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Breed-Dependent Differences in the Onset of Fear-Related Avoidance Behavior in Puppies

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Abstract

The onset of fear-related avoidance behavior occurs during and, to some extent, defines the sensitive period of development in the domestic dog. The objectives of this study were to identify the onset of fear-related avoidance behavior and examine breed differences in this behavioral development. Ninety-eight purebred puppies representing three breeds were tested: Cavalier King Charles spaniels (n=33), Yorkshire terriers (n=32), and German shepherd dogs (n=33). Data were collected weekly beginning 4-5 weeks after birth until 10 weeks of age. Puppies took part in four tests during each visit: a novel item, seesaw, step, and loud noise test. During each test, the presence or absence of fear-related avoidance behavior and crouched posture were noted. Saliva was also collected to measure salivary cortisol concentrations in the puppies before and after testing. A later onset of fear-related avoidance behavior was observed in Cavalier King Charles spaniels compared to German shepherd dog and Yorkshire terrier puppies ($F=11.78$, $N=29$, $P < 0.001$). The proportion of treatment puppies that exhibited fear in response to the testing was also different (Chi-Square=9.81, $N=56$, $P=0.007$): Yorkshire terriers ($N=14$, 78%), Cavalier King Charles spaniel ($N=10$, 53%), and German shepherd dogs ($N=5$, 26%). Cortisol concentrations decreased with age. Cavalier King Charles spaniel puppies that demonstrated fear-related avoidance behavior exhibited a greater ($t=2.133$, $N=79$, $P=0.036$) cortisol response than puppies that did not exhibit the behavior. Breed differences in the crouch response to the loud noise test, regardless of age, were observed ($F=18.26$, $N=98$, $P<0.001$). Cavalier King Charles spaniels demonstrated the highest incidence of crouching followed by the Yorkshire Terriers. Breed differences in puppy mobility were observed beginning at 6 weeks of age, with German shepherd dogs demonstrating the most mobility and Cavalier King Charles spaniels the least. The results of this study support the hypothesis that emotional and behavioral development, as well as the onset of fear-related avoidance behavior, varies among breeds of domestic dogs.

Keywords: Behavior, Development, Salivary Cortisol, Canine, Fear

Introduction

Fear and anxiety are emotional states induced by the perception of danger and threat to an individual's well-being (Boissy, 1995). Fearfulness is a very important emotional characteristic that predisposes an individual to perceive and react in a similar manner to a wide range of potentially dangerous events. Signs of fear in the domestic dog include physiologic responses, such as increased heart rate, hypersalivation, and elimination, and behavioral responses, such as escape, avoidance or defensiveness (Sherman and Mills, 2008). Many common canine behavioral problems, including stimulus specific fears and phobias, fear-related aggression toward humans and other animals, and some urination may be based in fearfulness (Sherman and Mills, 2008; Blackwell et al., 2013). The development of fear-related behavioral patterns in the domestic dog may be influenced by genetics, physiology, sensory perception, environmental exposure and experience (Overall, 2013).

Developmental changes in a dog's behavior and physiology begin at birth and extend throughout the dog's life with most change occurring before sexual and social maturity. Several broad time periods, including the "neonatal period", "transition period" and "critical period" (sometimes alternatively described as the sensitive period) in domestic canine development have been described (Scott and Fuller, 1965; Overall, 2013). During the “critical period”, which lasts from approximately three weeks through 12-14 weeks, pups will approach handlers and, if exposed to humans and other novel social and environmental interactions, will become socialized to new experiences (Coppinger & Coppinger, 2001; Freedman, King &Elliot, 1961; Scott and Fuller, 1965). The first expression of an adult-like fear response motor pattern begins during this time as well. Several factors likely play a role in its onset, including the neurophysiological capability to feel fear sufficiently to prompt an active response by the individual puppy, as well as the neuromuscular coordination to be able to carry out this response.

The specific time when the active avoidance of a fearful stimulus first occurs may vary among breeds. First expression of fear-related avoidance behavior patterns occurred at different ages in a study of German shepherd dog and Labrador retriever pups at Hampshire College[1]. At 5 weeks of age, 90% of German shepherd dogs showed moderate to extreme fear, while only 4% of Labrador retrievers showed even moderate fear. Further, a delay in the development of fear-related avoidance behaviors appears to be a result of domestication, since the onset of these responses is observed around 49 days of age in dogs, compared to 19 days of age in wolf pups (Coppinger and Coppinger, 2001). Domesticated foxes were found to have prolonged sensitive periods for socialization of over 60-65 days, compared to only 40-45 days in unselected foxes (Belyaev et al., 1984/85). Differential timing of development of fear response behaviors may influence breed-dependent temperament and behavioral responses in adult dogs.

[1] As reported in “Kennel Enrichment: Dog Studies Program and Lemelson Assistive Technology Development Center”, Lord, K. and Coppinger, R., Hampshire College, Amherst, Massachusetts

Over 50 breeds of dogs across all seven recognized American Kennel Club (AKC) groups have family lines in which “fear/shyness/nervousness/panic/anxiety” are major breeder-reported concerns (Overall et al., 2006). Although fear responses in adult dogs have been widely studied, there is very little published on “normal puppy behavior” and changes over time during puppy development, especially relating to the development of fear and fear-related adult behaviors (Godbout et al., 2007). “Nervous pointer dogs” provide the best example of genetic influence on fear development. This strain of dogs displays an intense and specific fear of humans that has been proposed as a model for human phobias. Beginning around three months of age, these dogs show extreme fear, including crouching, slinking away, and, if cornered, freezing in response to human interaction, regardless of environmental influence (Dykman, et al., 1979).

Work with foxes selected for varying degrees of tameness showed differing patterns of glucocorticoid concentrations by age. Foxes selected for enhanced aggressiveness had similar plasma cortisol concentrations as those selected for tameness at 30 and 60 days of age, but had significantly greater plasma cortisol on day 45 (Plyusnina et al., 1991). Other studies showed a peak in plasma cortisol at a later age in foxes selected for tameness (Trut et al., 2009). In human infants, different stressors activate the hypothalamic-pituitary-adrenal (HPA) axis, with physical stressors evoking a cortisol response, but with psychological stressors failing to do so (Jansen et al., 2010). Although cortisol has been used in a number of investigations of the fear response in dogs, very little research has been conducted on the HPA axis in young dogs.

