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Objective measurement of fear-associated learning in dogs

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body temperature;
dog

Abstract Attempts have been made in many studies to measure canine behavioral traits, but the results cannot be compared, because no standardized methods exist. The purpose of this study was to standardize the fear-eliciting stimuli (i.e., a conditioned stimulus) to assess a particular behavioral trait, namely fearfulness. We applied a Pavlovian aversive conditioning protocol and measured autonomic parameters, in addition to making behavioral observations. Fear-related autonomic responses, such as increased heart rate and increased body temperature, rose consistently in response to a conditioned stimulus, but behavioral changes did not consistently correlate with the physiological responses. Our findings show that dogs clearly respond to conditioned stimuli and that their autonomic reactions assessed in objective indices can be more reliable and consistent measures than some behavioral measures. Based on these results, we propose that associative learning between fear-inducing conditioned and unconditioned stimuli can be assessed accurately in dogs.

Introduction

Dogs coexist with human beings in many capacities. They serve as companions, guide dogs, service dogs, police dogs, drug detector dogs, and so on. As a result of the social expectations imposed on dogs and their use in the roles mentioned above, behavioral problems in dogs have received extensive attention, both publicly and academically. In addition, behavioral problems account for the most common cause of pets being relinquished in the United States, Australia, and Canada (Houpt et al., 1996).

Canine behavioral traits are developed to a major extent by both genetic factors and conditioning processes occurring in rearing environments. Recently, increased research has been undertaken to elucidate the genetic background of canine behavioral traits (van den Berg et al., 2003; Takeuchi et al., 2005). Careful phenotyping of genetic material is critical in the molecular analysis of behavioral traits (van den Berg et al., 2003). In genetic studies on human mental disorders, the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) and the International Classification of Diseases (ICD-10) are often applied (van den Berg et al., 2003), but no validated diagnostic criteria are available for studies on dogs. Therefore, questionnaire surveys are commonly used to assess canine temperaments and behavioral traits (Serpell and Hsu, 2001; Rooney et al., 2004). However, the subjectivity of this approach has often attracted criticism (van der Borg et al., 1991).

An alternative method is behavioral testing, whereby dogs' temperaments are scored in accordance with their behavioral responses to various stimuli across different sensory modalities (van der Borg et al., 1991; Netto and Planta, 1997; Svartberg, 2002; Svartberg and Forkman, 2002; Gazit

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et al., 2003). Behavioral tests are generally considered more objective than questionnaire-based surveys (van den Berg et al., 2003) and for several decades have been used in a wide range of contexts, from predicting the potential to become a successful guide dog (Goddard and Beilharz, 1986) to predicting problem behavior in dogs at animal shelters (van der Borg et al., 1991). However, as previous authors have noted (Jones and Gosling, 2005; Diederich and Jean-Marie, 2006), several points must be addressed to improve the reliability and validity of behavioral testing. For example, Diederich (2006) pointed out that any test is expected to meet 4 quality requirements:

1. Stimuli and notation of the test must be standardized.
2. A test must be reliable and the test-retest consistency must be verified. The measurement must be repeatable and free from random errors.
3. The scoring of the test must be sensitive and able to detect small differences.
4. The test must be valid. It must accurately measure a particular trait (eg, fearfulness).

We especially focused on 2 of these issues, 1) standardizing the fear-eliciting stimuli, and 4) assessing a reliable measurement of fear response. Many studies have tried to standardize the stimuli they use, for example by using novel items or strangers as stimuli (van der Borg et al., 1991; Netto and Planta, 1997; Svartberg, 2002; Svartberg and Forkman, 2002; Gazit et al., 2003), but it may be effectively impossible to achieve true standardization, given the likely range of previous experiences of all the dogs to be tested, and the unlikely outcome of finding something truly novel for every dog (Diederich and Jean-Marie, 2006). To minimize this concern, we paired a neutral stimulus with an aversive stimulus that would become a conditioned stimulus for all dogs in this study. Autonomic parameters such as heart rate and body temperature are common fear and anxiety indexes in many species (Hydbring-Sandberg et al., 2004; Kiyokawa et al., 2004; Van Reenen et al., 2005). Therefore, in this study, we examined whether such autonomic responses could be used as a standardized measure of canine behavior. Hence, in this study we assessed whether the conditioned stimulus induced a fear response that was measurable using autonomic parameters.

