VARIATION IN THE ONSET AND EXPRESSION OF HAZARD AVOIDANCE BEHAVIOR ACROSS THREE BREEDS OF DOMESTIC DOGS

A Thesis

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The onset of adult hazard avoidance behavior (fear motor patterns) occurs during the critical period of development in domesticated canines. In wolves, the onset of adult hazard avoidance behavior occurs at approximately 19 days (Coppinger and Coppinger, 2001). This onset is delayed in domestic dogs, taking place as late as day 72 in the Labrador Retriever (Coppinger and Coppinger, 2001). The objectives of this study were to identify, compare, and contrast the onset and expression of adult hazard avoidance behavior across three breeds of puppies between four and ten weeks of age and examine developmental breed differences in heart rate, behavior, and salivary cortisol concentrations. Ninety-eight puppies were tested within three purebred breeds: Cavalier King Charles Spaniels (n=33), Yorkshire Terriers (n=32), and German Shepherd Dogs (n=33). Data were collected weekly beginning when the puppies were 4-5 weeks of age and continuing until the treatment group puppies demonstrated hazard avoidance behavior, reached ten weeks of age, or left the breeder home. Puppies took part in four tests each visit: a Novel Item Test, Teeter-Totter Test, Step Test, and Loud Noise Test. During each test, the presence or absence of crouching and hazard avoidance behavior were noted. Heart rate was measured initially, following each test, and at the conclusion of the testing period. Saliva samples were also collected to measure salivary cortisol in the puppies prior to testing and 20 minutes following the final test.
Our results indicated a significantly (P < 0.05) later onset of hazard avoidance behavior in Cavalier King Charles Spaniels compared to both German Shepherd and Yorkshire Terrier puppies. The proportion of treatment puppies exhibiting hazard avoidance behavior at any point was also significantly (P < 0.01) different: Yorkshire Terriers (82%), Cavalier King Charles Spaniel (53%), and German Shepherds (26%). Yorkshire Terriers demonstrated the highest (P < 0.05) heart rates at five weeks of age, while Cavalier King Charles Spaniel heart rates were the lowest (P < 0.03) from six through eight weeks of age.

Significant (P < 0.05) breed differences in puppy mobility were found beginning at six weeks of age, with German Shepherds demonstrating the most mobility and Cavalier King Charles Spaniels the least. There were also significant breed (P < 0.01) differences in the crouch response to the Loud Noise Test, regardless of age, with Cavalier King Charles Spaniels demonstrating the highest incidence of crouching followed by the Yorkshire Terriers. Among treatment puppies of all breeds, the incidence of crouch was significantly (P < 0.01) greater from four to six weeks of age than seven through nine weeks of age, regardless of breed or gender.

Puppies that exhibited hazard avoidance behavior also demonstrated a tendency (P < .07) of higher concentrations of cortisol from the post-test salivary collection compared to the pre-test collection. No other puppies (treatment puppies not demonstrating hazard avoidance behavior and control puppies) showed this tendency.
The results of this study support the hypothesis that behavioral development and the onset of hazard avoidance behavior vary across breeds of domestic dogs.
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LIST OF ABBREVIATIONS

ACTH - Adrenocorticotropic Hormone
BPM - Beats per minute
CRH - Corticotropin-releasing Hormone
EEG - Electroencephalograph
HPA - Hypothalamic-Pituitary Adrenal Axis
HR - Heart Rate
LNT - Loud Noise Test
NIT - Novel Item Test
ST - Step Test
TTT - Teeter-Totter Test
LITERATURE REVIEW

Behavioral Development in the Domestic Dog (canis familiaris)

Ingrained emotional systems in the brain allow animals and humans to function as spontaneously active organisms with embedded values and goals that are further molded by life experiences (Panksepp, 1996). These genetically inherited systems are initially involved in organizing feelings and instinctual behavioral patterns to aid in survival. They are also involved in cognitive development and learning from external events. As an animal increases its experiences in life, learning and cognition assert a greater influence on behavioral responses to the environment, although the underlying emotional systems that were present at birth function as the core “centers of gravity” from which the learned behaviors are derived (Panksepp, 1996).

Scott and Fuller (1965) were the first to document critical periods in the behavioral development of the domestic dog. They conducted extensive studies on five breeds of dogs, (Shetland sheepdogs, basenjis, American cocker spaniels, wire-haired fox terriers, and beagles), making systematic daily observations throughout the first weeks of the puppies’ lives. Scott and Fuller observed that there were certain phases in the development of the dog when behaviors would change dramatically over a very short period of time and that these developmental changes could be placed into distinct and predictable stages (1965). The results of those studies demonstrated that canine
behavioral development begins in the neonatal period, peaks between six and eight weeks of age, and progresses more slowly as the dog matures (1965).

The canine neonate begins life completely dependent upon its mother for survival. Newborn puppies show very little activity or interactivity other than simple patterns of care-seeking, eliminative, and ingestive behavior. The characteristic behavior patterns of an adult dog are completely absent in the neonate (Scott and Fuller, 1965). Compared with an adult, the neonatal puppy is also deficient in most sensory capacities, having some of the greatest deficiencies in those senses which are most important to an adult dog (Scott and Fuller, 1965).

The newborn puppy is completely blind at birth. No sensory responses of the audiovisual system can be elicited until 2-4 days of age, when a slow and easily fatigued blink response to light can occur (Fox, 1971). In 1956, Charles and Fuller measured the electroencephalographic (EEG) output of the brain in developing puppies and found almost no brain waves and no differences between sleeping and waking states in the neonatal puppy. When tested with sudden loud noises, there was no reaction to either high or low tones (Scott and Fuller, 1965). Neonatal puppies demonstrate reliable reactions to negative odors, indicating that the sense of smell is at least functional from birth (Scott, 1958). The neonatal puppy reacts to other negative stimuli such as pain, cold, or hunger with a limited repertoire of yelping and moving at random (Scott and Fuller,
The only method of locomotion in a very young puppy is a slow crawl, usually in a circle, with head movements from side to side (Scott, 1958). When flipped onto its back, a puppy will immediately struggle to turn over and may vocalize in distress, demonstrating at least a low level of balance awareness (Scott and Fuller, 1965).

Early observations of newborn puppies indicate that neonatal puppies do not seem to learn by experience. According to Scott and Fuller (1965), a puppy will crawl to the edge of a scale platform, fall off, and begin to yelp in distress. When placed back on the middle of the platform, it will repeat the behavior again and again. A study by Cornwell and Fuller (1961), also found conditioned responses in young puppies to be extremely low and variable until approximately 15 days of age. Early physiological changes in the neonatal puppy are followed by dramatic emotional and behavioral changes, including the full expression of a startle response, the ability to approach or avoid individuals or objects, and the ability to make associations between events and emotions (Scott and Fuller, 1965).

The development of the central nervous system may be correlated with periods of behavioral development in the dog (Fox, 1971). Dramatic changes in EEG’s are observed in puppies at approximately three weeks of age. Brain waves increase in amplitude, and an EEG taken in sleep is noticeably different from one recorded when the puppy is awake. Nevertheless, the EEG does not resemble adult form until approximately seven or
eight weeks of age (Charles and Fuller, 1956). Examination of the spinal cord in the puppy indicates that from birth to three weeks of age the neurons are still immature and contain little Nissl (granular body) substance. At four weeks of age the features of the spinal cord almost resemble an adult dog and by five weeks of age the myelin and neurons are morphologically indistinguishable from those of an adult dog (Fox, 1965). Development of the brain during the first week of life is slow, but from the second or third week until approximately the sixth week of age, morphological changes are more rapid and correlate with the behavioral and neurological changes that are also dramatic at this time (Fox, 1965). Brain volume, size, and organization closely resemble that of an adult dog by the time the puppy reaches six weeks of age (Fox, 1971).

The complete opening of the eyes occurs at an average of 13 days (SD 2.3 days), with much breed and individual variation (Scott and Fuller, 1965). The first notable change in behavior occurs after the eyes open, when the puppy purposefully crawls backward as well as forward (Scott and Fuller, 1965). The opening of the eyes marks the end of the neonatal period and the beginning of the “transitional period” of puppy development, which lasts until approximately 21 days of age (Scott and Fuller, 1965).

In the third week of life, puppies are capable of orienting themselves in a room (Scott and Fuller, 1965). Puppies begin to sit upright on their forelimbs at approximately 20 days and are usually standing within a day or two after this (Fox, 1971). Fox (1971)
evaluated the sense of balance in young puppies and found that maintenance of posture when subjected to a seesaw tilting was initially poor until between 25 and 28 days of age. At 28 days, puppies enter a period of development from the poorly-controlled neonatal locomotor responses to more adult-like postural and equilibratory abilities (Fox, 1971). Puppies also demonstrate caution and awareness at 26 to 28 days of age when nearing an apparent edge in a visual cliff test (Fox, 1971). Study results by Scott and Fuller (1965), indicate that puppies develop the ability to make associations with painful experiences near the end of the transition period.

Puppies at 18 days of age may demonstrate a weak auditory startle response when asleep, but these responses appear variable to absent when the puppy is awake (Fox, 1971). The startle response to sound, which is the first indication that the sense of hearing has been developed, appears on the average at 19.5 days (SD 2.3 days) (Scott and Fuller, 1965). The startle response is easily recognizable and was determined to be the best indicator of the end of the transition period and the beginning of the socialization period of development in the dog. The socialization period begins at approximately three weeks of age and continues to approximately 12 weeks of age (Scott and Fuller, 1965).

The period of socialization is one of the most important critical periods of development in the life of an animal. This is the period in which the most critical social relationships are built (Scott and Fuller, 1965). In many animals there is an initial period
of imprinting and socialization which allows emotional attachment to any object (normally the mother), that is followed by a period of strong avoidance behavior to novel stimuli. The critical period of socialization in humans is from approximately six weeks to six months of age (Scott, 1963). This period is followed by a marked increase in fear reactions to strangers and being left alone. A study by Schaffer (1964) identified an interval of approximately one month between the attachment stage and fear-of-strangers stage in human infants. This study also found a significant tendency for infants who developed early specific attachments to also develop early fear of strangers (Schaffer, 1964). Studies of mallard ducks, (Ramsay, 1954), and white rock chicks (Hess, 1959), found imprinting to be strongest between 13 and 16 hours after hatching for both species, and noted that fear responses were not exhibited until 24 hours after hatching in the mallards and around 16 hours after hatching in the white rock chicks. In dogs, the period of socialization occurs between two and four months of age (Scott and Fuller, 1965). At the beginning of the period of socialization puppies begin to demonstrate responses to the sight or sound of people or other animals at a distance (Scott and Fuller, 1965). In the socialization period, the puppy also develops the capability to escape and avoid perceived hazards (Scott and Fuller, 1965).

