Article

Effects of a Nutritional Supplement (DiRelax™) on anxiety in dogs

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Simple Summary: The effects of a nutraceutical product, DiRelax™, were tested by the CBARQ questionnaire and by the impossible task paradigm in a cohort of anxious dogs. The treatment with DiRelax™ showed a positive effect on the dog's performances, with the decrease of the latency of task resolution during the solvable phases of the impossible task test. According to the results from the C-BARQ questionnaire, some of the behaviors appear improved. The results suggested that DiRelax™ can have an ameliorative effect on cognitive performances of anxious dogs.

Abstract: This study aimed to investigate the efficacy of DìRelax™, a nutraceutical formulated to reduce anxiety in dogs. The CBARQ questionnaire, some clinical investigations, and the impossible task test were performed in dogs before and after the treatment. Results showed an ameliorative effect on the performances of treated dogs during the solvable phases, with a significant decrease of the time needed to solve the task. No behavioral difference was found between treated and untreated anxious dogs during the unsolvable phase. According to the results from the C-BARQ questionnaire, some of the behaviors appear improved. In general, this study suggests that DiRelax™ can be safely administered with no adverse effects and can exercise a beneficial effect on anxious dogs by enhancing their cognitive abilities.

Keywords: dog; anxiety; nutraceuticals; impossible task; C-BARQ

1. Introduction

It is well known that inappropriate behaviours could represent a serious problem that menaces the physical and psychological integrity and welfare of the dog but also of the people around it [1-3].

Overall [4] proposed a link between stress and the beginning of anxiety, which is considered an important psychological disorder in dogs [5]. Anxiety is a condition induced when an environmental stimulus is perceived improperly as dangerous or threatening, and it becomes pathological when it is continued or occurs without environmental conditions justifying it [6].

Symptoms of anxiety such as excessive vocalization, destructive behaviour, restlessness, inappropriate defecation and urination, hyper-salivation, and escape attempts appear when dogs perceive that it is about to be left alone and become more obvious when the owner goes out or when it is exposed to unfamiliar persons or situation.

The most frequent anxiety-related effects are separation anxiety, generalized anxiety, aggressiveness, fears, phobias, and obsessive-compulsive disorders. Flannigan and Dodman [7] observed that dogs who live in a home with a single adult human were more predisposed to have separation anxiety compared with dogs from multiple owner homes.

Although several strategies and behavioural therapy approaches could reduce anxiety, in many cases a pharmacological approach is requested. Benzodiazepine in combination with fluoxetine (a selective inhibitor of the serotonin reuptake) is suggested to control signs of anxiety including fear, aggression, and separation-related problems [8].

In addition to pharmacological intervention, natural supplements may also be used and, indeed, their use in veterinary medicine showed a sharp increase in recent years [9]. Generally, supplements have potentially fewer side effects, and their use is not contraindicated with most drugs or disease processes (e.g., renal, hepatic, or cardiac dysfunction) in dogs. DìRelax™ is a commercial nutraceutical that includes 3a700 Vitamin E (RRR-alfa-tocopherol acetate), Vitamin B6 (Pyridoxine hydrochloride), and several substances that have been reported as acting analgesic and sedative properties. In particular, Escholtzia [10, 11], Hops [12], Whitania [13] and Passiflora [14] share anxiolytic properties while Krill oil, rich in polyunsaturated fatty acids (n-3 PUFAs), is involved in maintaining a healthy brain, enhancing brain functions such as reactivity, attention and cognitive performance [15] including memory and learning [16].

Apart from the anamnesis, there are several ways to measure anxiety in dogs. One of these is the use of the Canine Behavioural Assessment and Research Questionnaire (C-BARQ), originally designed to provide dog owners and professionals with standardized evaluations of canine temperament and behaviour. The C-BARQ is particularly useful for assessing the frequency and severity of problematic behaviours, such as fear and anxiety [17]. On the other hand, some validated behavioural tests allow to measure stressful responses in a strictly way. One of these is the impossible task paradigm providing insight into the decision-making processes in the realm of expectancy violation [18]. This experimental paradigm has already been used several times in dogs for studying canine social interactions with people [19-21]. However, Passalacqua et al. [22] demonstrated that anxiety could impair the performance of dogs and induce different behavioural patterns.

