been reported.[14,31,32] When "conditioned" dogs were compared to "unconditioned" dogs, LVIDdN, LVIDsN and LVmass/kg were higher in "conditioned" dogs. These findings suggest that the observed larger normalized LV diameter in systole and diastole and higher LV mass in "conditioned" dogs reflect true LV hypertrophy less evident in "unconditioned" dogs, rather than differences in body weight. In addition, the higher LVIDdN, LVIDsN, and EPSS but similar IVSdN and LVWdN suggest that the LV hypertrophy in "conditioned" dogs was eccentric, consistent with typical whippet training that involves various running events. The lack of significantly higher IVS and LVW thickness in the "conditioned" dogs may reflect an actual difference in this breed vs greyhounds[15] or, given a tendency toward thicker walls in the "conditioned" group, may reflect a milder degree of conditioning in these pet animals compared to greyhounds more rigorously trained for racing.

The eccentric LV hypertrophy, accentuated measures of LV filling and unchanged systolic function at rest in the "conditioned" dogs compared to "unconditioned" dogs are typical of human and canine athletes.[14,15,30] In studies of human athletes, the degree of LV

enlargement in athletes depended upon genetic background, but also body surface area, age, sex and type of sport. The "conditioned" dogs in our study were pet dogs and not bred exclusively for racing, and thus specific genetic "race-bred" background was less likely to have contributed to the changes seen. These "conditioned" dogs were pets competing in running or agility events rather than "professional" dogs trained for competitive track racing, but showed eccentric LV hypertrophy consistent with that seen in endurance athletes.[30] The similar volumetric measurements at rest between "conditioned" and "unconditioned" dogs while resting heart rates were lower in "conditioned" dogs were supportive of higher cardiac output "reserve," wherein the potential difference in cardiac output between rest and exercise is expected to be higher in athletes.[14] The higher E velocity in "conditioned" dogs also is consistent with the rapid early diastolic filling needed to achieve higher ventricular volumes.[30]

Whippets generally tend to have larger cardiac dimensions for their size than dogs of other breeds. This finding, in combination with modifications of LV dimensions that may occur because of athletic conditioning in competitive pet dogs, may lead to overdiagnosis of LV dilatation if nonbreed specific weight-normalized reference intervals are used. Specifically, the upper limits of the range of "normal" LVIDdN and LVIDsN in "conditioned" dogs of our study may fall outside of generic reference ranges for dogs. Use of breed-specific ventricular measurement reference ranges normalized to body weight is of particular importance when screening dogs for evidence of cardiomyopathy in a population of athletic dogs that may display eccentric LV hypertrophy at peak condition even when not used as "professional" racing animals.

Our study has strengths and limitations. It is the only study of whippets thus far that has attempted to examine the effect of conditioning on the echocardiographic findings of this athletic breed and served to develop echocardiographic reference ranges for a North American population of whippets. The study population did not include any dogs with definable cardiac abnormalities other than trivial valvular regurgitation, eliminating the possible effects of valvular regurgitation on findings. Our population was drawn specifically from a population examined before recent reports of increased risk of abnormal cardiac findings related to specialty meat-based non-grain diets,[4] and the values developed in our study can be used as reference values when diet-related or other changes in cardiac size and function are suspected.

Limitations of our study include the imprecise characterization of athletic conditioning. The owners were requested to select a conditioning level, but no attempt was made to standardize the amount of athletic training each dog had experienced. Personal bias by the owners may have obscured differences among groups, especially between C1 and C2 dogs. To maximize the difference in athletic conditioning, C1 dogs were directly compared to dogs not currently in training (C3 and C4), excluding the C2 dogs because that group contained a wide range of previous athletes, well-conditioned but not competing dogs and pet animals considered by their owners to be in "good but not great" condition; categorization of these dogs was problematic. The study group was drawn from healthy dogs made available by their owners or breeders for examination; unknown biases and motives of the owners and breeders in dog submission likely affected some characteristics of the population, particularly age. The overall number of dogs included in this analysis was relatively small, especially for subgroup comparisons, which may have affected reference intervals and limited ability to detect the true strength of the influence of C1 status using multiple regression. Because no accepted standards exist on how to generate reference intervals for echocardiography data in healthy dogs, we elected to use standards adopted by the Clinical Laboratory Standards Institute and the American Society of Veterinary Clinical Pathology.[13,20,35] Our reference intervals consist of central 95% reference values of the data set (dependent on Gaussian versus non-Gaussian distribution), each with 90% confidence intervals around the upper and lower limits. The latter help define the degree of uncertainty around the reference limits.

