The effect of odours and synthetic pheromones on reducing stress-related behaviour in the confined domestic dog

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A Critical Commentary

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ABSTRACT

Confinement can be a stressor for dogs and can be inherently stressful if it occurs in unpredictable and novel environments, such as kennels or laboratories. Stress can have deleterious psychological and physiological effects on canine health and, therefore, it is important to mitigate stress to avoid compromising welfare. A potential approach to reducing stress in confined dogs is the use of olfactory stimuli. Odours and synthetic pheromones are purported to improve wellbeing through alleviating stress and promoting behaviours indicative of relaxation. However, research into odours and pheromones marketed as stress relief products is understudied in dogs, which limits conclusions in the canine and veterinary industries on whether they are effective and cost efficient. Limited research has been conducted on the effects of odours, and no published research exists for the purported stress relief odour Pet Remedy® in pet dogs. Furthermore, no research exists for the synthetic pheromone Adaptil® spray in sheltered dogs, which is advertised as an effective strategy to reduce stress. Moreover, studies relating to the efficacy of the Adaptil® diffuser are equivocal due to contradictory findings and a lack of supporting behavioural and physiological indicators of reduced stress in pet dogs. This critical commentary discusses the efficacy of odours and synthetic pheromones as tools to reduce stress-related behaviour in confined pet and sheltered dogs.

A critical reflection on four peer-reviewed evidence sources was conducted to establish their contribution to canine olfactory research, and to provide an evidence base for future odour and pheromone research to build on. Odours such as coconut *(Cocos nucifera)*, ginger *(Zingiber officinale)*, vanilla *(Vanilla planifolia)* and valerian *(Valeriana officinalis)* were found to significantly reduce levels of vocalisations and activity in sheltered dogs indicating reduced stress, compared to control conditions. Coconut and ginger also increased levels of sleeping behaviour, suggestive of induced relaxation. However, valerian when combined with other odours found in the stress relief product Pet Remedy®, did not reduce behavioural indicators indicative of a stress response in pet dogs tested in a 'laboratory like' standardised condition. The research demonstrates that when applied on their own, coconut, ginger, vanilla and valerian may have some value in reducing stress-related behaviour in confined dogs. These odours could offer shelters a wider odour choice, aiding variation and interest while avoiding

habituation to repeated use of the same odours over time. Increasing understanding of the efficacy of odours could help benefit canine welfare through improved management practices and stress reduction. Though, longer term research and larger sample sizes that account for individual variation in dogs such as breed, previous experiences and differences in temperament is required to confirm this.

The research demonstrates that the synthetic pheromone Adaptil® spray did not markedly influence behaviours indicative of stress in sheltered dogs. Further, the Adaptil® diffuser did not reduce stress-related behaviour or eye temperature (º C) and did not influence heart rate (HR), heart rate variability (HRV) or ear temperature (º C) in pet dogs tested in a 'laboratory like' standardised condition when separated from their owners. The value of Adaptil® and whether it is at all warranted or only useful within certain contexts has yet to be fully determined, and future research that utilises placebo controlled, blinded experimental designs over a prolonged period is needed. Until there is a stronger evidentiary basis supporting the use of Adaptil®, veterinary professionals should be cautious about recommending such products to clients considering there are financial and ethical complications if products are ineffective. Future opportunities exist to determine odour and pheromone efficacy in confined dogs through incorporating cortisol and neurological measures such as functional magnetic resonance imaging (fMRI) alongside cognitive, behavioural, integumentary and cardiovascular parameters to further objectively measure stress.

Table of contents

List of tables

List of figures

List of appendices

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Declaration of authorship and training

I confirm that all of the work presented in this doctoral thesis, including the selected publications and accompanying commentary (except where stated), is the original work of the author.

I confirm that none of the published body of work included within this portfolio of publications has been submitted for another academic award in this or any other institution.

I confirm that the necessary training requirements have been met (60 – 120 credits, of which at least 60 are at Level M). Sixty credits of prior learning was achieved within the Masters of Animal Behaviour, University of Exeter.

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CHAPTER ONE

AN INTRODUCTION TO STRESS, ODOURS AND SYNTHETIC PHEROMONE USE IN DOGS

1.1 Focus of the critical review

This commentary focuses on the efficacy of odours and synthetic pheromones in reducing stress-related behaviour in the confined domestic dog *(Canis familiaris)*. The evidence sources (Appendix 1) demonstrate the potential efficacy of stress relief products, which could be taken forwards to the canine and veterinary industries to inform clinical practice. For example, informing veterinary professional decision making when recommending products to clients and whether odours and pheromones are a worthwhile investment for rescue shelters in reducing stress-related behaviours.

1.2 What is stress?

Stress is a biological response caused by intrinsic or extrinsic negative effects, defined as stressors that threaten homeostasis (Amaya *et al.*, 2020). When animals encounter stimuli that is perceived as a stressor, the sympathetic nervous system (SNS) is activated (Hennessy *et al.*, 1997; Beerda *et al.*, 2000) and catecholamines (adrenalin and noradrenalin) are released (Hydbring-Sandberg *et al.*, 2004; Lensen, Moons and Diederich, 2019). This leads to mydriasis (dilated pupils), alertness, vasoconstriction (Romero and Butler, 2007), increased heart rate and blood pressure (Ostrander *et al.*, 2009). The SNS is excitatory and responsible for inducing arousal which facilitates a fight or flight response (Polgar *et al.*, 2019), in turn allowing the animal to respond and adapt to challenges and thus aiding survival (Appelhans and Luecken, 2006). Some animals may be able to adapt and ‘cope’ with acute stressors, induced by single stressors or short-term exposure (Davis and Levine, 1982; Horváth *et al.*, 2007; Chmelíková *et al.*, 2020). However, prolonged exposure to stressors can induce distress and this is when welfare problems become apparent (Beerda *et al.*, 1997; Sapolsky, Romero and Munck, 2000). When animals are exposed to chronic stressors, the hypothalamic-pituitary-adrenal (HPA) axis is activated resulting in the release of the corticotrophin releasing hormone (CRH) which signals the pituitary gland to release the adrenocorticotrophic hormone (ACTH) into the blood stream. The adrenocorticotrophic hormone travels to the adrenal glands and prompts glucocorticoids (GCs) to be released from the adrenal cortex (Hekman, Karas and Sharp, 2014). This subsequently leads to circulation of GCs, such as cortisol and corticosterone (Ostrander *et al.,* 2009). During prolonged exposure to stress, the HPA system may become dysregulated and HPA responses maintained (Bhatnager and Vining, 2003; Hennessy, 2013), resulting in deleterious metabolic changes (Mills *et al.,* 2012). Thus, stress becomes maladaptive and results in an individual no longer being able to adapt to the environment (Nelson, 2005). Prolonged exposure to stress results in exhaustion (Bombail, 2017) and excess glucocorticoid production such as cortisol, which can alter biological function (Moberg and Mench, 2000; Bombail, 2017). These physiological alterations result in long term damage, including growth conditions, thyroid dysfunction and psychological disorders which may predispose animals to disease (Beerda *et al.,* 1999; Glaser and Kiecolt-Glaser, 2005; Dawkins, 2021). This suggests that when there is an inability to adapt to and manage stress, both behaviourally and physiologically, suffering may occur and welfare is compromised.

A universally accepted definition of animal welfare is lacking globally, in part due to disagreement on whether animals can experience conscious feelings (Dawkins, 2021). Welfare encompasses multidimensional aspects and comprises of an animal's ability to cope with the environment, quality of life as perceived by the animal and positive and negative mental states, with welfare varying over time from very bad to very good (Dawkins, 1988; Fraser and Broom, 1990; Sumner, 1996; Bracke *et al.,* 1999; Botreau *et al.,* 2007). Dawkins (2021) defines good welfare as "a state where an animal is both healthy and has what it wants, to include liking what it has". There is emphasis on the importance of considering valence, particularly as positive valence is generally associated with emotions such as happiness, pleasure and enhanced welfare (Dawkins, 2021). Whether an animal perceives an experience as positive or negative is important in trying to establish what the animal wants and ultimately the likelihood of improving animal welfare (Dawkins, 2021).

1.3 Stress triggers in dogs

Stressors for domestic dogs *(Canis familiaris)* can be generated through temporary and permanent confinement when housed in rescue shelters, vets, and research, boarding and breeding establishments. These environments can be inherently stressful (Hetts *et al.*, 1992; Beerda *et al.*, 1999; Beerda *et al.*, 2000), due to often being unpredictable, novel environments that may involve social isolation from people and other dogs (Tuber *et al.*, 1999; Willen, Schiml and Hennessy, 2019). Stressors caused by confinement may include: a lack of environmental control and behavioural expression (Bombail, 2017), high levels of noise, (Hennessy *et al.*, 2001; Willen, Schiml and Hennessey, 2019) exposure to aggressive dogs and changes to routine (Willen, Schiml and Hennessey, 2019). Furthermore, relinquished dogs separated from their owner may experience symptoms indicative of separation anxiety (Stephen and Ledger, 2006). These stressors can be experienced in all confined dogs, whether they are confined in shelters or working and research establishments (Beerda *et al.*, 2000; Rooney, Gaines and Bradshaw, 2007; Rooney, Gaines and Hiby, 2009). As there is an abundance of research that suggests confinement can be stressful, and consequently has an impact upon welfare, there is a need to identify stress responses in dogs using effective parameters that provide a broad picture of stress. These can then be used to determine whether stress relief strategies are effective in reducing stress-related behaviour in the dog.

1.4 Identifying stress responses in confined dogs

Confinement can be stressful for some dogs (Tuber *et al.*, 1999; Hennessey *et al.*, 2001; Rooney, Gaines and Bradshaw, 2007; Rooney, Gaines and Hiby, 2009; Willen, Schiml and Hennessey, 2019). Dogs experience acute stress on entering restrictive environments such as kennels or laboratories (Rooney, Gaines and Bradshaw, 2007; Dudley *et al.*, 2015) and also chronic stress in response to prolonged confinement (Beerda *et al.*, 2000). Responses to stress can be identified through changes in behaviour and physiology, including the endocrine, cardiovascular and integumentary systems (Bergamasco *et al.*, 2010). Furthermore, emotional state of an animal and whether they perceive stress as eustress (positive) or distress (negative) can be inferred through cognitive bias testing (Mendl *et al.*, 2009).

1.4.1 Behavioural indicators of stress

Numerous acute and chronic behavioural responses to stress have been proposed to reflect poor welfare in dogs (Table 1) (Beerda *et al.,* 1997). Acute stress responses, which are overtly expressed, include: vocalisation, paw-lifting and trembling of the body during short periods of confinement (Beerda *et al.,* 1997; Beerda, Schilder, van Hooff, de Vries and Mol, 1998; Beerda *et al.,* 2000). Excessive panting, when not used as a cooling mechanism, can also be an indicator of acute stress (Beerda *et al.,* 1997; Protopopova *et al.,* 2014), particularly if arousal levels caused by stressors result in increased body temperature (Overall, 1997). More subtle behaviours include snout or lip licking, lowered body posture, excretion, and yawning (Beerda *et al.,* 1997; Beerda, Schilder, van Hooff, de Vries and Mol, 1998; Beerda *et al.,* 1999; Beerda *et al.,* 2000). These aforementioned behaviours can also be observed when dogs are exposed to chronic stressors such as social restriction, but typically chronic responses include behaviours, which lack an obvious goal or function such as: stereotypic repetitive patterns (e.g. pacing) and auto-grooming, including self-mutilation (Beerda *et al.,* 1999; Beerda *et al.,* 2000). Increases in behavioural expression such as excessive barking and activity have also been suggested as stress responses to both short and longer term confinement (Beerda *et al.,* 2000).

Table 1: A summary of stress-related behaviours in the domestic dog, including whether they are acute or chronic, confounding factors and the confined environments the behaviours have been observed in (adapted from Polgar *et al.*, 2019).

