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2018-08

Höglund , K , Häggström , J , Hanås , S , Merveille , A -C , Gouni , V , Wiberg , M , Willesen , J L , Mc Entee , K , Sorensen , L M , Tiret , L , Seppälä , E H , Lohi , H , Chetboul , V , Fredholm , M , Lequarre , A -S & Ljungvall , I 2018 , ' Interbreed variation in serum serotonin (5-hydroxytryptamine) concentration in updates healthy dogs ' , Journal of Veterinary Cardiology , vol. 20 , no. 4 , pp. 244-253 . https://doi.org/10.1016/j.jvc.2018.05.002

http://hdl.handle.net/10138/305125
https://doi.org/10.1016/j.jvc.2018.05.002

publishedVersion

Journal of Veterinary Cardiology (2018) **20**, 244—253

Journal of Veterinary Cardiology
ESVC

www.elsevier.com/locate/jvc

Interbreed variation in serum serotonin (5-hydroxytryptamine) concentration in healthy dogs☆

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Planning of the study started in 2007. The study protocol was finalized in 2008. There was a follow-up meeting in 2010. The current manuscript was finalized and approved by the investigators in February 2018. The publication committee consisted of Katja Höglund, Ingrid Ljungvall, and Jens Häggström.
Presented in part at the 26th European College of Veterinary Internal Medicine—Companion Animal Congress, Gothenburg, Sweden, September 2016.

☆ A unique aspect of the Journal of Veterinary Cardiology is the emphasis of additional web-based materials permitting the detailing of procedures and diagnostics. These materials can be viewed (by those readers with subscription access) by going to http://www.sciencedirect.com/science/journal/17602734. The issue to be viewed is clicked and the available PDF and image downloading is available via the Summary Plus link. The supplementary material for a given article appears at the end of the page. To view the material is to go to http://www.doi.org and enter the doi number unique to this paper which is indicated at the end of the manuscript.

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https://doi.org/10.1016/j.jvc.2018.05.002

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Received 27 October 2017; received in revised form 4 May 2018; accepted 14 May 2018

KEYWORDS
Breed difference;
Biomarker;
Canine;
Heart valve;
5-HT

Abstract *Introduction:* Serotonin (5-hydroxytryptamine [5-HT]) has several biological functions. In different species, excessive 5-HT has been linked to valvular lesions, similar to those seen in dogs with myxomatous mitral valve disease. Previous studies suggest higher 5-HT in healthy Cavalier King Charles Spaniels (CKCSs), a breed highly affected by myxomatous mitral valve disease, compared to other breeds.
Objective: To investigate potential interbreed variation in serum 5-HT in healthy dogs.
Animals: 483 healthy dogs of nine breeds aged 1—7 years.
Methods: Dogs were examined at five European centers. Absence of cardiovascular, organ-related, or systemic diseases was ensured by thorough clinical investigations including echocardiography. Serum was frozen and later analyzed by enzyme-linked immunosorbent assay (ELISA).
Results: Median 5-HT concentration was 252.5 (interquartile range = 145.5—390.6) ng/mL. Overall breed difference was found ($p<0.0001$), and 42% of pairwise breed comparisons were significant. Univariate regression analysis showed association between serum 5-HT concentration and breed, center of examination, storage time, and sex, with higher 5-HT in females. In multiple regression analysis, the final model had an adjusted R^2 of 0.27 with breed ($p<0.0001$), center ($p<0.0001$), and storage time ($p=0.014$) remaining significant. Within centers, overall breed differences were found at 3/5 centers ($p\leq0.028$), and pairwise comparisons within those centers showed breed differences in 42% of comparisons. Among the included breeds, Newfoundlands, Belgian Shepherds and CKCSs had highest 5-HT concentrations.
Conclusions: Interbreed variation in serum 5-HT concentration was found in healthy dogs aged 1—7 years. These differences should be taken into account when designing clinical studies.

