

January 2016

ASSESSMENT OF SEPARATION ANXIETY IN DOGS: THE SEARCH FOR A DIAGNOSTIC METHOD

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**PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance**

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By Gahee Kim

Entitled

ASSESSMENT OF SEPARATION ANXIETY IN DOGS: THE SEARCH FOR A DIAGNOSTIC METHOD

For the degree of Master of Science

Is approved by the final examining committee:

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7/5/2016

Date

ASSESSMENT OF SEPARATION ANXIETY IN DOGS: THE SEARCH FOR A
DIAGNOSTIC METHOD

A Thesis

Submitted to the Faculty

of

Purdue University

by

Gahee Kim

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

August 2016

Purdue University

West Lafayette, Indiana

사랑하는 우리 가족에게

ACKNOWLEDGEMENTS

First and foremost, Dr. Ogata, I would like to thank you for everything you did for me. Your time, your understanding, your patience, your generosity, your advice and literally everything you did. I know how you cared for me to learn as much as possible, but you also cared for me outside of academia. Without your consideration, I would not have made it this far with my second language in this foreign country. Whenever I was stuck, I could recover due to your encouragement. I always think I am so lucky to have you as my advisor, and I know it was difficult to have a student like as strange as Lupa.

I also very appreciate the committee faculties who I worked with. Dr. Wilson, I really appreciate your time and help. Thank you for your wonderful guidance, I got to know how interesting it is to work in the laboratory. Dr. Weng, thank you for your work and time and advice for this study from start to finish. Thank you for all of the time you spent on me whenever I needed your help. I also really appreciated your cooperation even during your vacation far away from here.

Mindy, thank you for your help and friendship for the 2 years I was here. I was glad to have you in the behavior team. I would like to thank my family for always believing in and supporting my decisions. I know it is a rare blessing to be given an opportunity to study what I want abroad, and I believe it is a blessing to have a great family like you who has always supported me in doing what I wanted to do. I know being apart in a

different country is difficult yet you have given me endless love and support, and I thank you for this with all my heart.

Last, thank you to all the owners and their dogs who have participated in my study. I appreciate your participation and cooperation. You have made me want to be a better veterinarian.

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ABSTRACT

Kim, Gahee. M.S., Purdue University, August 2016. Assessment of Separation Anxiety in Dogs: The Search for a Diagnostic Method. Major Professor: Niwako Ogata.

The aims of this study were: (1) to explore the environmental factors and behaviors associated with the owners' perception of separation anxiety based on the questionnaires completed by the owners along with video recordings of their dogs during the owners' absence, and (2) to determine whether clotting conditions and hemolysis status affect canine serum BDNF levels. This study has two parts based on the two aims.

In the first part of study, the dogs were divided into a presumed separation anxiety group (P-SA group) and a presumed no-separation anxiety group (P-C group) based on owner's report via a questionnaire. Multiple logistic regressions analysis on the variables collected from questionnaire showed that the dogs without other dogs in the same household were 8.5 times more likely to be in the P-SA group (95% CI: 1.1 - 62.5). While in the video analysis, the duration of the behavior, called passive behavior, was significantly longer in the P-C group than in the P-SA group ($p = 0.008$ and, 0.004). When it comes to a behavior pattern, if the dog exhibited passive behavior only partially in the analyzed video of two sessions (T1 and T2), then these dogs were 15.2 times more likely to be in

the P-SA group compared to the one that exhibited passive behavior in both T1 and T2 sessions (95% CI:0.9 - 252.4). If the dog did not exhibit passive behavior in neither T1 nor T2, the dog was 25.2 times more likely to be in the P-SA group compared to the dog exhibit passive behavior in both of T1 and T2 (95% CI: 1.6 - 404.0).

In the second part of this study, various pre-analytical factors including clotting conditions and hemolysis conditions were compared to validate peripheral brain-derived neurotrophic factor (BDNF) as a possible biomarker. For clotting condition experiment, the clotting times ranged from 30 minutes to 2 hours at room temperature and from 30 minutes to 24 hours at 4°C. For the hemolysis study, three hemolysis concentrations, non-hemolysis, moderate hemolysis, and marked hemolysis were evaluated while adhering to the BDNF ELISA kit criteria. The clotting time, clotting temperature and sample hemolysis that are of concern in a clinical setting did not affect canine serum BDNF concentration.

Overall, this study found that the differences of owners' perception and the dogs' environment as well as dogs' behaviors between the P-SA group and the P-C group. However, more important thing is validity of patterns and duration of dog's behavior (i.e. passive behavior) during the owner's absence and it still needs careful interpretations. Considering the result from the second part of this study, a further investigation about canine peripheral BDNF as a potential biomarker, will supplement current diagnostic methods for separation anxiety.

CHAPTER 1. INTRODUCTION

Canine separation anxiety is the most common behavioral problem in dogs presented to animal behavior clinics in North America.¹ It occurs during both the owner's actual and virtual absence with various behavioral signs, including destruction, vocalization, and house soiling.¹ The consequences of separation anxiety often cause distress to owners and the collapse of the human-animal bond, which results in the abandonment of their dogs to shelters as well as a negative impact on the dog's own well-being.^{2,3}

Although the diagnosis of separation anxiety is often made based on owner-reported behavioral history and by ruling out other possible medical and behavioral problems, a definitive diagnostic method has not been established.⁴ If the clinical signs are either unspecific or not reported by the owner, it is possible for some cases to remain undiagnosed. Therefore, finding a clinically feasible and objective diagnostic method for canine separation anxiety is in need.

One possible solution may be the utilization of neurobiological measurements. Studies in humans have shown that a type of neurotrophin, the brain-derived neurotrophic factor (BDNF), promotes neuron health and survival in peripheral and central nervous systems.⁵ There have been reports that BDNF is involved in anxiety-related behaviors in

animal models using rodents, and many kinds of stressors have been reported to reduce the levels of BDNF.⁶ Because BDNF is a possible diagnostic tool for psychiatric diseases in humans, we speculate that it may also be a potential biomarker for canine behaviors, namely separation anxiety. According to studies in humans, pre-analytic factors, such as clotting conditions or storage temperature and duration, affect BDNF levels in the blood, while no studies have investigated canine BDNF in blood. Thus, as a first step we investigated whether pre-analytic conditions could potentially affect canine blood BDNF.^{7,8}

The aims of this study were: (1) to explore different factors and behaviors associated with self-reported separation anxiety with the traditional diagnosis methods which consist of questionnaires completed by the owners along with video recordings of their dogs during the owners' absence, and (2) to determine whether clotting conditions and hemolysis status affect canine serum BDNF levels.

The hypotheses of this study were: (1) There were differences of owner's perception, and the dog's environment when the owner presumed their dog had a separation anxiety, and (2) There were behavioral differences from video recordings between dogs with and without presumed separation anxiety. Last, (3) Clotting conditions and hemolysis status didn't affect canine serum BDNF levels. The first two hypotheses were tested in the study presented in Chapter 3 and the third hypothesis was tested in the study presented in Chapter 4.

CHAPTER 2. LITERATURE REVIEW

2.1. Problem definition

Canine separation anxiety is a behavioral problem in dogs that occurs when the dog is separated from its owner.¹ The most common chief complaints in dogs with separation anxiety presented to behavior clinics are destructive behavior, inappropriate elimination, and vocalization.⁹⁻¹² Destructive behavior is the most obvious sign detected by owners after returning home and the damage of property by destruction is also directly related with their economic concerns.^{3,11} One study distributed questionnaires to owners who had relinquished their dog to an animal shelter. Destruction of property during their absence was the second most common behavioral reason for surrendering ownership, with hyperactivity being the most common reason.² Another study analyzed videos of 20 dogs with separation anxiety and found that the dogs would destroy doors, windows, or other types of similar exits where the departure of their owner was directly observed.¹³ Vocalization is also a commonly displayed sign and is the frequent cause of complaints from neighbors.^{1,13} By analyzing video recordings of dogs with separation anxiety, Lund and Jørgensen (1999) found that barking tends to be caused by arousal and that most vocalization resulting from separation anxiety is a mix of barking, whining or howling rather than a single pure type of vocalization. Since vocalization can be triggered by non-

separation anxiety related factors ranging from arousal to external stimuli such as looking at strangers outside of windows, differential diagnoses for vocalization is required to rule out non-separation anxiety related cases.¹ Since inappropriate elimination is a response associated with excitement or fear, even properly house-trained dogs can exhibit house soiling during the owner's absence if afflicted by separation anxiety. They tend to urinate and/or defecate immediately after the owner's departure regardless of the time interval from their last most recent elimination time.¹⁴ Although some owners are still able to detect residual evidence of elimination after their return, this evidence is not always present due to the urination drying up or being wiped out by the dog's movement and detection is often times difficult or not possible.

In addition to the common symptomatic behaviors mentioned above, other signs including aggression towards their owners at the time of their departure, psychosomatic signs such as tachycardia, tachypnea, hyper-salivation, trembling, and an increase in motor activity such as restlessness such as pacing, circling, digging or excessive licking have also been reported. Separation anxiety is also known to manifest as behavioral depression, such as a lack of play, immobility, or decrease in appetite.^{4,10,14,15} Regardless of which of these various behavioral signs are exhibited, most will occur a short time after the owner's departure, reaching a peak during the first 5-30 minutes then repeating this behavior approximately every 25 minutes.^{13,14}

2.2. Demographic characteristics

Separation anxiety cases that are referred to animal behavior clinics in North America account for 20 to 40% of all cases.¹ Two surveys on separation anxiety revealed

that 14% (Allpoints Research, Winston-Salem, North Carolina 1997) to 17% (Lilly Market Research, Greenfield, Indiana, 2006) of dogs that receive regular veterinary care may also be affected, indicating that there might be a larger number of undiagnosed dogs.¹⁶ One study reported that up to 50% of all dogs have expressed separation anxiety related signs at least once in their lifetime.¹⁷

In several studies, male dogs were found to be more commonly associated with separation anxiety than female dogs.¹⁷⁻²⁰ However, other studies found that sex was not associated with separation anxiety.^{11,14}

Separation anxiety is generally believed to be exhibited at any age,^{14,16} although one study reported that the average age of dogs starting to show signs of separation anxiety is approximately 1.5 years old.¹⁹

One may consider genetic predisposition as it was suggested in other behavioral problems such as aggression or compulsive disorders,^{4,21,22} but the genetic contribution to the development of separation anxiety and the presence of predisposed breeds still remains inconclusive. Mixed-breeds were more likely to be presented at behavior clinics for treating separation anxiety than any pure-breed dogs, but this could also be a confounding variable as mixed dogs are likely to be adopted from shelters.^{10,11,20,23} This may suggest the cause is more due to preexisting environmental conditions rather than their particular breed type.