Materials and methods

Subjects

Twelve privately owned adult dogs of varying age (2–7 years old) were recruited for the study with owner's informed consent: seven females, six of whom were spayed, and five males, four of whom were castrated. The dogs were randomly divided into either the conditioned group or the control group.

The experiment was conducted at each dog's home or at a place familiar to the animal, such as a local indoor dog training club. Three dogs were subjected to the experimental procedures and tested at home, and 3 were subjected to the experimental procedures and tested at a local dog training club in both conditioned and control groups. All procedures were approved by the Animal Care and Use Committee of the University of Tokyo. The breeds of the dogs were the Labrador retriever (6), standard poodle (2), golden retriever (1), Doberman pinscher (1), collie (1), and American cocker spaniel (1). The dogs had no apparent physical or behavioral problems requiring treatment, as determined by a physical exam and an interview with the owner.

In the interview, we also asked the owner if the dog had any previous experience with the spray collar we used in this study. One dog (a Labrador retriever) in the control group had experienced wearing the spray collar for a few days about 3 years ago. This dog did not show any averse response when the device was attached, and there was no difference in terms of time needed to settle down as compared to other dogs. Therefore, we considered that all the dogs were equally conditioned at session 3.

Apparatus

A portable alarm buzzer (BH-204; National Co., Ltd., Osaka, Japan) was used as a conditioned stimulus (CS), and a remote controlled spray collar (Spray Commander; Multivet, Saint-Hyacinthe, Quebec, Canada) was used as a mildly aversive unconditioned stimulus (US). Heart rate (HR) was measured using a Polar heart rate monitor (Wear Link31; Polar Electro OY, Kempele, Finland), consisting of an electrode belt with built-in transmitter and remote monitoring receiver. In the study we tried as much as possible to avoid the influence of restraining a dog for measuring rectal temperature. We used the high-response thermometer, (Microprobe thermometer; Physitemp Instruments, Inc., Clifton, NJ), which requires approximately 2 seconds to measure temperature.

Experimental procedure

The overall procedural schedule is shown in Fig. 1. Each dog in each group was tested individually. The experimental procedure of Pavlovian aversive conditioning consisted of 3 sessions, with each session lasting 60 minutes. The 3 sessions were conducted in a single day. A baseline HR was measured before each session and then every minute during the first 5 minutes immediately after giving the CS in each session. Rectal temperatures were measured prior to the session and 20, 40, and 60 minutes after giving the CS in each session (Zethof et al., 1994).

The remote-controlled spray collar and chest band for monitoring heart rate were attached to each dog being tested outside the testing pen 30 minutes before the start of the first session. The unleashed dog and its owner entered the testing pen (2 m

