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Blood serotonin concentrations in phobic dogs fed a dissociated carbohydrate-based diet: a pilot study

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Abstract: Aim of this study was to evaluate the effects of a carbohydrate-based diet on serotonin blood concentrations in phobic dogs. For this study were recruited, from a public shelter, three dogs (2 neutered females and 1 male), weighing between 15 and 30 kg and living in the shelter for more than six months. Dogs received by a veterinary behaviorist a diagnosis of interspecific social phobia. The dogs were fed 2 daily meals (at 8.00 A.M. and 4.00 P.M.), the first meal was exclusively carbohydrate-based (puffed rice) whereas the second one was composed by the commercial diet.

Blood was collected every 21 days after 8 hours from carbohydrate meal to determine the levels of serotonin (5-HT), L-tryptophan (TRP) and cortisol. Statistical analysis did not reveal any significative difference between the serum concentrations of 5-HT, TRP and cortisol, at the different times, despite a tendency to increase during the time.

The results of this research are useful for directing further studies in the right direction, verifying the correctness of the hypotheses that can be formulated based on the analysis of these data.

Blood concentrations of cortisol suggest that there have been no particular episodes of stress. For this reason, it is possible to exclude that the reduced transformation of TRP in 5-HT is due to an increased activity of tryptophan 2,3-dioxygenase induced by cortisol. In conclusion, these results are to be considered as a further step to address, more correctly, further research on the effect of diet manipulation on serotonin blood and brain concentrations.

Key Words: serotonin, dog, phobia, dissociate diet, carbohydrates.

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Introduction

Domestic animals, especially the dogs, have shared their lives with humans for a long time (Savolainen et al., 2002). However, this relationship is sometimes negatively influenced by the exhibition of particular aspects of their ethology (Mengoli et al., 2013) or by behavioral problems. In dogs, intra and interspecific aggression, phobias and separation problems are the most frequent behavioral problems and reasons of relinquishment (Patronek et al., 1996).

Between these problems, attention has been recently paid to phobia, for its relevance in to affect dog welfare.

Phobia can be defined as a persistent and excessive fear of an object, individual or situation (Sherman & Mills, 2008). In dogs, different types of this behavioral pathology are signaled: noise, thunderstorm, social phobia and different therapeutic interventions have been proposed.

Phobia and other behavioral problems can result from many causes, including an altered functionality of serotoninergic pathways in the brain, characterized by serotonin (5-HT) deficiency (Sachs et al., 2015; Rosado et al., 2010 a,b). 5-HT and other neurotransmitters, mediating communication between cells and acting as trophic factors in the processes of neurogenesis, derive from precursors introduced with the diet. For this reason, in the last years some researchers have

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increasingly focused their attention on the possible effects of the diet on the behavior (e.g. Gatta et al., 2013).

As regarding the synthesis and release of 5-HT by brain, neurons are rapidly influenced by the local tryptophan (TRP) concentration (Fernstrom et al., 1990; Sharp et al., 1992). TRP passes through the blood–brain barrier by using carriers for which it competes with other large neutral amino acids (LNAAs), mainly leucine, isoleucine, valine, tyrosine and phenylalanine (Oldendorf & Szabo, 1976).

In the brain, TRP is hydroxylated to 5-hydroxytryptophan by the enzyme TRP hydroxylase, that is usually about half saturated with the amino acid (Carlsson, 1978). Therefore, increases in TRP in the brain can maximally double the 5-HT synthesis (Carlsson, 1978), but meals containing protein fail to raise brain TRP concentrations because of the contemporary increase of both serum TRP and LNAA concentrations by proportionally similar amounts, resulting in no net change in competition for uptake (Fernstrom, 1980).

Instead, previous studies have shown that the ingestion of a large carbohydrate, protein-free meal by fasting rats, rapidly raises brain TRP concentrations and stimulates 5-HT synthesis (Fernstrom, 1980). The carbohydrate meal is known to produce its effects on TRP via insulin secretion, which depresses the plasma concentrations of LNAAs in mammals, increasing their uptake into muscle, except TRP due to its bond with albumin (Fernstrom & Wurtman, 1972; Lotspeich, 1949).

