Health screening of old dogs before and after feeding an enriched diet

Diplomarbeit
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Vorgelegt von
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1. Introduction

The population of aged pet dogs is increasing and so are diseases associated with aging. People consider many physiological and behavioural changes of old dogs as a part of the normal aging process, however, it is important to differentiate between normal aging and pathologic aging. Most dog owners are informed about the physiological changes associated with normal aging, which includes changes in body composition and in metabolic rates as well as decline of various senses (Larsen, Farcas, 2014). In contrast, many of them are not aware about the pathological changes associated with brain aging that may result in severe cognitive dysfunction. Moreover, also in successfully aged dogs, pathologic processes in the brain, like oxidative damage, may lead to changes in gene expression and progressive accumulation of toxic proteins, which are leading to neuronal loss and dysfunction. These processes are linked to canine cognitive dysfunction and to symptoms, like disorientation, changes in the behaviour towards the owner or alteration in sleep-wake-cycles, which dog owners easily recognise in all day life (Head, 2010).

Schütt et al., (2015) suggest that blood analysis allows for screening disease progression in old dogs. By testing blood samples one can detect variations in the haematological parameters and in the chemical blood counts, which are indicators for certain diseases, e.g. the increase of glucose in diabetes mellitus, the increase of glucocorticoids in Cushing’s disease or the increase of inflammation values which can be caused by several diseases associated with age, like inflammation of the joints or tumours. More importantly, the increase of beta-amyloid in plasma has been considered as a biomarker for canine cognitive dysfunction (CCD) since the beta-amyloid values in plasma increases in dogs with cognitive impairment (Schütt et al., 2015; González-Martínez et al., 2013).

Several studies have shown that certain parameters, like IL-6 and C-reactive protein (CRP) in the blood correlates with CCD or other diseases accompanied by cognitive decline (Mongillo et al., 2015). Changes in peripheral immune cell populations of dogs with cognitive impairment, especially low numbers of CD4 lymphocytes and high numbers of monocytes, have also been found. Dunlap et al., (2005) reported that oxidative damage caused by free radicals is an important factor in several diseases correlated with age such as cancer, Alzheimer’s disease and heart disease. Therefore, it can be speculated that there might be a link between different physiological and cognitive parameters.
As mentioned above, CCD in dogs can be linked with several behavioural changes. Owners of affected pet dogs often report about specific problems in all day life, e.g. the failure of recognition of familiar people or even the owner her-/himself, stereotypic walking or circling, disorientation on walks or staring blankly at walls (Schütt et al., 2015). There are different approaches to counteract changes of different behaviours and cognitive functions in old dogs with the most commonly reported being dietary and behavioural intervention (Dodd et al., 2003; Harman 1969; Cotman et al., 2002; M. Fahnenstock et al, 2012). There are several studies suggesting that antioxidant-enriched diet in combination with behavioural enrichment hinder cognitive decline and slow down the pathological aging of the brain (Cotman et al., 2002; Fahnenstock et al, 2012). For example, in the study by Dunlap et al., 2005, one group of dogs was fed with antioxidant supplementation in the diet, whereas a control group was fed with a usual diet without antioxidants. They found, that antioxidant activity in the plasma was significantly higher for the group of dogs, which were fed antioxidant supplementation than the control group. The cell function in the brain can be severely impaired from oxidative damage caused by accumulation of reactive oxygen species e.g. free radicals. The results show that, antioxidants protect the cell membranes and keep them from getting damaged by losing their fluidity, thus, antioxidants act as a good prevention of neurodegenerative diseases like CCD. Vitamins, flavonoids and carotenoids have antioxidant characteristics and are used in veterinary prescription diet (Dodd et al., 2003). Another group of antioxidants are unsaturated fatty acids. One group of unsaturated fatty acids are omega fatty acids. They are essential for all mammals and must be supplied in the diet. Especially in ageing animals it is important to provide omega fatty acids in the diet to counteract cognitive deficits and inflammatory processes (J.M. Bouree, 2004). In a study of Mukaro et al., 2008, the leukocytes in general and lymphocytes decreased in humans, which received processed food with a higher dose of omega-3 long-chain polyunsaturated fatty acids.