The immediate response of a puppy to anything new in its environment is to run away or show a startle or freezing behavior. A very timid puppy will run away to the corner of the room, crouch, and give a high-pitched yelp (Scott and Fuller 1965). In Scott
and Fuller’s measurement of avoidance behavior, they found that some breeds are much more fearful (basenji in their case) than others at five weeks of age. As basenjis were originally bred as African village dogs, hazard avoidance behavior likely has survival value for both the individual dog and the continuation of the breed. In breeds of domestic dogs that have been bred primarily for companionship and that do not face the same dangers as puppies raised in African villages, this trait of early reactivity to perceived danger is unnecessary and even undesirable to most dog owners (Scott and Fuller, 1965).

The development of behavior in the domestic dog demonstrates the result of genetic selection against the development of the escape and avoidance behaviors normally found in wolves and other wild canids. This means that one or more of the factors limiting the period of socialization have been delayed in order that successful socialization can be achieved at a much later age in domestic dogs (Scott and Fuller, 1965). The rate at which individual behavioral and emotional systems develop in the domestic dog is dependent upon the dog’s genetic information, environmental exposure, hormonal secretions, motor coordination, neural connections, and sensory perception (Coppinger and Coppinger, 2001). The differences in emotional behavioral development between dogs form a prominent part of the unique characteristic temperaments of individuals and breeds (Scott and Fuller 1965).
Fear and Anxiety

Fear and anxiety are negative emotional states that are induced by the perception of actual or potential threats to the well-being of an individual (Boissy, 1995). Fearfulness is a characteristic of an individual that predisposes it to react relatively predictably to a large range of potentially dangerous events (Boissy, 1995). The degree of fearfulness can be highly variable among individuals. Fear-inducing stimuli initiate a complex interaction of behavioral and physiological changes that work together to optimize survival against a wide range of hazards (Mayes, 1979). Fearfulness has definite survival value, particularly for non-domesticated animals. The life expectancy of an animal (or human) is greatly increased if it can react quickly and appropriately to avoid threats. The selection of a suitable defensive reaction (e.g. freezing, fighting, fleeing, fainting, etc.) is the result of strong selection pressures as inappropriate defensive behaviors will usually produce rapid and disastrous consequences (Blanchard et al., 1990).

Overwhelming evidence points to the amygdala as the center of fear processing in the brain (Mayes, 1979). The amygdala is a small bilateral structure located in the limbic system, and is thought to be the location of the triggers for flight or fight responses (Mayes, 1979; Grandin and Deesing, 1998). Electrical stimulation of the amygdala is known to increase stress hormones in cats (Setekleiv et al., 1961), and evoke feelings of
fear in humans (Mayes, 1979). Lesions to specific parts of the amygdala have also been found to reduce defensiveness, reactivity, and flight behavior in rats (Blanchard and Blanchard, 1972; Kemble et al., 1984). The amygdala is conveniently located near the neocortex and hypothalamus of the brain, where sensory information is received and relayed, respectively. A feeling of fear is initiated by the amygdala in response to external (or internal) fear-inducing stimulation which activates the hypothalamus and a series of autonomic and behavioral processes throughout the body (Mayes, 1979). These systems all work together to reduce the threat or negative effects that may be caused by an ongoing state of fear or anxiety.

Fear and anxiety are generally considered undesirable emotional states in domestic animals as they can cause reduced welfare, growth and reproductive performance (Gray, 1971; Balm, 1999). Due to the negative effects of fear and stress on domestic animals, studies aimed at understanding and reducing fear and defensive behavior are of both economic and ethical significance (Boissy, 1995). The development of research to assess patterns of reactivity and factors that elicit fear and anxiety in animals could potentially offer strategies that would improve the well-being of animals and increase their production efficiency (Boissy, 1995).

An adaptive response of an animal to real or potential danger involves both behavioral changes that work to neutralize the effects of the triggering stimuli and
neuroendocrine adjustments that help maintain internal homeostasis (Boissy, 1995).

Fearful reactions may be exhibited differently depending on whether the threat stimulus is present (fear state) or potential (anxiety state) (Boissy, 1995). Fear in animals is often studied by observing the behavior of an animal and assessing related physiological changes that occur in response to potentially harmful stimuli (Gray, 1971; Salzen, 1979).

Gray (1971) noted that escape and avoidance behaviors are some of the best indicators of fear in animals. Nevertheless, the absence of avoidance behavior when facing possible fear-inducing stimuli does not necessarily indicate an absence of fear, as many other forms of behavior may be exhibited in a fear response. Active defense (attack, threat), active avoidance (flight, hiding, escape), or movement inhibition can all be behavioral expressions of a fear state (Boissy, 1995). Other behavioral responses that are often evaluated for indications of fear include expressive movements, or changes of the body such as facial expressions, posture, ear and tail position, vocalizations, piloerection, urination and defecation frequency or consistency, and the production of smells such as pheromones (Archer, 1979; Boissy, 1995).

Active avoidance or flight reactions vary according to the intensity of the stimuli inducing the fear (Archer, 1979). They may be as mild as an animal slowly moving a small distance away from a fear-invoking situation or as dramatic as a panic reaction, which often includes immediate, directed or undirected attempts to completely withdraw from the fear-inducing environment or stimulus (Archer, 1979). Describing active
avoidance or escape behavior as fearful behavior can be difficult as animals may move about their environment for a variety of reasons other than fear. Archer (1979) defines clear-cut active avoidance behavior as including the following: an animal’s normal locomotion method with higher intensity than normal, movement in a direction away from the source of potential danger (or undirected if no escape route is apparent or possible), and toward a place perceived as “safe”, accompanied by physiological changes (i.e. increased heart rate and respiration), and other behavioral demonstrations of fear, such as vocalizations or odor secretion.

Fear induced immobility reactions include the “freeze” behavior and tonic immobility and are demonstrated by a wide range of animal species (Archer, 1979). Just as active avoidance behaviors can be difficult to identify, classifying fear-induced immobility reactions can also be a challenge, especially since the reacting animal does not move in relation to the aversive stimuli (Archer, 1979). Freeze behaviors are usually short-lived, may include a crouched posture and response to touch, and are accompanied by normal physiological fear responses such as increased heart rate and respiration (Archer, 1979). Tonic immobility differs from freezing as it is generally longer and often follows a period of fight, flight, or struggle. Freezing is generally a survival response used to prevent notice or harm, whereas tonic immobility is frequently a final effort by an animal that has already been captured and is incapable of or has run out of other survival strategies (Archer, 1979).
Adaptive responses to fear-inducing stimuli are affected by preexisting characteristics of reactivity that are based on an individual’s genetic background, past environmental influences on development, and learning processes (Boissy, 1995). A complex interaction of these factors is responsible for the large range of fear or anxiety-related responses. The effectiveness of potentially harmful stimuli in eliciting a fear response is extremely variable among individuals and can be influenced by gender, age, state of the animal’s neuroendocrine system, and prevailing environmental conditions such as social context, properties of stressors, and the animal’s perception of controllability and predictability of the situation (Boissy, 1995). In a study by King et al. (2003), some dogs exhibited strong avoidance to a novel toy with obvious startle responses, while other dogs remained in close proximity to the stimulus. Differences in developmental maturity among animals may affect the reliability of results as well, as animals of different ages may react to potentially harmful stimuli in very different ways. Espmark and Langvatn (1985) found that deer would demonstrate a freeze response to fear-inducing stimuli from birth to approximately one week of age, but would react with a flight response just a week or two later.

Early environmental influences act especially during sensitive periods of brain development. It is thought that early-life experiences are involved in the programming of neuroendocrine function, including negative feedback systems, which later modify responses to stressor experiences (Anisman et al, 1998). Early environmental enrichment
and handling may also influence neuroanatomical development by increasing the number of hippocampal glucocorticoid receptors, which are implicated in both behavioral and endocrine regulations (Meaney et al., 2000). Chickens that received substantial stimulation in infancy, such as environmental enrichment or regular handling, were generally less reactive to fear-causing stimuli in adulthood (Jones and Waddington, 1992). Rats and mice handled in infancy also demonstrated lower fear responses and had lesser neuroendocrine reactions to novel stimuli and stressors later in life (Levine et al., 1967; Nunez et al., 1996; Anisman et al., 1998). Puppies that were handled early in life were found to be calmer, less vocal, and more confident in exploring novel environments (Gazzano et al., 2008). While gentle handling and environmental enrichment were found to be beneficial to animals in their later reactions to fear-causing stimuli, neonatal mice that were subjected to a more severe stressor, such as extended separation from the dam demonstrated exaggerated responses to fear-inducing stimuli as adults (Anisman et al., 1998; Aisa et al., 2008).

The results of many studies have shown that there are differences between genders in fear and defensive responses; however, the results are often conflicting across species. In one study, female rats exhibited faster recovery to stress than males (Conrad et al., 2003). In general, male rats have been reported to explore novel environments more slowly and demonstrate more freeze responses than females (Gray, 1971). Additionally, male dogs, foxes, minks, rats, cats, porcupines, and chickens have demonstrated more
reluctance to copulate in a novel, (and hence possibly threatening) environment compared to females of the same species (Gray, 1971). Nevertheless, Plutchik (1971) found that the females of all four breeds of dogs he studied were more likely to avoid the novel objects than the males, and female dogs were also more “afraid” than males in research by Goddard and Beilharz (1985).

Large differences in the behavioral and neuroendocrine reactions to aversive events are found between breeds or strains of rodents (Olivero and Castellano, 1990). In mice and rats, behavioral, hormonal, immunological, and neurochemical responses to environmental stressors may differ significantly among different genetic strains (Anisman et al., 1998; Anisman and Merali, 1999). Genetic factors have also been found to have a strong influence on the intensity of fear reactions in domestic animals and humans (Parsons, 1988).

Breed differences have been demonstrated in measures of fear and confidence in dogs as well (Mahut, 1958; Scott and Fuller; 1965; Plutchik, 1971). Plutchik (1971) found that terriers were most likely to approach novel objects and spent the longest amount of time in contact with them. Both beagles and basenjis in the Plutchik study showed very little interest in approaching or interacting with the novel objects. Basenjis had very high avoidance scores with most of the more timid dogs demonstrating a flight response. The Shetland sheepdogs in the study displayed a high level of conflict or
ambivalence, with high approach scores on some objects and high avoidance scores on others (Plutchik, 1971). Goddard and Beilharz (1985) found that boxers reacted with behavioral inhibition and decreased activity when exposed to fear-inducing stimuli, while kelpies responded with increased activity. Another study found that greyhounds were quicker to approach and spent more time near novel objects than other breeds evaluated (King et al., 2003). They also exhibited a significantly lower maximum heart rate following exposure to novelty (King et al., 2003). In general, the greyhounds did not show as much avoidance to stimuli when compared to the other breeds of dogs (King et al. 2003). Goddard and Beilharz (1985) examined fearfulness in four different breeds of dogs used for guiding the blind and found that German Shepherds demonstrated the highest level of fearfulness, whereas the Labrador retriever showed the lowest level of fearfulness. A study by Mahut (1958) categorized dogs into “fearful” and “fearless” groups, where the fearful dogs were primarily of the collie, German Shepherd, poodle, corgi, and dachshund breeds, and the fearless dogs included boxers and a variety of terrier breeds. Conversely, no significant differences in behavioral responses were found in the different breeds studied by Fox in 1971.