Studies performed on dogs have produced contrasting results: De Napoli (De Napoli et al., 2002) found that the addition of TRP to high protein diets or the switch to a low protein diet might reduce aggression in dogs displaying so-called dominance and territorial aggression. Plasma concentrations of 5-HT and TRP had consistent results in all phases of the study, despite different concentrations of dietary TRP. As suggested by the authors, this is most likely due to their inadequate analytic methods. Mugford reported that there was a reduction in aggressive behaviors in three of the seven aggressive Golden Retrievers after the introduction of a low-protein diet (15–18% of total energy) (Mugford, 1987). However, the composition of the experimental and previous diets was not reported.

In another study (Dodman et al., 1996), twelve dogs that exhibited either high territorial aggression dominance aggression or hyperactivity, and fourteen control dogs were fed each of three diets varying in protein content (180, 250 and 310 g crude protein/kg DM) for two weeks at living in-home situations. The low-protein diet and medium-protein diet decreased territorial aggression scores in comparison to the high-protein diet.

Our recent study has demonstrated that, in dog, a carbohydrate-based diet led to a decrease in LNAAs levels and consequently led to a significant higher TRP/LNAAs ratios 6 h after the provision of carbohydrates (Gazzano et al., 2018).

No one, until now, has investigated the effects of carbohydrate-based diets on serum serotonin concentrations, despite a study showed correlations between low levels of serotonin in blood and a behavioral pathology like aggressive behavior in English Cocker Spaniels (Amat et al., 2013).

In addition, serotonin synthetized in the brain can overcome the blood-brain barrier. As demonstrated by Nakatani et al. (2008), 5-HT transporters located on the brain endothelial cells may act as the efflux transport system for the 5-HT that crosses from the brain into the circulating blood.

Aim of this study was to evaluate the effects of a carbohydrate-based diet on serotonin blood concentrations in phobic dogs.

Animals, materials and methods

For this study, approved by the Ethical Committee of the University of Pisa, Italy (protocol n° 38/2016) in accordance with Directive 2010/63/EU, were recruited, from a public shelter, three

dogs (2 neutered females and 1 male), weighing between 15 and 30 kg and living in the shelter for more than six months. Dogs received by a veterinary behaviorist a diagnosis of interspecific social phobia. The study was conducted between January and April 2019.

The animals were fed two meals (at 8.00 A.M. and 4.00 P.M.) for 15 days with a commercial diet to standardize their metabolic status. After this period the dogs were fed 2 daily meals (at 8.00 A.M. and 4.00 P.M.), the morning meal was exclusively a carbohydrate-based one (puffed rice) whereas the evening meal was composed by the commercial diet previously eaten during the adaptation period. Ingredients and diet analytical constituents are reported in Table 1.

Table 1. Ingredients and diet analytical constituents (as fed basis).

		Commercial kibble	Puffed Rice	Diet
Moisture	%	10.0	5.5	7.9
CP	%	28.0	7.5	18.8
Fat	%	21.0	1.0	13.4
CF	%	2.6	2.5	2.5
NSC	%	31.9	83.0	53.0
Ash	%	6.5	0.5	3.8
EM	kcal/kg	3800	3250	3690

Blood was collected every 21 days after 8 hours from carbohydrate meal to determine the levels of serotonin (5-HT), L-tryptophan (TRP) and cortisol. Blood samples (4 ml) were left to coagulate at room temperature for 60 minutes, then centrifuged in ALC 4237R Refrigerated Centrifuge at 7000 rpm for 20' to 4°C to obtain the serum. The serum was divided into 200 µl aliquots and frozen until the time of analysis.

The extraction and quantification of 5-HT and TRP in serum samples were performed following an HPLC method, as previously described in the literature (Bearcroft et al., 1995; Atkinson et al., 2006) and based on fluorimetric detection. This method was slightly modified as follows: 200 µl $HCLO_4$ 4% v/v containing 2mM EDTA were added to 200 µl of serum or standard solution to precipitate proteins; the extract was mixed and centrifuged at 13000 rpm in micro centrifuge (ALC microCENTRIFUGETTE ® 4214) for 3 minutes. 50 µL of supernatant were taken with MICROLITER™ Syringes #705 and 20 µl injected into HPLC for analysis.