Many additional dogs were imaged, but our stringent requirement for exclusion of all but the most trivial valvular regurgitation and use of only the first examination per dog decreased the number of examinations eligible for inclusion. In some dogs, valvular regurgitation considered "mild" may not have reflected true disease but still required exclusion from analysis. Because of the large number of measurements gathered from each dog and limitations of imaging in some dogs, every animal was missing at least 1 of the recorded variables. The weight range of this single-breed population was relatively narrow, conceivably limiting the strength of associations documented, but these indexed values still are helpful to distinguish normal from abnormal in this population. We used echocardiographic views commonly available and frequently used when screening outwardly healthy dogs for subclinical cardiac disease. Volume measures and estimated ejection fractions using a modified Simpson's method of discs were obtained using right parasternal long axis views only (monoplane) rather than a biplane measurement. A previous study[7] found volume estimates from the right parasternal long axis view to be slightly smaller than those obtained from a left apical view, but the difference was judged not to be clinically relevant. Left ventricular mass estimates were based on methods validated in people, but do provide a basis for comparison between animals with different degrees of athletic conditioning as well as with previously published reports. Although every effort was made to obtain images according to published standards, the calculation of LVmass might have been impacted if any of the contributing values were inaccurate.

Our study provides echocardiographic reference intervals for cardiac structure and function for normal North American whippets and provides values indexed for body weight for the most frequently used measurements pertaining to cardiac chamber size and volume. These values may help distinguish normal dogs of this athletic breed from dogs with myocardial or other abnormalities. Whippets considered to be in peak athletic condition have larger hearts than do less conditioned whippets, but measures of systolic function are similar. Whippet athletes in peak condition have eccentric LV hypertrophy greater than would be expected based on body weight.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

The authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the University of Wisconsin School of Veterinary Medicine (#V01414-0-03-09).

HUMAN ETHICS APPROVAL DECLARATION

The authors declare human ethics approval was not needed for this study.

ORCID

Rebecca L. Stepien https://orcid.org/0000-0002-7210-2036
Heidi B. Kellihan https://orcid.org/0000-0002-9489-1187
Lance C. Visser https://orcid.org/0000-0002-3563-0737
Virginia Luis Fuentes https://orcid.org/0000-0001-8076-3806

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Basal plasma concentrations of N-terminal pro-B-type natriuretic peptide in clinically healthy adult small size dogs: Effect of body weight, age, gender and breed, and reference intervals

Charlotte Misbach [a,*], Valérie Chetboul [a,b], Didier Concordet [c], Philippe Gruet [d], Cindy Speranza [e], Anne-Cécile Hoffmann [a], Adriana Rocha [a], David Balouka [a], Amandine M.P. Petit [a], Emilie Trehiou-Sechi [a], Jean-Louis Pouchelon [a,b], Hervé P. Lefebvre [f]

[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] UMR 1331 Toxalim, INRA, Université de Toulouse, INP, Ecole Nationale Vétérinaire de Toulouse, F-31076 Toulouse cedex 03, France
[d] Novartis Animal Health Inc., Schwarzwaldallee 215, CH-4058 Basel, Switzerland
[e] Novartis Santé Animale, 14 Boulevard Richelieu, BP 430, 92845 Rueil-Malmaison cedex, France
[f] Unité de Recherche Clinique, Université de Toulouse, INP, Ecole Nationale Vétérinaire de Toulouse, F-31076 Toulouse cedex 03, France

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ABSTRACT

Plasma NT-proBNP has previously been evaluated in dogs with degenerative mitral valve disease (DMVD). However, reference intervals (RI) established according to the Clinical Laboratory and Standards Institute (CLSI) recommendations have never been provided. The objectives of this prospective study were to assess effects of breed, body weight, age, and sex on plasma NT-proBNP, and to establish RI according to CLSI for this biomarker in a large population of dogs predisposed to DMVD.

183 Healthy small-sized dogs from 7 breeds were included. Assays were performed by ELISA. Effects of covariates were tested using a general linear model. Although a sex effect was demonstrated ($P = 0.01$), no significant effect of breed, body weight or age was shown. The proposed RI was 157–2842 pmol/L. 7% of dogs had plasma NT-proBNP >2617 pmol/L, and were considered as outliers despite normal cardiovascular examination. In conclusion, plasma NT-proBNP may be high in a few healthy small-sized dogs.