The objective of this study was to identify, compare, and contrast the onset of fear-related avoidance behavior and concurrent concentrations of salivary cortisol among three specific breeds of purebred puppies between four and 10 weeks of age. Additionally, breed differences in development and mobility were identified. By studying canine development and onset of fear-related avoidance behavior, dog owners, breeders, veterinarians and other professionals gain a more reliable understanding of fear-related development and predictable breed differences. This knowledge could be further applied to improving welfare and standards of rearing, training, socialization, and behavioral modification techniques in domestic dogs of various breeds that are raised as pets and as working dogs. In addition, a broader understanding of the development of the fear response could lead to the prevention of fear-related behavioral problems, by increasing the potential for, and understanding of, appropriately timed exposure and socialization techniques for puppies in the sensitive periods of their development.

Materials and Methods

Research Design

The research was conducted in a randomized complete block design with repeated measures on puppies, which were blocked by litter, within breed. Ninety-eight puppies were tested within three purebred dog breeds: Cavalier King Charles spaniels (n=33, 19 treatment group puppies and 14 control group puppies), Yorkshire terriers (n=32, 18 treatment group puppies and 14 control group puppies), and German shepherd dogs (n=33, 19 treatment group puppies and 14 control group puppies). Seven litters of Cavalier King Charles spaniels (5 breeder locations), nine litters of Yorkshire terriers (3 breeder locations), and five litters of German shepherd dogs (3 breeder locations) were tested in the homes of volunteer cooperating dog breeders throughout the state of Ohio in the United States. Dog breeders were located and contacted through websites, dog breed clubs, shows and personal contacts. Breeders were provided with an overview of the study prior to the first testing of the litter and were asked to sign a voluntary consent form for each litter tested. Breeders were also notified that puppies or litters could be removed from the study at any time for any reason. Selection of the three breeds used in this study was based on breed background, personality, physical characteristics, and the availability of litters within the state of Ohio.

Cavalier King Charles spaniels, classified morphologically as a brachycephalic breed, have been bred to be "friendly, nonaggressive with no tendency toward nervousness or shyness" (American Kennel Club; AKC). Due to the availability of cooperative breeders within the central Ohio area, this was the first breed selected for the study. The Yorkshire terrier, with a smaller ratio of skull width to skull length (cephalic index) has a mesaticephalic skull. Although placed in the AKC Toy Group with the Cavalier King Charles spaniel, the AKC describes Yorkshire terriers as "… big personalities in a small package. Though members of the Toy Group, they are terriers by nature and are brave, determined, investigative and energetic." The Yorkshire terrier breed offered a strong contrasting personality and divergent genetic and morphological background from the Cavalier King Charles spaniel, yet is within the same AKC group, and was therefore selected as the second test breed.

The German shepherd dog has been the subject of many previous behavioral studies (Mackenzie et al., 1985; Ruefenacht et al., 2002; Strandberg et al., 2005; Svobodová et al., 2008). The German shepherd dog's breeding for herding and protectiveness, dolicocephalic morphology, and AKC classification in the Herding Group, made it a strong contrast to the first two breeds chosen for the study, and allowed for comparison to published experimental results.

Litters were selected for testing only from breeders with strong international (primarily Czech or German), working dog (law enforcement and protection) bloodlines, as German shepherd dog breeders informed us there can be distinct differences in personality and development among genetic lines within this breed.

Approximately one-half of the puppies in each litter were randomly assigned to the treatment group and the other half to the control group. Testing and data collection were performed once a week beginning when the puppies were between four and five weeks of age, based on convenience to the breeders' and tester's schedule. Due to lack of responses to any stimuli in Cavalier King Charles spaniel puppies at four weeks of age, we did not begin testing the majority of Cavalier puppies until five weeks of age. Testing and data collection continued until the treatment puppies demonstrated the onset of fear avoidance behavior, reached 10 weeks of age, or left the breeder's home, depending on which occurred first.

Puppies took part in four tests at each weekly visit: Novel Item Test, Seesaw Test, Step Test and Loud Noise Test. The test and puppy order was randomized each week. Fear-related avoidance behavior was defined as the immediate, rapid and pronounced movement away from the object or experience perceived as a hazard. "Crouched posture" was defined as a rapid pronounced lowering of the body. The presence or absence of these behaviors was noted during each test. The final physical location of each puppy was also noted at the conclusion of each test. The location of the puppy was defined by the region of the test mat on which the majority of the puppy's feet were placed. If the feet were evenly distributed between two areas, the location of the puppy's head was used to determine final location. Identical observations were made for all puppies in the control group who were tested on the same testing mats as the treatment puppies, however with no exposure to any of the stimuli.

All four tests for both treatment and control group puppies were videotaped to verify the data recorded at the initial time of testing, using a Sony Handycam Digital Video Camera Recorder DCR-SR45 with 30 GB hard disk drive (Sony Electronics, Inc., San Diego, CA). The video camera was mounted on a tripod (Sunpak 3300 Pro tripod, Sunpak ToCAD America, Inc. Rockaway, NJ) to allow for stable, hands-free recording. Prior to recording each segment, camera placement was checked to insure that the test area was in the field of view. Recording segments began prior to the puppy being placed in the test location, each test was timed with a wrist-held stopwatch, and the recording was stopped at the end of each test. Recording segments were identified by puppy, test, age, and date recorded. Additionally, the video recordings of 17 Cavalier King Charles spaniels (12 treatment and five control), 25 Yorkshire terrier (17 treatment and eight control), and 15 German shepherd dog (10 treatment and five control) puppies were evaluated by two outside personnel (an undergraduate student intern and a veterinary student) who were not present at the time of testing and were blind to the primary investigator's results. Results obtained from reviewing the videos were compared to those obtained during live testing to assess the reliability of the results.

Behavioral Testing

All tests were performed by the same person, who placed the puppy on the appropriate mat, stepped back approximately 4 feet, and initiated observations. Testing took place in a room or area near or adjacent to, but not directly beside or in the pen in which the puppies were being reared, so that other puppies in the litter could not observe testing prior to be tested. A puppy was removed one at a time from its pen, with the rest of the litter remaining in the pen. The puppy being tested progressed directly from one test to the next and was placed back in the puppy pen at the completion of the fourth test, at which time the next puppy was removed from the puppy pen for testing. The order of the puppies tested and order of the tests administered was randomized each visit.