Behaviour	**Acute/chronic**	**Confounding factors for individual behaviour as a measure of stress**	**Confined environment**	**Authors**	**Confounding factors for behaviour as a general measure of stress**
Paw lifting	Both	Can be related to posture, anxiety, playfulness, conflict or submission.	Individually housed in laboratory kennels; experimental room.	Beerda *et al.*, 1997; Beerda *et al.*, 1999; Beerda *et al.*, 2000	• Context dependent. The same behaviours can serve different functions e.g. the function of barking may serve as intraspecific communication, a sign of boredom or to alert owners of a potential threat (Yin and McCowan, 2004). • Type and intensity of threat perceived by the
Body trembling	Both	Can be used as a warming mechanism in cold extremes.	Individually housed in laboratory kennels; experimental room.	Beerda *et al.*, 1997; Beerda, Schilder, van Hooff, de Vries and Mol, 1998; Beerda *et al.*, 1999; Beerda *et al.*, 2000	
Excessive panting	Both	Can also be used as a cooling mechanism, increases with rising levels of activity or arousal.	Individually housed in shelter kennels; experimental room.	Beerda *et al.*, 1997; Hiby *et al.*, 2006; Protopopova *et al.*, 2014	

Lip licking	Both	Can occur after drinking or eating, can be under-reported due to subtle nature. Can be used as a calming signal to reduce arousal.	Experimental room.	Beerda *et al.*, 1997;	dog will affect its behavioural response (Beerda *et al.*, 1998). • Individual coping strategies e.g. reactive or proactive response by individual (Blackwell *et al.*, 2010; Rooney *et al.*, 2016). • Previous experiences (Appleby, Bradshaw and Casey, 2002; Horowitz, 2004). • Genetic factors; genetic predisposition including breed and sex (Horowitz, 2004; Serpell
Excretion	Both	A biological need, may occur when not stressed. May be due to medical, emotional, cognitive, or management-related.	Individually housed in laboratory kennels.	Beerda *et al.*, 1999; Beerda *et al.*, 2000	
Yawning	Both	Can be due to tiredness, displacement behaviour.	Individually housed in laboratory kennels.	Beerda *et al.*, 1999	
Excessive barking / vocalising	Both	Can be due to arousal, play, fear, boredom, response to strangers, and communication with other dogs or rewarded. May be breed specific.	Individually housed in laboratory kennels.	Hetts *et al.*, 1992	

Prolonged activity	Both	Difficult to decipher emotional valence e.g. arousal due to excitement or fear. Can be influenced by kennel design (e.g. mesh design offers broader visibility and increased stimulation).	Individually housed in laboratory kennels.	Hetts *et al.*, 1992; Beerda *et al.*, 2000	and Hsu, 2005; Stephen and Ledger, 2006). • Environmental factors including experience, rearing environment, temperament and neuter status (Appleby, Bradshaw and Casey, 2002; Horowitz, 2004; Jones and Gosling, 2005; Serpell and Hsu, 2005; Harvey *et al.*, 2016).
Prolonged resting	Both	Difficult to decipher emotional valence e.g. whether resting, boredom or learned helplessness.	Experimental room.	Beerda *et al.*, 1997;	
Pacing	Chronic	Behaviour can be repetitive if rewarded. Can be a coping mechanism. Can be due to neurological disorders e.g. vestibular dysfunction.	Individually and group housed in shelter kennels and laboratory kennels.	Hubrecht *et al.*, 1992; Hubrecht *et al.*, 1993; Beerda *et al.*, 1999; Beerda *et al.*, 2000	
Self-mutilation	Chronic	Behaviour can be due to presence of ectoparasites.	Individually housed in laboratory kennels.	Beerda *et al.*, 1999; Beerda *et al.*, 2000	

When animals are repeatedly exposed to stressors, pathological behaviours can be expressed (Bombail, 2017). For example, an increase in arousal levels and vigilance behaviour can lead to changes in salience to stimuli such as perception (Bombail, 2017), which can provoke fear and anxiety. Fear is an unpleasant emotional reaction to the presence of an actual threat, while anxiety occurs due to the imagined presence of a threat (Rhudy and Meagher, 2000). Both fear and anxiety are widely reported behaviours in confined dogs, and can signify negative welfare (Wells and Hepper, 1998). Increase in activities such as displacement behaviour and stereotypies have been linked with anxiety in sheltered dogs (Cafazzo *et al.,* 2014). More time spent walking and standing when observed in a highly stimulating environment, such as a shelter, can also indicate a dog is less relaxed (Amaya *et al.*, 2020). These behaviours are associated with increased arousal and form key elements of the stress response (Winsky-Sommerer, Boutrel and de Lecea, 2005).

Conversely, it has been suggested when animals are inactive and unresponsive they exhibit anhedonia, which is a reduction in feeling pleasure and considered depressive like behaviour (Luna *et al.,* 2020; MacLellan *et al.,* 2021). However, findings from Harvey *et al.*, (2019) suggest dogs that are awake but motionless (ABM) in a shelter environment may be showing signs of boredom rather than depression. Dogs that spent the most time ABM were found to be motivated to obtain stimulation and showed increased interest in a Kong filled toy, considered a form of desired stimulation. A further study by Harvey *et al.*, (2020) found time spent ABM in sheltered dogs was not associated with negative judgement of ambiguity, which is considered a proxy for low mood. Dogs showed more optimistic tendencies, suggesting kennelled dogs did not show 'depressive like' behaviour (Harvey *et al.,* 2020). However, further more refined research is required to confirm these findings and should focus on how inactive behaviour is quantified and other potential affective states associated with ABM, including validation of a behavioural indicator of "boredom" (Harvey *et al.,* 2019; Harvey *et al.,* 2020).

Inactivity may be associated with high levels of stress (Beerda *et al.,* 1997). Apathetic behaviours such as excessive resting and sleeping can occur, which can be indicative of a state of "learned helplessness" (Wells, 2002). Learned helplessness can occur when animals are repeatedly exposed to stressors they are unable to evade. Over time the animal does not try to remove itself from the stressor, even when the opportunity of escape presents itself (Overmeier and Seligman, 1967; Wells, 2002). Confinement inevitably leads to an inability to retreat from stressors. Subsequent behaviour observed suggest that an animal may be unable to adapt to the lack of control over the environment (Wells, Graham and Hepper, 2002), which can have profound effects upon animal welfare.

1.4.2 Physiological indicators of stress

Physiological stress responses include: elevated heart rate (HR), decreased heart rate variability (HRV), increase in core eye temperature, decrease in ear pinnae temperature and the production of cortisol, all these having been observed when dogs are introduced to novel environments (Table 2) (Beerda *et al.,* 1997; Beerda, Schilder, van Hooff, de Vries and Mol, 1998; Beerda *et al.,* 1999; Beerda *et al.,* 2000; Bergamasco *et al.,* 2010; Travain *et al.,* 2015; Riemer, 2016).

Heart rate and HRV have been used to indicate distress, with HRV considered a useful measure of autonomic nervous system (ANS) activity (Polgar *et al.,* 2019). Heart rate variability, when used as a stand-alone measure in dogs, has been typically found to decrease in response to stressors (Wormald *et al.,* 2017; Polgar *et al.,* 2019). Although, it has also been found to decrease in response to situations perceived as pleasant (such as obtaining food rewards or petting) (Zupan *et al.,* 2016). Heart rate is also influenced by activity, posture and orientation (Maros *et al.,* 2008). This suggests that, when HR and HRV are measured alone, they may not be useful assessments in determining the emotional state of dogs. Physiological responses alone are non-specific to emotion and do not account for whether stimuli is perceived as positive or negative (e.g. fear, joy, pain). Changes in core eye temperature and ear pinnae have been used to indirectly measure stress in stress inducing contexts. For example, during veterinary examinations internal core eye temperature significantly increased in dogs, which has been found to correlate with an increase in core body temperature, indicating a stress response (Travain *et al.,* 2015). Decreases in temperature in peripheral body locations, such as ear pinna, have been recorded in response to owner separation, suggesting that decreased ear temperature is associated with stress induced by social isolation (Riemer *et al.,* 2016). However, arousal can also cause changes in eye and ear temperature and therefore may not reflect emotional valence. Therefore HR, HRV and eye and ear temperature should be paired with behavioural indicators where they can be used as supplementary physiological data that further support behavioural indicators of stress (Katayama *et al.,* 2016; Bombail, 2017; Polgar *et al.,* 2019). When a range of welfare indicators are used in combination, behavioural and physiological parameters such as HR, HRV and temperature can help triangulate the level of stress and, together, have value in evidencing stress responses in dogs.

Salivary, plasma and urinary cortisol has been found in significantly higher concentrations in kennelled dogs after one day (Clark *et al.,* 1997; Dudley *et al.,* 2015), compared to baseline measurements (Rooney, Gaines and Bradshaw, 2007;) and home environments (Hennessy *et al.,* 1997; Stephen and Ledger, 2006), indicating kennels can be perceived by some dogs as stressful environments. Salivary cortisol has been found to be highly correlated with plasma cortisol; a measure of HPA activity. Obtaining saliva is also a non-invasive procedure (Bennett and Haysson, 2010; Lensen *et al.*, 2015), reducing the risk of elevating the dogs' stress levels during saliva collection. However, cortisol can be influenced by time of day (Beerda *et al.*, 1996), age (Fratkin *et al.,* 2013; Cobb *et al.,* 2016), sex (Garnier *et al.,* 1990; Taylor *et al.,* 2000) and climate (Ahrens *et al.,* 2005; Beerda *et al.*, 1999). Therefore these factors should be considered when designing studies, during statistical analysis and when interpreting study findings (Chmelíková *et al.*, 2020). Repeated samples could be taken over time leading to a cumulative measure of cortisol and has been suggested to provide a more representative overview of how an animal is responding to stressors (Koolhaas, *et al.,* 2011). A further major limitation of cortisol is it measures arousal (intensity of stimulus) rather than valence (positivity/negativity) and assesses responses to stimuli rather than whether an animal perceives the stimuli as positive or negative (Dawkins, 2021). Therefore, it is difficult to assess any emotional component of stress using cortisol as a standalone measure.

1.4.3 Emotional State

While conscious emotions in animals cannot be assessed directly (e.g. animals cannot use language to tell us how they feel), neural, behavioural and physiological indicators can be used to infer how animals respond to stimuli that induce positive or negative emotional states. Developing an objective approach to assess affective states and stress in animals is considered a crucial aspect of improving animal welfare (Dawkins, 2008).

An innovative 'affective state' framework to help decipher animal emotion was proposed by Mendl *et al.*, (2009), which considers emotions on two dimensions, valence (positivity/negativity) and arousal (how intense an emotion is). Drawing on findings from human psychology, a 'cognitive biases' approach was developed to assess animal emotion by measuring affect-induced biases in decision making through the use of ambiguous stimuli (Mendl *et al.*, 2009). This approach trains animals to discriminate between a stimulus that is associated with a positive experience (e.g. food) and a negative experience (e.g. fear). An ambiguous stimulus is then presented and how the animal reacts towards the stimulus is recorded (e.g. latency to approach and frequency of response). This response allows researchers to gauge whether the ambiguous stimulus is perceived as positive or negative (Bethall *et al.*, 2015).

Changes in cognitive bias have been found to determine whether an individual is experiencing stimuli or events that are perceived as positive (motivated to approach e.g. food) or negative (avoidance e.g. fearful stimulus) and therefore reflect affective valence and welfare (Uccheddu *et al.*, 2018). This approach has been widely used to infer affective states of animals, including humans (Mogg and Bradley, 1998; Richards *et al.*, 2002; Mogg *et al.*, 2006), birds (Bateson and Matheson, 2007); rats (Harding *et al.*, 2004; Burman *et al.*, 2008; Hales, Robinson and Houghton, 2016), farm animals (Baciadonna and McElliot, 2015) and dogs (Titulaer *et al.*, 2013; Wells *et al.*, 2017; Uccheddu *et al.*, 2018; Duranton *et al.*, 2019).

Cognitive biases can be categorised into different types; judgement and attention biases, the former having been more widely researched (Crump, Arnott and Bethell, 2018). In judgement bias testing, animals are tested using non-verbal paradigms, for example whether they are more likely to perceive an ambiguous situation with a positive outcome (optimistic bias, indicative of positive-valence states) or a negative outcome (pessimistic bias, indicated as negative-valence states) (Paul *et al.*, 2005; Mendl *et al.*, 2009; Mendl *et al.*, 2010; Mendl, Burman and Paul, 2010; Crump, Arnott and Bethell, 2018). A benefit of this approach is it assesses an animals mood rather than simply an animals response to stimuli (Dawkins, 2021).