The purpose of this research was to investigate the effectiveness of DiRelax™ (Dynamopet, Verona, Italy) in improving anxiety in dogs. To this aim, the C-BARQ questionnaire and the impossible task paradigm were used. Haematological and biochemical analyses were performed to assess possible adverse effects.

2. Materials and Methods

Dogs' owners gave their consent to house the animals into adequate facilities, to administer their dogs the DìRelax™ treatment, and to participate in the C-BARQ questionnaire before and after the treatment. DìRelax™ (Dynamopet, Verona, Italy) is mixture preparation of Krill Oil (3%), Vitamin E (RRR-alfa-tocoferil acetate) 24 mg, Vitamin B6 (pyridoxine hydrochloride) 100 mg/kg, Escholtzia (*Escholzia californica Cham.*) 55.2mg/kg, Hops (*Humulus lupulus L.*) 55.2mg/kg, Whitania (*Whitania somnifera L. Dunal*) 55.2 mg/kg, Passiflora (*Passiflora incarnata L.*) 8 mg/kg. The experiment, including owners' informed consent, housing, treatment, and questionnaire, was approved by the Ethical Animal Care and Use Committee of the University of Naples Federico II, (OPBA, CSV, University of Naples Federico II, PG/2021/0123753) following local and national law, regulations, and guidelines. This research avoided distress to the animals using proper clinical management.

2.1 Animals

Twenty-one dogs diagnosed with anxiety disorders were involved in the study. Participating dogs sample included 10 females (5 spayed) and 11 males (2 neutered) of different breeds, with the age ranging between 1 and 15 years (mean age ± SD: 5.95 ± 4.13 years; mean weight ± SD: 13.59 ± 8.77 kg), recruited from the client-owned referral

population of the Veterinary Teaching Hospital, Department of Veterinary Medicine and Animal Productions (University of Naples Federico II). To be included in the trial, dogs underwent a clinical and neurological examination and blood analysis including complete blood count, serum biochemistry, and thyroid profiles (TSH, total T4, and free T4) to assess the health status and to exclude neurological diseases and endocrinopathies that could influence dog's behavior. Dogs were randomly assigned to two groups, according to the type of treatment they had to undergo (i.e., supplement or placebo). It was projected to balance the number of samples in the groups. The final sample included thirteen dogs (5.46 ± 3.84 years) in the supplemented group (SU) and eight dogs (7.13 ± 4.19 years) in the placebo group (PL). No difference was found between the two groups for the mean age (P = 0.32). The treatment (i.e., supplement or placebo) had a total duration of 30 days. DiRelax™ Medium was orally administered to the supplement group at the dose of 0.5 mg/kg as indicated by the manufacturer.

2.2 C-BARQ questionnaire

The C-BARQ consists of several miscellaneous items as well as 14 different categories of behaviour—stranger-directed aggression, owner-directed aggression, dog-directed aggression, stranger-directed fear, non-social fear, dog-directed fear, separation-related behaviour, attachment and attention-seeking, obedience, trainability, chasing, excitability, touch sensitivity, energy level, and dog rivalry. In this research a questionnaire from the Veterinary Teaching Hospital of the University of Napoli based on the C-BARQ proposed by Serpell [23] was adopted.