Although the differences among breeds are quite marked in most respects, there are some individual animals whose behavior does not fit the typical pattern for their breed. For example, although most of the basenjis in the Plutchik (1971) study avoided all the novel objects and spent most of their time in one corner, there was one basenji
whose contact and approach scores were almost as high as those of the typical wire-haired terrier – a breed found to have more positive interactions around novel objects rather than avoidance. While this unpredictable variability may be a frustration in scientific research, it could be exactly what is needed to provide the animals necessary for selection programs aimed at changing the genetic factors that cause some of the undesirable fear-related behaviors in dogs (Plutchik, 1971).

Fear-related reactions are a common source of behavioral problems in dogs (Serpell and Jagoe, 1995). Fearfulness was also cited as the most common reason for the rejection of potential guide dogs (Goddard and Beilharz, 1985). Fear-related behavior problems can include anti-social behavior with other dogs or people, inappropriate urination, fleeing, and even aggression (Voith and Borchelt, 1996). Aggression related to fear can be triggered by an unfamiliar situation or a sudden change in the environment. Dogs exhibiting fearful body postures such as crouching, ears laid back, tail between the legs, or urination might respond with aggression if the fear-inducing threat continues to approach and the animal is unable to withdraw or escape.

Gray (1971) suggests that stimuli eliciting fear can be placed into four general categories: novel stimuli, intense stimuli (e.g. very loud or large), stimuli relating to specific survival hazards (such as predators, heights, isolation, etc.), and stimuli arising during social interactions with conspecifics. Animals will frequently react to novel and intense stimuli early in life, but can usually become adapted or habituated to them if they
are not followed by any disastrous consequences. If novel or intense stimuli are accompanied by, or associated with negative consequences however, a fifth fear-eliciting stimuli can occur: classically conditioned fear stimuli (Gray, 1971).

Novelty is anything new or strange in an animal’s environment. Novelty is a paradox because it is both fear-provoking and attractive to animals (Grandin and Deesing, 1998). A variety of authors have used novel stimuli such as mechanical toys, balls, and plastic bags to examine fear responses in dogs (Plutchik, 1971; Goddard and Beilharz, 1984; King et al., 2003; Ley et al., 2007; Haverbeke et al., 2008). Responses to novel stimuli have also been recorded in species such as rats (Izquierdo et al., 2003), mice, (Stenzel-Poore et al., 1994), sheep (Vandenheede et al., 1998), and chickens, (Jones and Waddington, 1992). Reactivity to novelty is of obvious adaptive value, as anything an animal has not previously experienced has the potential to cause harm. King (1966) noted that to the newborn animal, even the mother is initially unfamiliar stimuli. Nevertheless, offspring do not demonstrate fear responses to their mothers because there is a period after birth or hatching in which novel stimuli not only fail to elicit fear responses, but also elicit a bonding or imprinting response. The ability of novel stimuli to elicit fear comes later in the development of the infant (King, 1966). Interestingly, studies of rats, (Taylor, 1981), monkeys (Coe et al., 1982), and chicks (Jones and Merry, 1988) found that there is less behavioral disturbance when animals are exposed to novelty with a social partner than when they are alone.
Stimuli with intense physical characteristics such as large size, high speed, long duration, close proximity, or loudness produce fear responses comparable to those caused by novelty (Boissy, 1995; 1998). Dogs will tend to exhibit avoidance behavior in response to intense stimuli, such as sudden movement or sound, as demonstrated by King et al. (2003), Ley et al., (2007), and Plutchik, (1971). Stimuli relating to the special dangers in an animal’s physical environment such as heights or differences in lighting may also trigger specific innate fears in many mammals (Gray, 1971; Voith and Borchelt, 1996; King et al., 2003).

Fear invoked from interactions with conspecifics can include fear of strangers (common in humans), as well as an aspect of novelty-induced fear for animals or humans who have not been adequately socialized within their own species (Russell, 1979). A third cause of fear among conspecifics includes that resulting from territorial, protective, or aggressive behavior demonstrated by another animal of the same species (Russell, 1979).

Classically conditioned fear is developed by a combination of innate fears and learning through experiences (Gray, 1971). Classical conditioning involves an unconditioned stimulus which consistently elicits an unconditioned, innate response. For example, an electric foot-shock will consistently produce a fear reaction in the rat (Hunt and Otis, 1953). When an unconditioned stimulus (i.e. the foot-shock) is paired with a stimulus that initially causes little to no reaction in the animal (for example, flashes of
light), the animal will quickly begin to associate the conditioned stimulus (flashes of light) with the unconditioned stimulus (shock), and demonstrate a predictable conditioned response (in this case, a conditioned fear response of crouching and/or defecating) any time the conditioned stimulus is presented.

The study of fear must include evaluation of an animal’s response, intensity, and the strategy used to deal with the fear-inducing stimuli (Boissy, 1995). Fear-related responses are also dependent on, and may be modulated by, other motivational systems. For example, some stimuli intended to elicit fear in a testing situation may elicit predator- or play-related responses instead, confounding results (King et al., 2003). For this reason, it can be very difficult to attribute a given behavior to any single emotion or motivation. It is likely that the wide variety of fear-related responses exhibited within and across animal species is also representative of the wide range of individual coping strategies within a breed or species (Boissy, 1995). Further studies are needed to better understand the modifiability of emotional traits and the relative contributions that heredity, early environmental influences, learning, gender, age, neuroendocrine systems, and prevailing environmental conditions may have on the variability and modifiability of fear and anxiety-related responses (Boissy, 1995).
Physiological Fear Responses

Physiological changes in response to fear-inducing stimuli may include heart rate and blood pressure deviations, the redistribution of blood supply from the skin and viscera to the muscles and brain, bronchial dilation, and release of red blood cells, stored sugar, and hormones (Mayes, 1979; Engeland et al., 1990). All of these changes work together to prepare an animal for the appropriate behavioral reactions required to cope with an acute threat.

Heart rate

Cardiac changes are often used as a general psychophysiological indicator in domestic animals with the assumption that increased levels of stress are reflected by changes in heart rate (Maros et al., 2008). Heart rate has proven to be a useful objective measure of behavioral states associated with sympathetic stimulation, (e.g. excitement or stress), in species including the rat (Ashida, 1972), sheep (Palestrini et al., 1998), chicken (Candland et al., 1969) pig (White et al., 1995), wolf (Fox and Andrews, 1973), monkey (Weisbard and Graham, 1971), and dog (Gaebelein et al., 1977; Mayes, 1979; Palestrini et al., 2005). Observing heart rate changes in response to various stimuli are potentially more revealing than observing absolute levels as the reactivity of heart rate to different stimuli can be examined independent of basal levels and individual differences (Vincent and Leahy, 1997). The measurement of heart rate changes during environmental
stimulation has become more popular in ethological studies on fear and stress as non-invasive measurement usually causes less interference to the animal being studied and allows data to be collected over longer periods of time (Maros et al., 2008).

Heart rate changes may be influenced by bodily movement (ambulation and changes in body position) as well as psychological stimuli (Marchant et al., 1997; Maros et al., 2008). Some researchers have found that cardiac changes may reflect the “attentional state” of the animal as well. Orientation to novel but non-threatening stimuli can cause decreased heart rate in contrast to intense threatening stimulation which is accompanied by heart rate acceleration (Graham and Clifton, 1966; Lacey and Lacey, 1974). Based on the results of their studies, Lacey and Lacey (1974) propose that motivated attention toward external events or stimuli result in a deceleration and hypotensive heart response. Conversely, cognitive work involving motivated inattention, which may include defense or startle responses, gives rise to heart rate acceleration and hypertension.

In a study by Beerda et al., (1998), heart rate responses were not dependent upon the types of fear-inducing stimuli used, although behavioral and endocrine responses occurred more specifically according to the stimuli presented. As a result, Beerda (1998) believes that heart rate responses are indicative of behavioral arousal but that changes of heart rate cannot be used to distinguish between different types or levels of stress.


**Hypothalamic-Pituitary-Adrenal Axis**

The maintenance of homeostasis in an animal is constantly challenged by stressors such as illness, injury, unpleasant emotional states, and exposure to new environments. The brain appears to be the most important target organ for hormones released in challenging situations, although peripheral sites, such as the neuromuscular and cardiovascular systems are also important targets of stress-related hormones (Boissy, 1995). The body’s response to stressors is largely regulated by interactions among the hypothalamus, pituitary gland, and adrenal glands, which are collectively named the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1). The entire HPA system is highly responsive to both internal and external stressors and responds by transforming proteins into the energetic metabolites necessary for defense mechanisms used to cope with stressors (Mormede et al., 2007).
Figure 1. The Hypothalamic-Pituitary-Adrenal Axis

CRH = Corticotropin-releasing hormone; ACTH = Adrenocorticotropic Hormone

+ = stimulates  - = inhibits

(Adapted from Anisman and Merali, 1999)
The primary active hormone in the HPA axis is cortisol (in humans, dogs, cattle, sheep, pig, mink, fox, and fish), and corticosterone (in birds and laboratory rodents), which is released in response to a cascade of physiological events that begin with the brain’s recognition and reaction to a stressor (Mormede et al., 2007). The hypothalamus, which is located near the base of the brain, secretes corticotropin-releasing hormone (CRH) in response to stress, which travels directly to the anterior pituitary gland. CRH promotes the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH travels through the bloodstream to the adrenal glands, which are located on top of (anterior to) the kidneys. The adrenal glands respond to ACTH by releasing the glucocorticoid, cortisol into the bloodstream (Anisman and Merali, 1999).

Glucocorticoids act in combination with adrenal catecholamines to enable an organism to engage in rapid behavioral action by mobilizing available resources in the body. They inhibit glucose uptake and protein synthesis at storage sites, and stimulate the release of energy substrates such as glucose, amino acids, and insulin (Mormede et al., 2007). Cortisol exerts widespread physiological effects, helping direct oxygen and nutrients to the stressed site of the body while suppressing the immune response. Cortisol also influences appetite and satiety, arousal, vigilance, attention, and mood (Anisman and Merali, 1999). Under normal circumstances, the presence of cortisol in the bloodstream minimizes the effects of the stressor and sends negative feedback signals to the hypothalamus and pituitary gland to terminate CRH and ACTH secretion. If the stressor
continues or the negative feedback system fails to respond adequately, continuous high concentrations of CRH, ACTH, and cortisol can cause damage to the body, including suppression of growth, immune system dysfunction, and localized brain cell damage (Anisman and Merali, 1999).