Researchers have utilised judgement bias testing in an attempt to ascertain the affective state of pet dogs during odour based nose work activities (Duranton and Horowitz, 2019). Duranton and Horowitz (2019) found dogs latency to approach an ambiguous bowl reduced after two weeks of daily nose work training compared to heelwork training, suggesting the activity increased positive judgement bias and therefore the dogs were more 'optimistic' when they received nose work training. The authors proposed that olfaction-based activities contribute to dogs' welfare. Researchers have also utilised judgement bias testing to attempt to ascertain the affective state of short and long term housed sheltered dogs, alongside cortisol as an indicator of welfare (Titulaer *et al.*, 2013). Although null urinary cortisol: creatinine results were reported and cognitive measures of affective states were found not to vary between short and long term housed shelter dogs (Titulaer *et al.*, 2013). The authors proposed that the judgement bias test may not have been sensitive enough to detect differences. This was likely due to the wide variety of dogs housed at the shelter and the study excluding fearful and aggressive dogs. The researchers may have missed differences where a negative welfare state may have been more overt in these individuals. In some cases, opposite effects have been reported (Ligaya *et al.*, 2016; Raoult, Moser and Gygax, 2017). For example, fearful sheltered dogs perceived ambiguous stimuli with a positive outcome compared to non-fearful dogs (Willen, Schiml and Hennessey, 2019) while a pleasant experience (food) in laboratory housed dogs prompted a pessimistic bias (Burman *et al.*, 2011). Human presence has been found to influence latency to reach a food bowl (Kis *et al.,* 2015; Müller *et al.,* 2012; Uccheddu *et al.,* 2018), with researchers suggesting a shorter latency to reach a food bowl when a researcher is located behind it. This may suggest that dogs are potentially more interested in the researcher, rather than the actual food bowl itself, which may explain the inconsistency in findings.

Judgement bias testing has limitations including the need for animals to be trained, which is time consuming (Crump, Arnott and Bethell, 2018). Furthermore, the influence of stress on learning can create selection bias where fearful or anxious dogs are excluded from research. This can lead to differences being missed between individuals with more positive-valence states being recorded compared to dogs in more negative welfare states who are removed from the study (Mendl *et al.,* 2009). Extinction of learned behaviour through removal of reinforcement in judgement bias tests can also reduce the likelihood that dogs will respond to repeat testing (Doyle *et al.,* 2010), while repeat testing can also lead to fatigue and a reduction in responses (Arasaradnam *et al.,* 2011).

Attention bias testing can also be utilised, where the amount of visual attention and where that attention is directed is measured e.g. towards one stimulus compared to another (Crump, Arnott and Bethell, 2018). Affect-driven attention biases (ADABs) have been suggested to determine specific emotions, motivations, aversions, and preferences in animals (e.g. Lee *et al.,* 2016; Monk *et al.,* 2018; Lee *et al.,* 2018). In humans, it is known that anxiety is associated with biased attention towards stimuli that is perceived as threatening (Bar-Haim *et al.,* 2007). Attention biases should be generalisable to non-human species, as attention is considered to be pre-consciously influenced by anxiety (Lee *et al.,* 2018) and is a survival mechanism which is influenced by emotion (Crump *et al.,* 2020). In sheep, differences in anxiety linked to stress have been assessed through attention bias testing using a threat (e.g. presence of a dog) and pharmacological manipulation (Lee *et al.,* 2016). Attention was modulated according to drug, with sheep receiving an anxiogenic showing more attention towards the dog than those that received an anxiolytic (Lee *et al.,* 2016). This suggests that attention bias can assess different levels of anxiety in sheep (Lee *et al.,* 2016). Lee *et al.,* (2018) used a very similar experimental design and found the same findings in cattle, supporting the findings of Lee *et al.,* (2016). Taken together, these studies show that attention bias is influenced by changes in emotional state and is proposed a valid tool for assessing animal welfare (Howarth *et al.,* 2021). A benefit of ADAB is little or no training is required and it can therefore provide a rapid insight into affective state of animals (Lee *et al.,* 2018). As such, it is proposed that ADABs are an effective tool to measure animal welfare and should be utilised in future animal welfare research (Crump, Arnott and Bethell, 2018).

A major limitation of cognitive bias testing is it does not directly measure an animals choice. Humans provide the animals with stimuli, rather than the animals choosing what stimuli they want or do not want to interact with which can influence biases. As such, this approach needs careful interpretation and consideration as a complementary approach alongside other more direct measures (Dawkins, 2021), such as behaviour, physiology, preference and motivation testing and also where researcher influence is removed, to improve reliability and validity (Burani, Pelosi and Valsecchi, 2022).

Table 2: A summary of physiological measures of stress in the domestic dog, sampling method and strengths and weaknesses (adapted from Polgar *et al.*, 2019).

Physiological measure	**Sampling method**	**Strengths**	**Weaknesses**
Plasma Cortisol	Blood sampling via venepuncture	Reliable measure of hypothalamic-pituitary-adrenal (HPA) activity (Van der Laan, Vinke and Arndt, 2022).	Invasive procedure: handling and restraint required which can cause and exacerbate stress (Kobelt *et al.*, 2003; Bennett and Haysson, 2010; Cobb *et al.*, 2016; Chmelíková *et al.*, 2020). Expensive to analyse. Temperature, diurnal and circadian patterns and activity can confound results (Cobb *et al.*, 2016; Chmelíková *et al.*, 2020).
Salivary Cortisol	Saliva sampling via cotton dental rope	Highly correlated with plasma cortisol; measure of HPA activity. Non-invasive (Bennett and Haysson, 2010; Lensen *et al.*, 2015). Using a cotton swab dipped in ginger using a 30s sampling period has been found	Using food to induce salivary flow can produce spurious results (Granger *et al.*, 2007). Can fluctuate according to circadian rhythm (Kikkawa *et al.*, 2003), activity level (Hiby, Rooney and Bradshaw, 2006), stressor intensity

		to significantly increase salivary flow (Meunier *et al.*, 2021)	(Beerda *et al.*, 1998) and experience (Haverbeke *et al.*, 2008).
Urinary Cortisol	Urine sample	Non-invasive. Urinary cortisol accumulates over time which reduces the confound of natural variation in cortisol levels (Bryan *et al.*, 2013; Titulaer *et al.*, 2013; Chmelíková *et al.*, 2020).	Can be difficult to obtain sample e.g. timing, diurnal patterns, temperature and activity levels can confound results (Stephen and Ledger, 2006).
Hair cortisol	Hair shaft sample	Non-invasive (Siniscalchi *et al.*, 2013; Heimbürge, Kanitz and Otten, 2019). Easy to obtain. Cortisol accumulates over time which reduces the confound of natural variation in cortisol levels (Heimbürge, Kanitz and Otten, 2019; Van der Laan, Vinke and Arndt, 2022).	Effectiveness of analysis can be impacted by hair colour (Bennett and Haysson, 2010; Svendsen and Søndergaard, 2014), brushing and sunlight exposure (Mesavcova *et al.*, 2017).
Fecal cortisol	Fecal sample	Fecal steroid metabolites are less affected by diurnal patterns, compared to plasma cortisol (Uetake	Can be difficult to obtain sample e.g. timing in pet dogs that are not confined.

		et al., 2016; Righi *et al.*, 2019). Cortisol indication is slightly longer than found in plasma and saliva samples (Polgar *et al.*, 2019).	
Heart Rate (HR)	Heart rate monitor fitted to the dog; transmitter and receiver unit	Portable. Non-invasive once fitted and habituated to equipment.	Activity and posture can confound results (Maros *et al.*, 2008) and if dog is not habituated to wearing a monitor (Polgar *et al.*, 2019). Individuals can react differently to wearing the equipment.
Heart Rate Variability (HRV)	Heart rate monitor fitted to the dog; transmitter and receiver unit	Heart rate variability considered a useful measure of ANS activity (Polgar *et al.,* 2019). Portable. Non-invasive once fitted and habituated to equipment.	Activity, posture and orientation can confound results (Maros *et al.*, 2008) and if dog is not habituated to wearing a monitor (Polgar *et al.*, 2019). Individuals can react differently to wearing the equipment.
Core Eye Temperature	Infrared thermal imaging (IRT) camera	Non-invasive (Polgar *et al.*, 2019). Restraint not required and can be	Some individuals display aversive behaviour towards equipment (Travain *et al.*, 2015). Measurements can be

		used remotely without a person present.	confounded by the environment (e.g. heat reflection, temperature, cleanliness of coat) (Whitham and Miller, 2016).
Ear Pinnae Temperature	Infrared thermal imaging (IRT) camera	Non-invasive (Riemer *et al.*, 2016). Restraint not required and can be used remotely without a person present.	Some individuals display aversive behaviour towards equipment. Measurements can be confounded by the environment (e.g. heat reflection, temperature, cleanliness of coat). Difficulty in obtaining measurements in some dogs (e.g. ear hair structure and ear shape) (Riemer *et al.*, 2016).

1.5 The importance of mitigating stress in dogs

It is important to mitigate stress where distress occurs in order to avoid welfare being compromised. Stress can increase the frequency of undesirable behaviours in dogs, and when these occur in a shelter environment, dogs can be viewed as undesirable to adopters, leading to a reduction in the likelihood of being rehomed (Wells and Hepper, 1992). Furthermore, if behavioural problems occur after adoption, the dog is more likely to be returned to the shelter (Wells and Hepper, 2000; Mondelli *et al.,* 2004). Lengthy stays in shelters, which are renowned as inherently stressful environments, can compromise a dog's welfare (Tod *et al.*, 2005), and lead to an increased risk of euthanasia if not rehomed (Tuber *et al.,* 1999). Relinquishment to a shelter also inevitably leads to separation from a care-giver, which has been found to contribute to stress experienced by some dogs during confinement (Beerda *et al.,* 1997; Hennessey *et al.,* 2001; Stephen and Ledger, 2006). Manifestations of stress such as fear and frustration are also reported in similar confined environments, such as boarding kennels and laboratories, and are indicative of poor welfare (Stephen and Ledger, 2006). As a result, there has been increasing interest from researchers to identify stress in confined dogs, and to develop strategies to improve wellbeing, such as: increasing positive emotions and, as a result, experiences (Bergamasco *et al.,* 2010).

1.6 The use of odours and synthetic pheromones as tools to reduce stress-related behaviour in dogs

One potential approach to reducing stress and improving wellbeing in confined dogs is the use of olfactory stimuli: the addition of odours or odour impregnated materials that are non-biologically relevant or pheromonal in nature (Swaisgood and Shepherdson, 2005; Wells, 2009). The goal of odours and pheromones is the same; they are used to target stress-related behaviours and are purported to alleviate stress and promote behaviours associated with relaxation (Wells, 2009). In contrast, both operate using different mechanisms of the olfactory system but, however, are dispersed in similar manners (Mills *et al.,* 2012). They are vaporised into the air using diffusers, or are applied onto impregnated materials such as cloths (Wells, 2009), avoiding direct contact with individuals. The simple nature of applying olfactory stimuli in a non-invasive way, and the potential to use odours and pheromones as a tool to reduce stress-related behaviour in dogs, has presented the field of olfactory research with an opportunity to improve domestic dog wellbeing.

1.6.1 Odours

An odour is a mixture of molecules (known as odourants) that trigger a behavioural and/or physiological response in the receiving animal (Meunier and Rampin, 2017). In vertebrates, odour chemicals are detected by the main olfactory epithelium (Meunier and Rampin, 2017). In dogs, odours are inhaled through the nose and travel up the nasal cavity, entering the olfactory recess that houses the genes responsible for olfactory receptors (Jenkins, DeChant and Perry, 2018). The recess consists of an olfactory mucous membrane, often termed as a spatial odour map (Uchida *et al.*, 2000). This membrane spreads across bony structures called nasal turbinates (Green *et al.*, 2012), and it is within these turbinates where millions of tiny olfactory hairs capture odourants. Odour molecules dissolve and bind to protein receptor cells found on the sensory neurons in the mucous membrane (Green *et al.*, 2012). They are then transformed into electrical signals that travel-via the olfactory nerve- to the olfactory centre of the brain, where this information is then processed (Figure 1). The olfactory epithelium is sensitive to particular chemicals, and this determines a dog's ability to detect certain odours that are specifically non-pheromone based e.g. plant derived odours (Mills *et al.*, 2012).