2.3 Blood analysis

Blood samples were collected from each dog after 12 h fasting before and after the treatment from the jugular vein into tubes with and without ethylenediaminetetraacetic acid (K3EDTA) and immediately transported to the laboratory at the Department of Veterinary Medicine and Animal Productions, University of Naples Federico II. A complete blood count (CBC) was performed using a semi-automatic cell counter (Genius S, SEAC Radom Group, Calenzano, Italy). In addition, May–Grünwald–Giemsa-stained blood smears were evaluated for additional information and eventual evidence of platelet clumping. Serum samples, obtained by centrifugation at 1200×g for 15 minutes, were divided into aliquots and frozen at −80 °C. Blood chemistry analyses on serum aliquots were performed by an automatic biochemical analyser (Autolab, AMS Corporation, Rome, Italy) using reagents from Spinreact (Girona, Spain) to determine: haematocrit (HCT), haemoglobin (HB), red blood cells (RBC), white blood cells (WBC), platelets (PLT), total proteins (TP), urea (UREA), creatinine (CREA), glucose (GLU), alanine aminotransferase (ALT), total bilirubin (BIL), alkaline phosphatase (ALP), cholesterol (CHOL) and triglycerides (TRI). An AIA-360 Automated Immunoassay Analyzer and reagents from Tosoh (San Francisco, CA, USA) were used to assay TT4 and fT4, while TSH was assayed by the Immulite® 2000 Canine (Siemens Medical Solution Diagnostics, Los Angeles, CA, USA).

2.4 Impossible task test

Dogs were subjected to the impossible task test, before (round 1) and after (round 2) the treatment period. All tests were conducted at the Department of Veterinary Medicine and Animal Productions (University of Naples Federico II).

2.4.1 Experimental setting

The tests were conducted in an empty room of about 4 x 3 m, equipped with 2 cameras, placed in two different corners of the room, and the experimental apparatus (Fig. 1).
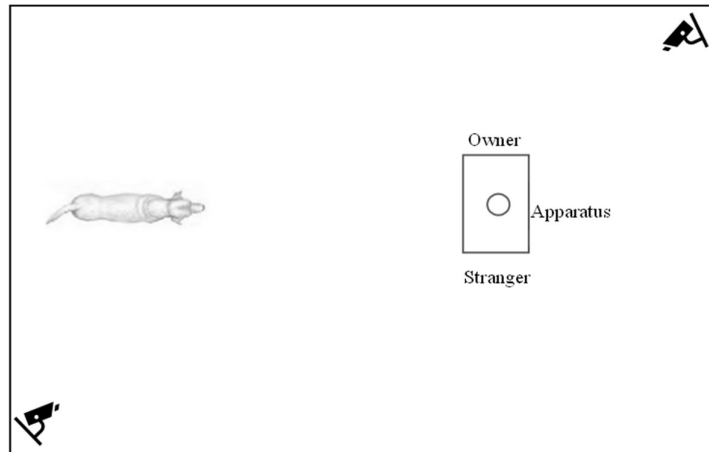
Figure 1. The figure shows the experimental room equipped with two cameras (in the corners) and the apparatus (in front of the dog). The owner and a stranger were placed on the two sides of the apparatus.

The experimental apparatus consisted of a glass feed container, placed on a rectangular wooden platform. The lid of the feed container was fixed on the platform, whereas the container was placed upside down on the tracks of the lid during the solvable task phase and was locked during the unsolvable task phase (Fig. 2).
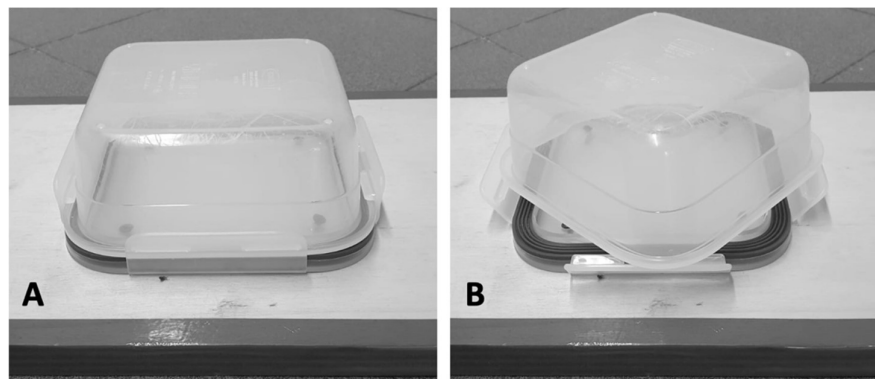
Figure 2. A: position of feed container during the solvable phase. B: locked food container during the unsolvable phase.