The assessment of cortisol concentration has become an established way to investigate stress since the hormone reflects the activity of the hypothalamic-pituitary-adrenal axis (Beerda, 1996). The most traditional and reliable method of analyzing cortisol is through blood plasma. However, blood collection is an invasive procedure that requires skilled technicians, a compliant subject, and the proper supplies and facilities for collection, processing, and storage of samples (Kirschbaum and Hellhammer, 1989; Dreschel and Granger, 2009). There has been an increasing preference for non-invasive measurements in physiological experiments, particularly in the study of stress, as invasive techniques may act as stressors themselves (Vincent and Michell, 1992). Venipuncture practices alone have been shown to increase blood cortisol levels, thereby confounding research aimed at examining other stressors (Kirschbaum and Hellhammer, 1989; Kirschbaum et al., 1989; Hennessy et al., 1998).

Various hormones, including steroids, can be detected in saliva (Mandel, 1990). Although not all salivary hormone measurements correlate well with plasma concentrations, salivary cortisol has been found to correlate significantly with free plasma
cortisol in dogs (Beerda, 1996). In human beings, salivary cortisol is currently regarded as one of the best physiological indicators of stress and is widely used in studies of psychobiology, endocrinology, behavioral medicine, and psychiatry (Bassett et al., 1987; Kirschbaum and Hellhammer, 1989; Schreinicke et al., 1990; Nierop et al., 2006; Simpson et al., 2008). Salivary measurements of cortisol are also widely used in stress-related research on farm animals (Fell et al., 1985; Fell et al., 1986; Geverink et al., 1998; Jarvis et al., 2006; Loberg et al., 2008; Gonzalez et al., 2009), and dogs, including studies on welfare, fear and anxiety, and human-animal interactions (Vincent and Michell, 1992; Beerda et al. 1996; 1997; 1998; Bergeron et al., 2002; Dreschel and Granger, 2005; Coppola et al., 2006; Jones and Josephs, 2006; Horvath et al., 2007).

Although a level of animal handling is required, saliva sampling has not demonstrated any negative behavioral or physiological effects and is well tolerated by many domestic animals (Kirschbaum et al., 1989; Dreschel and Granger, 2009). Kobelt et al. (2003) demonstrated that up to four minutes could be taken to collect a salivary sample from a dog without the effect of handling being reflected in cortisol concentrations.

Salivary cortisol is a useful measure of stress, however, a number of challenges associated with its collection and measurement have been identified, including obtaining adequate volume, avoiding contamination, and dealing with individual variability. Many
new assays only require a small volume of saliva (as little as 25 µl), however collecting even this amount may be difficult with small dogs or less compliant subjects (Dreschel and Granger, 2009). Low volume salivary samples limit the number of tests that can be run and the ability to duplicate tests for validity, while increasing the potential for a large error variance in measurement (Harmon et al., 2007; Dreschel and Granger, 2009). In addition, variation in cortisol concentrations has been found to exist among different breeds and ages of animals, as well as among individuals within a breed or of the same age (Dreschel and Granger, 2009).

Contamination of the saliva by food or drink, blood, or even saliva stimulants have been shown to dramatically impact cortisol measurement in humans (Granger et al., 2007; Dreschel and Granger, 2009). Previous studies have used salivary stimulants such as citric acid to induce salivation and increase sample volume (Beerda et al., 1998; Bergeron et al., 2002; Kobelt et al., 2003). Nevertheless, a study by Dreschel and Granger (2009) found that the use of citric acid for salivary stimulation in dogs can have effects on pH and cortisol concentration, particularly when used in larger amounts, and may not significantly increase salivation.

The magnitude of a neuroendocrine response to stressors depends on both the physical properties of the trigger stimulus and psychological factors of the subject. Psychological discomfort, which may result from uncertainty, conflict, frustration, or a
high degree of novelty, is frequently involved in HPA axis activation (Kirschbaum et al., 1989; Boissy, 1995).

A study by Beerda et al. (1998), found elevated cortisol in dogs in after exposure to electric shocks and a falling bag. Significant elevation of saliva cortisol was observed from 10 to 30 minutes after dogs were exposed to sound blasts (Beerda et al., 1998). In human beings subjected to acute acceleration stress, significant increases in salivary cortisol developed within 20 minutes (Tarui and Nakamura, 1987). Rats showed significant increases of corticosterone after 15 minutes of stress, although repeated and predictable exposure to the stressor provoked lower activation of the HPA axis (Muir and Pfister, 1987). Animals that have the option of moving and avoiding a stressor also tend to demonstrate decreased HPA axis arousal following exposure to a threatening situation (Dantzer and Mormede, 1983). While concentrations of cortisol cannot prove or disprove the emotional condition of animals or humans, they do provide important evidence toward interpreting emotional states in animals and understanding the influences of the environment around them (Vincent, 1992).
INTRODUCTION

More dogs and cats are euthanized for behavioral reasons than for all medical causes combined (Landsberg and Hunthausen, 2003). In a 1999 study by Patronek and Dodman, it was estimated that 224,000 of pets euthanized by veterinarians in the United States were euthanized for behavioral reasons alone. Voith and Borchelt (1996) suggest that the most common and probably the most serious behavioral problem in dogs is aggression.

Aggression associated with fear appears to be a major cause of dog attacks (Borchelt, 1983). Dogs exhibiting fearful body language such as a crouched posture, laid back ears, tail between the legs or urination might respond with aggression if a threat continues to approach and the animal is unable to withdraw or escape. Other fear-related behavioral problems include the inability to socialize with other dogs and people, inappropriate urination and difficulty in handling and training (King et al., 2003). Fearfulness, referring to responses associated with withdrawal and avoidance was also cited as the most common reason for rejecting potential guide dogs (Goddard and Beilharz, 1984).

In 1995, animal behavior researcher Alain Boissy stated that fearfulness is a basic psychological characteristic of an individual that predisposes it to perceive and react in a similar manner to a wide range of potentially frightening events. Boissy (1995) further
defined fear and anxiety as emotional states that are induced by the perception of an actual (or potential) danger that threatens the well-being of the individual. In all animals, genetic factors influence reactions to situations that may cause fear (Grandin and Deesing, 1998). Over fifty breeds of dogs across all seven recognized American Kennel Club groups have family lines in which “fear/shyness/nervousness/panic/anxiety” is a major breeder-reported concern (Overall et al., 2006). Despite the fact that fear responses in dogs have been studied frequently (Ogata et al., 2006), there is very little published on the range of “normal puppy behavior” and changes over time during puppy development, especially relating to the development of fear and fear-related behaviors (Godbout et al., 2007).

Drs. Scott and Fuller were the first to document critical periods in the behavioral development of the dog in 1965. The results of their studies demonstrated that critical periods in a dog's development begin at birth, peak between six and eight weeks, and extend to maturity. Physiological changes, such as the opening of the eyes and ears between approximately two and three weeks of age are followed by dramatic emotional and behavioral changes, including the startle response, ability to approach or avoid individuals or objects, and the ability to make associations between events and emotions (Scott and Fuller, 1965).
According to Coppinger and Coppinger (2001), domestic dogs have individual behavioral systems that are regulated by genetic information, environmental exposure, hormonal secretions, motor coordination, neural connections, and sensory perception. These behavioral systems develop at varying ages in different breeds of dogs. Additionally, behaviors that include predatory or fear-related motor patterns may be developed and exhibited to a greater or lesser extent depending on specific breed type and age.

The onset of adult hazard avoidance behavior (fear motor patterns) occurs during the critical period of development in domesticated canines. In wolves, the onset of adult hazard avoidance behavior occurs at approximately 19 days. This onset is delayed in domestic dogs, taking place as late as day 72 in the Labrador Retriever (Coppinger and Coppinger, 2001). The objective of this study was to identify, compare, and contrast the onset of adult hazard avoidance behavior across three specific breeds of purebred puppies between approximately four and ten weeks of age. In addition, we were interested in identifying and examining developmental differences over time in behavior, heart rate, and salivary cortisol concentrations across the three breeds studied.

By studying hazard avoidance behavior in greater detail and identifying the variation in times of onset in additional breeds of domestic dogs, it is hoped that dog breeders, current and future dog owners, veterinary professionals, behaviorists, and researchers will have a more reliable understanding of fear-related behavioral
development in young puppies. This knowledge could be further applied to rearing, training, socialization, and behavioral modification techniques in domestic dogs. In addition, a broader understanding of hazard avoidance behavior could potentially lead to the prevention of some 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

Study Design

The research was conducted as 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 Spaniel (n=33), Yorkshire Terriers (n=32), and German Shepherd Dogs (n=33). Five to nine litters of puppies within each of the breeds were tested in the homes of volunteer cooperating dog breeders throughout central Ohio and the surrounding area. Dog breeders were located and contacted through internet websites, dog breed clubs, shows, and personal contacts. Breeders were provided with an overview of the study in the form of the thesis proposal 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 if extreme behavioral reactions occurred, a health issue that could affect a puppy or litters’ development or welfare was exhibited, or the breeder requested to withdraw a puppy or litter from the study for any reason.

Selection of the three breeds used in this study was based on breed background and genetics, personality, physical characteristics, and the availability of litters within Ohio. The Cavalier King Charles Spaniel ranked as the 25th most registered dog breed out of 156 breeds in the American Kennel Club in 2008. (http://www.akc.org/reg/dogreg_stats.cfm. Accessed 7/28/09). According to the American Kennel Club, Cavalier King Charles Spaniels are bred to be “friendly, non-aggressive with no tendency towards nervousness or shyness” and have “sweet, gentle natures” (http://www.akc.org/breeds/cavalier_king_charles_spaniel/. Accessed 3/5/10). Due to the availability of cooperative breeders within the central Ohio area, this was the first breed selected for the study.

Yorkshire Terriers are slightly smaller than the Cavalier King Charles Spaniel and received the second highest number of American Kennel Club registrations in 2008 (http://www.akc.org/reg/dogreg_stats.cfm. Accessed 7/28/09). The Yorkshire Terrier has been placed in the American Kennel Club Toy Group, along with the Cavalier King Charles Spaniel. The American Kennel Club 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 background from the Cavalier King Charles Spaniel 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), and is also a popular breed among American pet owners, ranking third in number of registrations with the American Kennel Club in 2008 (http://www.akc.org/reg/dogreg_stats.cfm. Accessed 7/28/09). The German Shepherd Dog’s breeding for herding and protectiveness made it a strong contrast to the first two breeds chosen for the study. 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 told us there can be distinct differences in personality and development across genetic lines within the German Shepherd Dog breed.

Puppies within each litter were randomly assigned to either the control or treatment groups, with approximately one-half of the litter in the treatment group and the
other half in the control group. Testing and data collection were performed once a week beginning when the puppies were 4-5 weeks of age and continued until the treatment puppies demonstrated the onset of hazard avoidance behavior, reached ten weeks of age, or left the breeder’s home, depending on which occurred first.