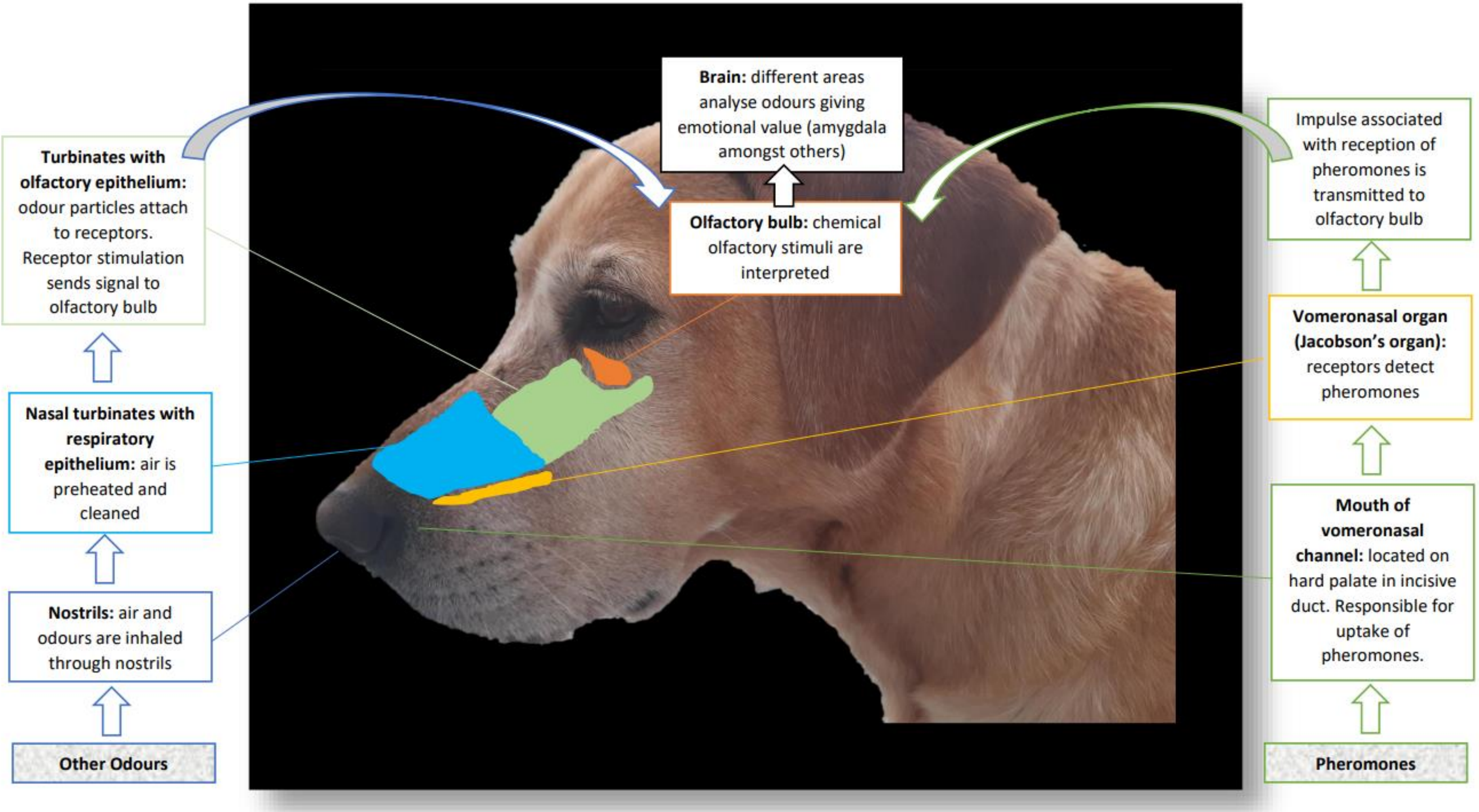

Figure 1: Canine odour and pheromone detection system (adapted from Kokocińska-Kusiak *et al.*, 2021).

A form of olfactory stimuli is the use of non-biologically relevant odours which are dispersed into the captive environment and inhaled (Bombail, 2017). Typically, odours are used in a therapeutic or stress relieving context, and include plant derived essential oils (Mills *et al.,* 2012), herbs, spices and synthetic fragrances (Clark and King, 2008). Odours have been found to influence behaviour in a range of captive species that include horses, rodents and zoo animals (de Almeida *et al.,* 2004; Komiya *et al.,* 2006; Ferguson *et al.,* 2013; Fay and Miller, 2015). In domestic dogs, research has been conducted on the effects of inhaled odours, but is largely restricted to odours that are commonly found to have anxiolytic effects on humans e.g: Lavender *(Lavandula angustifolia)*, Chamomile *(Anthennis nobilis)* and Valerian *(Valeriana officinalis)* (Itai *et al.,* 2000; Motomura *et al.,* 2001; Wheatley, 2001; Millot *et al.,* 2002; Cropley *et al.,* 2002; Becker *et al.,* 2014). Few studies have investigated the effects of odours on sheltered dogs, although studies that do exist largely report positive effects of the use of essential oils (e.g. Graham, Wells and Hepper, 2005; Amaya *et al.,* 2020). These studies have started to help build the evidence base to assist researchers in determining the efficacy of odours in reducing stress in confined sheltered dogs.

One of the first studies to investigate olfactory stimulation on the behaviour of sheltered dogs reported that Lavender *(Lavandula angustifolia)* and Chamomile *(Anthennis nobilis)* essential oils, known for their sedentary effects, reduced barking and activity in kennelled dogs which are perceived indicators of stress (Graham, Wells and Hepper, 2005). Any effects of time however could not be controlled for, due to the mesh kennel design and scent transfer to all dogs at the same time (Graham, Wells and Hepper, 2005). As such, a reduction in behaviour may have been time dependent, and could possibly have been outside of feeding times, where the dogs are prone to being more settled. More recently, lavender essential oil reduced arousal levels in sheltered dogs, although not to the same extent that music and dog appeasing pheromones associated with anxiolytic effects achieved (Amaya *et al.,* 2020). A reduction in arousal level is not surprising, considering the chemical compound terpineol found in lavender induces sleep in animals and humans (Buchbauer *et al.,* 1991). Odours such as lavender and chamomile may be beneficial in encouraging behaviours indicative of relaxation in dogs housed within shelters. This may result in improved welfare, in what is usually an over stimulating and stressful environment.

Other essential oils such as Rosemary *(Cymbopogon citrates)* and Peppermint *(Mentha piperata)* have been found to produce a contrasting effect compared to lavender and chamomile, with reports of increased vocalisations and alertness, thus suggestive of a stimulating effect (Graham, Wells and Hepper, 2005). This is an important finding because, although stimulating odours may be beneficial in some apathetic dogs through promotion of mental and physical stimulation, they may also induce stress in hyperactive dogs, which can negatively impact welfare (Graham, Wells and Hepper, 2005). Whether dogs adapted to the shelter environment over time could not be ascertained, as a control period at the end of the conditions was not implemented. However, this would be useful in future studies to determine whether length of time spent confined impacts behaviour. Further work is required to ascertain whether stimulating odours increase the abnormal stress-related behaviours that are linked with increased activity (Graham, Wells and Hepper, 2005). This will inform appropriate odour choice and application, consequently leading to improved management practices that are more likely to reduce stress within confined domestic dogs.

Odours such as Coconut *(Cocos nucifera)*, Ginger *(Zingiber officinale)* and Vanilla *(Vanilla planifolia)* have been less researched in dogs, but are reported to have potential welfare enhancing benefits in other captive species such as lions *(Panthera leo)*, sea lions *(Zalophus californianus)* and giraffe *(Giraffa camelopardalis rothschildi)* (Schuett and Frase, 2001; Clark and King, 2008; Hogan *et al.*, 2010; Fay and Miller, 2015), including decreases in stereotypic behaviour, suggestive of reduced stress (Samuelson *et al.*, 2017). In zoo animals, these odours have been found to enhance wellbeing by increasing species-specific behavioural repertoires and encouraging positive enclosure use (Schuett and Frase, 2001; Hogan *et al.*, 2010; Fay and Miller, 2015). However, these studies have small sample sizes (typically n = <10) and have not been tested in dogs, which consequently limits practical application in the canine industry. Further research is required to determine whether these odours also produce beneficial effects that are transferable to domestic dogs in captivity.

The common herb Valerian *(Valeriana officinalis)* is widely reported to have anxiolytic effects and a calming influence in humans (e.g. Wheatley, 2001; Cropley *et al.*, 2002) and non-human species e.g. mice and rats (Komori *et al.*, 2006; Hattesohl *et al.*, 2008; Murphy *et al.*, 2010). However, research for its effects upon dogs is scarce. Valerian is regularly used as a herbal remedy in the pet industry and forms the core anxiolytic ingredient of Pet Remedy®: a commercial, non-pharmacological intervention that is widely marketed as a stress relief product for pets (Pet Remedy, 2021). Smaller quantities of Vetiver *(Chrysopogon zizanioides)*, Basil Sweet *(Ocimum basilicum)* and Clary Sage *(Salvia sclaria)* form the rest of Pet Remedies® active ingredients, (Pet Remedy, 2021) having been reported as anxiolytics in rodents (Seol *et al.*, 2010; Rajasekhar, Kokila and Rajesh, 2014; Nemati *et al.*,2015; Nirwane *et al.*, 2015). There are many anecdotal reports of Pet Remedies® benefits, but only a small number of largely unpublished studies exist in pet dogs, cats and rabbits (Barrington, 2014; Unwin *et al.*, 2020; Pet Remedy, 2021). Given that Pet Remedies® active ingredient-valerian- has anxiolytic effects in other species, which points to welfare benefits, it seems prudent that any effects in confined pet dogs are published, where such effects may also be present.

There is a paucity of research that focuses on odours unlikely to be encountered in an animal's natural habitat, but that may be of potential value in enhancing captive canine wellbeing. Further research is warranted, and may widen the choice of stress reducing odours available for those involved in the care of captive dogs. Similarly, knowing which odours may have a stimulating, relaxing or indeed no effect, could help inform the decision-making process on the application of odours for those involved in caring for confined dogs. This will mean that some odours can be tailored for individuals, with potential benefits for canine wellbeing. Equally, where odours are found to have no effect, it may be more financially prudent for organisations to invest in, and implement, alternative stress relief measures or redirect funds to other areas of their work that strive to improve welfare.

1.6.2 Synthetic Pheromones

Pheromones are chemical signals that are used in intraspecific communication across a wide range of mammals, and are used as signals for social, sexual and parental communication (Coli *et al.,* 2016; Van den Berghe *et al.*, 2019). In dogs, pheromones are dispersed from the region of the ears, foot pads, genitalia, anal sacs or the intermammary sulcus, depending on their intended function (Mills *et al.,* 2012). An accessory signal, such as a distinctive odour, usually accompanies the release of the pheromone to help instigate detection by the receiving animal (Mills *et al.,* 2012). Pheromones are detected using a secondary olfaction system, the vomeronasal organ (VNO), also known as the Jacobson's organ (Mills *et al.*, 2012) (Figure 1). Pheromone detection is thought to be aided by the olfactory epithelia, although the epithelia is not the primary sensory target in pheromone detection (Leypold *et al.,* 2002; Mandiyan *et al.*, 2005; Mills *et al.,* 2012). The VNO nerve is responsible for information transference to the accessory olfactory bulb, and then to the amygdala through the limbic system (Pageat and Gaultier, 2003). This in turn induces either a behavioural or physiological response, and is key to regulating emotional processing in animals (Mills *et al.,* 2012; Santos *et al.,* 2020).

Pheromones regulate animal behaviour without involving conscious pathways (Christensen and White, 2000), although the specific function of only a limited number of mammalian pheromones have been identified (Frank *et al.,* 2010). In dogs, a focus on the region surrounding the mammary glands identified the mammary appeasing pheromone, also known as appeasines (Pageat and Gaultier, 2003). The appeasines are a mixture of esters of fatty acids including oleic, palmitic and linoleic acids combined with a species-specific signature, which are secreted by lactating bitches post parturition from the intermammary sebaceous glands (Pageat and Gaultier, 2003; Gaultier *et al.,* 2005; Mills *et al.,* 2012). Their primary function is to reassure and calm the puppies by helping them orient from the nest site into areas where the bitch has deposited the chemical, and so are perceived as safe by the puppies (Mills *et al.,* 2012).