Abbreviations

CKCS	Cavalier King Charles Spaniel
CV	coefficient of variation
IQR	interquartile range
MMVD	myxomatous mitral valve disease
5-HT	5-hydroxytryptamine

Introduction

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) has several important biological functions, including regulation of mood, sleep, energy homeostasis, platelet function, and cardiovascular tone [1—5], thus controlling diverse functions in the central as well as peripheral nervous system [2]. The 5-HT system is also important in cardiac valvular development and in maintaining normal valvular and myocardial function [6—9]. Altered 5-HT signaling has been suggested to be involved in the pathogenesis of myxomatous mitral valve disease (MMVD) in dogs [10—14] and might be especially important in the early development of the disease [15,16].

In the blood, 5-HT is mainly stored in dense platelet granules, and in healthy dogs and people, plasma concentrations are low [13,17,18]. In people receiving oral serotonergic medication, cardiac valvulopathy has been observed [19,20], and in animal models, rats injected with 5-HT developed myxomatous-like valvular changes [21,22]. In dogs with naturally occurring MMVD, 5-HT concentrations in mitral valve leaflets and left ventricular myocardium have been shown to be higher than in healthy

controls [13]. Studies have demonstrated higher 5-HT concentrations in serum and in platelet-rich plasma from dogs with MMVD compared with healthy controls [12,13], while no differences in plasma 5-HT concentrations have been reported between dogs with MMVD and controls [13,17]. These findings suggest that differences in serum 5-HT concentrations between dogs with or at risk of developing MMVD likely reside within the platelet 5-HT pool.

In dogs with MMVD, higher serum 5-HT concentrations have been reported in Cavalier King Charles Spaniels (CKCSs), a breed highly affected by MMVD at an early age [23], compared with other breeds [15]. In contrast, no difference in platelet or plasma 5-HT concentration was found between affected and non-affected non-CKCSs [17]. Canine reference values are lacking, but higher serum and platelet-rich plasma 5-HT concentrations have been found in healthy CKCS dogs compared with healthy dogs of other breeds [12,13]. In another study of healthy dogs, CKCS along with Scottish Terriers, Maltese, and Belgian Shepherds had higher serum 5-HT concentrations than two other terrier breeds and Labrador Retrievers [24].

The hypotheses of the present study were that breed differences in serum 5-HT concentrations exist in healthy dogs and that serum 5-HT concentrations differ among breeds of different morphotypes with higher concentrations in dog breeds predisposed to MMVD. The aim of the study was to investigate potential interbreed variation in serum 5-HT in a large cohort of healthy dogs of nine different breeds of different body sizes, morphology, and temperament.

Animals, materials, and methods

Animals

Examination of dogs took place at five centers: University of Liège, Belgium; University of Copenhagen, Denmark; National Veterinary School of Alfort, France; University of Helsinki, Finland; and Swedish University of Agricultural Sciences, Sweden. The examinations took place during the years 2009—2010. The study was approved by an ethical committee in each participating country. Dogs were privately owned, and informed owner consent was obtained. The study was performed as part of the EU-funded LUPA-project [25]. To be included in the study, dogs had to be healthy, purebred, and aged between 1 and 7 years. Dogs also had to have a normal body condition score (3—7, on a 9 point scale) [26] and be unrelated to each other at parental level. Each center could include two to five breeds, and within a center, each breed cohort included dogs of one sex only; either intact males or females that were spayed or in anestrus, according to the LUPA inclusion criteria. Dogs were excluded if any finding indicating systemic or organ-related disease was observed during the clinical examination described below.

Preparations

In preparation for the study, owners were instructed to feed their dogs only commercial dog food and no treats, the last 2 weeks before participation. On examination day, all dogs were fasted $\geq$12 h and had no access to water for at least 2 h before examination.

Characterization of health status

A morning urine sample from each dog, collected by the owner at home, was brought to the clinic. Standard urine analysis was performed using a dipstick chemistry test and refractometer for measurement of urine specific gravity. Each dog underwent a general physical examination including blood pressure measurement by high-definition oscillometry, according to published guidelines [27], and was assigned a body condition score [26]. A 5-min electrocardiographic (ECG) recording was performed along with an echocardiographic examination including two-dimensional, M-mode, and Doppler examinations. The echocardiographic examination was conducted with continuous ECG monitoring, and the dog was examined from right and left sides using standardized imaging planes [28]. After the health examination, blood was collected by venipuncture into 5-mL serum and ethylenediamine tetraacetic acid (EDTA) tubes. Routine hematology and biochemistry analyses were performed, including variables of liver and kidney function, glucose, and serum electrolyte concentrations. All dogs were examined unsedated.