2.3. Etiology

Even though separation anxiety has been studied for several of years, the etiology of separation anxiety is still elusive. Previous experiences of dogs being relinquished by

the owners could relate with separation anxiety, although it is not clear whether the abandonment was the cause or result.^{10,14} Flannigan and Dodman (2001) found that dogs adopted from shelters were more likely to have separation anxiety when compared to dogs adopted from other sources such as breeders, family or friends. Rehoming multiple times could also be a possible cause for canine separation anxiety.²⁴ Sudden change in the environment or daily routine including changes in a family member's schedule could also contribute to the development of separation anxiety.¹

2.4. Social and environmental factors

Some puppies can show stressed signs when they are left alone for the first time but these behaviors are generally less likely to persist.²⁵ However, experience during an early age does have an important role in canine behavioral development and could affect behavioral problems later during adult life.¹⁷ For example, early weaning or an illness between birth to 16 -weeks old have been suggested as a cause leading to separation anxiety.^{9,26}

It has also been reported that having other dogs in the same household does not have any significant impact on the anxiety, but having at least one cat does significantly decrease the occurrence of separation anxiety.²⁷ The relation between the composition of the owners' family members and separation anxiety is still controversial. In a study from Flannigan and Dodman (2001), dogs that had single adult owners were 2.5 times more likely to have separation anxiety. On the contrary, another study found that the majority of the dogs with separation anxiety resided with families consisting of two adults or

adults with children.²⁰ The gender of the owner did not seem to be a factor affecting separation anxiety.¹¹

Hyper-attachment to the owner has also been suggested, but is debated as to whether it is associated with separation anxiety. Several studies reported that dogs with separation anxiety usually tend to show hyper-attachment behaviors, such as excessive greeting responses after the owner's return, shadowing the owner at home, or sleeping with the owner.^{9,28,29} Other studies also found an association by evaluating hyper-attachment through questionnaires.^{11,20} However, two experimental studies that used video analysis concluded that hyper-attachment was not associated with separation anxiety in dogs.^{30,31} Parthasarathy and Crowell-Davis (2006) found that there was no significant difference between dogs with or without separation anxiety in the duration they spent near the owner's exit location. Konok et al. (2011) also analyzed video recordings of dogs greeting their owners. Through the evaluation of their proximity and visual focus direction with respect to the owner as well as behaviors such as tail-wagging or standing on hind legs and putting forelegs on the owner's body, they found that dogs with separation anxiety did not show more greeting affection than those without.

2.5. Treatment

Environmental management

Treatment for separation anxiety is comprised of environmental management, behavioral modification, and medication.¹

As an environmental management method, providing a place where the dog can settle and feel safe helps reduce anxiety.²⁹ To make the place more favorable, using long

lasting and high valued treats or toys can also help.¹ However, confining the dog in a crate before being trained to associate the crate as a safe location is not recommended as it does not help mediate anxiety, and might even worsen it.^{1,32} It has also been reported that regular exercise has positive effects on anxiety. Tiiara and Lohi (2015) found that dogs with separation anxiety appear to have less exercise than those without.

Behavior modification

Behavior modification focuses on building the dog's independence. A pet sitter or a daycare service could help reduce the duration of being alone until the dog gets used to being independent.¹ Establishing predictability in a dog's routine, not only in a daily schedule but also interacting with the owner, helps decrease their anxiety.³² The owner should initiate the interaction and should reward the dog when it behaves appropriately. This provides consistency and predictability which helps the dog learn independence and relaxation.³² Training such as taking obedience classes helps reduce problematic behaviors including separation anxiety.^{33,34} Systematic desensitization of being alone through the increase of isolation time from 5 minutes can also significantly reduce separation-related behaviors.³⁵ Takeuchi et al. (2000) conducted a cohort study to evaluate treatments for separation anxiety. They found treatment plans that the owners were most willing to continue were stopping punishment, followed by increasing exercise and providing toys before their departure.

Medication

The types of medication licensed in the United States to treat separation anxiety in dogs are the selective serotonin reuptake inhibitor (SSRI) fluoxetine and the tricyclic antidepressant (TCA) clomipramine. Fluoxetine affects serotonin by blocking the neuronal presynaptic membrane transporter and inhibits synaptic reuptake of serotonin, while clomipramine acts on neurotransmitters including serotonin, dopamine, norepinephrine and metabolites, all of which are involved in anxiety-related pathway and have anticholinergic effect. An oral administration of 1-2 mg/kg/day of fluoxetine has been demonstrated as an effective treatment for canine separation anxiety when it was administered along with behavior modifications.³⁶ In a larger study, Landsberg et al. (2008) found that an oral administration of 1 - 2mg/kg/day of fluoxetine without behavior modification also showed improvement while a standard dose (1 - 2mg/kg, q 12h) of clomipramine showed no undesirable long term effects for more than 13 months of administration.^{37,38} Since these medications take 4 to 6 weeks to reach clinical therapeutic levels, there are other medications that can be administered in combination with fluoxetine or clomipramine, although they are not licensed for canine separation anxiety. Examples of these medications that are commonly used in practice are benzodiazepines such as diazepam or alprazolam, an alpha-2 adrenergic receptor agonist such as clonidine, or a serotonin 2A antagonist/reuptake inhibitor such as trazodone.^{39,40}

2.6. Neurobiological approach to separation anxiety and peripheral brain-derived neurotrophic factor (BDNF)

The psychotropic medications mentioned above are proven to be an effective method of treatment in separation anxiety, which suggests there may be an underlying neurobiological mechanism of this condition. One such neurological mechanism that has been investigated includes involvement of brain-derived neurotrophic factor (BDNF). BDNF is one of the neurotrophins, which plays a crucial role in neuronal survival, death and neuroplasticity.⁵ It has been also reported that BDNF has a pivotal role in managing stress by regulating the hypothalamic-pituitary-adrenal (HPA) response as well as memory.^{41,42} One study postulated that modulated BDNF concentrations in the hippocampus and amygdala could account for stress-related psychiatric disorders.⁴³ BDNF passes the blood-brain barrier, so blood concentrations of BDNF are likely to reflect brain concentrations of BDNF.⁴⁴ Indeed, positive correlations between BDNF in brain as well as peripheral concentrations have been shown in humans, rodents and pigs.⁴⁴⁻⁴⁶ Based on the role BDNF plays in the central nervous system, it has been reported in rodent studies that the brain concentrations of BDNF are involved in anxiety behaviors.⁶ Studies in humans reported that affective disorders, such as obsessive-compulsive disorder, schizophrenia, eating disorders, and depression appear to be associated with decreased levels of BDNF.⁴⁷⁻⁵⁰ It has also been reported in other studies that the BDNF concentrations in depressive patients increased after they were administered antidepressants such as selective serotonin reuptake inhibitors, tricyclic antidepressants, serotonin norepinephrine reuptake inhibitors, and tetracyclic antidepressants.⁵¹⁻⁵³ In a meta-analysis study, it was shown that BDNF is regarded as a

potential, peripheral biomarker that can be used to diagnose psychiatric disorders as well as to evaluate the treatment efficacy of mood disorders.⁵⁴

The most common method of measuring peripheral BDNF concentrations in serum, plasma, and platelets from rats and humans is enzyme-linked-immunosorbent-assay (ELISA).^{55,56} It has been shown that blood platelets are the main reservoirs of blood BDNF, from which it can be released during platelet activation or coagulation processes, and that serum BDNF concentration was 10 times higher than that of plasma.⁵⁶ There are various factors in humans that can affect peripheral BDNF concentrations including age, weight, fasting state, drinking, smoking, exercise level, and living environment.^{8,57,58} Additionally, it has been suggested that pre-analytical factors, such as sample handling and storage conditions, can affect peripheral BDNF concentrations. However, it is unclear how these pre-analytical factors affect BDNF, and the results from studies and among species have been inconsistent.^{7,8,59-62} For example, Maffioletti et al. (2014) reported that in human samples at room temperature, serum BDNF concentrations continued to increase at 10 minutes, and continued to increase until 1 hour of clotting time¹⁷. Another study reported that BDNF was affected by the clotting temperature, and degraded unless the sera were kept at 4°C.⁸

Another factor that can affect peripheral BDNF concentrations is the degree to which a serum sample is hemolyzed. Hemolysis can occur due to mishandling the collection of blood in a clinical setting. Hemolysis can prevent analyzers from measuring the absorbance of light as a result of a color reaction, which is used to accurately quantify BDNF.⁶³ To date, there have been no studies in any species that have investigated to show if the effect of hemolysis on serum BDNF concentrations when measured by

ELISA and thus the effect of these factors on peripheral BDNF levels in dogs need to be validated.

CHAPTER 3. QUESTIONNAIRE AND VIDEO ANALYSIS OF DOGS WITH AND WITHOUT SEPARATION ANXIETY

3.1. Specific aims

There are two specific aims in this cross sectional study. The first specific aim of this study was to explore factors associated with owner's self-report of the presence of separation anxiety through the questionnaire and the second specific aim was to assess the differences in behavior categories of video analysis between dogs with and without the presumption of separation anxiety.

All study protocol was approved by the Purdue Animal Care and Use Committee (Approval # 1501001179).

3.2. Materials and methods

Subjects

Study dogs were recruited from March to November in 2015. All dogs in this study were recruited via fliers posted at local veterinary clinics in Lafayette and West Lafayette in Indiana, on the Purdue University West Lafayette campus, at pet-related businesses such as dog daycares, and pet friendly apartments and patients presented to the Animal Behavior Clinic at the Purdue University Veterinary Teaching Hospital. Inclusion criteria for the study dogs were of any breed, neutered in both sexes, and aged between

1.5 and 7 years. Other criteria stipulated that the current owner should have owned the dog for more than 1 month and that the dogs should be clinically healthy defined by blood work (i.e., complete blood count, and biochemistry panel). If no blood work had been done in the past 6 months, complete blood count (CBC), biochemistry panel, and thyroxine (T4) level were performed at their visit. Dogs who were currently not receiving any pharmacological treatments except for external and internal parasite preventions were included in the study. If the owners thought their dogs had separation anxiety, at least one of the separation anxiety signs should have been observed for a month or more without implementing any ongoing treatment.

Methods

An online-based behavior questionnaire and a 20-minute video recorded at home during owners' absence were used for this study. All dogs were divided into presumed separation anxiety group (P-SA group) and presumed no-separation anxiety group (P-C group) based on the owners' reports in the questionnaire and the dogs in the P-SA group should have no primary behavior problems other than the signs of possible separation anxiety. While the dogs in the P-C group should have no clinical behavior problems at the time of the study. At the end of recruitment, 16 dogs for the P-SA group and 17 dogs for the P-C group were included in the study.

