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# Supplementation with nutraceutical Calmina® (BuonaPET) improves anxiety status in shelter dogs: a pilot study

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*Abstract*: Shelter dogs may experience long-lasting stressful living conditions. As a result, veterinarians should use every tactic at their disposal to enhance these animals' comfort and well-being. In the present study, we enrolled dogs with an anxious profile, administered with a food supplement, based on fish protein hydrolysate (PEPTIDYSS®), L-theanine, Griffonia simplicifolia, Lactobacillus Helveticus, Vitamin B6, Zinc and Magnesium. At the end of the 60-day treatment period, we documented for the first time a significant improvement in anxiety-based behaviors, evaluated by means an increase of problem-solving skills and interspecific sociability. Overall, our preliminary data shed light on the tantalizing role of Calmina® in animal shelter, with the purpose of improving the adoption chance for dogs, who experience emotional discomforts.

*Key Words*: dog behavior; stress; serotonin; cortisol; human-dog relationship; emotions

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## Introduction

The quality of life at shelters is one of the primary issues surrounding the human-dog connection today, since dogs often live in conditions that do not respect their ethological needs, such as social interaction, exploration, physical activity and mental stimulation (d'Angelo et al., 2022). This can lead to stressful environments and experiencing negative emotions such as anxiety, fear, pain, and isolation; the effect of such an environment on dogs can also differ depending on the individual's past experiences and personality as well as kennel management (Corsetti et al., 2018; Iacopini & Gazzano, 2023). Stress represents a physiological response downstream the activation of the hypothalamic-pituitary-adrenal (HPA) axis which, in turn, regulates levels of glucocorticoid hormones, thus trying to adapt and keep on balance the neuroendocrine and behavioral system. In this respect, during stressful situations, the HPA axis causes the activation of the 'fight-or-flight' response (e.g. increased alertness and expression of stress behaviour) and the release of glucocorticoid hormones, including cortisol (Pariante et al., 2008). If stress fails to be addressed, it can result in serious health issues like anxiety, depression, burnout, and sleep disturbances (Kellendonk et al., 2002). Such detrimental effects on the emotional state becomes even worse in a time-dependent manner (Wells et al., 2002; Hiby et al., 2006). Together with glucocorticoid hormones, also serotonin (5-HT) is involved in the regulation of emotional state: is a neurotransmitter produced from tryptophan and is believed to be important in the aetiology and treatment of most behavioral disorders in dogs. Indeed, previous studies have reported that serotonin influences mood, cognition, and behavior, such as aggression, impulsivity, food selection, stimulation, sexual behavior, pain response, and emotional expressions (Ruddick et al., 2006; Cakiroglu et

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al., 2007; Berger et al., 2009; Bochis et al., 2022). Therefore, early intervention is needed to cope with the emotional alterations underlying the behavioral problems of dogs housed in kennels, thereby improving the chances of adoption (Gazzano et al., 2023). Growing evidence highlighted the importance of nutraceuticals based on alpha-casozepine, l-theanine, tryptophan, cannabidiol, cannabis, essential oil, melatonin and probiotics, in the treatment of behavioral disorders in dogs, since they have anxiolytic and antistress potentials, thus being regarded as an alternative to the conventional therapy (Sechi et al., 2017; Orlando, 2018; Tynes & Landsberg, 2021; Cannas et al., 2021; Ağan et al., 2022; Yu-Min et al., 2022; Sacchettino et al., 2023a). In addition, according to previous clinical cases, the administration of Calmina® in family dogs seems to reduce anxiety, stress and facilitate rehabilitation therapy in treating behavioral disorders (Sacchettino et al., 2023b). With this in mind, we sought to investigate in the present pilot study the potential role of daily administration of a Calmina® (BuonaPET) supplement in the anxiety management of nine mix-breed dogs, sheltered in an Italian kennel near Naples. This compound is enriched with different active ingredients: a protein hydrolysate from the by-products of sardine processing (PEPTIDYSS®), Zinc, Vitamin B6, L-theanine, Griffonia simplicifolia, Lactobacillus helveticus HA-122, and Magnesium, supplements that help promote the animal's state of calm and relaxation (Rahimi Niyyat et al., 2018; Araujoa et al., 2010; Titeaux et al., 2021; Yu-min et al., 2022).

## Materials and Methods

### *Participants*

All experimental protocols were approved by the Scientific Ethic Committee for Animal Experimentation on May 4th, 2023 (Reference number: PG/2023/0051881). The initial sampling involved 12 dogs. They were mixed-breed, both males (n = 6) and females (n = 6), all neutered, medium to large in size, according to the classification reported by Wilding (2018). Inclusion criteria: age two to eight years old, same commercial diet, time spent at least 12 months in shelter, and anxiety (value between 17-35) resulting from Evaluation of a Dog's Emotional Disorder (EDED Scale). As an exclusion criteria: the presence of organic and infectious diseases, and previous or ongoing behavioral rehabilitation (see Table 1).

| Dogs | Age(years) | Gender | EDED value |
|---|---|---|---|
| **Chicco** | 6 | M | 17 |
| **Serena** | 8 | F | 19 |
| **Blanco** | 6 | M | 17 |
| **Teo** | 6 | M | 19 |
| **Lady** | 8 | F | 24 |
| **Bella** | 6 | F | 17 |
| **Dino** | 6 | M | 18 |
| **Kocca** | 6 | F | 21 |
| **Sasà** | 2 | M | 17 |
| **Pupa** | 8 | F | 21 |
| **Spino** | 2 | M | 18 |
| **Sofy** | 2 | F | 21 |

**Table 1.** Characteristics of shelter dogs involved in the pilot study. The dogs involved were between two and eight years of age, fed the same commercial diet, had an anxious behavioural pattern resulting from the EDED (Evaluation of a Dog's Emotional Disorder) scale, and had to stay in the shelter for at least 12 months. All dogs were neutered. They had not undergone any previous or ongoing behavioural rehabilitation.

They were selected from the 80 guests of the Municipal Shelter of Pomigliano d'Arco (Naples), where they were housed after being caught on the territory by the local health authority, in order to limit the stray phenomenon. The structure of the kennel is organized in multiple boxes , based on intraspecific compatibility, to meet the social needs of the canine species. There is an open space as a walking area, in front of the boxes, where dogs could have a walk and interact with each other and with people.

The anxiety status was diagnosed by a veterinary behaviorist through a behavioral examination and using the EDED Scale of Pageat [as reported in d'Angelo et al., 2022], which allows the classification of dogs' behavior according to the presence/absence of centripetal and centrifugal behavior, and the expression of homeostasis or emotional disturbances. Social interactions, the ability to explore, and aggressiveness are centrifugal activities, whereas feeding, drinking, self-directed behavior, and sleep are centripetal activities. For each behavior considered, a specific score was attributed to each subject. Each dog then obtained a total score, which indicated its general emotional state. The dogs enrolled had a score from 17 to 24, corresponding to an anxious state, in particular in the presence of new stimuli, new people and/or loud noises. The animals underwent a complete blood test, which also included the evaluation of thyroid function, and protein electrophoresis, to rule out any co-morbidities, potentially related to behavioral issues (Camps et al., 2019; Amat et al., 2023). Three of them (Lady, Spino, Dino) showed organic pathologies; therefore, from an initial population of 12 animals, we administered the supplement to 9 healthy dog.

### *Procedures*

The research was structured over the 2-month period, as follows:

- dogs evaluations: behavioral and biochemical examinations, cortisol and serotonin analysis;
- daily administration of the nutraceutical Calmina® in dogs enrolled, according to the dosage/weight listed on the package, for 60 days ($T_0$);
- on the 60th day, behavioral follow-up by veterinarian expert in animal behavior and new blood sampling for cortisol and serotonin analysis ($T_{60}$).

### *Behavioral assesment*

To assess behavioral changes in dogs assuming Calmina®, the veterinary behaviorist tested the nine enrolled dogs at $T_0$ and $T_{60}$ using a modified temperament test (Valsecchi et al., 2011), by observation directly lasting 5 minutes per dog, between 8.30 and 10.30 a.m. before eating. The veterinary behaviorist (tester) who performed the behavioral assessment did not participate in any sampling collections. Behavioral assesment covered 5 behavioral domains: Approaching the kennel, Entering the kennel, Interspecific interaction (physical contact), the "come" command, Problem solving. Higher scores corresponded to a friendlier and more relaxed behaviour of the animal. For a description of the 5 domains, see Supplementary Materials.

### *Sample Collection and Cortisol- Serotonin Measurement in Dogs*

Blood sampling was performed between 8:30 am and 10:30 am before eating, as part of the health monitoring procedures routinely carried out by the kennel. A small patch of hair was shaved from the thoracic limb and topical anaesthesia [Eutectic Mixture of Local Anaesthetics (EMLA™) cream] applied to the area before collection of a 5 ml blood sample from the saphenous vein.

After the sampling, the blood samples were placed in a polystyrene container with ice and transported to the laboratory, where they were promptly centrifuged at 3000 rpm in a centrifuge for 10 min to obtain the serum for biochemistry analysis, cortisol and serotonin determination.

The samples obtained were stored at -20° C and then proceeded. Cortisol and serotonin levels were determined by immunoassay using the commercially available cortisol and serotonin ST/5-HT (5-hydroxytryptamine) canine kit (FineTest, Wuhan China), according to the manufacturer's indications.

### *Statistical Analysis*

Data were analysed through paired Student t-test in GraphPad Prism 10 software (La Jolla, California). Results were considered statistically significant for $p < 0.05$.

## Results

Here, we evaluated the serum levels of cortisol and serotonin in the dogs supplemented for two months with Calmina®: our data showed no main effect of the nutraceutical ($T_{60}$), when compared to the untreated ($T_0$) condition ($p > 0.05$, paired Student's t test), as reported in figure 1.

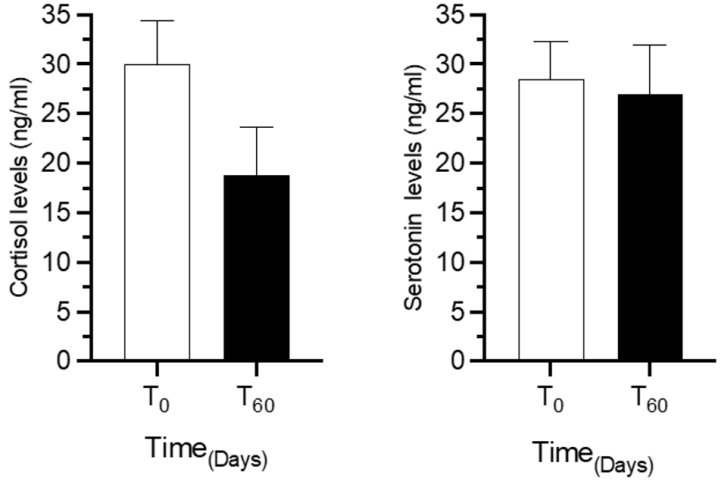


**Figure 1.** Effect Calmina® supplementation on the serum cortisol and serotonin levels in dogs. Cortisol and serotonin levels (ng/ml) following two-month Calmina® treatment. $p > 0.05$, compared to $T_0$ group (paired Student's t test). All values are expressed as mean ± SEM.

Then, to assess behavioral changes in dogs assuming Calmina®, we analyzed the behavioral observation score resulting in the modified temperament test at $T_0$ and $T_{60}$, on domains:

1) Approaching the kennel
2) Entering the kennel
3) Interspecific interaction (physical contact)
4) The "come" command
5) Problem solving.

