WELFARE IMPLICATIONS OF EARLY NEUROLOGICAL STIMULATION FOR PUPPIES IN COMMERCIAL BREEDING KENNELS

by

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To Trevor, for always believing in me and keeping me (mostly) sane!

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LIST OF ABBREVIATIONS

ENS =	early neurological stimulation
CB Kennel(s)=	commercial breeding kennel(s)
HPA axis =	hypothalamic-pituitary-adrenal axis
HRV =	heart rate variability
ACTH =	adrenocorticotropic hormone
PBK =	professional breeding kennel
USDA =	United States Department of Agriculture
BCS =	body condition score
RYG =	red, yellow, green categorical scoring of dog behavioral responses to stranger approach
RYG+ =	red, yellow, green categorical scoring of dog behavioral responses to multi-step stranger approach, plus eating of treats
AIC =	Akaike's information criterion
ML =	maximum likelihood
REML =	restricted maximum likelihood
SD =	standard deviation
SEM =	standard error of the mean
DF =	degrees of freedom
ICC =	Intraclass correlation coefficient

ABSTRACT

From birth and throughout their lives, dogs experience a variety of potentially stressful stimuli. Early neurologic stimulation (ENS) is believed to improve the ability of animals to handle stress, however its effects on dogs have not yet been fully explored. This study aimed to evaluate the effects and potential welfare implications of providing ENS to puppies in commercial breeding kennels. Seventy-six puppies, comprising two cohorts in one kennel were studied. Puppies were assigned to one of three treatment groups: ENS, held, or control, and then were marked for identification, and handled daily Monday through Saturday for 21 days, beginning on day three post-partum. ENS puppies received five “Bio Sensor” exercises (Battaglia, 2009). Puppies in the held treatment group were held for 30 seconds, which was the same length of time that was required to apply the Bio Sensor exercises to ENS treated puppies. Control puppies received identification marks daily and health assessments weekly, but otherwise were handled as normal for the breeder’s management plan. To evaluate treatment effects on physical health, all puppies received physical health assessments weekly, and additionally before and after transport to a distributor. To evaluate effects of treatment on behavioral responses to stressors, puppies were assessed shortly before and after transport (a known stressor), using three stranger approach tests and a 3-minute isolation test. Puppies were found to be generally healthy and clean throughout the study. A three-way interaction was observed between treatment, sex, and week of life, which affected puppies’ weights over the first eight weeks of life at the breeder’s kennel prior to the application of stressors (p = 0.006). Female ENS puppies were found to weigh more than their held and control counterparts, while for male puppies, held and control puppies weighed more than ENS puppies. A two-way interaction was observed between treatment and isolation on behavior for a single step of the multi-step stranger approach test performed at the breeder’s kennel (p = 0.025). While more puppies showed affiliative behavioral responses to the experimenter reaching for them after isolation than before, the change was greater in ENS and held treatment groups than controls. Treatment also directly affected the time puppies spent performing fearful behavior during the isolation test (p = 0.041). Handled puppies spent more time performing fearful behaviors than control puppies. No other significant effects of treatment were observed for the behavioral or physical health parameters measured. However, the finding that ENS and held-groups tended to show greater increases in the number of puppies displaying affiliative behavior

than controls (though it was only significant for one step) suggests that handling treatments primed puppies to view people as a form of social support during stress. The additional finding that ENS and held group puppies spent more time performing fearful behaviors (e.g., escape attempts, low postures) during isolation than control puppies, further supports this theory. While these results do not support the purported effects of ENS, they indicate that early handling may still benefit puppies by providing them positive interactions with humans. These interactions potentially prime developing puppies to view humans as safe sources of social support, perhaps increasing their likelihood of forming secure attachments with people later in life. Further, findings from this study suggest that simply holding puppies daily for short periods may be sufficient to produce beneficial effects. Future studies should incorporate measures of recovery in response to stress testing puppies receiving ENS treatment and should consider evaluating ENS in conjunction with attachment theory to provide more information on the potential welfare effects of early handling of puppies in commercial breeding and other kennel types.

CHAPTER 1. INTRODUCTION

Dogs are believed to be one of the earliest species of animals to be domesticated (Clutton-Brock and Jewell, 1993), and as such have lived together with humans for thousands of years. In fact, some of the earliest remains of domestic dogs have been found in burial sites together with human remains (Clutton-Brock and Jewell, 1993). Over time, humans have both intentionally and unintentionally shaped the evolutionary history of the dog, breeding them to fill many roles, such as lap dogs like the Cavalier King Charles Spaniel, hunting partners like the Labrador Retriever, and sled-pulling dogs such as the Siberian Husky. Thus, the dog has become an integral part of human society. The close bond between humans and dogs has continued. Today, high percentages of people who have at least one dog report that they view them as a member of the family. In fact, 85.1% of dog owners in the US (American Veterinary Medical Association, 2018), and 63% in Australia (Animal Medicines Australia, 2019) characterize dogs this way. Most owned companion dogs live primarily indoors, and some may even share a bed with their humans (Associated Press, 2009). People's spending on their companion animals has also increased, both in terms of expenditures on supplies and grooming, and on veterinary care and training (American Veterinary Medical Association, 2018; Animal Medicines Australia, 2019). Further, people who have dogs report a variety of benefits to their physical and psychological health, such as reduced loneliness and increased physical activity. These benefits could result both from the direct companionship of the dog and/or from getting more exercise due to having a dog, which tends to improve physical fitness and mood (Duvall Antonacopoulos, 2017; Knight and Edwards, 2008; The People's Dispensary for Sick Animals, 2019). The dog-human partnership is therefore firmly established and growing, as shown by the estimated 76.8 million pet dogs in the US, 8.2 million in Canada, and 9 million in the UK (American Veterinary Medical Association, 2018; Canadian Animal Health Institute, 2019; Pet Food Manufacturers' Association, n.d.). The large demand for dogs as companion animals coupled with an evolving understanding of animal sentience and welfare has led to debate about the ethics related to the breeding and raising of dogs, and concerns about the welfare of animals in high-volume commercial breeding (CB) kennels.

Currently in developed countries, most of us do not rely on our dogs for help moving our belongings, hunting, or keeping wild animals out of our homes. Instead, they are chosen to be companions either for the humans, or for other animals in the household (Animal Medicines

Australia, 2019; Duvall Antonacopoulos, 2017; Leslie et al., 1994). As a result, compatibility with owner lifestyle, the dog's behavior or temperament and physical health and appearance are all characteristics reported to be important when acquiring a dog (Bir et al., 2017; Weiss et al., 2012). When selecting a dog, a person may choose whether to purchase a mixed-breed dog or a purebred or designer breed. The selection of dogs based primarily on appearance and temperament may influence individuals in favor of purebred dogs or designer breeds, as people may expect the dog to look and behave within the range of a certain breed's standards. In the US, just under half the population of companion dogs are reported to be purebred, and the other half mixed-breed (American Veterinary Medical Association, 2018). In Australia, 40% of the dog population are estimated to be purebred dogs, 46% are mixed-breed, and 14% are 'designer breeds' (Animal Medicines Australia, 2019). In addition to selecting a certain type of dog, potential owners will also make choices about where to acquire a new dog. While about a third of pet dogs in the US are reported to come from rescue groups or shelters, almost one third come from breeders or pet shops (American Veterinary Medical Association, 2018). In Canada, 45% of pet dogs were reported to be acquired from breeders or pet stores with only 13% acquired from animal shelters (Perrin, 2009). In the UK, just over a third of pet dogs were reportedly acquired from rescues, while breeders made up 16% of the supply. However, another 31% of dogs were acquired either through private advertisements, the internet, or a pet shop (Pet Food Manufacturers' Association, n.d.). Note that dogs for sale in pet shops are often believed to come from commercial dog breeders, and it is difficult to identify the origin of dogs purchased online.

The rising popularity of dogs combined with the top reported sources of dog acquisition give a clue as to why commercial dog breeding operations exist in the first place. Many people want dogs and are willing to purchase them from breeders, pet shops or online sales venues where the source of the dog may be unclear.

There is a fair amount of concern regarding the welfare of dogs in commercial breeding kennels. This may stem from public concerns regarding the welfare of domestic livestock animals in many developed countries, including the United States and Europe, especially in larger and more intensely-managed commercial operations (Bennett and Blaney, 2003; European Commission, 2007; McEachern et al., 2007; McKendree et al., 2014). Part of the reason for this specific focus on larger commercial operations is the perception that any large-scale CB kennel is a "puppy mill", resulting in the conflation of the two terms (Croney, 2019). These operations are typically viewed as crowded and dirty, and full of malnourished dogs who do not get appropriate veterinary care, socialization or enrichment. Animal welfare organizations in the US and Canada have taken a stance against ‘puppy mills’, with the apparent goal of identifying welfare-compromised animals and shutting down these operations (Humane Canada, n.d.; Sinclair et al., 2015). However, it is important to point out that there is no established legal definition of a puppy mill. Often, the relative importance placed on the welfare of the dogs versus profit is used to differentiate between better and worse operations. Therefore, ‘puppy mills’ have been defined by some as operations where the profit margin is valued above all else, and especially at the expense of health and welfare of the animals (Candace Croney Research Group, n.d.). Welfare problems may include poor breeding decisions, lack of veterinary care, and lack of exercise and enrichment (National Companion Animal Coalition, n.d.).

In contrast to puppy mills, legally operating commercial breeding kennels are overseen by United States federal regulation. The Animal Welfare Act (AWA) regulates the care and treatment of many warm-blooded animals, including dogs. The AWA, enforced by the Animal and Plant Health Inspection Service (APHIS) branch of the US Department of Agriculture (USDA), requires anyone who maintains five or more breeding bitches and sells their offspring to be licensed or registered with APHIS and to meet certain minimum standards (United States Department of Agriculture (USDA), 2019). Minimum standards of care include, but are not limited to, requirements for specific levels of cleanliness and space for enclosures, a program of veterinary care and daily inspection by a caretaker, and identification and updated records for each animal (United States Department of Agriculture (USDA), 2019). While these regulations do not address every concern surrounding the commercial breeding of dogs, they do provide a baseline level of care for dogs in these facilities. Domestic animals, including livestock and companion animals are sentient beings, meaning they are able to experience happiness and enjoyment, but also pain and suffering (Singer, 1974). Good welfare for non-human animals provides ample opportunities for them to express normal behaviors and experience pleasure, but also ensures that negative experiences of pain, fear and suffering are minimized.

In addition to concerns about the welfare issues surrounding the commercial breeding of dogs, there is the perception that there are too many dogs already. However, consumers wish to have a variety of choices when acquiring a dog, creating demands that support commercial breeding. According to a 2015 survey conducted by Bir and colleagues, almost 53% of respondents agreed with the statement, “There is a dog overpopulation problem in the US”. Nevertheless, about 56% also agreed that “People should be able to buy purebred dogs” (Bir et al., 2017). In fact, though shelters in some areas of the country are full, an emphasis on spaying and neutering pets, and advertising such as the “Adopt don’t shop” campaign look to have been highly successful. Numbers of dogs admitted to shelters appears to be decreasing, while the percentage of dogs being adopted is on the rise, although these numbers can be difficult to determine (Humane Canada, n.d.; Rowan and Kartal, 2018). Interestingly, more of the dogs relinquished to shelters are reported to be mixed breed rather than purebred dogs, providing a counterpoint to the perception that dogs from breeders are the primary reason shelters are full (Diesel et al., 2010; Kwan and Bain, 2013). Additionally, illness and behavioral issues are commonly reported by owners to be reasons for relinquishment of a dog to shelters, suggesting that perhaps animals are not entering shelters solely due to an overpopulation problem, but rather due to owner dissatisfaction with their behavior or health, or other factors (Diesel et al., 2010; Kass et al., 2001; Kwan and Bain, 2013).

Finally, concerns have been raised regarding the behavioral health of dogs originating in commercial breeding kennels. Owner-report studies by McMillian and colleagues showed that dogs who were purchased from pet stores and therefore thought to come from large-scale commercial breeding kennels had higher rates of aggression and separation-related issues compared to dogs from noncommercial breeders (McMillan et al., 2013). In addition, retired breeding dogs were reported to have higher levels of health and fear-related problems, when compared to a control sample of other pet dogs (McMillan et al., 2011). Yet, recent studies using stranger approach tests conducted in commercial breeding kennels showed primarily affiliative responses to people from the dogs observed (Bauer et al., 2017; Mugenda et al., 2019), while another found that over half the dogs showed fearful responses when strangers interacted more closely with them in a multi-step approach test (Stella et al., 2019). Based on this information, it seems that the behavioral health and overall welfare of dogs in CB kennels is not yet fully understood and more research is needed in this area.

While a need exists for further research on the welfare state of dogs in commercial breeding kennels, public concern has already prompted several cities in the US as well as the state of California to enact bans on the retail sale of dogs, though in some cases dogs can still be sold if they were obtained from a shelter or other humane or rescue organization (American Veterinary Medical Association, 2017, 2011; Wilson, 2020). These rules in theory are supposed to put puppy mills out of business by cutting off demand, ostensibly saving many dogs from mistreatment. However, in practice, these actions fail to directly address welfare concerns of the adult dogs living in commercial kennels, and in fact may introduce new problems for these dogs by reducing breeders' financial ability to provide them care (Croney, 2019). Further issues may be created due to these rulings potentially sending buyers online (where regulation is lacking) or even driving a demand for a black market import of puppies (Croney, 2019; Gidda, 2018; Voris et al., 2011). In addition to concerns that smuggled puppies may face dangerous transportation conditions which can lead to illness, injury and death, improper vetting of imported animals has occurred, resulting in at least one instance of importing a dog suffering from rabies (Sinclair et al., 2015). While there are always risks of unintended consequences, sale bans on commercially bred puppies appear to introduce many health and welfare issues of their own, arguably more than they solve (Croney, 2019). Study and intervention at the level of the kennel rather than at the point of sale may be more effective in improving the welfare of dogs and puppies currently living in commercial breeding facilities.

CHAPTER 2. LITERATURE REVIEW

2.1 What is Animal Welfare and How is it Scientifically Assessed?

Animal welfare is the state of an animal in regards to its attempts to cope both physically and mentally, with the environment in which it is living (OIE World Organisation for Animal Health, 2013). Exact definitions of animal welfare have been debated over the years, but current definitions reflect a comprehensive view which considers both an animal's physical and mental health. This view of animal welfare entails minimizing negative experiences and mental states such as hunger, disease, fear or distress as much as possible, while also providing an animal with the ability to express normal behaviors and engage in pleasurable activities such as play (Mellor, 2016).

2.1.1 Assessment of Animal Welfare – Internal Physical Measures

Because animal welfare concerns both the physical and mental health of the animal, assessments of animal welfare involve both physical and behavioral measures. Often, the concept of stress is a key element in the evaluation of animal welfare, because both stress and welfare inherently deal with how an animal feels (Veissier and Boissy, 2007). Stress can be defined as a threat, real or perceived, to an organism's homeostasis, with homeostasis being the maintenance of physiological systems within the optimal range for life (McEwen and Wingfield, 2003). When an animal experiences a stressful event, that stressor acts on the hypothalamic-pituitary-adrenal (HPA) axis, which leads to a cascade of hormonal and physiological changes that are meant to assist the return to homeostasis, and which affect the whole body (Cannon, 1939; Selye, 1950). This process of achieving stability through change is termed allostasis (McEwen and Wingfield, 2003). The HPA axis is a collection of structures located in the central nervous system as well as peripheral tissues which forms the basis of the body's allostatic or stress response (Smith and Vale, 2006). Stress acts first on the hypothalamus causing it to release corticotropin-releasing hormone (CRH) to the pituitary. The pituitary then releases adrenocorticotropic hormone (ACTH) to the adrenal glands, which are located above the kidneys. The adrenals release stress hormones including glucocorticoids (e.g. cortisone, corticosterone), mineralocorticoids (e.g. aldosterone), and catecholamines (e.g. epinephrine and norepinephrine). These hormones act on nearly every system in the body, and result in a variety of physiological effects, including the release of stored energy, and the inhibition of growth, reproductive, and certain immune functions (Charmandari et al., 2005; Sapolsky et al., 2000). The wide range of effects resulting from stress have implications for physical and behavioral health and welfare. Importantly, the intensity and duration of stress impact ultimate effect. Short-term and mild stressors can be beneficial, leading to increases in immune function (Dhabhar and McEwen, 1997; Dhabhar and Viswanathan, 2005), and the enhancement of certain types of memory and learning (Shors et al., 1992; Yuen et al., 2009), among other things. While mild stress may benefit an animal, high intensity (severe) acute or long term (chronic) stressors can lead to detrimental effects including impairment of spatial memory (Luine et al., 1994), immunosuppression (Dhabhar and McEwen, 1997), increases in illness (Lehman et al., 1991), and cognitive abnormalities (Cohen et al., 2013; Rice et al., 2008). In other words, the effects of stress follow an "inverted U" curve, with levels that are either very high or low being detrimental, but mild levels showing positive effects (Sapolsky, 2015). Because the mechanisms of stress activation of the HPA axis and the resulting physiological effects are well understood, measurement of the level of activation of the HPA axis has been widely used as a physical measure to assess animal welfare. One way this can be accomplished is via measuring concentrations of glucocorticoids such as corticosterone (in birds and some rodents) or cortisol (in other mammals) (Broom and Fraser, 2007). Glucocorticoid concentrations can be measured in a variety of ways, including via the blood, urine, feces, saliva, and hair (Mormède et al., 2007; Sheriff et al., 2011). Because all kinds of stress can activate the HPA axis, not only those which may be detrimental to the animal, interpreting the relationship between glucocorticoid concentrations and overall welfare states can be challenging. It is therefore vital to take into account the context in which these concentrations were measured (Broom and Fraser, 2007).

Additional internal physical metrics can also be used to assess the welfare state of an animal, including heart rate, heart rate variability, immunological responses, and changes in body and skin temperature (Broom and Johnson, 2019). Heart rate has been shown to change in response to a variety of challenges, such as social stress (de Jong et al., 2000) and restraint (Chen and Herbert, 1995; McDougall et al., 2000). Often an increase is seen, but in some cases decreases have been observed, and it is important to consider the species being studied and its known unique characteristics in terms of heart rate when using this as a measure of welfare (Broom and Johnson, 2019). Because HPA axis activation usually increases blood pressure and heart rate, heart rate may be elevated for a variety of reasons which are not inherently indicators of poor welfare, such as exercise or sexual arousal. Therefore, heart rate measurements should be evaluated in combination with additional metrics of physiology and behavior before making interpretations of the welfare implications of heart rate changes.

Heart rate variability (HRV) is another way of measuring cardiac response to stressors, and is believed to be a more robust measure than heart rate alone, as it takes into account interactions between the sympathetic nervous system and the parasympathetic nervous system, which may provide some distinction between states of stress and arousal (von Borell et al., 2007). HRV has shown stronger correlations with some measures of well-being than heart rate alone in human studies, and is emerging as a suitable measure of stress responses in a variety of animals, including cows, horses, and sheep (von Borell et al., 2007). As mentioned above, immune function, often measured via leukocyte and/or neutrophil numbers and distribution, has been shown in studies in humans and a variety of non-human animals to often be enhanced by mild stressors, but impaired as a result of chronic stress (Dhabhar, 2014). Finally, core body temperature increases have been observed in response to a variety of psychological stressors such as handling, open field testing, and anticipation of a stressful event (Oka et al., 2001). While internal measures can provide detailed information about an animal's physiological stress response, they can sometimes be challenging and expensive to obtain, as they often require handling of the animals to procure a sample and specialized equipment to analyze it. Additionally, it may be challenging to interpret the results in relation to welfare. However, when assessed in context and combined with other metrics, such as behavioral measures, internal measures can be valuable indicators of an animal's welfare state.

2.1.2 Assessment of Animal Welfare – External Physical Measures

External physical metrics can also be a useful method of welfare assessment, and include assessments of the prevalence of disease and injury, body condition, weight and growth, reproductive measures (such as ability to breed & litter size), and life expectancy, among others (Broom and Fraser, 2007). Measurements of disease and injury rates can provide insight into the effects of housing and management practices. For example, a study in heifers observed significantly fewer and less severe hock injuries in straw yards, as compared to cubicles with rubber mats or mattresses (Livesey et al., 2002).

Due to the actions of stress on the immune system, animals who experience chronic stress may be more likely to fall ill. Further, it is not unreasonable to suppose that an animal who has contracted an illness may be experiencing worse welfare than their healthy conspecifics, simply due to being ill. Thus, measurements of disease prevalence, in conjunction with other measures, may give insight into the welfare status of animals.

Growth rates may also be used to evaluate potential welfare challenges, as activation of the stress response prompts the release of stored energy and inhibits digestion, which can lead to negative effects on growth. This link between stress and growth has been observed in animals, such as in a study by Hemsworth and Barnett (1991), in which young pigs who were subjected to aversive handling had lower weights and rates of gain in early life, as compared to conspecifics who received pleasant handling.

2.1.3 Assessment of Animal Welfare – Behavioral Measures

In addition to the utilization of internal and external physical metrics to assess animal welfare, many studies also measure behavioral parameters. Behavioral metrics are very useful to assessments of welfare because they provide insight into an animal's perception of a potential stressor. When the central nervous system perceives a stimulus as a threat, it will prompt a stress response regardless of whether the stimulus is truly a threat (Moberg, 2000). The level of perceived threat determines the category and intensity of the stress response, and this perception will be influenced by an animal's life history, including its genetics, experience, and physiological state (Moberg, 2000). Behavioral metrics that have been used to assess welfare in animals include the measurement of fear/avoidance, pain, and sickness behaviors; evaluation of changes in patterns of normal behaviors such as eating, drinking, sleeping, and grooming (Broom, 1988); and the presence of abnormal behaviors or stereotypies.

It is important to note that measures must be taken to minimize observer bias in evaluations of behavior. For example, blinding the observer to the animal's treatment group can help minimize assessment biases stemming from knowledge of the animal's treatment and perceptions about its effects. Additionally, inter- and intra-rater reliabilities can be assessed to evaluate both the ability of independent observers use identical behavioral ethograms in the same way, and the consistency of a single observer, respectively. While these approaches are considered powerful ways to minimize bias, reviews of published animal behavior studies have often failed to include use of these methods (Burghardt et al., 2012).

Fear and avoidance behaviors may be expressed as a reaction to painful stimuli. For example, angus calves showed significant increases in escape-avoidance behavior as a response to hot-iron branding, as compared to both freeze-branded or control (unbranded) calves (Lay et al., 1992). This suggests that the hot branding procedure itself is painful and aversive, not simply the handling. Aversion behaviors can also result from handling alone if handling methods are aversive or painful. For example, gilts who received aversive handling (slapping, shocking) demonstrated higher levels of fear and aversion to humans than those who received pleasant handling (stroking) instead (Hemsworth and Barnett, 1991). Similarly, young pigs who received pleasant handling in addition to regular management showed much less fear of their familiar handler than pigs who received only routine management handling (Tanida et al., 1995). In a similar study with kittens, those handled by one person showed similar levels of fear in response to a stranger as kittens that were unhandled, while kittens who were handled by five different people showed less fear of the stranger (Collard, 1967). These studies indicate the importance of exposing animals to a variety of stimuli, in order to promote generalized acclimation, which will be discussed later in more detail in relation to dogs.

Assessment of pain is another means by which to evaluate situations that may negatively impact an animal's welfare. Since pain is a subjective experience, evaluations of the painfulness of different management factors or physical health problems often require observation of behaviors (Gregory, 2004; Mellor, 2012). To validate that certain behaviors are in fact indicative of pain, researchers may implement a sham procedure or provide analgesics and evaluate differences in behavior. For example, a study evaluating pain behaviors in dairy calves following dehorning used both approaches, and found that head shaking and ear flicking are behaviors which indicate pain resulting from dehorning (Faulkner and Weary, 2000). Another study administered a local anesthetic to healthy and lame cows to validate gait scoring and weight scale measurements as indicators of lameness (Rushen et al., 2007). There can be considerable variation in expressions of pain in different species and over different developmental stages (Gregory, 2004), all of which must be taken into account when assessing pain as a measurement of welfare.

Sickness behaviors, such as lethargy and inappetence can also be used as external identifiers of poor welfare states. In a study of dairy cows, behaviors such as reduction of lying time and lowered food consumption preceded diagnoses of uterine infection (Neave et al., 2018). Interestingly, a study in cats showed increases in sickness behaviors as a result of unusual external events, but not in relation to actual health status (Stella et al., 2011). Based on this information, it appears that sickness behaviors could indicate a variety of potential poor welfare states, but without other measurements, it may be difficult to discern the exact cause of the behaviors and thus the severity of its impact on welfare.