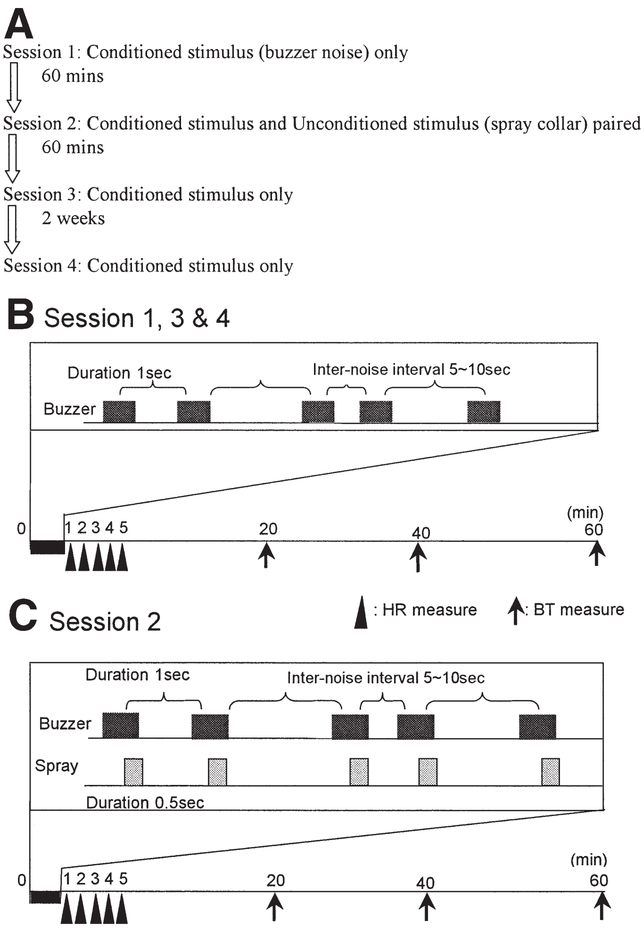

Figure 1 (A) Experimental procedure. (B) Experimental setting at session 1, 3, and 4. (C) Experimental setting at session 2, where unconditioned stimulus was paired with conditioned stimulus.

x 3.5 m), and the dog was allowed to have as much time as necessary to settle down, which was confirmed by a heart rate and rectal temperature of less than 100 beats per minute (bpm) and 38.5°C, respectively. Then, session 1 began. The intervals between sessions were also noted, because some dogs needed time to settle down between sessions.

The buzzer noise and remote controlled spray collar stimulus were paired 5 times to ensure conditioning in session 2. During all sessions, physiological data such as heart rate and rectal temperature were measured at the aforementioned intervals. During sessions 2 and 3, the first 6 minutes, including the time when giving the CS, were also recorded on video. We analyzed the behavior for 1 minute during CS in each session and for the first 1 minute after the CS in each session for session 2 and 3.

In session 1, the buzzer noise (CS) was sounded 5 times, with an interval of 5 to 10 seconds. The volume of the buzzer noise was approximately 120 dB, and the duration was approximately 1 second.

In session 2, the buzzer noise (CS), with a duration of 1 second, and the spray stimulation (US), with a duration of 0.5 second, were paired and repeated 5 times. The interval between the pairs of buzzer noise and spray stimulation was 5 to 10 seconds. These 2 stimuli were proactively paired (i.e., the last 0.5 second of buzzer noise overlapped with 0.5 second of spray stimulation).

In session 3, only CS (the buzzer noise) was provided, again 5 times with an interval of 5 to 10 seconds, as in the first session.

In session 4, two weeks after the day of the experiment, the dogs were exposed to the buzzer noise (CS) again to determine if the fear conditioning had been forgotten.

Owners were allowed to talk to and touch their dogs in a usual manner during the experiments, but they were not allowed to give food or toys. In the control group, the dogs were subjected to exactly the same procedure, except that in session 2, the buzzer noise (CS) was not paired with the remote-controlled spray stimulation (US); instead, the 2 stimuli were administered randomly during the first 1 minute of the session. The heart rate and rectal temperature between sessions 1 and 3 were compared to assess fear response induced by the conditioning.

Statistical analysis

Data analysis was performed with Stat View J 5.0 (SAS Institute, Cary, NC; no longer available). The data were expressed as means (±SEM) and significance was set at $P = 0.05$ for all statistical tests. Heart rate and body temperature were expressed as the change from baseline for group comparison, and were analyzed by 2-way repeated ANOVA measures in each session.

For all sessions, the area under the curve (AUC) for both heart rate and body temperature changes from the baseline in each group were compared using 2-way repeated ANOVA measures, and t-tests were performed for comparison in each session between groups. Also, the intersession AUC change in each group was compared using paired t-tests.

Results

Heart rate and body temperature for the 3 test sessions, and for the fourth session held 2 weeks later, are shown in Figure 2. In session 1, in which only the neutral conditioned stimulus of buzzer noise was provided, neither the heart rate nor the body temperature changed significantly compared to baseline measurements in either the conditioned or the control groups. In session 2, in which the subjects were provided with the unconditioned stimulus (spray), the heart rate and body temperature increased in both groups. In session 3, the heart rate and body temperature of those in the conditioned group increased significantly (2-way repeated ANOVA, $F[1,45]=4.88$, $P=0.055$, and $F[1,30]=9.245$, $P<0.05$, respectively) in response to the fear-conditioned cue of the buzzer noise compared to those in the control group. Heart rate increased significantly after receiving the buzzer noise in session 3 and returned to the baseline within the first 5 minutes. Body temperature peaked at either 20 minutes or 40 minutes after the