The wooden platform was fixed on the floor by double-sided adhesive tape. All parts of the experimental apparatus were washed with a slightly perfumed and non-toxic disinfectant after each test. The owners were asked not to feed their dogs during the four hours before testing to enhance the interest of the dogs toward the feed used as bait during the test. The feed palatability was ascertained by administering dog small bits of feed before the test.

2.4.2 Procedure

The test consisted of three solvable trials in which the dogs could obtain the feed by manipulating the container followed by an unsolvable trial, in which the container was fixed onto the wooden board. In all the trials, the owner and an unfamiliar person were present and maintained the identical position standing at either side of and one step back (30 cm) from the apparatus. All participants were previously instructed by the experimenter to look straight ahead and ignored the dog (e.g., neither spoke, looked at, or touched the dog) during the test. Two different researchers were involved in the dog's management during the test: one researcher held the dog and the other placed the feed below the glass

container during solvable trials, ensuring that the dog observed the procedure and blocked the apparatus in the unsolvable trial. At the beginning of the unsolvable trial, both researchers left the room. The duration of the unsolvable trial was 60 s.

2.4.3 Data collection

The impossible task tests were video recorded and analysed with the Solomon Coder beta® 14.05.19 (ELTE TTK, Budapest, Hungary). For the solvable trials, the latency of the resolution (i.e., the time in seconds from the beginning of the trial until the resolution) was observed. The behaviours of dogs during the unsolvable trial were coded according to a specific ethogram (Table 1). Stress behaviours (i.e., yawning, vocalization, licking, scroll-ing, and scratching) were also recorded. All data were collected in frequency (number of occurrences), duration (time in seconds), and latency.

Table 1. Ethogram adopted for the analysis of dog's behaviour.

Target	Behaviour	Description
Apparatus, Owner, Stranger	Gaze	Look at the target from a stationary position
	Interact	Physical interaction with the target
	Towards	Go toward the target

A second observer collected the same data for the inter-observer reliability and there was no difference between the two observers.

2.5 Statistics

The CBARQ data were statistically evaluated using the Wilcoxon non-parametric test.

For blood analysis, the effects of sampling times and between groups were analyzed by ANOVA according to the following model:

$$y_{ijk} = \mu + G_i + S_j + \varepsilon_{ij}$$

where y is the dependent variable, μ is the mean, G is the group effect (i = SU, PL), S is the sampling effect (j = 0, 30) and ε is the error effect.

As regards the impossible task paradigm, Due to the no normal distribution of the data, non-parametric statistics were adopted. Comparisons were made between the two experimental groups (i.e., supplement vs. placebo) using the Mann-Whitney U-test, and, in each group, between the data obtained before and after treatment (i.e., round 1 vs round 2) using the Wilcoxon signed-rank test. All analyses were performed with GraphPad Prism® software 5.01.

3. Results

3.1 Clinical scores

As depicted in table 2, only 6 over 42 questions from the C-BARQ questionnaire showed significant differences between groups after the treatment with DiRelax™.

Table 2 – Improved behaviours according to the C-BARQ questionnaire between groups.

Improved behaviour	P
When his/her food is taken away by a household member	*0.004*
When approached while eating by another (familiar) household dog	*0.050*
When barked, growled, or lunged at by an unfamiliar dog	*0.023*
When groomed or bathed by a household member	*0.010*

|	
Tends to follow you (or other members of the household) about the house, from room to room	*0.024*
Urinates when left alone at night, or during the daytime.	*0.000*

3.2 Blood analysis

Regarding haematology and blood biochemistry before and after the treatment with DiRelax™, no statistical difference was seen between the groups, and no time effect was recorded within the groups (data not shown).