Questionnaire: The questionnaire (Appendix A) used in this study was modified from the one used in a previous study by Tiira and Lohi (2014).⁶⁰ The 23 questions in the questionnaire included general information on the dog's breed, current age, sex,

background information (age at adoption, origin), environment (other dogs or cats in the household) and daily schedule (frequencies of regular walk and amount of other exercise in addition to a regular walk, consistency of daily schedule, hours being left alone daily, confinement during the owner's absence as well as at night, training history). The questions related to the presence of aggressive behaviors towards strangers, familiar persons including the owner and family members, unfamiliar dogs, and familiar dogs (including housemate dogs) were also asked. If the owner thought their dogs had separation anxiety, questions were asked regarding the age of onset and separation anxiety signs were asked. Additionally, the specific questions about the presence of certain behaviors such as presence of fear and anxiety towards noise, strangers, unfamiliar dogs, new environment or situation, and during owner's absence were asked. It was also asked whether they had sought out advice/help from a primary veterinarian, trainer or the internet for anxiety signs.

Video film: The owners were asked to record their dogs from the owners' departure to return, for a minimum of 20 minutes. The owners used their own equipment such as an iPad or a camera (Kodak Zi8 Camcorder®) provided by us for recording. The owners were asked to turn on the camera right before they left their house and to turn it off right after their return. The video camera was set in the room where the dog usually stayed during the owner's absence. If the dog was left out of a crate and roamed freely in the house, the video camera was set in a space where the dog was likely to stay for most of its time. After the owners filmed their dogs, they uploaded the videos on YouTube, Dropbox, Google drive, or a USB drive or saved the file on the camera provided by the

investigators. At the end of the study, when the owners reported that they thought their dogs had separation anxiety or if we found the dogs showed anxious signs in the videos, we provided the owners with advice on how to manage their dogs' anxiety.

Data analysis

The statistical significance for all analyses were set at $P < 0.05$ unless otherwise stated, and the analyses were performed using IBM SPSS Statistics for Windows (Version 22.0, Armonk, NY: IBM Corp).

Questionnaire: The answers to the behavior-related questions were coded as the absolute and proportional frequencies. Breed, adopted age, having other animals such as dogs or cats in the same household were dichotomized (Table 3.1). All information related with age except for current age was calculated in months. Birth date derived age was basically used for current age. When the date of birth was not clear, the owner reported current age based on the estimated age by the dog's veterinarian or the shelter where they adopted the dog from. The number of daily walks was divided into 3 categories: less than once a day, once a day, and more than twice a day. For amount of exercises other than daily walks, dogs were assigned to one of the 3 following groups: Up to 30 minutes, 30 minutes to 1 hour, and > 1 hour. Attending puppy training and adult training classes were dichotomized (Table 3.1). If the dogs were only trained by the owner at home, they were categorized as not attending any training classes. The total length of time that the dogs were left alone at home was divided into three categories: Up to 4 hours, 4 to 8 hours, and > 8 hours. The presence of the confinement place during its sleep and the owner's

absence was also asked and dichotomized (Table 3.1). If the dog was limited access to the rest of the house, it was regarded as confinement even though the dog might not be in a crate. To determine associated factors of owner's self-report of separation anxiety, all answers including general information, environment and daily schedule, the presence of aggressive behaviors, the presence of fear and anxiety signs, and the presence of separation anxiety signs from the questionnaire were analyzed in this study.

Descriptive analyses were performed on the answers to general information of the dogs and the dogs' behavior information on the questionnaires. In order to investigate factors associated with self-report separation anxiety through the questionnaire, each answer from questionnaire was initially investigated using simple logistic regression. In order to analyze the association between the multiple variables and presumed separation anxiety, multiple logistic regression analysis with a forward stepwise selection was also used. The variables that resulted in a $p < 0.1$ in the simple logistic regression were used as an independent variable while the presence of separation anxiety according to the owner's report was used as a dependent variable in multiple logistic regression.

Video film: Recorded video was segregated by the first 5 minutes (T1) and the last 5 minutes (T2) out of the 20-minute recording for data analysis (Figure. 3.2). The modified ethogram from the previous study by Cannas et al. (2014) consisted of 22 behavior categories was used (Table 3.2).⁶¹ A focal animal sampling and continuous recording method (Martin and Bateson, 2007) were used to code the dogs' behaviors.⁶² Eighteen behavioral categories (exploration, locomotion, passive behavior, orientation to environment, scratching, oral behavior, play, panting, not visible, grooming, barking,

whining, howling, growling, trembling, circling, drinking, and eating) were recorded as a duration in seconds and four behavioral categories (pawing up, yawning, lip licking, and elimination) were recorded in frequency. If the dog was out of the video frame for longer than 1 minute in each of T1 or T2, that video clip was excluded from the evaluation. Two observers were trained to analyze the video recordings. The values of each observer were compared by the Wilcoxon signed-rank test to evaluate reliability of the values of observers.

Four behaviors (oriented to environment, passive behavior, play, panting) in which the average duration of the dogs in each group was longer than 30 seconds in each clip (T1 or T2) were selected for further analysis. Then, based on whether each of those 4 behaviors occurred in (1) none, (2) either, or (3) both of T1 and T2 the dogs were categorized into 3 groups, the behavior was considered occurring if it was observed for more than 30 seconds in one session.

Statistical analysis: To test the second hypothesis, which is “there were behavioral differences from video recordings between dogs with and without presumed separation anxiety”, the duration of each behavior between the P-SA group and the P-C group at T1 and T2 was separately compared using the Mann-Whitney U test. Also, logistic regression was used to evaluate correlation between separation anxiety and selected behavior categories. Crate usage was adjusted as a confounder in the logistic regression as it could limit movement of dog which might affect type and duration of behavior exhibited by dog. Median and range of the duration for each recorded behavior was reported and used in the comparison between the P-SA group and the P-C group.

In separate analysis from the P-SA and the P-C group, based on the crate usage from video clips, all dogs were divided into the “in-crate” group (IC group) and the “out-of-crate” group (OC group) to compare the behavior differences between dogs’ in-crate (IC group) and out-of-crate (OC group) were also compared.

3.3. Results

3.3.1. Questionnaire

Based on the owners’ report of the presence of separation anxiety signs, the dogs were divided into two groups, the presumed separation anxiety group (P-SA group) and the presumed control group (P-C group). A total number of 33 dogs were recruited for this study in which 16 dogs were placed in the P-SA group and 17 dogs in the P-C group.

Descriptive statistics for questionnaire

Signalment: In the P-SA group, 7 dogs (44%) were pure breeds and 9 dogs (56%) were mixed breed. In the P-C group, 11 dogs (65%) were pure breeds and 6 dogs (36%) were mixed breed. The median age was 2.6 years old (ranging from 1.0 to 7.6 years) for the P-SA group and 3.2 years old (ranging from 1.9 to 6.7 years) for the P-C group (Fig. 3.2). All dogs were neutered, with 7 (44%) female and 9 (56%) male dogs in the P-SA group, while 10 (59%) females and 7 (41%) males in the P-C group.

Background information: The median age at adoption for the P-SA group was 15.5 months (range from 2 to 42 months) and 2.5 months (range from 2 to 60 months) for the P-C group (Figure 3.3). 3 dogs (19%) in the P-SA group were adopted at an early age

(i.e. adopted younger than 3-month old) while the number of dogs in the P-C group was 10 (59%). Two dogs (13%) in the P-SA group and 9 dogs (53%) in the P-C group were acquired from breeders while 13 dogs (81%) in the P-SA group and 7 dogs (41%) in the P-C group were from shelters or rescues.

Social and environmental information: In the P-SA group, 5 owners (31%) had at least two dogs in the same household while 3 owners (19%) had at least one cat. In the P-C group, 13 dogs (77%) had at least one other dog and about half ($n = 9$, 53%) had at least one cat in the same household. Among total 33 dogs, 6 pairs of the dogs were from the same household in this study. The number of pairs, both of which were included in the P-C group was 3. The other 3 pairs were composed of one in the P-SA group and the other one was in the P-C group. There were 2 dogs (13%) in the P-SA group and 7 dogs (41%) in the P-C group that were trained as an adult. Regarding puppy training, none of the dogs in the P-SA group had received any training as a puppy while 2 dogs (12%) in the P-C group had received puppy training.

Nine dogs in both the P-SA group (56%) as well as in the P-C group (53%) had a consistent schedule. During the owner's absence, 9 dogs (56%) in the P-SA group and 9 dogs (53%) in the P-C group were allowed to roam freely in the house, and 5 dogs (31%) in the P-SA group and 6 dogs (35%) in the P-C group were confined during the night. Lastly, 13 dogs (81%) in the P-SA group and 13 dogs (77%) in the P-C group experienced changes in their environment or schedule such as moving since they were adopted.

Other information coded as ordinal variables were presented in Table 3.3.

Separation anxiety profile: For the 16 dogs in the P-SA group, the median age when the signs were first observed was 18 months (ranging from 2.5 to 36 months). Of these dogs, 7 (44%) had sought out advice/help from a primary veterinarian. The number of dogs who had sought out advice/help from a trainer was 1 (6%), and from the internet was 2 (13%).

Fear of noises: Ten dogs (63%) in the P-SA group and 7 dogs (41%) in the P-C group exhibited fear of noises. Half of the dogs in the P-SA group ($n = 8$, 50%) had fear of fireworks and thunderstorms while 5 dogs (29%) had the same fear in the P-C group.

Aggression: The number of the dogs that showed aggression toward strangers was 5 (31%) in the P-SA group and 7 (41%) in the P-C group. Three dogs (19%) in the P-SA group and 11 dogs (65%) in the P-C group showed aggression toward familiar person including owners. The number of the dogs that showed aggression towards an unfamiliar dog was 7 (44%) in the P-SA group and 11 (65%) in the P-C group. Three dogs (19%) in the P-SA group and 7 dogs (41%) in the P-C group also exhibited aggression towards familiar dogs.

Variables related to separation anxiety

The simple logistic regression analysis on the individual variables showed that most of the variables were not associated with presumption of the presence of separation anxiety (i.e., $p \geq 0.1$). The list of p-value results is shown in Table 3.4. Four variables were shown to be associated with self-report separation anxiety (i.e., $p < 0.1$). Those

variables were: having at least one other dog in the house ($p = 0.012$), having at least one cat in the house ($p = 0.024$), age when the dog was adopted ($p = 0.051$), and whether the age of adaption was younger than 3 months old ($p = 0.024$). These four variables were further analyzed in the multivariate logistic regression, and one had significant effect to pursue the final model (i.e., $p < 0.05$). The result showed that the dogs that did not share homes with another dog were 8.5 times more likely to be in the P-SA group (95% CI: 1.1 - 62.5). This answered the first aim of this study, which is that there were associated factors of the presence of separation anxiety from the owner's self-report.

3.3.2. Video film

Descriptive statistics for videos

Among the 33 owners who had filled out the questionnaire, 30 had also submitted video recordings of their dogs. In 7 of these video submissions, the dogs were not visible for more than 1 minute in either the first (T1) or last (T2) 5 minutes of the recording, and were therefore excluded from further analysis. As the result, a total of 11 dogs in the P-SA group, and 12 dogs in the P-C group were used in the video analysis. Among 22 behaviors based on the ethogram (Table 3.2), the average duration which were summed each dog's 4 main behaviors (Oriented to the environment (OE), passive behavior (PA), play (PL), panting (PT)) were more than 30 seconds of total 5 min of time session (T1 or T2) in each group, and therefore were selected for further video data analysis. A comparison of the duration of the 4 main behaviors of individual dogs between the P-SA

group and the P-C group, and between the IC group and the OC group is shown in Figure 3.4 and Figure 3.5.