A synthetic analogue Adaptil®, formerly known as Dog Appeasing Pheromone (DAP) (Adaptil; Ceva Santé Animale), has been developed to replicate the pheromones released by the bitch. To date, it is the only canine pheromone-based product sold in the United Kingdom (UK) (BSAVA, 2017), and is widely used commercially to regulate stress and modify undesirable behaviour in all ages of dogs (Mills *et al.,* 2012; Hewson, 2014; BSAVA 2017). Adaptil® is frequently used as a complimentary therapy, often alongside behavioural modification (Mills, 2012). It can be delivered by means of an electronic diffuser, collar or spray (Adaptil, 2020). The product works by changing the animal's perception of the environment and, in turn, its response to specific stimuli, by providing an environment that reassures the animal (Mills *et al.,* 2012). This creation of bias, rather than neurological control of behaviour, can replace anxiety resulting in an environment that helps facilitate behavioural modification (Mills *et al.,* 2012).

Using pheromones to manage behavioural problems and stress in dogs has been the subject of a number of studies over the years. However, studies are equivocal in their findings with many reporting contradictories (Mooney, 2020). A wealth of research has reported upon Adaptil's® stress reducing properties in clinically stress inducing environments. Improvements in behaviour have been reported during noise exposure to fireworks and sound induced fears (Sheppard and Mills, 2003; Levine, Ramos and Mills, 2007; Levine and Mills, 2008; Landsberg *et al.,* 2015), owner separation (Gaultier *et al.,* 2005), rescue shelters and post adoption (Tod *et al.,* 2005; Osella *et al.,* 2015; Amaya *et al.,* 2020), veterinary facilities and pre-operative stress (Mills *et al.,* 2006; Young-Mee *et al.,* 2010; Siracusa *et al.,* 2010), transport (Estellés and Mills, 2006), hospitalised dogs (Kim *et al.,* 2010), puppy training (Denenberg and Landsberg, 2008) and dogs undergoing surgery (Siracusa *et al.,* 2010). However, many of these studies lack blinded, placebo controlled and randomised clinical trials, which introduces biases affecting internal validity (efficacy) and external validity (effectiveness) (Frank *et al.,* 2010). Some researchers have reported no reduction in behaviours indicative of stress when military dogs were exposed to Adaptil® during the transition from foster homes to training kennels (Broach and Dunham, 2016). Equally, no reduction in stress-related behaviours has been found in long term kennelled dogs (Grigg and Piehler, 2015). More recently, Adaptil® and the success rates of similar products recorded by dog owners were only as successful as results described for a placebo effect (Reimer, 2020). Contrasting findings may be due to differences in the aforementioned methodological approaches and limitations, which are inherent in pheromone research (Frank *et al.*, 2010; Hewson, 2014). This makes it difficult to come to any firm conclusions regarding efficacy from the current literature base (Frank *et al.*, 2010).

A further limitation of pheromone research is the literature base, which has concentrated on reporting behavioural responses as a single measure of stress in dogs when investigating the effects of Adaptil®. Measuring behaviour as a single indicator of stress does not take into account any changes at the physiological level as a result of stress. When a range of welfare indicators are used in combination, behavioural and physiological parameters such as HR, HRV and temperature can help triangulate the level of stress and, together, have value in evidencing stress responses in dogs. Consequently, there is an opportunity to combine both physiological and behavioural parameters with blinded, randomised and controlled experimental designs. This research will help obtain a more objective and broader measure of stress in dogs and their responses to Adaptil®.

An area of pheromone research that still remains unknown is the efficacy of Adaptil® spray in reducing stress-related behaviour in sheltered dogs. Much of the existing body of research has focused on Adaptil® diffusers and collars. A limitation of diffusers and collars is that they can take up to 24 hours to diffuse into the environment, and so take a considerable amount of time to become effective (Adaptil, 2021). Contrastingly, a spray provides a rapid application, and may be more useful in eliciting a prompt effect in reducing stress-related behaviours, such as those observed when entering a kennel environment, a surrounding regarded as an acute stressor (Rooney, Gaines and Bradshaw, 2007; Dudley *et al.*, 2015). It seems prudent then, that research should investigate any effects that the Adaptil® spray has upon stress-related behaviours in confined sheltered dogs.

It is important to understand whether pheromones are effective, given that much of the current body of research reports equivocal findings (Mooney, 2020). Stress-related behaviour can impact welfare, and make husbandry practices harder for staff in organisations that care for dogs e.g. shelters, veterinary practices and laboratories (Hewson, 2014). Therefore, as organisations commonly use pheromones, they should be effective to ensure that they are a worthwhile investment (Hewson, 2014). A better understanding of Adaptil® could be used to inform the decision making of veterinary professionals when recommending such products to clients. Veterinary professionals working in the industry have indicated that they would like to provide their clients with evidence-based advice to support their practice (Buckley, 2019); however this is currently lacking. Furthermore, rescue shelters could use this information to evaluate the efficacy of pheromone products and assess whether the use of Adaptil® is an effective and cost-efficient method of reducing stress-related behaviour in dogs. This is especially important given that shelters are often run on restricted financial budgets.

1.7 Research aim

There is a lack of an evidence base in the use of odours and equivocal findings relating to the use of synthetic pheromones as effective strategies to reduce stress-related behaviour in dogs. Therefore, the overarching aim of the research was to investigate the effects of odours and synthetic pheromones on reducing stress-related behaviour in confined pet and sheltered dogs, with there being potential to improve captive canine welfare.

1.8 Research objectives

The work presented aimed to achieve the following research objectives:

1) To investigate whether Pet Remedy®, a Valerian based odour, reduced behaviours indicative of stress in confined pet dogs tested in a 'laboratory like' standardised condition.

2) To investigate whether odours (vanilla, coconut, ginger and valerian) affect the behaviour of confined sheltered dogs.

3) To investigate whether Adaptil® spray, a synthetic pheromone, reduced vocalisation intensity and frequency of stress-related behaviours of confined sheltered dogs.

4) To investigate whether Adaptil® diffuser reduced behavioural and physiological stress responses in confined pet dogs when separated from the owner and tested in a ‘laboratory like’ standardised condition.

1.9 Structure of the thesis

The underpinning research of this submission focuses on four research projects and their outputs (Tables 3 and 4, Appendix 1). They share a common focus: investigating the efficacy of olfactory stimuli that are purported to reduce stress-related behaviour in captive, domestic dogs. In total, four works published between 2016 and 2020 are submitted, three of which were led by the author of the submission (the author supervised one of the projects in their capacity as dissertation supervisor). The works are submitted alongside a critical commentary that explores the efficacy of odours and synthetic pheromones in reducing stress-related behaviour. The submission also includes an overview of wider research achievements to date, as well as a reflection on personal development. An introduction to the use of odours and synthetic pheromones, current evidence base and also the research aims and objectives are stated in this chapter. Chapter two introduces the odours Valerian and Pet Remedy®, and examines further opportunities to investigate odours in dogs and the need for efficacy data in the field of olfactory research. Chapter three investigates the odours coconut, ginger and vanilla and their potential value in relieving stress in dogs. Chapter four introduces pheromonatherapy and examines the efficacy of Adaptil® in reducing stress-related behaviour in dogs. This chapter also discusses methodological limitations of Adaptil® research. Chapter five evaluates behavioural and physiological parameters of stress and reviews the use of these parameters to objectively measure the effects of Adaptil® in reducing stress in dogs. Chapter 6 critically assesses odours and synthetic pheromones in reducing stress-related behaviour in dogs and discusses limitations, challenges and the future of odour and pheromone research. The thesis ends with a summary of the conclusions of the research.

Table 3: Citations of published works submitted in support of DPhil by publication award; including validation, number of citations and journal esteem

Evidence Source	Citation	Type of research validation	Citations and Journal Impact Factor**
1	**Taylor, S**. and Madden, J. (2016) 'The effect of pet remedy on the behaviour of the domestic dog *(Canis familiaris)*', *Animals*, 6(11), p.64.	Single blind peer review	Citations: 4 Impact Factor: 1.654
2	Binks, J., **Taylor, S**., Wills, A. and Montrose, V.T. (2018) 'The behavioural effects of olfactory stimulation on dogs at a rescue shelter', *Applied Animal Behaviour Science*, 202, pp.69-76.	Single blind peer review	Citations: 14 Impact Factor: 2.448
3	Hermiston, C., Montrose, V.T. and **Taylor, S**. (2018) 'The effects of dog-appeasing pheromone spray upon canine vocalizations and stress-related behaviors in a rescue shelter', *Journal of Veterinary Behavior*, 26, pp.11-16.	Single blind peer review	Citations: 15 Impact Factor: 1.975
4	**Taylor, S**., Webb, L., Montrose, V.T. and Williams, J. (2020) 'The behavioral and physiological effects of dog appeasing pheromone upon canine behavior during separation from owner', *Journal of Veterinary Behavior*, 40, pp.36-42.	Single blind peer review	Citations: 7 Impact Factor: 1.975

Note: See Appendix 1 for links to copies of submitted works. Single blind peer review = the author does not know who the reviewers are, but the reviewers are aware of who the author is (Wiley, 2020). **Citations according to Google Scholar and Impact Factor up to 03/12/21.

Table 4: Author contribution submitted in support of DPhil by publication award

Evidence Source	Collaborative Project	Lead Researcher	Research Team	Research Method	Data Collection		Analysis		Interpretation of Results		Write Manuscript		Dissemination			
					Pilot	**Main**	**Lead**	**Team**	**Lead**	**Team**	**Lead**	**Team**	**J**	**P**	**LA**	**RI**
1	N	Y	Y	OBS	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y		Y
2	N	N	Y	OBS						Y		Y	Y		Y	
3	N	Y	Y	OBS			Y	Y	Y	Y	Y	Y	Y	Y		
4	N	Y	Y	OBS			Y	Y	Y	Y	Y	Y	Y		Y	

N = No, Y = yes, confirming personal contribution, OBS = Observational, J = Journal article, P = Poster, LA = Lay Article, RI = Radio Interview

CHAPTER TWO

VALERIAN AND PET REMEDY®

2.1 Focus of the critical review

Limited research has been conducted on the effects of Valerian *(Valeriana officinalis)* in dogs. Valerian is widely purported to induce an anxiolytic effect suggestive of relaxation and positive welfare in captive mammals, including mice and rats (Komori *et al.,* 2006; Hattesohl *et al.,* 2008; Murphy *et al.*, 2010). Therefore, investigating any anxiolytic effects in dogs which may have similar welfare benefits seems prudent, considering dogs are invariably subjected to stressors when housed in captivity (e.g. kennels and laboratories) (Hetts *et al.,* 1992; Beerda *et al.,* 1999; Tuber *et al.,* 1999;; Beerda *et al.,* 2000; Willen, Schiml and Hennessy, 2019). Chapter 2 introduces Valerian as well as Pet Remedy®, a valerian-based odour widely sold on the pet market as a non-pharmacological stress relief for pets. Opportunities have been identified for further investigation on the effects of valerian-based odours, specifically focusing on the commercially marketed product Pet Remedy®. The importance of testing odours, as well as the need for data (which is significantly lacking for non-pharmacological stress relief products), is also discussed.

2.2 Introduction to Valerian *(Valeriana officinalis)*

Valerian is a herb that is derived from the root of the plant *Valeriana officinalis* (Murphy *et al.,* 2010), and is widely used to treat anxiety across a plethora of animals including pigs, mice, rats (Peeters *et al.,* 2004; Komori *et al.,* 2006; Khom *et al.,* 2010; Murphy *et al.,* 2010; Casal-Plana *et al.,* 2017) and also humans (Gupta, Srivastava and Lall, 2019). Valerian's anxiolytic effect is believed to be caused by the affinity between valerenic acid (an extract from the root) and gamma amino butyric acid (GABA) receptors (Murphy *et al.,* 2010). Valerenic acid binds with $GABA_A$ receptors, thus resulting in a stimulation process that produces chloride channels of which induce an inhibitory or 'calming effect' upon the nerve (Komori *et al.,* 2006; Murphy *et al.,* 2010). Other valerian extracts may also inhibit GABA transaminase activity, which prevents the enzymatic breakdown of GABA in the central nervous system (CNS) (Ortiz, Nieves-Natal and Chavez, 1999; Murphy *et al.,* 2010), resulting in a further state of calm (Santos *et al.,* 1994). Valerian is considered a safe herb by the US Food and

Drug Administration (FDA) (Wynn and Fougere, 2006), and is widely used in the pet industry as an alternative to pharmacological drugs, which are prone to side effects (Gupta, Srivastava and Lall, 2019).