The statistics were made by paired Student t-test. Results were considered statistically significant for $p < 0.05$. The data of these behavioral observations allow us to understand how sociability and the ability to solve a problem increased (Figure 2).

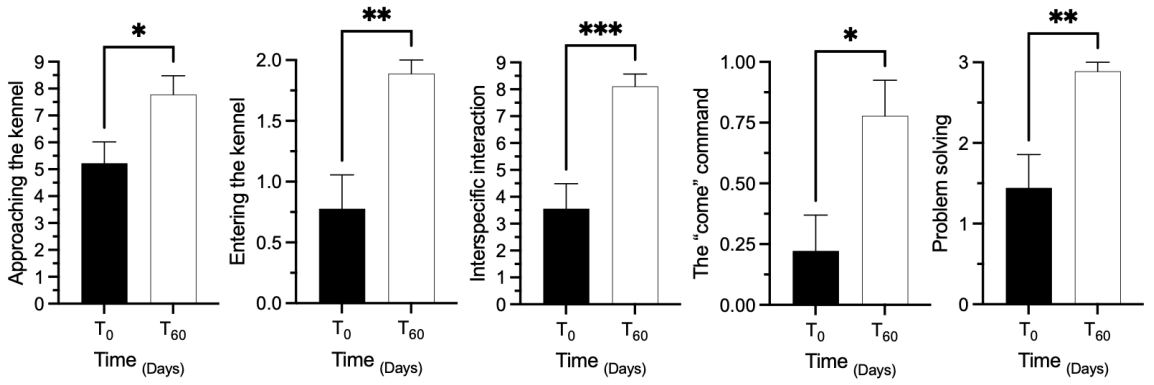


**Figure 2.** Behavioral observation score. Behavioral changes in dogs assuming Calmina® at modified temperament test performed by the behaviorist at $T_0$ and $T_{60}$. *, **, ***: $p < 0.05$, 0.01, 0.001, respectively; paired Student t-test.

## Discussion

The results of our pilot study allowed us to observe an improvement in anxious behaviors in dogs treated with Calmina® for the 60-day supplementation, although serum cortisol and serotonin levels did not change significantly.

In Italy, there are several laws on stray dogs, including the regional regulation (Regional Law 11 April 2019, n. 3.) that obliges the shelter to employ the figure of the dog trainer, since consider that a well-planned and tailored training programme can increase emotional and cognitive abilities in dogs, and this represent a crucial factor in restoring a better human-dog relationship (d'Angelo et al., 2022). However, when dogs have altered levels of anxiety, their abilities to learn news skills and behavior, or experience positive emotions are impaired, making the behavioral rehabilitation program more complicated (Sacchettino et al., 2023). Shelter dogs frequently experience negative emotions due to living in socially unsatisfactory and spatially restricted environments; such conditions generally result in increased arousal, intraspecific issues and instability in dogs, thus making them less suitable for adoption (Breeda et al., 1999; d'Ingeo et al., 2021). In our results, the dogs improved on sociability, evaluated by the domains "Approaching the kennel", "Entering the kennel", "Interspecific interaction (physical contact)" and the "Come" command, showing themselves more prone to social interactions. Dogs' adoptability in shelters could be enhanced by this increased social friendliness, on account of the adopters believe that animals with adequate social behavior are more approachable and less aggressive (Wright et al., 2007). Despite the lack of information on the influence of social interaction on adoption success, dogs that typically act withdrawn, antisocial, or fearful have a lower chance of being adopted (Tuber et al., 1999; Protopopova et al., 2014). Moreover, the reduction of anxiety should also be considered from a canine welfare and life impact perspective; in fact, Dreschel (2010) showed that dogs with extreme fear directed by the stranger hesitated in a shorter lifespan of six months than dogs without fear directed by the stranger. In addition, fearful dogs may show more discomfort in a hospital situation where they are exposed to and handled by strangers, negatively impacting their physical and emotional health (Dreschel et al., 2010). Therefore, all canine therapeutic interventions – including those based on nutraceuticals – could be useful to improve the level of interspecific social interaction in dogs housed in kennels. Our dogs have significantly improved in the 'Problem-solving' domain, demonstrating their ability to stay on a task by using their olfactory intelligence. In our view, this improvement is related to a reduction of the anxiety's state, as this emotional discomfort in dogs can manifest in various ways, such as decrease of appetite and of olfactory – exploratory activity, hypervigilance, avoidance, hiding, escape attempts (Gazzano et al., 2020; Landsberg, 2023). In line with this, previous research

indicated that intermediate degrees of arousal are essential for optimal performance; conversely, too low or high arousal levels (as in anxiety status) can adversely affect and hinder performance (Mills, 2010; Bray et al., 2015). Furthermore, in recent study sniffing is potentially defined as an explicit cognitive behavior (Kokocinska-Kusiak et al., 2021), which requires concentration and an intermediate level of arousal. It's no surprise, since the anxiety and the concentration are negatively related: in human medicine, anxiety symptoms in adolescents are linked to poor concentration, influencing on their school performance over time (Leigh et al., 2021). Even in dogs, as shown by d'Angelo and collaborators in (2022) the ability to remain concentrate on olfactory research is related to the state of emotional activation (arousal); therefore, the improvement of olfactory behavior could depend on reducing anxiety levels and on achieving an appropriate emotional level for the learning process.

Regarding serum cortisol levels, our research shows a trend – not statistically significant – of decreasing levels in all enrolled dogs. It is possible that nutraceuticals may have contributed to reduced cortisol levels and improved stress resilience in dogs, as reported by Sechi and colleguas in 2017. In addition, a further explanation is that even though blood cortisol measurement is a very precise method of stress assessment, the invasive nature of sampling could lead to temporary spikes in cortisol (Hanna et al., 2019; Marza et al., 2024), which Calmina® supplementation may not have significantly prevented. Furthermore, environmental stress may have played a role in sampling: the blood samples were taken at the kennel's veterinary clinic, place that was not conducive to the emotional comfort of dogs (Gazzano et al., 2025). Lastly, the small number of dogs involved may not have been sufficient for a statistically significant change in blood cortisol levels.

Regarding serotonin levels, despite the observed improvements in behaviors, no significant change in canine serum was observed. Previous research by De Napoli and Dodman (2000) reported an improvement in the aggressiveness of dogs with tryptophan supplementation, a precursor of serotonin. Although Calmina® is enriched in griffonia as a source of tryptophan, our results do not confirm a significant variation in serotonin levels, in the canine state of anxiety. One possible explanation is that we investigated a different disorder (aggression vs anxiety), and this would explain the discordant results, since other neurotransmitter systems – besides the serotonergic system – may be involved in anxiety (i.e. GABAergic System). Nevertheless, also Riggio and colleagues observed no association between serum levels of tryptophan and serotonin in their study on shelter dogs (Riggio et al., 2021). Whereas the blood-brain barrier controls the flow of serotonin in an opposite way to that of tryptophan – from peripheral circulation to central circulation – it could help explain the negative results. Despite the continuing desire to try to draw a link between behaviour, peripheral serotonin level and tryptophan, future research is needed to explain their mechanism and correlation. According to these findings, behavioral assessment could be a more accurate way to measure canine stress than just relying on hormonal or physiological parameters. In addition, it is important to consider the personality of dogs with behavioral dysfunction so that an individualized rehabilitation plan can be implemented, taking into account context and relationships, together with nutrition, gut-brain axis and reproductive status (Tynes & Landsberg, 2021; Landsberg, 2023; Ogi et al., 2024; Sacchettino et al., 2025).

## Conclusions

The results of this pilot study showed that supplementation with the nutraceutical Calmina® (BuonaPET) in shelter dogs improved anxiety-related behaviors and sociability, although serum cortisol and serotonin levels did not show statistically significant changes. It's worth noting that there were no behavioral programs employed during the supplementation period, which highlights the effects of the nutraceutical without any additional variables. Supplementing nutraceuticals can be beneficial in protecting the emotional well-being of dogs in kennels and increasing their sociability. Further research is recommended to investigate the role of nutraceuticals on anxiety behaviors in sheltered dogs and the neurotransmitter pathways targeted by nutraceuticals.

## Limitations

Despite the encouraging behavioral results of this pilot study, it is plausible that a few limitations might have influenced the results obtained. To begin with, we recognize that our study involved a small number of patients, due to the tightening factors we have chosen as inclusion criteria in the recruitment and in the analyses performed in the kennel's environment; indeed, we decided to exclude dogs that had signs of organic or infectious disease, which could have altered the results. In addition, this pilot study covered two months of supplementation: this short interval may not have been sufficient to induce neurotransmitter changes. In conclusion, the lack of a control group without treatment, as we evaluated variations within the same sample group. Further studies are needed to investigate the relationship between dysfunctional behaviour and nutraceuticals in kennelled dogs. The effect of nutraceuticals on behavioural modification should also be investigated in conjunction with the rehabilitation programme.

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## L'integrazione con il nutraceutico Calmina® (BuonaPET) migliora lo stato d'ansia nei cani dei rifugi: uno studio pilota

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[†] *Questi autori hanno contribuito in egual misura a questo lavoro e condividono la paternità*

*Sintesi*

I cani dei rifugi possono vivere in condizioni di stress prolungate. Di conseguenza, i veterinari dovrebbero utilizzare ogni strategia a loro disposizione per migliorare il comfort e il benessere di questi animali. Nel presente studio, abbiamo arruolato cani con un profilo ansioso, ai quali è stato somministrato un integratore alimentare a base di idrolizzato proteico di pesce (PEPTIDYSS®), L-teanina, Griffonia simplicifolia, Lactobacillus Helveticus, Vitamina B6, Zinco e Magnesio. Al termine del periodo di trattamento di 60 giorni, abbiamo documentato per la prima volta un significativo miglioramento dei comportamenti ansiosi, valutato attraverso un aumento delle capacità di problem-solving e della socialità interspecifica. Nel complesso, i nostri dati preliminari hanno fatto luce sul ruolo allettante di Calmina® nei rifugi per animali, con l'obiettivo di migliorare le possibilità di adozione per i cani che sperimentano disagio emotivo.

## Supplementary Material

Behavioral assesment covered 5 behavioral domains, described as follows:

1) Approaching the kennel: the observer quietly approaches the kennel with a neutral posture, avoiding direct eye contact, and stops a few centimeters from the fence. Postures and behaviors were labeled for simplicity as: friendly (tail wagging, nonaggressive barking, exuberant, and/or calm approach seeking contact with the observer), neutral (i.e., the dog holds still, showing neither threatening nor friendly behaviors), fearful (a crouched posture, including ears, and tails held low, avoiding eye gaze, shaking, whimpering), or threatening (barking, growling, lunging towards the mesh, stiff posture, piloerection, etc.). This behavior was observed for 3 minutes.

*Score*

Friendly and calm dog, approaching the tester asking for contact = 9;
Friendly dog, still, inside the kennel, not asking for contact = 8
Approaching exuberant and excited dog, hyperactive = 7
Neutral dog, calmly approaching the tester = 6
Neutral and still dog, only looking at the tester or barking = 5
Neutral dog avoiding contact, moving away from the tester = 4
Fearful dog, approaching the tester in low posture = 3
Fearful dog, still, in the center of the kennel= 2
Fearful dog avoiding the tester, moving away, and/or hiding in its shed/hut =1
Aggressive and threatening dog = 0

2)Entering the kennel: the observer walks into the kennel and closes the door. For 30 seconds the observer stands still, arms held loosely along the side, ignoring the dog completely, taking care not to cross its' eye gaze.