Stereotypic behaviors are an additional metric that can be used to assess welfare in animals. The widely accepted general definition is that these behaviors are repetitive, show little variance, and have no apparent function (Broom and Johnson, 2019). More specifically, these repetitive behaviors are believed to be the result of "frustration, repeated attempts to cope, and/or CNS [central nervous system] dysfunction" (Mason and Rushen, 2006), and are thus often used as indicators of poor welfare. A wide variety of stereotypic behaviors have been observed in animals, including weaving, cribbing, and self-mutilation in horses (Houpt and McDonell, 1993), bar-biting and head-waving in pigs (Rushen, 1985), and tongue-rolling and bar-biting in cows. In both horses and cows, stereotypic behaviors that are present in confined systems disappear when animals are housed on pasture, indicating that housing systems can affect animal welfare (Redbo, 1990; Ruet et al., 2020). However, while stereotypies may indicate welfare-compromising situations, and in some cases are clearly detrimental to the animal or its companions, in other instances they appear to assist animals in coping with sub-optimal conditions (Mason and Rushen, 2006). Therefore, simply preventing animals from preforming stereotypies without addressing their underlying causes is unlikely to meaningfully improve their welfare and may even worsen it.

In summary, there are many metrics which can be utilized in assessments of animal welfare. Often, studies will include both measures of physiology and behavior in order to gain a more complete picture of an individual's responses to potential challenges and evaluate the potential effects of management and other factors on animals' overall welfare state.

2.2 Welfare Assessment in Dogs

Many of the metrics which are used in other animals to evaluate their welfare states can also be used to evaluate the welfare of dogs. Primarily, studies using these measures have been focused on assessing the welfare of dogs in shelters or research kennels. Physiological measurements of cortisol, heart rate, overall health status, and immune function have all been utilized. Behavioral measurements including the evaluation of changes in normal behavior patterns and the performance of stereotypic behaviors have been assessed as well.

2.2.1 Physiological Measures

Validated means of measuring cortisol in dogs include via blood plasma, saliva (Beerda et al., 1996; Vincent and Michell, 1992), urine (Beerda et al., 1996; Rooney et al., 2007), feces (Schatz and Palme, 2001), and hair (Accorsi et al., 2008; Bennett and Hayssen, 2010; Bryan et al., 2013). Many studies compare cortisol concentrations between kenneled dogs and dogs living in homes, to investigate whether the kennel environment itself is stressful to dogs. For example, plasma cortisol has been shown to be significantly more concentrated in shelter dogs as compared to dogs sampled in their homes (Hennessy et al., 1997), and higher concentrations of urinary cortisol were observed in kenneled shelter dogs as compared to dogs who had been rehomed (Stephen and Ledger, 2006). Additionally, higher cortisol concentrations and lower surface temperatures have been observed in pet dogs during kenneling as compared to when they were at home (Part et al., 2014). Interestingly, some studies have also shown differential cortisol responses over time in kenneled dogs based on their previous histories (i.e. habituation to kenneling), which highlights the potential for individual variability on these metrics (Hiby et al., 2006; Rooney et al., 2007). In addition to plasma and urinary cortisol, salivary cortisol sampling is a popular method, since collection is fairly simple and non-invasive, in comparison with measures such as plasma, which requires obtaining a blood sample. However, variability between dogs and over time indicates the need for multiple measurements (Kobelt et al., 2003). Hair cortisol sampling is a newer method for evaluating these hormone concentrations in animals, and in dogs has been found to correlate well with salivary cortisol. Thus it is an additional non-invasive way of determining cortisol concentrations in dogs (Bennett and Hayssen, 2010). Hair cortisol also has the added benefit of providing a longer-term picture of stress over time, eliminating some of the need for multiple measures in studies requiring longer-term measurements of HPA activation.

Heart rate and heart rate variability are also useful physiological measures of arousal in dogs, though as mentioned previously, they cannot indicate poor welfare states on their own. Some studies have shown heart rate increases in dogs in response to stressful situations, as compared to non-stressed controls (e.g. Galosy et al., 1979). However others have not observed specificity of heart rate responses to different stimuli (e.g. Beerda et al., 1998). Heart rate variability patterns, which as mentioned previously may show clearer correlations with stress responses, have also been linked to dogs' temperaments, where calmer dogs were observed to show less variability than excitable dogs (Vincent and Leahy, 1997). Again, this emphasizes the potential for individual differences, and the need to take both context of the measurement and the dog's prior history into account when utilizing these metrics to measure welfare in dogs.

Various measures of immune function, such as amounts of neutrophils and leukocytes (types of white blood cells), have also been utilized in concert with other behavioral and physiological metrics to assess the stress responses of dogs subjected to spatial and social isolation (Beerda et al., 1999a) and air travel (Bergeron et al., 2002). Further, measures such as growth rates and weight gain, which are more typically used as metrics of production in livestock, may still be used to evaluate the health and welfare of dogs. As mentioned briefly above, the stress response prompts physiological changes which release energy and inhibit digestion. Therefore, an animal with no other medical issues who experiences visible weight loss or who struggles to gain weight may be experiencing a state of chronic stress and impaired welfare (Rooney et al., 2009). Finally, in intact dogs used for breeding, such as those in commercial breeding kennels, measures of reproductive health such as ability to conceive and litter size may also be appropriate as indicators of welfare status.

2.2.2 Behavioral Measures

In addition to physiological metrics, many behavioral metrics utilized in other animals may also be used to assess the welfare of domestic dogs. Changes in expression of normal behaviors such as grooming & play behaviors, vocalization, and posture, among others, can all potentially indicate stress and compromised welfare (Beerda et al., 1997; Protopopova, 2016; Rooney et al., 2009). For example, profound increases or decreases in activity level can represent a visible sign of anxiety in animals (Overall et al., 2001). The earlier study by Hiby and colleagues (2006) also observed that, on average, dogs with increasing cortisol showed higher activity levels than those with decreasing cortisol, although the relationship was reversed when examined within-dog, indicating a complex relationship between cortisol concentration and activity level (Hiby et al., 2006). The above study by Part and colleagues (2014) also observed that dogs spent significantly less time resting, and more time alert and moving when kenneled compared to when at home.

However, there was no relationship between these behavioral metrics and physiological metrics such as cortisol (Part et al., 2014).

Increases in self-grooming and vocalizing have also been observed in dogs subjected to spatial and social restriction (Beerda et al., 1999b; Hetts et al., 1992). It is important to note that dogs may vocalize in a variety of contexts, not all of which indicate poor welfare, and there are breed differences in vocalization frequency (Pongrácz et al., 2010), so it would be difficult to ascertain welfare state from a measurement of vocalization alone.

In addition to altered patterns of normal behaviors, the presence of abnormal behaviors or stereotypies such as circling, pacing, wall-bouncing, tail chasing, or chewing on walls may indicate compromised welfare (Stephen and Ledger, 2005). In a study of shelter and laboratory dogs in the UK, dogs who were group housed showed lower levels of non-social repetitive behaviors such as pacing and circling behaviors than dogs who were housed individually (Hubrecht et al., 1992). However, high variability in individual expression of stereotypic behaviors has been observed in dogs, and the relationship with welfare state is not entirely clear (Denham et al., 2014).

The presence of anxiety-related behaviors such as urination or defecation, trembling, paw-lifting, lip- or nose-licking, head shaking, cowering or hiding, and yawning can also be measured to assess dog welfare (Rooney et al., 2009; Sonntag and Overall, 2014). For example, a study exposing dogs to variety of potentially stressful stimuli observed a tendency for low postures and increased salivary cortisol to occur concomitantly in response to certain stimuli, such as shocks and loud noises, though there was considerable individual variation in responses (Beerda et al., 1998). Positive correlations between cortisol concentration and behaviors such as circling and urination have been observed in dogs (Beerda et al., 2000), indicating the potential for these to serve as markers of impaired welfare. However, other studies have demonstrated behavioral changes over time in response to kenneling, such as increases in grooming and decreases in locomotion and paw-lifting, though these lacked clear correlations to cortisol concentration (Rooney et al., 2007). This could in part be because a variety of situations will cause activation of the HPA axis and resulting increases in cortisol and other hormone concentrations, as previously discussed.

In conclusion, many physiological and behavioral parameters can be measured to assess the welfare of dogs. Dogs present a wide variety of individual variation on some metrics but evaluating changes within an individual and considering the context in which measurements were obtained can help researchers gain a clearer picture of the welfare implications of various situations for dogs.

2.3 Welfare of Dogs in Commercial Breeding Kennels

When discussing dogs living in commercial breeding (CB) kennels, many similarities exist between this population and shelter or laboratory dogs. All these populations live in kennel environments, and thus have similar welfare concerns. Some factors of concern to all these environments include elevated levels of noise, limited human contact, and limited environmental enrichment. Additionally, most dogs in these populations will undergo transport in a vehicle with other dogs at least once in their lives, a circumstance which has a high potential to be stressful. Finally, some factors create the potential for unique welfare challenges, including the development of behavioral problems. Important elements to consider include the role of genetic selection and screening, and early socialization, which all play a role in puppies' development. While genetics and socialization impact all dogs, they are particularly relevant in the breeding dog population, as breeders have significant control here.

2.3.1 Effects of Noise Levels on Dog Welfare

Dogs who are raised and kept for breeding stock in CB kennels will typically spend the majority of their lives living in some type of kennel environment. Many of these dogs are group housed, but some may be housed singly. Kennel environments may be indoor, outdoor, or both, and can have a variety of sizes and flooring substrates. One potential concern in any kennel environment is the noise level. High noise levels (above 100dBA) can cause hearing loss in humans with extended exposure (Centers for Disease Control and Prevention, 2019) and noise levels in a variety of kennel environments, including shelters, training centers, and laboratories have been measured to exceed this level frequently (Coppola et al., 2006a; Sales et al., 1997). Since dogs have more sensitive hearing than humans, this level of noise may be even more detrimental to them (Beerda et al., 1997; Sales et al., 1997). High levels of noise often result from barking, whining, and other vocalizations from the dogs housed in the kennel. Additionally, dog kennels are often constructed from materials such as concrete, which facilitate easy cleaning and disinfection. However, these materials do not absorb noise and may in fact amplify it. Cleaning and feeding procedures may also add to the noise, due to high-pressure sprayers, banging of food tins and scoops, and/or excitement of dogs due to this stimulation. Further, dog doors providing access to the outside portions of indoor-outdoor CB kennel runs, while likely beneficial to the dogs, are often constructed of metal or hard plastic and may produce a loud noise any time a dog passes through, adding to the general cacophony. Finally, while the ability of dogs to see each other could be valuable for their social enrichment, this stimulation can prompt dogs to bark at each other (Solarz, 1970). Currently, noise at the decibel levels commonly recorded in kennels is likely to constitute a physical stressor for dogs with the possibility of permanent hearing damage (Scheifele et al., 2012). A high level of noise in dog kennels has the potential to cause frustration in human caretakers as well (Glass and Singer, 1972). As such, noise levels in kennels are a significant welfare concern, both for dogs and their humans.

2.3.2 Welfare Implications of Limited Human Interaction

In addition to the potential welfare impacts of consistently high noise levels, many dogs raised in CB kennels may experience limited human interaction. This could happen in a very large kennel with a high number of dogs, where the size of the operation constrains time a breeder may have to spend with each individual dog. However, it could also occur in smaller kennels, when caretakers have many other responsibilities to manage.

Because biosecurity is also a concern in any type of kennel operation, some breeders may choose to limit the time they or others spend in the kennel or interacting with the dogs in order to minimize the risks of spreading disease. Human contact seems to be valuable to dogs however, and has shown some tendency to lower cortisol concentrations in shelter dogs, though the gender of the human may have an effect (Coppola et al., 2006b; Hennessy et al., 1997; Shiverdecker et al., 2013). Additionally, the presence of a familiar human caretaker during exposure to a novel environment has been shown to lessen the resulting elevation of plasma glucocorticoid concentrations in dogs (Tuber et al., 1996). Finally, just two minutes of human interaction with toys resulted in some increase in sociability towards humans in a population of small shelter dogs (Conley et al., 2014). Based on this information, it is likely in dogs' best interests to have consistent positive interactions with their caretakers, though care should be taken to ensure these interactions are safe for both parties.

2.3.3 Lack of Complexity Within the Kennel: Implications for Welfare

A further potential for poor welfare of dogs living in kennels exists in the form of the kennel environment itself, if it is perceived as barren by the dogs. Outdoor exercise and playtime have shown benefits in shelter dogs (Menor-Campos et al., 2011); therefore, providing dogs with access to the outdoors via indoor-outdoor runs and/or having play yards is likely beneficial to their welfare in most cases.

The quality of the social environment dogs experience in kennels also impacts their welfare. Dogs are social animals, so housing them in pairs or small groups, rather than alone, is likely to benefit them. In fact, group housing has been observed to increase investigative behaviors and decrease social confrontations between dogs, behavioral problems and stereotypies (Mertens and Unshelm, 1996). A recent pilot study also showed decreased barking and repetitive behaviors in some dogs when transitioned from individual to pair housing (Grigg et al., 2017).

Besides interacting with conspecifics, dogs also utilize objects they can access to entertain themselves. Within their own enclosures, dogs may make use of any available objects as targets for play, chewing, and pawing behaviors, and will carry around loose items (Hetts et al., 1992). This suggests that these types of activities are also behaviorally important to dogs, and a lack of access to appropriate items could lead to frustration or injury. The provision of toys may help promote safe play. However, adult dogs acclimate fairly quickly to new toys, so it has been recommended that toys are rotated/changed out frequently to provide novelty and encourage use (Wells, 2004). Dogs seem to show a preference for toys that can be chewed on and moved around, as opposed to very large toys or ones that are tied down (Wells, 2004). Providing adequate toys may relieve some stress and help to prevent boredom and the development of destructive or stereotypic behaviors. Welfare implications of boredom in animals have not yet been fully explored, but animals' tendencies to work to obtain stimulation imply that boredom may be a welfare concern (Meagher, 2019).

2.3.4 Genetics

In addition to the environmental factors of kennels, a dog's welfare can be profoundly affected by their genetics. For example, certain breeds such as golden retrievers are prone to hip problems which can be painful and may require surgical repair (McGreevy, 2007). Other breeds may suffer from congenital issues with the eyes (collies) or back (dachshunds) (Grandin and Deesing, 2014). Brachycephalic (short-nosed) dogs such as pugs and bulldogs have difficulty breathing which in some cases is so severe that it reduces their quality of life to the point where they are humanely euthanized (McGreevy, 2007). Clearly, inherited issues have the potential to impact functioning and welfare states, sometimes so extremely that the consequences are fatal. Additionally, some breeds, such as pointers, suffer from high reactivity levels due to breeding selections for hunting traits (Grandin and Deesing, 2014). High reactivity may cause a dog to view a wider variety of stimuli as threatening, leading to impaired welfare for an animal in situations that might otherwise be innocuous.

2.3.5 Socialization

Finally, proper socialization is important for the normal development of dogs and other animals, but it can be challenging to accomplish in a CB kennel. One very important developmental stage in a puppy's life, the socialization period, occurs from about 3-10 or 12 weeks of age (Braastad and Bakken, 2002; Scott and Fuller, 1965). This is not the only time puppies need socialization, but it is highly important, as this window of time is when puppies most effectively learn which stimuli are safe and which are not, and develop related fear responses (Scott and Fuller, 1965). This is also the period during which puppies develop social relationships with humans and other dogs (Scott and Fuller, 1965). Thus, interactions of puppies within their litters and with their dams, as well as with a variety of people are important factors for young dogs at this stage. A wider range of experiences during the socialization period can improve puppies' abilities to cope with novel challenges later in life (Braastad and Bakken, 2002). Therefore, providing puppies with a variety of non-threatening situations in which to explore stimuli they may be exposed to later in life, such as grass and unfamiliar objects such as stairs, is key during this time. In short, a puppy that does not receive adequate socialization early in its development may struggle to adapt later when placed in its new home.

2.3.6 Behavioral Problems and Transport

Several owner-report studies have noted higher rates of behavioral problems in puppies sold through pet stores and assumed to originate in high-volume commercial breeding kennels compared to puppies that came from other sources, as summarized in a review by McMillan (2017). Behavioral problems in dogs can develop from a variety of interactions between genetics and environment, and currently, it is unclear where the fearful responses observed in these studies originate.

One experience which could lead to some of the problematic fear behaviors reported in puppies from pet stores is transport. While transport is not inherently bad for welfare, it does have the potential to serve as a major stressor in animals (Broom, 2005; Fisher et al., 2008), and it is something which is common for puppies originating in CB kennels to undergo at least once in their lifetime. Generally, puppies from CB kennels will be transported after eight weeks of age to a distributor, and from there to pet stores or new owners. Retired breeding dogs may also undergo long transports for sale or rehoming (Stella et al., 2019). Several aspects of transport have the potential to challenge the welfare of transported animals. Risk areas include handling and transport conditions which may lead to stress and fear, fatigue and dehydration, and thermal or physical injury, among others (Fisher et al., 2008). The effects of transportation on stress and welfare have been studied in a variety of domesticated social species, such as pigs (e.g. Becerril-Herrera et al., 2010; Sutherland et al., 2010), cows (e.g. Earley et al., 2012; Hong et al., 2019) sheep (e.g. Wickham et al., 2012), and horses (e.g. Padalino and Raidal, 2020). In pigs, transportation has been observed to cause increases in lesions (wounds), cortisol concentrations, and white blood cell counts, and decreases in body weight (Sutherland et al., 2010). Certain studies of transportation in cows have observed increases in cortisol and white blood cell counts (Hong et al., 2019), while others have seen decreases in immunological parameters and transient decreases in weight, but no changes in cortisol (Earley et al., 2012). Studies in horses indicate that travel length and accommodations can affect the behavioral and physiological stress response (Padalino and Raidal, 2020; Tateo et al., 2012). Studies examining physiological and behavioral responses to transport in sheep and cattle have observed changes consistent with stress in blood cell variables, as well as elevated cortisol, heart rate, and core body temperature (Stockman et al., 2011; Wickham et al., 2012). Nearly all of these changes were greater in a first (naïve) transport event as compared to a habituated one, and were significantly correlated with qualitative behavioral assessment (QBA) scores, in that more animals appeared ‘anxious’ or ‘agitated’ (as opposed to ‘confident’ or ‘calm) in the naïve transport than the habituated. Also, animals who appeared behaviorally stressed also had greater physiological indications of stress (Stockman et al., 2011; Wickham et al., 2012).

These findings indicate that an animal's prior experiences affect how they perceive and react to transport.

In dogs, there have been relatively few studies on the effects of transport. A study by Bergeron and colleagues (2002) examining air transport of dogs observed changes in cortisol and white blood cells in response to both the ground and air portions of the travel that indicate these forms of transport are stressful to dogs. Another study on car transport for working dogs observed changes in heart rate and heart rate variability as well as the presence of behavioral stress indicators such as whining, lip-licking, and tremor (Skånberg et al., 2018). More study is needed in this area but based on the existing literature, it is reasonable to conclude that the experience of transport can be stressful for dogs.

In aiming to improve the welfare of dogs living in commercial breeding kennels and their puppies, there are many areas which require further research. Additionally, there is a need to find effective ways to intervene to improve welfare of dogs in CB kennels. The role of early handling as a potential means of protecting dogs from the effects of the various stressors they may encounter in kennels and later in homes requires consideration. This study therefore explores the use of early neurological stimulation (ENS) as a method to potentially increase puppies' ability to cope with stressors and therefore improve their overall welfare.

CHAPTER 3. EARLY HANDLING AND ENS

As discussed previously, dogs and puppies in CB kennels may be exposed to many potentially stressful situations common to kennel environments, including high noise levels, limited human interaction, lack of environmental enrichment, and social isolation (Taylor and Mills, 2007). Additionally, dogs from CB kennels are likely to undergo transport at least once in their lives, which may be a stressful experience (Broom, 2005; Fisher et al., 2008). If the kennel or transport situations are perceived as stressful by the animal, a stress response will be evoked, which will vary in type and intensity according to the animal's perception of the stressor, and this response may have widespread physical and behavioral effects on the individual (Charmandari et al., 2005; Moberg, 2000), as previously discussed. It is also well established that early experiences have the potential to alter development and create neurological and behavioral changes in an individual.

3.1 The Importance of Early Life Experiences in Influencing Animals' Stress Responses

Adverse experiences can influence an organism at any age or developmental stage but may have an especially profound effect during early life. Not only is early life a period of heightened development, it is believed early life experiences inform the programming of certain functions, such as the stress response (Maras and Baram, 2015; Tarry-Adkins and Ozanne, 2011). In other words, an organism may use clues present in the early environment to predict what the future environment will be and adapt accordingly (Boyce and Ellis, 2005). In the case of early life stressors, differential effects have been observed, with some experiences leading to greater vulnerability while others lead to greater resilience to adversity experienced later in life. Many factors likely affect the ultimate outcome, and exactly which mechanisms are responsible is still unclear (Hartmann and Schmidt, 2019). Hypotheses which have been proposed to make sense of the existing data include the cumulative and match/mismatch hypotheses. The cumulative hypothesis states that negative effects of stress accumulate or add up over time, leading to an increased allostatic load and a greater potential for detrimental effects (Nederhof and Schmidt, 2012). On the other hand, the match/mismatch hypothesis states that resilient outcomes are more likely when the early environment is similar to the later environment, but when the two are mismatched, there is an increased risk of detrimental effects instead (Schmidt, 2011). Evidence for the cumulative hypothesis has been observed, for example in a study by Uchida and colleagues (2010), which found that rats who were subjected to daily maternal separation periods in early life had higher post-restraint test corticosterone concentrations. There is also evidence to support the match-mismatch hypothesis. One such example is a study by Santarelli and colleagues (2017), where male mice subjected to an impoverished environment in both early life and adult life, as well as mice with matched stimulating environments showed lower levels of anxiety in a dark-light box test, as compared to mice with mismatched environments. An integrated model of both hypotheses which additionally accounts for individual sensitivity has also been proposed (Nederhof and Schmidt, 2012).

3.2 Effects of Early Handling on Animal Development and Welfare

Like many great scientific discoveries, the idea that early life stressors may provide animals an "inoculation effect" against later stressors seems to have been discovered almost by accident. Seymour Levine, a pioneer of early development studies, in an attempt to test the effects of early life trauma in rat pups using shock, found that both handled 'controls' (placed in shock pen but not shocked) and shocked pups showed *improved* performance on later tests of avoidance learning and extinction (Levine et al., 1956). The curious discovery that early stress stimulations were not universally negative and might attenuate later stress responses led to an explosion of study in the area. Effects of early handling have been explored in several species, including rats (e.g. Castelli et al., 2020; Levine et al., 1967; Plotsky and Meaney, 1993), mice (e.g. Tremml et al., 2002), non-human primates (e.g. Parker et al., 2007, 2005, 2004), cats (e.g. McCune, 1995; Meier, 1961), and pigs (e.g. Zupan et al., 2016). Handling interventions and brief maternal separations are common methods employed as stressors in early life. Various protocols for early handling and maternal separation to facilitate stress inoculation have been developed and are commonly referred to as early neurological stimulation or ENS. Many studies have demonstrated changes which seem to indicate improved modulation of the stress response in handled animals as compared to unhandled controls (for a review, see Raineki et al., 2014). In other words, ENS is the process of applying gentle stressors to an animal during its critical early developmental period as a way of stimulating the HPA axis, with the goal of ultimately shaping the animal's behavioral and neurological development to enable better handling of later stressors. Studies examining ENS were initially carried out in rodents due to their ability to be a good model for many other mammals, and the large numbers readily available living in research housing.

There is some evidence that the removal of neonatal animals leads to changes in maternal behavior, which may be responsible for the resulting benefits of ENS on stress responses. An early study in rats observed increased licking and grooming, and more active (vs. passive) nursing postures in dams with handled pups as compared to dams with unhandled pups (Liu et al., 1997). They also demonstrated that dams with naturally high levels of licking, grooming, and active nursing produced pups with similarly reduced HPA reactivity to stress as handled rat pups (Liu et al., 1997). Further, pups from dams with these increased measures of maternal care were also more explorative in an open field test than pups from dams with lower levels of maternal care (Caldji et al., 1998). However, the relationship appears to be non-linear and may involve other factors, such as environmental stress level (Macrì and Würbel, 2007, 2006).

Another early study in rats observed that a reduction in temperature was both necessary and sufficient to produce effects analogous to those produced via early handling, as measured by ascorbic acid depletion (Schaefer, 1963). This idea has not yet been fully explored however, and based on the complex relationship seen with maternal care, it is possible that temperature may constitute one of many contributing factors to the effects of ENS.

3.3 ENS in Rodents

Early studies of ENS in rodents utilized treatments where pups were removed from their nests daily. For example, a study by Levine and colleagues (1967) demonstrated that rat pups who received brief daily handling and separation from their dams from day 1-20 of life showed lower levels of behavioral and physiological reactivity on an open-field test than undisturbed pups.