Results of 4 main behaviors at each time session

(1) Main behaviors observed in the P-SA group and the P-C group in T1

The median duration of the 4 main behaviors of each group in T1 is shown in Table 3.5. OE was observed in all 11 dogs in the P-SA group and 10 out of the 12 dogs in the P-C group. In the P-SA group, PA was observed by 2 dogs out of the 11 and in 9 out of the 12 dogs in the P-C group. PL was observed by 4 dogs in the P-SA group but none of the dog in the P-C group during T1. PT was observed by 1 dog in the P-SA group and by 2 dogs in the P-C group.

(2) Main behaviors observed in P-SA group and P-C group in T2

The median duration of the 4 main behaviors of each group in T2 is shown in Table 3.6. In the P-SA group, OE was observed by 8 out of the 11 dogs and was observed by 6 out of the 12 dogs in the P-C group. PA was observed by 6 out of 11 dogs in the P-SA group and by 11 out of 12 dogs in the P-C group. PL was observed by 3 dogs in the P-SA group and by none of the dogs in the P-C group. PT was observed by 4 dogs in the P-SA group and by 2 dogs in the P-C group.

(3) Usage of crate

Based on the usage of crates observed in the video recordings, all dogs were divided into 2 groups as an alternative analysis: 15 dogs were included in the “in-crate”

group (IC group) and 8 dogs were included in the “out-of-crate” group (OC group). This variable was investigated as a confounder in the multivariable analysis.

(4) Main behaviors observed in IC group and OC group in T1

The median duration of the 4 main behaviors of two groups in T1 is shown in Table 3.7. OE was observed by 14 out of 15 dogs in the IC group and by all 8 dogs in the OC group. PA was observed by 6 out of the 15 dogs in the IC group and 5 out of the 8 dogs in the OC group. PL was observed by 2 dogs in the IC group and by 2 in the OC group, and while PT was observed by 3 dogs in the IC group, none of the dog in the OC group had PT.

(5) Main behaviors observed in IC group and OC group in T2

The median duration of the 4 main behaviors of each group in T2 is shown in Table 3.8. OE was observed by 9 out of 15 dogs in the IC group and by 5 out of 8 dogs in the OC group. PA was observed by 12 dogs in the IC group and 5 dogs in the OC group. In the IC group, only 1 dog was observed to display PL and in the OC, 2 dogs were observed to display PL. PT was observed by 4 dogs and by 2 dogs in the IC group and OC group respectively.

Behavioral differences from video between the P-SA group and the P-C group

The duration of PA in T1 was significantly longer in the P-C group than the P-SA group ($p = 0.008$). PA in T2 was also significantly longer in the P-C group compared to the P-SA group ($p = 0.044$). However, there were no significant differences between the

IC group and the OC group in the duration of the 4 behaviors for either T1 or T2. With 3 categorized groups based on whether a behavior occurred in none, either, or both T1 and T2, the result showed that there was a negative association between how many sessions the dog exhibited PA in and separation anxiety after adjusting for crate usage ($p = 0.047$). If the dog exhibited PA in either of T1 or T2, then the dog was 15.2 times more likely to be in the P-SA group compared to the dog that exhibited PA in both of T1 and T2 (95% CI: 0.9 - 252.5). If the dog did not exhibit PA in either of T1 or T2, the dog was 25.2 times more likely to be in the P-SA group compared to the dog exhibit PA in both of T1 and T2 (95% CI: 1.6 - 404.0). These results support the second aim which is “there were behavioral differences from video recordings between dogs with and without presumed separation anxiety.”.

3.4. Discussion

The aims of this cross-sectional study were; (1) to explore factors associated with the presence of separation anxiety from the owner’s report of the questionnaire, and (2) to assess the differences in behavior categories of video analysis between dogs with and without the presumption of separation anxiety. The influence of crate usage at the owner’s absence was also investigated as a confounding variable in statistical analysis. To collect variables between the presumed separation anxiety group (P-SA group) and the presumed control group (P-C group) from a total of 33 dogs, the traditional diagnostic method was used that was the questionnaire filled by owners. Several previous studies have reported that the information from the owner-filled questionnaire were reliable.^{31,64,67} For example, according to Hsu and Serpell (2003), behavior information

provided by the owner through questionnaire was positively associated with behavioral diagnoses by a behaviorist.⁶⁷ These diagnoses were aggression towards owner and stranger, stranger directed fear, and separation behavior. Konok et al., (2011) and Tiira and Lohi (2014) also reported that owners' report was reliable based on the correlation analysis between the questionnaires and the behavioral tests regarding separation anxiety, fear toward strangers, and fear toward novel objects.^{31,64}

In our study, the multiple logistic regression was performed to find significant associated factors for the presence of separation anxiety in data obtained from self-report questionnaire. As a result, one variable (i.e. "having other dog in the same household") had significant differences between the P-SA group and the P-C group. Based on the odds ratio, it was interpreted that the single dog in the household was 8.5 times more likely to be in the P-SA group ($p = 0.036$). Having another dog during the owner's absence could help to reduce the signs of separation anxiety that would have been apparent if the dog was alone, however, our result regarding having multiple dogs in the household is inconsistent from the previous studies. McBride et al. (1995) followed up via phone or mail for 197 dogs that were adopted from 2 shelters in southern England, and found there was no association between having other dogs in the same house and separation anxiety.²⁷ A retrospective case-control study based on the medical records comparing 200 dogs with separation anxiety and 200 control dogs with other behavioral problems from the Behavior Clinic at Tufts University, School of Veterinary Medicine also showed that having other pets in the same house was not associated with separation anxiety although it was not clearly defined what species of pets they were that lived with the dog in the study.¹¹ This study had included all other non-canine species which could

have included those such as cats and that could have resulted in the discrepancy between the results from this study and ours.¹¹

The other study which was conducted by analyzing questionnaire data from 3,264 family dogs in Finland investigated that the associations between environmental factors and canine anxiety and found that the number of dogs in the house was negatively associated with fearfulness including fear of noise and separation anxiety, however, the direct association between the number of dogs in the household and the presence of separation anxiety was not found.⁶⁴

It is critical to note that our result shows the association between having other dog in the same household and separation anxiety. Therefore, to deeply understand the causal factor between having other dog and the presence of separation anxiety, more detailed information should be collected from questionnaire. Questions should be asked would be how long the dogs have been together in the same house, and their social relationship, and the number of dogs in the house with larger sample size in future study.

The dogs in the P-C group were supposed to have no clinical signs of any behavior problems but answers in the questionnaires showed 7 (41%) of them exhibited aggression toward strangers and 11 dogs (65%) exhibited aggression toward familiar person including owners. Eleven dogs (65%) in the P-C group showed aggression toward an unfamiliar dog and 7 dogs (41%) in the P-C group exhibited aggression towards familiar dogs. But other than aggression at unfamiliar dogs of 2 dogs, their aggression was scored not severe by the owner.

Due to our exclusion criteria no dogs received any ongoing treatment for their self-reported separation anxiety behaviors in our samples, however, 63% of the owners in

the P-SA group had sought out help or advice through primary veterinarians, trainers or the internet and specifically 44% of the owners in the P-SA group had sought out advice from their primary veterinarian. This might imply that regardless to seek advice from the veterinarian, the clinical signs were not too severe to start any treatments. Alternatively, if most owners' early inquiry wasn't received well by their primary veterinarian to the point to provide an early intervention to prevent worsening the problem. If it is the case, this could lead having the case worsen and the case might end up with a break of the bond between the dog and its owner. Moreover, 38% of the owners in the P-SA group had never tried anything for separation anxiety signs of their dogs although they thought their dogs had separation anxiety. Thus, this result showed the important role of primary veterinarians as they could be the frontline to give an owner a guidance of the treatment to the right direction when early signs of separation anxiety were reported. Based on this result, we would suggest including a list of questions about separation anxiety signs as a part of the routine health examination in dogs at general practices. It would be helpful to monitor the progress of the clinical signs to make an earlier intervention of separation anxiety when it is necessary.

In our video analysis, the result showed that duration of PA of the P-C group was significantly longer than the P-SA group in both of T1 and T2 sessions out of total 20-minute video. The duration of 4 behaviors were also compared by the crate usage and the result did not show any association between usage of a crate and the duration of each behavior. However, the usage of crate was regarded as a confounding variables in further study to investigate the difference in behavioral patterns between the P-SA group and the P-C group because it was expected that a crate could physically restrict the dogs' activity

as a result of which might affect behaviors which the dog could exhibit during the owner's absence. In the result of the difference in behavioral pattern between the P-SA group and the P-C group, we found that the dog that was less likely to exhibit PA was more likely to be included in the P-SA group. After adjusting for crate usage, the results showed that there was an inverse relation between the number of sessions PA occurred in and the likelihood of self-reported separation anxiety.

Our result of comparing durations of behaviors, which showed the duration of PA was the main difference between the P-SA group and the P-C groups, is in accordance with the previous study by Scaglia et al., (2013).⁶⁸ In their study, 20 to 60 minute videos of 30 dogs with non-separation anxiety determined by the owner during their absence were analyzed and it was found that dogs without presumed separation anxiety spent most of their time with passive behavior during alone.⁶⁸ Another study with separation related problems that was diagnosed found that eight dogs out of 23 dogs had passive behavior and 2 of them exhibited passive behavior with trembling.⁶⁹ These imply passive behavior could have two interpretations; exhibited by behavioral inhibition or relaxed state which means PA could be explained to both of anxious signs and relaxed sign.^{68,69}

Oriented to Environment (OE) has been another clinical sign that is difficult to determine the underlying motivation.^{65,68,69} Palestrini et al. (2010) analyzed 20 to 60 minute videos of 23 dogs with separation related problems and found that among 14 categorical behaviors, OE was the second most exhibited behavior following vocalization.⁶⁹ However, Scaglia et al. (2013) studied these behaviors in dogs with self-reported non-separation anxiety raised the question that if OE was a significant behavior to indicate having separation anxiety.^{68,69} Cannas et al. (2014) supported the result of

Palestrini et al. (2010) based on the 22 - 90 minute video evaluation of 23 dogs with diagnosed separation anxiety, finding that among various categorical behaviors the proportion of the duration of OE was the longest, followed by vocalization.⁶⁵

With current knowledge OE can also be interpreted in two ways; as a specific sign of separation anxiety or as a behavioral sign common in dogs with or without anxiety.