NOVEL ITEM TEST: Treatment puppies were placed on the marked center of a mat (61 x 122 cm; Figure 1A) facing a forward-moving, light and noise-emitting stuffed toy (duck; 20 x 24 cm) that was activated at the moment the puppy was placed on the mat. The test was limited to five seconds to minimize the chances of desensitization to the novel object each week. Control group puppies were also placed in the same marked location on the mat for five seconds, but were not exposed to the novel stimulus. Each puppy received a score of 0 (remained in neutral position), +1 (moved into approach section of the mat) or -1 (moved into avoid section of the mat).

SEESAW TEST: Treatment group puppies were placed on the marked center of a board balanced on a central fulcrum (seesaw, covered with rubber, anti-slip matting, 141 x 122 cm, 12 cm off the ground; Figure 1B), for 10 seconds. Observations were also made on control group puppies, which were placed on an identically marked and matted stable, floor-level surface for 10 seconds. Each puppy received a score of 0 (remained in neutral position), +1 (moved into mid-region of seesaw or marked mat) or +2 (moved to edge or off seesaw or mat).

STEP TEST: Treatment group puppies were placed on the marked center of a small platform (covered with rubber, anti-slip matting) for 10 seconds. The platforms were made proportional in surface size and height to the size, age and mobility of the puppies, such that the puppies would perceive being on a small ledge (23 cm in length and width, 5 cm high for the Cavalier King Charles Spaniel and Yorkshire Terrier puppies, and 20 x 41 cm x 10 cm high for the German Shepherd Dog puppies; Figure 1C). Control group puppies were observed on an identically marked flat surface for 10 seconds. Each puppy received a score of 0 (remained on the step) or +1 (moved off the step).

LOUD NOISE TEST: Treatment group puppies were placed on the marked center of a mat. Within three seconds after being placed on the mat, the 23 x 23 x 5 cm step from the Step Test was dropped approximately 41 cm in front of the puppy from a height of approximately 51 cm, resulting in a short, sudden loud noise (Figure 1D). To determine the intensity of the loud noise, a 3M Quest Technologies sound level indicator, precalibrated prior to use at 114 dB, was used to measure the sound level in 10 repetitions of dropping the step. The mean reading was 95.36 dB with a SE of 0.45 (SD=1.43). The Loud Noise Test also included visual stimulus because the puppies could see the step, but visual reaction was not recorded because many puppies were not oriented in the direction of the falling object and responded only to the sound. The immediate reaction of the puppy was observed for five seconds. Control group puppies were placed in the same marked location on the mat for approximately seven seconds but were not exposed to the sudden noise. Each puppy received a score of 0 (remained in neutral position), +1 (moved into approach section of the mat) or -1 (moved into avoid section of the mat).

Salivary Cortisol Collection and Assay

Saliva was initially collected on all three breeds studied but the majority of samples from the Yorkshire terriers did not yield enough saliva for assay, so this breed was ultimately not used for salivary cortisol evaluation. Puppy saliva was collected on Salimetrics, L.L.C. (State College, Pennsylvania, USA) sorbettes using two sorbettes per puppy for each collection. Sorbettes were placed in each puppy's mouth and moved over and under the tongue and inside each cheek for approximately 1-2 min. The first salivary sample was collected prior to any testing. The second sample was collected in the same manner as the first, approximately 20 min after the conclusion of the final test, which has been demonstrated as an appropriate length of time to detect a stress-induced elevation of salivary cortisol (Dreschel and Granger, 2005). Saliva-soaked sorbettes were placed in 1.5 ml microcentrifuge tubes, labeled and placed on ice within 20 min. Samples were frozen in a -20 degrees Celsius freezer within 12 h. For assay, samples were thawed to room temperature for centrifugation and sorting. Samples were centrifuged at 10,000 x g for 20-30 min, at four degrees Celsius, and sorbettes were removed from the tubes. Samples were then assayed or refrozen until a later date for assay. Salivary samples that were refrozen were thawed to room temperature and centrifuged again at 10,000 x g for 15 min at four degrees Celsius on the day of assay to precipitate mucins. Free cortisol was assayed using an expanded range high sensitivity salivary cortisol enzyme immunoassay kit from Salimetrics, L.L.C. (State College, Pennsylvania, USA). The salivary cortisol assay was performed following the directions of the Salimetrics kit. The minimal concentration of free cortisol that our assay could distinguish from 0 was 0.007 μg/dl (i.e., 2 standard deviations from the 0 standard).

Statistical Analyses

Data were analyzed using the Statistical Analysis System (SAS) 9.2 TS Level 2 MO. Parameters were estimated using restricted maximum likelihood (REML) and Fisher's Protected LSD was used for means separation when the F-test indicated significant differences. Reported means are least-squared means unless otherwise noted. Mixed models with repeated measures across weeks were used to analyze the dependent variables of puppy final location and the presence or absence of a crouched posture in each test.

The proportion of treatment group puppies expressing fear-related avoidance behavior at some point during testing was analyzed as contingency tables with three proportions (Chi-square test with 2 df). When significant, this test was followed with 2 x 2 contingency tables (Chi-square test with 1 df) to compare the proportions between each of the 3 pairs of breeds. The significance threshold for these latter tests was penalized using a Bonferroni inequality to ensure that the familywise error rate remained under 5% (Milliken and Johnson, 2009).

For continuous dependent variables, the mixed models included the fixed effects of test, breed, gender, group (treatment or control), age, and reviewer, with the random effects of litter within breed and puppy within litter within breed. Residual plots were produced and did not indicate violation of the normality of errors assumption.

For cortisol analyses, the treatment structure of this experiment was a 2 x 2 x 2 x 4 factorial arrangement (2 breeds, 2 genders, 2 treatments, and 4 ages) as defined by Milliken and Johnson (2009). The design structure was a split block design (where the litters within breed served as blocks) with repeated measures (the weekly measurements on the puppies). The model for basal cortisol concentrations (cortisol measurements made before puppies were subjected to the stimuli or placebo tests) and the cortisol response (post stimuli minus pre stimuli) included the fixed effects of breed (1 df), gender (1 df), treatment (1 df), age (3 df), and all 2-way and three-way interactions terms except for gender, and the random effects of litter within breed and puppy within breed x litter x gender. The error correlation matrix due to the repeated measures on each puppy was modeled as a heterogeneous autoregressive structure, since it yielded the smallest Bayesian information criterion of a variety of error structures investigated. The interactions of fixed effect factors with age were tested using the SLICE option of the LSMEANS statement of the MIXED procedure.