Further studies used similar protocols of early stress inoculation, such as Plotsky and Meany (1993), who applied ENS to male rat pups via gentle daily removal of the pups from their home cages to a separate container for a 15-minute separation from their dams on days 2-14 of life. Rats were tested using a 20-minute restraint stressor at 3-4 months of age. Handled animals showed lower concentrations of the rodent stress hormone corticosterone in response to stress and a quicker return to baseline, as compared to non-handled pups or those who were maternally separated for 180 minutes (Plotsky and Meaney, 1993).

Nunez and colleagues (1996) also applied a nearly identical protocol of ENS to rat pups daily from days 1-22 of life. Rats were tested at various points between 4 and 11 months of age using tests of their exploration new areas, weighing, punished lick suppression, and an open field test. This study found that handled animals showed less resistance to capture and/or handling, and lower concentrations of stress hormones versus untreated control animals. No significant differences were observed between groups in exploratory behavior (Núñez et al., 1996).

Effects of neonatal handling have not always been straightforward, however. Researchers employing a 20-minute daily handling and removal of pups from their dams over the first three weeks of life observed some mediating effects of handling on corticosterone responses to the stress of an elevated plus-maze, but found no significant effects on rats' behavior in the maze, or on several hippocampal parameters (Durand et al., 1998). Further, some studies have indicated that early handling may lead to detrimental outcomes on certain behavioral and physiological parameters, such as social behavior (Karkow and Lucion, 2013) and reproduction (Raineki et al., 2013), as well as leading to alterations in feeding habits (Silveira et al., 2008).

Despite the many various outcomes observed over time, recent studies have continued to demonstrate what appear to be beneficial effects of early handling. For example, a study conducted by Castelli and colleagues (2020), found that early handling as a 15-minute separation from days 2-21 of life (analogous to earlier studies) prompted increases in maternal care and offset the detrimental effects on pups of administering prenatal glucocorticoid to dams. Pups who were handled showed increased locomotor activity, less immobility and more swimming in a forced swim test, as well as lowered concentrations of plasma corticosterone in both shock exposure and non-shocked tests, as compared to unhandled pups (Castelli et al., 2020).

3.4 ENS in Other Species

Similar ENS work has also been carried out in non-human primates (Levine and Mody, 2003; Parker et al., 2007, 2005, 2004) domestic cats (McCune, 1995; Meier, 1961), and pigs (Zupan et al., 2016). Studies of ENS in squirrel monkeys have shown the potential for lasting effects of early life stimulation (Parker et al., 2007), while studies in cats have highlighted the interactions of genetics and experience in shaping animals' ultimate responses to stressors (McCune, 1995).

Parker and colleagues conducted a longitudinal examination of the effects of early stress inoculation in squirrel monkeys. The first part of the study found decreases in plasma concentrations of stress hormones (ACTH & cortisol) and maternal clinging, as well as increased explorative and ingestive behaviors in nine month old squirrel monkeys who had received an intermittent stress inoculation (1 hour social separation) once a week from weeks 17-27 of life (as compared to uninoculated controls), in two different novel environment situations (Parker et al., 2004). A second study on the same animals at one and a half years old showed that stress-inoculated animals still had lower baseline concentrations of cortisol and significantly lower baseline concentrations of ACTH, in addition to showing increased cognitive control of behavior on a marshmallow retrieval test, as compared to controls (Parker et al., 2005). Finally, a third study of the same monkeys at two and a half years of age continued to show greater novelty-seeking explorative behavior in stress-inoculated monkeys, as compared to controls, but no significant differences in cortisol concentrations were observed in any of the animals (Parker et al., 2007). Other studies in squirrel monkeys have shown similar results, with intermittent four to six hour social separations prior to weaning leading to decreased cortisol and vocalization responses to later social separations at two and three years of age (Levine and Mody, 2003).

In domestic cats, kittens who received five hours of gentle handling (petting and soft talking) weekly from 2-12 weeks of age were tested at one year old and found to be friendlier (as measured by latency to approach, body language, and vocalizations, among other things) towards both familiar and unfamiliar people than unhandled controls (McCune, 1995). McCune observed that the extent of the differences in friendliness created by handling were affected by genetic factors, namely, the friendliness of the father (1995). They also noted that responses to a novel object varied almost entirely due to father-friendliness; handling had little effect on this parameter (McCune, 1995). Another study in Siamese kittens applied gentle handling (petting) for 10 minutes twice daily and observed indications of accelerated development such as earlier opening of eyes, venturing from the nesting box, and development of coloration points in handled kittens, as compared to controls (Meier, 1961).

A recent study in pigs also observed that early handling increased certain types of play behavior and exploration in piglets where either half or all the litter was handled, as compared to piglets from non-handled litters (Zupan et al., 2016). Handled piglets also vocalized less than non-handled piglets during an isolation test, and more readily accepted stroking by a human (de

Oliveira et al., 2015). Handled piglets received two minutes of handling per day for 23 sessions between five days of age and weaning. Results varied based on the type of exploration and play, as well as the sex and body weight of the piglet, so the authors suggest that genetics could influence the ultimate effects of the handling treatment (Zupan et al., 2016).

In summary, early handling of animals has been explored in several species, with indications that certain handling procedures may provide animals with a greater resiliency when face with stressors later in life. Various forms of ENS have been tested, with many experiments utilizing brief maternal separation and handling procedures. Interventions have often resulted in reduced physiological and behavioral indications of stress for animals facing potentially stressful situations, as compared to unhandled controls. These results suggest that ENS has important implications for young animals' development as well as for their abilities to cope with stressors that ultimately influence their welfare. The extent to which these impacts hold for dogs therefore warrants consideration.

CHAPTER 4. EFFECTS OF EARLY NEUROLOGICAL STIMULATION ON PUPPIES RAISED IN COMMERCIAL BREEDING KENNELS

The effects of ENS have been demonstrated with non-human primates (Levine and Mody, 2003; Parker et al., 2007, 2005, 2004), rodents (Durand et al., 1998; Levine et al., 1967; Núñez et al., 1996; Plotsky and Meaney, 1993), and cats (McCune, 1995; Meier, 1961). Relatively little has been published on this type of early handling with dogs.

4.1 ENS in puppies

Early studies of the effects of ENS in canines were conducted by researchers Fox and Stelzner (1966). They employed a method of ENS that entailed an hour of various stimulations, applied daily from the first day to week five of life, to three treatment groups: handled puppies, controls, and partially socially isolated puppies (Fox and Stelzner, 1966). Puppies were then tested at five weeks of age using a 15-minute arena test with objects. Puppies' specific interactions with each stimulus were measured, as well as their non-specific exploratory behavior, random activities, and distress vocalizations. Puppies' body weights, brain weights and reflexes were also measured at three and four weeks of age. This study found that handled puppies were generally hyperactive, highly exploratory, very social to humans, and dominant socially with their peers (Fox and Stelzner, 1966). Handled puppies were also better at problem-solving barrier tasks than unhandled or partially socially isolated puppies, but showed the most distress vocalizing after the handler removed objects from the arena, in contrast to control puppies who did not seem to mind the removal of objects but were very emotionally aroused when first placed in the arena (Fox and Stelzner, 1966). Non-specific exploratory activity was found to be highest in handled puppies when objects were present in the arena, while in control puppies, levels of non-specific exploratory activity were highest when the arena was empty (Fox and Stelzner, 1966). There was a trend of better standing and walking coordination at four weeks of age in handled puppies but the difference was non-significant, and there were no significant differences between treatment groups in body weight, brain weight, or reflexes (Fox and Stelzner, 1966).

The effects of ENS in dogs have not yet been fully explored, but a study by Gazzano and colleagues (2008) suggests that its effects may be more apparent when puppies are raised in a relatively barren environment. This study used seven litters of puppies, four of which (23 puppies) were raised in professional breeding kennels (PBKs) and three (20 puppies) raised in homes. Their ENS "consisted of a daily 5-min session of very gentle tactile stimulation [massage] involving the animal's entire body, held alternately in the prone and the supine position", and was applied from days 3-21 of life (Gazzano et al., 2008). Puppies were tested at eight weeks of age using a three-minute isolation test and a five-minute arena test. During the isolation test, researchers measured puppies' vocalizations and their exploratory activity. Heart rates were also measured directly before and after isolation. For the arena test, they measured the amount of time puppies spent in the first square in which they were placed, the number of entrances into and time spent in a central circle which contained a person sitting in a neutral position, the number of squares the puppy crossed, and the number of objects investigated and the time spent exploring the objects. Latency to first yelp was observed to be longest in handled puppies raised in a PBK. Non-handled puppies from a PBK also took longer to vocalize than handled or non-handled puppies raised in a home environment. There was no significant difference in latency to vocalize between handled and non-handled puppies raised in a home. Duration of vocalizations was observed to be shorter in PBK puppies than puppies raised in a home. No significant differences were seen in the duration of vocalizations between handled and non-handled puppies who were raised in the same environment. Handled puppies from both environments spent more time in locomotor exploratory activity than non-handled puppies from the same environment. There was no significant difference between environments when treatment was the same. No significant heart rate differences were observed between the four groups. There were also no significant differences between groups on any of the arena parameters measured (Gazzano et al., 2008).

Further interest in puppy ENS was sparked by a review of a protocol developed for military working dogs (MWDs) called "Bio Sensor" (Battaglia, 2009). The Bio Sensor program was intended to enhance puppies' ability to perform as MWDs and included six stimulation exercises of which five are described in Battaglia's review. The five exercises described are:

1. "Tactile stimulation - holding the pup in 1 hand, the handler gently stimulates (tickles) the pup between the toes on any one foot using a Q-tip." (Battaglia, 2009).
2. "Head held erect - using both hands, the pup is held perpendicular to the ground, (straight up), so that its head is directly above its tail." (Battaglia, 2009).

3. “Head pointed down - holding the pup firmly with both hands the head is reversed and is pointed downward so that it is pointing toward the ground.” (Battaglia, 2009).
4. “Supine position - hold the pup so that its back is resting in the palm of both hands with its muzzle facing the ceiling.” (Battaglia, 2009).
5. “Thermal stimulation - use a damp towel (wash cloth) that has been cooled in a refrigerator for at least 5 minutes. Place the pup with all 4 feet down on the towel. Do not restrain it from moving.” (Battaglia, 2009).

According to Battaglia’s 2009 review, these exercises were applied to puppies from days 3-16 of life and each exercise was given for 3-5 seconds. Battaglia interviewed the former director of the Bio Sensor program, as well as one of their geneticists, and reported their claims that ENS enhanced dogs’ cardiovascular and adrenal performance, and increased their stress tolerance and disease resistance (Battaglia, 2009). However, it is not clear from the report what specific metrics were used to make these determinations.

The Bio Sensor protocol was further explored by researchers Schoon and Berntsen (2011), who applied the five Bio Sensor stimulations Battaglia described to a cohort of puppies who were being raised to become mine detection dogs. Exercises were applied within the first three weeks of life. A handling control group was also used, in which puppies were simply held for the duration of the ENS exercises to control for the effect of human contact (Schoon and Berntsen, 2011). Puppies’ developmental parameters were measured, including eye opening, tail wagging, and walking, among others. Puppies’ social parameters were also measured, including such metrics as mounting, barking, and playing, among other things. Puppies were reportedly tested by week 10 on a variety of tests, including their environmental skills and search behavior, and their reactions to a novel person and objects. This study found no significant differences between puppies who received ENS exercises and handled controls on any of the developmental parameters measured. Additionally, no significant differences were observed between treatment groups regarding performance on a standard mine dog puppy test, further training, nor final deployment. The authors attributed the lack of effects of ENS on puppies in this study in part to the rich socialization program already in place for them. This socialization program spanned the first 10 weeks of life and included exposure to radio, playtime with toys, handling beginning at three weeks of age, exposure to a variety of people and the outdoors, introduction to a variety of environments including an obstacle course, simple search exercises, and exposure to riding in a vehicle (Schoon and Berntsen, 2011).

In summary, ENS has been explored in a variety of species, and a variety of ENS interventions have been employed, with indications that it could be a promising way to reduce an animal's negative stress reactions. In dogs, some studies have shown benefits while others have not demonstrated any effects of treatment, though the differences in the types of protocols employed and the environments utilized may be responsible for some of this disagreement. Given the inconsistencies in findings to date and the small number of studies that exist, the efficacy of ENS as a potential intervention to improve welfare of puppies in commercial breeding kennels warrants further investigation. The objective of the current study was therefore to determine if providing ENS to puppies in CB kennels changes their health and/or behavioral responses to stressors in ways that could improve their welfare. The hypotheses were that puppies who received ENS would show differences in their physical health metrics (e.g. less illness, increased weight) and in their fear and exploratory behaviors (e.g. more affiliative towards strangers, more exploration of a novel area when alone) as compared to matched controls.

4.2 Subjects and Facilities

This study followed puppies through transport, and therefore included breeders who already worked with distributors on shipping dogs; to be included in the study, both the breeders and distributors had to be located in areas that were accessible to the researchers. Three USDA licensed, Amish-owned CB kennels, located in Ohio, USA, qualified for the study and volunteered to participate. Two kennels were originally selected from the three that qualified, based on the number of expected litters, and a single kennel was ultimately utilized after the discovery of illness in one of the two kennels. Puppies (n = 76; 40 males, 36 females) born between July 29 and September 9, 2019, representing 16 litters and six different breeds (**Table 1**), were included in this study. These puppies comprised two cohorts, consisting of litters born within about one week of each other that were shipped to a distributor at the same time. The first five litters comprised cohort one, and the last 11 litters comprised cohort two. Puppies born by cesarean section or those who were cross-fostered were excluded from the study population. The procedures outlined in this study were approved by the Purdue University Institutional Animal care and Use Committee.

Table 1: Breeds represented, and number of puppies per breed and treatment group.

Breed	Number of Puppies	Control	ENS	Held
Bichon/Toy Poodle cross	8	3	2	3
Miniature Pinscher	13	4	5	4
Miniature Schnauzer	16	6	5	5
Pomeranian	15	6	4	5
Bichon/Shih Tzu cross	11	3	4	4
Toy Poodle	13	3	5	5

4.3 Handling Procedures

All puppies were assigned to one of three treatment groups (ENS, held, and control) using a balanced, random assignment (random UX app for Android). All puppies were marked for identification by researcher 1 (GB) along the back using non-toxic marking pens (Mosaiz, Amazon) beginning on day three of life and continuing every handling day thereafter. Some dams cleaned their puppies too much for this marking to be effective, so those puppies were marked in the armpits with a Sharpie marker instead. Breeders were instructed to refresh markings only if necessary, and to disturb puppies as little as possible when doing so, preferably combining the marking with other routine kennel procedures which required handling of puppies (e.g. cleaning pens). Microchips were not implanted in puppies less than 6-7 weeks of age, consistent with normal practices in the breeding kennel.

Handling began on day three postpartum and was carried out for 21 days. In order to minimize stress on dams, all puppies were retrieved from the litter by a known handler and then handed to researcher 1, who applied the treatments described below. On two days, a known handler was not available to assist the researchers; on these two days, researcher 1 retrieved the puppies herself and applied the handling treatments. Treatments were carried out daily Monday through Saturday. Researchers were unable to handle puppies on Sundays due to facility restrictions. A smartphone timer (Google clock app) was used to time the handling treatments to ensure they were all approximately 30 seconds long.

Puppy order for retrieval and handling within litter was randomly assigned and varied each day. Due to difficulties with identification, one litter of nine puppies was simply retrieved randomly without assignment, and each puppy was identified and recorded as they were retrieved, in order to avoid extra handling that would have been necessary to retrieve them in a specific order. Litters themselves were handled in birth order.

In order to protect the puppies and other dogs on-site from disease transmission, strict biosecurity procedures were implemented. The procedures required all research personnel to wash or sanitize hands and don protective boot covers or facility-specific shoes which were disinfected using Rescue™ cleaner (Virox Animal Health) prior to or upon entry to the kennel. The known handler who retrieved puppies for the researchers wore a disposable protective gown and gloves to retrieve puppies for handling and changed the gloves between litters. Additionally, researcher 1 washed and sanitized her hands before the handling sessions, wore a disposable scrub top or gown and gloves during handling, and sanitized hands and changed gloves between litters. Finally, all items which contacted puppies or were utilized during the handling session (markers, scale, smartphone) were disinfected between litters with Rescue™.

4.3.1 ENS Treatment Group

The ENS group received five exercises on each treatment day based on the "Bio Sensor" protocol as described in Battaglia (2009), and outlined in the previous chapter. Each exercise was applied for five seconds, in the following order: tactile stimulation (**Figure 1**), head erect (**Figure 2**), head down (**Figure 3**), supine (**Figure 4**), and thermal stimulation (**Figure 5**).

Figure 1: Tactile stimulation – the puppy's toes were tickled gently with a Q-tip.

Figure 2: Head held erect – the puppy was held so the head is directly above the tail.

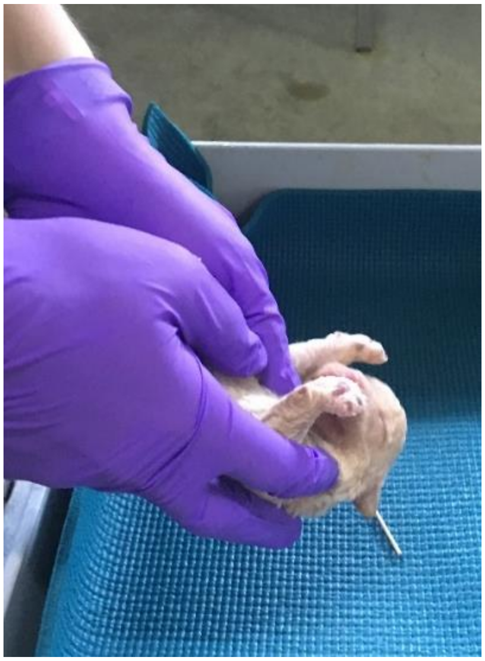

Figure 3: Head pointed down – the puppy was held so the tail is above the head (this puppy is moving into or out of the final position).

Figure 4: Supine position – the puppy was held on their back.

Figure 5: Thermal stimulation – the puppy was placed on a cool, damp washcloth.

4.3.2 Held Treatment Group

Puppies in the held treatment group were held in a sternal position (**Figure 6**) for 30 seconds to approximate the amount of time ENS puppies were receiving exercises. This allowed for evaluation of the effects of ENS exercises beyond the effects of removal from the nest and human handling.

Figure 6: Held – the puppy was held in a sternal position.

4.3.3 Control Group

Control group puppies were marked for identification each treatment day while being held by the known handler. They were weighed weekly and assessed for health (detailed below), but otherwise received no additional handling besides that in place for regular care and cleaning by the breeder.

4.4 Physical Health Assessments

All puppies were weighed within a day of birth by the breeder. Subsequently, the researchers weighed puppies and assessed their physical health weekly, beginning on the seventh day postpartum and continuing through eight weeks of age. Puppies were given a body condition score (BCS) using a novel 1-3 scale created for this study. This scale measured body condition for puppies as thin (1), normal (2), or obese (3). It was developed because no BCS system is currently established for use in puppies. Puppies' cleanliness was also scored using a 0-4 scale which denotes the percentage of the body covered in debris (0 = 0%: clean/no debris, 1 = 1-25%, 2 = 26-50% , 3

= 51-75%, 4 = > 76%) (Bauer et al., 2017). Nasal and ocular discharge, sneezing, coughing, symptoms of upper respiratory infection, missing fur or poor coat, wounds, sores, or lesions, diarrhea, and pyoderma (skin infection), were all recorded as present or absent (Y/N).

Researchers conducted all puppy health assessments for cohort one, and through weeks 3-5 for cohort two, after which they were done by the breeder and known handler using scoring sheets provided by the researchers. Inter-rater reliability training was conducted with the breeder and known handler as follows: researchers first scored puppies and explained the process while the breeder and known handler observed; breeder, known handler, and researchers scored puppies together with breeder and handler stating their scores first. This process was repeated until there was at least 90% agreement between the researchers and the breeder for 10 puppies. After training was complete, the breeder and known handler scored puppies on their own once the researchers departed. On health assessment days, researcher 1 weighed and assessed each puppy following handling, or marking for controls. Researcher 2 (TS) verified the assessments and recorded all physical health metrics. Puppy health assessments were repeated on all puppies at eight weeks of age by researcher 3 (AR) following behavioral testing pre- and post-transport.

4.5 Testing Procedures

Puppies in this study underwent transport to a distributor at eight weeks of age. Puppies were assessed in their home kennels 2-3 days prior to transport and again at the distributor 48 hours after transport. Testing protocols at the breeder’s kennel and at the distributor were kept as identical as possible to allow for comparison. All puppies were collared by the breeder (who was male) or a male researcher for visual identification and given a 30-minute acclimation period between collaring and testing. Collar placement and the acclimation period were video recorded for possible analysis but are not included in this study. Puppies were given a random assignment within litter for individual testing. All testing was video recorded. Stranger approach tests and health assessments were scored live by researcher 3 and the scores were recorded immediately. Isolation tests were scored later from video by researcher 1. For scent minimization and biosecurity purposes, the isolation pen was spot cleaned in between puppies if soiled, and fully disinfected between litters, using towels and Rescue ™ cleaner (Virox Animal Health).

4.5.1 Stranger Approach Tests

All stranger approach tests were conducted using an unfamiliar person (researcher 3) who was blinded to the treatment group assignment for each puppy. Following the 30-minute collar acclimation period, a one-step stranger approach test was conducted using a modified version of the Field Instantaneous Dog Observation (FIDO) tool (Bauer et al., 2017). This modified one-step test was conducted on all puppies while in their home pens or distributor pens with their littermates and will be referred to as the group RYG test throughout the remainder of the text. The unfamiliar person (researcher 3) approached each pen door, stood quietly directly in front of the pen, turned to the side to avoid direct eye contact with the puppies, and extended a hand towards the pen. She immediately scored the response of the puppies as red (R), yellow (Y), or green (G). Red responses indicate fear and include defensive behaviors such as low postures, freezing, and running away, as well as offensive fear-based behaviors such as barking and lunging. Green responses include relaxed body language, soliciting attention from, or remaining undisturbed by, the unfamiliar person. Finally, yellow responses are those which are ambivalent (e.g., approach and avoid) or a response that was not clearly red or green (Bauer et al., 2017). RYG scores were reported verbally by researcher 3 and recorded by one of the other researchers.

Puppies were given a three-minute break between the end of stranger approach scoring and the beginning of individual testing. Each puppy in its turn was retrieved from the litter by a familiar handler (at the breeder’s kennel), or by a researcher (at the distributor) and individually placed into a 12-ft. circumference (six-24 in. panels) isolation pen (Precision Pet Products) with rubber mat flooring (Rubber-Cal Armor-Lock 3/8 in. x 20 in. x 20 in. Black Interlocking Rubber Tiles). The puppy was then immediately scored by researcher 3 using a four-step stranger approach test, adapted from the four-step FIDO test outlined by Stella and colleagues (2019). The adapted test scored puppies on three levels of stranger approach, as well as their willingness to take treats, as a proxy measure of their distress level. It will be referred to as the RYG+ test (RYG plus treats) throughout the remainder of the text. The procedures included:

1. The unfamiliar person approached the pen door and squatted directly in front of the pen, turned to the side and avoiding direct eye contact. The puppy was scored as red yellow, or green (Approach RYG) as described above.

2. The unfamiliar person tossed a treat to the puppy in the pen, maintaining side orientation and avoiding direct eye contact. Whether or not the puppy ate the treat was recorded (Y/N) (Approach Treat).
3. The unfamiliar person remained squatting, maintaining side orientation and avoiding direct eye contact, and opened the door of the pen. The puppy was again scored as red, yellow, or green (Open RYG). The unfamiliar person then offered a food treat directly from their hand to the puppy while maintaining the same orientation and avoiding direct eye contact. Whether or not the puppy ate the treat from the hand was recorded (Open Treat).
4. The unfamiliar person reached out and attempted to touch the puppy (e.g. under the chin, on the chest, on the side of the neck) maintaining side orientation and avoiding direct eye contact. The puppy was again scored as red, yellow, or green (Reach RYG), and whether the puppy allowed the researcher to touch them was recorded (Y/N) (TOUCH). The unfamiliar person offered a final food treat directly from their hand to the puppy while maintaining the same orientation and avoiding direct eye contact. Whether or not the puppy ate the treat from the hand was again recorded (Reach Treat).