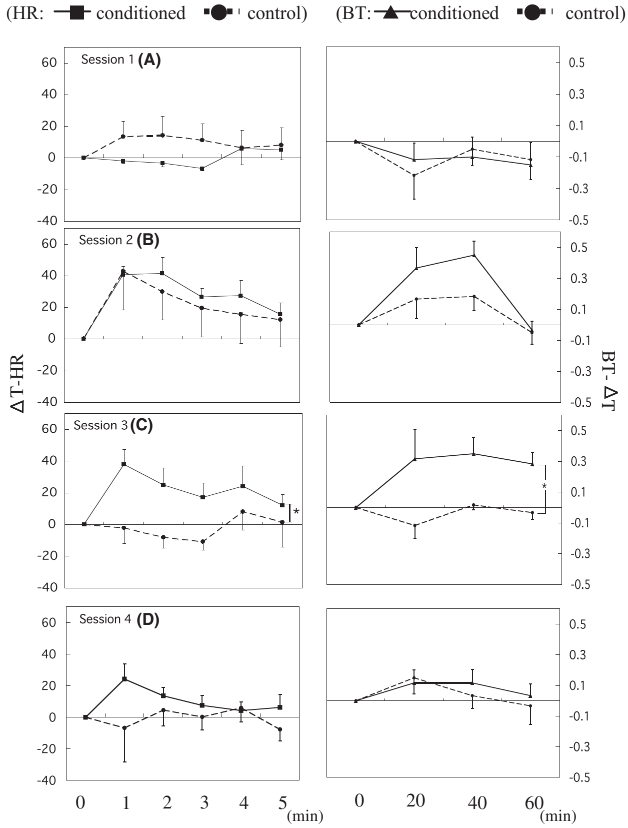

Figure 2 Mean (± SEM) changes in heart rate (HR) and body temperature (BT) of conditioned and control groups of dogs. Both HR and BT in session 3 (C), there were significant differences between two groups. *$P < 0.05$, two-way repeated ANOVA.

buzzer, depending on the individual, and remained at a high level for 60 minutes. In the control group, the individual dogs experienced a remote control spray collar (US) equal to the conditioned dogs in the session 2, but did not respond to the buzzer noise in session 3, indicating that they were not conditioned by the buzzer noise (CS). Group differences were observed 1, 2, and 3 minutes after the stimulation in the heart rate response, and 40 and 60 minutes after the stimulation in the body temperature (t-test, $P<0.05$). When tested again 2 weeks after the conditioning, neither heart rate nor body temperature increased significantly in either the conditioned or control groups.

The area under the curve (AUC) of heart rate and body temperature for all 4 sessions is shown in Figure 3. Although no difference was observed for overall change in heart rate, a significant difference was found for body temperature between the conditioned and control groups (2-way repeated ANOVA, $F[1,30]=6.04$, $P<0.05$). The AUCs for all sessions were compared, and significant differences were found between the conditioned and control groups for both body temperature and heart rate in session 3 (heart rate, $P=0.05$, body temperature, $P<0.05$, t-test). As shown in Figure 3, the AUC for heart rate and body temperature in session 2 were equally high in both groups. Comparing the intersession changes of AUC in the conditioned group, the AUC for heart rate in sessions 2 and 3 differed significantly from that found in session 1 ($P=0.01$ and $P=0.05$, respectively). Similarly, the AUC for body temperature in the conditioned group tended to differ between sessions 2 and 3, and sessions 1 and 2 ($P=0.08$ for both). These differences were not observed in the AUC of the control group for either heart rate or body temperature.

Video recordings of the sessions revealed various patterns of behavior in each dog. In sessions 2 and 3, the first 1 minute when CS was given, the conditioned group showed behaviors such as tail down, running, whining, freezing, looking in the direction from which the sound was coming, licking their mouths, panting, and jumping (see Table 1). However, the behavioral responses of each individual dog were not all the same in session 3 as in session 2. In the control group, the dogs showed behaviors similar to the conditioned dogs in session 2 when they received the spray stimulation, but none of these behaviors in session 3, when they received only the buzzer noise.