Ethical Approval

Research design and protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University.

Results

Behavioral Tests

There were no effects of sex, litter or reviewer in any of the analyses. The onset of fear-related avoidance behavior was indicated for any puppy that exhibited immediate, rapid and pronounced movement away from the object or experience perceived as a hazard in any single test during a testing session. Results from two external video reviewers were not significantly different from the results of the original tester's on-site evaluations of the same puppies ($F=0.10$, $N=57$, $P=0.907$). Representative videos showing the response of the same Yorkshire terrier puppy in the Novel Item Test before (at 31 days of age) and after (at 38 days of age) the onset of fear-related avoidance behavior may be viewed in Supplementary Videos 1 and 2, respectively.

The proportion of treatment group puppies that exhibited fear-related avoidance behavior in any test varied significantly among the three breeds and there was a significant breed effect on the age at onset of this behavior (Table 1). The onset of fear-related avoidance behavior was later in Cavalier King Charles spaniel (N=10) compared with German shepherd dog (N=5) (t=4.85, P<0.001) and Yorkshire terrier (N=14) (t=3.04, P=0.014) puppies (Table 1). There was no significant difference between German shepherd dog and Yorkshire terrier puppies in the age at onset of fear-related avoidance behavior.

The Step Test data were determined by whether the puppy remained on the step or moved off the step (or step-marked region on the mat for control puppies) during the ten-second test period. There was a significant treatment by breed interaction (F=4.36, N=98, P=0.014) on the final location of puppies in the Step Test. No effects of treatment were observed in Yorkshire terrier puppies (Figure 2). Treatment and control group German shepherd dog and Cavalier King Charles spaniel puppies demonstrated significant differences in their behavioral responses to the Step Test (Figure 2). Control group German shepherd dog (t=2.01, N=33, P=0.045) and Cavalier King Charles spaniel (t=3.17, N=33, P=0.002) puppies of all ages were significantly more likely to move out of the designated “step area” than treatment puppies of the same breed were to move off of the step. There was also a significant breed effect on the final Step Test location (F=18.24, N=98, P<0.001). All (treatment and control) German shepherd dog and Yorkshire terrier puppies were significantly more likely to move off the step (treatment) or “step area” (controls) than Cavalier King Charles spaniel treatment or control puppies.

Figure 3 illustrates results from the Novel Item Test. Age by breed results from the Loud Noise Test were identical in pattern to the Novel Item Test. There was a significant breed by age interaction (F=2.87, N=98, P=0.003) in the final location of puppies in the Novel Item Test. There were no significant breed differences from four to six weeks of age. At seven weeks of age, all (treatment and control) German shepherd dog and Yorkshire terrier puppies were more likely to move into the "Approach" section of the mat than Cavalier King Charles spaniel puppies of the same age. German shepherd dog puppies also became significantly more likely to move into the "Approach" section of the mat at seven weeks compared to six weeks of age (t=2.50, N=33, P=0.013). Differences between German shepherd dog and Yorkshire terrier puppies in final location to the Novel Item Test was observed at eight (t=3.16, N=55, P=0.002) and nine (t=3.67, N=55, P<0.001) weeks of age, when the German shepherd dog puppies became significantly more likely to move into the "Approach" section of the mat than the Yorkshire terrier puppies of the same age.

There was a significant age x breed interaction (Figure 4; F=3.90, N=98, P<0.001) in the Seesaw Test. Beginning at six weeks of age, the German shepherd dog and Yorkshire terrier puppies became significantly more likely to move to the mid-region, edge or completely off the seesaw or identically marked region on the mat (control puppies) compared with the Cavalier King Charles spaniel puppies of the same ages (Figure 4; German shepherd dog, $t \geq 3.94$, P<0.001; Yorkshire terrier, $t \geq 2.23$, P<0.05). Also, comparisons of age within breed revealed that German shepherd dog ($t \geq 5.10$, P<0.001) and Yorkshire terrier ($t \geq 3.03$, P<0.01) puppies six to nine weeks of age were more likely to move from the starting location than those within breed at four and five weeks of age.

There was a significant breed x treatment interaction on the incidence of crouched posture in the Loud Noise Test (Table 2; F=18.26, N=98, P<0.001). A greater proportion of

Cavalier King Charles spaniel treatment puppies demonstrated crouched posture compared to both the German shepherd dog (t=6.85, P <0.001) and Yorkshire terrier (t=4.46, P<0.001) treatment group puppies, which also differed significantly from one another (t=2.87, P=0.005). In addition, there was an age x treatment interaction in the incidence of crouched posture in the Loud Noise Test (F=2.55, P=0.020). The incidence of crouching in control group puppies remained similar among all ages, however, the incidence of crouching among treatment group puppies became significantly less after six weeks of age, regardless of breed or sex (t≥2.88, P<0.004).

Cortisol Measurements

Mean salivary cortisol concentration in German shepherd dog and Cavalier King Charles spaniel puppies of all ages and treatments was 0.439 μg/dl. The ANOVA showed no main effects of gender on salivary cortisol. As it was not always possible to collect sufficient saliva from the young puppies, the total data set contained a number of missing values. To evaluate cortisol concentrations most accurately over time, data were analyzed from only those individuals for whom at least three weeks of cortisol values were obtained. Cortisol decreased with age similarly in both breeds, with cortisol concentrations in week 8 being significantly less than those at weeks 5 and 6 (Figure 5; t=2.37, N=19, P<0.020). German shepherd dog puppies tended (F=3.80, N=19, P=0.060) to have greater concentrations of cortisol than Cavalier King Charles spaniel puppies (Figure 5).

There was an acute response of cortisol to treatment in Cavalier King Charles spaniels (t=2.26, P=0.020), but the response was not significant in German shepherd dogs (t=0.58;

P>0.050; Figure 6A). Cavalier King Charles spaniel puppies that exhibited fear-related avoidance behavior had a greater cortisol response than control and treatment puppies that did not exhibit the behavior (t=2.133, N=79, P=0.036; Figure 6B). Cortisol responses among groups of German shepherd dog puppies did not differ significantly (t=1.088, N=108, P=0.279).