4.5.2 Isolation Test

Immediately following completion of the RYG+ test, researcher 3 picked up any uneaten treats, and all researchers walked out of sight of the puppy, leaving it alone for a three-minute isolation test, which was timed using a stopwatch and video recorded for later scoring. Behaviors scored from video included exploratory and non-exploratory locomotion, stationary behaviors such as sitting or standing, self-grooming, shaking head or body, escape attempts, vocalizations, eliminations, and overall postures (**Table 2**). When three minutes had elapsed, researcher 3 returned to the isolation pen and performed another RYG+ test on the puppy. Upon conclusion of the second RYG+ test, researcher 3 proceeded with the puppy health assessment as outlined previously, and then returned the puppy to the known handler. The known handler returned the puppy to its home pen and retrieved the next puppy for testing.

Video coding was conducted using the Behavioral Observation Research Interactive Software (BORIS) version 7.9.7 (Friard and Gamba, 2016). Table 2 lists the behavioral ethogram used for video coding. Researcher 1 coded all videos. Six videos had to be dropped due to interruptions during the testing process, leaving 114 coded videos. State behaviors included durations and frequencies, while point events included only frequencies. Posture (low, neutral, high) was recorded as a modifier for each behavior observed.

Table 2: Ethogram used for Isolation Video Scoring

Category	**Behavior**	**Description**
Locomotory behaviors (States)	Exploratory locomotion	Puppy engaged in motor activity (e.g., walking, trotting, running) involved in targeted investigation of the environment (sniffing, pawing, licking, etc.) (adapted from Gazzano et al., 2008 & Scaglia et al., 2013)
	Non-exploratory locomotion	Puppy engaged in motor activity (e.g., walking, trotting, running) without exploration of the environment. (adapted from Scaglia et al., 2013)
	Repetitive behaviors	Movement repeated along the same path (imaginary line, along the fence, in a circle), or any stereotypic behaviors (repeated bouncing off pen walls, jumping up and down on the same spot with 2 or 4 legs in the air, pivoting on hind legs) (adapted from Denham et al., 2014; Lefebvre et al., 2010)
Stationary Behaviors	Sit (State)	"Front legs straight, rear end lowered, and resting on "hocks" and perineum" or floor (adapted from Flint et al., 2018; Overall, 2014)
	Stand (State)	Puppy is in a upright position supported by 3 or 4 legs (adapted from Lefebvre et al., 2010; Overall, 2014)
	Lie down (State)	Puppy's body is in contact with the ground, not supported by its legs. Head may be lifted or resting on the ground, and the eyes may be open or closed. (adapted from Flint et al., 2018; Lefebvre et al., 2010)
	Stationary exploration (State)	Targeted investigation of the environment (sniffing, pawing, licking, etc.) while in a stationary position (sit, stand, lie).
	Self-grooming (State)	"Action of cleaning of the body surface by licking, nibbling, picking, rubbing, scratching, and so on directed toward the animal's body" (Scaglia et al., 2013)
	Shaking head/body (Point)	Rotation of the head, or of the entire body beginning with the head and moving towards the tail. (Overall, 2014)
	Escape attempt (State)	Puppy attempts to leave test pen by pawing at pen walls, sticking nose through pen bars, or biting pen bars. (adapted from Mugenda, 2018)

Table 2 continued

Vocalizations (Points)	Any vocalization e.g. bark, growl, howl, whine	Bark: "Sharp vocalization, often loud and repetitive" Growl: "Low-pitched grumble, with or without exposed teeth" Howl: "Low pitched, long duration vocalization" Whine: "High-pitched vocalization" (Flint et al., 2018)
Eliminations (Points)	Urination	Puppy expels urine from the body
	Defecation	Puppy expels feces from the body
Posture (States-modification)	High	"the breed specific posture as shown by dogs under neutral conditions, but in addition, the tail is positioned higher, or the position of the head is elevated and the ears are pointed forwards, or the animal is standing extremely erect" (Beerda et al., 1998)
	Neutral	"the breed specific posture shown by dogs under neutral conditions" (Beerda et al., 1998)
	Low	A dog shows any or all of the following: a lowered tail position (as compared to a neutral posture, may be curled forward between hind legs), "a backward positioning of the ears", and/or bent legs. (Beerda et al., 1998)
Behavioral Category (States)	Red	"Fearful body language (e.g. ears back, whale eye, scanning, tail tuck, low and back posture); Flight; Fight (e.g. barking, lunging, growling, hard & forward body language); Frozen or catatonic; Stereotypic behaviors Note if aggression was seen. (Bauer et al., 2017)
	Yellow	"Ambivalent body language (e.g. body language/postures are a mix of green & red); Ambivalent approach (e.g. approach and avoid); Ambivalent behaviors (e.g. behaviors are a mix of green & red) Note: clearly not red or green" (Bauer et al., 2017)
	Green	"Relaxed body language (e.g. soft, loose, wiggle, neutral eyes/ears/posture); Affiliative approach; Solicits attention (e.g. scrabbling at cage door or attempting to sniff/lick observer); Neutral (undisturbed from behavior occurring prior to observer approach, e.g. eating/ drinking, play, rest) Note frantic/overstimulated or stereotypic behaviors. (Bauer et al., 2017)

4.6 Data Analysis

Initial exploratory analysis was performed using descriptive statistics and graphic visualizations were utilized to guide further statistical analysis. Means, standard errors of the means, percentages, chi-square tests, and basic graphs for visualization were created in Excel. Remaining analyses were performed in RStudio version 4.0.2 (RStudio Team, 2020), utilizing the "nlme", "lme4", "car", and "DescTools" packages.

4.6.1 Physical Health

Descriptive statistics were utilized to determine the percentage of puppies from each treatment group that showed signs of any health problem over the first eight weeks of life (i.e. at the breeder) and prior to the application of stressors (i.e. testing and transport to the distributor). Descriptive statistics are also presented for puppies that showed a health problem at either the breeder's kennel (eight weeks of age) or the distributor (i.e. after transport stressor) and to show the percentages of puppies in each cleanliness category over the first eight weeks of life, as well as on the testing days at the breeder's kennel and at the distributor.

Weight and weight gain were assessed for the first eight weeks and for the testing days at the breeder's kennel and at the distributor using separate general linear mixed effects models (GLMEs). Treatment group (ENS, held, control), timepoint (week), sex, and breed were included in the model as fixed effects. All relevant interaction effects were also included. Cohort, litter, and puppy ID were included as nested random factors to account for non-independence of puppies across measurements as well as litter and cohort effects. Model residuals were checked for normality and homoscedasticity, as per the assumptions of a GLME. A backward stepwise approach was used to remove interactions and factors and increase model fit, assessed via Akaike's information criterion (AIC) values. Model fit was evaluated using maximum likelihood (ML) and test statistics (χ^2 and p) were extracted from the best fitting model using restricted maximum likelihood (REML) and a Wald's test. Results for weight and weight gain during the transport and testing week are listed in Appendix A.

4.6.2 Stranger Approach Tests

Puppies' responses to the group RYG at the breeder's kennel were evaluated to provide a baseline measure of response to stranger approach for each puppy, before any stressors were applied. Percentages of puppies scored as red, yellow, and green were calculated for each treatment group, and a chi-square test was used to compare numbers of green puppies in each group with what would be expected by chance (i.e. 33%). Chi-square tests were unsuitable for red and yellow, as only one red and six yellow dogs were observed.

To evaluate whether treatment group affected puppies' responses to the initial RYG+ test, percentages of red, yellow, and green for each treatment group at each step of the test were compared using paired t-tests. Additionally, each step of all RYG+ tests was assessed via separate binomial generalized linear mixed effects models (GLMMEs) using a binomial distribution and a log-it link function. For the RYG steps, puppies that were recorded as red or yellow were analyzed together in order to form two binomial categories. Yellow and red were grouped and scored as 0, and green was scored as 1. For the treat and touch steps, yes or no were the binomial responses. Each model contained treatment group and time point (pre- or post-isolation) as fixed effects as well as the interaction between the two. Cohort, litter, and puppy ID were also included in these models as nested random effects. Normality and homoscedasticity of model residuals were evaluated, and a backward stepwise model selection approach using ML and AIC values was again used to indicate the best fitting model. Interactions and explanatory variables were sequentially removed to increase model fit. The best fitting model was run with REML and the test statistics (χ^2 and p) were extracted using a Wald's test.

4.6.3 Isolation Test

Means, standard deviations (SD), and standard errors of the means (SEM) were calculated for the behaviors scored during the three-minute isolation tests, and cross tabulations were used to identify the most common behaviors (longest duration) for each treatment group. Behaviors were grouped into categories (fearful, non-fearful, stationary, active) for analysis. Fearful behaviors included escape attempts, and stationary or locomotive behaviors displayed with a low posture. Non-fearful behaviors included locomotive and stationary behaviors displayed with a neutral posture. Stationary behaviors included stand, sit and lie, with all postures grouped. Active behaviors included all locomotive behaviors, both exploratory and non-exploratory. All self-grooming and high postures were dropped from the groupings due to ambivalence of interpretation. Repetitive behaviors, shaking off and elimination were dropped due to minimal to no observations of these. Each behavioral category (fearful, non-fearful, stationary, active) along with number of vocalizations was compared using separate GLMEs which included treatment group and time point (pre/post-transport), along with their interaction as fixed effects, and the nested random effects of cohort, litter, and puppy ID. Model residuals were checked for normality and homoscedasticity. Square root transformations were utilized when needed to improve normality (stationary behavior only). A backward stepwise model selection method using ML and based off AIC values was again used to increase model fit. Models were fitted using ML and the test statistics (χ^2 and *p*) were extracted from the best fitting models using a Wald's test and restricted maximum likelihood (REML). To assess intra-rater reliability, researcher 1 recoded a subsample of videos four months after initial video coding was complete. Intraclass correlation coefficient (ICC) estimates and their 95% confidence intervals were calculated based on a two-way, mixed effects, single rater model (Koo and Li, 2016).

CHAPTER 5. RESULTS

5.1 Physical Health

Over the course of this study, the majority of puppies were observed to be clean and healthy. Puppies' health issues for the first eight weeks were summed and the mean health score for each group was compared. Over the first eight weeks of life (i.e. at the breeder's kennel) and prior to the application of stressors (i.e. isolation testing and transport to the distributor), only 3.3% of all puppies showed evidence of any health problems (n = 20). Percentages of puppies showing each health problem for each treatment group are shown in **Figure 7**.

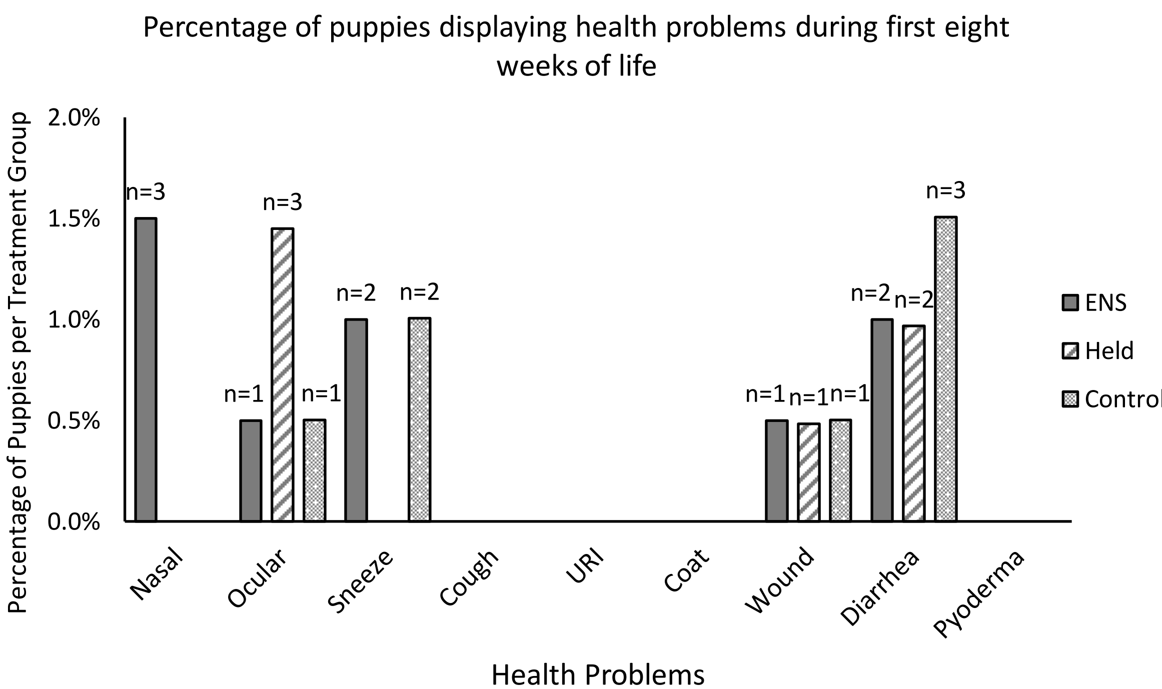

Figure 7: Percentage of puppies in each treatment group (ENS, held, control) showing each health problem measured across the first eight weeks of life prior to the application of stressors.

Over the first eight weeks of life and prior to the application of stressors, most puppies (92.4%) were scored as 'clean', with less than 25% of their bodies covered in debris. A small number of puppies (n = 19) were scored as 26-50% 'soiled' for at least one time point, however, 11 of these puppies were scored 26-50% at just a single time point during this period. Only eight puppies were scored 26-50% on more than one assessment over this time. No puppies were scored higher than 50%. Using our novel three-point body condition score, all puppies were scored as having a ‘normal’ body condition, with the exception of one male ENS puppy (scored as underweight) who was sick and died in the second week of life.

Only two puppies had observable health problems at the breeder’s kennel two to three days prior to transport (a small wound was noted for one ENS puppy and one control puppy). Similarly, only two puppies showed evidence of health problems at the distributor 48 hours following transport (a different control puppy was found to have a minor wound, and another control puppy was observed to have a cough).

During the transport week, most puppies (93.4%) were scored as ‘clean’, with less than 25% of their bodies covered in debris. A small number of puppies (n = 8) were scored as 26-50% ‘soiled’ at the breeder’s kennel prior to transport, however, all puppies scored 0-25% at the distributor following transport. On our novel three-point body condition score, all puppies were a normal body condition.

5.2 Weight and Weight Gain

As expected, week and breed were found to significantly affect puppy weight over the first eight weeks ($\chi^2 = 6560.73$, $p < 0.001$ and $\chi^2 = 20.15$, $p = 0.001$, respectively). Sex was also found to significantly affect puppy weight over this time period, with males weighing significantly more than females ($\chi^2 = 5.12$, $p = 0.024$). A two-way interaction between week and sex ($\chi^2 = 16.65$, $p < 0.001$) and a three-way interaction between treatment group, week, and sex ($\chi^2 = 10.21$, $p = 0.006$) revealed that this difference was influenced by treatment and varied between weeks. For females, ENS puppies consistently weighed more than their held and control counterparts, while for males, held and control puppies were observed to be heavier than ENS puppies by approximately two weeks of age. At around seven weeks of age, male held puppies began to weigh more than male controls (**Figure 8**).

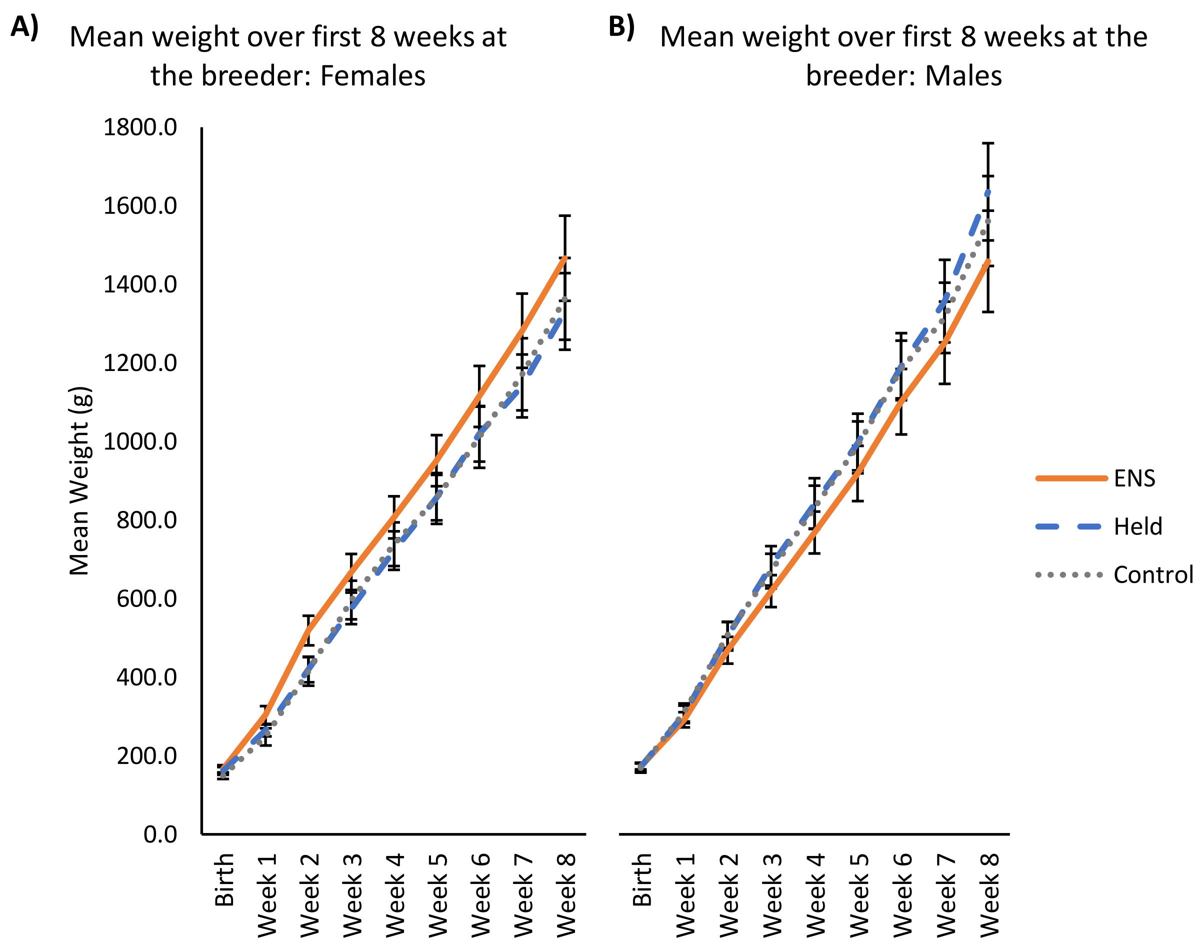

Figure 8: Mean weight for A) female and B) male puppies in each treatment group over the first eight weeks at the breeder's kennel, prior to application of stressors. Error bars represent the standard errors of the means.

Neither treatment group nor any interaction had a significant effect on weight *gain* over the first eight weeks of life. Puppy weight gain was, however, significantly affected by week ($\chi^2 = 26.71$, $p < 0.001$), sex ($\chi^2 = 5.62$, $p = 0.02$), and breed ($\chi^2 = 23.74$, $p < 0.001$). Details of these results are provided in Appendix A.

5.3 RYG and RYG+ Tests at the Breeder

Puppies were primarily scored as "green" on the single-step RYG (**Figure 9**) taken while puppies were in pens with their littermates (hereafter referred to as group RYG). Treatment did not significantly affect the likelihood of a puppy scoring “green” when scored within their litter while at the breeder’s kennel prior to testing ($\chi^2 = 0.24$, DF = 2, $p = 0.89$).

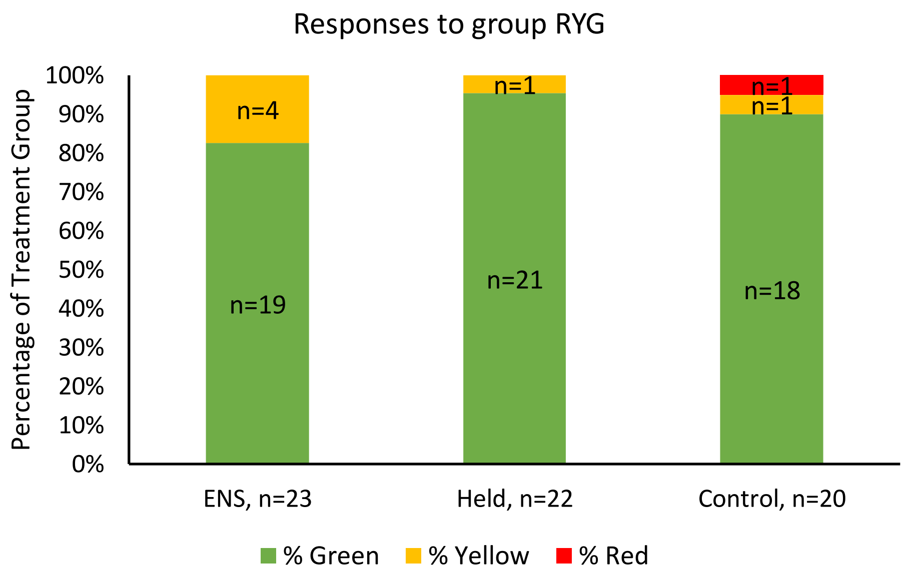

Figure 9: Percentage of puppies in each treatment group scoring red, yellow, and green at eight weeks of age when scored within their litter while at the breeder’s kennel prior to the application of stressors.

Puppies were primarily scored as “yellow” at all three RYG steps of the initial RYG+ (**Figure 10**) following separation from littermates and prior to the three-minute isolation test conducted at the breeder’s kennel before transport (hereafter referred to as pre-isolation breeder RYG+). Treatment group did not significantly affect puppies’ scores in the pre-isolation breeder RYG+. There was no significant difference in scores between ENS and held ($t = -0.04$, $p = 0.97$), ENS and control ($t < -0.001$, $p = 1$) or held and control treatments ($t = 0.05$, $p = 0.96$).

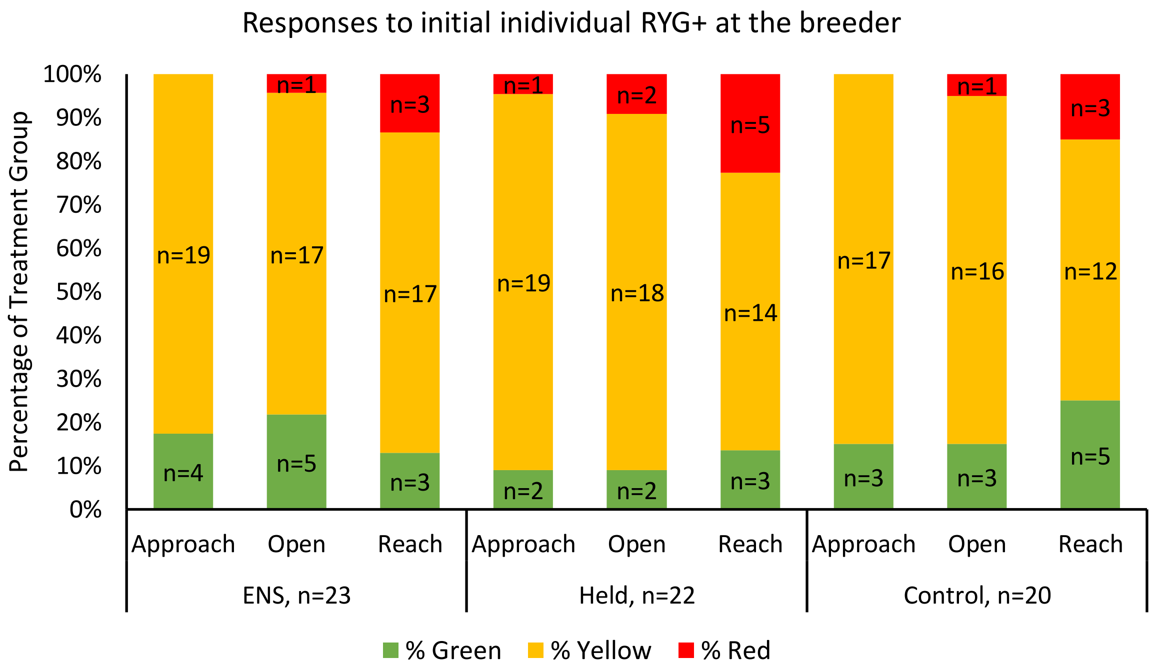

Figure 10: Puppies' responses to the initial RYG+ test following separation from littermates, and prior to the isolation test at the breeder.

At the breeder's kennel prior to transport, there was no significant effect of treatment on puppies' responses to the stranger approach step of the RYG+ test ($\chi^2 = 0.46$, $p = 0.794$). However, isolation was found to significantly affect puppies' responses to this step of the test, in that more puppies showed affiliative behavior after isolation than before ($\chi^2 = 6.2$, $p = 0.013$; **Figure 11**). ENS and held groups showed a greater increase in the number of puppies displaying affiliative behavior at the approach step following isolation than the control group. However no significant interaction effect between treatment and time point was observed ($\chi^2 = 1.21$, $p = 0.546$).