HPLC analyses were performed using a RP Gemini C18 column (250 mm x 4.6 mm, 5 um) (Phenomenex, Torrance, CA, USA) and a Jasco HPLC apparatus (Jasco Corporation, Ishikawa-Machi Hachioji-Shi, Tokyo, Japan) equipped with 2 gradient pumps (PU-1580), a mixer unit (HG-2080-03) and a fluorescence detector (FP-920).

The mobile phase consisted of methanol (CH_3OH) and ammonium acetate (CH_3COONH_4) 100 mM (20:250 v/v), pH 4.5, degassed and filtered with 0.2 µm diameter filters and eluted at a flow rate of 0.800 ml/min.

Fluorescence detector was set at 290 nm excitation wavelength and 337 nm emission wavelength. Data was acquired using Jasco Borwin 1.5.0 software (Jasco Corporation, Ishikawa-machi Hachioji-shi, Tokyo, Japan). The interface between chromatography instruments and a PC based data acquisition is the JMBS electronic interface box HERCULE 2000 VI.0.

Serotonin creatinine sulfate monohydrate and L-tryptophan (TRP) were purchased from Sigma-Aldrich Inc. (Saint Louis, MO, USA).

Stock solution (10 mM) of 5-HT and stock solution (100 mM) of TRP were prepared in 10 ml $HClO_4$ 10%, divided in aliquots of 1 ml and stored at -20°C. Diluted standard solutions in

$HClO_4$ 4% were prepared daily and employed to identify chromatographic peaks and to calculate calibration curves.

Cortisol concentrations from canine serum were measured using an ELISA kit (Diametra®, Segrate, Italy), according to the manufacturer's instructions. Briefly, the antigen cortisol in the sample competes with the antigenic cortisol conjugated with horseradish peroxidase-cortisol (HRP) for binding to the limited number of antibodies anti cortisol coated on the microplate. After incubation and washing, the enzyme HRP in the bound fraction reacts with the substrate (H_2O_2) and the TMB substrate and develops a blue color that changes into yellow when the stop solution (H_2SO_4) is added. Cortisol concentration in the sample is calculated based on a series of standards and the color intensity is inversely proportional to the cortisol concentration in the sample. The method allows the determination of cortisol from 0 to 500 ng/ml.

Data were statistically analyzed applying Wilcoxon test, by using SPSS® STATISTICS 17.0.

Results

In figure 1, 2, 3 are reported the serum concentrations of 5-HT, TRP and cortisol of the three dogs examined. Statistical analysis (Table 2) did not reveal any significative difference between the serum concentrations of 5-HT, TRP and cortisol, at the different times, despite a tendency to increase during the time.

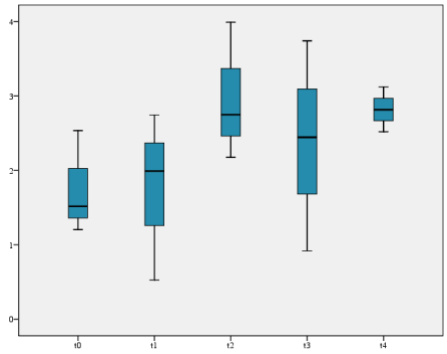

Figure 1. Mean serum 5-HT concentration (µM/ml ± S.D.) at different time (T0=baseline, T1=3 week, T2=6 week, T3=9 week, T4=12 week).

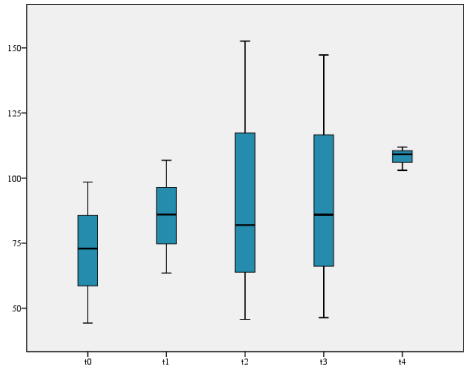

Figure 2. Mean serum TRP concentration (µM/ ml ± S.D.) at different time (T0=baseline, T1=3 week, T2=6 week, T3=9 week, T4=12 week).