In the present study we aimed to look at the link between an enriched diet and cognitive decline by feeding one group of dogs with enriched diet (e.g. supplementation of broad-based antioxidants and omega fatty acids) for one year, whereas a control group received a normal diet from the same company. We aimed to see if the diet shows an effect on the performance of dogs in behavioural tests and on the blood parameters. Using a blood test after one year of feeding the diet, we aimed to detect any differences in blood parameters between the test and control groups and investigate if there are behavioural changes measured with a cognitive task in accordance with these changes.
In the behavioural test we measured dogs’ manipulative behaviour and the persistency in the form of duration and frequency of that behaviour. This test is part of the “Vienna Canine Cognitive Battery”, which is sensitive to show age differences (Chapagain et al., 2017).

2. Hypothesis

As the behaviour test is measuring motivation and persistency we hypothesized that the dogs fed with an enriched diet will be more motivated and persistent in manipulating the toy. Furthermore, the inflammatory parameters in the blood are decreased and so are the diseases correlated with oxidative stress like orthopaedic problems (arthritis). Broadly the quality of life of dogs will be improved.

3. Material and Methods

3.1 Subjects

We used 120 pet dogs of different breeds including mixed breeds of size 7-42 kg. The age of the dogs ranges from 6-14 yrs. All of the participating dogs are living together with their respective owners in one household. The dogs were recruited using the dog-database of the Clever Dog Lab at the University of Veterinary Medicine, Vienna and through distributing flyers. All dogs used in the study were recruited for the PhD project “Cognitive aging in pet dogs”. The test that we used for this diploma thesis is a part of the “Vienna Canine Cognitive Battery Tests”, consisting of 10 different tests.

3.2 Procedure:

3.2.1 Veterinary examination and blood test:

All dogs went through a health screening, including physical, neurological, orthopaedic and ophthalmologic tests to rule out e.g. painful diseases of the locomotor system or blindness, which were exclusion criteria. Blood tests should rule out any dogs suffering from other severe diseases, severe mobility problems or sensory impairments not classified as canine cognitive dysfunction, which would hinder the dogs from participating in the study. Veterinary examination is done two times; once before starting the dietary intervention with
the test food and then after one year of feeding the respective diet to the two groups (for
details see below).
All examinations were implemented by veterinarians of the University of Veterinary
Medicine of Vienna, within the context of our study.

**Detailed description of blood test:**

The blood count is a common and very effective tool in modern medicine. It helps
veterinarians to detect many diseases and further take the right medical decisions. Reversely,
each parameter can also help to rule out some diseases. Regarding research on ageing dogs
and the influence of feeding enriched diet, the following parameters play a crucial role.

- The haematocrit is given as a percentage of blood cells in a volume of blood i.e. the
test measures the amount of space (volume) red blood cells take up in the blood.
Haemoglobin is a molecule within erythrocytes which is binding and carrying oxygen.
Both parameters are important for the supply of oxygen in the blood. If these two
parameters are low, the patient may have anaemia. The erythrocyte indices show the
size (MCV), the amount of haemoglobin (MCH) and the concentration of
haemoglobin (MCHC) in the red blood cells. Red cell distribution width (RDW)
shows, whether the cells have the same shapes and sizes. These parameters can help to
differentiate between the different forms of anaemia.

- The total amount of leukocytes is a marker for inflammations. The differential blood
count shows the different white cells. Each cell type gives important information, e.g.
a high number of eosinophils is often caused by parasites, allergies or neoplasia
whereas neutrophils, another subgroup of leucocytes, can be subdivided in segmented
cells and banded cells. An increased number of segmented cells can be observed in
stressed animals, at the time of blood sampling, as well as in dogs with
hyperadrenocorticism, steroid therapy or infection. Whereas banded cells are a strong
indicator of inflammation or infection. Lymphocytes decreases or increases in viral
infections, severe bacterial infection and endotoxemia. Chronic inflammations, foreign
body reactions and immune mediated diseases, induce an increase of monocytes in the
peripheral blood.
- Creatinine is a parameter for the kidney function. It is a waste product of the muscle metabolism and further filtered and excreted in urine. If the parameter is high, it is an indication of a decreasing kidney function.