These findings indicate the current questions and limitation of interpretation of behavior signs that may appear identical but have different underlying causes when diagnosing of separation anxiety based on video analysis. Behavioral patterns of dogs with separation anxiety has been investigated with previous studies but the number of studies were limited. Lund and Jørgensen (1999) analyzed 4 hour long videos from 20 dogs with separation anxiety with general activity which were defined as bout of transitions between behaviors in 5-min intervals.¹³ They found trends in the time course of activity which were periodic distribution of activity with exponential model. Palestrini et al., (2010) analyzed 40 minute of video from 17 dogs with separation anxiety dogs.⁶⁹ With 10 minute of intervals for the 40 minute, they found barking and OE tended to decrease and PT tended to increase over time. Our study also analyzed pattern of behavior along with those previous studies and investigate the probability of the dog being included in the P-SA group based on the number of sessions which the dog showed PA. However, as noted above, PA could be regarded as both of separation anxiety signs and nonclinical sign, it should be carefully interpreted. The lack of standardized method to evaluate pattern of behavior results in the difficulty to understand the association between behavioral pattern and separation anxiety, by restricting to compare the results

from other studies. While further study is in need on the current diagnostic tools, having an additional diagnostic method could supplement the available diagnostic methods.

One possible solution would be a neurobiological approach where a potential biomarker such as Brain-derived neurotrophic factor (BDNF) is considered to a diagnostic and an evaluation of treatment efficacy in psychiatric field. The psychotropic medication such as fluoxetine and clomipramine has been shown effects on canine separation anxiety, the neurobiological mechanism might be shared between human and canines. Thus, BDNF could enable if it could be a potential objective biomarker for canine behaviors as well.

Table 3.1. Variables dichotomized which were collected from the questionnaire

Variables	Dichotomization
Breeds	Dichotomized to pure breeds and mixed breeds
Adopted age	Dichotomized to being adopted before 3 months old or after 3 months old.
Having other dogs in the same household	Dichotomized to having at least one dog in the same household or not.
Having other cats in the same household	Dichotomized to having at least one cat in the same household or not.
Attending puppy training	Attending training classes when the dog was less than 4 months old
Attending adult training	Attending training classes when the dog was
The presence of the confinement place during sleep	Dichotomized to if the dog was limited access to the rest of the house or not.
The presence of the confinement place during the owner's absence	Dichotomized to if the dog was limited access to the rest of the house or not.

Table 3.2. Behavioral categories and definitions

Behavioral category recorded as duration	Definition
Exploration - EX	Motor activity directed toward physical aspects of the environment, including sniffing, and gentle oral examination such as licking
Locomotion - LO	Walking or running around without exploring the environment (pacing). Moving all 4 legs.
Passive behavior - PA	Lying down with the head on ground without any obvious orientation toward the physical or social environment
Oriented to the environment - OE	Sitting, standing, or lying down (the head does not rest on the ground) with obvious orientation toward the physical or social environment, including sniffing, close visual inspection, distant visual inspection (vigilance or scanning)
Scratching - SC	All active behaviors resulting in physical contact with the cage/door, including scratching the cage/door with the paws, jumping on the cage/door, handling with the forelimbs
Oral behavior - OB	Any vigorous behavior directed toward the environment/cage using the mouth (including chewing, biting, shaking, pulling with the mouth)
Play - PL	Any vigorous or galloping gaited behavior directed toward a toy; including chewing, biting, shaking from side to side, scratching or batting with the paw, chasing rolling balls, and tossing using the mouth. Although the dog may take the objects into its mouth, destruction is not included in this category.
Panting - PT	Panting

(Table 3.2 continued)

Not visible - NV	Not visible (during these periods, activities like barking, whining, scratching, chewing were identified and recorded by the sound of the activity). Only the time when the dog is out of angle and we can't hear the dog is included in this category.	
Grooming - GR	Action of cleaning the body surface by licking, nibbling, picking, rubbing, scratching, and so on, directed toward the animal's body (self-grooming)	
Barking - BA	Majority time of a bout with barking and the bout starts with barking	
Whining - WH	Majority time of a bout with barking and the bout starts with whining	If a type of vocalization is less than 10% of the bout, then it could be ignored.
Howling - HO	Majority time of a bout with barking and the bout starts with howling. Longer duration with heading up.	
Growling-GW	Majority time of a bout with growling and the bout starts with growling	
Trembling - TR	Trembling/shaking movements of the body or head	
Circling - CL	Movement of the dog in circles	
Drinking - DR	Drinking	
Eating - ET	Eating	
Behavioral category recorded as frequency	Definition	
Paw up - PU	Front limb raised	
Yawning - YA	Yawning	
Lip licking - LL	Part of tongue is shown and moved along the upper lip	
Elimination - EL	Defecation or urination in sitting or standing position	

Table 3.3. Descriptive statistics (% and the number of dogs) of ordinal variables from social and environmental information

Variables		P-SA Group (n=16)	P-C Group (n=17)
Number of daily walk	Less than once a day	25% (n=4)	5% (n=1)
	Once a day	44% (n=7)	35% (n=6)
	More than twice a day	31% (n=5)	59% (n=10)
Additional exercise	≤ 30min	13% (n=2)	35% (n=6)
	30min <, ≤ 1hr	44% (n=7)	18% (n=3)
	> 1hr	44% (n=7)	47% (n=8)
Left alone	0 to 4 hrs	31% (n=5)	18% (n=3)
	4 to 8 hrs	25% (n=4)	35% (n=6)
	> 8 hrs	44% (n=7)	47% (n=8)

Table 3.4. P-value results from simple logistic regression for questionnaire survey

Variables	p-value
Breed	0.231
Age	0.287
Sex	0.388
Origin	0.143
The number of daily walk	0.766
Duration of other exercise	0.186
Adult training	0.141
Puppy training	0.999
Duration of the dog being left alone	0.592
Consistency of schedule	0.849
Confinement during being left alone	0.723
Confinement during sleeping	0.806
Recent change in schedule or environment	0.738
Fear of loud noise	0.224
Feat of firework or thunder	0.231
Aggressiveness at familiar person	0.293
Aggressiveness at stranger	0.861
Aggressiveness at unfamiliar dogs	0.231
Aggressiveness at familiar dogs	0.170

Table 3.5. Median (range) duration (in seconds) of 4 main behaviors of the P-SA group and the P-C group in T1

Behaviors	P-SA group (n=11)	P-C group (n=12)
OE: Oriented to the environment	117 (5–273)	114 (0-243)
PA: Passive behavior	0 (0-223)	151.5 (0-253)
PL: Play	0 (0-291)	0 (0-0)
PT: Panting	0 (0-19)	0 (0-219)

Table 3.6. Median (range) duration (in seconds) of 4 main behaviors of the P-SA group and the P-C group in T2

Behaviors	P-SA group (n=11)	P-C group (n=12)
OE	12 (0-280)	0.5 (0-226)
PA	35 (0-300)	298.5 (0-300)
PL	0 (0-300)	0 (0-0)
PT	0 (0-89)	0 (0-168)

Table 3.7. Median (range) duration (in seconds) of 4 main behaviors of the IC group and the OC group in T1

Behaviors	IC group (n=15)	OC group (n=8)
OE	133 (0-273)	67 (5-151)
PA	0 (0-253)	119 (0-228)
PL	0 (0-280)	0 (0-291)
PT	0 (0-219)	0 (0-0)

Table 3.8. Median (range) duration (in seconds) of 4 main behaviors of the IC group and the OC group in T2

Behaviors (seconds)	IC group (n=15)	OC group (n=8)
OE	7 (0-226)	7 (0-300)
PA	258 (0-300)	261 (0-280)
PL	0 (0-296)	0 (0-300)
PT	0 (0-168)	0 (0-40)

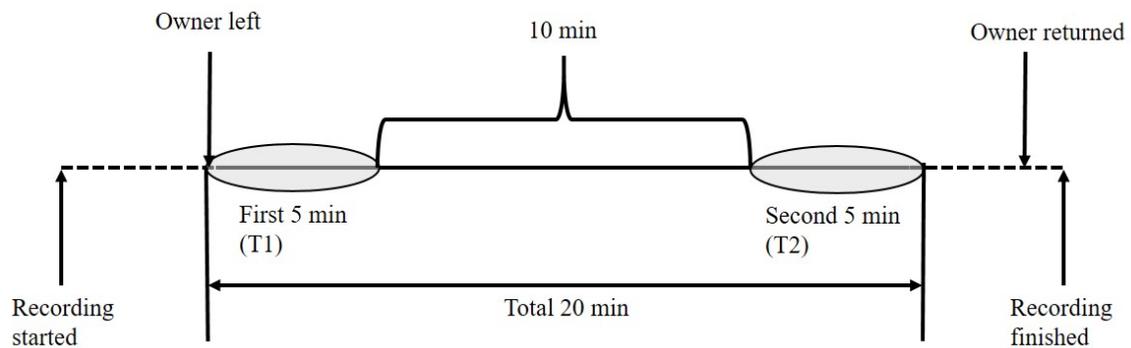


Figure 3.1. Two sessions (T1 and T2) for video analysis

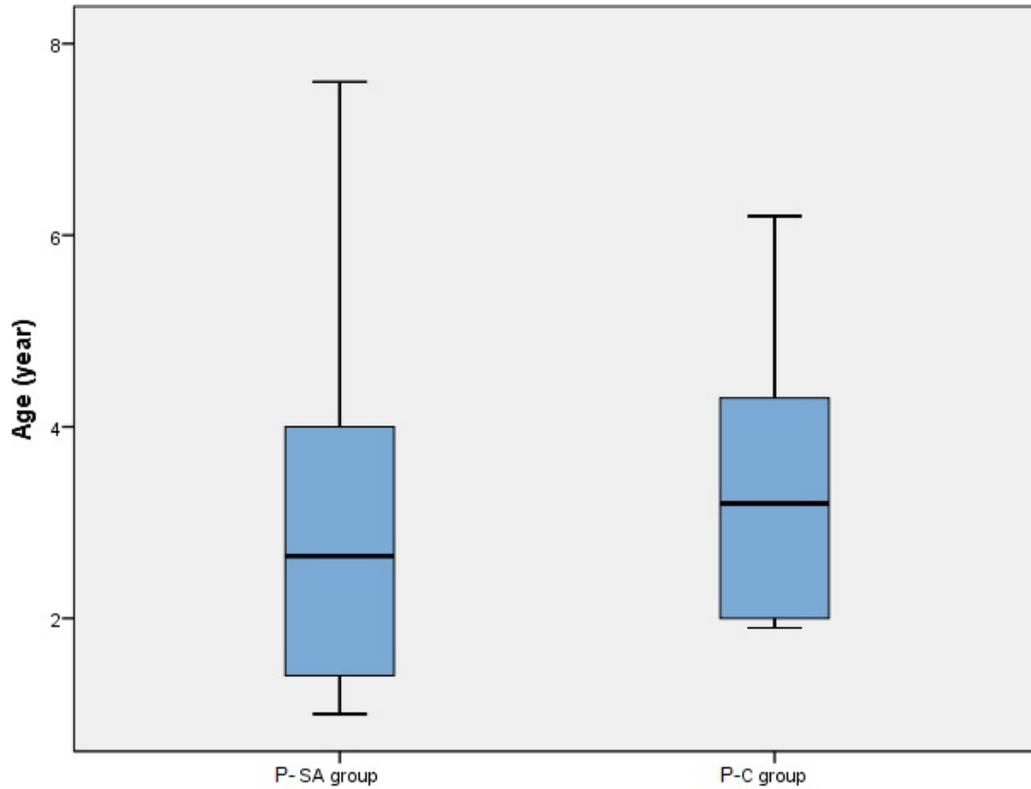


Figure 3.2. Descriptive statistics (median and range) for differences in current age (year) between the P-SA group and the P-C group