2.2.1 Pet Remedy®

Valerian is the active ingredient of Pet Remedy® (Unex Designs Ltd., Torquay, Devon, UK), a commercial non-pharmacological intervention that is widely available in pet stores and veterinary practices (Pet Remedy, 2021). Pet Remedy® is advertised as a “natural de-stress and calming” product for pets; comprising of mammals, birds, and reptiles including cats, dogs, rabbits, rodents, horses and parrots (Pet Remedy, 2021). Pet Remedies® ingredients consist of a concentration of 5.37% essential oils formulation: Valerian and smaller quantities of Vetiver *(Chrysopogon zizanioides)*, Basil Sweet *(Ocimum basilicum)* and Clary Sage *(Salvia sclaria)* (Pet Remedy, 2021). The oils are mixed with 7.85% water and 86.78% Di Propylene Glycol (DPG) (Pet Remedy, 2021). The product can be applied using an electronically heated plug-in diffuser which disperses Pet Remedy® into the environment, via a spray, applied either on bedding or clothing or a wipe applied on a bandana (Pet Remedy, 2021). There are positive anecdotal reports of Pet Remedies® stress relieving benefits for pets (Unwin *et al.,* 2020). However, to date, limited published clinical studies exist that test the efficacy of Pet Remedy®.

One article which has been published investigated whether Pet Remedy® ameliorated fear of handling in domestic rabbits *(Oryctolagus cuniculus)*. When rabbits were exposed to Pet Remedy® in a novel arena test, decreases in heart rate were reported when handled by an experimenter, suggesting Pet Remedy® potentially encourages relaxed behaviour (Unwin *et al.,* 2020). While the authors employed a double-blind placebo-controlled study design, measures such as scented masks and nose clips may have been insufficient to fully blind the tester from Pet Remedies® distinctive smell, derived from the valerian component of the product (Pet Remedy, 2021). Similarly, the rabbits may have responded to the smell. The study lacked full blinding which may have introduced observer bias, limiting conclusions drawn on the effectiveness of Pet Remedy®. It is a challenge in all areas of olfactory research to ensure adequate blinding of researchers, given the nature of research relating to odours and the ability to discriminate odour conditions versus a placebo or no odour control. The problem of conducting blinded studies with valerian is particularly ubiquitous, given its strong odour (Krystal and Ressler, 2001). Moreover, it is possible that individual effects of the herbs used in Pet Remedy® may influence behaviour. However, it is also impossible to disentangle the influence of the other active components of Pet Remedy® (e.g. vetiver, basil and sage), as well as those effects induced when valerian itself combines in a single product. Because of this, it would be beneficial to test valerian and other odours on their own individual merits.

The effects of Pet Remedies® key component, valerian, has been reported in other species. In human research, valerian has been found to reduce physiological reactivity during stress (e.g. Cropley, 2002). Though, a placebo was not used which limits whether valerian had any effect, therefore disputing its credibility. Using a placebo is key in olfactory research to ensure any 'placebo effects' are measured, which is where there is an observable improvement that is not attributable to the odour being tested (McMillan, 1999). Similarly, Wheatley (2001) reported a decrease in insomnia when humans were given valerian but did not implement double-blinded or placebo-controlled trials which potentially introduces observer bias, and questions whether the treatment actually had any effect.

Further, in mice and rats, inhaled valerian was found to have anxiety reducing properties (Komori *et al.,* 2006; Murphy *et al.,* 2010). In pigs, decreased heart rate was recorded when subjected to transport induced stress with valerian given orally (Peeters *et al.,* 2004). More recently, valerian has been found to influence sheltered domestic cats *(Felis catus)* by stimulating behaviour (Bol *et al.,* 2017), which contrasts previous research citing anxiolytic effects in other species. The delivery mechanisms of exposure differed (inhalation versus oral administration), which may have altered pharmacokinetics and subsequently the susceptibility and effect of valerian. Further, species differences in pharmacokinetics have been found, and may account for these contrasting findings (Toutain, Ferran, Bousquet-Mélou, 2010).

Future research on odours that relieve stress in all species would be beneficial, in particular on species housed in confinement, such as dogs, who frequently find restrictive environments (such as shelters and laboratories) challenging. It is key that research should include placebo-controlled and fully blinded experiments, as these are currently lacking in the field. Placebo designed experiments may result in more conclusive findings relating to the effects of valerian on stress (Cropley *et al.*, 2002), while double-blind trials would remove any bias towards results. Future research undertaken in controlled environments is also required. To address limitations of current research, screens should be used to block odours from reaching experimenters, naïve experimenters should be used to reduce observer bias and placebo-controls should be introduced to account for any placebo effects. This will enable valerian-based stress relief products to be objectively and rigorously tested.

Efficacy data in the field of non-pharmacological products is severely lacking (Unwin *et al.*, 2020). Limited published clinical studies exist that test the efficacy of the valerian-based stress relief product Pet Remedy®. Research in rabbits (e.g. Unwin *et al.*, 2020) shows positive behavioural effects of Pet Remedy® in reducing fear which enables knowledge transfer relevant to rabbit welfare, however it limits generalisation to other species such as dogs. There are no published data existing for dogs and the effects of Pet Remedy® on behaviour, despite anecdotal reports of its effectiveness at reducing stress and being widely used in the pet industry (Gupta, Srivastava and Lall, 2019; Pet Remedy, 2021). Given that dogs often experience fear and stress in captivity, research on the efficacy of Pet Remedy® in reducing stress is warranted.

A lack of empirical evidence may pose potential problems for researchers, veterinary professionals and those using or recommending stress relief products. Products that are ineffective may drain valuable funds and potentially waste money (Unwin *et al.*, 2020), which could be better spent on products where data supports their effectiveness using a strong evidentiary basis, as opposed to anecdotal reports. This is particularly relevant in dog shelters, where funds are often restricted. Furthermore, if products that are ineffective are being used as attempts to reduce stress-related behaviour (this having been linked with behavioural problems), the issue may become intensified. This causes concern as, if behavioural problems are left untreated, they can escalate, and potentially result in the animal being euthanised (Miller *et al.,* 1996; Mondelli *et al.,* 2004; Gonzalez Martinez *et al.*, 2011). More effective treatments may also be underutilised or overlooked, leading to a delay in usage (Unwin *et al.,* 2020). There is a need for more empirical research to be undertaken to counteract these potential issues, with future research building upon methodologically rigorous studies where anxiolytic odour effects are known (e.g. valerian) but less widely tested odours as well, to determine whether they may also have potential benefits to animal welfare.

Within Evidence Source 1 and Appendix 1, the efficacy of Pet Remedy® will be explored by gathering data from confined pet dogs tested in a 'laboratory like' standardised condition. A repeated measures, double blinded, placebo-controlled study design will be utilised and taken forward to address limitations of current research that underpin the evidence base for future odour research to build on. The impact of this research can then be used as knowledge transfer to inform at all levels, including veterinary professionals and those involved in the care of all captive dogs. Not only would this inform professionals on the efficacy of odours in reducing stress, but the resultant effect could also potentially benefit canine welfare through improved management and stress reduction practices.

2.3 EVIDENCE SOURCE 1

Taylor, S. and Madden, J. (2016) 'The effect of pet remedy on the behaviour of the domestic dog *(Canis familiaris)*', *Animals,* 6(11), p.64.

2.3.1 Summary:

Taylor and Madden (2016) (Appendix 1) aimed to investigate whether the valerian-based product Pet Remedy® reduced behaviours indicative of acute stress in dogs already reported as suffering from stress-related disorders. Twenty-eight pet dogs were tested in a 'laboratory like' standardised condition, which was considered a brief (30 minute), non-familiar, "stressful" environment that would induce an acute stress response (Dhabhar, 2009). Behavioural responses of the dogs were video recorded using a double blind, placebo-controlled, and counterbalanced repeated measures design. Dogs were exposed to both a placebo and Pet Remedy® plug-in diffuser for 30 minutes with an intervening period of approximately 7 days between conditions. There were no significant differences ($p > 0.05$) in a reduction of stress-related behaviour in either the Pet Remedy® or placebo condition. The findings suggest that Pet Remedy® did not reduce behavioural indicators indicative of a stress response.

2.3.2 Rationale:

Pet Remedy® is widely available in pet stores and veterinary practices and is used by veterinary professionals, rescue shelters and the general public (Pet Remedy, 2021). Despite being widely used with anecdotal reports of its effectiveness in reducing stress-related behaviour, no published studies exist for pet dogs. In captive rabbits, Pet Remedy® had been purported to reduce behavioural indicators of fear and stress (e.g. Unwin *et al.*, 2020), but no work had been undertaken in dogs who may also suffer from stress in captivity. This study was undertaken to address the lack of published clinical studies and efficacy data relating to Pet Remedy® in reducing stress-related behaviour in pet dogs. This study addressed the gap in knowledge which can be used to inform at all levels, including veterinary professionals and those involved in the care of all captive dogs regarding the efficacy of the product. The study also addressed the need for a methodologically rigorous experimental design which was lacking in the field of olfactory research to strengthen the validity of results in the field (e.g. employing double blinded, placebo controlled experimental design).

2.3.3 Contribution to the field of odour research

The study was the first published scientific study to test the efficacy of Pet Remedy® on the behaviour of pet dogs, and offered a unique insight into the effectiveness of a commercially available stress relief product. Before the publication of this study, any measurable effects of Pet Remedy® on canine behaviour were unknown and there was a lack of published clinical studies in the field (Unwin *et al.*, 2020). This may have led to industry professionals relying on anecdotal reports of the efficacy of Pet Remedy®, rather than being informed by science. The study contributed to the field by incorporating a robust methodology which had previously been lacking in the literature base. A double-blinded, placebo controlled experimental design (which used a naïve experimenter) was employed to address the need to strengthen the validity of results in the field. This may have helped produce more conclusive findings relating to the efficacy of Pet Remedy® in reducing stress, which in turn strengthened internal validity while also removing bias and reducing observer effects, thus improving external validity. This allowed an objective and rigorous study design whilst testing the efficacy of Pet Remedy®. The study also contributed to the field of odour research by filling in a gap in knowledge and has provided an opportunity for future research to build on the study findings to further test the efficacy of Pet Remedy®.

2.3.4 Research methodologies and limitations

Prior to the main study, a pilot study was conducted to trial physiological and behavioural recording, overcome any practical issues (Lensen *et al.,* 2015) and make any changes required to the study design. Data collected included the use of physiological parameters, such as heart rate and salivary cortisol, alongside behavioural parameters in an effort to establish a broader picture of stress (Grigg and Piehler, 2015; Broach and Dunham, 2016). The pilot study consisted of three dogs and ran smoothly, with only minor changes to the fitting of the heart rate monitor required. Therefore, it was decided to run the main study as per the pilot study.

Cortisol was chosen as a parameter for the study because it is a key component of stress (Bennett and Hayssen, 2010). It is also considered a reliable indicator of stress when paired with other parameters across a range of domestic species including guinea pigs (Künzl *et al.*, 2003), rats (Radahmadi *et al.*, 2006), pigs (Turner *et al.*, 2005), sheep (Smith and Dobson, 2002) and dogs (Pastore *et al.*, 2011; Part *et al.*, 2014). Salivary cortisol was considered the most appropriate medium due to being largely non-invasive (Bennett and Haysson, 2010; Lensen *et al.*, 2015), reducing the risk of elevating the dogs' stress levels during saliva collection.

Collecting salivary cortisol from dogs in the pilot study was problem free, however taking samples from stressed dogs in the main study was sporadic and obtaining enough saliva to analyse was difficult, in particular in smaller and less compliant dogs (Dreschel and Granger, 2009). A 6" cotton braided rope was the medium used in the study (Salimetrics Europe Ltd) and was chosen because comparable studies had successfully used the medium to obtain saliva for analysis (e.g. Dreschel and Granger, 2005; Bennett and Hayssen, 2010). To ensure standardisation and obtain comparable results, the same collection method was used throughout (Strazdins *et al.*, 2005; Golatowski *et al.*, 2013; Lensen *et al.*, 2015). A treat placed under the nose to attempt to aid salivary flow had some success (as used by Dreschel and Granger, 2005 and Bennett and Hayssen, 2010), although the treat was not given to the dog as the effect of food contamination can impact cortisol measurement and lead to spurious results (Granger *et al.*, 2007). Citric acid has been used in attempts to increase salivary flow; however, it is an aversive substance for dogs (Dreschel and Granger, 2009) and, if used, could potentially induce stress so was discounted. The recommended volume of saliva for the assay was 25 µl (Chmelíková *et al.*, 2020; Salimetrics, 2021), but often the sample collected was far below this recommendation, which rendered many of the samples void and unable to be analysed. Low volume can result in error variance (Chmelíková *et al.*, 2020), so it was therefore decided to focus on reporting behavioural parameters in the published Taylor *et al.,* (2016) study.