- GLDH is an enzyme, which is present in the cells of the liver and if they are destroyed for any reasons (e.g. hepatitis) then the enzyme is free in the blood and their activity measureable. Usually measured liver parameters are ALT (alanine transaminase), AST (aspartate transaminase), GGT (gamma-glutamyltransferase) and ALP (alkaline phosphatase). These parameters are indicators for the enzyme activity of the liver, kidney, muscles or pancreas. Other parameters measures liver function, like ammonia and bile. If the liver is damaged, its ability to clean the blood from ammonia is impaired. Ammonia is a small molecule, which is able to pass through the blood brain barrier and can accumulate; this can lead to hepatic encephalopathy, severe neurological damage and symptoms which can look similar to CCD. A frequent control of these parameters is reasonable in old dogs.

- Total protein is the amount of protein in blood consisting of two major groups, albumin and globulin. Albumin is made in the liver and is important for tissue growth and healing, also to keep up the oncotic pressure in the vessels. Globulins are primarily made by the immune system and also from the liver and help to fight against infections, act as transport proteins or as acute phase proteins.

- Alkaline phosphatase is made by the liver, the bones and a few other organs. A high level of this enzyme is caused by bone growth (normal in young animals), damage of liver cells or bone disease like tumours.

All the parameters listed above help to interpret the clinical signs of a patient in the right way and diagnose specific disease.
Table 1:
The table below shows different parameters that are analysed in the blood:

<table>
<thead>
<tr>
<th>Haematology</th>
<th>Differential blood count</th>
<th>Biochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes per µl</td>
<td>Stab cells (%)</td>
<td>Creatinine</td>
</tr>
<tr>
<td>Haemoglobin in g/dl</td>
<td>Segmented cells (%)</td>
<td>Total protein</td>
</tr>
<tr>
<td>Haematocrit (packed cell volume)</td>
<td>Lymphocytes (%)</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>MCV (mean corpuscular volume)</td>
<td>Eosinophils (%)</td>
<td>GLDH</td>
</tr>
<tr>
<td>MCH (mean corpuscular haemoglobin)</td>
<td>Neutrophils (%)</td>
<td></td>
</tr>
<tr>
<td>MCHC (mean corpuscular haemoglobin concentration)</td>
<td>Basophiles (%)</td>
<td></td>
</tr>
<tr>
<td>Leucocytes per µl</td>
<td>Large unstained cells (%)</td>
<td></td>
</tr>
<tr>
<td>CHCM (Corpuscular Haemoglobin Concentration Mean)</td>
<td>Lymphoblasts (%)</td>
<td></td>
</tr>
<tr>
<td>MPXI (myeloperoxidase index)</td>
<td>Juveniles (%)</td>
<td></td>
</tr>
<tr>
<td>RDW (red cell distribution width)</td>
<td>Stab cells per µl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Segmented cells per µl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphocytes per µl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eosinophils per µl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neutrophils per µl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basophiles per µl</td>
<td></td>
</tr>
</tbody>
</table>
3.2.2 Treatment and testing

The dogs were divided into two groups balanced for age, sex, training level, weight and breed. The groups received either the test or the control food for one whole year. Since this was a double-blind study, both owner and experimenter were kept ignorant about which food was the test and which one is the control food. All dogs were tested in a manipulative persistency task before and after one year of treatment.

3.2.3 Description of behaviour test:

We used two different kinds of toys for this test; 1) Kong© and 2) “Twist and treat” by PetSafe©.

For the test done before the diet feeding period, the dog is provided with a manipulative toy “Kong”, filled with dry food. In the first phase, the toy was filled with small pieces of food. If the dog manipulates the toy, food easily comes out. In the second phase, the toy was filled with bigger pieces of food which do not fall out during toy manipulation. In both phases, the dog was given the toy for 2 minutes. For the test done after one year, the procedure was the same but a different type of toy (“twist and treat”) was used to rule out a learning effect. The dogs should not remember the exact way of using the toy to have immediate success. In this study the manipulation and persistency behaviour was examined thus, the dogs should not show memory abilities but almost the same behaviour as in the first test, which was a new situation for the dogs.