*Score*

The dog approaches the tester = 2
The dog stands still = 1
The dog goes away from the tester = 0

3) Interspecific interaction (physical contact): the observer stands still in the kennel, bends forward slightly, calling the dog in a gentle, relaxed manner. If there is no response, the observer may hold his/her hand out toward the dog, palm-side up, and even seek physical contact. The dog's behavior in the subsequent 30 seconds is observed.

*Score*

Friendly and calm dog, approaching the tester asking for contact = 9;
Friendly dog, still, inside the kennel, not asking for contact = 8
Approaching exuberant and excited dog, hyperactive = 7
Neutral dog, calmly approaching the tester = 6
Neutral and still dog, only looking at the tester or barking = 5
Neutral dog avoiding contact, moving away from the tester = 4
Fearful dog, approaching the tester in low posture = 3
Fearful dog, still, in the center of the kennel= 2
Fearful dog avoiding the tester, moving away, and/or hiding in its shed/hut =1
Aggressive and threatening dog = 0

4) "Come" command: the dog is off leash, the observer stands a minimum of 5 m from the dog, and calls the dog with a calm voice for approximately 30 seconds. If the dog is reticent, the observer may kneel down and call the dog once more holding his/her hand out, palm up.

Score:

The dog goes toward the tester after being called = 1
The dog does not follow the command = 0

5) Problem solving: the dog is off leash, the observer gives a titbit of food to the dog to evaluate its interest in the food reward; the observer approaches the dog, shows a second titbit, and making sure that the dog is still watching, places it on the floor. The observer moves away and notes whether the dog explores the object and attempts to retrieve the titbit within 30 seconds.

*Score*

The dog accomplishes the task = 3
The dog interacts with the box but does not accomplish the task = 2
The dog is interested in the food but looks at the tester not interacting = 1
The dog is not interested in the food or in the task = 0.

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# Behavioral analysis of the Czechoslovakian Wolfdog using the C-BARQ questionnaire

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*Abstract*: The Czechoslovakian Wolfdog (CWD) is a recently established hybrid breed with limited behavioral data available. Using the validated Italian version of the C-BARQ questionnaire, this study assessed 204 CWDs (103 males, 101 females; 74% intact, 26% neutered/spayed). Results indicate high trainability (mean 2.86/5) and very low owner-directed aggression (0.28/4). However, sex and reproductive status influenced several traits. Males were more possessive of objects ($p = 0.0065$), excitable when visitors arrived ($p = 0.011$), and prone to attachment behaviors, such as following the owner ($p = 0.045$) or reacting to owner affection toward others ($p = 0.0006$). Neutered males displayed significantly higher stranger-directed aggression ($p = 0.0019$), while dog-directed aggression averaged 2.05/4, mainly expressed by males toward unfamiliar males ($p < 0.0001$). Fear-related behaviors were generally low across groups. Overall, the CWD emerges as a trainable, adaptable breed, though management should consider sex- and status-related differences, particularly in male aggression and attachment.

*Key Words*: behavior, Czechoslovakian Wolfdog, C-barq questionnaire, sex difference, castration

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## Introduction

The Czechoslovakian Wolfdog is a relatively recent breed, officially recognized by the Fédération Cynologique Internationale (FCI) in 1999. Its establishment began in 1955 in former Czechoslovakia under the direction of Colonel Jan Hartl and Dr. Heiri Machat. The breeding program involved controlled crosses between working-line German Shepherds (Canis lupus familiaris) and Carpathian grey wolves (Canis lupus lupus), with the objective of combining desirable behavioral traits of the German Shepherd (trainability, social cohesion, and cooperative temperament) with morphological and physiological characteristics of the wolf (strength, endurance, and robustness). The breed was initially employed for military purposes, particularly border patrol, and subsequently in search and rescue operations. In Italy, the population experienced a marked expansion during the 2010s, peaking in 2016 with more than 1,400 new registrations in the Italian Kennel Club (ENCI). Since then, numbers have progressively declined, reaching approximately 500 registrations in 2024 (Caniglia et al., 2008).

Given the recent establishment of this breed, limited information is currently available regarding its behavior in the domestic context and potential sex-related differences. It is well established that sex hormones influence behavior in both males and females. In females, reproductive behavior is regulated by cyclic hormonal fluctuations, which first induce estrous behaviors and subsequently, after parturition, maternal care (Lezama-García 2019). These hormonal dynamics are also responsible for the frequent occurrence of pseudopregnancy in bitches (Root 2018). In males, testosterone plays a central role and is strongly associated with behaviors such as inter-male aggression and urine marking (Hart & Eckstein, 1997).

The first studies on the effect of sex hormones on behavior are due to the pioneering research of Scott and Fuller in the 1960s (Scott & Fuller, 1965), followed by the research of Hopkins (Hopkins et al., 1976) and then Brochelt (Brochelt, 1983).

Early investigations into this topic showed several important shortcomings. One of the most evident was the absence of control groups of intact dogs, which prevented clear attribution of behavioral changes to neutering rather than to external influences such as household modifications or new activities like training. Many works were retrospective in design, depending on historical records and owners' recollections, both of which increase the likelihood of bias and inaccuracies (Wright J.C. & Nesselrote M.S, 1987). Moreover, unwanted behaviors were not always differentiated from those linked to medical issues, so similar actions could be reported without recognizing their distinct origins. The samples themselves were

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often skewed, with a higher representation of dogs already displaying aggression—as in Borchelt's 1983 study—or selected for particular outcomes. Small numbers of subjects further limited the strength of the conclusions, while factors such as breed, age, and timing of castration were frequently ignored. Finally, inconsistencies in the classification of behaviors, such as treating aggression as a single undifferentiated category rather than considering underlying motivations like fear or pain, reduced the reliability and comparability of results across studies (Arroube & Pereora, 2025)

The use of questionnaires in canine behavioral research has evolved considerably. Early studies often relied on open-ended owner reports and follow-up phone interviews, an approach prone to subjective interpretation and inconsistencies in questioning across researchers. In contrast, more recent work has employed standardized and rigorously developed instruments, providing more reliable and comparable data. Before the 2000s, no validated tools were available to systematically assess canine behavior. From that period onward, structured questionnaires were introduced to evaluate behavior in relation to factors such as reproductive status. Among the most widely recognized is the Canine Behavioral Assessment and Research Questionnaire (C-BARQ).

The C-BARQ gathers behavioral information indirectly through reports provided by owners, handlers, or caregivers, under the assumption that individuals most familiar with a dog are best suited to accurately describe its typical behavior [14]. It currently consists of 100 items that assess a dog's usual responses to a broad range of everyday situations and recent stimuli. Responses are recorded using two ordinal rating scales: a five-point scale (0–4) reflecting the severity or intensity of specific behaviors (e.g., aggression, fear), and a seven-point scale (0–6) indicating the frequency of behaviors (e.g., barking, howling). This dual-scale format enables a more precise evaluation of both the occurrence and magnitude of behavioral traits.

The objective of the present study was to characterize the baseline behavioral profile of the Czechoslovakian Wolfdog in a family setting and to examine potential differences associated with sex and reproductive status.

## Materials and methods

For this study, the validated Italian version of the C-BARQ, as described by Broseghini et al. (2023), was employed, with some modifications consisting of the addition of three questions (two in Section 1 and one in Section 6) and the removal of Section 7. The questionnaire was distributed online via social media platforms and breed club pages and further circulated among Czechoslovakian Wolfdog breeders and owners through word of mouth. An introductory section was included to collect demographic information (name, breed, date of birth, sex, and, where applicable, date of castration/neutering).

The C-BARQ uses two response formats depending on the behavior assessed. Items in sections 1, 4, 6, and 7 measure the frequency of specific behaviors on a six-point ordinal scale (0 = not applicable, 1 = never, 2 = rarely, 3 = sometimes, 4 = often, 5 = always), providing an index of behavioral consistency over time. Items in sections 2, 3, and 5 assess the intensity or severity of behaviors, regardless of frequency, using a five-point scale (0 = none, 1 = mild, 2 = moderate, 3 = intense, 4 = severe). Higher scores generally reflect more problematic behaviors, with the exception of trainability, where higher scores denote a desirable trait.

The original 100 C-BARQ items were grouped into 13 behavioral subscales. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). After verifying normal data distribution, a one-way ANOVA was applied to compare behavioral scores across the four coat-color groups, followed by Tukey's post hoc test for multiple comparisons. For each item, both the overall mean score and the mean score by coat-color group were calculated. Internal consistency of the questionnaire was evaluated using Cronbach's alpha.

## Results

A total of 204 questionnaires were collected for the study. Of the dogs represented, 103 (50.5%) were male and 101 (49.5%) were female. Regarding reproductive status, 151 (74.0%) were intact and 53 (26.0%)

were neutered or spayed. Specifically, the sample included 60 intact females, 41 spayed females, 91 intact males, and 12 neutered males.

The results for each of the 13 behavioral subscales are presented in Table 1

| | | Males | Females | Intact males | Castrated males | Intact females | Neutered females | General mean |
|---|---|---|---|---|---|---|---|---|
| 1 | Trainability Frequency scale (0-5) | 2.89±0.44 | 2.83 ±0.46 | 2.90± 0.43 | 2.84±0.49 | 2.85±0.47 | 2.81±0.45 | 2.86±0.45 |
| 2 | Strange Direct Aggression Intensity scale (0-4) | 1.21±0.98 | 1.10±0.96 | 1.20 ±0.99 | 1.29±0.97 | 1.04±0.93 | 1.18±1.00 | 1.18±0.97 |
| 3 | Owner-directed aggression Intensity scale (0-4) | 0.33±0.51 | 0.25±0.38 | 0.35 ±0.53 | 0.19±0.31 | 0.30±0.43 | 0.18±0.27 | 0.28±0.42 |
| 4 | Dog-directed aggression Intensity scale (0-4) | 2.18±0.92 | 1.84±1.01 | 2.16 ±0.84 | 2.31±1.40 | 1.73±1.11 | 2.01±0.82 | 2.05±1.00 |
| 5 | Dog rivalry Intensity scale (0-4) | 0.67±0.85 | 0.84±0.93 | 0.68 ±0.87 | 0.55±0.57 | 0.77±0.87 | 0.95±1.04 | 0.76±0.88 |
| 6 | Stranger-directed Fear Intensity scale (0-4) | 0.69±0.99 | 0.85±0.97 | 0.60 ±0.93 | 1.50±1.10 | 1.00±1.01 | 0.62±0.89 | 0.86±0.99 |
| 7 | Nonsocial fear Intensity scale (0-4) | 1.03±0.81 | 0.93±0.69 | 0.99±0.81 | 1.36±0.74 | 0.93±0.69 | 0.93±0.71 | 1.02±0.73 |
| 8 | Dog-directed Fear Intensity scale (0-4) | 0.67±1.10 | 0.62±0.71 | 0.60±1.03 | 1.25±1.44 | 0.64±0.77 | 0.59±0.63 | 0.71±0.94 |
| 9 | Separation-Related Behaviors Frequency scale (0-5) | 1.26±0.89 | 1.08±0.86 | 1.28±0.89 | 1.17±0.90 | 1.05±0.87 | 1.13±0.86 | 1.17±0.88 |
| 10 | Excitability Intensity scale (0-4) | 2.29±0.86 | 2.09±0.87 | 2.25±0.87 | 2.53±0.78 | 2.06±0.96 | 2.15±0.72 | 2.23±0.84 |
| 11 | Attachment/ Attention-seeking Frequency scale (0-5) | 3.19±0.99 | 2.78 ±1.00 | 3.19±0.95 | 3.20±1.31 | 2.92±0.99 | 2.56±0.99 | 3.01±1.08 |
| 12 | Chasing Frequency scale (0-5) | 1.63±1.06 | 1.48±1.00 | 1.69±1.05 | 1.21±1.10 | 1.36±1.10 | 1.66±0.81 | 1.53±1.02 |
| 13 | Touch Sensitivity Intensity scale (0-4) | 0.60±0.60 | 0.54±0.59 | 0.57±0.60 | 0.81±0.50 | 0.53±0.64 | 0.56±0.50 | 0.59±0.57 |

The responses indicate that the breed is generally characterized by a high level of trainability and low levels of owner-directed aggression. However, males showed greater possessiveness over objects compared to females ($p = 0.0065$). With regard to stranger-directed aggression, a significant effect of reproductive status was observed when dogs were approached directly by an unfamiliar child while on a leash, as illustrated in Figure 1.