Discussion

This study demonstrates the association of fear-related autonomic responses with conditioned stimuli in dogs. This is the first time this type of study has been done in dogs. Previous

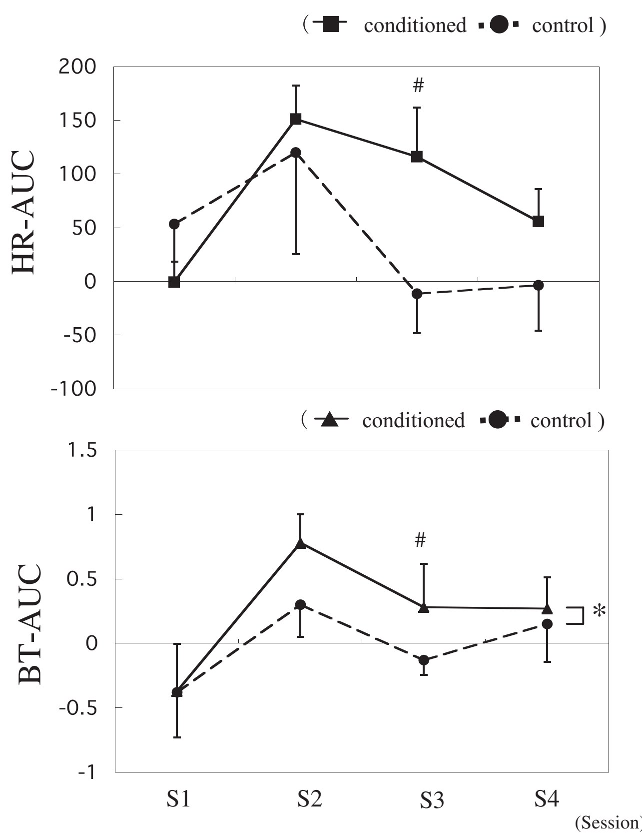

Figure 3 Area under the curve (AUC) of heart rate and body temperature through all of 4 sessions. *$P < 0.05$, compared to the control group (two-way repeated ANOVA). $^{\#}P \leqq 0.05$, compared to the control group (t-test).

Table 1 Dog behaviors of the conditioned group in the 1 minute of the session 2 and 3. Total duration for each behavior was written in a parenthesis by seconds.

Breed	Session 2, during CS	Session 2, after CS
Standard Poodle 1	Jump & pace & Tail (16) Freeze (44)	Freeze & Tail (60)
Standard Poodle 2	Jump & Run & Tail (06) Freeze (54)	Freeze & Tail (60)
Labrador retriever 1	Bark & Run & Tail (60)	Freeze & Pant & Tail (60)
Labrador retriever 2	Jump & Tail (60)	Stay (60)
Golden retriever	Lick & Stay (60)	Stay (60)
Doberman Pincher	WD & Pace & Tail (60)	Tail & Pace (60)
Breed	**Session 3, during CS**	**Session 3, after CS**
Standard Poodle 1	Freeze & Tail (60)	Freeze & Tail (60)
Standard Poodle 2	Freeze & Tail (60)	Freeze & Tail (60)
Labrador retriever 1	WH & Run & Tail (20) Freeze & Pant (40)	Freeze & Pant (60)
Labrador retriever 2	Look (60)	Stay (60)
Golden retriever	Beg (60)	Stay (60)
Doberman Pincher	Freeze & Tail (60)	Freeze & Tail (60)

Note. Jump = Jumping, Pace = Pacing, Tail = Tail down, Run = Running, Bark = Barking, Lick = Licking, Stay = Staying with owner, WD = Withdrawing, Freeze = Freezing, WH = Whining, Pant = Panting, Look = Looking to the sound, Beg = Begging to the owner