Detailed procedure of the two testing phases:

Phase one: The experimenter (E) and the owner with the dog met in front of the testing room. E was waiting and watching with cameras (see below “test recording and coding”) outside while the owner walked with the dog to the centre of the testing room, removed the leash, and sit down on one of the two chairs, which were before placed in the room. The owner let the dog free to explore the room. E entered the testing room and walked to a prepared table in a corner of the room. E puts smaller pieces of dry food in the prepared Kong© wobbler and makes it ready. E places the toy in the centre of the room and then sits on the second chair next to the owner. The dog has 2 minutes to manipulate the toy. If the dog loses interest in the toy, the owner can encourage it verbally but not move from their chair.
Phase two: After two minutes of the first phase, E took the toy and then refilled it with a large “marky” dry dog treat and closed it so that the food could not leak when the dog manipulated the toy. Just like in phase 1 the dog can play with this now blocked toy for 2 minutes and the owner can encourage it verbally but not move from their chair if the dog loses interest in the toy.

3.2.4  Test recording and coding:

To do a detailed analysis of the behaviors of the dogs in the abovementioned phase 1 and 2, digital video cameras were located in the room and the tests were recorded. The videos generated from the tests were coded afterwards and analyzed using the video-coding software Solomon Coder beta 15.11.19 (by Andras Peter; http://solomoncoder.com) with a continuous sampling technique. The technique includes two variables termed manipulate toy and search food. The coder observed the behavior of the dog on the video and coded the respective variable. A randomly chosen set of 20 dogs were coded by a second coder, and inter observer reliability was calculated by estimating the intraclass correlation coefficient for each of the variables. Hereinafter, a detailed description of the two variables.

Manipulate toy:
Manipulation is defined as the behaviour, where the dog is in actual contact with the toy and moves it with the nose or the paw. The behaviour is also coded as “manipulate” when the interruption between two contact phases is less than 4 frames (approximately 0.4 seconds) in the coding program, because it is defined as one bout of manipulation. The parameter “manipulate” is measured as duration of manipulation and frequency of manipulation. We tested, whether the dogs show differences in duration and/ or frequency of manipulation. As aforementioned the analysis was done on data generated from before diet and after diet.

Search food:
After the dog stops manipulating the toy, it searches out the small food pieces either using its nose, or by sight, and eats the food. We will code this behaviour as search food.
3.3 Statistical analyses

As all dogs finished the tests and blood sampling, the results were pooled in Excel and statistically analysed with SPSS. Therefore, we extracted relevant blood parameters from the blood counts and transformed the data from Solomon Coder into an SPSS sheet.

3.3.1 Extracted blood parameters

As already introduced in table 1, five certain parameters were selected to analyse inflammation processes. We examined leucocytes in general (number/µl before and after diet) and within this group of cells, lymphocytes (percentage (%) and absolute number (number/µl) before and after diet), monocytes (percentage (%) and absolute number (number/µl) before and after diet). Furthermore, we analysed total protein (before and after diet in g/dl). Notwithstanding, neutrophil bands are a strong indicator of acute inflammation, they did not occur at all (< 0,01/µl) in the blood samples of the examined dogs in our research.

To test for normality, we used Kolmogorov-Smirnov-test. Not normally distributed values were absolute number and percentage of monocytes, segmented cells, leucocytes and total protein. To determine differences between pre- and posttest, we used Wilcoxon test for not normally distributed values. For normally distributed values (percentage and absolute number of lymphocytes), we used t-test, because of the higher statistical power.

To investigate, whether the control and experimental group differed from each other, the difference between measured values of pre- and posttest was calculated and was further used for analysis.

For normally distributed values (difference of percentage number of segmented cells, difference of percentage number of lymphocytes and difference of number of leucocytes), ANOVA (analysis of variance; a common statistical tool to compare two means from two unrelated groups (Petrie A., Watson P., 2006)) was used. For not normally distributed values (difference of percentage number of monocytes, difference of absolute number of lymphocytes, difference of absolute number of monocytes and difference of total protein), Mann-Whitney-U-test was used.
3.3.2 Data extracted from video coding of behaviour test:

Both groups of dogs, which were fed with the respective diet were labelled for the statistical analysis. One food group was labelled as food group 1= “round” and the other group as food group 2= “square”. Since it is a double-blind study, we did not know, which group received the test or the control food. The results before receiving the food, we labelled as time 1 and after one year of feeding, was labelled as time 2. Each test with a total of four minutes was divided in two consecutive phases, like it is described above. First phase of two minutes with unblocked toy, we labelled as play mode 1 and the second phase of two minutes with blocked toy we labelled as play mode 2. Each phase was examined of the described parameters manipulate toy and search for food.