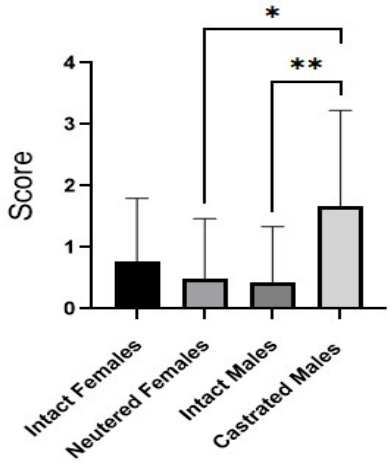


**Figure 1.** Responses to the item "Is the dog aggressive when approached directly by an unfamiliar child, outside the home?" A significant difference was detected using the Mann–Whitney test (p = 0.0019). Post hoc analysis revealed significant differences between neutered males, intact males, and spayed females. (*p= 0.02 **p= 0.006)

Furthermore, neutered males exhibited higher levels of aggression when approached by a stranger attempting to touch or pet them (Figure 2).

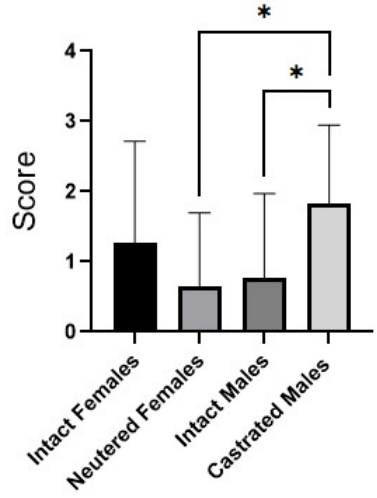


**Figure 2.** Responses to the item "Is the dog aggressive when a stranger attempts to touch or pet it?" A significant effect was detected with the Mann–Whitney test (p = 0.0015). Post hoc analysis revealed differences between neutered males, intact males, and spayed females. (*p=0.011)

With respect to interactions with other dogs, no significant rivalry was observed among cohabiting individuals. However, a slight increase in aggression was noted toward dogs of the same sex during on-leash walks. Differences related to reproductive status were also identified and are presented in Figure 3.

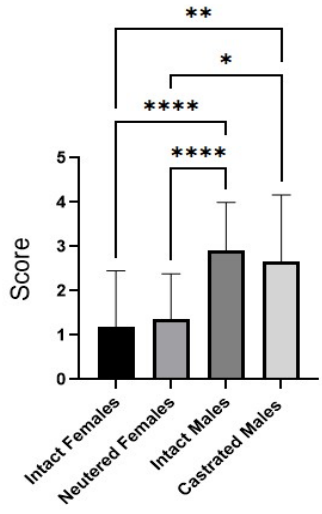


**Figure 3.** Responses to the item "Is the dog aggressive when approached directly by an unfamiliar male dog while walking on a leash?" A significant effect was detected with the Mann–Whitney test ($p < 0.0001$). Post hoc analysis revealed differences between neutered males, intact males, and both spayed and intact females. (*p=0.025, **p= 0.005, ****p= <0.0001)

Sex-related differences were also observed in separation-related behaviors. Males showed a greater tendency to howl compared to females ($p = 0.0364$), exhibited stronger attachment to a specific family member ($p = 0.049$), and were more likely to follow the owner or household members from room to room ($p = 0.045$). In addition, males were more prone to whining or attempting to intervene when the owner or others displayed affection toward another person ($p = 0.0006$), a behavior predominantly expressed by intact males (Figure 4).

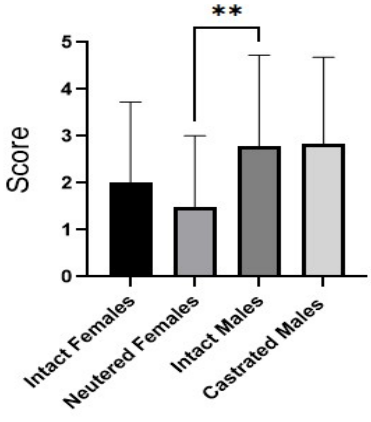


**Figure 4.** Responses to the item "Does your dog become agitated (whines, jumps, tries to get in the way) when the owner or others show affection toward another person?" A significant effect was identified using the Mann–Whitney test ($p = 0.0009$). Post hoc analysis revealed a difference between intact males and spayed females. (**p= 0.0027)

Males also exhibited higher levels of excitability when visitors arrived at the home ($p = 0.011$), (Fig.5).

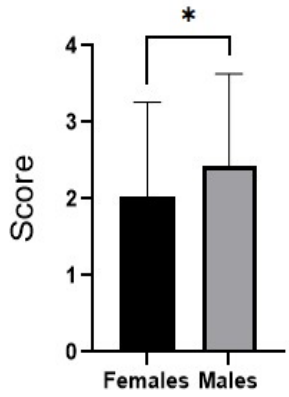


**Figure 5.** Responses to the question "Is the dog excited when do people come to the house?" A significant result was found using the Mann-Whitney test (P = 0.0109). The test showed a significant difference between males and females. (*p=0.0109)

## Discussion

The findings of this research on the Czechoslovakian Wolfdog provide a clearer and more detailed profile of the breed. These dogs are highly trainable, which explains their use in both research and sporting activities. They also adapt well to family life, showing no signs of aggression toward their owners. Coexistence with other dogs in the same household appears to be generally positive, as scores in the rivalry category remain very low—well below 1 on a scale of 0 to 4. However, this result should be interpreted with caution, since it is not always clear how many Czechoslovakian Wolfdogs actually live with other dogs or interact with them regularly.

The picture changes when considering aggression toward unfamiliar dogs. With an average score of 2.05, the data suggest a moderate level of reactivity, most commonly expressed by males (both intact and neutered) toward other males. For this reason, owners should be mindful of such tendencies and take appropriate precautions during encounters with unknown dogs. A structured socialization and training program, begun at an early age, can play a crucial role in reducing this issue.

With regard to fear-related behaviors, the Czechoslovakian Wolfdogs in our sample showed a low tendency to be intimidated by either social or physical stimuli. Scores for fear of unfamiliar people or dogs were consistently low, as were those related to non-social fears: the breed generally shows little reactivity to novel objects, sudden noises, thunder, or fireworks. In contrast to findings reported in other studies (BIBLIO Mengoli), no significant differences emerged between sexes or reproductive status.

Although often described as an independent breed, the Czechoslovakian Wolfdog is capable of forming a strong emotional bond with its owner and may display signs of distress when separated. Behaviors linked to attention-seeking and physical contact with the owner are common, with an average score of 3.01 on a 5-point scale. The development of this attachment bond—even in early life (Mariti et al.; Carlone et al.)—is a crucial factor, providing the dog with the sense of security needed to thrive in a human environment. The importance of relational factors in modulating behavioral responses is also confirmed by recent studies on shelter dogs, in which oxytocin was found to be associated with a lower physiological response to stress during veterinary examinations (Gazzano et al., 2025). However, the intensity of this motivation can become problematic if not properly managed. Dogs that rely heavily on attachment are at greater risk of developing separation-related issues, including separation anxiety, which can be challenging to treat. For this reason, it is vital that prospective owners are informed of this predisposition—particularly marked in intact males—and that they seek guidance from qualified dog trainers and veterinary behaviorists to prevent the emergence of problematic behaviors and to safeguard the human–dog relationship.

## Conclusion

In conclusion, this study enhances our understanding of the behavioral characteristics of the Czechoslovakian Wolfdog, drawing attention to differences linked to sex and reproductive status. These insights provide valuable guidance for owners, trainers, and veterinarians, ultimately supporting the development of a more harmonious and fulfilling bond between humans and this unique breed.

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### Analisi comportamentale del Cane Lupo Cecoslovacco mediante il questionario C-BARQ

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*Sintesi*

Il Cane Lupo Cecoslovacco (CLC) è una razza ibrida di recente formazione, per la quale sono disponibili dati comportamentali limitati. Utilizzando la versione italiana validata del questionario C-BARQ, questo studio ha analizzato

204 soggetti (103 maschi, 101 femmine; 74% interi, 26% sterilizzati). I risultati mostrano un'elevata addestrabilità (media 2,86/5) e livelli molto bassi di aggressività diretta verso il proprietario (0,28/4). Tuttavia, sesso e stato riproduttivo influenzano diversi tratti. I maschi si sono mostrati più possessivi verso gli oggetti ($p = 0,0065$), più eccitabili all'arrivo di visitatori ($p = 0,011$) e più inclini a comportamenti di attaccamento, come seguire il proprietario ($p = 0,045$) o reagire quando questo mostrava affetto verso altre persone ($p = 0,0006$). I maschi castrati hanno evidenziato una maggiore aggressività verso gli estranei ($p = 0,0019$), mentre l'aggressività verso altri cani ha raggiunto una media di 2,05/4, soprattutto tra maschi verso conspecifici maschi non familiari ($p < 0,0001$). I comportamenti legati alla paura sono risultati generalmente bassi in tutti i gruppi. Nel complesso, il CLC appare una razza addestrabile e adattabile, sebbene la gestione debba considerare differenze legate a sesso e stato riproduttivo, in particolare per aggressività e attaccamento nei maschi.

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# Preliminary insights into β-Amyloid, phospho-tau and inbreeding in Labrador Retriever dogs

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*Abstract*: Serum concentrations of β-amyloid peptides (Aβ40 and Aβ42) and phosphorylated tau (p-tau) are emerging as potential biomarkers of age-related neurodegenerative processes in dogs, particularly in relation to canine cognitive dysfunction. At the same time, the genetic background, especially inbreeding, may influence aging trajectories and brain pathology. This paper reports preliminary observations on the relationship between plasma Aβ40, Aβ42, and p-tau levels and the inbreeding coefficient in 24 healthy Labrador Retriever dogs. Blood samples were collected and analyzed using ELISA kits specific for canine Aβ and phosphorylated tau, and inbreeding coefficients were calculated based on pedigree data. In addition, the Canine Dementia Scale (CADES) was administered. Although the small sample size limits the strength of statistical inference, initial findings suggest potential associations between age, biomarker concentrations, and sex. However, the inbreeding coefficient was too low to detect possible correlations with biomarker levels. These results primarily provide preliminary insight into the effects of neurobiological aging in dogs, while the contribution of genetic factors remains to be clarified in larger cohorts.