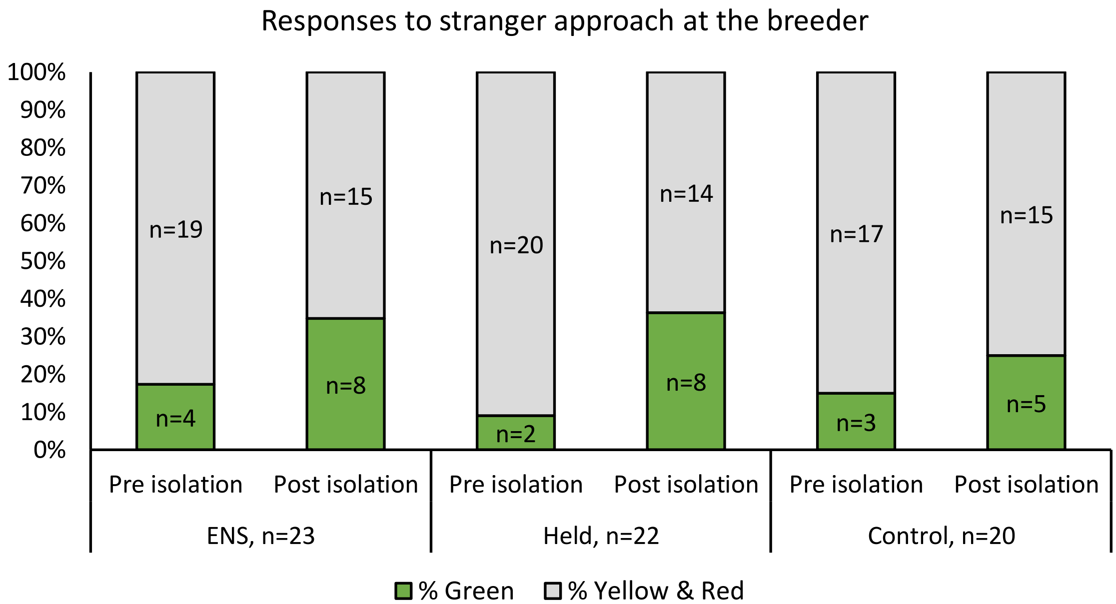

Figure 11: Responses to stranger approach for each treatment (ENS, held, control), before and after the 3-minute isolation test at the breeder's kennel prior to transport.

There was no significant effect of treatment (χ^2 = 0.10, p = 0.949), isolation (χ^2 = 0.99, p = 0.32), or their interaction ($p > 0.05$) on puppies' willingness to eat the treat (tossed into pen) immediately following the stranger approach portion of the RYG+ test at the breeder's kennel prior to transport (**Figure 12**).

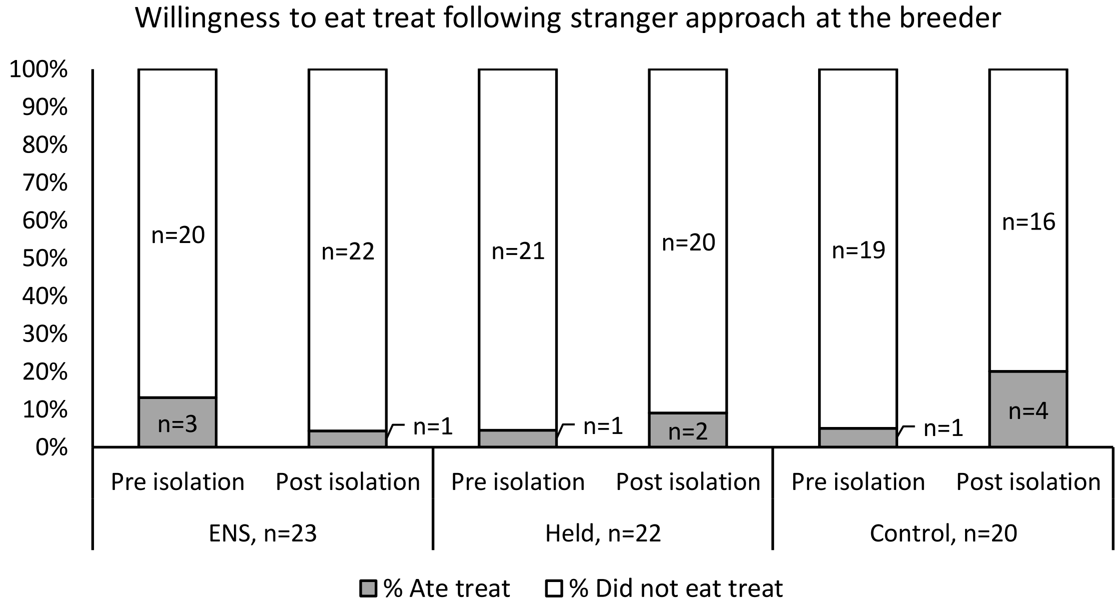

Figure 12: Willingness to eat the treat (tossed into pen) immediately following stranger approach for each treatment (ENS, held, control), before and after the 3-minute isolation test at the breeder's kennel prior to transport.

Treatment group did not significantly affect puppies' responses to the stranger opening the pen door during the RYG+ test at the breeder's kennel prior to transport (χ^2 = 2.16, p = 0.34). However, isolation did significantly affect puppies' responses to this step of the test (χ^2 = 6.69, p = 0.01). More puppies were observed to be affiliative after isolation than before **(Figure 13**). There was no significant effect of the interaction between treatment and time point (χ^2 = 0.04, p = 0.978).

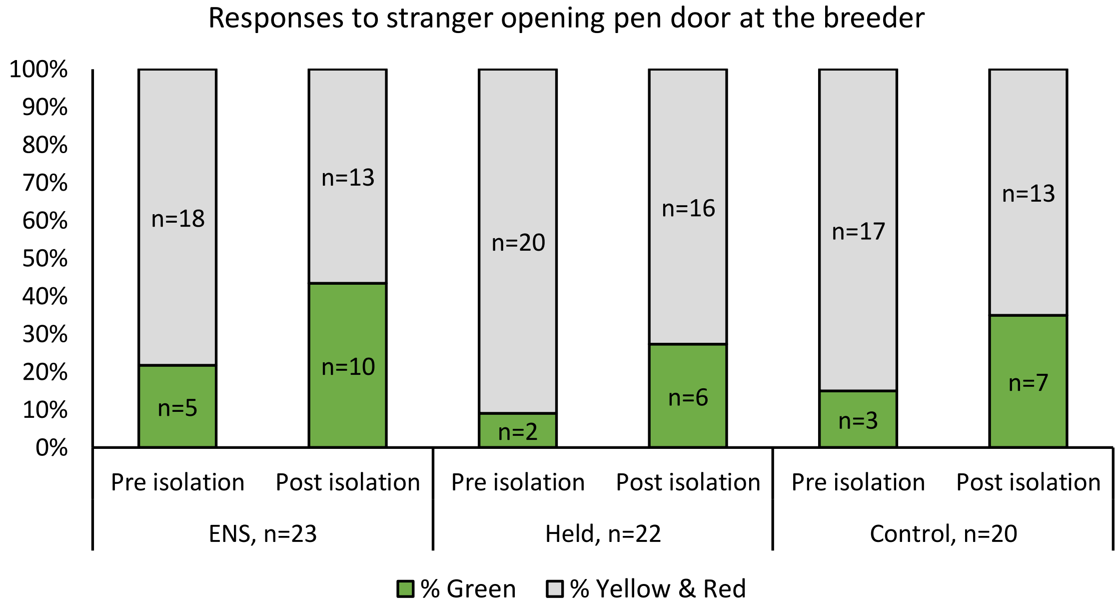

Figure 13: Responses to stranger opening the pen door for each treatment (ENS, held, control), before and after the 3-minute isolation test at the breeder's kennel prior to transport.

Treatment group had no significant effect on puppies' willingness to eat the treat (hand-offered) immediately following the open-door portion of the RYG+ test at the breeder's kennel prior to transport ($\chi^2 = 1.16$, $p = 0.561$). Willingness to eat this treat was significantly affected by isolation ($\chi^2 = 9.38$, $p = 0.002$; **Figure 14**). More puppies were willing to eat the treat after isolation than before. While greater numbers of held and control puppies appeared to be willing to eat the treat following isolation than ENS puppies, no significant interaction effect between treatment and time point was observed ($\chi^2 = 1.52$, $p = 0.467$).

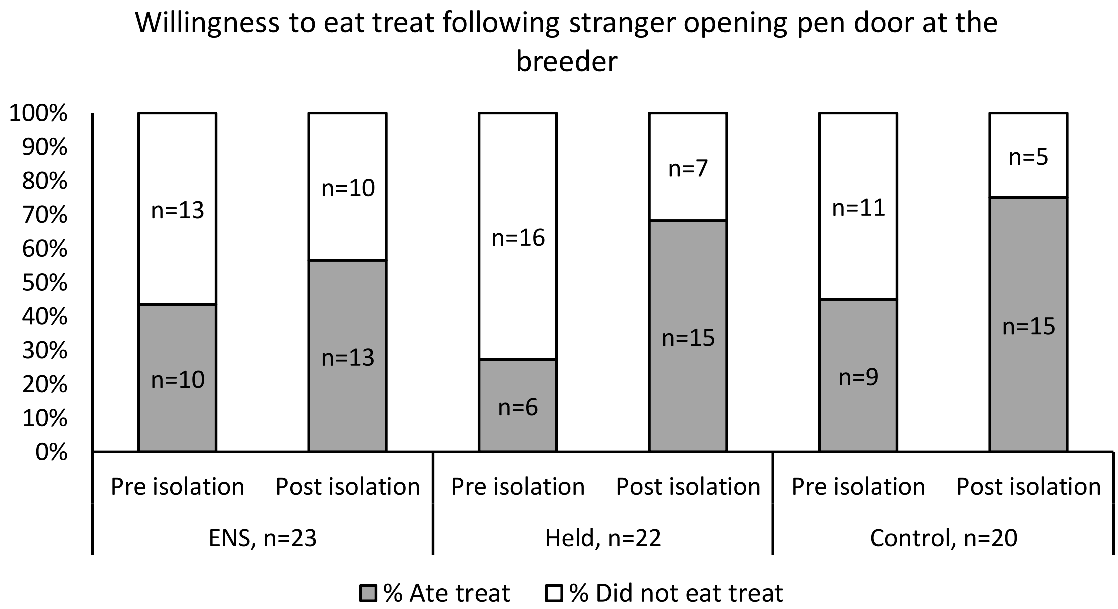

Figure 14: Willingness to eat treat (hand-offered) immediately following opening of the pen door for each treatment (ENS, held, control), before and after the 3-minute isolation test at the breeder's kennel prior to transport.

At the breeder's kennel prior to transport, puppies' responses to the portion of the RYG+ test where the experimenter reached for the puppy and attempted to touch them were not significantly affected by treatment group alone ($\chi^2 = 0.25$, $p = 0.881$). However, responses were significantly affected by isolation ($\chi^2 = 11.83$, $p < 0.001$), with more puppies showing affiliative responses to the stranger's reach attempt after isolation than before. A two-way interaction between isolation and treatment group revealed that this change varied by treatment ($\chi^2 = 7.34$, $p = 0.025$). ENS and held groups had a greater increase in the number of puppies showing affiliative behavior when the experimenter reached for them following isolation than controls (**Figure 15**).

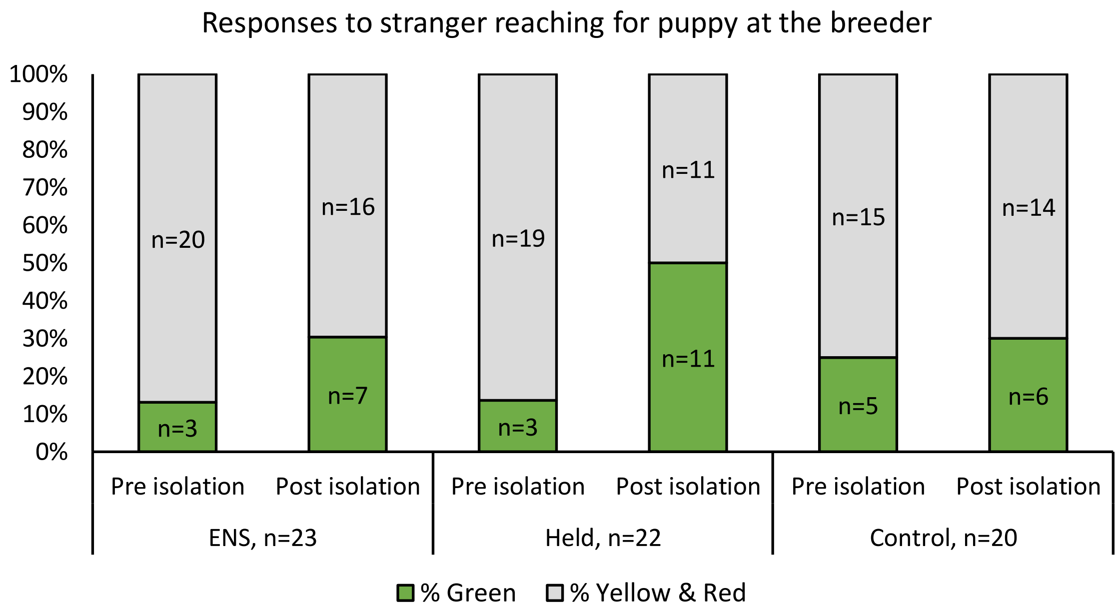

Figure 15: Responses to stranger reaching toward puppy for each treatment (ENS, held, control), before and after the 3-minute isolation test at the breeder's kennel prior to transport.

There was no significant effect of treatment (χ^2 = 1.74, p = 0.418), isolation (χ^2 = 0.07, p = 0.795), or their interaction (p > 0.05) on puppies' willingness to eat the treat (hand-offered) immediately following the reach portion of the RYG+ test at the breeder's kennel prior to transport (**Figure 16**).

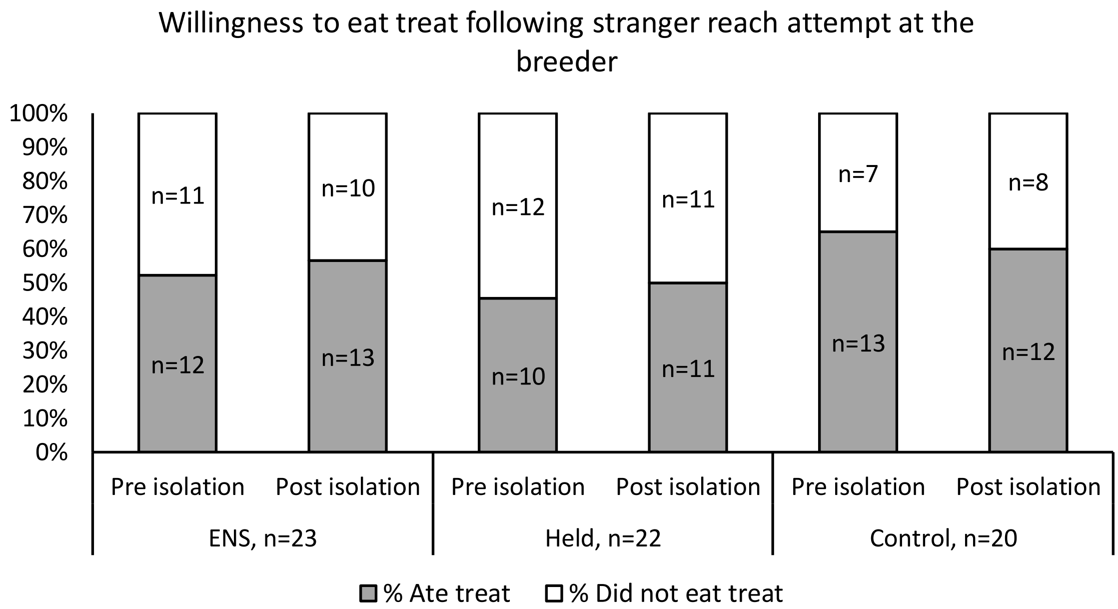

Figure 16: Willingness to eat treat (hand-offered) immediately following the stranger's reach attempt for each treatment (ENS, held, control), before and after the 3-minute isolation test at the breeder's kennel prior to transport.

At the breeder's kennel prior to transport, puppies' willingness to allow the unfamiliar person to touch them was not significantly impacted by treatment ($\chi^2 = 0.43$, $p = 0.805$). This step of the RYG+ test was significantly affected by isolation, where more puppies allowed touch after isolation than before ($\chi^2 = 7.44$, $p = 0.006$; **Figure 17**). There was a greater increase in the number of puppies from the held treatment group who accepted the unfamiliar person's touch following isolation compared to ENS and control groups, although no significant interaction effect between treatment and time point was observed ($\chi^2 = 3.83$, $p = 0.147$).

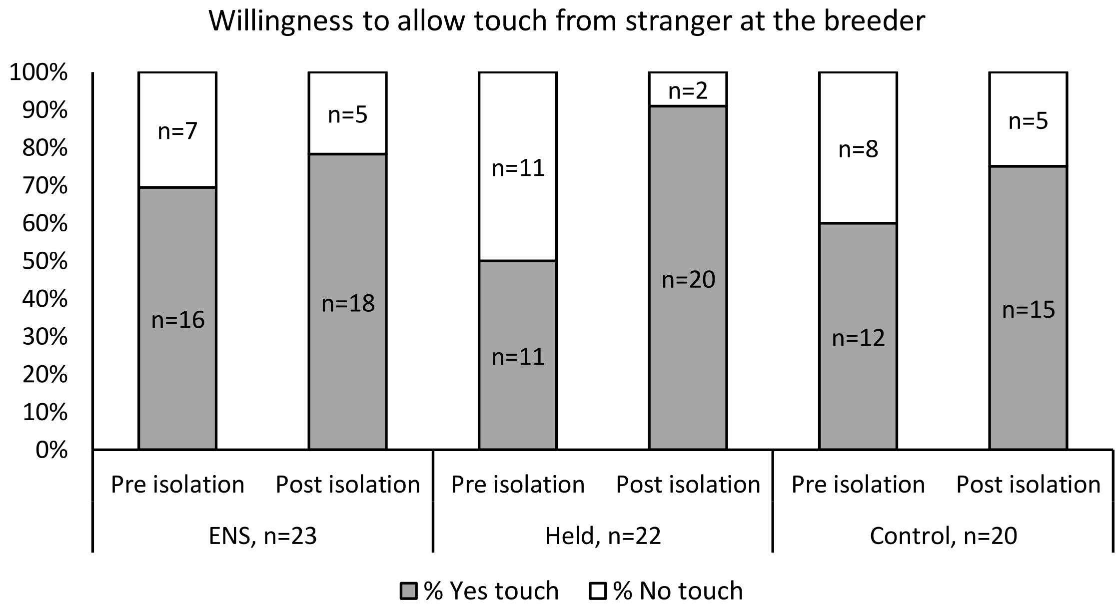

Figure 17: Willingness to allow touch from the stranger for each treatment (ENS, held, control), before and after the 3-minute isolation test at the breeder's kennel prior to transport.

5.4 RYG+ Tests at the Distributor

At the distributor following transport, treatment group did not significantly affect puppies' responses to the stranger approach portion of the RYG+ test done at the distributor following transport ($\chi^2 = 1.99$, $p = 0.37$). Isolation was found to significantly affect responses to this step of the test, in that more puppies were observed to be affiliative after isolation than before ($\chi^2 = 11.24$, $p < 0.001$). ENS and held groups showed a greater increase in the number of puppies displaying affiliative behavior than the control group on the approach step following isolation at the distributor (**Figure 18**), but no significant interaction effect between treatment and time point was observed ($p > 0.05$).

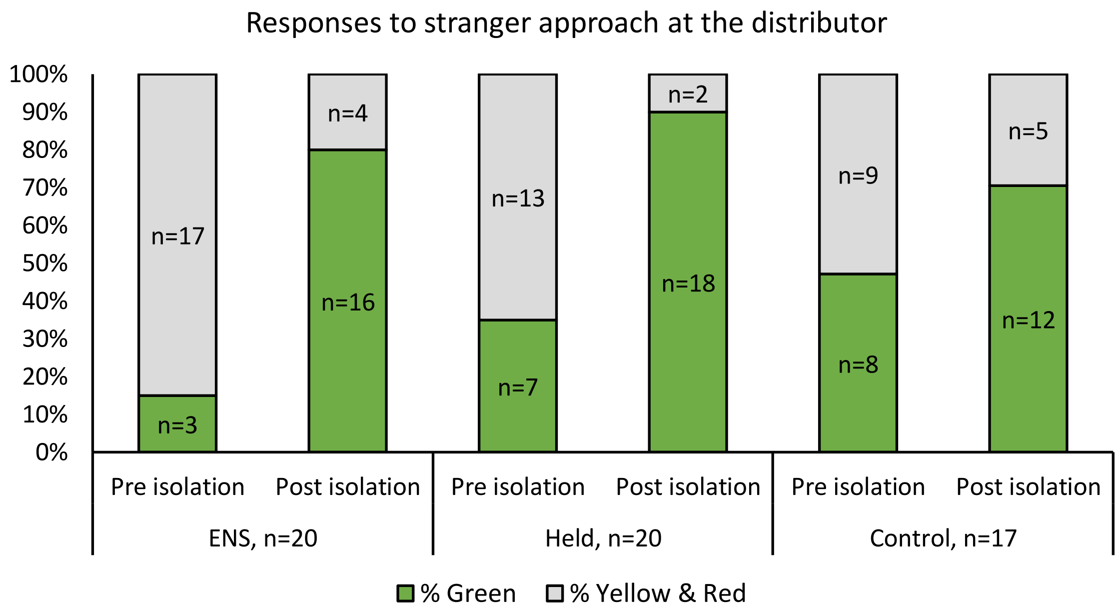

Figure 18: Responses to stranger approach for each treatment (ENS, held, control), before and after the 3-minute isolation test at the distributor following transport.

There was no significant effect of treatment ($\chi^2 = 0.21$, $p = 0.901$), isolation ($\chi^2 = 0.08$, $p = 0.782$), or their interaction ($\chi^2 = 0.16$, $p = 0.925$) on puppies' willingness to eat the treat (tossed into pen) immediately after the stranger approach portion of the RYG+ test at the distributor following transport (**Figure 19**).

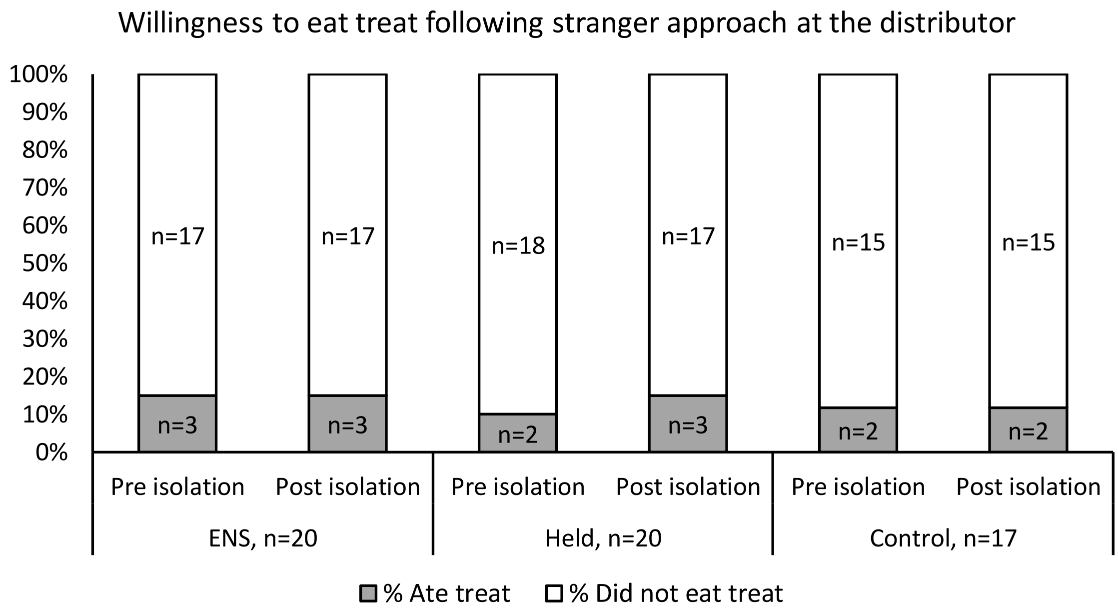

Figure 19: Willingness to eat the treat (tossed into pen) immediately following stranger approach for each treatment (ENS, held, control), before and after the 3-minute isolation test at the distributor following transport.