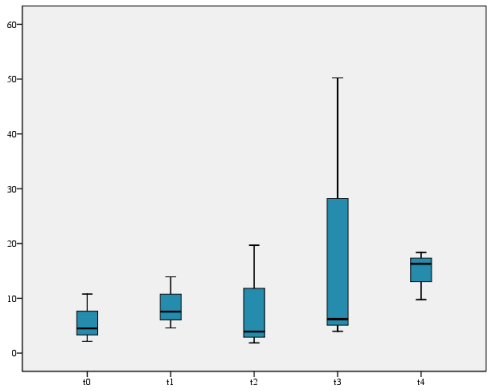

Figure 3. Mean serum cortisol concentration (ng/ml ± S.D.) at different time (T0=baseline, T1=3 week, T2=6 week, T3=9 week, T4=12 week).

Table 2. Z values obtained with Wilcoxon test for 5-HT, TRP and Cortisol, comparing their concentration between different times analyzed.

Z	T1 *vs* T0	T2 *vs* T0	T3 *vs* T0	T4 *vs* T0
5-HT	0; *n.s.*	-1.604; *n.s.*	0; *n.s.*	-1.604; *n.s.*
TRP	-1.604; *n.s.*	-1.604; *n.s.*	-1.604; *n.s.*	-1.604; *n.s.*
Cortisol	-0.535; *n.s.*	0; *n.s.*	0; *n.s.*	-1.604; *n.s.*

Discussion

Phobia is one of the most serious behavioral problem in dogs because this pathology can deeply affect the quality of life, limiting the possibility of this animal to participate to the activities of the owner.

Among the different forms of phobia, the social, interspecific, towards people, is certainly the most serious for its consequences on the relationship with the human being and for the difficulty of management of the animal that it entails. If a dog that presents a phobia of noises can, with special care, be managed in an acceptable way for its welfare, this becomes practically impossible in the case of interspecific social phobia. In fact, the dog lives in a strongly anthropized social environment in which it is necessary that it can fit properly.

The causes of social phobia are certainly due to deficiencies during the development of the behavior of the puppy that has not been adequately socialized towards the human being. Since Scott & Fuller's pioneering researches in the sixty (Scott & Fuller, 1965), we know that in the period of socialization, puppies must be exposed to the greatest possible number of stimuli (Gazzano et al., 2008a).

Maternal care (Guardini et al., 2015; 2016; 2017) and neonatal stimulations (Gazzano et al., 2008b) can also have a positive effect in a normal development of the puppies.

However, it has been pointed out that phobia could have genetic bases. Some studies have, in fact, found a greater incidence of phobia, especially regarding noise, in certain breeds (Mengoli et al., 2012). Moreover, the standards of some breeds, that carry generic annotations regarding suspiciousness towards strangers, make to suppose that diffidence, if not a true phobia, is typical and, in the past, sought after as it is considered positive for the working use of the dog.

The manipulation of the diet can be an easy tool to use by the owner and well accepted even by people who exclude the use of psychotropic drugs in the therapy of behavioral pathologies of their animal.

It is therefore of considerable interest to check whether there is the possibility of using special diets to change the behavior of the dog.

The results of previous research have shown a link between serotonin blood levels and behavioral pathologies, such as aggression (Amat et al., 2013), a behavior often present in interspecific social phobia. Other studies conducted also by our research group (Gazzano et al., 2018), have shown how the manipulation of the diet can modify the blood relationship between TRP and other neutral amino acids, thus increasing its bioavailability.

The data of this first preliminary research does not clarify whether the increased bioavailability of TRP contributes to increasing the synthesis of serotonin. In fact, serotonin concentrations, although showing a tendency to increase with respect to the initial value, did not present statistically significant variations between the different times considered.

The major limitation of this study obviously lies in the reduced number of subjects examined, even though they constitute a homogeneous group with regards to living environment, time spent in shelters, management and diet.

However, these results are useful for directing further studies in the right direction, verifying the correctness of the hypotheses that can be formulated based on the analysis of these data.