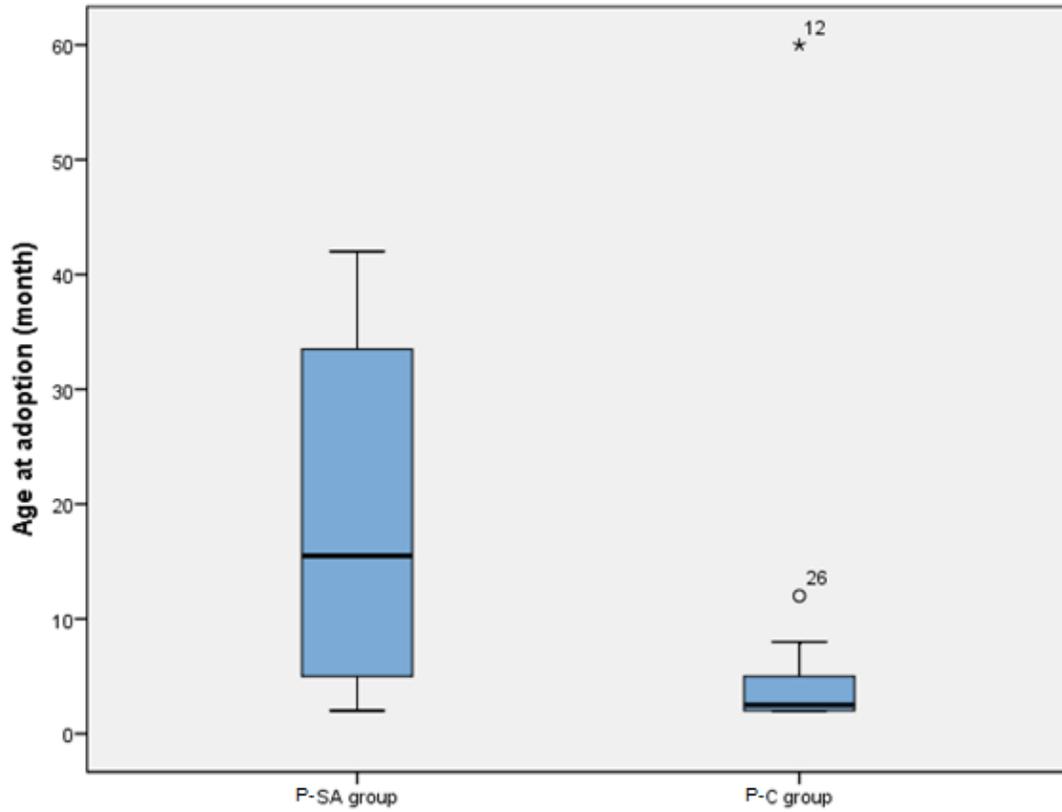


Figure 3.3. Descriptive statistics (median and range) for differences in age at adoption (month) between the P-SA group and the P-C group

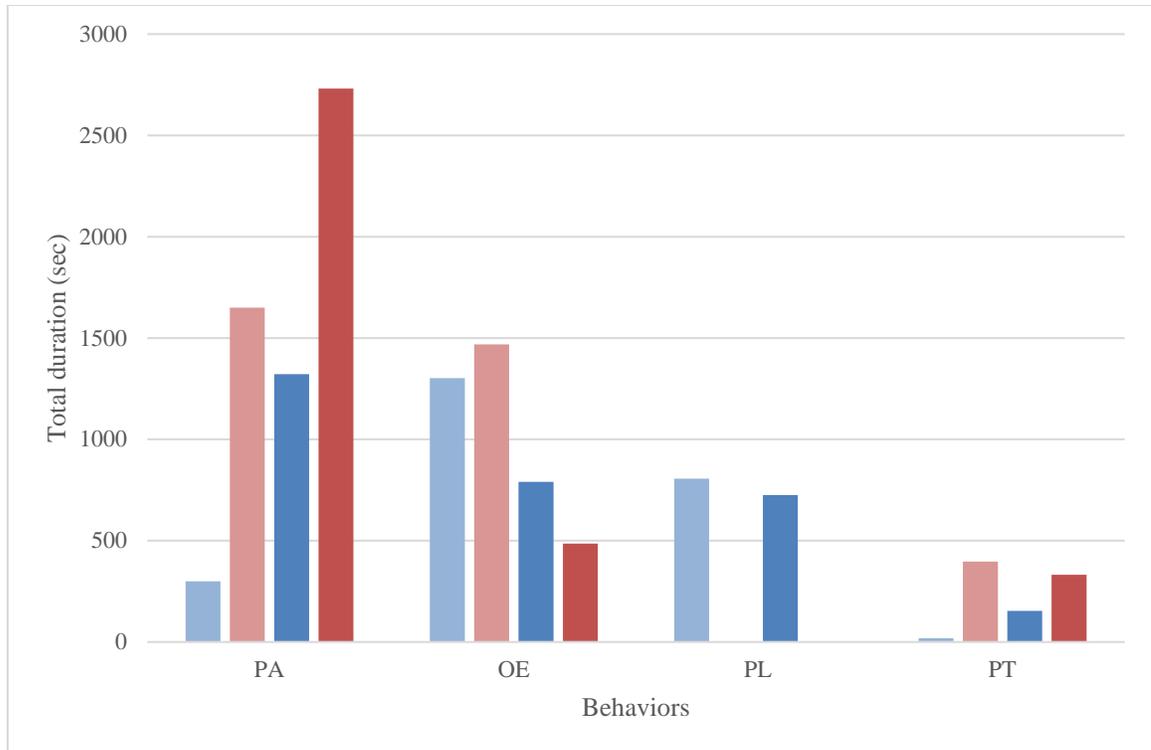


Figure 3.4. Descriptive statistics for total summed duration of the 4 behaviors (duration in seconds) between the P-SA group and the P-C group for T1 and T2

Light blue: the P-SA group in T1, Dark blue: the P-SA group in T2, Light red: the P-C group in T1, Dark red: the P-C group in T2

OE: Oriented to the environment, PA: Passive behavior, PL: Play, PT: Panting

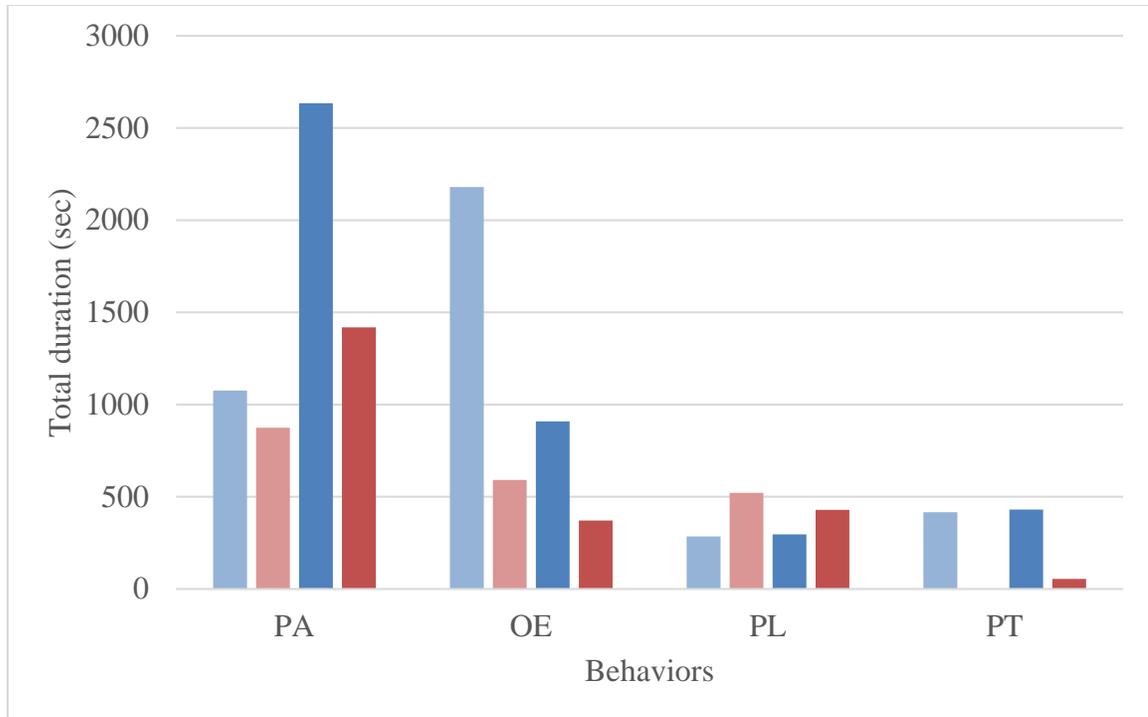


Figure. 3.5. Descriptive statistics for total summed duration of 4 behaviors (duration in seconds) between the IC group and the OC group for T1 and T2

Light blue: the IC in T1, Dark blue: the IC in T2, Light red: the OC in T1, Dark red: the OC in T2

CHAPTER 4. COMPARISON OF VARIOUS PRE-ANALYTICAL FACTORS TO VALIDATE SERUM BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) IN DOGS

4.1. Specific aims

The aim of this study was to compare various pre-analytical factors including clotting conditions and hemolysis conditions while adhering to the BDNF ELISA kit criteria.

All study protocol was approved by the Purdue Animal Care and Use Committee (Approval # 1501001179).

4.2. Materials and methods

4.2.1. Subjects

Experiment 1

The first experiment was designed to evaluate the effect of clotting conditions on canine serum BDNF concentrations. Serum samples from 11 clinically healthy dogs were used for this study. Among these dogs, 5 were purebred and 6 were mixed breed dogs. Their median age was 5.7 years old (range: 1 - 11 years). All of the dogs (4 females and 7 males) had been neutered or spayed.

Experiment 2:

The second experiment was designed to evaluate the effect of hemolysis on canine serum BDNF concentrations. Serum from 11 dogs were used. Of these 11 dogs, 10 were clinically healthy dogs, 8 were purebred and 2 were mixed breed dogs. Their median age was 5.9 years old (range: 1 - 10 years). Nine of the dogs had been neutered or spayed (4 females and 5 males) and 1 dog was an intact male. Additionally, commercially available lyophilized powdered canine serum (Sigma-Aldrich, Saint Louis, MO, USA) was reconstituted and used in this experiment as the 11th sample.