At the distributor following transport, treatment did not significantly affect puppies' responses to the stranger opening the pen door during the RYG+ test ($\chi^2 = 0.53$, $p = 0.767$). Responses to this step were again found to be significantly affected by isolation, with more puppies showing affiliative behavior after isolation ($\chi^2 = 11.46$, $p < 0.01$). The held treatment group showed a greater increase in the number of puppies behaving affiliatively at the open-door step following isolation than ENS and control groups (**Figure 20**), but no significant interaction effect between treatment and time point was observed ($\chi^2 = 3.52$, $p = 0.172$).

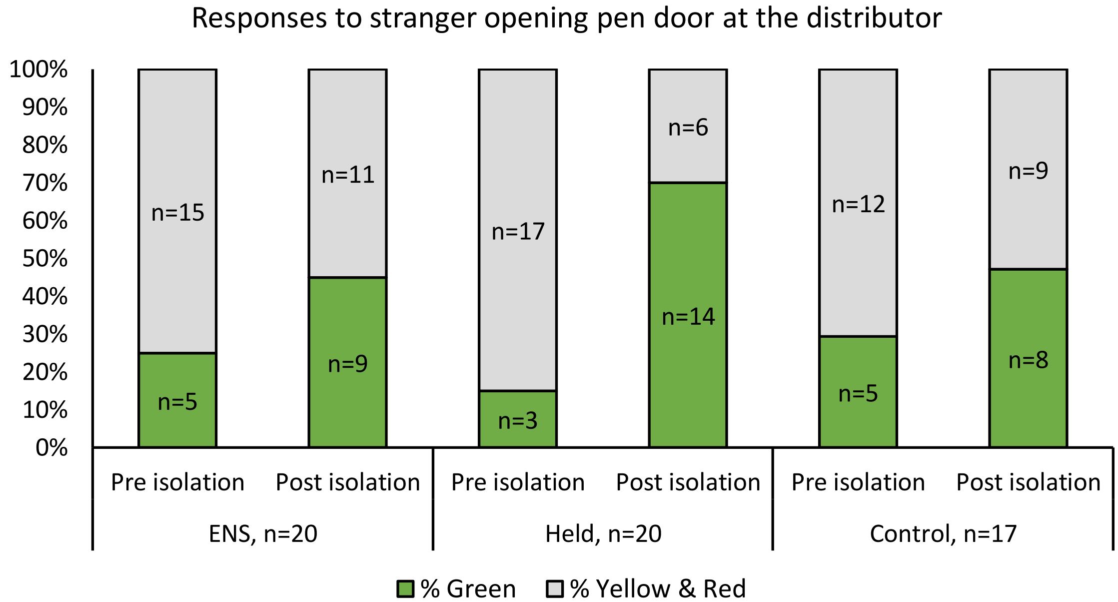

Figure 20: Responses to stranger opening the pen door for each treatment (ENS, held, control), before and after the 3-minute isolation test at the distributor following transport.

There was no significant effect of treatment (χ^2 = 0.38, p = 0.826), isolation (χ^2 = 0.38, p = 0.538), or their interaction (χ^2 = 1.31, p = 0.52) on puppies' willingness to eat the treat (hand-offered) immediately following the open-door portion of the RYG+ test at the distributor following transport (**Figure 21**).

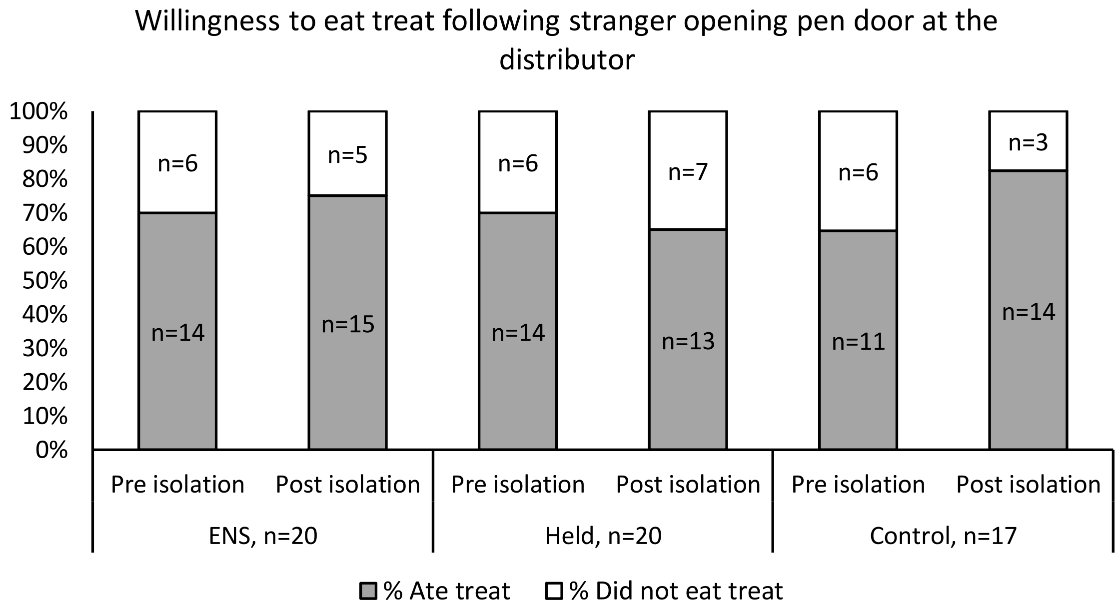

Figure 21: Willingness to eat treat (hand-offered) immediately following opening of the pen door for each treatment (ENS, held, control), before and after the 3-minute isolation test at the distributor following transport.

At the distributor following transport, no significant effect of treatment was observed on puppies' responses to the portion of the RYG+ test where the experimenter reached for the puppy and attempted to touch them (χ^2 = 2.24, p = 0.326). This step of the test was again significantly affected by isolation (χ^2 = 5.78, p = 0.016) where more puppies showed affiliative responses after isolation than before (**Figure 22**). The ENS group showed the greatest increase in the number of puppies displaying affiliative behavior and the control group the least, with the held group falling in between. However, no significant interaction effect between treatment and time point was observed (χ^2 = 1.22, p = 0.543).

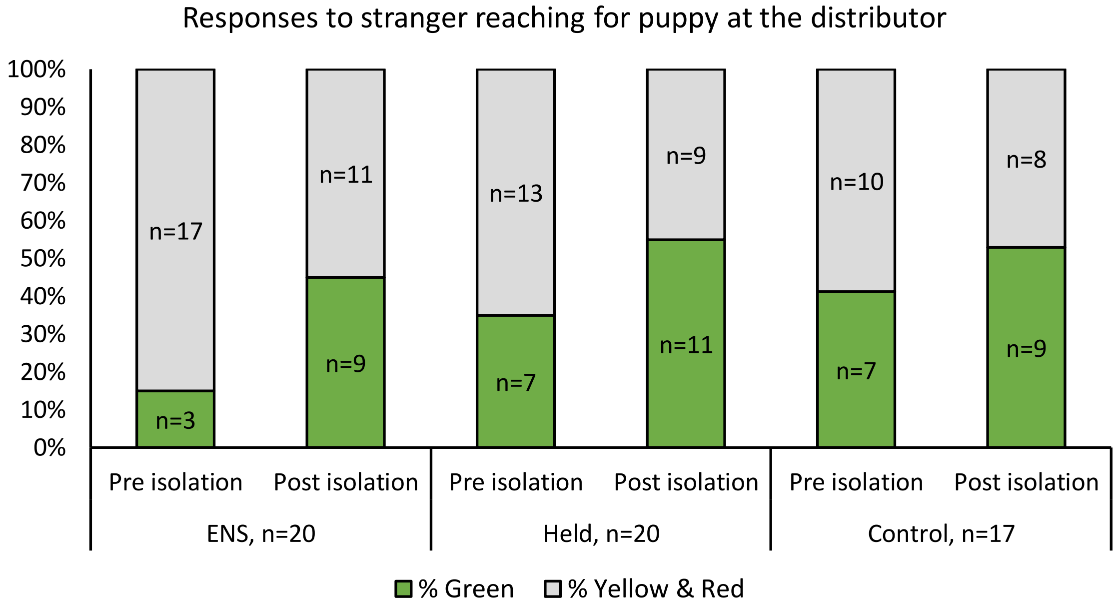

Figure 22: Responses to stranger reaching toward puppy for each treatment (ENS, held, control), before and after the 3-minute isolation test at the distributor following transport.

There was no significant effect of treatment (χ^2 = 0.17, p = 0.92), isolation (χ^2 = 0.18, p = 0.676), or their interaction (χ^2 = 2.45, p = 0.294) on puppies' willingness to eat the treat (hand-offered) immediately following the reach portion of the RYG+ test at the distributor following transport (**Figure 23**).

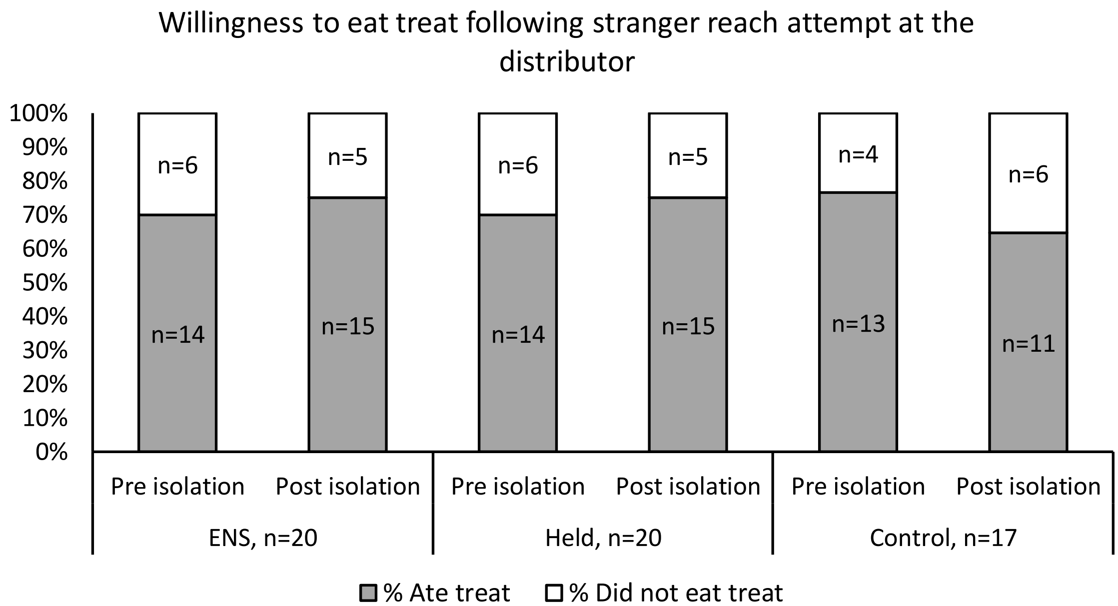

Figure 23: Willingness to eat treat (hand-offered) immediately following reach attempt for each treatment (ENS, held, control), before and after the 3-minute isolation test at the distributor following transport.

At the distributor following transport, no significant effects of treatment (χ^2 = 0.04, p = 0.981) or the interaction between treatment and isolation (p > 0.05) were found on puppies' willingness to allow the unfamiliar person to touch them. Isolation was found to significantly affect puppies' willingness to allow stranger touch (χ^2 = 10.09, p = 0.001). The numbers of puppies accepting touch only changed for the held group, where more puppies accepted the touch after isolation than before (**Figure 24**).

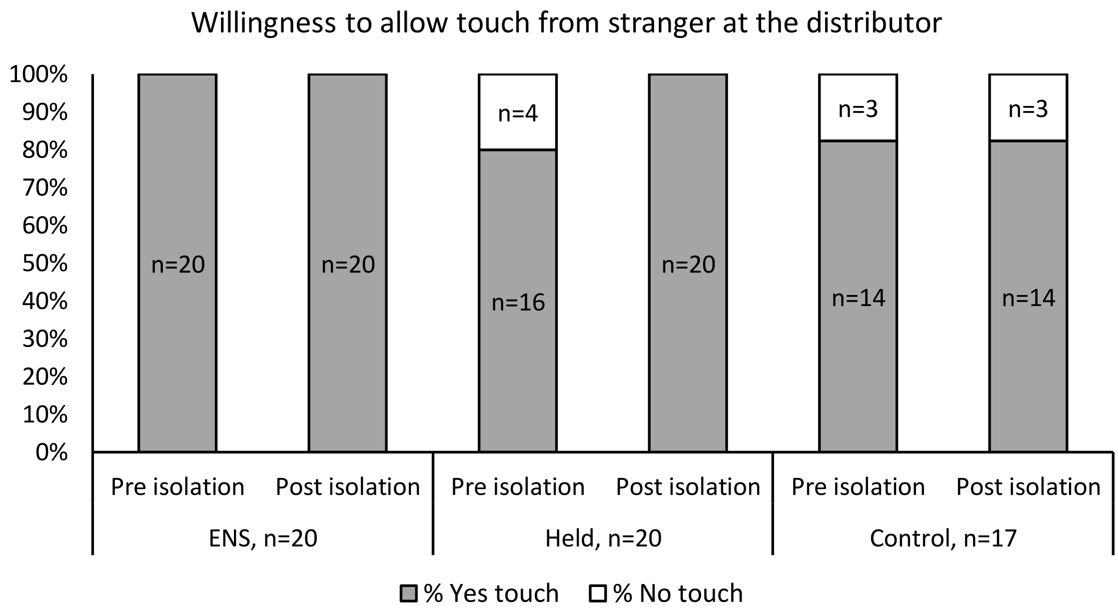

Figure 24: Willingness to allow touch from the stranger for each treatment (ENS, held, control), before and after the 3-minute isolation test at the distributor following transport.

5.5 Isolation Test Behavior

Twelve videos (10.5%) were selected at random from the 114 isolation videos and were re-coded by researcher 1. Intraclass correlation coefficient (ICC) estimates fell between 0.87 and 0.96. According to the suggested interpretation of ICC values by Koo and Li (2016), this indicated good to excellent reliability for all behavioral categories. A table of ICC estimates, their 95% confidence intervals, and p-values is included in Appendix C.

Treatment group was found to significantly affect time spent performing fearful behaviors during the isolation test ($\chi^2 = 6.37$, $p = 0.041$; **Figure 25**), regardless of location (i.e. the effect of location was insignificant: $\chi^2 = 0.26$, $p = 0.608$). ENS puppies spent the most time performing fearful behaviors and control puppies spent the least time performing them, while held puppies showed an amount of fearful behavior between ENS and control groups (**Table 3**). The interaction of treatment and isolation did not significantly affect time spent performing fearful behaviors ($\chi^2 = 0.47$, $p = 0.792$). A complete table of means and standard deviations for all behaviors is included in Appendix B.

Table 3: Means and standard deviations for time each treatment group spent performing fearful behaviors during the isolation test.

Treatment Group	Time point	Mean (s)	Standard Deviation
ENS	Breeder	54.74	22.37
	Distributor	51.74	26.94
Held	Breeder	48.87	26.39
	Distributor	43.46	17.06
Control	Breeder	38.33	25.48
	Distributor	40.60	24.46

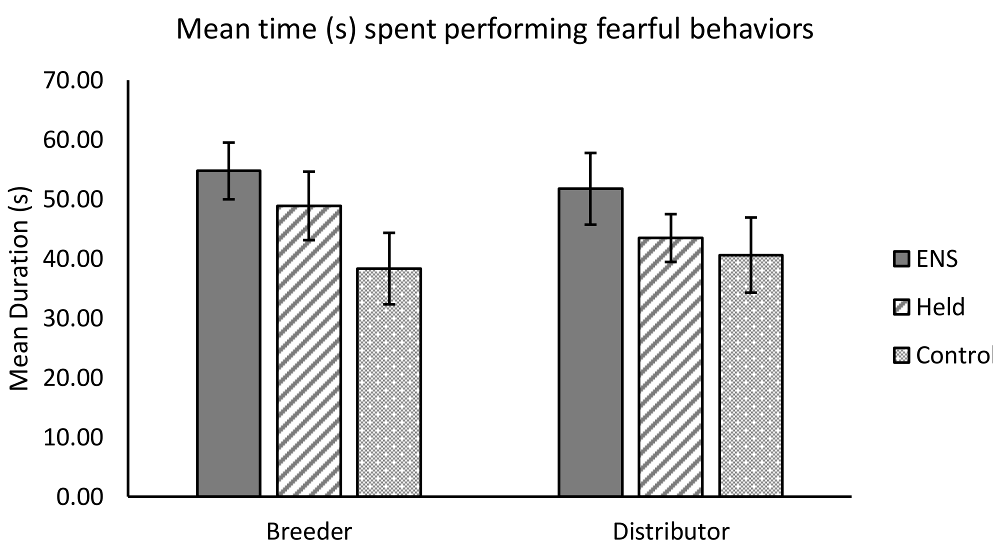

Figure 25: Mean time (seconds) spent performing fearful behaviors for each treatment group during the 3-minute isolation test at the breeder's kennel (prior to transport) and at the distributor (following transport). Error bars represent standard errors of the means.

Treatment was not found to significantly affect time spent performing non-fearful behaviors during the isolation test (χ^2 = 2.29, p = 0.319). Time spent performing non-fearful behaviors was, however, significantly affected by the test location, which embeds the stress of transport (χ^2 = 4.05, p = 0.044; **Figure 26**). Puppies spent significantly less time performing non-fearful behaviors at the distributor following transport than at the breeder's kennel prior to transport. The interaction of treatment and isolation did not significantly affect time spent performing non-fearful behaviors (χ^2 = 3.74, p = 0.154).

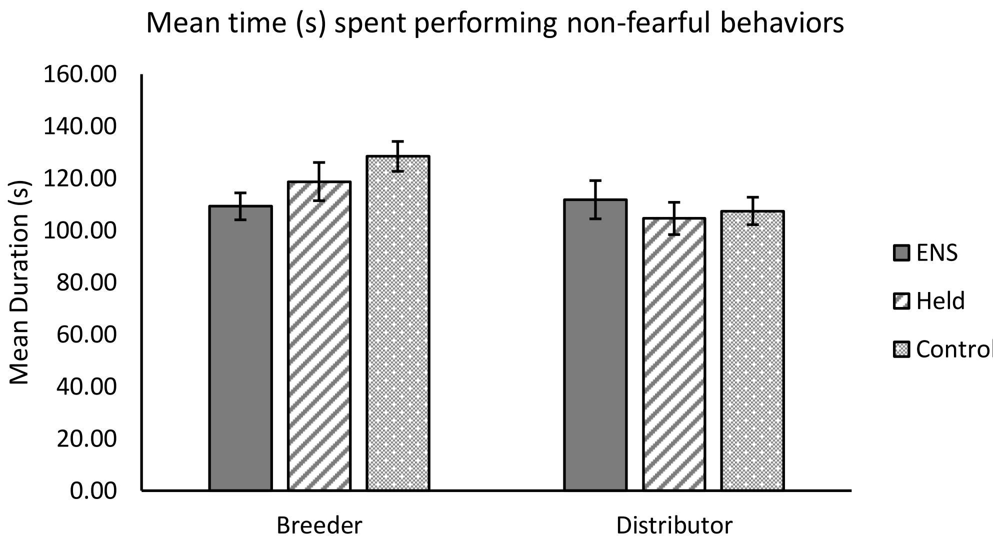

Figure 26: Mean time (seconds) spent performing non-fearful behaviors for each treatment group during the 3-minute isolation test at the breeder's kennel (prior to transport) and at the distributor (following transport). Error bars represent standard errors of the means.

There were no significant effects of treatment ($\chi^2 = 0.49$, $p = 0.785$), location ($\chi^2 = 0.04$, $p = 0.844$), or their interaction ($\chi^2 = 0.4$, $p = 0.817$) on time spent performing stationary behaviors.

Treatment group did not significantly affect time spent performing active behaviors ($\chi^2 = 4.17$, $p = 0.124$). Location, which included transport stress, was found to significantly affect time spent performing active behaviors during the isolation test ($\chi^2 = 17.22$, $p < 0.001$; **Figure 27**). The interaction of treatment and isolation did not significantly affect time spent performing active behaviors ($\chi^2 = 0.13$, $p = 0.937$). Puppies spent less time performing active behaviors at the distributor following transport than at the breeder's kennel prior to transport.

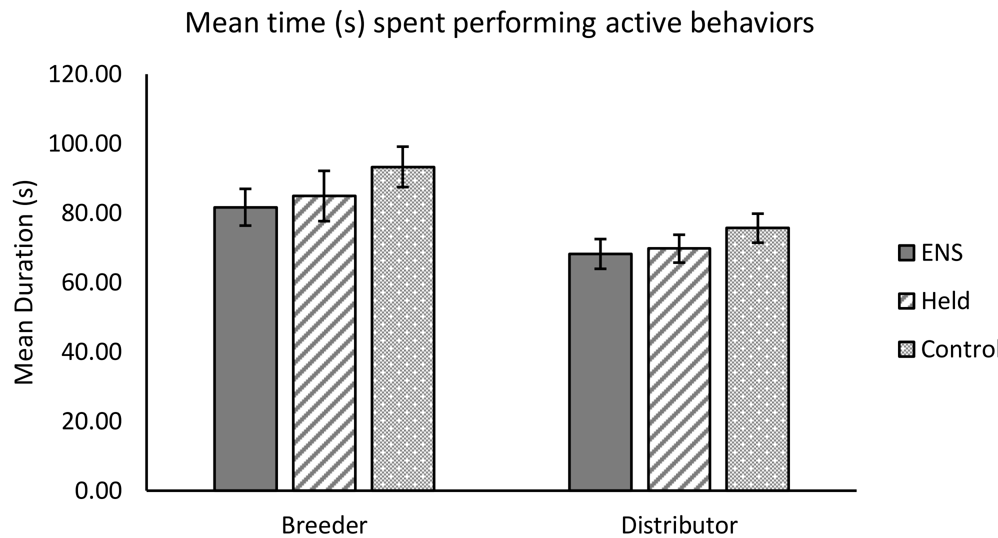

Figure 27: Mean time (seconds) spent performing active behaviors for each treatment group during the 3-minute isolation test at the breeder's kennel (prior to transport) and at the distributor (following transport). Error bars represent standard errors of the means.

Treatment group had no significant effect on the number of vocalizations made during the isolation test (χ^2 = 0.81, p = 0.668). Location, and embedded transport stress, was found to significantly affect the number of vocalizations made (χ^2 = 6.51, p = 0.011; **Figure 28**). Puppies vocalized more at the distributor following transport than at the breeder's kennel prior to transport. Puppies' number of vocalizations was not significantly affected by the interaction of treatment and location (χ^2 = 0.80, p = 0.669).

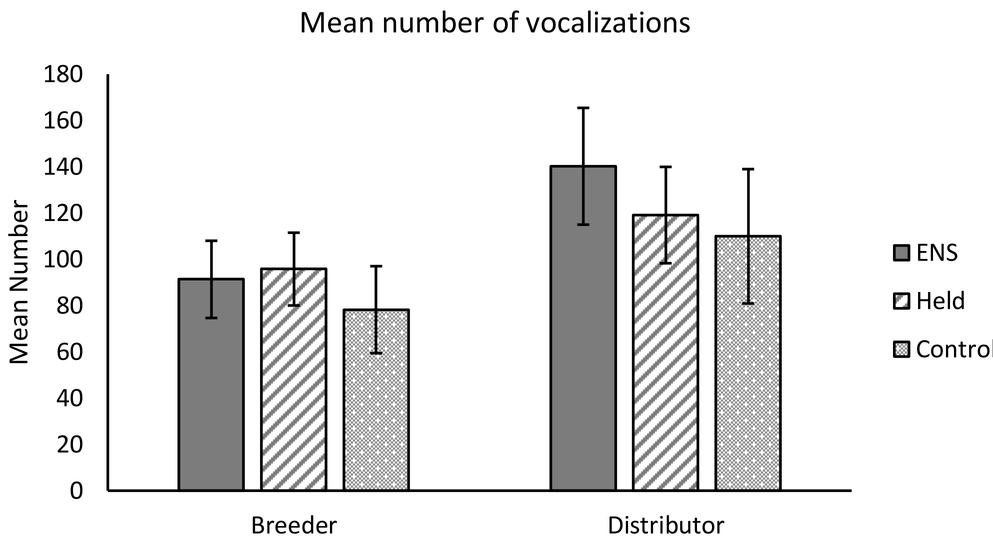

Figure 28: Mean number of vocalizations for each treatment group during the 3-minute isolation test at the breeder's kennel (prior to transport) and at the distributor (following transport). Error bars represent standard errors of the means.

CHAPTER 6. DISCUSSION

Based on previous studies of ENS in dogs and other animals, (e.g. Castelli et al., 2020; de Oliveira et al., 2015; Fox and Stelzner, 1966; Gazzano et al., 2008; Levine et al., 1967; McCune, 1995; Parker et al., 2004), the hypotheses tested in this study included that puppies who received ENS would show differences in their physical health metrics, as well as in their fear and exploratory behaviors, as compared to matched controls. It was hypothesized that puppies who received ENS would exhibit indications of improved health, such as less illness and increased growth, related to their held and control counterparts. Further, it was expected that puppies in the ENS treatment group would demonstrate increased affiliative behavior towards strangers as well as less fear and more exploration of a novel environment when socially isolated, as compared to held and control group puppies.