*Key Words*: dog; labrador; inbreeding coefficient; pTau; Amyloid β; CADES

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## Introduction

Dogs are increasingly recognized as a valuable model for studying age-related cognitive decline and neurodegeneration, due to similarities with human aging processes. Among the most studied biomarkers are amyloid-beta (Aβ) peptides and Tau proteins, which show age-dependent changes in both healthy and cognitively impaired dogs. In particular, plasma Aβ40 and Aβ42 levels tend to increase with age in healthy dogs, while significantly lower concentrations have been reported in dogs affected by canine cognitive dysfunction syndrome (CDS) (Panek et al., 2020). Tau-related synaptic impairment and neuroinflammation have also been associated with cognitive decline (Smolek et al., 2016), and recent studies have further characterized age-related behavioral and cognitive changes in dogs (Ciurli et al., 2023a; 2023b). Despite this growing knowledge, the possible influence of genetic factors, especially inbreeding, on these neurobiological aging markers remains poorly understood. Inbreeding is commonly used in selective breeding to stabilize desirable traits, but it can also lead to increased expression of recessive genetic disorders (Comparini et al., 2019; Cecchi et al., 2020; Bannasch et al., 2021; Barsotti et al., 2024), reduced genetic diversity, and inbreeding depression, a condition increasingly recognized as relevant to animal welfare (Keller & Waller, 2002; Brzeski et al., 2014; Cecchi et al., 2016) that has been linked to reduced fertility (Langlois & Blouin, 2004; Gonzales-Recio et al., 2007), changes in morphological traits in dog (Cecchi et al., 2018) and in other species (Bussiman et al., 2018; Hossein-Zadeh, 2012; Gómez et al., 2009), and a lower response to selection. In humans, inbreeding has also been associated with an increased risk of Alzheimer's disease (Vardarajan et al., 2015; Moreno-Grau et al., 2021; Ghani et al., 2015), suggesting a potential role of genetic background in cognitive decline.

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Based on this framework, the present study aimed to evaluate serum concentrations of Aβ40, Aβ42 and phosphorylated Tau (pTau) in a small cohort of Labrador Retrievers, and to explore their relationship with inbreeding coefficients and cognitive status, as assessed through the CAnine DEmentia Scale (CADES) (Madari et al. 2015). Although preliminary, these findings may offer useful insights into the complex interplay between genetics and brain aging in dogs.

## Material and methods

The study included 24 clinically healthy Labrador Retriever dogs (7 males and 17 females), aged 6 to 15 years, obtained from family-owned breeders and currently living as privately owned pets. For each dog, the following parameters were considered:

*Tau and Beta.* From each dog, a blood sample of 5 ml was collected to evaluate, in serum, β amlyoid 40 and 42, and phosphotau 181, by using ELISA kits, respectively: Canine Amyloid Beta Protein 40 (cat.No: MBS013429), Canine Amyloid Beta Peptide 1-42 (Cat.No: MBS742661) and Canine Phosphorylated Tau 181 (Cat.No: MBS006469), MyBioSource, San Diego, CA 92195-3308 USA.

*Behavioural examination* - In this study, the CADES assessment scale, developed and validated by Madari et al. (2015), was chosen. This scale evaluates four behavioral domains associated with cognitive changes in dogs: spatial orientation (Domain A), social interactions (Domain B), sleep-wake cycle (Domain C), and house soiling (Domain D). Its use allows for the quantification of cognitive decline in dogs and the classification of individuals into different cognitive states based on their scores: Normal Aging (NA, 0–7 points), Mild Cognitive Impairment (MiCI, 8–23 points), Moderate Cognitive Impairment (MoCI, 24–44 points), and Severe Cognitive Impairment or Canine Dementia (CD, 45–95 points).

Due to its high sensitivity in detecting early cognitive changes, the CADES scale is considered a valuable tool for both diagnosing and monitoring cognitive decline in dogs.

*Inbreeding coefficient (F)* - the pedigree of each dog was also collected, and a database was built. We used Pedigree Viewer software (Kinghorn, 1994) to verify whether there were any errors left in the data files. The individual and average inbreeding coefficient (F), both overall and by sex, was computed using the CFC software (Sargolzaei et al., 2006). The inbreeding coefficient is the probability that at any randomly drawn locus of a given individual has two identical by-descent alleles (Wright, 1922). This parameter was calculated by the tabular method described by Meuwissen & Luo (1992).

## Statistical Analysis

To highlight the relationships between F and both β-amyloid 40 and phospho-tau 181, the non-parametric Spearman's rank correlation test was performed using Jamovi version 2.5 (The Jamovi Project, 2024), retrieved from https://www.jamovi.org and accessed on 7 July 2025. The Spearman test was chosen after verifying, through the Shapiro–Wilk test, that the data were not normally distributed. All parameters were also evaluated for differences between male and female dogs. Regarding CADES scores, to reduce the impact of outliers, scores were winsorized, and a small amount of noise was added to zero values.

Moreover, a cluster analysis, an exploratory multivariate statistical method, was performed with JMP software version 5.0 (SAS, JMP) to identify possible groups of animals sharing similar characteristics across variables such as age, F, Aβ40, tau, and scores obtained from the CADES assessment. This type of analysis allows for the grouping of individuals based on patterns of similarity, facilitating the detection of naturally occurring subgroups within the dataset. A one-way analysis of variance (ANOVA) was used to identify potential differences between the groups,

with a sufficiently large number of dogs included to support statistical comparison. To assess whether there was a significant association between cluster membership and the sex of the dogs, a chi-square test of independence was performed. The number of males and females within each cluster was tabulated, and expected frequencies were calculated based on the overall sex distribution in the sample. Due to the limited sample size and small expected cell counts, Fisher's exact test was also considered to validate the robustness of the results.

## Results and discussion

Results showed that, out of the 24 purebred animals, only 5 females were found to be inbred (mean inbreeding coefficient F = 0.046; min = 0.002, max = 0.125), with one individual having F = 0.125 and the remaining four with F < 0.05.

An inbreeding coefficient of 0.05 is considered the maximum acceptable threshold (Ciampolini et al., 2013). As Beuchat (2015) explains, while adverse effects of inbreeding typically begin to appear at around 5%, a marked decline in offspring vitality and an increased risk of expressing deleterious recessive mutations are observed when the coefficient exceeds 10% (Marín Navas et al., 2021).

Low inbreeding levels have also been reported in previous studies, such as the one conducted on the same breed at the Guide Dog School for the Blind in Scandicci (Cecchi et al., 2009).

Mean values, both overall and by sex, for age (in years), and for Aβ40, pTau, and CADES scores in the analyzed dogs are reported in Table 1. The dogs were all clinically healthy and privately owned, with an overall mean age of 11.54 ± 1.87 years. The age distribution was similar between sexes, with no substantial difference between females and males. Although some sex-related trends were observed in biomarker levels and cognitive scores, no statistically significant differences were found. In particular, pTau concentrations and CADES scores were higher in males, suggesting a possible tendency toward greater cognitive impairment in this group. However, these trends did not reach statistical significance and should be interpreted cautiously. Further research with a larger sample size may help clarify these observations.

It is also noteworthy that Aβ42 was not detected or quantifiable in any of the analyzed samples, a finding that may suggest either a limitation of detection sensitivity or genuinely low circulating levels in this population. A similar result was reported in aged horses, where Aβ42 was undetectable in all subjects despite the presence of measurable Aβ40 and pTau181 levels, and no signs of cognitive decline were observed. This absence of Aβ42, considered a key isoform in initiating Tau phosphorylation, was hypothesized to reflect species- or population-specific differences in amyloid metabolism (Gazzano et al., 2025). These findings raise the possibility that undetectable Aβ42 might be associated with the preservation of cognitive function, although further studies are needed to clarify its biological significance in dogs.

**Table 1.** Mean values, both overall and by sex, for age (in years), and the levels of Aβ40, pTau, and CADES scores in the analyzed dogs. Aβ42 was not detected or quantified in the analyzed samples.

| | Total sample | Females | Males |
|---|---|---|---|
| N° | 24 | 17 | 7 |
| Age (years) | 11.54±1.87 | 11.53±0.47 | 11.57±0.73 |
| Aβ40 (pg/ml) T0 | 21.63±2.85 | 23.81±3.36 | 16.35±5.22 |
| Tau pg/ml | 11.80±2.66 | 9.25±3.07 | 18.00±4.79 |
| CADES score | 4.29±2.14 | 2.53±2.11 | 8.57±3.91 |

When investigating possible correlations among the analyzed parameters, statistical analysis revealed only one significant association, between age and the CADES score ($r = 0.614$; $P < 0.01$), suggesting a possible age-related cognitive decline. However, given the limited sample size, this finding should be interpreted with caution.

Using multivariate analysis, four distinct clusters of dogs were identified based on age, inbreeding coefficient (F), Aβ40 and Tau concentrations, and CADES scores (Figure 1). The differences among dogs belonging to the different clusters are reported in Table 2.

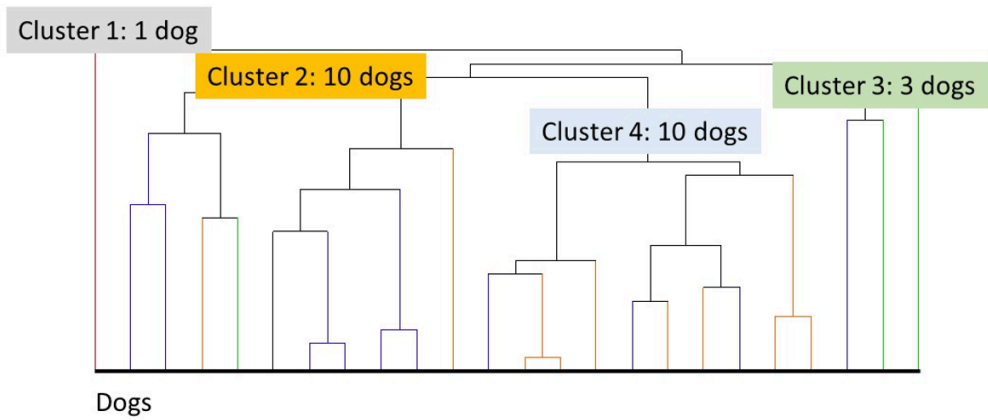


**Figure 1.** Multivariate clustering of dogs based on age, F coefficient, Aβ40 and Tau levels, and CADES score. The figure shows the distribution of individual dogs within the four identified clusters, highlighting differences in biomarker profiles and cognitive outcomes.

**Table 2.** Descriptive data for each cluster, including number and sex of dogs, mean age, inbreeding coefficient (F), plasma Aβ40 and Tau concentrations, and CADES scores.

| Cluster | Dogs (n°) | Sex | Age (years) | F | Aβ40 (pg/ml) T0 | Tau pg/ml | CADES score |
|---|---|---|---|---|---|---|---|
| 1 | 1 | Female | 6 | 0.125 | 0 | 13.76 | 0 |
| 3 | 3 | 2 Females and 1 Males | 14.30 | 0.001 | 13.30 | 1.88 | 27.00 |
| 2 | 10 | 5 Females and 5 Males | 10.9 B | 0.006 | 21.92 B | 24.95 A | 1.40 B |
| 4 | 10 | 9 Females and 1 Males | 11.9 B | 0.008 | 25.98 B | 1.43 B | 0.88 B |

Letters (A, B) on the same column indicate statistically significant differences ($p < 0.01$) between clusters, based on one-way ANOVA and post hoc comparisons.