4.2.2. Sample preparation

Experiment 1:

Six clotting conditions, including clotting times and temperatures, were performed after the collection of the blood samples. The coagulation times for the room temperature (25°C) incubation experiment were 30 minutes, 1 hour, and 2 hours. The coagulation times for the 4°C incubation experiment were 30 minutes, 1 hour, and 24 hours. After samples completed their incubation times, the blood was centrifuged at approximately 15,000 g for 15 minutes at 4°C. All serum samples were stored at -80°C until the BDNF measurement.

Experiment 2:

After the blood samples were collected, they were stored at -80°C until the BDNF measurement in this study. The hemolysis conditions created for this study were conducted according to previous hemolysis studies in which specific hemoglobin

concentrations were achieved by adding washed canine red blood cells (Innovative Research, Inc., Novi, MI, USA) in a serum pool.⁷¹ Three hemoglobin concentrations were prepared; non-hemolysis (0 g/L), moderate hemolysis (2.5 g/L), and marked hemolysis (10 g/L). These conditions were created by adding the washed canine red blood cells to the 10 non-hemolyzed sera and to the commercially purchased serum.

4.2.3. BDNF ELISA measurement

In both experiments, the serum BDNF concentrations were measured within 2 months of the time when the blood was sampled, using a canine BDNF ELISA (Cloud-Clone Corp., Houston, TX, USA). The absorbance was measured using a microtiter plate reader at 450 nm (Molecular Devices, CA, Sunnyvale, CA, USA). The concentration of BDNF in the samples was expressed as nanograms of BDNF per milliliter (ng/mL) of serum using an external BDNF standard curve provided with the ELISA. According to the manufacturer, the detection range of the BDNF ELISA kit is 0.156 -10 ng/mL, and the sensitivity is 0.061 ng/mL.

4.2.4. Statistical analyses

Experiment 1:

To evaluate the effect of the clotting conditions on the canine serum BDNF concentrations, the BDNF concentrations were compared among the different clotting durations at the same temperature by Friedman's test. BDNF concentration was also compared between the temperatures at the same clotting duration using the Wilcoxon signed ranks test.

Experiment 2:

To evaluate the effect of hemolysis on canine serum BDNF concentrations, BDNF concentrations for the three hemolysis concentrations were compared using a linear mixed model.

The statistical significance for all analyses was set at $p < 0.05$, and the analyses were performed using IBM SPSS Statistics for Windows (Version 22.0, Armonk, NY: IBM Corp).

4.3. Results

The result from experiment 1 where the median serum BDNF concentrations at different clotting conditions in the 11 dogs studied are shown in Table 4.1. There were no significant differences in BDNF concentrations among the three groups (30 minutes, 1 hour and 2 hours) at room temperature ($p = 0.913$) or at 4°C ($p = 0.148$). No significant differences in BDNF concentrations were observed between the two temperatures at 30 minutes (room temperature, 4°C) ($p = 0.091$) or at 1 hour ($p = 0.722$). Additionally, there were no significant differences observed in BDNF concentrations between the 2-hour clotting time at room temperature and the clotting time at 24 hours at 4°C ($p = 0.374$).

Furthermore, the result from experiment 2 where the mean serum BDNF concentrations for each hemolysis level are shown in Figure 4.1. For non-hemolyzed and marked hemolyzed samples, 10 dogs and 1 reconstituted canine sera were analyzed. For moderate hemolyzed samples, 6 dogs and 1 reconstituted canine serum were analyzed due to small volume of samples available from the other 4 dogs. There were no

significant differences in BDNF concentrations among the three hemolysis concentrations ($p = 0.528$).

4.4 Discussion

To our knowledge, no research has been published regarding how canine peripheral BDNF concentrations can be affected by determinants such as age, weight, fasting state, exercise level or pre-analytical factors. In an attempt to support behavioral medicine's exploration of serum BDNF as a potential biomarker for canine behavioral disorders such as separation anxiety, this study investigated whether or not the pre-analytical conditions such as clotting time, clotting temperature, and sample hemolysis can affect canine serum BDNF concentrations.

In the first experiment, the results showed that there was no statistical difference between the clotting time and the temperature in canine serum BDNF concentrations at the times and temperatures used. These results were slightly different from those of a previous study in humans which reported that a significant difference was observed between 30 min and 1 hour of clotting time at room temperature, although the same study reported that the serum concentration of BDNF at 30 minutes clotting time at room temperature reached 91.8% of the BDNF concentrations at 1 hour or longer clotting time at room temperature.⁷ No statistical differences were observed in canine serum BDNF concentrations between clotting at room temperature and at 4°C for a duration of 30 minutes, or for 1 hour, which does not agree with the conclusion of a previous study in human samples.⁸ Although it was small sample size, the subjects in both experiments of the current study were compared to themselves therefore; sex, age and individual

signalment were all controlled in our study. The difference between previous studies and ours might be attributed by a species difference. Therefore, based on the results from this study, it is shown that clotting time and temperature would not affect the BDNF concentrations in dogs. It would be probably recommended that each experiment to set the consistent protocol of clotting time and temperature that is manageable in the clinical setting for further study in dogs.

In the second experiment, canine serum BDNF concentrations among three different hemolysis concentrations were compared. A previous study emphasized the importance of pre-analytical conditions in clinical chemistry analyses.⁷² Specifically, hemolysis could decrease the quality of the laboratory test result if the assay measures the amount of absorbance of light, such as is the case with ELISA analyses.⁶³ The results of this study, however, showed no statistical differences among the three different hemolysis concentrations. Findings from this study provide essential information needed to justify measuring BDNF concentrations in canine serum.

In conclusion, this study showed that clotting time, clotting temperature and sample hemolysis that are of concern in a clinical setting did not affect canine serum BDNF concentrations. The current study contributed the essential information that will lead for further investigation about peripheral BDNF as a potential biomarker in dogs.

Table 4.1. Median and range of serum BDNF concentrations (ng/mL) at different clotting conditions

Clotting Temperature	Clotting time			
	30 minutes	1 hour	2 hours	24 hours
4°C	0.232 (0.042-3.193)	0.141 (0.048-3.267)		0.543 (0.050-2.783)
Room Temperature (25°C)	0.141 (0.055-2.068)	0.110 (0.061-2.168)	0.128 (0.055-4.043)	

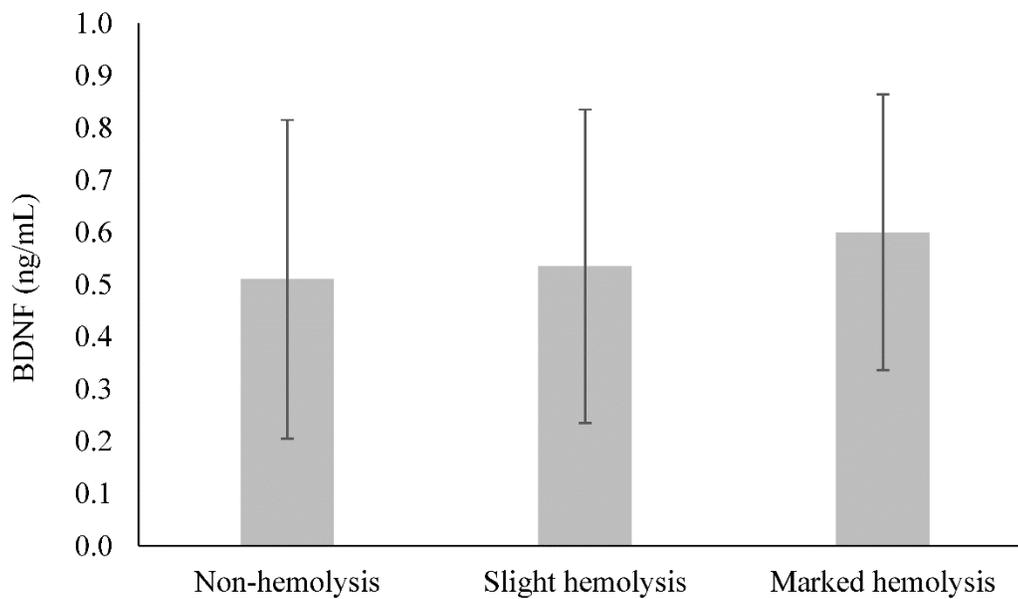


Figure 4.1. Mean canine serum BDNF concentration (ng/mL) for different hemolysis conditions. Error bars are standard deviation

CHAPTER 5. CONCLUSION

Despite the most common behavior problems in dogs, separation anxiety is easily undiagnosed due to the non-specific nature of the problematic behavioral signs, and the lack of an established definitive diagnostic method. This study has two parts: (1) To assess the questionnaire and video data in dogs with and without self-reported separation anxiety, and (2) To evaluate the feasibility of measuring canine serum BDNF levels in a clinical setting in regards to pre-analytical sample conditions such as clotting and hemolysis.

The first part of the study presented in Chapter 3 used traditional diagnosis of self-reported questionnaire and analyzing dog's behavior by recorded video during the owner's absence. Questionnaire analysis showed that having another dog in the same house was a significant associated factor to the owner's presumption. All other environmental as well as background factors have no difference. We found that dogs without any other dog in the same household were 8.5 times more likely to be in the presumed separation anxiety (P-SA) group, however, it needs to be studied with more detailed information in further study with larger sample size to understand if there is a causal factor between separation anxiety and having other dog in the same household. From the video analysis, we found significant difference in the duration of passive behavior between the P-SA and P-C groups. The duration of passive behavior in the P-C

group was significantly longer than that of the P-SA group. However, the current literature suggested prudent interpretation of passive behavior for video analysis as it could result from either separation anxiety or relaxed status. In this study self-reported questionnaire and video data were analyzed separately and its result was not compared with the diagnosis of the presence of separation anxiety in each dog. It would be interesting to know how these traditional methods provide reliable information for clinical diagnosis and which behaviors can be significant signs of diagnosed separation anxiety cases.

The second part of the study presented in Chapter 4, evaluated whether pre-analytical factors that are determined during handling the samples, especially clotting and hemolysis, could affect serum BDNF. The results of our study showed that neither the clotting condition nor hemolysis status affect serum BDNF levels in dogs. To date, no studies have investigated canine peripheral BDNF concentrations; thus, our results provide important information in further studying BDNF as a potential biomarker for separation anxiety in dogs.

This study provided the limitation and future application of the current diagnostic method and possibility of the application of neurobiological diagnostic method in separation anxiety. While the questionnaire as well as the video recording of the behavior during owner's absence are inevitable and the current most available information to help diagnosis, this study showed their limitations of interpretation as well. In the next step, it needs to be assessed that the validity of each and both tools by comparing the clinical diagnosis. Furthermore, it would be important to assess if addition of measuring BDNF

enhances the current diagnostic methods as it may lead the new diagnostic method based on a neurobiological mechanisms of separation anxiety.