The first hypothesis, that puppies who received ENS would be healthier than held and control puppies was only partially met. The only area where the ENS puppies showed changes in physical health metrics consistent with study hypotheses was on weight. While previous studies of ENS in dogs have not demonstrated effects on weight or weight gain (e.g. Fox and Stelzner, 1966), these metrics were included in the physical health measures for this study due to their value as measurements of overall health (ill puppies are likely to be underweight or struggle to gain weight), and welfare (stress activation of the HPA axis inhibits growth and mobilizes stored fat). In contrast to previous studies of ENS in dogs, this study did observe some effects of treatment on puppy weights. Puppies' weights throughout the first eight weeks of life varied according to a three-way interaction between treatment, sex, and week. The findings were consistent with the hypothesis for female puppies but ran counter to the hypothesis for male puppies. In female puppies, those in the ENS treatment group consistently weighed more than their held and control counterparts, while for males, held and control puppies weighed more than ENS puppies. However, for the males, the differences were not evident until about two weeks of age. It is unclear why treatment led to opposite results in male and female puppies. Comparisons of weights within treatment groups and across sexes during the first eight weeks of life at the breeder's kennel revealed that male held and control puppies consistently weighed more than female held and control puppies respectively, while both ENS groups weighed about the same. This suggests that male puppies in this study may have had an initial tendency to weigh more than female puppies, and ENS helped female puppies match weight with their male counterparts. Previous work has shown that adult male dogs tend to weigh more than females (Chase et al., 2005; Helmink et al., 2000), but recent work has shown that this is not consistent across breeds (Schrank et al., 2019). Specifically, Schrank and colleagues (2019) found that male Tibetan Terriers gained more weight than females, but female Bernese Mountain Dogs gained more weight than males. One might anticipate that sex-differences in weight gain in the breeds used in this study may have contributed to the differences observed. However, while the current study did find significant differences in both weight and weight gain between breeds, no significant interaction of breed and sex was found for either weight or weight gain. Visual inspection of the data suggested that males had higher weights and weight gains than females for all breeds except for Bichon/Shih Tzu-cross puppies (see Appendix A), where females seemed to show higher weights and weight gains. This suggests that the effect of breed on the results was probably minimal. It is unclear why male ENS puppies in the current study did not experience an advantage over held and control males, as did the female puppies. While it is possible that male puppies already weighed near the expected capacity for their breeds, and therefore had limited room for improvement in this area, this does not explain why male ENS puppies actually weighed less than their held and control counterparts.

Because most puppies were healthy throughout the entire study period, and each treatment group showed very few health problems over the first eight weeks of life (control = 3.51%, held = 2.89%, ENS = 4.5%), there were not enough of these to evaluate the effects of treatment on puppies' physical health. While the inability to analyze the health data was disappointing, this finding suggests that the welfare of these puppies, in relation to their health status was likely not compromised in a significant way. In fact, the relative health of the puppies in this study counters previous reports of dogs thought to have originated in CB kennels (McMillan et al., 2011), as well as reported public perceptions of puppies from these establishments (Bateson, 2010). These discrepancies may reflect differences in information obtained via owner reports (e.g. McMillan et al., 2011) as compared to direct on-site studies. This theory is supported by the fact that findings of the current study are consistent with recent investigations also conducted on-site in U.S. commercial breeding kennels, which found no significant problems in dental, ear, or foot health in the dogs evaluated (Stella et al., 2018).

Puppies' body condition scores were also measured throughout the study as a physical health metric since both extremely low and extremely high body condition scores may be indicative of poor health, inappropriate nutrition, or mismanagement (Barnard et al., 2016; Grandin, 2010). To evaluate puppies' body conditions, a novel body condition scoring system was developed for this study. The need to do so reflects deficits in the existing literature as well as opportunities for further areas of study needed to validate the scoring. Similarly to the other measures of puppy health, all puppies had a normal body condition score (except the one ill ENS puppy who was scored as underweight and died), so it was not possible to evaluate the effects of treatment on puppies' body condition.

While not directly related to ENS, puppies' cleanliness was also evaluated, as body cleanliness is a useful proxy measure for management factors which can affect dog welfare, such as kennel cleaning protocols. Another management factor which can be indirectly evaluated via body cleanliness is the adequacy of pen sizes, as dirty dogs may indicate that pen sizes are insufficient (Barnard et al., 2016). The majority of puppies were found to be less than 25% soiled throughout the entire study, and over half of those who scored 26-50% soiled only received that score at a single time point. For the puppies that scored 26-50% across multiple time points, all but one came from the same litter and the difference in cleanliness for this litter may be attributed to differences in maternal care. This litter's dam was not as fastidious as other dams in the study, something the breeder had noted for her previous litter as well (the litter evaluated for the current study was only her second). This finding suggests that while management factors certainly affect puppy cleanliness, maternal care factors likely have a strong influence as well. Individual variation in maternal care is well established in rodent literature (e.g. Francis and Meaney, 1999; Liu et al., 2013; Meaney, 2001; Pan et al., 2014) and has also been demonstrated in dogs (Bray et al., 2017; Foyer et al., 2016; Guardini et al., 2017, 2016). Another factor affecting puppy cleanliness in this study was that puppies were bathed and groomed in the few days prior to transport. This may have influenced the cleanliness scores of the puppies at the distributor following transport. However, the finding that most puppies were quite clean throughout the entire study suggests that overall management practices for these litters was sufficient to maintain a reasonable level of body cleanliness for the puppies. While this finding contradicts public opinion pertaining to these dogs, it aligns with findings from a recent study conducted at other breeding kennels located in the United States (Stella et al., 2018).

The second hypothesis, that puppies who received ENS would be more affiliative toward strangers and display more exploratory behaviors when alone was also only partially met. The

RYG+ tests revealed differences between treatment groups only on responses to the stranger reaching for the puppy in the breeder's kennel and the isolation tests showed differences between treatment groups only in fearful behavior. Furthermore, there was some indication that where differences between treatment groups were observed, the held treatment group received similar benefits from handling to the ENS group, and possibly more benefit.

The first test of puppies' behavioral responses to ENS evaluated their responses to stranger approach (RYG) while in their home pens at the breeder's kennel with their littermates. Treatment group (ENS, held, control) was not found to significantly affect the likelihood of a puppy scoring "green" (affiliative) on this test. This lack of effect is not entirely clear, but it is likely due to the high percentage of puppies scoring "green" overall. The overall percentage of affiliative behavior found may have been too high for ENS to show a marked effect.

Comparison between puppies' scores on the group RYG and their individual responses to approach during the subsequent initial RYG+ test revealed a shift from a majority of "green" affiliative responses to largely "yellow" ambivalent responses following separation from littermates. The change in scores suggests that puppies felt more secure with their littermates in a familiar environment, than alone in a novel environment. This aligns with previous findings that isolation from conspecifics is associated with a variety of stress responses in social species, including cows, pigs, and dogs (Boissy and Le Neindre, 1997; Hennessy, 1997; Herskin and Jensen, 2000; Walker et al., 2014). Unfortunately, treatment also failed to significantly affect puppies' responses on the approach step of the initial RYG+ test at the breeder, so neither ENS nor held interventions appear to have helped puppies cope with this stressor.

The only step of the RYG+ test affected in any way by treatment group was when the unfamiliar person reached for the puppy prior to trying to touch them during the RYG+ test at the breeder's kennel. For this step of the test, more puppies were generally affiliative after isolation than before isolation, and treatment group interacted with isolation to affect puppies' responses. Both ENS and held treatment groups appeared to show a greater increase than controls in the number of puppies showing affiliative behavior at this step of the test. While this result is consistent with expectations that ENS would increase affiliative behaviors, it also indicates that held treatments may have helped puppies cope more effectively or be more confident with an unfamiliar person reaching for them. Interestingly, the held treatment group displayed the largest increase in the number of puppies showing affiliative behaviors on this step of the test, so it is possible that the held treatment was even more effective than ENS at eliciting the intended benefits. These findings are noteworthy, because the only study of ENS in dogs to date which included a held control group (Schoon and Berntsen, 2011) found no differences between held controls and puppies who received ENS, though their study did *not* include a control group with no additional human handling.

Based on previous research (Fox and Stelzner, 1966), it was hypothesized that ENS puppies would show more affiliative behavior than held and control puppies on multiple steps of the RYG+ test, as was observed for the "reach" step. Additionally, puppies in the held treatment were expected to show affiliative behaviors intermediate to ENS and control puppies, since puppies in the held group were also removed from the nest and given a basic level of gentle handling. However, none of these expectations were met for the other steps of the RYG+ test done at the breeder's kennel, nor any steps of the RYG+ test carried out at the distributor. Several factors may have led to the lack of differences found between treatment groups. One possible explanation is that all puppies in the study received enough positive interactions with people during their first eight weeks of life to mask the effects of additional handling from ENS or held treatments. This could be a result of standard interactions with caretakers, since normal kennel procedures were continued for all puppies in this study. It could also be due to an artifact of the study design, because all puppies were marked daily for identification and given a health assessment weekly. Alternatively, the additional handling provided through ENS and held treatments may not have been perceived as sufficiently positive by puppies to lead to a change in their behavioral responses to humans as compared to control puppies.

Curiously, some steps of the RYG+ tests were affected by isolation alone. At the breeder's kennel, portions of the test which were affected by isolation included: responses to stranger approach, opening of the pen door, offering a treat while the door was open, and the attempt by the stranger to touch the puppy. At the distributor, isolation affected puppies' responses to stranger approach, opening of the pen door, the stranger reaching towards the puppy, and the stranger attempting to touch the puppy. For all these steps, regardless of location, more affiliative responses were seen after isolation than before. For the one treat step affected, more puppies were willing to eat the treat after isolation than before. Puppies' interactions with the researcher during early handling treatments (ENS and held) may have primed their brains to view people as social buffers during stressful experiences, which could explain why puppies displayed more affiliative behavior towards the unfamiliar person upon their return to the pen than on their initial approach, assuming that the stressor of isolation was perceived by puppies as more aversive than being approached by a stranger. This explanation would be consistent with existing knowledge that early experiences shape development, which is one of the underlying principles used to support theories of ENS, as discussed previously (e.g. Maras and Baram, 2015; Raineki et al., 2014).

Interestingly, not only did more puppies demonstrate “green” or affiliative responses after isolation than before, more affiliative responses were observed after transport than before. It is possible that this gradual change in responses reflects habituation to the researcher conducting the stranger approach tests, or to the tests themselves. The same researcher was utilized for all stranger-approach testing to maintain consistency in scoring and minimize potential changes in puppies’ responses to the stranger. Differences in physical characteristics, such as gender, have been shown to affect dogs’ responses to humans (Wells and Hepper, 1999). Additionally, there is some evidence that dogs have the ability to differentiate between different human faces and correctly match voice and gender (Racca et al., 2010; Ratcliffe et al., 2014). Due to the limited contact puppies experienced with this individual, risk of acclimation was expected to be small and of a lesser concern than potential confounds that could have arisen from the use of different individuals. Additionally, the transport week contained multiple events that may have been stressful for puppies, including bathing and grooming, multiple handling events with unfamiliar people, transport, and introduction to the novel environment of the distributor. It may be that the cumulative effect of stressors which puppies experienced during the transport week was so profound that it masked some of the differences that testing was meant to elucidate.

One such difference that may have been masked was the eating of treats during the RYG+ tests. For all but the treat offered following the opening of the pen door at the breeder (which was affected by isolation only), puppies’ willingness to eat treats during the RYG+ testing was not significantly affected by treatment, isolation, or their interaction. It is worth noting that there was a substantial increase in the number of puppies who ate the hand-offered treats compared to the number of puppies who ate the tossed treats. This suggests that the method of treat presentation may have had a greater influence on whether puppies ate it than other factors. One possible explanation for this result is that puppies were somewhat overstimulated during testing, and therefore less likely to have noticed the tossed treats than the hand-offered treats. Caution should be exercised in interpreting this finding, though, because only one puppy was recorded as being frantic or overstimulated and only on one test. It is also possible that young puppies may be less aware of their surroundings, or that these puppies were more familiar with having treats offered by hand rather than tossed to them.

The final test of the second hypothesis examined puppies' behaviors during isolation. Specifically, puppies who received ENS were expected to show less fear and greater exploration than held and control puppies. Surprisingly, ENS puppies spent *more* time performing fearful behaviors during the isolation test than the other two treatment groups, regardless of location (i.e. breeder or distributor). Held puppies showed levels of fearful behavior intermediate between ENS and control groups, and control puppies spent the least time performing fearful behaviors during isolation. It is possible that this rather contradictory result is consistent with the explanation for the changes observed in puppies' responses to RYG+ before and after isolation. As stated above, handling during early life may have primed puppies that the presence and social support of a human would offset the stress of social separation. If this was the case, it would then be expected that handled puppies would have demonstrated a stronger reaction to separation than controls, which is indeed what was observed. Findings from prior ENS research may support this explanation. For instance, studies of ENS by Gazzano and colleagues (2008), found that puppies raised in a professional kennel vocalized later and less than those raised in a home, regardless of treatment. They hypothesized that, since puppies in their study who were raised in a professional kennel were more accustomed to lower levels of human interaction than those raised in a home, the conditions of isolation were less upsetting to them. Similarly, control puppies in the current study may also have been more accustomed to being alone than handled puppies, and not primed to expect human interaction in stressful situations. Thus, control puppies may have been relatively less disturbed by the conditions of isolation than ENS or held puppies, which could explain why they spent a smaller amount of time exhibiting fearful behaviors during the isolation test than their handled counterparts. The fact that responses were tiered –i.e., ENS puppies (more complex early handling) showed the greatest amount of fear behaviors, held puppies (simple handling) showed a moderate amount, and controls (least handling) spent the least time performing fear behaviors– appears to support this theory.

Another way to consider these results is in the context of the match/mismatch hypothesis of disease. This hypothesis relates to the ability of organisms to use early life conditions to make predictions about the future environment and prepare for it, often via altering homeostatic set points (Schmidt, 2011). When the early environment accurately predicts the later environment, they are "matched", and organisms tend to do well. On the other hand, "mismatched" early and late environments tend to lead to dysfunction, as demonstrated in rats by Santarelli and colleagues (2017). This theory also fits the observed results for puppies' fear behaviors during isolation in the current study. According to this reasoning, the consistent pattern of removal from the nest paired with human handling throughout the social separation, and performed during puppies' critical developmental period, would essentially have programmed puppies' nervous systems to expect a similar pattern of events in the future. When these puppies later experienced separation from their litter for the isolation test but did not receive human handling throughout the social separation, their previous situational adaptation was "mismatched" with the current challenge, leading to increased distress. Conversely, control puppies had not received this pattern of interaction in early life and thus did not display a "mismatched" response to isolation. Puppies from the held group, who received an intermediate level of handling, showed an intermediate distress response, between what was seen in ENS or control groups, which further supports this explanation. While handled puppies' responses seemed to indicate that they were more attached to humans, this should not be interpreted to mean that handled puppies necessarily suffer more separation-related issues. On the contrary, the general increase in affiliative responses on the RYG+ tests following isolation, and puppies' continued willingness to accept treats indicated that they were able to return to a positive emotional state following isolation.

Unlike some previous studies of ENS (e.g. Fox and Stelzner, 1966; Gazzano et al., 2008), and inconsistent with the results observed for fear behaviors, this study found no effect of treatment group on the amount of time puppies spent performing non-fearful or active behaviors during isolation, nor on the number of vocalizations they made. However, test location (i.e. breeder vs. distributor) which embeds the stress of transport, did have a significant effect on these parameters. As a group, puppies spent significantly less time performing non-fearful and active behaviors at the distributor than at the breeder. They also vocalized more at the distributor following transport than at the breeder prior to transport. These changes may have resulted from the overall stress of being in a new and unfamiliar environment when at the distributor, or residual effects of transport stress, both of which may have led to heightened awareness and reactivity.

Finally, none of the explanatory factors measured (treatment, test location, or their interaction) impacted the amount of time puppies spent performing stationary behaviors during the isolation tests. The reasons for this result are not yet entirely understood. Potentially, the cumulative effect of all the stressors experienced by puppies throughout the transport week was great enough that neither held nor ENS treatments sufficiently prepared puppies, and therefore limited the number of treatment effects observed. It is also possible that stationary behavior did not happen to reflect fear as well as expected for these puppies, and therefore did not change measurably between the breeder's kennel and the distributor.

Some limitations of the current study exist. For example, while the sample size for this study was robust in comparison to other ENS studies in dogs, a larger sample size would have increased the ability to detect statistically significant differences between treatment groups. Because of the nature of this study, enrolling and testing a truly representative sample size for the entire population of CB dogs would have been logistically and financially impossible to achieve due to limitations associated with recruiting criteria, the numbers of litters born within a week of each other, and the time investment required from caretakers.

While physiological metrics such as cortisol concentrations, heart rate, or heart rate variability would have been potentially valuable measures of welfare to add to those collected, there were several challenges associated with collecting such information with the puppies studied. These additional metrics were not included because it was of utmost importance to minimize puppies' exposure on test days to additional stressors that were not a part of the study's primary testing, in order to avoid confounding of the results. For measurements of cortisol concentrations specifically, the small physical size of many puppies in the study made it difficult and potentially problematic to try to obtain the repeated blood or saliva samples necessary. For measures of heart rate and heart rate variability, the additional handling and training required to acclimate puppies to testing equipment would have been a potentially significant confounding factor.

Finally, marking individual puppies for identification proved quite challenging. In large part, this was due to dams cleaning their puppies, thus removing the marks. It was not possible to use long-term colorings such as livestock paint on puppies, as this would impact their appearance and therefore their ability to be sold to the public. The short-term durability of marking crayons and pens used therefore required all puppies to be handled briefly each day to be re-marked. This introduced slightly more handling for control puppies than originally intended, but since the process was the same for all puppies, the differences between treatment groups with regards to overall amounts of handling were maintained. However, the overall increase in handling for all puppies may have masked some effects of treatment, as previously noted.

Despite the limitations described above, findings of this study suggest that ENS had very little effect on the health and behavioral measures assessed. It must be considered that the Bio Sensor protocol for ENS may not have the effects originally reported by Battaglia (2009), since neither the current study, nor the only other published study to evaluate Bio Sensor (Schoon and Berntsen, 2011) were able to replicate their results. In fact, the current study not only implemented the exact Bio Sensor protocols specified in the 2009 paper, but applied them for the maximum period (five seconds) specified in the Bio Sensor protocol, and for 21 days as opposed to 13 days as reported in Battaglia (2009). Schoon and Berntsen found no effect from the Bio Sensor protocols in their 2011 study of mine detection puppies but proposed that it might have been due to the elaborate socialization program already in place for their population of dogs masking the effect. From this, the current study would have been expected to show more substantial effects as the puppies in this study had a significantly less elaborate socialization protocol. Yet, ENS only showed minor effects. These assertions notwithstanding, it must be noted that the parameters measured in this study differed somewhat from those listed in Battaglia's report of Bio Sensor. According to the report, the military claimed that Bio Sensor ENS improved cardiovascular and adrenal performance, stress tolerance, and disease resistance (Battaglia, 2009). The report unfortunately did not specify how these benefits were measured. While the current study did not measure heart rates or adrenal function, measures of puppy health and responses to stress largely revealed no effects of treatment. Future work incorporating measures of heart and adrenal function could provide additional clarity as to whether Bio Sensor can benefit these organs.

Future studies should also consider investigating ENS in conjunction with assessments of attachment styles, as this could provide information on whether ENS is able to affect puppies' attachment styles in ways that are beneficial to them. The behavioral patterns that puppies demonstrated in this study (i.e. returning to affiliative and ingestive behaviors after isolation) may be evidence of secure attachment styles. Secure attachments are characterized by a preference for the caretaker and some distress upon separation, but the ability to return to a calm state when they are reunited (Udell and Brubaker, 2016). Studies of attachment originated in human parent-child research, and four main styles of attachment have been identified: Secure, Insecure-Avoidant, Insecure-Resistant, and Insecure-Disorganized (Fearon and Roisman, 2017; Udell and Brubaker, 2016; see also Solomon et al., 2019 for detailed descriptions of the styles). Recent research in dogs supports the existence of similar attachment styles between dogs and their caretakers (Konok et al., 2019; Solomon et al., 2019). Incorporating attachment theories into further ENS work, particularly when social isolations tests are used might be useful in providing further insight into dogs' responses to such stressors. It would likewise be sensible for future studies to include additional measures of recovery after testing, to elucidate the effects of ENS on puppies' return to baseline following a stress test. Measurements of activity levels, numbers of vocalizations, and a second group RYG test after puppies have been returned to their pens could all provide valuable additional information on the effects of ENS interventions.

CHAPTER 7. CONCLUSIONS

The objective of this study was to determine the effects of early neurological stimulation (ENS) on the welfare of puppies from commercial breeding (CB) kennels. Based on a review of previous literature regarding the welfare of kenneled dogs, intervention at the level of the kennel seemed a promising avenue for exploration. Relatively few studies have examined ENS in dogs, and no studies to date have explored ENS in this population. Therefore, this study aimed to examine ENS as a potential in-kennel intervention for puppies in CB kennels to alter their stress responses in ways which could improve their ultimate welfare outcomes as measured via health and behavioral metrics. The study was designed with three treatment groups (ENS, held, and control) as previously detailed, to enable exploration in this population of both the effects of Bio Sensor ENS exercises (outlined in Battaglia, 2009) and holding puppies (akin to Schoon and Berntsen, 2011) for 21 days within the first 4 weeks of life, in comparison to controls.

Contrary to the hypotheses put forth at the outset of this project, ENS alone did not have significant effects on the health or behaviors of puppies in this study. Findings indicated that ENS neither improved puppies' health nor decreased their responses to stress as purported. However, it did improve weights in female puppies. Additionally, the results suggest that both ENS and held treatments may have primed puppies to view humans as safe, and as potential sources of social support during stressors involving some degree of social isolation.

Collectively, the results of this study suggest that early handling may be useful in helping puppies to form positive associations with people, but it is not clear whether the ENS exercises themselves are more beneficial than simply holding puppies for a short period of time. In fact, while many test steps did not show significant differences between treatment groups, the data showed that puppies who were simply held for the same length of time as ENS-treated puppies often displayed greater increases in the number of puppies showing affiliative behaviors than either ENS-treated or control groups. This finding suggests that short daily sessions of just picking up and holding puppies may be a sufficient intervention to achieve social/behavioral benefits, and perhaps may even be a more effective form of ENS than Bio Sensor-exercise application.

APPENDIX A. ADDITIONAL FINDINGS FOR WEIGHT AND WEIGHT GAIN

Weight and weight gains were assessed for the first eight weeks and for the testing days at the breeder and the distributor using separate general linear mixed effects models (GLMEs) as described in the methods section.

Breed (χ^2 = 20.15, p = 0.001) and sex (χ^2 = 5.12, p = 0.024) both significantly affected puppy weights over the first eight weeks of life at the breeder and prior to the application of stressors. Overall, males weighed significantly more than females.

Post hoc testing (Tukey's Honest Significant Difference Test, Table A1) and visualization of the data (Figure A1), revealed that Pomeranian (z = -3.51, p = 0.006) and Toy Poodle (z = -2.92, p = 0.04) puppies weighed significantly less than Miniature Schnauzer puppies. Pomeranian puppies also weighed significantly less than Miniature Pinscher puppies (z = -2.94, p = 0.038).

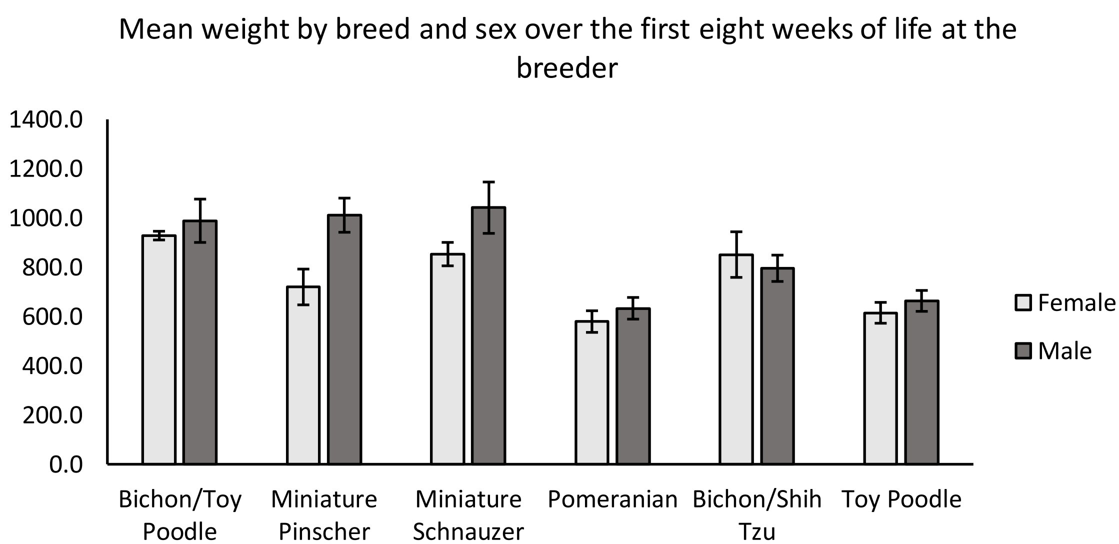

Figure A 1: Mean weights (grams) for females and males of each breed over the first eight weeks at the breeder's kennel, prior to application of stressors. Error bars represent standard errors of the means.