Sample handling

Within 30 min of sample collection, serum tubes were centrifuged at 2400 rpm for 15 min. Serum was harvested and divided into several aliquots per dog. Samples were frozen in plastic cryotubes and stored at −80°C at each center for up to 1 year. They were thereafter transported frozen to a commercial laboratory[l] for analysis of biochemical metabolic

[l] Laboratoire Vebio, Arcueil, France.

Table 1 Distribution of included dogs by center of examination, breed, and sex.

Breed	Belgium	Denmark	Finland	France	Sweden	Total
Boxer					15M	15
Belgian Shepherd	65M			24M		89
CKCS					33M	33
Dachshund			25M		16M	41
Doberman Pinscher				14M		14
Finnish Lapphund			50M			50
German Shepherd	15M		60M			75
Labrador Retriever	5M	44F		28F	45M	122
Newfoundland		44F				44
Total	85	88	135	66	109	483

CKCS = Cavalier King Charles Spaniel; M = male; F = female.

variables, according to the LUPA protocol [25,29,30]. After these analyses, performed in batches during the years 2009–2010, samples were immediately refrozen and stored at the laboratory at −20°C. Samples were thereafter transported frozen to the Swedish University of Agricultural Sciences, Uppsala, Sweden, where 5-HT analyses were performed in spring 2015. The samples used for the serum 5-HT analyses had been thawed and refrozen once.

Analysis of serum 5-hydroxytryptamine

Analysis of serum 5-HT concentration was performed at the Laboratory of Clinical Sciences, Swedish University of Agricultural Sciences, by an experienced laboratory technician blinded to dog identity. Samples were analyzed according to manufacturers' instructions using a commercially available enzyme-linked immunosorbent assay (ELISA) assay[m] validated for dogs [12,15].

All samples were analyzed in duplicate, and on each ELISA plate, dogs from different examination centers were mixed in order to reduce interassay variation that might confound results. Samples with >12% intra-assay coefficient of variation (CV) were rerun and samples with concentrations <20 ng/mL were assigned the concentration 19.9 ng/mL. The in-house interassay CVs were calculated from results of the low and high control samples included in each of the ELISA plates giving mean CVs of 6.0% (51–118 ng/mL) and 8.4% (168–313 ng/mL). The in-house intra-assay CV, calculated from duplicates was 6.4% (values between 20 and 1396 ng/mL). Mean values of the duplicates were used for data analysis. Storage time was calculated from date of sample collection to date of ELISA analysis.

Statistical analyses

Statistical analyses were performed using commercially available software.[n] Data are presented as medians and interquartile ranges (IQRs). A p-value < 0.05 was considered significant, unless otherwise indicated.

The non-parametric Kruskal-Wallis test was used to investigate overall differences among breeds in serum 5-HT concentration. If a significant difference was detected, pairwise breed comparisons were performed by Mann-Whitney U-test with Bonferroni adjustment (adjusted significance level was $p<0.0014$).

Owing to uneven breed distribution among the centers, breed was highly covariate with the center. Kruskal-Wallis test was therefore also used to investigate breed differences in serum 5-HT concentration within each center. At centers including more than two breeds, pairwise breed comparisons were performed using Mann-Whitney U-test with Bonferroni adjustment if an overall significant difference was detected.

Sex difference in serum 5-HT concentration in all dogs was evaluated by Kruskal–Wallis test.

Univariate regression analyses were performed to evaluate potential associations between breed, age, body weight, examination center, sex, storage time, and serum 5-HT concentration. To compensate for influence of confounding factors on serum 5-HT concentration, multiple regression analysis was performed, including variables that reached $p<0.2$ in the univariate regression analysis. Owing to the covariance between breed and body weight, the latter was not included in the multiple regression analysis. The analyses were performed in a reverse stepwise manner [31], starting with all included variables and removing the variable with

[m] Serotonin ELISA (RE59121), IBL International GmbH, Hamburg, Germany.

[n] JMP Pro, version 11.2.0, SAS Institute Inc, Cary, NC, USA.