Improvements to the study could have been achieved by focusing on different cortisol mediums, although each has its constraints (Table 2). A cotton swab dipped in ginger has been found to increase salivary flow and could be implemented in future studies to enhance effectiveness of collecting salivary samples and subsequent analysis (Meunier *et al.*, 2021). Recording adrenal activity using hair samples and analysing cortisol is a non-invasive (Siniscalchi *et al.,* 2013), simple and quick technique that overcomes the issues with collecting saliva, and has reliably analysed adrenal activity in dogs for an extended period of time (Siniscalchi *et al.,* 2013; Bryan *et al.,* 2013; Grigg *et al.,* 2017; Heimbürge, Kanitz and Otten, 2019; Van der Laan, Vinke and Arndt, 2022). However, some studies have suggested hair colour (Bennett and Hayssen, 2010; Svendsen and Søndergaard, 2014), sunlight exposure and brushing may impact the amount of cortisol that can be obtained from the hair (Mesarcova *et al.,* 2017) which limits its effectiveness, although can be controlled to some extent, making it a more reliable medium. Venous blood samples could have been taken but this method was not considered appropriate, due to being an invasive procedure which may have increased stress and elevated cortisol (Kobelt *et al.,* 2003; Bennett and Hayssen, 2010), potentially skewing results. Urine samples are difficult to obtain, risking incomplete datasets (Stephen and Ledger, 2006).

Heart rate was recorded using a Polar® RS800CX heart rate monitor which has been validated for use in dogs that are locomotory (Essner *et al.,* 2013; Essner *et al.,* 2015). Therefore, dogs were not restricted in the study. The dogs in the study were also unrestrained to enable them to perform a wide behavioural repertoire including lying down, eating, drinking and locomotor behaviour. This was because the observation period (30 minutes) was lengthy, and being unrestrained enabled the dogs to use a bed for comfort if desired. As heart rate (HR) is highly dependent on activity levels, this made their interpretation problematic (Jonckheer-Sheehy *et al.,* 2012), despite recent studies validating the heart rate monitor for use in dogs that are locomotory (e.g. Essner *et al.,* 2013; Essner *et al.,* 2015). Heart rate may have been influenced due to exercise and movement rather than stress. Likewise, there were issues with poor electrode contact linked with movement of the dog, which may have resulted in loss of connectivity and data, evidenced through invalid traces (Jonckheer-Sheehy *et al.,* 2012). Improvements to the study to increase conductivity could have included clipping the coat, although this was not feasible as was deemed undesirable by the dogs' owners. However, the dogs' chest area and the electrodes on the strap were moistened with water before fitting to increase electrical conduction to counteract this. An elasticated strap was also securely fitted containing the two electrodes, and fastened with Vetrap 3M™ to reduce slipping and increase connectivity. Nevertheless, it is important to ensure data reliability, and therefore, due to the aforementioned issues, only behavioural parameters were reported in the published study.

A comprehensive range of behaviours were used in the study (Table 5), which were informed using previous canine research reporting behaviours indicative of stress (e.g. Serpell, 1995; Voith and Borchelt, 1996; Beerda *et al.,* 1997; Beerda *et al.,* 1998; Beerda *et al.,* 1999; Beerda *et al.,* 2000; Palestrini *et al.,* 2005; Tod *et al.,* 2005; Hiby *et al.,* 2006; Mills *et al.,* 2006; Rooney *et al.,* 2007; Villa *et al.,* 2013). These provided a broad picture of stress-related behaviour. Recording behavioural parameters was also an inexpensive and non-invasive method (Pastore *et al.,* 2011), thus reducing observer effects. A blinded placebo-controlled design which employed counterbalancing was also implemented, improving reliability and validity through reducing potential bias and assessing any treatment effects. The sample size ($n = 28$) was slightly lower than previous studies in the field-which are typically $< n = 60$ (e.g. Graham *et al.,* 2005; Amaya *et al.,* 2020) and therefore limits generalising the findings to the wider population of confined dogs.

Table 5: Ethogram of behaviours, measured as frequencies (*f*) (events) or durations (*d*) (states) (Taylor and Madden, 2016).

Behaviour	**Definition**	**Measurement Method *(f or d)***
Auto-grooming	Time spent in behaviours directed to own body including licking and scratching.	*d*
Digging	Time spent with one or both front paws in a digging motion, on floor or wall of holding pen.	*d*
Drinking	Time spent drinking water from bowl.	*d*
Lying down	Time spent ventral/lateral lying on ground with all four legs resting and in contact with ground. Eyes may be open or closed.	*d*
Nosing	Time spent investigating surroundings whilst sniffing and with nose held in contact with the edge of the holding pen surface.	*d*
Locomotion	Time spent in forward movement with legs resulting in shift of whole body to a new position in the pen.	*d*
Hind legs	Time spent with hind limbs in contact with the ground and fore limbs in air. Front legs do not touch pen sides or pen exit.	*d*
Sitting	Time spent with hind quarters on ground with two front legs being used for support.	*d*
Standing	Time spent with all four paws on ground and legs upright and extended supporting body.	*d*
Circling	Time spent in circling locomotion, often repetitive (performed more than once).	*d*
Chewing	Time spent chewing non-nutritive material.	*d*

Stretching	Time spent with front or hind legs outstretched.	*d*
Exit "rear"	Time spent standing on hind legs with front legs resting or digging against exit.	*d*
Wall bouncing	Time spent standing on rear legs with front legs rebounding off, resting on or digging on pen walls other than exit. Often repetitive (performed more than once).	*d*
Barking	Number of times dog makes staccato vocalisation.	*f*
Howling	Number of times dog makes loud prolonged vocalisation with mouth open and head extended.	*f*
Nose licking	Number of times dog extends its tongue upwards to cover nose, before retracting into mouth.	*f*
Paw lifting	Number of times dog raises a single paw and holds it above the ground without moving.	*f*
Sighing	Number of times dog intakes a deep breath and blows out loudly.	*f*
Whining	Number of times dog makes high pitched vocalisation.	*f*
Panting	Frequency of episodes spent with mouth open with tongue extended accompanied with rapid breathing and expansion/contraction of chest.	*f*
Yawning	Number of times dog opens its mouth wide open for a period of a few seconds and then closes.	*f*
Urination/defecation	Number of times dog either urinates or defecates in the pen.	*f*

2.3.5 Implications and questions generated

Longitudinal studies using a larger sample of pet dogs combining physiological and behavioural parameters are required to confirm the results of preliminary investigations. The study timescale and short exposure to the stressful novel conditions may not have been long enough to induce an effect, therefore a longer study duration over a period of months rather than weeks is recommended. Obtaining and analysing hair cortisol as opposed to salivary would be more feasible from a practical point of view and potentially more suitable for a longer term study, given cortisol in hair accumulates over time which would help reduce the confound of natural variation in cortisol levels (Heimbürge, Kanitz and Otten, 2019; Van der Laan, Vinke and Arndt, 2022). Fecal cortisol could also be utilised, as fecal cortisol indication is slightly longer than found in plasma and saliva samples and is also non-invasive (Polgar *et al.*, 2019). Fecal samples would also be relatively accessible, if research took place in a confined environment such as a shelter.

It is also possible that an effect was not detected due to low power therefore, future research should be conducted over a longer duration and with a larger sample size. A sample size calculation was conducted for future studies, with a recommended sample size of 383 dogs (Survey System, 2022). In the current study, pet dogs were largely easy to recruit however, the number of participants was constrained by the short study duration. In an ideal scenario, given enough time and resources, a larger sample size compared to the current study, should be feasible for future research given pet dogs in the current study were straight forward to recruit. Collaboration across institutions may also increase sample size. A larger evidence-base will increase knowledge and understanding of the efficacy of Pet Remedy® in reducing stress in dogs. Practical application of the study findings can be used to underpin future research and provide an evidence base to build on to further test the efficacy of Pet Remedy®.

While the 'laboratory like' environment used to test Pet Remedy® was novel and likely to induce stress, behaviours observed may have been different to those seen if the study had been replicated in a real life setting. To increase the ecological validity of the study, the research could be repeated in a more applied setting. For example, a veterinary examination room where sham examinations are conducted. This would be more representative of a real veterinary examination, which are known to produce acute stress responses in dogs (Csoltova *et al.,* 2017). Study findings could be used to guide practitioners as to whether Pet Remedy® is effective in reducing stress during consultations and whether there is potential for the well-being of dogs to be improved during veterinary visits. Further areas for research have been highlighted and will be of value to future projects.

Future research could focus on individual components of Pet Remedy®. Pet Remedy® is a product which combines multiple ingredients (e.g. valerian, vetiver, basil and sage) and it is unknown whether these components, when combined, have a greater effect than the sum of their separate effects. Furthermore, they may produce effects when combined that are different to when applied individually (Galindo *et al.,* 2010). Likewise, there are various methods of delivery including a diffuser, spray and wipe format (Pet Remedy, 2021). The diffuser delivery method was chosen for this study due to being supplied for the study by the manufacturer. However, the formats in which the odour is applied may differ in their effect on behaviour when influenced by confounding factors in the environment. For example, if odours are sprayed on bedding, cleaning products such as detergents may interfere with the action of the product (Hewson, 2014). Likewise, odours evaporated into the environment using diffusers rely on owner compliance with manufacture instructions on how to use the product most effectively. The individual components of Pet Remedy®, including valerian (addressed by Evidence Source 2) and effectiveness of delivery method, are areas that warrant further research.

It has been proposed that the most effective, long-term solution for behavioural problems, where products such as Pet Remedy® may be employed, is combining stress relief products with systematic desensitisation training (Levine, Ramos and Mills, 2007), which may reduce stress. However, when used in combination with other interventions, it is difficult to determine what aspect may cause a reduction in stress. It is possible that any effects of the odour may be a conditioned response to training for example, and this needs to be further determined. Odour discrimination is also an issue, and scope exists for further research to include placebo conditions that have an identical odour to Pet Remedy®. This may avoid researchers discriminating between placebo and Pet Remedy® trials in the future (Unwin *et al.,* 2020). A placebo condition containing an identical odour to valerian has been trialled in human studies. A placebo tablet containing the whole root *V.eduClis,* a species of valerian, gave the same appearance and distinctive odour, but in small non-pharmacologically significant quantities (Francis and Dempster, 2002). A placebo containing *V.educlis* could be applied to future valerian-based animal studies to overcome odour discrimination.

Individual variation is also problematic, with detection and responses to odours likely differing between individuals and breed (Jezierski *et al.*, 2014). The breeds tested in this study may not have detected the odours as effectively as others or responded in a similar manner. Pseudogenes and specific gene polymorphisms vary by breed, suggesting internal genetic factors may account for why some breeds of dogs are better able to detect odours (Kokocińska-Kusiak *et al.,* 2021). Going forward, future research is required using a standardised breed type which may reduce breed-specific variability in behavioural data. Scent hounds such as the Bloodhound (Dahlgren *et al.,* 2012), Basset Hound and Beagle, which are all known to have high olfactory acuity and exploratory behaviour (Hamilton, 2013), could be utilised in an attempt to reduce breed specific variability relating to detection and response to odours. Other breeds such as Pugs have been found to perform well in a olfactory discrimination task, despite being a brachycephalic breed and having a reduced rostrum size which is proposed to reduce olfaction, although this has yet to be scientifically tested (Hall *et al.,* 2015). If Pugs have a higher rate of odour detection compared to other breeds, it may be beneficial to test the efficacy of Pet Remedy® in this breed, where an affect may be more prominent. The Staffordshire Bull Terrier, one of the most commonly relinquished dog breeds in the UK (Carter and Martin, 2021), could also be included in future shelter dog research to minimise breed-specific variability. Individuals are of a similar size, which may help standardise factors that alter pharmacokinetics, such as susceptibility, which is influenced by size and weight (Nair and Jacob, 2016).

The breed is also widely available in shelter environments, which are considered stressful, and has potential to increase sample sizes.