1. Introduction

Degenerative mitral valve disease (DMVD) is the most common acquired heart disease in small size dogs, and is characterized by degenerative valvular lesions resulting in systolic mitral regurgitation with potential hemodynamic consequences, such as reduced forward cardiac output and increased intracardiac pressures (Kvart and Häggström, 2005; Borgarelli et al., 2008). As demonstrated in humans (Francis, 1998; Ferrari et al., 1998), such hemodynamic alterations may result in complex neurohormonal activation (especially adrenergic nervous and renin–angiotensin–aldosterone system activation with overexpression of natriuretic peptides), in order to maintain adequate cardiac output, blood pressure, and tissue perfusion. To date, natriuretic peptides, including the inactive aminoterminal portion of brain natriuretic peptide (N-terminal pro-B-type natriuretic peptide, NT-proBNP), are considered as one of the most reliable neurohormonal markers of heart diseases in dogs (Sisson, 2009; Boswood, 2009; Oyama, 2009a). Plasma NT-proBNP is correlated with canine DMVD severity, and can be used in combination with clinical status to predict outcome in both asymptomatic dogs and dogs with congestive heart failure (CHF) (Oyama et al., 2008; Serres et al., 2009; Chetboul et al., 2009; Reynolds et al., 2012). Moreover, this biomarker has also been used to discriminate between CHF and primary pulmonary disease in dogs with cough or dyspnea (Fine et al., 2008; Oyama et al., 2009b). Finally, plasma NT-proBNP has been shown to decrease with treatment of CHF (Atkinson et al., 2009; Schöber et al., 2011) and the reduction in plasma NT-proBNP after initiation of treatment is considered as a useful predictor of overall cardiac survival (Wolf et al., 2012). Plasma NT-proBNP has been previously evaluated in a large population of healthy dogs ($n = 550$), including small and large breed dogs from 9 different breeds (Häggström et al., 2012). Highly significant breed differences were found and the authors concluded that breed-specific reference ranges might therefore be necessary for optimal clinical use of natriuretic peptides as cardiac biomarkers.

* Corresponding author. Tel./fax: +33 670941623.
E-mail address: cmisbach@vet-alfort.fr (C. Misbach).

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However, to the best of the authors' knowledge, no study has specifically documented reference intervals (RI), nor the potential effects of physiological covariates (e.g., breed, body weight, age, and sex) on plasma NT-proBNP concentration in a large population of healthy adult small size dogs from different breeds predisposed to DMVD.

The aims of this prospective study were therefore (1) to evaluate plasma concentrations of NT-proBNP, (2) to identify potential effects of breed, body weight, age, and sex and (3) to establish tentative RI according to the statistical procedures recommended by the Clinical Laboratory and Standards Institute guidelines (CLSI, 2008), in a large population of healthy adult small size dogs from different breeds known to be predisposed to DMVD.

2. Material and methods

2.1. Animals

The study population consisted of healthy non-neutered adult (age >10 months and <8 years) dogs of 7 different breeds predisposed to DMVD (Bichon (B), Cavalier King Charles (CKC) and King Charles (KC) Spaniels, miniature Poodle (MP), Shih-Tzu (ST), Yorkshire Terrier (YT), and Dachshund (D)), prospectively recruited in the Paris area (France). Breeder's consent was obtained for each animal before its enrolment in the study and all procedures were approved by a local ethics committee and in compliance with the Procedures and Principles of Good Clinical Practice (Food and Drug Administration Good Clinical Practice, 2012). An animal information form (including breed, sex, date of birth, history, diet, and also reproductive, vaccination and deworming status) and a copy of the pedigree were obtained for each dog before inclusion. Dogs from different breeds could be owned by the same breeder. However, specific attention was paid to avoid including dogs from the same family (e.g., siblings, parents, and littermates), and a maximum of 10 dogs were recruited from a given breeder in order to avoid any bias due to breeder-dependent environmental effects (housing, diet, exercise, etc.).

Animals were assessed as healthy on the basis of a complete physical examination, history, and routine plasma biochemistry. Occurrence of clinical signs and past history of medical events (e.g. infectious disease, lameness, etc.) did not lead to exclusion of a dog from the study if the animal had totally recovered and if the treatment had been stopped at least 3 months before blood sampling. Occurrence of clinical signs between the time of blood collection and 2 months later was assessed by a phone call interview with the breeder.

Dogs had to be fasted for at least 10 h before sampling and the diet should not have been changed during the previous 15 days. Females had to be in anoestrus, and neither lactating nor pregnant.

Non-fasted dogs, overweight dogs, dogs with an abnormal clinical examination, and dogs on medication at the time of blood sampling were excluded from the study. Other exclusion criteria were antiparasitic drug administration or vaccination during the 15 days preceding blood sampling.

2.2. Blood sample collection

To avoid any potential circadian periodicity, blood was collected during the same period of the day (between 10.00 AM and 2.00 PM) and all the dogs were sampled within an 8-week period (July–August 2011). Fasted dogs were acclimated to the investigator and the room for 10 min before sampling. Blood was drawn with a 20 G needle and a 5 mL syringe from the jugular vein of awake animals, which were always in the same position (sitting position). Two mL of blood were collected and placed in a plastic tube containing K3-EDTA (Venosafe VF-052STK, Terumo France, Guyancourt, France). Blood was centrifuged (15 min, 1500*g*) (EBA 20, Hettich, Tuttlingen, Germany) at room temperature within 30 min of blood sampling. The plasma (at least 0.5 mL) was placed into transport tubes containing proteases (Cardiopet, Idexx, Alfort, France) provided by the commercial laboratory (Laboratoire Idexx, Alfort, France), transferred at 4 °C within 45 min of blood sampling and then stored at −80 °C (less than 6 h after blood sampling).