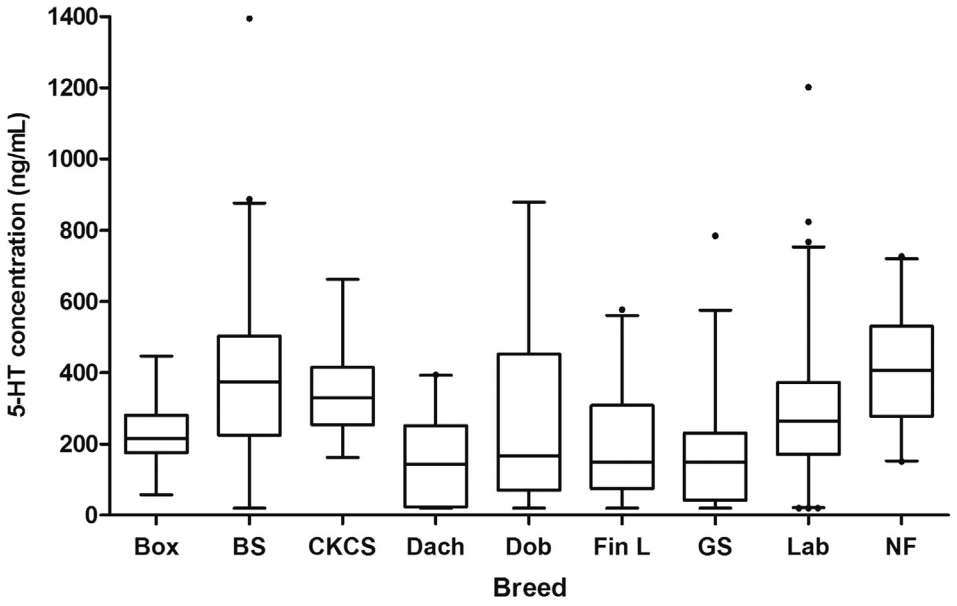

Figure 1 Boxplot showing serum 5-HT concentration by breed. The top, bottom, and line within each box correspond to the 75th percentile (top quartile), the 25th percentile (bottom quartile), and the 50th percentile (median), respectively. The whiskers extend from the bottom 2.5th percentile to the top 97.5th percentile. Outliers, represented by black dots, were included in statistical analyses. There was an overall significant breed difference ($p<0.0001$). For information on which breeds that differed significantly, see Table 2. Box = Boxer; BS = Belgian Shepherd; CKCS = Cavalier King Charles Spaniel; Dach = Dachshund; Dob = Doberman Pinscher; FinL = Finnish Lapphund; GS = German Shepherd; Lab = Labrador Retriever; NF, Newfoundland; 5-HT = 5-hydroxytryptamine.

highest *p*-value until all remaining variables reached $p<0.05$. All variables were assessed only as main effects; no interaction terms were considered in the model. Distribution of residuals in the multiple regression analysis was tested for normality using Shapiro-Wilk W test. The adjusted R^2 is defined as the percentage of the total sum of squares explained by the regression and also considers the degrees of freedom for variables added.

Results

In total, 483 dogs of nine breeds were included in the study, while 26 examined dogs were excluded due to findings of clinically relevant organ-related or systemic diseases. Distribution of breeds and dogs included at the different centers is shown in Table 1. Each center examined dogs from two to four breeds. Some breeds were examined at more than one center, and Labrador Retrievers were included at four of five centers. Sex distribution was uneven with 116 female dogs and 367 males (Table 1). Median age (n = 483) was 3.3 years (IQR = 2.6–4.3) and median body weight (n = 444) was 30.0 kg (IQR = 22.5–35.5). Median storage time (n = 481) was 5.6 years (IQR = 5.3–6.3).