Puppies took part in four tests at each visit: Novel Item Test (NIT), Teeter-Totter Test (TTT), Step Test (ST), and Loud Noise Test (LNT). During each test, the presence (YES) or absence (NO) of the following fear-related arousal signals were noted:

“Crouched Posture” – A rapid, pronounced lowering of the posture, sometimes in combination with movements that enlarge the distance from the eliciting stimuli (Beerda et al., 1998).

“Trembling” - A clear shivering of the body that is ongoing (Beerda et al., 1998).

“Vocalization” - Barking, growling, whining, yelping, and any other vocal emissions of varying pitch or persistency (Beerda et al., 1998).

“Hazard Avoidance Behavior” – The immediate, rapid, purposeful, and pronounced movement away from the object or experience perceived as a hazard. May include other behavioral indicators of fear such as tail tucked between legs, ears pinned against head, vocalizations, lowered posture, and seeking of “safety” (human, hiding spot, nest, etc.).
The final physical location of each puppy was also noted at the conclusion of each test. The physical location of the puppy was defined by the region of the mat on which the majority of the puppy’s paws 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. Additionally, 17 Cavalier (12 treatment and 5 control), 25 Yorkshire Terrier (17 treatment and 8 control), and 15 German Shepherd Dogs (10 treatment and 5 control) puppies had their video-recorded testing evaluated by two outside personnel (an Animal Science undergraduate student intern and an Ohio State University work-study veterinary school student) who had not been present at the time of testing and were blind to the primary investigator’s data results. The results of their data on behavioral responses from reviewing the videos were compared to the primary investigator’s results in order to increase the reliability of the results.

*Behavior Testing*

Puppies were given at least two minutes to acclimate to the tester’s presence before initial heart rate measurements were recorded. All puppies (treatment and control)
had initial heart rate measurements taken prior to testing and while in their familiar nest environment. All puppies were awake at the time of measurement to avoid confounding effects of conscious state on heart rate. The order puppies were assigned for initial heart rate and all testing was randomized with each visit. Heart rate was recorded for all puppies immediately (within 10 seconds) following each of the four experimental tests for the treatment group puppies or control time periods for the control group puppies. Finally, heart rate of each puppy was recorded in the familiar nest environment after the completion of all testing. All heart rates recorded throughout the experimental period were measured non-invasively via a 10-second reading through a stethoscope warmed to room temperature that was placed on the left ribcage of the puppy. The ten-second measurements were multiplied by six to obtain a beats per minute (BPM) value.

**NOVEL ITEM TEST (NIT):** Treatment puppies were placed on the marked center of a mat (24 x 48 inches) facing a forward-moving, light and noise-emitting stuffed toy (duck) (8 x 9 ½ inches) 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. During this five second period, the presence (YES) or absence (NO) of the previously mentioned fear-related arousal signals were noted, as well as each puppy’s final physical location. 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.
Location: APPROACH - The marked one-third of the mat that included the novel item or any location off the mat that crossed over the line separating the “Approach” and “Neutral” sections of the test.

AVOID – The marked one-third of the mat that was furthest away from the novel item or any location off the mat that crossed over the line separating the “Avoid” and “Neutral” sections of the test.

NEUTRAL – The marked middle of the mat where the puppy was initially placed, separating the “Approach” and “Avoid” sections.

![Figure 2. Novel Item Test Mat Dimension](image-url)
TEETER-TOTTER TEST (TTT): Treatment group puppies were placed on the marked center of an initially balanced teeter-totter (covered with rubber, anti-slip matting, and 16 x 48 inches, 5 inches off the ground), for ten seconds, during which the presence (YES) or absence (NO) of the previously mentioned fear-related arousal signals were noted. Observations were also made on control group puppies, which were placed on an identically marked and matted stable surface for ten seconds.

Location: CENTER - The labeled middle of the teeter-totter or mat which contained the marked location that the puppy was initially placed on at the beginning of the test.

MID – The marked areas on either side of the “Center” portion of the teeter-totter or identically marked mat.

EDGE/OFF – The marked areas on each edge of the teeter-totter or mat or the movement of the puppy completely off of the teeter-totter or teeter-totter marked mat.
**Figure 3. Teeter-Totter Test Dimensions**

<table>
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<td>10”</td>
<td>8”</td>
<td>5”</td>
<td>10”</td>
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**STEP TEST (ST):** Treatment group puppies were placed on the marked center of a small step (covered with rubber, anti-slip matting). The steps were made proportional in surface size and height to the size, age, and mobility of the puppies: 2 x 9 x 9 inches for the Cavalier King Charles Spaniels and Yorkshire Terriers, and 4 x 8 x 16 inches for the German Shepherd puppies.

Puppies spent ten seconds in this test, during which the presence (YES) or absence (NO) of the specific fear-related arousal signals was noted (crouching, vocalizing, trembling, and hazard avoidance behavior). Control group puppies were placed on an identically marked flat surface for ten seconds with the same observations made.
The final physical location of the puppy in this test was also noted.

**Location:**

- **ON BOX** – The puppy remained where it was originally placed at the beginning of the test.
- **OFF BOX** – The puppy moved off the step or step-marked region (control puppies) at some point during the test.

![Diagram of Step Test Dimensions](image)

**Figure 4.** *Step Test Dimensions* - (A.) View from above mat, (B) View from floor
LOUD NOISE TEST (LNT): Treatment group puppies were placed on the marked center of a mat. Within three seconds after being placed on the mat, the 2 x 9 x 9 inch step from the Step Test was dropped approximately 20 inches in front of the puppy from a height of approximately 20 inches, resulting in a short, sudden loud noise. The immediate reaction of the puppy was observed for five seconds. The presence (YES) or absence (NO) of the previously mentioned fear-related arousal signals was noted as well as each puppy’s final physical location. 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.

Location:

APPROACH - The marked one-third of the mat that included the dropped step or any location off the mat that crossed over the line separating the “Approach” and “Neutral” sections of the test.

AVOID – The marked one-third of the mat that was furthest away from the dropped step or any location off the mat that crossed over the line separating the “Avoid” and “Neutral” sections of the test.

NEUTRAL – The marked middle of the mat where the puppy was initially placed, separating the “Approach” and “Avoid” sections.
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, PA) sorbettes, using two sorbettes per puppy for each collection. Sorbettes were placed in the puppies’ mouths and moved over and under the tongue and each cheek pouch for approximately 1-2 minutes. The first salivary sample was collected immediately following the initial heart rate measurement and prior to any testing. The second saliva sample was collected in the same manner as the first, approximately 20 minutes after the conclusion of the final test. The saliva soaked sorbettes were placed in 1.5 ml. microcentrifuge tubes, labeled, and placed in ice within

Figure 5. Loud Noise Test Mat Dimensions
20 minutes. Samples were frozen in a -20°C freezer within 12 hours and remained there until centrifugation. Several weeks to months later, samples were removed from the freezer and thawed to room temperature for centrifugation and sorting. Samples were centrifuged at 10,000 x g for 20-30 minutes, at 4°C, and sorbettes were removed from the tubes. Samples were then assayed or re-frozen until a later date for assay. Saliva samples that were re-frozen were thawed to room temperature and centrifuged again at 10,000 x g for 15 minutes at 4°C on the day of assay to ensure removal of extraneous debris. Free cortisol was assayed using an expanded range high sensitivity salivary cortisol enzyme immunoassay kit from Salimetrics, L.L.C. (State College, PA). The salivary cortisol assay was performed following the directions of the Salimetrics kit. The lower limit of assay sensitivity was determined with 14 sets of duplicates of zero standard. Sensitivity was defined as the mean of the zero standard samples plus two standard deviations of the mean. As such, the minimal concentration of free cortisol that our assay could distinguish from 0 was 0.007 µg/dL. In addition, linearity of dilution was established in four duplicate samples at a 1:2 dilution (94% recovery) and 1:4 dilution ratio (96% recovery). Intra-assay and inter-assay precision was established with 11% and 13.6% coefficients of variation, respectively. Four saliva samples containing different concentrations of endogenous cortisol were also spiked with known quantities (3.0 ug./dL and 1.0 ug/dL) of cortisol and assayed, demonstrating a mean of 109% recovery (SD 9%).
**Statistical Analyses**

Data were analyzed using Statistical Analysis Software (SAS) 9.2 TS Level 2 MO. The Mixed procedure of SAS was used as we had unbalanced data across the three breeds. Parameters were estimated using a restricted maximum likelihood method. Reported means are least-squared means unless otherwise noted. In addition, the Chi-Square tests were used on some of the proportional data to determine significant differences among groups. Mixed models with repeated measures each week were used to analyze the dependent variables of puppy heart rate, final locations, and the presence or absence of a crouched posture in each test, as well as salivary cortisol concentration differences between the first and second collection. Puppies were evaluated for any trembling and vocalization in each test, but these behaviors were expressed too infrequently to find any significance (< 10%), and were subsequently removed from our analyses. The mean onset age (in days) of hazard avoidance behavior was also evaluated using a mixed model without repeated measures on puppies. The proportion of treatment group puppies expressing hazard avoidance behavior at some point during testing was analyzed with repeated measures in a Chi-Square Analysis. Mixed models included the fixed effects of test (NIT, TTT, ST, LNT), breed, sex, 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 across the three breeds tested.
RESULTS

Differences in the age at onset of hazard avoidance behavior were analyzed across the three breeds used in this study. Our results indicated a significantly later onset of hazard avoidance behavior in Cavalier King Charles Spaniel puppies compared to both German Shepherd (P < 0.05) and Yorkshire Terrier (P < 0.01) puppies (Table 1). There was not a significant difference between German Shepherd and Yorkshire Terrier puppies in the onset of hazard avoidance behavior, however the proportion of treatment group puppies that exhibited hazard avoidance behavior at any point in response to our testing varied significantly (P < 0.01): Yorkshire Terriers (82%), Cavalier King Charles Spaniel (53%), and German Shepherds (26%) (Table 1).