studies (King et al., 2003; Hydbring-Sandberg et al., 2004; Palestrini et al., 2005) have focused on autonomic reactions to fear stimuli in dogs, using items such as gunshot, an opening umbrella, an unfamiliar environment, approaches of strangers, and so on as aversive stimuli. However, the individual dog's experiences of these stimuli may have affected the degree of fear response observed. In the present study, we measured fear-conditioned responses to a buzzer noise and found that the dogs' autonomic responses were clearly distinguishable from those of the nonconditioned dogs. All dogs used in the study were pet dogs, so we allowed the owners to be with dogs and interact normally with them except for providing food or toys. This design may have affected some of the behavioral or autonomic responses to the CS; however, the opportunity to be with the owner and to be touched by the owner was equal in both groups. Accordingly, the results obtained in this study were mainly because of conditioning processes, in which the conditioned group experienced pairing of CS and US, whereas the control group experienced lack of pairing of CS and US. In addition, the data from session 1, in which none of the dogs showed a fear response, provided support for the view that the autonomic responses we measured were not influenced by rearing experience. Based on these results, we propose that measurement of a fear-conditioned response could be a reliable index for evaluating traits of dogs that are not influenced by the dog's rearing experience with the stimuli.

Fear response in dogs has been studied frequently, because fear is a potential cause of behavioral problems such as aggression and inability to socialize with other dogs and people (King et al., 2003). Fearfulness is also considered to be an unsuitable trait for dogs training to be guide dogs (Goddard and Beilharz, 1985). However, measure of a dog's response to a fear-eliciting stimulus frequently overlaps with measure of its reactivity (Jones and Gosling, 2005) because it is hard to determine objectively whether behaviors like pacing, running around, or barking signal fearfulness or a more general reactivity.

In this study, we used a variety of breeds. Differences in behavioral characteristics between breeds have been reported in other studies (Bradshaw et al., 1996; Goodwin et al., 1997; Wilsson and Sundgren, 1997) and, as expected, the behavioral responses to the CS in sessions 2 and 3 varied among the individual dogs. Evaluation of variation in behavioral traits is difficult, especially given potential breed variation. Objective determination of the degree of fearfulness as demonstrated by the dog's behavior may be complex. For example, a dog that tries to escape by running may be more—or less—fearful than a dog that shows freezing to the spot. So, in an attempt to avoid individual differences emanating from rearing environments and breed, we measured autonomic responses in combination with behavioral observations.

In the behavioral observations, we found that the 6 individuals in the conditioned group, consisting of 4 different breeds, showed various phenotypes, and each behavior showed varying degrees of associated physiological reaction. It has previously been observed that behavioral changes in dogs do not always parallel changes in physiological response (Diederich and Jean-Marie, 2006). Similar results were obtained in the present experiment. For example, some dogs exhibited no obvious fear-related behaviors such as freezing or panting, but these dogs, nevertheless, displayed autonomic changes. These individual differences in behavior in the conditioned group may reflect differences in coping style (Van Reenen et al., 2005). Although the behavioral responses of each dog varied between sessions 2 and 3 (Table 1), the physiological reactions measured (heart rate and body temperature) were much more consistent among individuals within the conditioned group. In session 1, none of the dogs showed any obvious physiological reactions when compared to the control group (Fig. 2A), indicating that buzzer noise was initially an equally neutral stimulus for all of the dogs tested. Subsequent changes in physiological reactions showed that this neutral stimulus changed to a conditioned stimulus in session 2 for all individuals (Fig. 2B). In this study the number of dogs was small, and the breed, age, and sex distribution between the two groups was unequal. The use of more dogs with equally distributed sexes and breeds would be required for further experiments to clarify any effects of sex, breed, and age. However, the finding that all dogs showed similar autonomic responses in the conditioning session 2 (Fig. 2B) may suggest the possibility that autonomic reactions are less influenced by breed, sex, and age differences.

Body temperature increases known as stress-induced hyperthermia (SIH) have been observed in various species, both prior to and during exposure to anxiogenic or stress-inducing stimuli (Kluger et al., 1987). Stress-induced hyperthermia occurs when animals undergo physiological stress, which might result from factors such as a loud noise, heat or handling, or psychological stress that might result from a perceived threat. There are at least two different mechanisms of SIH: a PGE_2 (prostaglandin)-dependent mechanism and a PGE_2-independent, serotonin-mediated mechanism (Oka et al., 2001). It has been shown that SIH is not caused by increased physical activity (Oka et al., 2001), nor by increased ambient temperature (Long et al., 1990), and that the measuring procedures required are noninvasive; therefore, SIH reflects animals' responses to stressors relatively precisely. Also, SIH can be measured repeatedly in different contexts using the same animal, and the results are consistent over time, suggesting that this parameter is suitable for within-subject experimental design (Olivier et al., 2003).