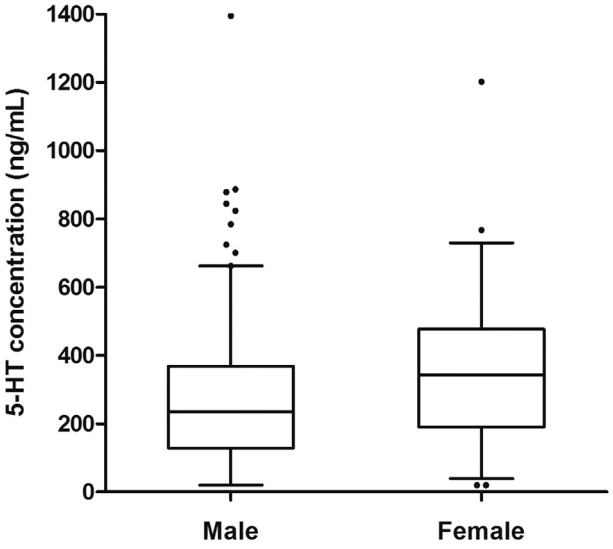

Figure 2 Boxplot showing serum 5-HT concentration by sex. The top, bottom, and line within each box correspond to the 75th percentile (top quartile), the 25th percentile (bottom quartile), and the 50th percentile (median), respectively. The whiskers extend from the bottom 2.5th percentile to the top 97.5th percentile. Outliers, represented by black dots, were included in statistical analysis. Female dogs had significantly higher serum 5-HT concentration compared with male dogs ($p<0.0001$). 5-HT = 5-hydroxytryptamine.

Table 2 Pairwise comparisons between breeds in serum 5-HT concentration.

Breed	Box	CKCS	Dach	Dob	FinL	GS	Lab	NF
BS			*		*	*	*	
	Box							*
		CKCS	*		*	*		
			Dach				*	*
				Dob				
					FinL		*	*
						GS	*	*
							Lab	*

Box = Boxer, BS = Belgian Shepherd, CKCS = Cavalier King Charles Spaniel, Dach = Dachshund, Dob = Doberman Pinscher, FinL = Finnish Lapphund, GS = German Shepherd, Lab = Labrador Retriever, NF= Newfoundland; 5-HT = 5-hydroxytryptamine.
The asterisks denote significant differences using a Bonferroni corrected *p*-value of <0.014.

Serum 5-hydroxytryptamine concentration

Median 5-HT concentration was 252 ng/mL (IQR = 146−391). Serum 5-HT concentrations by breed are shown in Fig. 1 and by sex in Fig. 2. Median serum 5-HT concentration was higher in female dogs, 342 ng/mL (IQR = 190−477), compared to males, 235 ng/mL (IQR 128−368), $p<0.0001$.

Group-wise breed comparisons in all dogs and within centers

An overall breed difference in serum 5-HT concentration was found, $p<0.0001$. Pairwise breed comparisons were significantly different in 15 of 36 comparisons (42%), Table 2. Serum 5-HT concentration was highest in Newfoundlands, followed by Belgian Shepherds and CKCSs. These three breeds had median concentrations approximately 2.5 times higher than median values in Dachshunds, Finnish Lapphunds, and German Shepherds, which had lowest concentrations, Fig. 1.

In the group-wise breed comparisons within each center, overall breed differences were found at three of the five centers, $p\leq0.028$. Pairwise comparisons within these centers were significant in five of 12 comparisons (42%), $p\leq0.017$.

Univariate and multiple regression analyses

The univariate regression analysis showed association between serum 5-HT concentration and breed ($R^2 = 0.22$, $p<0.0001$) and center of examination ($R^2 = 0.23$, $p<0.0001$). There was also an association with sex ($R^2 = 0.03$, $p<0.0001$) with higher serum 5-HT concentrations in female dogs than in males. There were positive associations between 5-HT concentration and storage time ($R^2 = 0.08$, $p<0.0001$) and body weight ($R^2 = 0.02$, $p=0.002$). Age was not associated with serum 5-HT concentration ($p=0.72$). In the multiple regression analysis, breed ($p<0.0001$), center of examination ($p<0.0001$), and storage time ($p=0.014$) remained significant in the final model with an adjusted model R^2 of 0.27.

Discussion

In the present study, including almost 500 healthy dogs of nine breeds with different morphotypes, overall interbreed variation in serum 5-HT concentration was found in dogs aged between 1 and 7 years. Pairwise comparisons showed significant differences in 42% of breed comparisons, with Newfoundlands, Belgian Shepherds and CKCSs having the highest serum 5-HT concentrations. Higher serum and platelet-rich plasma 5-HT concentrations have previously been shown in healthy CKCSs compared with other breeds combined [12,13], and interestingly, in a recent study including approximately 100 healthy dogs, CKCSs along with Belgian Shepherds were also among the breeds with the highest serum 5-HT concentrations [24]. However, the Dachshund, which like the CKCS is a breed at risk of developing MMVD [32], was among the breeds with lowest serum 5-HT concentrations in the present study; whereas, the two other breeds with higher 5-HT, Newfoundlands and Belgian Shepherds, are not high-risk breeds for MMVD.