Results in Table 1 represent the data of the initial tester, however, two outside personnel (an Animal Science undergraduate student intern and an Ohio State University work-study veterinary student) reviewed videos from the testing of 57 puppies used in the study. Because the reviewers analyzed a percentage (58%) and not the complete representation of the dogs tested, reviewer least squared means are slightly different from those expressed in the final test results. Nevertheless, reviewer results on the onset and expression of hazard avoidance behavior in the puppy videos analyzed were not significantly different (P > 0.90) from the results of the original tester’s evaluations of the same dogs (Table 2; Table 3).
<table>
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<th>Standard Error</th>
<th>% of Treatment Puppies Demonstrating HAB Behavior</th>
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<td>55.1 (^a)</td>
<td>3.1</td>
<td>53 (^a)</td>
</tr>
<tr>
<td>German Shepherd</td>
<td>39.4 (^b)</td>
<td>6.5</td>
<td>26 (^b)</td>
</tr>
<tr>
<td>Yorkshire Terrier</td>
<td>42.2 (^b)</td>
<td>2.5</td>
<td>82 (^c)</td>
</tr>
</tbody>
</table>

\(^1\) Data expressed as least squared means

**Table 1. Onset of Hazard Avoidance Behavior (HAB) Across Breeds**

Values in a column with uncommon superscripts are significantly different (P < 0.05)
### Table 2. Reviewer Comparisons – Onset Age and Incidence of Expression of Hazard Avoidance Behavior (HAB)

Results were obtained from the 57 puppies randomly selected to validate inter-reviewer reliability. These means do not include the data from the additional 41 puppies evaluated in the study and therefore do not represent our final results.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Onset Age (Days)</th>
<th>Primary Tester</th>
<th>Standard Error</th>
<th>Reviewer 1</th>
<th>Standard Error</th>
<th>Reviewer 2</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavalier K.C. Spaniel HAB</td>
<td>51.3</td>
<td>3.5</td>
<td>51.3</td>
<td>3.5</td>
<td>50.1</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>German Shepherd HAB</td>
<td>39.4</td>
<td>6.2</td>
<td>38.2</td>
<td>6.5</td>
<td>37.1</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>Yorkshire Terrier HAB</td>
<td>42.1</td>
<td>2.4</td>
<td>40.1</td>
<td>2.4</td>
<td>41.1</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>Cavalier K.C. Spaniels</td>
<td>18.0</td>
<td>6.9</td>
<td>16.0</td>
<td>6.9</td>
<td>15.0</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>German Shepherds</td>
<td>17.0</td>
<td>7.5</td>
<td>15.0</td>
<td>7.5</td>
<td>19.0</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>Yorkshire Terriers</td>
<td>30.0</td>
<td>6.1</td>
<td>35.0</td>
<td>6.1</td>
<td>35.0</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>Control Puppies</td>
<td>2.0</td>
<td>6.7</td>
<td>1.0</td>
<td>6.7</td>
<td>0.0</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>Treatment Puppies</td>
<td>41.0</td>
<td>5.0</td>
<td>43.0</td>
<td>5.0</td>
<td>47.0</td>
<td>5.0</td>
<td></td>
</tr>
</tbody>
</table>

1 Data expressed as least squared means
<table>
<thead>
<tr>
<th>Week</th>
<th>Primary Tester (% of Puppies)</th>
<th>Standard Error</th>
<th>Reviewer 1 (% of Puppies)</th>
<th>Standard Error</th>
<th>Reviewer 2 (% of Puppies)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>10</td>
<td>0.05</td>
<td>8</td>
<td>0.05</td>
<td>4</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td>19</td>
<td>0.05</td>
<td>16</td>
<td>0.05</td>
<td>20</td>
<td>0.05</td>
</tr>
<tr>
<td>7</td>
<td>21</td>
<td>0.06</td>
<td>19</td>
<td>0.06</td>
<td>30</td>
<td>0.06</td>
</tr>
<tr>
<td>8</td>
<td>27</td>
<td>0.07</td>
<td>29</td>
<td>0.07</td>
<td>29</td>
<td>0.07</td>
</tr>
<tr>
<td>9</td>
<td>31</td>
<td>0.09</td>
<td>37</td>
<td>0.09</td>
<td>25</td>
<td>0.09</td>
</tr>
</tbody>
</table>

**Table 3. Reviewer Comparisons – Incidence of Hazard Avoidance Behavior by Age (HAB)**

*Results were obtained from the 57 puppies randomly selected to validate inter-reviewer reliability. These means do not include the data from the additional 41 puppies evaluated in the study and therefore do not represent our final results.*
Heart rate data were analyzed across breed, age, treatment versus control groups, gender, and pre and post-treatments. There were no significant differences in heart rate response to any specific test or between genders (Table 4; Table 5). There was a significant (P < 0.02) interaction effect of age, treatment group, and breed on heart rate. At five weeks of age, both treatment and control group Yorkshire Terriers demonstrated significantly higher (P < 0.05) heart rates compared to all German Shepherd and Cavalier King Charles Spaniel puppies of the same age. All Cavalier King Charles Spaniel puppies’ heart rates were significantly lower (P < 0.03) than German Shepherd and Yorkshire Terrier puppy heart rates from six through eight weeks of age. Additionally, the Cavalier King Charles Spaniel puppies showed no significant differences between treatment and control groups across all ages, with very little variation of heart rate throughout the testing period. All German Shepherd puppies and the treatment group Yorkshire Terrier puppies demonstrated a significant (P < 0.02) increase in mean heart rate from five to six and six to seven weeks of age, respectively (Figure 6). Significant differences (P < 0.02) in heart rate between treatment and control group German Shepherd and Yorkshire Terrier puppies were observed at seven weeks of age.
Figure 6. Interaction of Age, Breed, and Treatment on Heart Rate

Values expressed as least squared means in beats per minute. Vertical bars represent the standard errors of the means.
<table>
<thead>
<tr>
<th>Breed</th>
<th>Female (BPM)</th>
<th>Standard Error</th>
<th>Male (BPM)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavalier K.C. Spaniel</td>
<td>189.1</td>
<td>0.88</td>
<td>186.6</td>
<td>1.00</td>
</tr>
<tr>
<td>German Shepherd</td>
<td>203.5</td>
<td>3.02</td>
<td>197.8</td>
<td>1.13</td>
</tr>
<tr>
<td>Yorkshire Terrier</td>
<td>215.0</td>
<td>1.27</td>
<td>207.4</td>
<td>1.23</td>
</tr>
</tbody>
</table>

**Table 4. Mean Heart Rate – Female and Male Within Breed**

<table>
<thead>
<tr>
<th>Test</th>
<th>Cavalier K.C. Spaniel (BPM)</th>
<th>Standard Error</th>
<th>German Shepherd (BPM)</th>
<th>Standard Error</th>
<th>Yorkshire Terrier (BPM)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>189.5</td>
<td>1.4</td>
<td>198.3</td>
<td>1.9</td>
<td>209.3</td>
<td>2.0</td>
</tr>
<tr>
<td>NIT</td>
<td>189.1</td>
<td>1.7</td>
<td>201.2</td>
<td>2.2</td>
<td>213.4</td>
<td>2.2</td>
</tr>
<tr>
<td>TTT</td>
<td>186.0</td>
<td>1.6</td>
<td>200.2</td>
<td>2.0</td>
<td>213.4</td>
<td>2.1</td>
</tr>
<tr>
<td>ST</td>
<td>187.0</td>
<td>1.5</td>
<td>201.0</td>
<td>2.1</td>
<td>213.4</td>
<td>2.1</td>
</tr>
<tr>
<td>LNT</td>
<td>187.5</td>
<td>1.9</td>
<td>200.0</td>
<td>2.0</td>
<td>212.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Final</td>
<td>188.0</td>
<td>1.6</td>
<td>194.5</td>
<td>2.0</td>
<td>205.7</td>
<td>2.6</td>
</tr>
</tbody>
</table>

**Table 5. Mean Heart Rate in Response to Each Test Within Breed**
The movement of puppies in response to the testing stimulus was measured by recording the location of the puppies at the conclusion of each test. In the Novel Item Test, numerical values were assigned to each of the possible locations with a “0” designating that the puppy remained in the “Neutral” area, “1” indicating the puppy moved into the “Approach” section of the mat, and “-1” indicating that the puppy moved into the “Avoid” section of the mat.

The data include all puppies regardless of expression of hazard avoidance behavior. Results indicated no significant differences between treatment and control puppies or gender (Table 6), and no differences across breeds from four to six weeks of age. At seven weeks of age, German Shepherd and Yorkshire Terrier puppies were significantly (P < 0.05) more likely to move into the “Approach” section of the mat than Cavalier King Charles Spaniel puppies of the same age. By eight and nine weeks of age, the German Shepherd puppies were significantly (P < 0.01) more likely to move into the “Approach” section of the mat than any puppies six weeks of age and younger, and both the Cavalier King Charles Spaniel and Yorkshire Terrier puppies of the same age, which demonstrated statistically similar responses to each other at eight and nine weeks of age (Figure 7).
Figure 7. Breed Differences in Final Location – Novel Item Test

Data include treatment and control puppies. Results are expressed in least squared means based on assigned values:
1 = moved into approach section of mat, 0 = remained neutral, -1 = moved into avoid section of mat

Due to lack of responses to any stimuli in Cavalier King Charles Spaniel puppies at four weeks of age, the majority of Cavalier puppies were not tested until five weeks of age, resulting in no Cavalier data reported for the four week category.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Standard Error</th>
<th>Treatment</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cavalier K. C. Spaniel</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.04</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
</tr>
<tr>
<td>Male</td>
<td>-0.06</td>
<td>0.06</td>
<td>0.00</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>German Shepherd</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.32</td>
<td>0.08</td>
<td>0.16</td>
<td>0.09</td>
</tr>
<tr>
<td>Male</td>
<td>0.17</td>
<td>0.09</td>
<td>0.22</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Yorkshire Terrier</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.07</td>
<td>0.09</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>Male</td>
<td>0.14</td>
<td>0.08</td>
<td>0.00</td>
<td>0.09</td>
</tr>
</tbody>
</table>

**Table 6.** Mean Location – Novel Item Test – Gender and Treatment Effects

Means expressed based on assigned values:
1 = moved into approach section of mat, 0 = remained neutral, -1 = moved into avoid section of mat
The Loud Noise Test was coded identically to the Novel Item Test. If the puppy remained in the area it was placed in at the beginning of the test (“Neutral”), it was coded as “0”, with “1” indicating the puppy moved into the “Approach” section of the mat, and “-1” indicating that the puppy moved into the “Avoid” section of the mat.

Results indicated no significant differences in final location between treatment and control puppies and genders (Table 7) and no differences across breeds from four to six weeks of age. At seven weeks of age, German Shepherd and Yorkshire Terrier puppies were significantly (P < 0.01) more likely to move into the “Approach” section of the mat than Cavalier King Charles Spaniel puppies of the same age. By eight and nine weeks of age, the German Shepherd puppies were significantly (P < 0.05) more likely to move into the “Approach” section of the mat than any puppies six weeks of age and younger, and both the Cavalier King Charles Spaniel and Yorkshire Terrier puppies of the same age, which demonstrated statistically similar responses to each other at eight and nine weeks of age (Figure 8).
Figure 8. Breed Differences in Final Location – Loud Noise Test

Data include treatment and control puppies.
Results are expressed in least squared means based on assigned values:
1 = moved into approach section of mat, 0 = remained neutral, -1 = moved into avoid section of mat

Due to lack of responses to any stimuli in Cavalier King Charles Spaniel puppies at four weeks of age, the majority of Cavalier puppies were not tested until five weeks of age, resulting in no Cavalier data reported for the four week category.
<table>
<thead>
<tr>
<th>Breed</th>
<th>Control</th>
<th>Standard Error</th>
<th>Treatment</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cavalier K.C. Spaniel</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.14</td>
<td>0.07</td>
<td>-0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>Male</td>
<td>0.11</td>
<td>0.08</td>
<td>-0.05</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>German Shepherd</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.26</td>
<td>0.08</td>
<td>0.19</td>
<td>0.08</td>
</tr>
<tr>
<td>Male</td>
<td>0.28</td>
<td>0.10</td>
<td>0.26</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Yorkshire Terrier</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.15</td>
<td>0.10</td>
<td>0.03</td>
<td>0.10</td>
</tr>
<tr>
<td>Male</td>
<td>0.14</td>
<td>0.10</td>
<td>0.19</td>
<td>0.13</td>
</tr>
</tbody>
</table>

**Table 7. Mean Location – Loud Noise Test – Gender and Treatment Effects**

Means expressed based on assigned values:
1 = moved into approach section of mat, 0 = remained neutral, -1 = moved into avoid section of mat
The final locations of puppies from the Teeter-Totter Test were coded with “0” designating that the puppy remained in the area it was placed on at the beginning of the test (“Center”), “1” indicating the puppy moved slightly, into the “mid” section, and “2” indicating that the puppy moved to either of the distant edges or completely off the teeter-totter or teeter-totter marked region on the mat (control puppies).