2.3. Plasma NT-proBNP assay

Plasma NT-proBNP concentration was measured using EDTA-potassium samples and a commercially available canine specific assay (Cardiopet, Idexx, Alfort, France). This sandwich ELISA assay has already been used and validated for diagnostic purposes in the dog (Boswood et al., 2008; Zieba et al., 2008). The inter- and intra-assay coefficients of variation (CV) were 5.3, 6.6, 9.2%, and 10.7, 2.1, 4.2% for low (600 pmol/L), medium (1200 pmol/L) and high (2400 pmol/L) concentrations, respectively. Samples were sent every two weeks to the commercial laboratory (Laboratoire Idexx, Alfort, France).

2.4. Statistical analysis

A value of $P < 0.05$ was considered significant. Identification of outliers and determination of RI were performed according to the current CLSI guidelines (2008). Native and Box-Cox transformed data were first tested for normality by use of the Anderson–Darling test. When the data distribution remained non-Gaussian after Box-Cox transformation, visual inspection of values in both tails of the distribution was used. Identification of outliers was performed using the Tukey method. When an outlier was identified, further examinations (see below) were specifically scheduled. The results of these examinations were used as criteria to decide whether an outlier dog should be removed or not from the study.

Effects of breed and other covariates on plasma NT-proBNP concentration were tested using a statistical software package (Systat version 8.0, SPSS Inc, Chicago, IL). Age and body weight in each breed were compared by ANOVA. The effects of breed, sex, age, and body weight on plasma NT-proBNP concentration were tested using the following linear mixed effects model:

$$Y = \mu + \text{Breed} + \text{Sex} + a\text{Age} + b\text{Body weight} + (\text{Breed} \times \text{Age}) + (\text{Breed} \times \text{Body weight}) + (\text{Breed} \times \text{Sex}) + \varepsilon,$$

where Y is the value of the plasma variable; μ is a constant term; a and b are the slope coefficients for age and body weight irrespective of the breed. The other terms denote interactions between breed and age, breed and body weight, and breed and sex. ε is the residual term of the model.

Reference intervals were defined as central 95% intervals bounded by the 2.5th and 97.5th percentiles. The upper and lower limits of the RI with their 90% confidence intervals (CI) were determined in the global population using a non-parametric approach (Geffré et al., 2011). Data were expressed as median, [interquartile ranges], except for results regarding systolic arterial blood pressure and plasma BUN and creatinine (mean ± SD and [ranges] as minimum and maximum) in outlier dogs.

2.5. Further examinations for NT-proBNP outliers

After the Tukey method was applied, some of the tested dogs appeared as outliers (see statistical analysis). A complete cardiovascular examination, including conventional echocardiography and Doppler examinations as well as systemic arterial blood pressure measurement and plasma NT-proBNP, blood urea nitrogen (BUN) and creatinine determinations were specifically scheduled for these outliers 3 months after the first assay.

Systolic arterial blood pressure was indirectly measured before each echocardiographic examination in conscious dogs, in accordance with the ACVIM consensus statement (Brown et al., 2007) by the same trained observer (CM) using the Doppler method (811-BL, Parks Medical Electronics Inc. Aloha, Ore, USA). Dogs were gently held by the owner in sternal recumbency. An inflatable cuff (Soft-cuf, Ref 2422, 2 cm large, Parks Medical, USA) of appropriate-size was placed on the tail, as previously described (Chetboul et al., 2010). A period of acclimatization was allowed for each patient before blood pressure was measured. Several measurements were performed over 5–10 min to obtain a stable set of 5 values, the mean of which was taken as the patient's systolic blood pressure.

Conventional echocardiographic and Doppler examinations were performed by the same trained observer (CM) in awake dogs gently restrained in standing position, using continuous ECG monitoring with an ultrasound unit (Vivid i BT 10 SW appl. R 10.3.0, GE Healthcare, 9900 Innovation Drive, Wauwatosa, WI 53226, USA) equipped with a 5S (2–5 MHz) phased-array transducer, as previously described (Chetboul et al., 2004, 2005). Echocardiographic variables included the left ventricular diameters, the left ventricular free wall and interventricular septum thicknesses at end-diastole and end-systole as well as the fractional shortening for M-mode, and the left atrium on aorta ratio for the two-dimensional mode. Conventional Doppler variables included the maximal systolic aortic and pulmonary velocities, maximal early (E) and late (A) diastolic mitral flow velocities, as well as systolic pulmonary arterial pressure and diastolic pulmonic artery-to-right ventricle pressure gradient, when tricuspid and pulmonary regurgitations were identified, respectively. Echocardiographic and Doppler variables were compared with the previously established reference ranges (Gonçalves et al., 2002; Chetboul et al., 2005).

3. Results

3.1. Population

Twenty-nine of the 183 dogs selected by breeders for examination during the kennel visit were excluded from blood sample collection. Twelve of these dogs presented with a heart murmur, 5 females were in estrus, 5 dogs had been vaccinated 2 days before examination, 3 dogs were non-fasted, 2 females were having a pseudolactation, one dog had hyperthermia with severe neck dermatitis, and one dog was overweight.