Center of examination was associated with serum 5-HT concentration in the present study. Because each center could include two to four breeds according to the LUPA criteria [25], breed distribution was skewed with certain breeds represented at one center alone and certain breeds shared between several centers. Hence, with the present study design, breed and center were partly covariate. In order to try to separate the effect of center from the effect of breed, breed comparisons were also performed within each center, showing overall breed differences at three of the five centers and significant differences in five of the 12 pairwise breed comparisons. Included breeds represented dogs of varying body sizes, morphology, and temperament. During the development of different dog breeds, strong selection for specific physiologic, morphologic, and behavioral traits has created breeds with great diversity, and certain features are inherited closely within breeds [33]. In the same population of dogs, we have previously

shown breed differences in several blood variables, including natriuretic peptides and endothelin-1, and in urinary concentrations of epinephrine and norepinephrine [30,34,35], differences which are likely influenced by genetic factors. In addition to heterogeneity between breeds, selection during breeding has led to homogeneity within dog breeds [33]. However, even within breeds, genetic variation between geographic locations and subpopulations might exist [36], which could contribute to the effect of center in the present study. Furthermore, the association between serum 5-HT concentration and center could partly be because of differences in procedures between centers. Such influences were minimized by use of standardized examination protocol, sampling, and sample handling between centers, but minor differences might have occurred, which could have affected the results.

There was a weak association with sex, with higher serum 5-HT concentrations in females compared to males, which is in accordance with findings in people [37]. In dogs, higher serum 5-HT concentrations have also been found in females compared with males in previous studies [12,15], while a recent study did not find an effect of sex on serum 5-HT concentration [24]. The present study was, however, not designed to study sex differences, and the results should be interpreted cautiously. In contrast to serum, a previous small study on plasma 5-HT concentrations in dogs showed higher concentrations in males than in females, while sex differences in plasma 5-HT concentrations have not been found in people [38].

Among the two breeds predisposed to MMVD included in the study, the CKCS had significantly higher 5-HT concentration compared to Dachshunds. Development of MMVD in dogs is age-dependent, with the CKCS being genetically predisposed to early development of the disease [23,39], while the Dachshund is affected at a higher age [40]. 5-hydroxytryptamine has been suggested to be involved in the pathogenesis of MMVD, and higher serum 5-HT concentrations have previously been shown in CKCS compared with other breeds [15]. Interestingly, studies show higher serum and plasma concentrations in dogs with mild disease or in healthy dogs of predisposed breeds compared with dogs in late stage MMVD [15,16], suggesting that 5-HT could be specifically important in the early development of the disease. The present study only included young healthy dogs with a median age of 3.3 years. Whether included CKCS or Dachshunds have or will go on to develop MMVD is currently unknown, but it is possible that the higher serum 5-HT in the CKCS could be connected to a risk of earlier development of the disease. Among five small size dog breeds in a previous study of healthy dogs, the CKCS was also among the breeds with highest serum 5-HT concentration, along with Scottish Terriers and Maltese, while West Highland White Terriers and Jack Russell Terriers had lower concentrations [24]. That study included two largesize breeds, and in accordance with our findings, the Belgian Shepherd showed high serum 5-HT concentrations [24]. In their study, the majority of Belgian Shepherds were male, and in our study all Belgian Shepherds were male. Hence, sex cannot explain the high concentration in that breed, while the high 5-HT concentration in Newfoundlands in our study might partly be explained by the higher values found in females in previous studies [12,15]. Furthermore, because the 5-HT system, along with other neurochemical systems, has been suggested to control various temperamental traits [41], breed differences in 5-HT concentrations might be connected to differences in temperament. However, the present study only investigated peripheral, and not central, 5-HT concentrations, therefore no conclusions can be made concerning potential associations between 5-HT concentrations and temperament of included breeds.