Results indicated no significant differences in final location between treatment and control puppies and genders (Table 8) and no differences across breeds from four to five weeks of age. From six through nine weeks of age, however, the German Shepherd (P < 0.01) and Yorkshire Terrier (P < 0.05) puppies were significantly more likely to move to the mid-region, edge, or completely off the teeter-totter or identically marked region on the mat (control puppies) than the Cavalier King Charles Spaniel puppies of the same ages (Figure 9).
Figure 9. Breed Differences in Final Location – Teeter-Totter Test

Data include treatment and control puppies. Results are expressed in least squared means based on assigned values:
0 = remained where placed at beginning of test, 1 = moved to mid-region of teeter-totter or marked mat, 2 = moved to edge or off teeter-totter or mat.

Due to lack of responses to any stimuli in Cavalier King Charles Spaniel puppies at four weeks of age, the majority of Cavalier puppies were not tested until five weeks of age, resulting in no Cavalier data reported for the four week category.
<table>
<thead>
<tr>
<th>Breed</th>
<th>Control</th>
<th>Standard Error</th>
<th>Treatment</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavalier K. C. Spaniel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.52</td>
<td>0.16</td>
<td>0.34</td>
<td>0.11</td>
</tr>
<tr>
<td>Male</td>
<td>0.86</td>
<td>0.16</td>
<td>0.26</td>
<td>0.10</td>
</tr>
<tr>
<td>German Shepherd</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.15</td>
<td>0.17</td>
<td>0.94</td>
<td>0.17</td>
</tr>
<tr>
<td>Male</td>
<td>1.00</td>
<td>0.19</td>
<td>0.85</td>
<td>0.14</td>
</tr>
<tr>
<td>Yorkshire Terrier</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.07</td>
<td>0.19</td>
<td>1.21</td>
<td>0.17</td>
</tr>
<tr>
<td>Male</td>
<td>0.89</td>
<td>0.19</td>
<td>0.81</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Table 8. Mean Location – Teeter-Totter Test – Gender and Treatment Effects

Means expressed based on assigned values:
0 = remained where placed at beginning of test, 1 = moved to mid-region of teeter-totter or marked mat, 2 = moved to edge or off teeter-totter or mat.
The Step Test data were determined by whether the puppy remained on the step or moved off the step during the ten-second test period. Results indicated no significant differences in final location between genders (Table 9) or control or treatment Yorkshire Terrier puppies. German Shepherd and Cavalier King Charles Spaniel control and treatment puppies, however, demonstrated significant differences in their behavioral responses to the Step Test. Control group puppies of both breeds, regardless of age, were significantly (P < 0.05) more likely to move out of the designated “step area” than treatment puppies were to move off of the step (Figure 10).

**Figure 10.** Breed and Group Differences in Final Location – Step Test

Values expressed as least squared means. Based on assigned values:
1 = off the step, 0 = remained on the step
There was a significant ($P < 0.01$) effect of age on the final location of puppies in the Step Test. Figure 11 demonstrates the changes in the proportions of puppies that moved off the step (treatment group puppies) or designated step area (control group puppies) with increasing age. Significant differences in the puppies’ final location were found between five and six weeks of age and six and seven weeks of age, regardless of breed, group, or gender.

Figure 11. Age Differences in Final Location – Step Test

Values are expressed as proportions of puppies on or off the step.
Table 9. Mean Location – Step Test – Gender Effects

Means are based on assigned values: 1 = off the step, 0 = remained on the step
There was a significant (P < 0.01) effect of group on the incidence of crouched posture in the Teeter-Totter Test. Table 10 demonstrates the proportions of control and treatment group puppies within each breed that demonstrated a crouched posture in response to the Teeter-Totter Test. There were no significant interactions between breed, gender, and age on the incidence of crouching in the Teeter-Totter Test (Table 11).

<table>
<thead>
<tr>
<th>Breed</th>
<th>Control (%)</th>
<th>Treatment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavalier King Charles Spaniel</td>
<td>3.0 \textsuperscript{a}</td>
<td>27.1 \textsuperscript{b}</td>
</tr>
<tr>
<td>German Shepherd</td>
<td>5.0 \textsuperscript{a}</td>
<td>17.3 \textsuperscript{b}</td>
</tr>
<tr>
<td>Yorkshire Terrier</td>
<td>3.6 \textsuperscript{a}</td>
<td>19.7 \textsuperscript{b}</td>
</tr>
</tbody>
</table>

Table 10. Incidence of Crouch Exhibited in Teeter-Totter Test

Values with uncommon superscripts are significantly different (P < 0.01).
<table>
<thead>
<tr>
<th>Breed</th>
<th>Incidence of Crouch (%)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavalier K.C. Spaniel</td>
<td>16.1</td>
<td>4.5</td>
</tr>
<tr>
<td>German Shepherd</td>
<td>11.4</td>
<td>5.2</td>
</tr>
<tr>
<td>Yorkshire Terrier</td>
<td>12.4</td>
<td>4.7</td>
</tr>
<tr>
<td>Female</td>
<td>11.6</td>
<td>4.1</td>
</tr>
<tr>
<td>Male</td>
<td>15.0</td>
<td>3.8</td>
</tr>
<tr>
<td>4 Weeks</td>
<td>16.4</td>
<td>5.4</td>
</tr>
<tr>
<td>5 Weeks</td>
<td>10.5</td>
<td>3.9</td>
</tr>
<tr>
<td>6 Weeks</td>
<td>18.2</td>
<td>3.9</td>
</tr>
<tr>
<td>7 Weeks</td>
<td>8.8</td>
<td>4.1</td>
</tr>
<tr>
<td>8 Weeks</td>
<td>6.9</td>
<td>4.6</td>
</tr>
<tr>
<td>9 Weeks</td>
<td>13.3</td>
<td>6.5</td>
</tr>
<tr>
<td>10 Weeks</td>
<td>19.0</td>
<td>11.3</td>
</tr>
</tbody>
</table>

Table 11. Incidence of Crouch Exhibited in Teeter-Totter Test – Non-significant Differences Across Breed, Gender, and Age
There was a significant ($P < 0.05$) breed by group interaction on the incidence of crouched posture in the Loud Noise Test. A significantly ($P < 0.01$) higher proportion of Cavalier King Charles Spaniel treatment puppies demonstrated crouched posture compared to both the German Shepherd and Yorkshire Terrier treatment group puppies, which also differed significantly ($P < 0.01$) from one another. Table 12 demonstrates the proportions of control and treatment group puppies within each breed that demonstrated a crouched posture in response to the Loud Noise Test.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Control (%)</th>
<th>Treatment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavalier King Charles Spaniel</td>
<td>2.5 $^a$</td>
<td>79.7 $^b$</td>
</tr>
<tr>
<td>German Shepherd</td>
<td>5.0 $^a$</td>
<td>28.4 $^c$</td>
</tr>
<tr>
<td>Yorkshire Terrier</td>
<td>1.8 $^a$</td>
<td>45.5 $^d$</td>
</tr>
</tbody>
</table>

Table 12. Incidence of Crouch in Loud Noise Test – Breed by Group Interaction

Values with uncommon superscripts are significantly different ($P < 0.01$).
There was also a significant (P < 0.01) age by group interaction on the incidence of crouched posture in the Loud Noise Test. Incidence of crouch in control group puppies remained similar across all ages. Among treatment group puppies, however, the incidence of crouch was significantly (P < 0.01) higher from four to six weeks of age than seven through nine weeks of age, regardless of breed or gender (Table 13).

<table>
<thead>
<tr>
<th>Age (Weeks)</th>
<th>Control (%)</th>
<th>Treatment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>5.3 a</td>
<td>50.0 b</td>
</tr>
<tr>
<td>5</td>
<td>5.7 a</td>
<td>63.3 b</td>
</tr>
<tr>
<td>6</td>
<td>2.8 a</td>
<td>54.2 b</td>
</tr>
<tr>
<td>7</td>
<td>0.0 a</td>
<td>36.6 c</td>
</tr>
<tr>
<td>8</td>
<td>4.4 a</td>
<td>25.9 c</td>
</tr>
<tr>
<td>9</td>
<td>0.00 a</td>
<td>36.4 c</td>
</tr>
</tbody>
</table>

Table 13. Incidence of Crouch Exhibited in Loud Noise Test – Age by Group Interaction

Values with uncommon superscripts are significantly different (P < 0.01).
There were no significant differences between male and female puppies of all three breeds tested in the incidence of crouch response to the Loud Noise Test (Table 14).

<table>
<thead>
<tr>
<th>Breed</th>
<th>Female</th>
<th>Standard Error</th>
<th>Male</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavalier K. C. Spaniel</td>
<td>0.49</td>
<td>0.07</td>
<td>0.48</td>
<td>0.08</td>
</tr>
<tr>
<td>German Shepherd</td>
<td>0.11</td>
<td>0.04</td>
<td>0.25</td>
<td>0.05</td>
</tr>
<tr>
<td>Yorkshire Terrier</td>
<td>0.28</td>
<td>0.06</td>
<td>0.23</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Table 14. Incidence of Crouch – Loud Noise Test – Means Across Genders
Puppies that exhibited hazard avoidance behavior at testing demonstrated a tendency \((P < .07)\) to have an increase in the concentration of cortisol from the post-test salivary collection compared to their pre-test collection on the day of testing (Table 15). No other puppies (treatment puppies not demonstrating hazard avoidance behavior and control puppies) showed this tendency.