To analyse the data of the behaviour test, a value was calculated. To do so, following formula was applied:

Percentage time of manipulation = duration of manipulation time (seconds)*100/ (120 – duration of time searching for food (seconds))

The formula was created to receive the actual percentage of time, the dog manipulated the toy. Percentage time was calculated for both phases of each trial (before and after diet with toy blocked and unblocked).

The values were tested for normality with Kolmogorov-Smirnov-test. Since the calculated values were not normally distributed, all values were transformed with arcussinus for the following analyses. The aim of the transformation was to use parametric tests for the analyses, because of the higher statistical power.

To show an interaction between time (one year of feeding the distributed food), play mode (blocked or unblocked toy) and food group (control and testing group), we used ANOVA¹ and analysed each parameter particularly for differences and different combinations of these parameters (play mode with time, play mode with food group, time with food group, time with play mode and food group).

Graphs were created with estimated marginal means, to illustrate the results. For all analyses we assumed an α=.05.
4. Results

4.1 Inflammation parameters in blood

The difference between pre- and posttest of percentage number of monocytes, absolute number of segmented cells, absolute number of lymphocytes and number of leucocytes was significant (see table 2+3). The monocytes significantly increased over one year (Fig. 2), whereas the absolute number of segmented cells (Fig. 3), absolute number of lymphocytes (Fig. 4) and the number of leucocytes (Fig. 1) significantly decreased over one year.

Table 2:

Wilcoxon-test and Wilcoxon rank test for not normally distributed values (just significant values illustrated)

<table>
<thead>
<tr>
<th>source</th>
<th>Z</th>
<th>Asymp. Sig. (2-tailed)</th>
<th>N</th>
<th>Mean Rank</th>
<th>Sum of Ranks</th>
</tr>
</thead>
<tbody>
<tr>
<td>monocytes_percentage_ad - monocytes_percentage_bd</td>
<td>-3.182</td>
<td>.001</td>
<td>13</td>
<td>20.27</td>
<td>263.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25.43</td>
<td>864.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>segmented_cells_absolute_ad - segmented_cells_absolute_bd</td>
<td>-2.721</td>
<td>.007</td>
<td>34</td>
<td>26.06</td>
<td>886.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>22.60</td>
<td>339.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>leucocytes_absolute_ad - leucocytes_absolute_bd</td>
<td>-3.029</td>
<td>.002</td>
<td>32</td>
<td>28.66</td>
<td>917.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18.12</td>
<td>308.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17</td>
<td>49</td>
<td></td>
</tr>
</tbody>
</table>
Table 3:

Paired samples test (t-test) for normally distributed values (just significant value illustrated)

<table>
<thead>
<tr>
<th></th>
<th>lymphocytes_absolute_bd - lymphocytes_absolute_ad</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>321,85918</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>584,58586</td>
</tr>
<tr>
<td>t</td>
<td>3,854</td>
</tr>
<tr>
<td>df</td>
<td>48</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>.000</td>
</tr>
</tbody>
</table>

Figure 1:

Line chart with error bars +/- 1 standard deviation: decrease of number of leucocytes over one year; x-axis: time (blood sampling before and after diet), y-axis: mean concentration of leukocytes in cells/µl
**Figure 2:**

Line chart with error bars $\pm$ 1 standard deviation: Increase of percentage number of monocytes over one year; x-axis: time (blood sampling before and after diet), y-axis: mean percentage of monocytes.

![Monocytes Line Chart](image)

**Figure 3:**

Line chart with error bars $\pm$ 1 standard deviation: Decrease of absolute number of segmented cells over one year; x-axis: time (blood sampling before and after diet), y-axis: mean concentration of segmented cells in cells/µl.

![Segmented Cells Line Chart](image)
Figure 4:

Line chart with error bars +/- 1 standard deviation: Decrease of number of lymphocytes over one year; x-axis: time (blood sampling before and after diet), y-axis: mean concentration of lymphocytes in cells/µl

_Difference between food groups:_

The results show no significant differences between the control and the experimental group in any of the analysed parameters (see tables 4+5).