One hundred and fifty-four dogs belonging to 28 different breeders were therefore included in the study (see characteristics in Table 1). Significant differences between breeds were found for body weight ($P < 0.001$) as expected, but not for age ($P = 0.411$). The greatest difference in body weight was observed between CKC and YT (8.4 kg [7.8–8.9] and 3.2 kg [2.3–3.3], respectively).

3.2. Plasma NT-proBNP concentration

The commercial laboratory (Laboratoire Idexx, Alfort, France) did not quantify the NT-proBNP concentration when it exceeded 2842 pmol/L. In 9 out of the 154 tested dogs, plasma NT-proBNP exceeded 2842 pmol/L, so their NT-proBNP value was set at 2842 pmol/L for the statistical analysis.

The plasma NT-proBNP values obtained in the global tested population and in each of the 7 tested breeds are given in Table 2. Distribution of plasma NT-proBNP concentrations among the reference sample group is illustrated in Fig. 1. Although no age or breed effect was observed on plasma NT-proBNP, a significant sex effect ($P = 0.01$) was shown (males: 683 pmol/L [404–892]; females: 844 pmol/L [550–1327]).

3.3. Characteristics of NT-proBNP outliers

After the Tukey method was applied, 11 of the 154 dogs included in the study were considered as outliers (i.e., plasma NT-proBNP concentration $\geqslant$2617 pmol/L). The plasma NT-proBNP concentration was >2842 pmol/L for 9 dogs, and 2816 and 2829 pmol/L for the 2 remaining dogs. As described above, a complete cardiovascular examination, as well as plasma BUN and creatinine determination were specifically scheduled 3 months after the first blood sampling.

The outlier population (8 females and 3 males, age: 3.2 years [2.1–5.2]; body weight: 6.5 kg [5.4–9.1]) included 4 CKCS, 3 ST, 1 B, 1 D, 1 KC, and 1 MP. All conventional echocardiographic and Doppler variables were within the reference ranges (Gonçalves et al., 2002; Chetboul et al., 2005). Similarly, none of them were hypo- or hypertensive (mean ± SD [ranges] systemic systolic arterial blood pressure: 148 ± 10 mmHg [130–158]) (Brown et al., 2007), and no arrhythmia was detected during the echocardiographic examinations, using concomitant ECG tracing (mean ± SD [ranges] heart rate: 115 ± 18 bpm [90–140]).

The 11 outlier dogs were also rechecked 3 months after the first blood sampling for plasma NT-proBNP concentrations using the same procedure described above. At recheck, the plasma NT-proBNP concentration was 2040 pmol/l [775–2842] *versus* 2842 pmol/l [2842–2842] at the first visit. Only 4/11 dogs still had NT-proBNP concentrations >2842 pmol/L, and those of the 7 remaining dogs

Table 1
Epidemiologic characteristics of the reference sample group including 154 healthy adult small size dogs from 7 different breeds.

Variable	Global	Breed						
		B	CKC	KC	D	MP	ST	YT
n dogs (%)	154	8 (5.2)	36 (23.4)	17 (11.0)	27 (17.5)	20 (13.0)	28 (18.2)	18 (11.7)
n breeders*	28	2	8	4	4	5	5	5
Sex (%)								
F	96 (62.3)	4 (4.2)	21 (21.9)	14 (14.6)	15 (15.6)	12 (12.5)	17 (17.7)	13 (13.5)
M	58 (37.7)	4 (6.9)	15 (25.9)	3 (5.2)	12 (20.7)	8 (13.8)	11 (19.0)	5 (8.6)
Age (years)								
Median	3.2	4.2	2.3	3.7	2.9	3.4	3.4	3.9
IQR	1.9–4.8	2.7–5.5	1.7–3.8	2.4–6.4	1.6–4.2	1.8–4.9	1.9–5.6	2.4–5.9
BW (kg)								
Median	6.5	4.4	8.4	7.5	5.0	5.5	6.5	3.2
IQR	4.6–8.3	3.1–6.8	7.8–8.9	6.6–8.3	4.3–8.9	4.4–6.9	5.7–8.0	2.3–3.3

n: Number of dogs; Global: the tested overall population; F: female; M: male; B: Bichon; CKC: Cavalier King Charles spaniel; D: Dachshund; KC: King Charles spaniel; MP: miniature Poodle; ST: Shih-Tzu; YT: Yorkshire Terrier; BW: body weight; IQR: interquartile range.
* Animals from different breeds could belong to the same breeder.

Table 2
Descriptive statistics for plasma NT-proBNP assessed in 154 healthy adult small size dogs from 7 different breeds.