<table>
<thead>
<tr>
<th>Treatment Puppies - Without HAB</th>
<th>Mean Changes in Cortisol Concentration (Collection 2 – Collection 1)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- 0.13</td>
<td>0.09</td>
</tr>
<tr>
<td>Treatment Puppies - With HAB</td>
<td>0.27</td>
<td>0.12</td>
</tr>
<tr>
<td>Treatment Puppies (No HAB - puppies who previously or later showed HAB)</td>
<td>- 0.12</td>
<td>0.13</td>
</tr>
<tr>
<td>Control</td>
<td>- 0.06</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 15. Mean Changes in Cortisol Concentration Across Groups
Mean cortisol concentrations from the first (pre-test) and second (post-test) collection of all breeds, ages, treatments, and genders are listed in Table 16. No significant differences in salivary cortisol concentrations were found across breeds, treatments and controls, or ages (Table 17; Table 18). Despite non-significant differences in salivary cortisol concentrations across groups, the number of samples with detectable levels of cortisol was extremely high. Of the 368 saliva samples analyzed, only four (1.1\%) were below our level of detectability (0.007 ug/dL).

<table>
<thead>
<tr>
<th></th>
<th>Concentration (ug/dL)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of Collection 1</td>
<td>0.48</td>
<td>0.05</td>
</tr>
<tr>
<td>Mean of Collection 2</td>
<td>0.40</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Table 16.** Mean Salivary Cortisol Concentrations

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Mean Concentration (ug/dL)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.51</td>
<td>0.08</td>
</tr>
<tr>
<td>5</td>
<td>0.44</td>
<td>0.04</td>
</tr>
<tr>
<td>6</td>
<td>0.47</td>
<td>0.06</td>
</tr>
<tr>
<td>7</td>
<td>0.49</td>
<td>0.07</td>
</tr>
<tr>
<td>8</td>
<td>0.44</td>
<td>0.16</td>
</tr>
<tr>
<td>9</td>
<td>0.18</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Table 17.** Mean Salivary Cortisol Concentrations by Age
Table 18. Mean Salivary Cortisol Concentrations Across Breed, Gender, and Group

<table>
<thead>
<tr>
<th>Breed</th>
<th>Control (ug/dL)</th>
<th>Standard Error</th>
<th>Treatment (ug/dL)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavalier K.C. Spaniel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.34</td>
<td>0.04</td>
<td>0.37</td>
<td>0.05</td>
</tr>
<tr>
<td>Male</td>
<td>0.28</td>
<td>0.04</td>
<td>0.55</td>
<td>0.06</td>
</tr>
<tr>
<td>German Shepherd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.43</td>
<td>0.04</td>
<td>0.66</td>
<td>0.10</td>
</tr>
<tr>
<td>Male</td>
<td>0.34</td>
<td>0.21</td>
<td>0.46</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Table 19 provides information regarding the random effects of litter within breed and puppy within litter within breed, as well as the residual effects for each analysis. These data indicate how similar puppies within a litter were in their responses compared to those across litters within the same breed, as well as what the individual variance components were across puppies within a litter and breed.
<table>
<thead>
<tr>
<th></th>
<th>Litter within Breed</th>
<th>Standard Error</th>
<th>Puppy Within Litter Within Breed</th>
<th>Standard Error</th>
<th>Residual</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hazard Avoidance Behavior Onset</td>
<td>35.78</td>
<td>24.24</td>
<td>N/A</td>
<td>N/A</td>
<td>31.80</td>
<td>12.48</td>
</tr>
<tr>
<td>Video Reviewers – HAB Onset</td>
<td>32.50</td>
<td>20.96</td>
<td>23.32</td>
<td>10.05</td>
<td>7.31</td>
<td>1.60</td>
</tr>
<tr>
<td>Heart Rate</td>
<td>56.90</td>
<td>34.21</td>
<td>141.94</td>
<td>29.97</td>
<td>773.35</td>
<td>23.03</td>
</tr>
<tr>
<td>Location - Novel Item Test</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.01</td>
<td>0.16</td>
<td>0.01</td>
</tr>
<tr>
<td>Location - Teeter-Totter Test</td>
<td>0.07</td>
<td>0.04</td>
<td>0.06</td>
<td>0.03</td>
<td>0.43</td>
<td>0.04</td>
</tr>
<tr>
<td>Location - Step Test</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.14</td>
<td>0.01</td>
</tr>
<tr>
<td>Location - Loud Noise Test</td>
<td>0.00</td>
<td>0.01</td>
<td>0.03</td>
<td>0.01</td>
<td>0.20</td>
<td>0.02</td>
</tr>
<tr>
<td>Crouch - Teeter-Totter Test</td>
<td>0.01</td>
<td>0.00</td>
<td>0.01</td>
<td>0.01</td>
<td>0.10</td>
<td>0.01</td>
</tr>
<tr>
<td>Crouch - Loud Noise Test</td>
<td>0.01</td>
<td>0.01</td>
<td>0.00</td>
<td>N/A</td>
<td>0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>Salivary Cortisol Concentration</td>
<td>0.00</td>
<td>N/A</td>
<td>0.00</td>
<td>N/A</td>
<td>0.53</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**Table 19.** Variance Components – Random Effects and Residuals
DISCUSSION

The results of this study support the hypothesis that emotional and behavioral development and the age of the onset of hazard avoidance behavior vary across breeds of domestic dogs. These results are in agreement with previous research by Coppinger and Coppinger (2001), which demonstrated that specific breeds of dogs have a defined and predictable hazard avoidance behavior onset. Coppinger and Coppinger (2001) indicated a hazard avoidance onset of 35 days in the German Shepherd. The results from our study are comparable to those published by Coppinger and Coppinger (2001), with a least squared means estimate for hazard avoidance behavior onset in the German Shepherd at 39.4 days (SD 3.57 days). In mice and rats, behavioral and hormonal responses to environmental stressors have been found to differ significantly among different genetic strains (Anisman et al., 1998; Anisman and Merali, 1999), and genetic factors have also been found to have a strong influence on the intensity of fear reactions in domestic animals and humans (Parsons, 1988). It is not known what the genetic background was of the German Shepherd Dogs in the Coppinger and Coppinger (2001) study. The German Shepherd puppies used in the current study were specifically selected from purpose-bred, working-line dogs of international (primarily German and Czech) bloodlines and did not appear to differ significantly in their development of hazard avoidance behavior from those studied by Coppinger and Coppinger (2001).
The heart rate values and changes in relation to puppy age in this study were consistent with those identified in previous research. In 1965, Scott and Fuller found that heart rate in puppies reached a low point at approximately four to five weeks of age. This was followed by an increase in heart rate, reaching a high point between six and eight weeks of age. The increased heart rate was maintained in most breeds for several weeks. Scott and Fuller (1965) presumed that this increase was associated with the puppies’ development of the ability to form conditioned responses or a change in their emotional responses to the environment which also occur around this age. Although the Cavalier King Charles Spaniel puppies in this study did not follow this pattern, significant increases in mean heart rate were demonstrated by all the German Shepherd puppies and the treatment group Yorkshire Terrier puppies at six and seven weeks of age, respectively.

Studies by Mahut (1958), Scott and Fuller (1965), Plutchik (1971), Goddard and Beilharz (1985), and King et al., (2003) have all identified breed differences in fear responses in adult dogs. Their studies examined movement, toward or away from novelty or in general, as well as time spent near a novel item and behavioral fear indicators such as the freeze response, crouching, trembling, vocalizing, etc. This study revealed no significant breed differences in behaviors demonstrated across puppies at four and five weeks of age. Breed differences in mobility were identified at six weeks of age when the
Cavalier King Charles Spaniel puppies demonstrated significantly less movement in the Teeter-Totter Test compared to the German Shepherd and Yorkshire Terrier puppies.

Puppies of all three breeds were also significantly more likely to remain on the step in the Step Test at four and five weeks of age compared to puppies six weeks of age and older. By seven weeks of age the Cavalier King Charles Spaniel puppies showed significantly less movement in the Novel Item, Loud Noise, and Teeter Totter Test compared to the other two breeds studied. At eight and nine weeks of age Yorkshire Terriers and Cavalier King Charles Spaniels were similar in their movement during the Novel Item and Loud Noise Test, however the Cavalier King Charles Spaniel remained the least mobile in the Teeter-Totter Test for the duration of the study. Significant breed differences, regardless of age, were also found in the proportions of treatment group puppies that demonstrated hazard avoidance behavior, responded to the Loud Noise Test with a crouch, and moved off the step in the Step Test.

Regardless of breed, all treatment group puppies between four and six weeks of age showed significantly higher 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 on the average at 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 related to the expression of the adult hazard avoidance
behavior motor patterns. In fact, beginning at seven weeks of age, treatment group puppies of all breeds showed significantly lower 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 4-5 weeks of age.

Previous studies have demonstrated differences in fear responses among genders (Gray, 1971; Plutchik, 1971; Goddard and Beilharz, 1985; Conrad et al., 2003), however the results of this study did not exhibit any significant differences between the fear reactions or development of male and female puppies. Previous research findings have been conflicting about which of the genders is the more “fearful” or “reactive” gender, although in dogs, females were generally recognized as the more fearful gender (Plutchik, 1971; Goddard and Beilharz, 1985). It is possible that if a gender difference in fear reactivity exists in dogs, it does not become apparent until after puberty, when other notable differences in personality and physiology also become apparent.

Significant increases in salivary cortisol concentrations have been identified 10-30 minutes after exposure to fear-inducing stimuli in humans, rats, and adult dogs (Muir and Pfister, 1987; Tarui and Nakamura, 1987; Beerda et al., 1998; Dreschel and Granger, 2005). There was a tendency for puppies in the current study that behaviorally showed hazard avoidance behavior in response to the testing to also demonstrate increases in
cortisol concentrations in post-test saliva samples compared to pre-test samples. The increase in cortisol is a physiological change that may be associated with the onset and expression of hazard avoidance behavior in puppies. There were no significant differences in salivary cortisol concentrations after exposure to fear-inducing stimuli across breeds, ages, or genders. The results of the analyses of salivary cortisol demonstrate that puppies release detectable concentrations of cortisol from a very young age. Interestingly, the mean cortisol concentrations of puppies in this study were two to four times the baseline concentrations of adult dogs studied by Dreschel and Granger (2005; 2009). The results of human and primate studies indicate that the infant adrenal gland is larger in size than the adult adrenal (Oppenheimer et al., 1983). At birth, the primate adrenal cortex contains a large “fetal zone” that occupies about eighty percent of the adrenal gland, with a much smaller peripheral region that later becomes the adult adrenal. After birth, the fetal zone of the adrenal gradually undergoes involution, being replaced by connective tissue and resulting in adult-like adrenals by one year of age (Oppenheimer et al., 1983). The puppies in this study may have had larger neonatal adrenal glands that were responsible for the increased mean concentrations of cortisol.

Research on breed differences in behavior has been conducted on dogs; however few studies have specifically looked at differences in the development of fear motor patterns in young puppies across different breeds of dogs. The results of this study indicate that there are significant differences among breeds in the onset and expression of
hazard avoidance behavior. Additionally, the results demonstrate significant breed differences in heart rate in relation to age, development of mobility related to age, and incidence of crouching in response to a sudden noise. The results of this study indicate a need for further research in this area, as a more reliable understanding of breed-specific, fear-related development in young puppies could be applicable to breeding, rearing, training, socialization, and behavioral modification practices in domestic dogs.


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