Discussion

The results of this study support the hypothesis that the onset of fear-related avoidance behavior in domestic dogs varies by breed. Significant breed differences were observed in both the age at onset of fear-related avoidance behavior and the proportion of puppies that demonstrated this behavior. In addition, behavioral responses to the tests and changes in mobility during early puppy development differed among breeds.

These results are in agreement with previous research by Coppinger[2], who demonstrated a defined and predictable age of onset of fear in working German shepherd dogs to be about 35 days of age. The results from this study are similar, with a least squared means estimate for the onset of fear-related avoidance behavior in the German shepherd dog of 39.4 ± 6.5 days in those dogs that exhibited the behavior. Additionally, a mean onset age of fear-related avoidance behavior was identified in two other breeds of dogs, the Cavalier King Charles spaniel (55.1 ± 3.1 days SEM) and the Yorkshire terrier (42.2 ± 2.5 days SEM). The Cavalier King Charles spaniel puppies demonstrated a significantly later onset of fear-related avoidance behavior compared with both the Yorkshire terrier and German shepherd dog puppies. This delayed onset of fear-related avoidance behavior may be related to a slower developmental rate of the Cavalier

[2] See footnote 1

King Charles spaniel, which is also reflected in the results of the development of mobility among breeds and ages. In a study by Goodwin and colleagues (1997), the Cavalier King Charles spaniel ranked least similar to the wolf in physical characteristics, and showed the fewest number of wolf-like agonistic visual signals. This indicates a physical and behavioral paedomorphosis, or underdevelopment, compared to other breeds, such as the German shepherd dog, which ranked close in appearance and agonistic signaling to the wolf. The results of the current study also indicate that behavioral development proceeds at a slower rate than in some other breeds. In fact, due to lack of responses to any stimuli in Cavalier King Charles Spaniel puppies at four weeks of age, we did not begin testing the majority of Cavalier puppies until five weeks of age.

The breeders of puppies used in this study anecdotally report that the Cavalier King Charles spaniel puppies tend to have their eyes open and begin weaning and exploration at a later age than many other breeds. The breed demonstrates the most neoteny of the three breeds selected with their high ratio of skull width to skull length (cephalic index). Just as dogs and domesticated foxes have longer critical socialization periods than wolves (Lord, 2012) and non-selected foxes (Belyaev, et al, 1984/85) respectively, perhaps more neotenous breeds of dogs such as the Cavalier King Charles spaniel would also have a longer socialization period. Trut and colleagues (2009) note that neotenization of behavioral and morphological traits are correlated with delayed developmental rates even *in utero*. Selection pressures have varied for the three breeds in this study, with the Cavalier King Charles spaniels selected primarily as a royalty "luxury item" or house pet, the German shepherd dog selected from lines of working dogs, and the Yorkshire terrier with origins as a working rat catcher belonging to the middle class. A recent study has noted that behavioral characteristics of dogs can be related to height, bodyweight and skull shape (McGreevy, et al, 2013). The morphological differences among the three breeds in the present study provide an interesting contrast in studying the covariation of behavior and body structure.

Analysis of salivary cortisol demonstrated that young puppies release detectable concentrations of cortisol as early as four weeks of age. Interestingly, the mean cortisol concentrations of puppies tested were two to four times the baseline concentrations of adult dogs studied by Dreschel and Granger (2005, 2009). Studies of human infants over time show a decrease in basal cortisol over the first year of development (Tollenaar et al., 2010). It is likely that the adrenal glands of the dog develop in a similar fashion, accounting for the greater concentration of cortisol found in very young puppies compared to adult dogs (Beerda et al., 1998; Dreschel and Granger, 2005).

Significant increases in salivary cortisol concentrations have been identified 10-30 min after exposure to fear-inducing stimuli in dogs, humans, and rats (Beerda et al., 1998; Dreschel and Granger, 2005; Muir and Pfister, 1987; Tarui and Nakamura, 1987). In the present study, Cavalier King Charles spaniel puppies that showed fear-related avoidance behavior in response to the testing had a greater percentage cortisol change from pre-test to post-test samples than those puppies that did not exhibit the behavior. The increase in cortisol is a physiological change that may be associated with the onset and expression of fear-related avoidance behavior in puppies.

Goddard and Beilharz (1985), King et al. (2003), Mahut (1958), Plutchik (1971), and Scott and Fuller (1965) have all identified breed differences in behavioral fear responses in adult dogs. They examined movement toward or away from novelty, time spent near a novel item, as well as behavioral fear indicators, such as the freeze response, crouching, trembling and vocalizing. The current study revealed no significant breed effects on behaviors demonstrated among puppies at four or five weeks of age. Breed differences in mobility were first identified at six weeks of age when both the German shepherd dog and Yorkshire terrier puppies demonstrated significantly more movement in the Seesaw Test compared to the Cavalier King Charles spaniel puppies. By seven weeks of age the German shepherd dog and Yorkshire terrier puppies showed significantly greater movement in the Novel Item, Loud Noise, and Seesaw Test compared to the Cavalier King Charles spaniel puppies of the same age. At eight and nine weeks of age Yorkshire terriers and Cavalier King Charles spaniels were similar in their mobility during the Novel Item and Loud Noise Test, however the Cavalier King Charles spaniel demonstrated the least amount of movement in the Seesaw Test for the duration of the study, while German shepherd dog puppies consistently demonstrated a high level of mobility that began at six weeks of age and continued through the final day of testing.

Regardless of breed, all treatment group puppies between four and six weeks of age exhibited significantly greater incidences of crouching in response to the Loud Noise Test compared to puppies older than six weeks of age. According to Scott and Fuller (1965), the startle response to sound appears at an average of 19.5 days (SD 2.3 days), so it is likely that the crouching at this age in response to the sudden, loud noise was a reflexive startle reaction and not an adult-like, active fear-related response. Lord (2012) also makes this distinction between a startle reaction and a more permanent avoidance of fear. In fact, beginning at seven weeks of age, treatment group puppies of all breeds showed significantly fewer crouching reactions to the Loud Noise Test. It is also possible that the decline in the incidence of crouch response to the

Loud Noise Test was related to an acclimation over time of the puppies to the sudden noise, as the test was repeated weekly, starting when the puppies were four to five weeks of age. Habituation is described as a type of learning where the response to a stimulus decreases over time, with repeated exposure to the stimulus (Grissom and Bhatnagar, 2009). Using puppies that were naïve to the tests at each time point would eliminate that possibility, but would require a much larger sample population, introducing much greater variability between and within litters. While habituation to the loud noise stimulus could play a role, the results showing that puppies exhibited a dramatic avoidance response to the fearful stimuli at a later time, indicates that habituation to the stimulus did not significantly influence our ability to detect the onset of a fear response.