Blood concentrations of cortisol, which are stable during the period examined, suggest that there have been no episodes of stress. This result is of particular importance because it allows us to hypothesize that the reduced transformation of TRP in 5HT is not due to an increased activity, in the liver, of tryptophan 2,3-dioxygenase induced by cortisol. In fact, in mammals more than 90% of the total TRP is degraded in the liver through the kynureninase pathway (Young et al., 1978) and the first enzyme of tryptophan oxidation, tryptophan 2,3-dioxygenase, is regulated by glucocorticoids (Knox & Mehler, 1951; Voigt & Sekeris, 1980) and by tryptophan itself (Knox, 1951, 1966). In an in-vitro experiment with rat liver cells, replacement of glucocorticoid with low doses of dexamethasone phosphate resulted in a 7-8-fold increase in the total activity of tryptophan 2,3-dioxygenase (Salter & Pogson, 1985).

Further studies will be necessary to rule out, with certainty, this hypothesis and to verify, instead, if the lack of increase in the production of serotonin depends on an insufficient passage of TRP through the blood-brain barrier, caused by a reduced presence of the amino acid in the carbohydrate-based diet: the addition of TRP to the diet will allow us to evaluate the validity of this hypothesis.

Finally, it will be necessary to verify if the little increase in blood serotonin, observed at the different times of collection and maintained for three weeks, even after the suspension of the diet, is due to the positive effect of the exposure of the animals to a photoperiod longer. Bright light is, in fact, a standard treatment for human seasonal depression, but some studies also suggest that it is an effective treatment for nonseasonal depression (Golden et al., 2005) and also reduces depressed mood in women with premenstrual dysphoric disorder (Lam et al. 1999), and in pregnant women suffering from depression (Epperson, 2004).

In human postmortem brain, serotonin levels are higher in those who died in summer than in those who died in winter (Carlsson, 1980). A similar conclusion came from a study on healthy volunteers, in which serotonin synthesis was assessed by measurements of the serotonin metabolite 5-hydroxyindoleacetic acid in the venous outflow from the brain (Lambert et al., 2002). There was also a positive correlation between serotonin synthesis and the hours of sunlight on the day the measurements were made, independent of season.

In conclusion, these results are to be considered as a first step to address, more correctly, further research on the effect of diet manipulation on serotonin blood and brain concentrations.

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Concentrazioni ematiche di serotonina in cani fobici alimentati con una dieta dissociata a base di carboidrati: uno studio pilota

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Sintesi

Lo scopo di questo studio è stato quello di valutare gli effetti di una dieta a base di carboidrati sulla concentrazione ematica in cani fobici. Per questo studio sono stati reclutati da un canile pubblico, 3 cani (2 femmine castrate ed 1 maschio), di peso compreso tra 15 e 30 Kg e ospitati in canile da almeno 6 mesi. Ai cani è stata diagnosticata, da un veterinario esperto in comportamento, una fobia sociale interspecifica. I cani sono stati alimentati con 2 pasti giornalieri (alle 8,00 e alle 16,00), il primo esclusivamente a base di carboidrati (riso soffiato) ed il secondo composto dalla dieta commerciale con cui erano solitamente alimentati.

Il sangue è stato prelevato ogni 21 giorni, dopo 8 ore dal pasto di carboidrati per determinare i livelli di serotonina, L-triptofano e cortisolo.

L'analisi statistica non ha rivelato alcuna differenza statisticamente significativa tra le concentrazioni di serotonina, L-triptofano e cortisolo, ai differenti tempi analizzati, nonostante una tendenza all'aumento durante il tempo.

I risultati di questa ricerca sono utili per indirizzare ulteriori studi nella giusta direzione, verificando la correttezza delle ipotesi che possono essere formulate sulla base dell'analisi di questi dati.

Le concentrazioni ematiche di cortisolo suggeriscono che non ci siano stati particolari episodi di stress. Per questa ragione è possibile escludere che la ridotta di triptofano in serotonina sia dovuta ad un'aumentata attività della triptofano-2,3-deossigenasi, indotta dal cortisolo.

In conclusione, questi risultati sono da considerarsi come un primo step per indirizzare, in modo più corretto, ulteriori ricerche sull'effetto della manipolazione della dieta culle concentrazioni ematiche e cerebrali di serotonina.