Cluster 1 included a single young dog (6 years old) with a high inbreeding coefficient ($F = 0.125$), undetectable Aβ40, elevated Tau levels (13.76 pg/ml), and a CADES score of 0. The unique biomarker and cognitive profile of this dog prevents meaningful comparisons with the other clusters. Its distinctive pattern likely reflects individual variability rather than group-level trends, as this subject was the youngest and had the highest inbreeding coefficient, with a markedly different profile compared to the others. In the remaining clusters (2, 3, and 4), inbreeding coefficients (F) were generally low and showed limited variability. This narrow distribution hampers the assessment of the potential influence of inbreeding on biomarker expression or cognitive outcomes. While F was included in the clustering process, its contribution to group differentiation appears minimal in this dataset.

Cluster 3 (n = 3) included the oldest dogs (mean age: 14.3 years), characterized by the lowest F values (0.001), low Aβ40 (13.30 pg/ml), low Tau (1.88 pg/ml), and the highest CADES scores (27.00), indicating pronounced cognitive impairment. This cluster appears to differ from the two largest clusters with respect to age, Aβ40 concentrations, and CADES scores. Nevertheless, the limited number of subjects did not allow for a statistical significance evaluation.

Cluster 2 (n = 10) comprised slightly younger dogs (mean age: 10.9 years), with low F (0.006), elevated Aβ40 (21.92 pg/ml), high Tau (24.95 pg/ml), and low CADES scores (1.40), suggesting preserved cognitive function despite elevated biomarker levels.

Cluster 4 (n = 10) included dogs with a mean age of 11.9 years, low F (0.008), high Aβ40 (25.98 pg/ml), low Tau (1.43 pg/ml), and the lowest CADES scores (0.88), indicating intact cognitive abilities. This Cluster consisted of 9 females and only 1 male, whose values were similar to those of the females.

The only significant difference between the two largest clusters (2 and 4) concerns Tau levels; all other variables included in the multivariate analysis are comparable, thus making it challenging to interpret this finding.

No significant association was found between the four clusters and the sex of the animals. Although this distribution did not reach statistical significance, the pattern observed in Cluster 4, where females predominated and the only male showed values comparable to those of the females, could tentatively point to sex-related influences on cognitive preservation or biomarker expression. Further research with larger and more balanced samples is needed to explore potential sex-related effects on cognitive aging in dogs (Schütt et al. 2015). These findings highlight the heterogeneity of cognitive aging in dogs and suggest a potential dissociation between biomarker profiles and behavioral outcomes. It is possible that individual cognitive reserve, lifestyle, or unmeasured genetic and environmental factors modulate this relationship.

Future studies involving larger and genetically more diverse populations, with longitudinal follow-up, are needed to clarify the trajectories of biomarker changes and their clinical relevance. Additionally, the role of inbreeding should be further explored in samples with a broader F distribution to better assess its potential impact on neurodegeneration and cognitive resilience.

## Conclusions

This preliminary study represents an initial attempt to explore potential correlations between inbreeding and biomarkers of cognitive degeneration in dogs. In the present population, the inbreeding coefficients derived from pedigree analysis were relatively low, limiting the ability to detect clear associations with pTau and β-amyloid (Aβ40) levels. Expanding the sample to include a greater number of dogs with higher inbreeding coefficients would allow for more robust and informative analyses. This approach is more feasible in the canine model than in human studies, where identifying inbred individuals is considerably more difficult.

Moreover, increasing the number of older dogs is equally important. The biomarker and cognitive profiles observed in Cluster 3, characterized by advanced age, low Aβ and Tau levels, and high CADES scores, suggest a distinct neurocognitive trajectory that warrants further investigation. Including more aged subjects may help clarify the progression of cognitive decline and its relationship with neurodegeneration in dogs, enhancing our understanding of canine aging and its translational relevance to human conditions. Our findings also highlight the need to further investigate potential sex-related influences on cognitive aging and biomarker profiles in dogs.

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## Osservazioni preliminari su β-Amiloide, fosfo-tau e consanguineità nel Labrador Retriever

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*Sintesi*

Le concentrazioni plasmatiche di β-amiloide (Aβ40, Aβ42) e tau fosforilata (p-tau) sono potenziali biomarcatori di neurodegenerazione nel cane. Questo studio ha esaminato 24 Labrador Retriever sani per valutare l'associazione tra

questi biomarcatori e il coefficiente di consanguineità, calcolato dai dati genealogici. I campioni di sangue sono stati analizzati con kit ELISA e i cani valutati tramite *Canine Dementia Scale* (CADES). I dati preliminari indicano possibili correlazioni tra età, sesso e livelli dei biomarcatori, ma non tra questi ultimi e il coefficiente di consanguineità, risultato troppo basso per evidenziare legami significativi. Questi risultati confermano l'interesse di approfondire il rapporto tra invecchiamento cerebrale e fattori genetici in studi su campioni più ampi.

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# Melatonin in dogs: mechanisms and applications for behavioral issues. Commentary on current state of art and future perspectives

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*Abstract*: Melatonin is a multifaceted neurohormone primarily synthesized by the pineal gland. It offers the capacity to enhance the management of behavioral issues in domestic canines. Scientific data supporting its therapeutic value is scarce and varied, despite its widespread off-label clinical application. This commentary summarizes current knowledge regarding the clinical uses and mechanisms of action of melatonin in addressing behavioral disorders in dogs. Clinical evidence supports the potential impact in managing preoperative anxiety, noise phobia, separation-related disorders, compulsive behaviors, and canine cognitive disorders. The recommended dosage is in a range of 1.5 to 6 mg, depending on the dog's weight; the dosage needs to be delivered every 8 to 24 hours, contingent upon the therapeutic circumstances. The safety profile is favorable with negligible side effects. Although melatonin shows promise as an adjuvant treatment for canine behavioral problems, the existing evidence is predominantly based on case reports and anecdotal experiences. To establish evidence-based dose guidelines, long-term efficacy, and safety profiles, thorough randomized controlled trials in dogs are essential.

*Key Words*: behavior, melatonin, dog, noise phobia.

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## Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a multifaceted neurohormone primarily produced by the pineal gland, with additional contributions to circulating levels from extrapineal sources such as the gastrointestinal tract and retina. Melatonin is generated via intricate enzymatic processes, commencing with the conversion of the amino acid tryptophan. Tryptophan is taken into the bloodstream, conveyed to the pineal gland, and used for the synthesis of proteins essential for melatonin generation (Parmar & Daya, 2001). Melatonin production exhibits a circadian rhythm, reaching its zenith at night due to diminished light exposure, thereby informing the body of alterations in environmental photoperiods and modulating biological rhythms accordingly (Fang et al., 2018; Gao et al., 2022). Melatonin's biological functions primarily occur through the activation of two high-affinity G-protein coupled receptors, MT1 and MT2, which are predominantly expressed in the suprachiasmatic nucleus (SCN) of the hypothalamus, the central circadian pacemaker, as well as in various other brain regions and peripheral tissues. Activation of the MT1 receptor predominantly inhibits adenylate cyclase, leading to a decrease in cAMP levels, whereas the MT2 receptor controls cyclic GMP pathways, each initiating unique intracellular cascades that regulate physiological responses (Gao et al., 2022; Salavati et al., 2023). In addition to receptor-mediated activities, melatonin demonstrates significant antioxidant properties by neutralizing reactive oxygen species (ROS) and enhancing the activity of endogenous antioxidant enzymes, including superoxide dismutase and glutathione peroxidase. This safeguards cerebral tissue from oxidative damage associated with neurodegenerative processes (Paradies et al., 2015). In dogs, melatonin affects the modulation of neurotransmitters like as serotonin, dopamine, and gamma-aminobutyric acid (GABA), which are essential in regulating mood, anxiety, and circadian sleep-wake patterns. Moreover, melatonin regulates the hypothalamic-pituitary-adrenal

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(HPA) axis, diminishing excessive cortisol secretion under stress, thereby aiding in the reduction of anxiety-like behaviors (Salavati et al., 2023; Song & Yoon, 2025). Despite the intricate nature of these systems rendering melatonin a promising therapy option for behavioral disorders in dogs, scientific study remains limited, and data supporting its therapeutic efficacy is derived from model animals, humans, and case reports involving dogs. The heterogeneity in dosage regimens and result measurements hinders broad accord.

## Behavioral disorders in dogs and melatonin

Behavioral disorders are a significant welfare concern for domestic dogs and their caregivers, affecting a considerable portion of the global population (Campbell, 1986; Yamada et al., 2019; Dinwoodie et al., 2019; Salonen et al., 2020; Yang et al., 2021). Prevalent disorders encompass generalized anxiety, aggression, noise phobias (such as thunderstorms and fireworks), separation-related disorders, compulsive behaviors, and age-associated cognitive impairment (Landsberg et al., 2023). These conditions frequently indicate dysregulation in neurochemical systems, altered circadian rhythms, and inappropriate stress responses. Anxiety disorders in dogs have similar neurophysiological mechanisms to those in humans, characterized by abnormalities in serotonergic and dopaminergic neurotransmission, together with hyperactivity of the HPA axis, which intensifies sympathetic nervous system arousal. Furthermore, sleep problems exacerbate behavioral dysregulation, establishing a detrimental cycle (Song & Yoon, 2025). Melatonin's capacity to regulate circadian rhythms and influence central neurotransmission provides a biological basis for its therapeutic use in various illnesses. Indeed, melatonin together with adenosine facilitates the restoration of behavioral equilibrium by enhancing synchronization of the sleep-wake cycle and diminishing nocturnal cortisol peaks (Ruiz-Cano et al. 2022; Masson et al., 2024). Experimental and clinical investigations in rodents and animals illustrate melatonin's anxiolytic effects by increasing serotonergic activity and promoting GABAergic inhibitory neurotransmission. This dual action assists in mitigating fear and stress reactions, which are fundamental to numerous canine behavioral issues (Wang et al., 2021; Song & Yoon, 2025).

## Clinical use of melatonin in canine behavioral management

In veterinary clinical practice, melatonin is widely utilized off-label to address behavioral issues, owing to its advantageous safety profile and convenience of administration; preoperative anxiety represents a specific situation. Niggemann et al. (2019) performed a clinical trial including 50 dogs to assess the efficacy of melatonin as pre-medication in healthy dogs waiting for elective surgery. Their main goal was dual: to ascertain if oral melatonin administration elicited a calming effect in dogs prior to anesthesia, and to assess whether this effect facilitated a reduction in the required dose of Propofol for anesthesia induction and intubation. The treatment group administered 5 mg/kg of melatonin orally, two hours before to the induction of anesthesia. The researchers discovered that dogs designated as "skeptical" in the melatonin group exhibited more calmness 90 minutes post-administration compared to "skeptical" dogs in the placebo group. This indicates a main advantage for the most anxious animals. Moreover, dogs categorized as "trustful" in the melatonin group necessitated a markedly reduced dosage of propofol for anesthesia induction in contrast to "trustful" dogs in the placebo group. Researchers showed that melatonin is effective in veterinary pre-medication to mitigate alertness and anxiety before to general anesthesia, especially in more agitated dogs, and to decrease the necessary dosage of Propofol for anesthesia induction in calmer dogs (Niggermann et al., 2019).

In 2023, Salavati and colleagues performed a veterinary clinical experiment with 25 healthy dogs to examine the effects of orally administered melatonin on female dogs having ovariohysterectomy (OHE). The study aimed to investigate if melatonin administration may regulate and alleviate post-operative inflammatory and oxidative effects, given its recognized anti-inflammatory and antioxidant properties. The authors administered melatonin at an oral dosage of 0.3 mg/kg. This administration was place on the days preceding and following the surgery (days -1, 0, 1, 2, and 3). To attain the aim, the researchers quantified the levels of various biomarkers in the dogs' blood, including cortisol and serotonin (for the behavioral aspect) and cytokines (such as CRP, SAA, IL-10, for the inflammatory component). The findings indicated that the administration of melatonin (both pre- and post-operatively) resulted in a substantial reduction in cortisol levels and primary inflammatory indicators in the group of female dogs treated with melatonin following OHE, in contrast to those that had surgery without treatment. Furthermore, serotonin levels elevated in females that were administered melatonin. The research concluded that oral administration of melatonin prior to and following ovariohysterectomy is an efficient strategy for managing elevated inflammation and cortisol levels generated by surgery in female dogs.