**Table 4:**

ANOVA test of between-subject effects (food group), N= 49

<table>
<thead>
<tr>
<th>source</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>percentage of segmented cells</td>
<td>.423</td>
</tr>
<tr>
<td>percentage of lymphocytes</td>
<td>.375</td>
</tr>
<tr>
<td>number of leucocytes</td>
<td>.760</td>
</tr>
</tbody>
</table>
Table 5:

Mann-Whitney test with food group as grouping variable, N=49

<table>
<thead>
<tr>
<th></th>
<th>Mann-Whitney U</th>
<th>Wilcoxon W</th>
<th>Z</th>
<th>Asymp. Sig. (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>percentage of monocytes</td>
<td>216.500</td>
<td>567.500</td>
<td>-1.654</td>
<td>.098</td>
</tr>
<tr>
<td>absolute number of lymphocytes</td>
<td>266.000</td>
<td>617.000</td>
<td>-0.661</td>
<td>.509</td>
</tr>
<tr>
<td>absolute number of monocytes</td>
<td>257.000</td>
<td>608.000</td>
<td>-0.841</td>
<td>.400</td>
</tr>
<tr>
<td>absolute number of segmented cells</td>
<td>270.000</td>
<td>546.000</td>
<td>-0.581</td>
<td>.561</td>
</tr>
<tr>
<td>total protein</td>
<td>243.000</td>
<td>594.000</td>
<td>-0.642</td>
<td>.521</td>
</tr>
</tbody>
</table>

4.2 **Manipulation persistency test**

While we found no significant difference between the experimental and control group and no interactions with play mode (see Table 6 “food group”, “play mode*food group”) and time period (see Table 6 “food group*time”), we found a significant interaction between the percentage animals manipulated the unblocked toy and the blocked toy and whether they were tested before or after receiving the diet (see Table 6 “play mode” and “time”). When analysing the data separately, we found that animals spend slightly less time manipulating the unblocked toy than manipulating the blocked toy before receiving the diet. After receiving the diet for one year, the dogs spent less time manipulating the blocked toy than the unblocked toy (see Fig. 5). Both food groups spent more time manipulating the toy after receiving the respective diet, independently from play mode (see Fig. 6, 7, 8).
Table 6:

ANOVA with calculated variables and interactions

<table>
<thead>
<tr>
<th>source</th>
<th>Type III sum of squares</th>
<th>df</th>
<th>Mean of squares</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>play mode</td>
<td>3,87</td>
<td>1</td>
<td>3,87</td>
<td>8,733</td>
<td>.004</td>
</tr>
<tr>
<td>play mode * food_group</td>
<td>.008</td>
<td>1</td>
<td>.008</td>
<td>.182</td>
<td>.671</td>
</tr>
<tr>
<td>error (play mode)</td>
<td>3,900</td>
<td>88</td>
<td></td>
<td>.044</td>
<td></td>
</tr>
<tr>
<td>time</td>
<td>6,065</td>
<td>1</td>
<td>6,065</td>
<td>46,604</td>
<td>.000</td>
</tr>
<tr>
<td>time * food_group</td>
<td>.033</td>
<td>1</td>
<td>.033</td>
<td>.250</td>
<td>.619</td>
</tr>
<tr>
<td>error (time)</td>
<td>11,453</td>
<td>88</td>
<td></td>
<td>.130</td>
<td></td>
</tr>
<tr>
<td>play mode * time</td>
<td>.661</td>
<td>1</td>
<td>.661</td>
<td>23,712</td>
<td>.000</td>
</tr>
<tr>
<td>play mode * time * food_group</td>
<td>2,281E-5</td>
<td>1</td>
<td>2,281E-5</td>
<td>.001</td>
<td>.977</td>
</tr>
<tr>
<td>error (play mode*time)</td>
<td>2,452</td>
<td>88</td>
<td></td>
<td>.028</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5:

Graph with estimated marginal means of the calculated values from the behavior test (for details see methods) on y-axis and time on x-axis (1= before receiving diet, 2= after receiving diet, time range= one year) Grey line: play mode with unblocked toy; red line: play mode with blocked toy
Figure 6:
Graph with estimated marginal means of calculated values from the behavior test (for details see methods) on y-axis and time on x-axis (1= before receiving diet, 2= after receiving diet, time range= one year)
Grey line: food group round; red line: food group square; just play mode with unblocked toy illustrated