The age range of included dogs was narrow with IQR between 2.6 and 4.3 years. Hence it is not surprising that age was not associated with serum 5-HT concentration. In a previous study, serum 5-HT concentration decreased with increasing age in healthy dogs, but the correlation was weak [12], and in other studies, age has not been proven to be associated with serum 5-HT concentration [15,24].

The majority of peripheral 5-HT is synthesized in intestinal enterochromaffin cells, and upon secretion into the blood stream, it is rapidly taken up and stored in platelets [42]. Because plasma concentrations of 5-HT are generally low [13,21,22], analysis of 5-HT in serum samples, as in the present study, might mainly represent platelet content released during coagulation of blood samples. In a previous study, serum 5-HT concentration has been shown to be approximately 40 times higher compared to plasma in healthy CKCSs [16]. Serum 5-HT concentration has been suggested to indirectly represent the sum of platelet and plasma 5-HT concentrations in people as well as dogs [43,44], and serum 5-HT has thus been proposed a reliable and simple sample type for assessment of 5-HT in dogs [44].

Platelets are attracted to injured cardiac tissue and based on in vitro studies; 5-HT release by platelets appears to be a major contributor of

platelet effects, such as fibroblast activation [45]. It has therefore been suggested that platelets attracted to injured mitral valves release 5-HT, leading to increased local 5-HT signaling and progression of valvular changes during development of MMVD [12,46]. However, different studies have shown decreased [47] or increased [48] aggregation potential of platelets in dogs with MMVD, and further studies are needed to investigate the potential role of in vivo platelet 5-HT release in the pathogenesis of MMVD. In addition to 5-HT production in enterochromaffin cells, there is also evidence of local cardiac 5-HT synthesis in different species including dogs [10,49,50]. Findings of increased trypthophane hydroxylase-1, 5-HT-receptor 2B, and transforming growth factor β in MMVD-affected valves [10,51] might support the role for local 5-HT production in the pathogenesis of MMVD. In previous studies, higher 5-HT concentration has been found in platelets [13] as well as serum samples of healthy CKCSs compared to non-CKCS controls [12,13], and high plasma and platelet 5-HT has been suggested to be a familial trait among CKCSs, which could contribute to the early onset of MMVD in the breed [16].

Tryptophan, the precursor for 5-HT, is an essential amino acid abundant in certain food, such as nuts, seeds, soya foods, and cheese. Food has been shown to affect 5-HT concentrations in people, with lower values after a tryptophan-poor diet [37]. The dogs in the present study were fasted for at least 12 h on examination day to minimize the effect of food intake. Furthermore, they were only allowed to eat commercial dog food and no treats for 2 weeks before participation. The food should therefore not have had any major effect on the results.

The study has limitations. The storage time was long, and for the majority of that time, samples were stored at −20°C. In a previous study, storage time did not affect serum 5-HT concentration in samples stored up to 2 years at −80°C [15]. Being a neurotransmitter [2], 5-HT should be more stable than a protein, but median concentration in the present study is approximately 50% of median concentration observed in previous studies on healthy dogs by use of the same ELISA [12,16]. In the univariate regression analysis, storage time showed a weak ($R^2 = 0.08$) but significant association with serum 5-HT concentration. In the final model of the multiple regression analysis, storage time also remained significant, but with a modest effect ($p=0.014$) compared to breed and center of examination (both $p<0.0001$). The comparably long storage time should, therefore, be regarded as a study limitation. Furthermore, samples had been thawed once, which could have affected results. For these reasons and because the study only included nine breeds, with uneven sex representation and narrow age span, results should not be interpreted as reference values.

Conclusions

Interbreed variation in serum 5-HT concentration was found in healthy dogs aged between 1 and 7 years, with highest concentrations in Newfoundlands, Belgian Shepherds and CKCSs. These breed differences should be taken into account when designing clinical studies.

Conflicts of Interest Statement

The authors declare no conflicts of interest.

Acknowledgment

The study was funded by the European Commission (FP7-LUPA, GA-201370), the Agria Research Foundation, and the Swedish Kennel Club Research Foundation. The authors also thank Annlouise Jansson and Anna Svensson for excellent laboratory work. They thank the universities involved for allowing completion of the study and dog owners for their willingness to enroll their dogs in this study.

Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jvc.2018.05.002.

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