Although there is an absence of controlled and long-term studies, case reports indicate that melatonin may be beneficial in addressing anxiety, fear of fireworks and thunder, sleep cycle disorders in dogs and cats (Casey et al., 2022; Landsberg, 2005; Landsberg et al., 2023), noise phobia (Aranson, 1999), separation anxiety, compulsive disorder (Sacchettino et al., 2023), and cognitive dysfunction syndrome (Landsberg, 2005; Paradies et al., 2015; Zhao et al., 2024). To the authors' knowledge, the first reported case report in dogs was in 1998, when melatonin was administered in combination with amitriptyline to treat noise phobia at a dose of 0.1 mg/kg per day and up to every 8 hours during fireworks and storms.

In 2023, Sacchettino and colleagues published a case report regarding a 5-year-old mixed-breed dog suffering with a compulsive disorder that was refractory to conventional antidepressant therapies. The researchers employed an interdisciplinary and integrative approach that combined three components: a behavioral program, the administration of cannabis products, and melatonin supplementation (2 mg/day). Clinical observations and owner reports indicated that the integrated strategy resulted in a decrease in the frequency of compulsive episodes and improved overall management of the dog compared to prior therapy with paroxetine, without any short-term side effects reported. Additionally, the authors indicated that sustaining a decrease in dysfunctional behavior to a level deemed acceptable by caregivers was accomplished during a subsequent four-month follow-up period.

Melatonin was recently suggested "by Costa (2023) in the "Chill protocol" for evaluating a pre-visit sedative treatment. This prospective research trial aimed to assess the sedative and behavioral effects of a regimen administered by the caregivers before to a veterinary appointment, referred to as the GMA protocol (gabapentin, melatonin, and acepromazine). The investigation encompassed 45 client-owned dogs exhibiting symptoms of anxiety, fear, and/or aggression during veterinary appointments. Caregivers were instructed to administer gabapentin, melatonin (3-5 mg per dog based on weight), and acepromazine (administered orally-transmucosally) 90-120 minutes prior to the subsequent appointment. Researchers noted a substantial decrease in stress scores and a notable increase in sedation scores following the implementation of the GMA regimen, in comparison to baseline measurements. The GMA technique significantly diminished indicators of anxiety, tension, and fear-induced aggression during visits. The findings indicated that the approach enhanced the manageability of dogs, facilitating the completion of tests on those that were previously too aggressive or fearful to be inspected safely. A notable association was observed between advancing age and diminished post-GMA stress levels, indicating that older dogs may have a more pronounced response. The research indicates that this multimodal approach serves as an extra resource for veterinarians to enhance care quality and mitigate the risk of injury to both staff and animals.

## Dosage, safety, and side effects of melatonin in dogs

There is currently no clear consensus regarding the dosage or formulation (e.g., conjugated or not) in behavioral medicine. The authors generally recommend a range of 1.5 mg for small dogs, 3 mg for dogs weighing 15 to 50 kg, and 3-6 mg for dogs weighing more than 50 kg, administered every 8-24 hours (Aranson, 1999; Landsberg et al., 2023). The frequency and timing of treatment are contingent upon the clinical context—typically administered 30 to 60 minutes prior to stimulus exposure or bedtime for anxiety and sleep disturbances, as pharmacokinetic analysis indicates rapid and complete absorption following oral administration, with peak plasma concentrations reached within 30-60 minutes (Sääf et al., 1980; Salavati et al., 2023).

Sharman and Bondy (2016) reported that melatonin has minimal toxicity, even at elevated dosages, rendering it an even safer supplement in comparison to other pharmacological drugs. Its efficacy seems to stem not from a singular mechanism, but from its capacity to implement several neutralizing measures against diverse danger aspects. Pharmacodynamic investigations validated melatonin's dose-dependent effects on calm and sedation, while preserving cardiac and respiratory function, hence promoting its incorporation into multimodal treatment regimens (Peace et al., 2019; Salavati et al., 2023). Even though caregivers may regard melatonin as natural and safe, it contains pharmacologically active compounds that can lead to harmful effects and/or interact with medications. The main interaction issue is drugs that are strong inhibitors of the CYP1A2 enzyme, such as antidepressants (Papagiannidou et al., 2014; Li et al., 2018), which leads to an augmentation of standard treatment. This outcome was additionally emphasized by a clinical case in which a patient exhibited profound sedation following the simultaneous administration of melatonin with two antidepressants (citalopram and nortriptyline) and one opioid analgesic (oxycodone), likely due to the inhibitory effect of melatonin on hepatic enzymes (Foster et al., 2015). Adverse effects are often modest, encompassing fatigue, dizziness, headache, irritability and gastrointestinal disturbances (Seabra et al., 2000). However, due to the presence of excipients harmful to dogs in certain human melatonin products (e.g., xylitol), it is advisable to utilize veterinary-specific formulations.

## Conclusion

Melatonin, recognized for its efficacy as a circadian synchronizer and potent antioxidant, has garnered increasing interest in dog behavioral therapy. The physiological mechanism of melatonin in dogs is intricate, involving interactions with the hormonal systems (particularly the hypothalamic-pituitary-adrenal axis) and neurotransmitters. The hormone is hypothesized to affect not only the sleep but also indirectly regulate issues such as separation anxiety, psychogenic alopecia, and compulsive behavior. Specifically, Zhao et al. (2024) highlighted melatonin's role as an antioxidant that mitigates oxidative damage, offering neuroprotective effects in various animal models. This perspective suggests melatonin could be beneficial in elderly dogs suffering from cognitive dysfunction (Landsberg, 2005), also aiding in the regulation of the circadian rhythm—a parallel observed in human patients with cognitive decline (Magri et al., 2004).

Moreover, the association between melatonin and stress management pertains to essential welfare settings, including shelter surroundings. Research on shelter dogs, which generally exhibit heightened stress and cortisol levels, suggests that the administration of agents that regulate the HPA axis may be advantageous (Giuliano et al., 2024). The research by Gazzano et al. (2025), which demonstrated a substantial negative association between oxytocin and cortisol during stressful veterinarian handling, reinforces the notion that hormonal support can mitigate the physiological stress response. Given the proposed antagonistic effect of melatonin on the HPA axis and its ability to reduce stress markers, its incorporation alongside behavioral management

strategies (e.g., environmental enrichment, human interaction) may serve as a beneficial support for enhancing the short-term and long-term welfare of dogs experiencing the persistent stress of shelter.

Despite these optimistic future perspectives, is essential to emphasize that most of the current evidence regarding the use of melatonin in dogs is derived from anecdotal experiences or isolated case reports within an integrated approach (Aronson, 1999; Denenberg, 2021; Sacchettino et al., 2023). Pharmacodynamic research and randomized clinical trials, which are essential for establishing doses, long-term efficacy, and safety, remain scarce.

Future studies must concentrate on accurately delineating specific molecular processes in dogs, including melatonin's impact on serotoninergic, dopaminergic, and GABAergic circuits, as observed in other species (Miguez et al., 1994; Haduch et al., 2016).

However, given the current limitations in evidence-based pharmacological data, a more immediately strategic and functional perspective is required for patient care. Accordingly, an integrated and multidisciplinary strategy for managing canine behavioral diseases is paramount (Sacchettino et al., 2023). This approach, which necessitates robust collaboration among veterinarians, behavior specialists, nutritionists, neurologists, and other relevant experts, ensures that all influencing factors—from stress and physiology to environment and diet—are simultaneously addressed.

Only through this enhanced multidisciplinary framework, supported by ongoing research and scientific rigor, will the full therapeutic potential of melatonin be elucidated, providing clinicians and caregivers with more credible, evidence-based resources to enhance the emotional well-being and behavioral health of dogs.

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## Melatonina nei cani: meccanismi e applicazioni per i problemi comportamentali. Analisi sullo stato dell'arte attuale e prospettive future

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*Sintesi*

La melatonina è un neuroormone multifattoriale sintetizzato primariamente dalla ghiandola pineale, che offre la possibilità di migliorare la gestione dei problemi comportamentali nei cani domestici. Nonostante la sua diffusa applicazione clinica off-label, i dati scientifici a supporto del suo valore terapeutico sono scarsi e disomogenei. Questo commentary riassume le attuali conoscenze relative agli usi clinici e ai meccanismi d'azione della melatonina nell'affrontare i disturbi comportamentali nel cane. L'evidenza clinica ne supporta il potenziale impatto nella gestione dell'ansia preoperatoria, delle fobie da rumore, dei disturbi correlati alla separazione, dei comportamenti compulsivi e della disfunzione cognitive canina. Il dosaggio consigliato è di 1,5 mg per i cani di piccola taglia e di 3-6 mg per i cani di grande taglia. Il dosaggio può essere somministrato ogni 8-24 ore, a seconda delle circostanze terapeutiche. Il profilo di sicurezza è favorevole, con effetti collaterali trascurabili. Sebbene la melatonina si dimostri promettente come trattamento adiuvante per i problemi comportamentali canini, l'evidenza esistente si basa prevalentemente su case report ed esperienze aneddotiche. Per stabilire linee guida basate sull'evidenza per il dosaggio, l'efficacia a lungo termine e i profili di sicurezza, sono essenziali studi randomizzati controllati approfonditi sui cani.

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# Integrating the gut-brain axis, CBD, and melatonin in the management of anxiety and frustration-driven displacement behavior in a Lagotto Romagnolo: An integrated approach

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*Abstract*: Ettore, a two-and-a-half-year-old male Lagotto Romagnolo, underwent a behavioral visit due to difficulty staying calm at home in the presence of the female caregiver. The manifested symptoms—hyperactivity, constant demands for interaction, and displacement behaviors (e.g., scratching, pacing, destructiveness)—were interpreted as the expression of an underlying anxiety exacerbated by chronic frustration, typical of high-drive dogs. The onset coincided with a modification in the female caregiver schedule and diminished availability. The therapeutic protocol integrated melatonin and cannabidiol (CBD) for their anxiolytic and wellness-enhancing effects, alongside focused support for the gut-brain axis, together with a targeted behavioral and environmental modification intervention. Subsequent follow-ups showed a significant decrease in restlessness and an enhancement in autonomy, validating the effectiveness of a multimodal management strategy that incorporates medication, emotional support, and a focus on gut health.

*Key Words*: anxiety disorder; microbiota; nutraceutical; working dog, combined approach.