	Global	Breed						
		B	CKC	KC	D	MP	ST	YT
Number of dogs	154	8	36	17	27	20	28	18
NT-proBNP (pmol/L)								
Median	783	804	1053	964	395	791	868	602
IQR	476–1232	700–1329	791–1446	637–1317	278–566	553–1436	533–1426	465–930

Global: the overall tested population; F: female; M: male; B: Bichon; CKC: Cavalier King Charles spaniel; D: Dachshund; KC: King Charles spaniel; MP: miniature Poodle; ST: Shih-Tzu; YT: Yorkshire Terrier; IQR: interquartile range.

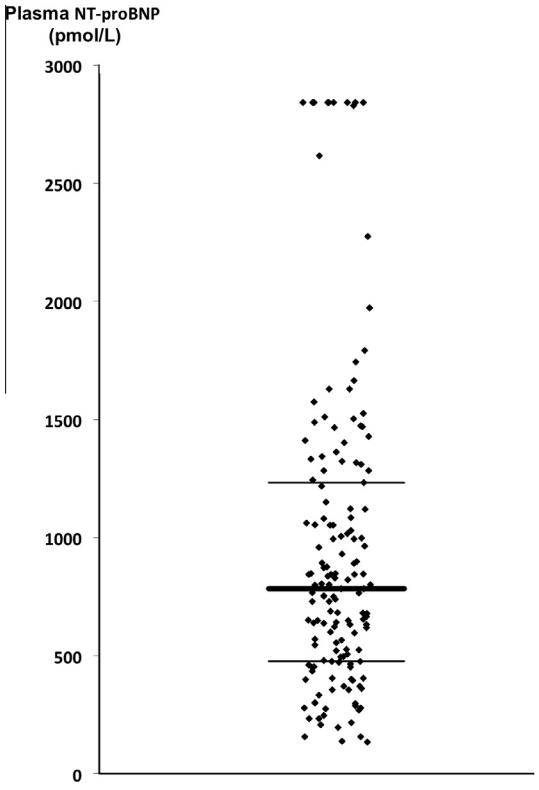

Fig. 1. Distribution of plasma NT-proBNP concentrations among the 154 healthy small size dog population. The thick horizontal bar represents the median value (i.e., 783 pmol/L) and the thin horizontal bars represent the first and third quartiles (i.e., 476 and 1232 pmol/L, respectively).

were between 365 and 2455 pmol/L (Fig. 2). None of the 11 outlier dogs showed an increase in plasma BUN and creatinine, i.e., mean ± SD [ranges] 4.83 ± 1.33 mmol/L [3.33–8.34] and 47.7 ± 10.6 µmol/L [26.5–70.7], respectively (RI provided by the laboratory (Laboratoire Vebiotel, Arcueil, France): 3.33–8.34 mmol/L and 53.0–132.6 µmol/L, for BUN and creatinine, respectively). Owing to these results, none of the 11 outliers were excluded for RI establishment.

3.4. Reference intervals for plasma NT-proBNP

Reference intervals were established in the whole reference sample group (n = 154 dogs). The distribution of plasma NT-proBNP concentration was tested for normality and could not be transformed to fit a Gaussian distribution. The corresponding lower (LL) and upper (UL) limits of the RI with 90% CI were, respectively, 157 [134–233] and 2842 [2842–2842] pmol/L.

4. Discussion

In the present study, plasma NT-proBNP concentration was measured in a large healthy adult small size dog population using standardized procedures with strict inclusion and exclusion criteria, in order to minimize sources of pre-analytical variations. Moreover, RI determination was performed according to the statistical procedures recommended by the CLSI guidelines, to which the American Society for Veterinary Clinical Pathology recently recommended adherence (ASVCP Quality Assurance and Laboratory Standards Committee, 2012). Despite these precautions, the plasma NT-proBNP concentrations exhibited a wide range of values, suggesting inter-individual variability, although all dogs belonged to the same size category. Hence, in the general population, the inter-individual CV was 96% and the within-breed inter-individual CVs ranged from 62% (CKC) to 100% (MP). When considering several other studies (Table 3) on plasma/serum NT-proBNP including groups of healthy dogs (Oyama et al., 2008; Atkinson et al., 2009; Kellihan et al., 2009, 2011; Raffan et al., 2009; Schmidt et al., 2009; Chetboul et al., 2009; Collins et al., 2010; Wess et al., 2011; Cunningham et al., 2012; Hezzell et al., 2012), two reports included healthy comparable breeds as here (i.e., small size dogs), but had a too small sample size (i.e., less than 31, *versus* 154 in the present study) to allow any conclusion on inter-individual variability (Chetboul et al., 2009; Hezzel et al., 2012). In one report including a high number of dogs (n = 196) from the same large breed (i.e., Doberman Pinscher), inter-individual CV according to age category reached 53% in dogs ⩽3 years (Wess et al., 2011). Nevertheless, in the latter studies, the material and methods used for sample handling and assays were different as in our study, which represents a limitation for such comparisons.