Table A 1: Results of post hoc Tukey's Honest Significant Difference Testing of breed differences in weight across the first eight weeks of life at the breeder prior to the application of stressors.

Linear Hypotheses:	Estimate	SEM	*z*-value	*p*-value
Miniature Pinscher - Bichon/Toy Poodle = 0	-49.77	152.45	-0.326	0.99949
Miniature Schnauzer - Bichon/Toy Poodle = 0	11.23	152.53	0.074	1
Pomeranian - Bichon/Toy Poodle = 0	-357.35	147.34	-2.425	0.14343
Bichon/Shih Tzu - Bichon/Toy Poodle = 0	-175.62	161.38	-1.088	0.88325
Toy Poodle - Bichon/Toy Poodle = 0	-315.9	152.2	-2.076	0.29388
Miniature Schnauzer - Miniature Pinscher	61	112.57	0.542	0.99424
Pomeranian - Miniature Pinscher = 0	-307.58	104.73	-2.937	0.03764
Bichon/Shih Tzu - Miniature Pinscher = 0	-125.84	123	-1.023	0.90791
Toy Poodle - Miniature Pinscher = 0	-266.13	111.15	-2.394	0.1541
Pomeranian - Miniature Schnauzer = 0	-368.58	105.15	-3.505	0.00582
Bichon/Shih Tzu - Miniature Schnauzer = 0	-186.84	124.57	-1.5	0.65805
Toy Poodle - Miniature Schnauzer = 0	-327.13	112.13	-2.917	0.0396
Bichon/Shih Tzu - Pomeranian = 0	181.74	117.3	1.549	0.62548
Toy Poodle - Pomeranian = 0	41.45	104.46	0.397	0.99869
Toy Poodle - Bichon/Shih Tzu = 0	-140.29	122.84	-1.142	0.86037

Neither treatment group nor any interaction had a significant effect on weight gain over the first eight weeks of life. Puppy weight gain was, however, significantly affected by week (χ^2 = 26.71, p < 0.001, Figure A2), sex (χ^2 = 5.62, p = 0.02, Figures A3 & A4), and breed (χ^2 = 23.74, p < 0.001, Figures A4 & A5). Overall, male puppies gained more weight than females. Post hoc testing (Tukey's Honest Significant Difference Test, Table A2) and visualization of the data (Figure A5), revealed that Pomeranian (z = -3.46, p = 0.007) and Toy Poodle (z = -3.92, p = 0.001) puppies gained significantly less weight than Miniature Schnauzer puppies. There was also a trend for Toy Poodle puppies to gain less weight than Bichon/Poodle (z = -2.75, p = 0.063) and Miniature Pinscher (z = -2.83, p = 0.052) puppies.

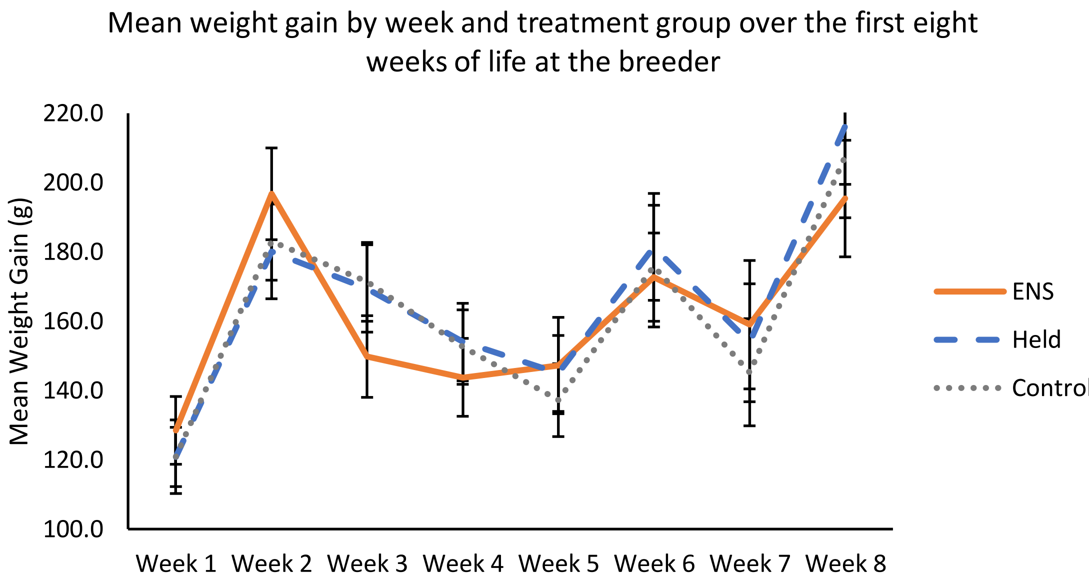

Figure A 2: Mean weight gain (grams) for each treatment group over the first eight weeks at the breeder's kennel, prior to application of stressors. Error bars represent standard errors of the means.

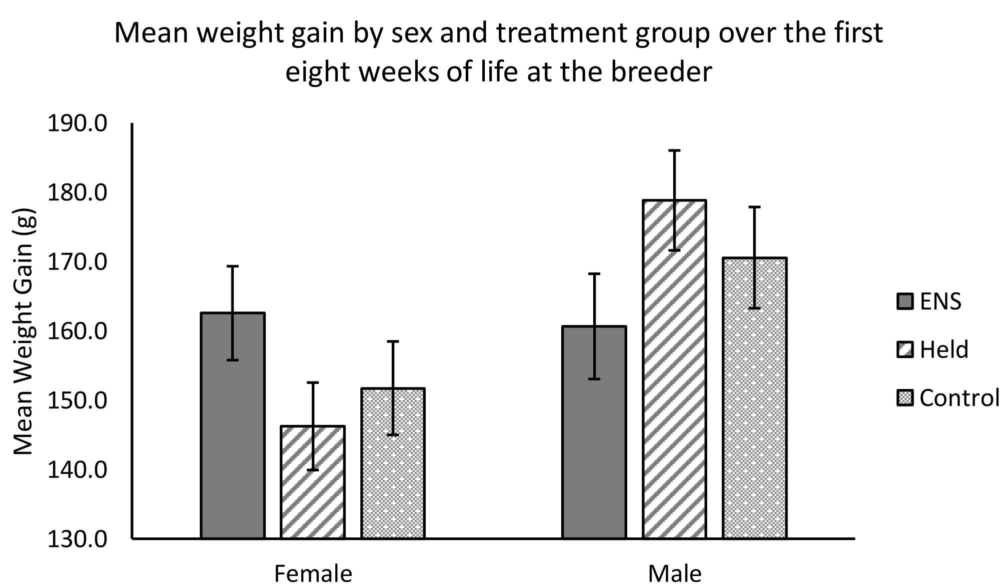

Figure A 3: Mean weight gain (grams) for female and male puppies in each treatment group over the first eight weeks of life at the breeder prior to the application of stressors. Error bars represent standard errors of the means.

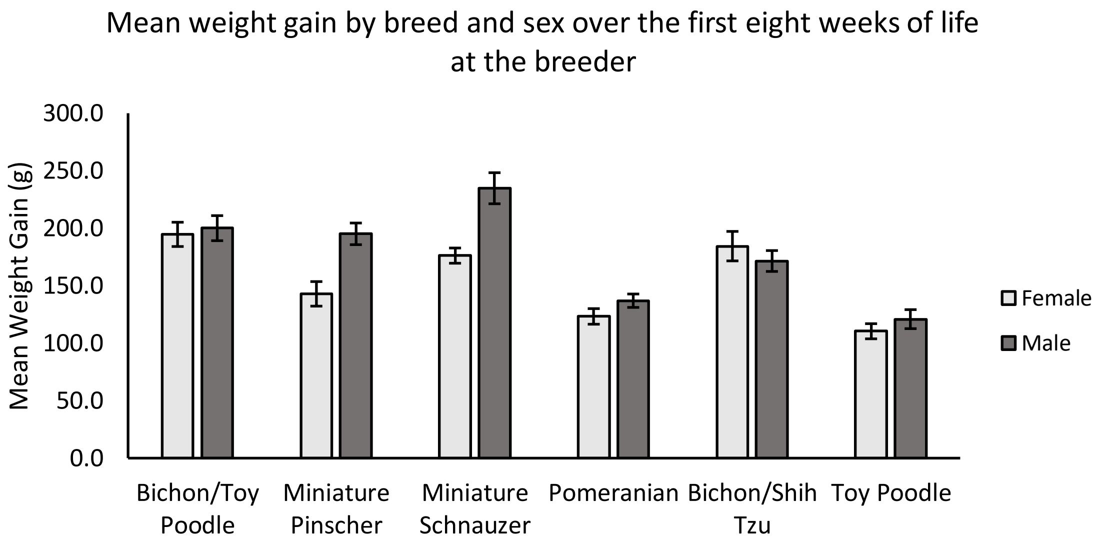

Figure A 4: Mean weight gain (grams) for female and male puppies in each breed over the first eight weeks of life at the breeder prior to the application of stressors. Error bars represent standard errors of the means.

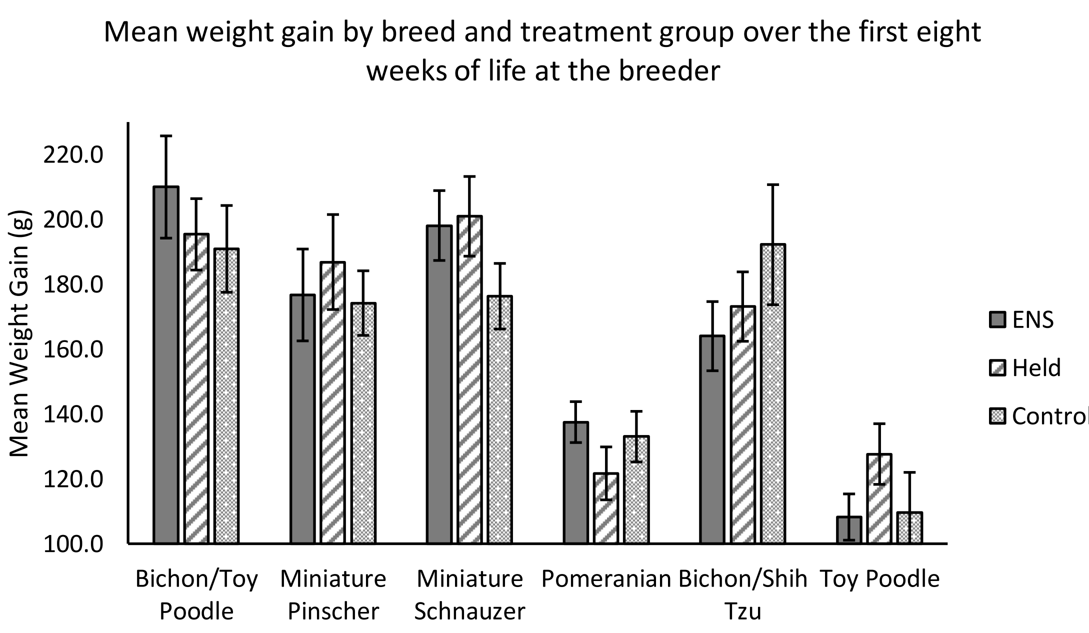

Figure A 5: Mean weight gains for each treatment group and breed throughout the first eight weeks of life at the breeder prior to the application of stressors. Error bars represent standard errors of the means.

Table A 2: Results of post hoc Tukey's Honest Significant Difference Testing of breed differences in weight gain across the first eight weeks of life at the breeder prior to the application of stressors.

Linear Hypotheses:	Estimate	SEM	*z*-value	*p*-value
Miniature Pinscher - Bichon/Toy Poodle = 0	18.401	29.894	0.616	0.98966
Miniature Schnauzer - Bichon/Toy Poodle = 0	6.243	29.866	0.209	0.99994
Pomeranian - Bichon/Toy Poodle = 0	67.499	28.899	2.336	0.17572
Bichon/Shih Tzu - Bichon/Toy Poodle = 0	28.811	31.522	0.914	0.94166
Toy Poodle - Bichon/Toy Poodle = 0	81.981	29.782	2.753	0.06322
Miniature Schnauzer - Miniature Pinscher	24.644	22.793	1.081	0.88659
Pomeranian - Miniature Pinscher = 0	49.098	21.306	2.304	0.18792
Bichon/Shih Tzu - Miniature Pinscher = 0	10.41	24.626	0.423	0.99824
Toy Poodle - Miniature Pinscher = 0	63.58	22.485	2.828	0.05176
Pomeranian - Miniature Schnauzer = 0	73.742	21.326	3.458	0.00707
Bichon/Shih Tzu - Miniature Schnauzer = 0	35.055	24.889	1.408	0.71714
Toy Poodle - Miniature Schnauzer = 0	88.225	22.51	3.919	0.00118
Bichon/Shih Tzu - Pomeranian = 0	38.687	23.536	1.644	0.56346
Toy Poodle - Pomeranian = 0	14.482	21.172	0.684	0.98331
Toy Poodle - Bichon/Shih Tzu = 0	53.17	24.608	2.161	0.25167

Treatment group did not have a significant effect on puppy weight during the test week ($\chi^2 = 0.83$, $p = 0.66$). Timepoint (i.e. breeder vs distributor, $\chi^2 = 121.72$, $p < 0.001$, Figure A6) and sex ($\chi^2 = 5.15$, $p = 0.023$, Figure A7) however, did significantly affect puppy weight during this time period. Puppies weighed more at the distributor than at the breeder, and as before male puppies weighed more than female puppies.

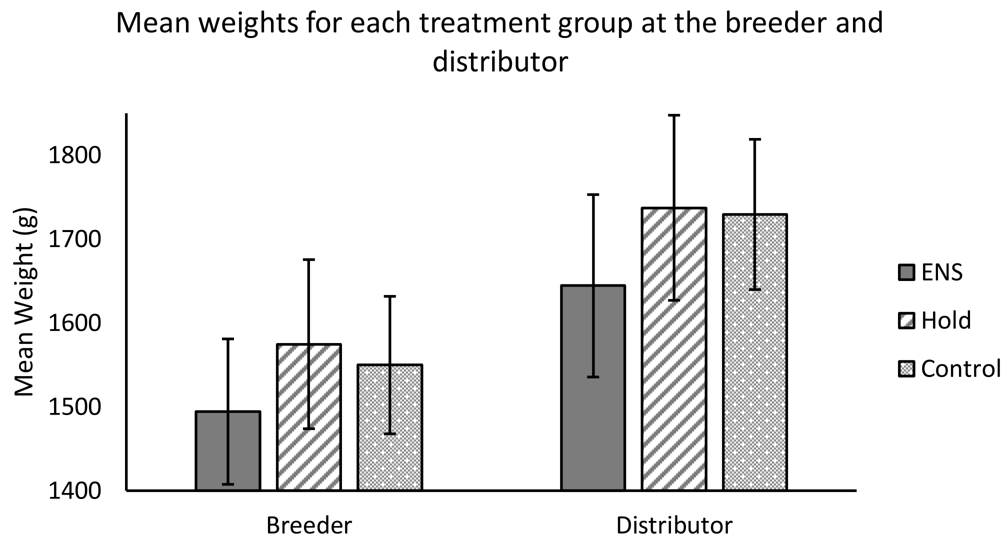

Figure A 6*:* Mean weights for puppies in each treatment group at the breeder's kennel prior to transport and at the distributor following transport. Error bars represent standard errors of the means.

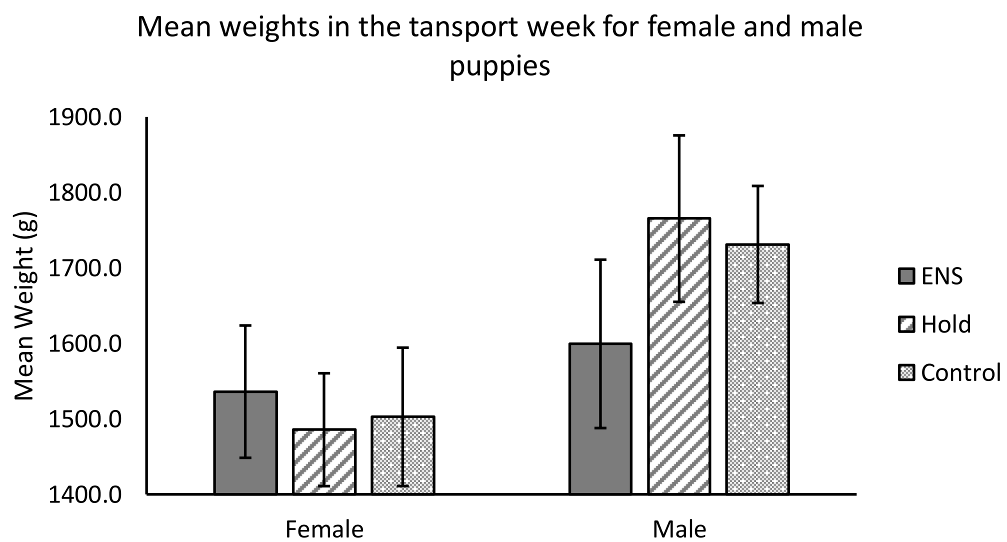

Figure A 7*:* Mean weights for male and female puppies of each treatment group across timepoints (i.e. breeder and distributor) during the transport week. Error bars represent standard errors of the means.

Again, breed significantly affected weight gain between the breeder and the distributor (χ^2 = 18.04, p = 0.003. Figure A8), and post hoc testing (Tukey's Honest Significant Difference Test, Table A3) revealed that Bichon/Toy Poodle puppies had significantly greater weight gains than other breeds ($p < 0.05$, Table A3).

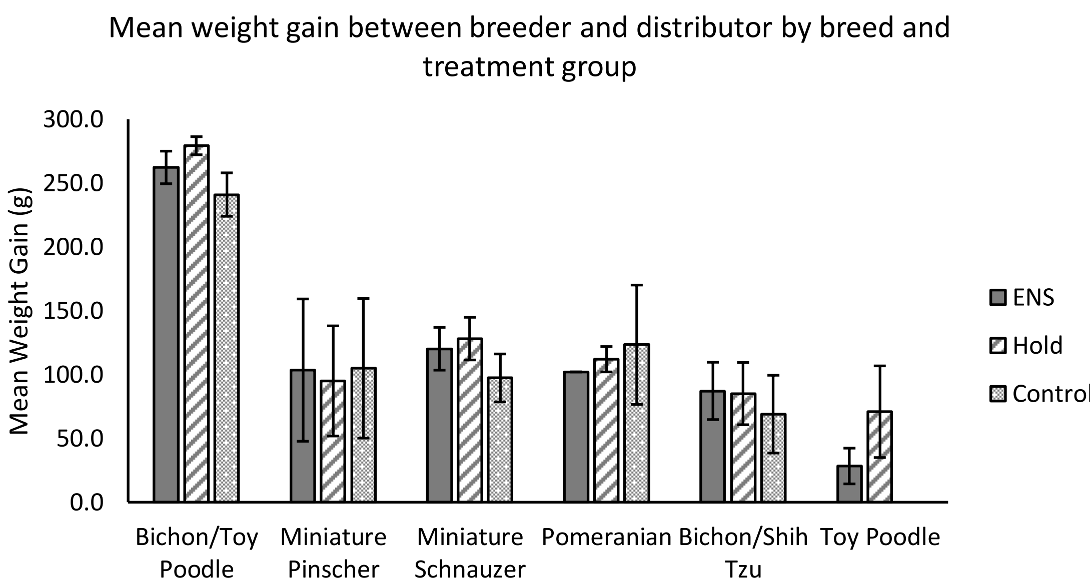

Figure A 8: Mean weight gains for each treatment group and breed between the breeder and distributor over the transport week. Error bars represent standard errors of the means.

Table A 3*:* Results of post hoc Tukey's Honest Significant Difference Testing of breed differences in weight gain between the breeder and the distributor over the transport week.

Linear Hypotheses:	**Estimate**	**SEM**	***z*-value**	***p*-value**
Miniature Pinscher - Bichon/Toy Poodle = 0	-161.749	49.542	-3.265	0.01366
Miniature Schnauzer - Bichon/Toy Poodle = 0	-142.893	49.33	-2.897	0.04276
Pomeranian - Bichon/Toy Poodle = 0	-156.752	54.142	-2.895	0.04262
Bichon/Shih Tzu - Bichon/Toy Poodle = 0	-188.68	51.986	-3.629	0.00382
Toy Poodle - Bichon/Toy Poodle = 0	-210.273	52.818	-3.981	<0.001
Miniature Schnauzer - Miniature Pinscher = 0	18.856	36.858	0.512	0.99565
Pomeranian - Miniature Pinscher = 0	4.997	42.439	0.118	1
Bichon/Shih Tzu - Miniature Pinscher = 0	-26.931	39.762	-0.677	0.98411
Toy Poodle - Miniature Pinscher = 0	-48.524	41.178	-1.178	0.84469
Pomeranian - Miniature Schnauzer = 0	-13.859	42.932	-0.323	0.99952
Bichon/Shih Tzu - Miniature Schnauzer = 0	-45.787	40.069	-1.143	0.86111
Toy Poodle - Miniature Schnauzer = 0	-67.38	40.802	-1.651	0.5595
Bichon/Shih Tzu - Pomeranian = 0	-31.928	45.285	-0.705	0.98098
Toy Poodle - Pomeranian = 0	-53.521	46.627	-1.148	0.85879
Toy Poodle - Bichon/Shih Tzu = 0	-21.593	44.085	-0.49	0.99646

APPENDIX B. ADDITIONAL DATA FOR BEHAVIORAL CATEGORIES MEASURED DURING ISOLATION

Table B 1: Means and standard deviations for amount of time each treatment group spent performing each behavioral category measured during the isolation test.

Behavioral Category	Treatment Group	Location	Mean (s)	Standard Deviation
Fearful	ENS	*Breeder*	54.74	22.37
		Distributor	51.74	26.94
	Held	*Breeder*	48.87	26.39
		Distributor	43.46	17.06
	Control	*Breeder*	38.33	25.48
		Distributor	40.60	24.46
Non-fearful	ENS	*Breeder*	109.23	24.24
		Distributor	111.77	32.84
	Held	*Breeder*	118.74	33.61
		Distributor	104.58	26.29
	Control	*Breeder*	128.41	24.39
		Distributor	107.46	20.41
Active	ENS	*Breeder*	81.67	24.82
		Distributor	68.21	19.15
	Held	*Breeder*	84.90	33.19
		Distributor	69.72	17.03
	Control	*Breeder*	93.28	24.75
		Distributor	75.63	16.20
Stationary	ENS	*Breeder*	38.63	33.44
		Distributor	44.49	24.81
	Held	*Breeder*	36.74	36.72
		Distributor	36.22	15.57
	Control	*Breeder*	37.48	31.42
		Distributor	35.72	21.73

Table B 2: Means and standard deviations for number of vocalizations from each treatment group measured during the isolation test.

Treatment Group	Location	Mean	Standard Deviation
ENS	Breeder	91.3	78.3
	Distributor	140.2	112.8
Held	Breeder	95.8	72.1
	Distributor	119.1	88.4
Control	Breeder	78.2	79.7
	Distributor	109.9	112.4

APPENDIX C. INTRACLASS CORRELATION COEFFICIENT DATA

Table C 1: Intraclass correlation coefficient (ICC) estimates for intra-rater reliability on coding of behaviors during isolation, including upper and lower 95% confidence intervals (CI), and p-values.

Behavior	ICC estimate	Lower 95% CI	Upper 95% CI	p-value
Fearful	0.897	0.893	0.901	<0.001
Non-fearful	0.865	0.860	0.870	<0.001
Stationary	0.907	0.903	0.910	<0.001
Active	0.916	0.913	0.919	<0.001
Vocalizations	0.963	0.962	0.965	<0.001

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