Figure 7:
Graph with estimated marginal means of calculated values on y-axis and time on x-axis (1= before receiving diet, 2= after receiving diet, time range= one year)
Grey line: food group round; red line: food group square; just play mode with blocked toy illustrated
Figure 8:

Graph with estimated marginal means of calculated values on y-axis and time on x-axis (1= before receiving diet, 2= after receiving diet, time range= one year)
Grey line: food group round; red line: food group square)
5. Conclusion and Discussion

5.1 Blood analyses:

In this study we could show a significant decrease of leukocytes in general, in the absolute number of segmented cells and lymphocytes in the blood samples of the tested dogs over one year. The percentage of monocytes increased over one year. The tested food did not influence the tested blood parameters. Our results suggest that age has an increasing effect on the percentage of monocytes. Lawrence et al., 2013 found, that monocyte count and percentage decreased in dogs of 1-6 years of age, only to increase afterwards and then return to the levels of 1-year old dogs or even increase slightly. In a study of Mongillo et al., 2015 the examined blood parameters showed a higher count and percentage of monocytes in cognitively impaired dogs. The authors collected blood samples of 45 physically healthy pet dogs, an almost similar sample size as was collected in the present study (N=49). In regard of monocyte count, the results of Mongillo et al., 2015 and Lawrence et al., 2013, show a reverse outcome, compared to our study. In regard of percentage of monocytes, the studies showed a comparable outcome to our results. Although the percentage of monocytes in our study even increased over one year, the blood sampling stayed above the reference levels on both cases, like described below. Consequently, the level was high throughout the whole year.

Conversely the absolute number of segmented cells and lymphocytes and the number of leucocytes in general decreased in our study over one year. As introduced in the methods section, detailed description of blood test, it is unusual that the inflammation parameters are falling with advanced age, since old dogs tend to contract diseases more easily. We expected a converse effect in ageing dogs.

Additionally, we had to consider the diagnostic reference levels of the examined parameters. The reference level of percentage of monocytes is < 5% of the totality of leucocytes, absolute number of lymphocytes 780-4500/µl, absolute number of segmented cells 3300-11250/µl, leucocytes in general 6000-15000/µl and total protein 6-7,5g/dl. As mentioned above, the dogs in the study are slightly above the reference level of percentage of monocytes (on average 5,24% before diet and 5,81% after one year). It could be assumed, that chronic inflammations or diseases associated with increased numbers of monocytes accumulated in
the tested dogs. However, it is rather unlikely, because the absolute numbers of monocytes are within reference level. Consequently, it can be inferred that the percentage of monocytes just shifted within all leukocyte distribution.

We should rather discuss the possibility of a high incidence of so called “stress leukograms” in the dogs. The latter could be caused by a raise of stress level during the actual blood sampling. In this case the blood shows typical deviations like neutrophilia, monocytosis and/or lymphopenia. The other significantly different blood parameters (absolute number of segmented cells and lymphocytes and the number of leucocytes) are within reference levels and therefore do not give evidence of associated diseases.

In a study by Mukaro et al., 2008, a decreased absolute number of leukocytes and lymphocytes could be detected in humans, which received processed food with a higher dose of omega-3 long-chain polyunsaturated fatty acids. We also used an omega-3 fatty acids enriched diet for the treatment group in our study. The decrease of the leukocytes is related to our results, but nevertheless, we did not find a correlation to the enriched diet.

Another study investigated intracellular redox processes, which are closely related to antioxidant substances, like mentioned in the introduction. The leukocyte populations in the study show an improvement of function and recovery of the redox processes after feeding an enriched diet with Vitamin C, E and other antioxidants (Alvarado et al., 2006). Since intracellular processes were not considered in the present study, this information was lost. It is possible, that the blood cells of the dogs in our study showed different intracellular actions after feeding the enriched diet and this circumstance was not recognised.

As a conclusion and referring to the hypothesis of the study, we did not find any significant results, which could have been caused by the tested food.