Eleven NT-proBNP outliers were detected in the present study. All 11 dogs were confirmed to be free of cardiovascular diseases using a complete standard echocardiography and Doppler examination as well as systemic arterial blood pressure measurement, and were therefore kept for statistical analysis. When they were re-tested for plasma NT-proBNP 3 months later using the same procedures, only 4/11 dogs had plasma NT-proBNP values that still exceeded the upper limit of detection of the assay, and the 7 remaining dogs showed a 1.2 to 6-fold decrease from their initial NT-proBNP value. These findings are in accordance with a study performed on the weekly variability of plasma NT-proBNP in 28 healthy dogs (Kellihan et al., 2009), showing that the difference between maximal and minimal NT-proBNP values was >500 pmol/L, and between 100 and 200 pmol/L in 20% and 40% of the recruited dogs, respectively. In healthy humans, NT-proBNP intra-individual variability may be high, i.e., between 26% and 130% according to studies (Melzi d'Eril et al., 2003; Wu et al., 2003), which thus hampers the interpretation of changes in this biomarker with disease progression and therapy adjustments in patients with cardiovascular diseases (Bruins et al., 2004; Miller et al., 2009). Nevertheless, owing to the very small number of outlier dogs

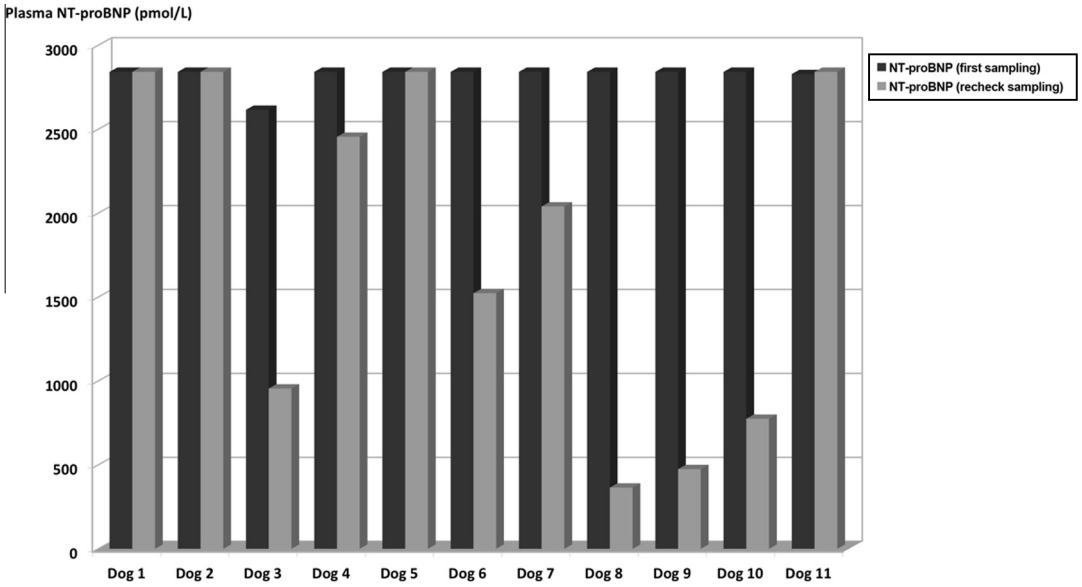

Fig. 2. Comparison between plasma N-terminal pro-B-type natriuretic peptide (NT-proBNP) concentrations assessed in the 11 outlier dogs at first sampling and 3 months later.

Table 3
Comparison of plasma/serum N-terminal pro-B-type natriuretic peptide values in healthy dogs from 11 studies.

Authors	*n*	Age (year)	Body weight (kg)	Breeds	Plasma/serum NT-proBNP (pmol/L)
Cunningham et al. (2012)*	17	Mean ± SD 5.8 ± 2.9	Mean ± SD 30.0 ± 6.9	Mixed breed, Doberman Pinscher, Golden Retriever and other breeds	Median [ranges] 462 [38–1210]
Hezzell et al. (2012)	30	Median (IQR) 9.0 (6–11)	Median (IQR) 11 (7.2–13.8)	Cross-breeds, Cavalier King Charles Spaniel (*n* = 7), Miniature Poodle, Yorkshire Terrier, Border Collie	Median (IQR) 324 (167–530)
Kellihan et al. (2011)	8	Median [ranges] 6 [1–9]	Median [ranges] 28.5 [3.4–55.0]	Great Dane, German shepherd dog, Rottweiler, Dachshund, Boxer, Maltese, Bearded Collie, Cocker spaniel.	Median [ranges] 744 [531–2710]
Wess et al. (2011)	196	Mean 4.4	Mean 34.4	Doberman Pinscher	Median [ranges] 303 [22–1325]
Collins et al. (2010)	10	Data not shown	Data not shown	Cavalier King Charles Spaniels, crossbreds, Boxers, Lurchers, English Springer and Cocker Spaniels, Doberman Pinscher, Bull Terriers, Labrador and Golden Retriever, Great Dane, Irish, Wolfhound, Gordon and Irish Setter, Whippet, Miniature Schnauzer, Fox Terrier	Median [ranges] 413 [356–487] before freezing 709 [378–825] after freezing
Atkinson et al. (2009)	9	Median 5	Median 19.8	Data not shown	Median [ranges] 373 [209–738]
Chetboul et al. (2009)	22	Mean ± SD 9.1 ± 1.6	Mean ± SD 6.8 ± 3.6	Small size breeds: King Charles and Cavalier King Charles Spaniel, Bichon, Yorkshire Terrier (*n* = 12); Other small breeds (*n* = 10)	Median [ranges] 278 [68–515]
Kellihan et al. (2009)	28	Median 7	Median 20.4	Mixed breed, Labrador and Golden Retrievers, American Staffordshire Terriers, other breeds	Median [ranges] Week 1: 377 [159–631] Week 2: 320 [159–632] Week 3: 358 [159–650]
Raffan et al. (2009)	39	Mean 6.0	Data not shown	Unknown	Median [ranges] 118 [2–673]
Schmidt et al. (2009)	23	Median (IQR) 7 (5–8)	Median (IQR) 17.7 (10–30)	Mixed breed (48%), Golden Retriever, Keeshonds and other breeds	Mean (95%CI) 261 (225–303)
Oyama et al. (2008)	40	Median (IQR) 7.0 (4.3–8.0)	Median (IQR) 19.9 (8.5–30.5)	Mixed breed (40%), Great Dane, Golden retriever and other breeds	Median (IQR) 290 (478–598)