Other studies have demonstrated differences in fear responses between sexes (Döring, et al., 2009; Goddard and Beilharz, 1985; Plutchik, 1971; Wells and Hepper, 2000). No sex differences in development or expression of fear reactions were observed in the current study. However, if a sex difference in fear reactivity exists in dogs, it is possible that it is not identifiable until after puberty when other behavioral and phenotypic sexual dimorphisms also become more apparent.

Conclusion

The results of this study indicate that there are significant differences among the three breeds studied in the onset and expression of fear-related avoidance behavior. There were also statistically significant breed differences in development of mobility related to age and incidence of crouching in response to a sudden noise. An expanded and more reliable understanding of breed-specific development has applications in the breeding, rearing, training, socialization and behavioral modification of domestic dogs. Understanding developmental behavioral responses to fear-inducing stimuli may help to prevent fear-related behavioral problems and the high costs and diminished welfare that accompany them.

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Authorship Statement

The idea for the paper was conceived by Peter Neville and Joy Pate. The experiments were designed by Mary Morrow, with significant input from Peter Neville, Joy Pate, and Nancy Dreschel. The experiments were performed by Mary Morrow, Ann Ottobre and Joe Ottobre. The data were analyzed by Mary Morrow, Normand St-Pierre, Ann Ottobre and Joe Ottobre. The paper was written by Mary Morrow, Joy Pate and Nancy Dreschel, with significant input from all other authors.

Conflict Statement

The authors have no conflicts of interest to disclose related to this manuscript

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Figure Legends

Figure 1. Mat dimensions, design and photographs for the Novel Item (A), Seesaw (B), Step (C) and Loud Noise (D) tests. In the Step Test, the size of the box was dependent on the breed (See methods text).

Figure. 2. Breed and group differences in final location in the Step Test. Each puppy received a score of 0 (remained on the step) or 1 (off the step). Values expressed as least squared means ± SEM. N=33 German shepherd dogs (GSD), N=33 Cavalier King Charles spaniels (CKCS); N=32 Yorkshire terriers (YT). *indicates a significant difference from control within breed. Breed effect on final location is indicated by horizontal lines, $F=18.24$, $P<0.001$.

Figure. 3. Breed by age differences in final location in the Novel Item Test. Each puppy received a score of 0 (remained neutral), 1 (moved into approach section of mat) or -1 (moved into avoid section of mat). CKCS = Cavalier King Charles spaniels, GSD = German shepherd dogs, YT = Yorkshire terriers. Data include treatment and control puppies and values are expressed as least squared means ± SEM. [a,b]Values with different superscripts are significantly different among breeds within week ($P<0.010$). * Indicates a significant difference from weeks 4-6 within breed (German shepherd dogs; $t \geq 2.5$, $P \leq 0.013$, N=33)

Figure. 4. Breed differences in final location in the Seesaw Test. Each puppy received a score of 0 (remained where placed at beginning of test), 1 (moved to mid-region of seesaw or marked mat) or 2 (moved to edge or off seesaw or mat). Data include treatment and control puppies (N=98) and results are expressed as least squared means ± SEM. *

Indicates a significant difference among breeds within age (Cavalier King Charles spaniels (CKCS) vs German shepherd dogs (GSD), t>3.94,P<0.001; Cavalier King Charles spaniels vs. Yorkshire terriers (YT), t>2.23, P<0.050). . $^{+}$Indicates a significant difference among ages within breed, values for German shepherd dog (t>5.10, P<0.001) and Yorkshire terrier (t>3.03, P<0.010) puppies weeks 6-9 were greater than those on weeks 4 and 5.

Figure 5. Baseline (pretreatment samples) salivary cortisol concentrations among German shepherd dog (GSD, N=10) and Cavalier King Charles spaniel (CKCS, N=9) puppies for which cortisol values were obtained on at least 3 different weeks. Data are presented as least squared means ± SEM.

Figure 6. Cortisol response to treatment in Cavalier King Charles spaniel (CKCS) and German shepherd dog (GSD) puppies. A) Percent change in cortisol 20 minutes following treatment in Control (N=34) and Treatment (N=45) CKCS and Control (N=45) and Treatment (N=63) GSD. Treatment group includes puppies that did, and those that did not, exhibit fear-related avoidance behavior. *Indicates difference from Control, within breed, (t=2.26, P=0.020). B) Percent change in cortisol in puppies that did not (No Fear Response; N=72 CKCS, N=105 GSD) or did (Fear Response; N=7 CKCS, N=3 GSD) exhibit fear-related avoidance behavior. Cavalier King Charles spaniel puppies that exhibited fear-related avoidance behavior had significantly (t=2.133, N=79, P=0.036) greater cortisol response than all puppies (control and treated) that did not exhibit the behavior. *Indicates difference from Control, within breed, (P<0.05).

Table 1. Onset of fear-related avoidance behavior among breeds.

Breed	% of Treatment Puppies Demonstrating the Behavior[1]	Days of Age at Onset [2]
Cavalier King Charles spaniel	53[ab]	54.8 ± 2.74[a]
German shepherd dog	26[a]	39.4 ± 1.60[b]
Yorkshire terrier	78[b]	43.6 ± 2.48[b]

[1] Chi-Square=9.81, N=56, P=0.007; Pair-wise comparisons: CKCS vs GSD: Chi-Square=2.75, N=38, P=0.097; CKCS vs YT: Chi-Square=2.56, N=37, P=0.109; GSD vs YT: Chi-Square=9.80, N=37, P=0.002

[2] F=11.78, N=29, P<0.001, Data expressed as least squared means ± SEM

[a,b] Values within a column with uncommon superscripts are significantly different

Table 2. Incidence of crouching in loud noise test, breed by treatment interaction*

Breed	Control % (N)	Treatment % (N)
Cavalier King Charles spaniel	0.2 ± 7.2 (14)	79.5 ± 6.3[a] (19)
German shepherd dog	2.9 ± 7.2 (14)	22.4 ± 6.3[b] (19)
Yorkshire terrier	1.2 ± 6.7 (14)	43.8 ± 5.8[c] (18)

Data expressed as least squared means ± SEM

*F=18.26, N=98, P<0.001

[a,b,c] Values with uncommon superscripts are significantly different (P<0.01).