5.2 **Manipulation persistency test:**

The dogs in the present study showed a significantly higher level of persistency in the second test after one year, in comparison to the initial test before feeding the diet. Furthermore, we could show an increased persistency with the unblocked toy compared to the blocked toy. For the test dogs it is necessary to persist to finally solve the given problem. Furthermore, behavioural and cognitive flexibility is a very important tool, that leads to success in problem solving tasks (Chow et al., 2016). In the present study we used a two-step task. The first step with the unblocked toy should primarily introduce the tested dogs to the concept of the toy
and lead to success. This first step was necessary to motivate the dogs in an unfamiliar location to perform and to relieve anxieties, but also to compare the persistency afterwards, when they were in a non-solving task. The second step was performed to measure the persistency in a non-solving task, with the blocked toy. Referring to our study, the time (one year in between the tests) has a strong effect on the manipulation time, which is significantly higher in the second test after one year. Thus, the dogs were more persistent in the second than in the first test, independent from play mode (toy unblocked or blocked). It could be speculated, if there is some learning or memory effect, in regards to the function of the toy in the second test and that was the reason why more time was spent manipulating the toy. This would correspond to the theory of Chow et al., 2016, that problem-solving abilities improve in a row of problem-solving tasks. Consequently, the animals can solve the problem quicker. In our study this would mean, that they were able to get more food in a shorter time period and present themselves more motivated. We used different types of toys for pre- and posttest to reduce the memory effect, but it is unclear, whether the examined dogs remembered the general concept of a food toy or whether they got more comfortable with the location and the procedure. This phenomenon is also discussed in Brubaker et al., 2018. Unfamiliar persons and testing conditions normally have an influence on a dog’s performance. The persistence of the dogs increased in the second test, if there was encouragement from the owner in the first test. It is possible, that the dogs in our study felt more secure in the second test, because they got encouragement from the owner in the first test.

However, Milgram et al., 2005, detected an improvement of learning in a discrimination task in old dogs, which received behavioural enrichment and enriched food. In our study, the play mode has a strong effect on the time spent manipulating. With the unblocked toy, dogs spent significantly more time manipulating the toy in comparison to the blocked toy. Success in form of food falling out of the unblocked toy, can be used as an explanation for the higher persistency of the dogs. As seen in the examination of inflammation parameters, the results of the behaviour test did not show a correlation between the examined diet and the persistency in the tested dogs.

For future projects it would be relevant to compare the neurological status before and after an enriched diet and to measure other inflammation parameters in the blood e.g. c-reactive protein, because it is a strong indicator of inflammation, also associated with CCD. It would also be interesting to examine the intracellular processes of redox reactions. Furthermore, it is still unclear whether the enriched food has a stronger effect on cognitive function if combined with behavioural enrichment.
6. Summary

Ageing in dogs is characterized by many behavioral and physiological changes (Larsen, Farcas, 2014). An important factor in several diseases correlated with age, such as the Canine Cognitive Dysfunction Syndrome and arthritis, is oxidative damage caused by free radicals (Dunlap et al., 2005). The function of cells can be severely impaired from oxidative damage of cell membranes however, antioxidants that protect the cells may prevent or slow down the development of neurodegenerative diseases (Dunlap et al., 2005). Previous studies suggest that an antioxidant-enriched diet in combination with behavioural enrichment can slow cognitive decline and the pathological aging of the brain (Cotman et al., 2002; Fahnenstock et al., 2012).

In the present study we aimed to look at the link between an enriched diet and cognitive decline by feeding one group of dogs an enriched diet for one year, whereas the control group received a normal diet. We aimed to see if the diet would show an effect on the performance of dogs in behavioural tests and on the blood parameters. Using a blood test after one year of feeding the diet, we aimed to detect differences in blood parameters between the test and control groups and behavioural changes, that would be measured with a cognitive task in accordance with these changes. We found a significant decrease of leukocytes in general, in the absolute number of segmented cells and lymphocytes in the blood irrespective of the assigned group. The percentage of monocytes increased over one year. However, the behaviour tests showed a significantly higher level of persistency in the second test after one year, in comparison to the initial test before feeding the diet. Furthermore, we could show an increased persistency with the unblocked toy compared to the blocked toy. The results did not show a correlation between the examined diet and the persistency in the tested dogs.
7. Zusammenfassung:


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