n: number of dogs; IQR: interquartile range; CI: confidence interval; SD: standard deviation.
* Protocol using protease tubes.

(*n* = 11), intra-individual variability of plasma NT-proBNP could not be assessed in the present study.

As described in humans (Loke et al., 2003) and in one recent report in dogs (Wolf et al., 2013), a sex effect on plasma NT-proBNP was observed in the present study, with females having higher median plasma NT-proBNP values than males. Conversely, no age or body weight effects were found, in accordance with several other reports (Boswood et al., 2008; Tarnow et al., 2009; Kellihan et al., 2009; Oyama et al., 2008; Ettinger et al., 2012). However plasma NT-proBNP concentration has been shown to be

significantly increased in healthy Doberman Pinschers >8 years as compared with younger dogs of the same breed (Wess et al., 2011), and to be inversely related to body weight in a group of 39 asymptomatic CKCS with DMVD (Tarnow et al., 2009). Nevertheless, the absence of a significant effect of age on plasma NT-proBNP may be attributable to the fact that all recruited dogs were < 10 years old, and this could represent a limitation of the present study.

Two studies reported a significant breed effect for plasma NT-proBNP (Oyama et al., 2008; Häggström et al., 2012). However, no breed effect was observed in the present study. This may be explained by the fact that all dogs belonged to the same size category.

This study presents several limitations. Firstly, plasma NT-proBNP concentrations were only measured on a second occasion in dogs that were found to have exceptionally high concentrations at the first time of measurement. Since these dogs were outliers, it is likely that when measured on a second occasion, the variability in their concentrations would be greater than that seen by more typical members of the population (Bland and Altman, 1994a,b). Therefore, conclusions cannot be drawn about natural variability of plasma NT-proBNP concentration in typical individual dogs from this study. Another limitation is that the UL of the RI determined in the present study corresponds to the limit of quantification, as the commercial laboratory responsible for the NT-proBNP assays did not assess plasma NT-proBNP values >2842 pmol/L. Therefore, the UL of the RI should be interpreted with caution. Moreover, complementary cardiovascular examinations were only scheduled for the outliers, and not for all the recruited dogs. However, one important step, as for any study of reference intervals, was to define the criteria for health. Since echocardiography is not currently considered as a prerequisite for NT-proBNP testing, the present study was designed so that the conditions were similar to those encountered in routine clinical practice, and dogs were considered healthy on the basis of a complete physical examination, history and routine plasma biochemistry. Finally, effect of sample handling and storage could have affected plasma NT-proBNP concentrations, as serum NT-proBNP has been shown to decrease at room temperature and increase after storage at −20 °C (Collins et al., 2010). Nevertheless, according to the latter results, the authors recommend that samples should be separated and frozen within 1 h of blood collection and sent frozen to the laboratory until assays, and this was the case in the present study.

In conclusion, this study suggests that plasma NT-proBNP concentration is characterized by a high inter-individual variability in healthy adult small size dogs. Therefore, a single plasma value in a healthy small size dog should be interpreted with caution. Moreover, a sex effect has been demonstrated, with females having higher concentrations than males, but the clinical relevance of partitioning the RI according to sex needs further investigations. Prospective studies including a larger sample size and other breeds are therefore needed to better understand NT-proBNP physiology in dogs.

Conflict of interest

The Vivid i ultrasound system used in the study was lent by Scil Animal Care Company (67120 Altorf, France). Novartis Animal Health supported C. Misbach's PhD program.

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