Figure 1

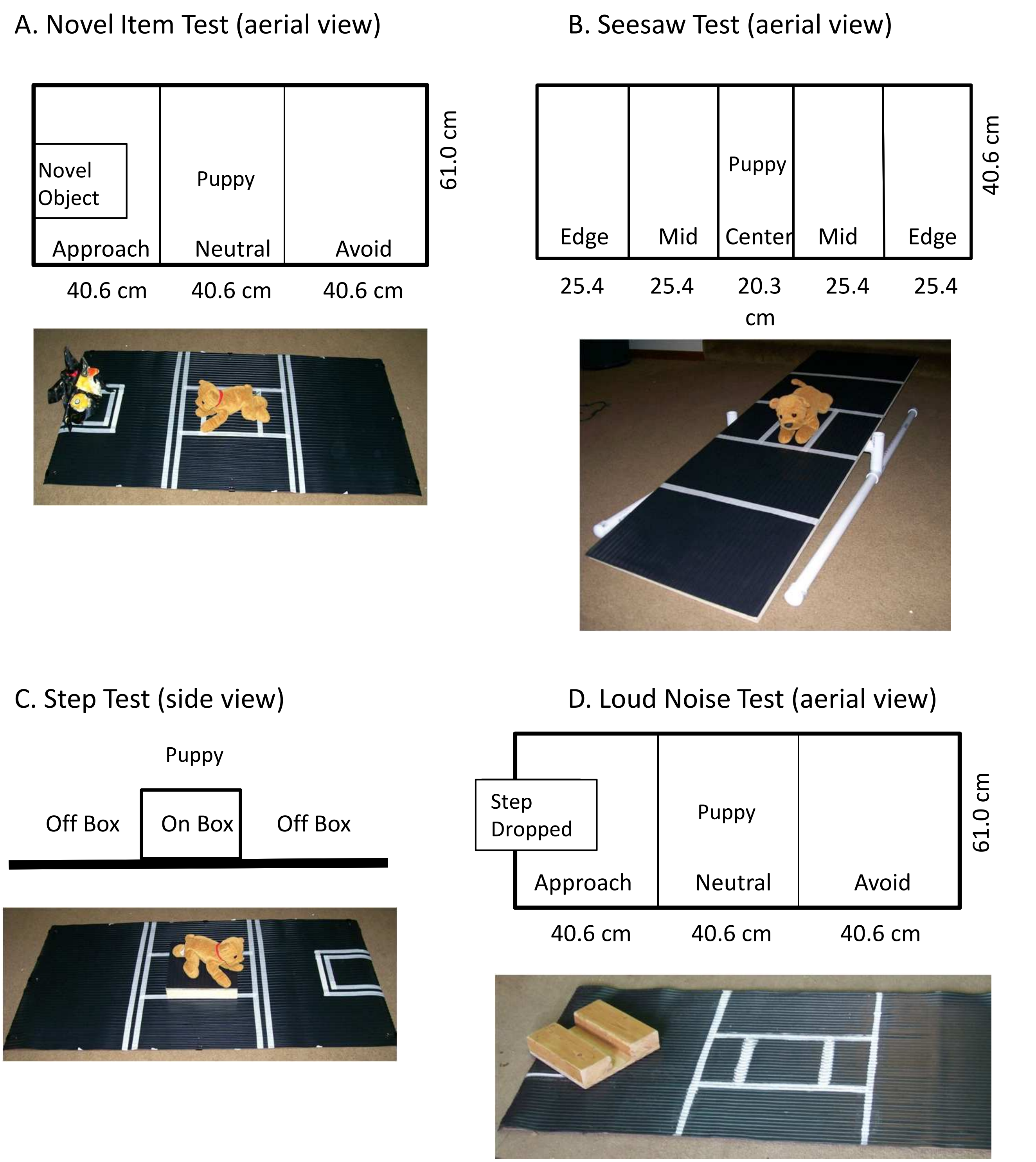

A. Novel Item Test (aerial view)
Novel Object
Puppy
Approach
Neutral
Avoid
61.0 cm
40.6 cm
40.6 cm
40.6 cm
B. Seesaw Test (aerial view)
Puppy
Edge
Mid
Center
Mid
Edge
40.6 cm
25.4
25.4
20.3 cm
25.4
25.4
C. Step Test (side view)
Puppy
Off Box
On Box
Off Box
D. Loud Noise Test (aerial view)
Step Dropped
Puppy
Approach
Neutral
Avoid
61.0 cm
40.6 cm
40.6 cm
40.6 cm

Figure 2

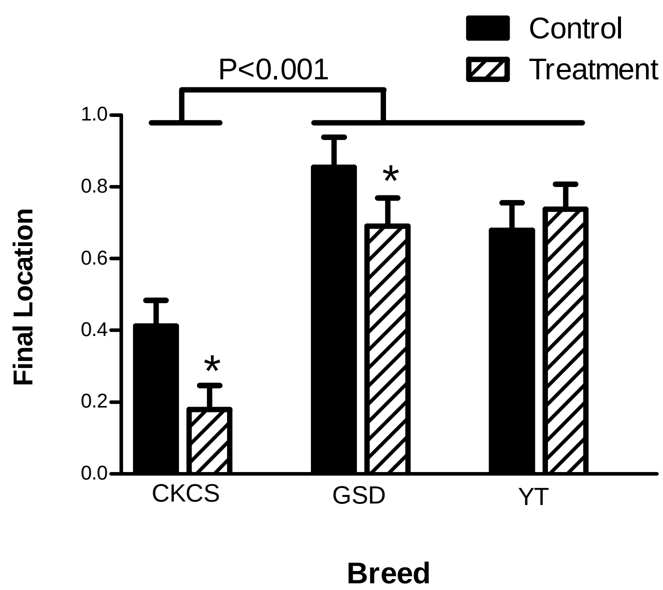

Control
Treatment
P<0.001
Final Location
1.0
0.8
0.6
0.4
0.2
0.0
*
*
CKCS
GSD
YT
Breed