3.2 Impossible task test

Comparing the latencies of task resolution during the solvable phase between round 1 and round 2, it emerged that there are no significant differences in the times recorded for the dogs of the placebo group, while a significant decrease was observed in the dogs of the supplement group (Wilcoxon test, W = 72, P = 0.0024; Fig. 3). No significant difference was recorded in the behaviours expressed by the dogs of both groups (supplement and placebo) during the unsolvable phase, neither towards the owner or the stranger nor in the interaction with the experimental apparatus. The two groups behaved similarly in both round 1 and round 2.

Figure 3. The latency of task resolution during the solvable trials in placebo and supplement (DiRelax) groups. Horizontal lines inside boxes: medians; boxes: from 25% to 75% quartiles; thin vertical lines: minimum and maximum values. Black boxes: Round 1; Gray boxes: Round 2. P-value: *P = 0.024
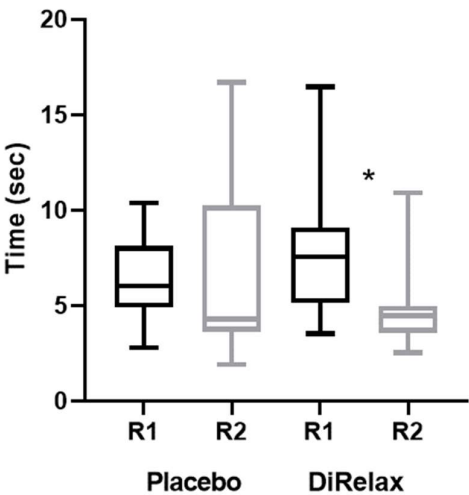
4. Discussion

Dogs are the oldest domesticated animals establishing over the years a cooperative working link with humans, sharing both foraging mode and a similar social system. They represent currently family members [24, 25]. This intense and singular relationship reflects the way dogs and humans communicate with each other in the right way and failing this goal negatively affects the common welfare [26, 27]. Of course, anxiety negatively impacts the welfare of dogs which in turn involves their human family. Therefore, any intervention aimed to reduce anxious responses should be welcomed. In recent years, the use of natural remedies in alternative or together with the recommended drug therapy has been widely proposed in western countries. Different herbal remedies are often used together to achieve several beneficial effects. Indeed, some authors suggested that a synergic effect may occur using different substances both of natural and/or industrial origin. Moreover, many attempts to identify the active components of herbal remedies have concluded that in general no one component is responsible for the therapeutic capacity, but rather a

complex and intricate interaction of various herbs may result in therapeutic efficacy [28, 29]. The results of the current study seem to underline this aspect showing an ameliorative effect during the solvable part of the impossible task paradigm. Particularly, after the treatment with the nutraceutical DiRelax™, the dogs in our study showed a significant decrease in the latency of the task resolution during the solvable trials. In the previous study in the impossible task paradigm [22], anxious dogs showed a lower ability to solve the task, greater dependence on the human, as well as avoidance behaviors towards the task, compared to non-anxious dogs. The significant difference found in the ability to resolve during the solvable task could indicates a relationship between cognitive performance and problems due to anxiety disorders, as demonstrated in other species [30, 31]. The intake of the feed supplement may have led to improvements in the anxiety allowing dogs to reduce the time to solve the task and to reach the reward earlier. On the other hand, no differences emerged when the feed reward was not attainable. This suggests that, although the supplement was effective in improving dogs' cognitive performance, the improvement was not strong enough to afford the absence of reward. Together with our testing procedure an ameliorative effect was also perceived by the owners. Indeed, the analysis of the questionnaire score showed significant favorable responses to the treatment in 6 questions. Moreover, a positive trend was observed for the remaining questions. Importantly, the lack of differences in hematology and biochemical chemistry shows that DiRelax™ was well tolerated and can be safely administered to dogs using the protocol recommended by the manufacturer.

5. Conclusions

Taken together, the results suggest that DìRelax™ possess beneficial effects in improving anxiety in dogs with no adverse effects on learning or physiology. However, further studies using higher doses and or longer administration should be performed to explore a possible higher efficacy and long-lasting effects of this natural supplement.

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