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## Introduction

Anxiety and hyperactivity within the domestic setting constitute a prevalent issue in veterinary behavioral medicine, especially in breeds chosen for their elevated work drive and physical activity, such as the Lagotto Romagnolo. In these individuals, an insufficient or deteriorating routine may induce persistent frustration, which might further aggravate an underlying anxiety condition (Casey et al., 2022). The observed clinical indications, including displacement behaviors (shaving, turning) and hyperactivity, frequently stem from psychological stress and the dog's coping mechanisms. To tackle this intricate issue, behavioral medicine increasingly employs multimodal strategies that combine behavioral therapy with nutraceutical and pharmaceutical support (Niyyat et al., 2018; d'Angelo et al., 2022; Scandurra et al., 2022; Sacchettino et al., 2023b; Giuliano et al., 2024). Indeed, cannabidiol (CBD) and melatonin are experiencing significant success in veterinary medicine for stress control. CBD has garnered attention for potential antistress and anxiolytic effects, modulating the hypothalamic-pituitary-adrenal (HPA) axis while fostering an overall sense of well-being (Di Salvo et al., 2023; Flint al., 2024; Opyd, 2025). In veterinary clinical practice, melatonin is widely utilized off-label to address emotional discomfort, owing to its advantageous safety profile and convenience of administration, diminishing preoperative alertness and anxiety (Niggemann et al., 2019) while additionally lowering cortisol levels in response to physiological stress (Salavati et al., 2023; Song&Yoon,2025). Furthermore, scientific interest in the gut-brain axis and behavior modulation is increasing, as recent data associate altered microbial profiles (i.e., dysbiosis) with anxiety and fear (Sacchettino et al., 2025, Sacoor et al., 2024). Bidirectional interactions along this axis are known to significantly influence canine anxiety disorders, presenting new challenges for veterinary medicine in using psychobiotics to regulate emotional dominance (Sacoor et al., 2024). The purpose of this clinical case study is

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to describe the response to the one-year integrated treatment given to a Lagotto Romagnolo who was diagnosed with an anxiety disorder and chronic frustration.

## Case Presentation

Ettore is a two-and-a-half-year-old intact male Lagotto Romagnolo. He was adopted, at two months from a working-line breeder At present, he resides with a couple in a suburban setting, near a forest. The caregivers requested a behavioral examination since Ettore struggles to remain calm in a home setting and consistently seeks interaction when the female caregiver is present. His routine was primarily focused on fulfilling his high breed drive, in line with the high working drive suggested by the breeder, incorporating long daily walks with his caregivers (some reaching up to 15 km) and including a significant element of performative activities and olfactory research. During walks, Ettore is quite exploratory and engages his sense of smell intensely. The routine also featured caregivers-managed morning chewing sessions, which included food toys, rabbit ears, and pig ears. He often vomited after consuming food too quickly. The caregivers observed that while Ettore stays calm during the chewing activity, he quickly transitions into a state of constant exploration once it concludes. In addition, Ettore had suffered multiple episodes of gastroenteritis, which were treated with antibiotics, and there has been a recent adjustment in his diet. The behavioral history linked the onset of symptoms to a decrease in the time the female caregiver spent with the dog after starting a new job, disrupting the previous routine's balance. Clinical signs observed in the home included hyperactivity, scratching (at surfaces), digging, spinning, and attention-seeking requests, which were often manifested through destructive acts (such as damaging the couch cover) predominantly performed in the female caregiver's presence. The caregivers reported no issues with sleep, except when they rose during the night to use the bathroom. In such instances, Ettore ensured they safely reached the bathroom before returning to sleep, demonstrating a keen sense of social vigilance.

## Diagnostic Evaluation and Treatment

### *Diagnostic hypothesis*

The diagnostic hypothesis was anxiety disorder (intermittent anxious state) (Masson et al., 2024), with situational anxiety and phobia being ruled out (Gazzano & Ogi, 2020; Landsberg et al., 2023). The clinical signs were severely amplified by frustration (Casey et al., 2022; Landsberg et al., 2013) related to his high working drive and reduced social interaction, resulting in secondary reinforcement-maintained attention-seeking behaviors.

### *Treatment*

At the end of the behavioral visit, it was prescribed to administer melatonin (2 tablets twice daily) plus CBD 10% full spectrum (2 drop in the morning and 2 in the evening) plus psychobiotics based on Lactobacillus rhamnosus, Lactobacillus acidophilus, and Lactobacillus reuteri (Psicobrain®, 1 tablet in the evening). Initial dosages were calculated based on the dog's body weight and adjusted during follow-up, in line with the "Start low and go slow" approach (MacCallum&Russo, 2018). Behavioral prescriptions were provided to enhance calm environments and diminish stimulating activities, particularly those associated with search tasks in wooded areas.

### *Follow-up*

Followed up in 30 days after initiating therapy, the caregivers reported that Ettore was significantly more relaxed; they observed many more instances of calmness and independence. Addi-

tionally, they monitored hematobiochemical parameters, all of which were within normal limits. It was agreed upon to continue with the same plan of treatment for an additional month, warning caregivers that relapses could occur because of the Christmas holiday. Followed up in two months, the caregivers reported that Ettore had experienced a period of decline due to changes in holiday habits during Christmas, compounded by the time spent apart from his female caregiver at home due to COVID-19. Nevertheless, at the time of follow-up, the caregivers reported that the condition had shown considerable improvement since the holidays. He experienced brief periods of evening restlessness, during which he tended to chew his bed. Therefore, it was essential to promote the practice of evening chewing (Uccheddu et al., 2024) by incorporating a Kong stuffed with moist gastrointestinal food. It was also recommended to substitute Psicobrain® with the probiotic Enterelle® (Saccharomyces cerevisiae sub. boulardii, Lactobacillus acidophilus, Enterococcus faecium) for a duration of 15 days, as Ettore was required to take antibiotics for medical purposes. The remaining therapy was based on the prescribed melatonin and CBD. During the follow-up, six months after the initiation of integrated and interdisciplinary therapy, the female caregiver indicated that Ettore appeared significantly calmer and more manageable. Additionally, regarding the issue of frustration, they noted a marked improvement, as he had ceased to exhibit destructive behaviors. At this point, the therapy plan was stabilized to: melatonin (1 tablet twice daily) and CBD 10 % full spectrum (1 drop in the morning and 1 in the evening). After 6 further months of stable treatment, the positive clinical condition was maintained at follow-up.

## Discussion

This case report elucidates the intricate therapy of behavioral disorders in high-driving dogs, exemplified by the Lagotto Romagnolo (working dogs). The caregivers noticed a worsening of behavior following the interruption of the routine, and a decrease in social interaction due to the new employment of the female caregiver. The widespread hyperactivity, heightened social vigilance, and engagement in displacement behaviors (such as spinning and scratching) indicate an underlying intermittent anxious state (Masson et al., 2024). Anxiety has been significantly increased by frustration (Casey et al., 2022) resulting from excessive stimulation, such as excessively long walks (up to 15 km) and performative olfactory games in the woods, which probably intensified his state of excitement rather than alleviating it. The destructive behaviors were systematically recognised as reinforcement-maintained attention-seeking behaviors, indicating that the caregivers' response, regardless of its negativity, functioned as an unintentional form of positive reinforcement (Landsberg et al., 2013). Therefore, modifications to the environment and daily routines—such as the establishment of a consistent and predictable routine, the minimization of stimulating factors, and the incorporation of calming recreational activities—have been prescribed as a fundamental aspect of therapy. Such measures are essential for high-energy dogs exhibiting hyperactivity and frustration, as demonstrated by Casey et al. (2022), since environmental clarity and the restriction of non-functional stimuli are critical for mitigating chronic arousal and social vigilance.

The combined effect of melatonin and CBD full-spectrum has been crucial for symptom relief and the regulation of the neuroendocrine axis. CBD, through its interaction with the endocannabinoid system, has been utilized for its anxiolytic effects, as well as to enhance overall satisfaction and increase patient well-being (Di Salvo et al., 2023), alleviating discomfort and feelings of frustration and the trouble in calming (Marliani et al, 2024). Concurrently, melatonin was utilized for its established mood-stabilizing properties and for the control of excitement (Salavati et al., 2023; Song et al., 2025). As evidence of their combined positive effects, Sacchettino et al. (2023a) published a case report concerning a 5-year-old mixed-breed dog afflicted with a compulsive behavior unresponsive to conventional antidepressant treatments. The researchers utilized

an interdisciplinary and integrative methodology that incorporated three elements: a behavioral program, the administration of cannabis products, and melatonin supplementation (2 mg/day). Clinical observations and caregivers reports suggested that the integrated strategy led to a reduction in the frequency of compulsive episodes and enhanced overall management of the dog compared to previous therapy with paroxetine, with no short-term side effects noted. The authors noted that maintaining a reduction in dysfunctional behavior to a level considered acceptable by caregivers was achieved during a subsequent four-month follow-up period. Thus, it is likely that the combined melatonin-CBD treatment of our patient effectively regulated arousal, utilizing the synergistic effects of both substances for emotional stabilization.

The end of undesirable behaviors in the initial months was directly attributable to the effective application of involuntary reinforcement management (Landsberg et al., 2023). By removing all forms of caregiver attention, including reprimands, and promptly diverting the dog to suitable calming activities, such as a Kong, the reinforcement loop was disrupted. This suggests that the damaging activity was not compulsive but rather instrumental and part of an ineffective coping strategy for frustration.

The administration of psychobiotics was crucial, especially considering the patient's medical history of recurrent gastroenteritis episodes and antibiotic use; lactobacilli-based probiotics offered continuous support to the emotional axis and dysfunctional behaviors (Yeh et al., 2022; Bijaoui&Zimmerman, 2025), in addition to the recolonization of the intestinal microbiota after antibiotic treatment. This underscores the important and interrelated functions of intestinal health, inflammation, and the gut-brain axis in the manifestation and treatment of canine behavioral disorders (Gorzelanna & Miszczak, 2024; Kiełbik & Witkowska-Piłaszewicz, 2024).

## Conclusion

This clinical case study described the response to the one-year integrated treatment given to a Lagotto Romagnolo who was diagnosed with an intermittent anxiety status and chronic frustration. The enduring remission of clinical symptoms was not solely attributable to the administration of melatonin and CBD but rather to the concurrent stabilization of various components. Although integrated neuro-emotional support provided the necessary physiological basis, the key to success lies in the rigorous implementation of behavioral prescriptions. The implementation of attention management and the reduction of excitatory activities were essential in disrupting the negative learning cycle that perpetuated destructive and hyperactive behaviors. This, along with the management of the chewing ritual and the support of the gut-brain axis, facilitated the transformation of the dog's environment and relational routine. This clinical case supports a model wherein pharmacological or nutraceutical therapy does not replace behavioral and environmental modifications but serves as a catalyst that reduces the patient's arousal, enhancing receptivity to learning and facilitating relational changes and routine consolidation into sustained clinical remission.

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## Integrare l'asse intestino-cervello, il CBD e la melatonina nella gestione dell'ansia e dei comportamenti disfunzionali guidati dalla frustrazione in un Lagotto Romagnolo: un caso clinico in approccio integrato

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*Sintesi*

Ettore, un Lagotto Romagnolo maschio di due anni e mezzo, è stato sottoposto a una visita comportamentale a causa della difficoltà a mantenere la calma in casa in presenza della sua caregiver. I sintomi manifestati, quali iperattività, richieste costanti di interazione e comportamenti di distruzione, sono stati interpretati come l'espressione di un'ansia sottostante esacerbata da una frustrazione cronica, tipica dei cani da lavoro. L'insorgenza è coincisa con una modifica degli orari della caregiver e una sua ridotta disponibilità. Il protocollo terapeutico ha integrato melatonina e cannabidiolo (CBD) per i loro effetti ansiolitici e di potenziamento del benessere, affiancati da un supporto mirato per l'asse intestino-cervello, insieme a un intervento di modificazione comportamentale e ambientale specifico. I follow-up successivi hanno mostrato una significativa diminuzione dell'irrequietezza e un aumento dell'autonomia, convalidando l'efficacia di una strategia di gestione multimodale che incorpora terapia farmacologica, supporto emotivo e attenzione alla salute intestinale.