Heart rate, which is a well-established response to stress and emotional stimuli (Beerda et al., 1998; Palestrini et al., 2005), is known to represent the autonomic net effect of the parasympathetic nerves that slow the heart and the sympathetic nerves that accelerate the heart. Heart rate allows for the calculation of alternative indices of stress (Pagani et al., 1991), such as exposing dogs to sound blasts or novel objects (Galosy et al., 1979; Beerda et al., 1998; King et al., 2003; Hydbring-Sandberg et al., 2004). The response of heart rate in this study showed the same tendency as SIH. The increases and subsequent decreases in heart rate occurred relatively quickly, in less than 5 minutes in most cases. Heart rate changes are thought to be a nonspecific characteristic to different types and/or levels of stress (Beerda et al., 1998). A significant difference in heart rate was found between the conditioned group and the control group; therefore, if individual differences in time taken to recover to the baseline were compared, heart rate might be useful for evaluating individual differences in response to stimuli. Beerda et al (1998) reported that differences in heart rate recovery depend on the type and/or level of the stimulus. We propose that evaluating a dog's physiological responses, such as heart rate and body temperature, to a fear-conditioned stimulus could provide an objective index of anxiety in dogs, particularly when various breeds are tested together.

When subsequently retested 2 weeks later, the responses of the dogs in the conditioned group had significantly decreased, whereas no significant difference was found in the responses of the control group. This result suggests that the dogs used in our experiment were in the process of forgetting the fear conditioning involving the pairing of the buzzer noise and spray stimulation, and that the spray collar used was a relatively weak aversive stimulus. However, in this study we deliberately chose dogs that did not show behavior problems, especially those related to fear and anxiety. Responses and their persistence could differ for dogs that show fear-related problems. We also observed that during the preparation for the test (putting on the spray collar and the chest band for heart rate measurement), dogs who were subjected to the test at home showed a tendency to be less active than did the dogs at the local training club. To avoid such influences as much as possible, the test was started after the dog settled down, which was confirmed by a heart rate (less than 100 bpm) and rectal temperature (lower than 38.5 degrees centigrade). However, it would be preferable to conduct the test at the same place for all dogs. That said, a comparison of home vs. training center test results may show differential responses for dogs with problems associated with anxiety and fear. Such information would be useful in managing and treating anxious and fearful dogs.

In this study, we showed that autonomic responses to a fear-conditioned stimulus were consistent in 6 dogs in a conditioned group, when compared to the control group. This result provides clear evidence that dogs respond to conditioned stimuli, as suggested by anecdotal evidence from the real world. For example, a dog that shows distress about the owner's absence (i.e., separation anxiety) commonly displays nervous responses not only to the owner's absence itself, but also to cues relating to the owner's absence, such as the wearing of a jacket or the sounds of car keys, which were initially nonaversive and neutral stimuli to the dog (Appleby, 2003; Horwitz, 2003). Our findings suggest that we could more reliably assess individual differences in vulnerability and resistance to fear conditioning in clinics. Doing so would allow us to use such data as a diagnostic tool, such as in the evaluation of intensity of fear response and of the effect of the anxiolytic drug administration in practice. It has been thought that more extreme individuals should be more consistent in their behavior than should intermediate individuals (Wilson et al., 1994). So, if we add these data to behavioral observations in practice, we could get more reliable information about individuals.

Individual response to fear conditioning is important, not only for understanding behavioral problems in dogs, but also for the performance of working dogs. For example, Svartberg (2002) noted that success at high levels of performance in working dog trials requires a certain "boldness score" on the shyness-boldness axis, which is independent of the dog's breed or sex. It will be important in future studies to determine how individual differences for fear conditioning affect fear-related behavioral problems in dogs. It is possible that applying the same spray collar paradigm used in this study as an unconditional stimulus would create too strong a stimulus for more fearful individuals. If we were to manipulate the experimental procedures by changing the intensity of the unconditioned stimulus, the frequency of conditioning, or the interval of measuring the physiological parameters and/or contextual fear-conditioning forgetting time (i.e., more or less than the 2 weeks used in this study), we could assess individual differences for fear conditioning in dogs. These individual differences would describe the dog's behavioral traits more accurately.

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