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APPENDIX

APPENDIX A Questionnaire

Separation anxiety study

Q1 Separation Anxiety Study Form Today's Date

Q2 Owner Information Owner's Name (First, MI, Last)

Q3 Address (Street, City, State, Zip)

Q4 Phone Number:

Q5 E-Mail:

Q7 Pet Information Pet's Name:

Q8 Pet's Breed:

Q9 Pet's Color:

Q10 Pet's Current Age:

Q11 Pet's Date of Birth:

Q12 Pet's Sex:

- Female
- Spayed female
- Male
- Neutered male

Q13 If your pet is spayed/ neutered, at what age was she/he spayed/neutered (years/months or Unknown)?

Q14 Household Information Number of adults in the household:

_____ Number of female adults in household

_____ Number of male adults in household

Q15 Number of children (younger than 16 years old) in the household:

_____ Number of female children in household

_____ Number of male children in household

Q16 Ages of the girls (younger than 16 years old):

Q17 Ages of the boys (younger than 16 years old):

Q18 Number of other dogs (please write name, breed, age, and sex of each dog) in the household:

Q19 Number of other animals (Please write name and species):

Q20 Background Information Age your dog was obtained (years & Months)

Q21 Origin

- Bred at your residence
- Breeder
- Shelter/Rescue
- Other _____
- Don't Know

Q22 If your pet was obtained as a puppy (younger than 6 months old), how did you select your dog from a litter? (Skip this and following 2 questions, if it was obtained as an adult)

- Biggest/dominant
- Breeder selected
- Looks
- No choice
- Most outgoing
- Most timid
- Smallest/Submissive
- Other _____

Q23 What was the personality of the dog as a puppy (younger than 6 months old)? Please check all that apply.

- Aggressive to Owner/familiar people
- Aggressive to Strangers
- Aggressive over food/treats (edible)
- Aggressive over toy/objects (non-edible)
- Aloof
- Cautious
- Fearful (new/unfamiliar environment)
- Fear of Noises
- Happy, outgoing
- Hyper-excitable
- Shy of Strangers
- Super-submissive
- Other _____

Q24 Did you meet your dog's parents or do you have information about littermates? If yes, please describe:

Q25 How would you generally describe your dog's current personality? Please check all that apply.

- Aggressive to Owner/familiar people
- Aggressive to Strangers
- Aggressive over food/treats (edible)
- Aggressive over toy/objects (non-edible)
- Aloof
- Anxious
- Fearful (new/unfamiliar environment)
- Fear of Noises
- Happy, outgoing
- Hyperexcitable
- Shy with Strangers
- Supersubmissive
- Other _____

Q26 What was the first age (by months) your dog met each of the following? Never=0,

Unknown=13

_____ Unfamiliar adult male

_____ Unfamiliar adult female

_____ Unfamiliar children

_____ Unfamiliar dog

Q27 Medical Information List any existing medical conditions of the dog:

Q28 List any current medications and/or supplements with doses:

Q29 General Information Exercise: On average, how many times does your dog get a regular walk (either on or off leash)?

- Three times or more per day
- Twice a day
- Once a day
- No walk
- The dog is outside all the time
- Others _____

Q30 Does your dog get any other exercise (excluding the regular walk)?

- Yes
 - No
- 

Q31 On average, how many hours/minutes does your dog get other exercise (excluding the regular walk)?

- 2 hours or more per day
- 1-2 hours
- 30 minutes-1 hour
- less than 30 minutes

Q32 What sorts of training did the dog receive and at what age?

- Attended obedience classes (please write the age when it started)

- Attended puppy classes (please write the age when it started)

- Others _____
- None

Q33 Diet: What type of food does your dog lately (for past 6 months to current) eat?

Please write brand, type, etc...

Q34 How many meals a day does the dog receive?

_____ Number of feedings

Q35 Do you pick up the food bowl after each meal time, if he/she does not finish it?

- Yes (1)
- No, leave it on the floor at all times

Q36 Daily Schedule: Average hours that the dog is left alone per day? (Choose 13 if it is longer than 12 hours)

_____ Hours

Q37 Dog's schedule on weekdays is:

- Consistent
- Varies

Q38 Where is the dog when left alone? (Please specify e.g. in a crate, or free in a house, etc.)

Q39 Where does your dog sleep at night?

Q40 Have there been any major changes in your dog's environment/schedule since you obtained the dog? If so, please write when and what kind of changes have occurred and how you think they impacted your dog.

Q41 Separation Anxiety Profile If your dog shows behaviors below, please mark % that best describes the frequency.

	Never (0%) (1)	Rarely (Often (40- 60%) (3)	Almost always (60- 90%) (4)	Always (>90%) (5)
Follows you around the house	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Loses appetite after you leave	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Excessive drinking in your absence	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Eliminates in home only in your absence	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Excessive salivation in your absence	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Shaking when you leave	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Pacing when you leave	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Panting when you leave	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Becomes aggressive when you leave	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Barks/whines excessively within 30 minutes of your departure	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Destroys property only in your absence	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Repetitive behavior (chases tail, licks self, etc) when you leave	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Decrease activity after you leave	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

Exhibits an excessive greeting on your return	<input type="radio"/>				
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Q42 Age at which separation anxiety was observed?

Q43 Frequency of the problem

How many days per week has the problem been observed? (1)

How many days per month has the problem been observed? (2)

Q44 Severity of the problem?

Very Serious (1)

Serious (2)

Not Serious (3)

Q41 Do you think your dog has separation anxiety?

Yes (1)

No (2)

Q46 Have you gotten any professional help before for his/her separation anxiety? If so,

who did you consult with?

Internet information (3)

Local dog trainer (2)

Primary veterinarian (1)

Animal behaviorist (4)

Others (5) _____

No, I have not consulted with anybody (6)

Q47 Fear & Anxiety Profile If your dog shows fear or anxiety towards any noise(s)

below, please mark all that apply.

- No, my dog does not typically show anxiety or fear to any type of noises (1)
- Fireworks (2)
- Thunderstorms (3)
- Noises, please write the example(s) (4) _____

Q48 Under the trigger you marked on the previous question (noises), please mark % that

best describes the frequency.

	Never (0%) (1)	Rarely (Often (40-60%) (3)	Almost always (60-90%) (4)	Always (>90%) (5)
Barks or whines	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Destroys property	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Loses appetite	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Excessive drinking	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Frequently eliminates	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Salivation	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Hiding	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Shaking	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Pacing	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Panting	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Remains near you	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

Q49 If your dog does NOT show fear or anxiety towards any noise(s) above, please specify how your dog behaves under loud noises including thunderstorms, and fireworks.

Please mark all that apply.

- Tail in high position (1)
- Tail below the back line, but not between the legs (2)
- Has appetite and is playful (3)
- Is calm, and no change in his/her behavior (4)
- Others (6) _____

Q50. If your dog shows fear or anxiety towards any trigger(s) below , please mark all that apply.

- No, my dog does not typically show anxiety or fear to strangers (4)
- Stranger(adult) (1)
- Stranger (children) (2)

Q51 Under the trigger you marked on the previous question (stranger), please mark % that best describes the frequency.

	Never (0%) (1)	Rarely (Often (40- 60%) (3)	Almost always (60- 90%) (4)	Always (>90%) (5)
Avoids/withdraws	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Barks (is not going towards stranger)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Growls (is not going towards stranger)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Shaking	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Tail low/between legs	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Pacing	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Panting	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Remains near you	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Loses appetite around stranger	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Barks/growls and goes towards stranger	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

Q52 If your dog does NOT show fear or anxiety towards any unfamiliar humans above, please specify how your dog behaves when meeting a stranger. Please mark all that apply.

- If allowed, always goes to greet the person (1)
- Jumps, licks, is very excited (2)
- Takes relaxed contact, sniffs and tail is wagging (3)
- Sniffs, but loses its interest soon (4)
- Is not interested in people, but does not bark or mind if a person wants to pet the dog (5)
- Barks, growls (7)
- Others (6) _____

Q53. Does your dog show fear or anxiety towards an unfamiliar dog ?

- No, my dog does not typically show anxiety or fear to an unfamiliar dog (5)
 Yes (3)

Q54 Under the trigger you marked on the previous question, please mark % that best describes the frequency.

	Never (0%) (1)	Rarely (Often (40- 60%) (3)	Almost always (60- 90%) (4)	Always (>90%) (5)
Avoids/withdraws	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Barks (is not going towards unfamiliar dog)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Growls (is not going towards unfamiliar dog)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Shaking	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Tail low/between legs	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Pacing	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Panting	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Remains near you	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Loses appetite around unfamiliar dog	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Barks/growls and goes towards unfamiliar dog	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

Q55 If your dog does NOT show fear or anxiety towards an unfamiliar dog, Please specify how your dog behaves when meeting an unfamiliar dog. Please mark all that apply.

- Eager to meet, always friendly (1)
- Eager to meet, but loses its interest soon (2)
- Usually friendly, but responds aggressively if another dog shows aggressive behavior (3)
- Usually friendly, but sometimes starts a fight (4)
- Usually barks/growls (5)
- Barks/growls and goes towards a dog (9)
- Not interested in other dogs (10)
- Others (6) _____

Q56 If your dog shows fear or anxiety towards any trigger(s) below , please mark all that apply.

- No, my dog does not typically show anxiety or fear (3)
- New situation, new environment (1)
- Others (2) _____

Q57 Under the trigger you marked on the previous question (new situation/environment), please mark % that best describes the frequency.

	Never (0%) (1)	Rarely (Often (40-60%) (3)	Almost always (60-90%) (4)	Always (>90%) (5)
Remains near you	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Wants out of the situation/new space	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Barks/whines	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Tail low/between the legs	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Decreases activity, does not explore	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Loses appetite in the situation/new space	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Excessive drinking	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Frequently eliminates in the situation/new space	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Salivation	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Shaking	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Pacing	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Panting	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

Q58 If your dog does NOT show fear or anxiety in a new situation/environment above, please specify how your dog behaves instead. Please mark all that apply.

- Tail in high position (1)
- Tail below the backline, but not between the legs (2)
- Is curious and eager to explore (3)
- Is able to eat and sleep at the new place (4)
- Is calm as being in an usual place (5)
- Others (6) _____

Q59 Aggression Profile

Please write the frequency of that all behaviors below on the scale 1-4. For example, if your dog almost always barks aggressively toward strangers and snapped at them one time (rarely), then mark number 3 under "bark aggressively" and mark 1 under "snap/bite" at the target of stranger. Alternatively, however, if your dog has never showed aggressive behavior to some or any targets below, then mark number 4 under "No aggressive reaction" at the target the dog does not show aggression

- . 1. Rarely (less than 40% of the time), 2. Often (40-60%), 3. Almost Always (60-90%), 4. Always (more than 90% of the time)

	No aggressive reaction (1)	Growl (2)	Bark aggressively (3)	Try to snap/bite (no skin contact even though he/she could) (4)	Snap/bite (skin contact) (5)
Stranger					
Familiar person					
Owner, family member					
Unfamiliar dog					
Familiar dog					
Housemate dog					

Q60 Additional comments if you have any?