Figure 3

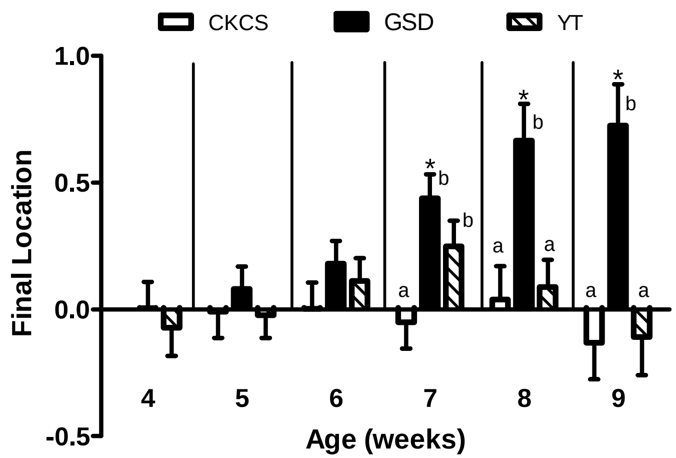

CKCS
GSD
YT
1.0
0.5
0.0
-0.5
Final Location
*
b
b
a
a
a
b
a
a
4
5
6
7
8
9
Age (weeks)

Figure 4

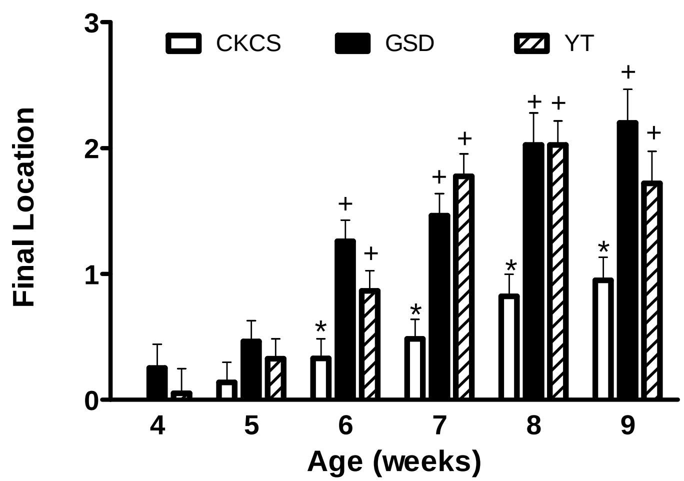


Figure 5

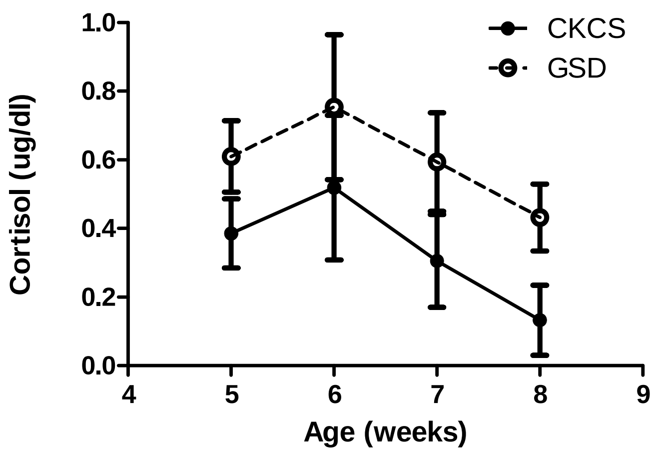
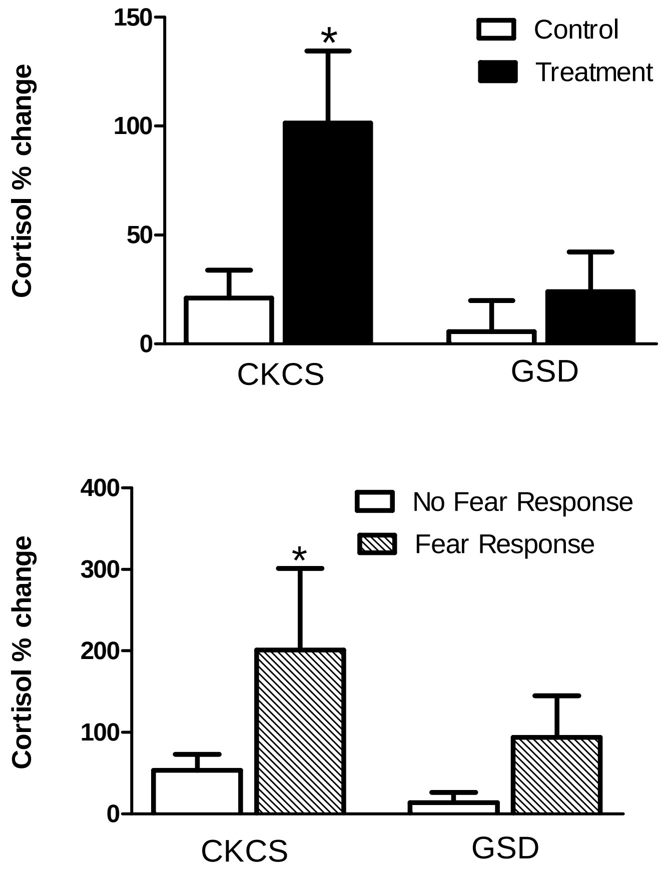


Figure 6


Cortisol % change
150
100
50
0
*
Control
Treatment
CKCS
GSD
Cortisol % change
400
300
200
100
0
*
No Fear Response
Fear Response
CKCS
GSD

Highlights

- Puppies of three breeds were tested for active avoidance of perceived hazards and for cortisol reactivity using novel stimuli from four to nine weeks of age
- The onset of fear-related avoidance behavior, as well as other indicators of fearfulness differed by breed
- Cortisol decreased with age in puppies, and a greater cortisol response occurred in puppies that exhibited fear-related avoidance behavior than in those that did not
- Prediction of developmental responses to fear-inducing stimuli may increase the potential for preventing fear-related behavioral problems