Given that there are individual differences in the behavioural expression of stress (Rooney, Gaines and Bradshaw, 2007), it may be that Pet Remedy® works for some dogs, but not others and may be dependent on temperament (Jones and Gosling, 2005). Temperament can be defined as differences in behaviour that are stable when tested under similar situations (Diederich and Giffroy, 2006) and has been found to influence how dogs respond to the environment (Jones and Gosling, 2005). Siniscalchi *et al.,* (2016) investigated whether dog nostril laterality was influenced by human and canine odours during different emotional events. Owners were asked to rate their dogs temperament using the Canine Behaviour Assessment and Research Questionnaire (CBARQ). Dogs that were more aggressive and fearful towards other dogs, were more likely to use their right nostril to sniff a conspecific odour that was collected during a stressful situation (isolation from owner in unfamiliar environment). As negative emotions are associated with the right hemisphere (Davidson, 1992), this implies that temperament could influence behavioural responses to emotional odours (Siniscalchi *et al.,* 2016). It would be interesting to incorporate CBARQ into future odour research to evaluate temperament of dogs and compare lateralised behavioural responses to different odours. This may aid future research in determining individual variations in dogs and their responses to odours. For example, if different temperaments are linked with whether dogs respond to odours or not.

It was challenging to publish a study which reports negative results, given that positive publication bias is a current issue in the field (van der Schot and Phillips, 2013; Bombail, 2017; Reimer, 2020). Similarly, it was difficult to compare the small effect size with other similar published studies due to the lack of reporting that is inherent in the field (Funder and Ozer, 2019). In some cases, study findings have been prevented from publication due to being unfavourable to the pharmaceutical and pet product industry (Yaun *et al.,* 2011). Yet, it is important to publish less favourable findings to avoid researchers unnecessarily repeating studies, but also to develop understanding in the field (van der Schot and Phillips, 2013). The under reporting of studies with negative findings also risks introducing bias into the field (Mlinarić *et al.,* 2017), which can misinform canine welfare and veterinary industries and, subsequently, clinical practice. Therefore, it is important that studies are published where an effect is not found, such as Taylor and Madden (2016). The study findings may enable reasoned judgements on the efficacy of odours for industry practitioners. For example, the study findings can be used alongside future research to help inform veterinary professionals and those involved in the care of all captive dogs in the efficacy of Pet Remedy®, and help to inform their decision in whether to recommend or use the product for reducing stress in captive dogs. Dog owners, shelter staff and veterinarians could consider seeking other approaches that are supported by peer reviewed studies which can be used in combination with Pet Remedy®, considering no harmful effects have been reported. Furthermore, given there has historically been a lack of research on olfactory stimuli effects in dogs (Murtagh *et al.,* 2020), there is also scope for investigating less widely researched odours in reducing stress, which may potentially have value in enhancing canine wellbeing (e.g. coconut, ginger and vanilla – addressed by Evidence Source 2).

CHAPTER THREE

COCONUT, GINGER AND VANILLA

3.1 Focus of the critical review

Reports on the effects of Coconut *(Cocos nucifera)*, Ginger *(Zingiber officinale)* and Vanilla *(Vanilla planifolia)* odours in dogs are scarce, despite the potential welfare enhancing benefits found in other captive species. Investigating these odours may provide an opportunity to widen the choice of stress reducing odours available for those involved in the care of captive dogs and, simultaneously, improve canine welfare. Chapter 3 introduces the odours Coconut, Ginger and Vanilla and their use in reducing stress in captive animals. Their potential value and application in changing behaviour through relieving stress in sheltered dogs is discussed.

3.2 Introduction to Coconut *(Cocos nucifera)*, Ginger *(Zingiber officinale)* and Vanilla *(Vanilla planifolia)*.

Essential oils such as Coconut *(Cocos nucifera)*, Ginger *(Zingiber officinale)* and Vanilla *(Vanilla planifolia)* have been investigated in humans. Vanilla is used as a relaxant to aid sleep disorders (Meghani *et al.*, 2017), with more citrus based odours acting as stimulants (Hwang and Shin, 2015). Coconut has been found to decrease sympathetic responses in people exposed to stress (Mezzacappa *et al.*, 2010), while ginger is reported to enhance immune function (Carrasco *et al.*, 2009). When investigating coconut, ginger and vanilla odours in animal research, there is a clear bias towards zoo animals (Clark and King, 2008), and much less is known about their effects in other captive species. A strong bias towards zoo-based research is likely due to the number of stereotypic behaviours found in zoo housed species indicating poor welfare, as well as increased public concern and interest in the welfare of zoo housed animals (Sherwen and Hemsworth, 2019).

In zoo housed animals, the efficacy of vanilla and coconut in reducing abnormal behaviour has been tested in wombats using scented logs *(Lasiorhinus latifrons)*, but were found to be unsuccessful in reducing stereotypic behaviour (Hogan *et al.*, 2010). The authors suggest that the underlying motivation for the behaviour may not have been addressed by scented logs, or the behaviour was too ingrained for the enrichment to have a marked effect. Instead, an increase in foraging and explorative behaviours were recorded, suggesting an enhanced behavioural repertoire and positive use of the environment (Hogan *et al.,* 2010). These behaviours were perceived as indicating enhanced wellbeing through addressing the goals of enrichment, for example increasing the range of species-specific behaviour and encouraging interaction with the captive environment (Hogan *et al.,* 2010).

In zoo housed lemurs *(Lemur catta)* no marked influence of coconut impregnated cloths on behaviour was found, although a more general effect of olfactory stimulation versus no odour condition was noted with more resting, sleeping and locomotion recorded (Baker, Taylor and Montrose, 2018). It is possible that coconut is not a biologically meaningful odour for Lemurs. Odours such as faeces or urine from other Lemurs may be more relevant in inducing activity that increases enclosure utilisation and behavioural repertoire resulting in positive use of the captive environment. By contrast, others have suggested vanilla reduces stereotypic behaviour in sea lions (Samuelson *et al.,* 2017) such as repetitive swimming and also increased use of the enclosure. Although statistical differences in a reduction in stereotypic behaviour was not observed, large effect sizes reported do suggest a trend in the reduction of problematic behaviours. However, the small sample size (n = 4) limits conclusions drawn to the wider population of captive sea lions and, although no difference in behaviour was found between natural and non-natural odour conditions, sea lions responded positively to both conditions, potentially suggesting novelty effects. Conversely, Vanilla impregnated cloths had no effect on zoo housed western lowland Gorilla *(Gorilla gorilla)* behaviour, with the authors suggesting more biologically relevant conspecific odours potentially being a more pertinent option when incorporating odours as enrichment (Wells *et al.,* 2007). The delivery mechanism of using a cloth for odour presentation may also not have been species appropriate, since primates may be more driven by objects that encourage foraging behaviour (Wells *et al.,* 2007). Ginger in lions and Rothschilds giraffe created a stimulating effect by increasing activity levels, potentially as an effect of odour novelty (Schuett and Frase, 2001; Fay and Miller 2015). Increases in locomotory activity were deemed beneficial for the species studied, who are known to display sedentary behaviour in captivity (Schuett and Frase, 2001; Fay and Miller 2015).

The wider implications of research in odours is the potential for odours to also impact visitor behaviour. Novel odours may lead to more positive visitor experiences, if species are engaging in the natural species-specific behaviours that are deemed more desirable by the public in zoo housed species (Fay and Miller, 2015). An increase in visits, and consequently revenue, will support conservation efforts (Fay and Miller, 2015). Potential benefits of odour use may also be applicable for other species, for example those housed in rescue shelters where increased likelihood of adoption may be influenced by more desirable species behaviour and interactions with adopters. This may be particularly applicable to shelter housed dogs, with many spending lengthy periods of time in a stressful environment awaiting adoption. Likewise, the benefits of a reduction in stress-related behaviours seen in confined zoo species (Schuett and Frase, 2001; Hogan *et al.,* 2010; Fay and Miller 2015) may be transferred to confined sheltered dogs and improve welfare in the process.

Quantifying meaningful impacts of odours is complex. There are inconsistencies relating to specifically what effects coconut, ginger and vanilla odours have in reducing stress-related behaviour. Some authors report odour enrichment has little effect in stress inducing environments such as zoos (e.g. Wells *et al.,* 2007; Myles and Montrose, 2015). One possible explanation is the diversity of species used in zoo studies, where primary senses are likely to differ. For example, primates have been considered "microsmatic" whereby sense of smell is limited (Heymann, 2006), and they therefore rely on sight instead (Wells and Hepper, 2017). More olfactory driven species such as Lemurs, where olfaction is highly developed (Schilling, 1974; Kappeler, 1998; Drea and Scordato, 2008), may be more intrinsically driven to respond to odours, similar to dogs. It is also possible that the odours used in zoo-based studies have no biological relevance for some animals (Wells and Hepper, 2017), and changes in behaviour may have been attributed to novelty effects. Nonetheless, given that many zoo-based studies have reported positive behavioural effects in captive species, there is value in investigating their effects in other species which are also housed in potentially stressful captive environments. To the authors' knowledge, no studies have focused on the use of coconut, ginger and vanilla in domestic dogs housed in stressful environments such as rescue shelters. Given dogs' high olfactory acuity (Carlone *et al.,* 2018), these odours may have some practical application in reducing behaviours that are detrimental to welfare.

3.3 The value and application of coconut, ginger and vanilla odours in reducing stress in sheltered dogs

In 2009 it was estimated that 129,473 dogs were relinquished to rescue shelters in the UK (Clark, Gruffydd-Jones and Murray, 2012). Rescue shelters are inherently stress inducing environments for dogs. On entering shelters acute stress can occur (Rooney, Gaines and Bradshaw, 2007; Dudley *et al.,* 2015), while prolonged stays can induce chronic stress which becomes maladaptive (Beerda *et al.,* 2000; Nelson, 2005). Odours are simple to apply, non-invasive, cheap and easy to store (Nielsen *et al.*, 2015) and may have value in reducing stress in dogs, given positive effects reported in other captive species. Investigating less well known odours such as coconut, ginger and vanilla could provide opportunities to widen the choice of odours already used in shelters if they are effective at encouraging positive behavioural effects. Odour use is currently limited in shelters where only odours known to have anxiolytic effects in people are commonly used e.g. Lavender and Chamomile. Animals may become habituated to repeated use of the same odours over time (Graham, Wells and Hepper, 2005; Melfi *et al.,* 2007). This is particularly the case when there is an absence of consequences due to stimulus exposure. This can occur when an odour may no longer produce an association with positive consequences and over time any beneficial behaviour associated with the odour decreases. Moreover, olfactory receptors can become saturated with an odour leading to olfactory fatigue (Arasaradnam *et al.,* 2011). Therefore, a rotation of an assortment of different, less conventional odours could be used to aid variation and interest. Melfi *et al.,* (2007) suggests different odours are used at intervals of three weeks, although this research is based in zoo housed Sumatran Tigers *(Panthera tigris sumatrae),* and an appropriate period would need to be determined in other species such as dogs.

Within Evidence Source 2 and Appendix 1, the effects of coconut, ginger, vanilla and valerian on the behaviour of confined sheltered dogs will be explored by gathering data from rescue shelter dogs in a quasi-experimental, fixed treatment order, controlled study. These will be taken forward to address the lack of research into less established odours and whether they have value in reducing stress-related behaviour in sheltered dogs, where positive results seen in zoo housed species may be transferable to dogs who similarly experience stress in confinement. The impact of this research can then be used to widen the choice and rotation of stress reducing odours available for those involved in the care of captive dogs, if there are beneficial effects. Similarly, knowing which odours may have a stimulating, relaxing or indeed no effect, could help inform the decision-making process on the application of odours for those involved in caring for confined dogs. This will mean that some odours can be tailored for individuals, with potential benefits for canine wellbeing. Equally, where odours are found to have no effect, it may be financially more prudent for organisations to invest in and implement alternative stress relief measures, or redirect funds to other areas of their work that aim to improve welfare.

3.4 EVIDENCE SOURCE 2

Binks, J., **Taylor, S**., Wills, A. and Montrose, V.T. (2018) 'The behavioural effects of olfactory stimulation on dogs at a rescue shelter', *Applied Animal Behaviour Science*, 202, pp.69-76.

3.4.1 Summary

Binks *et al.*, (2018) (Appendix 1) aimed to investigate the effects of olfactory stimulation via coconut, ginger, vanilla and valerian on the behaviour of 15 sheltered dogs. The dogs were simultaneously exposed to six olfactory conditions using scented cloths following a fixed order (cloth control, coconut, vanilla, valerian, ginger and odour control) for 2 hours a day for 3 days, with an intervening period of 2 days between conditions. Acute and chronic stress responses were recorded, induced by exposure to stressors typically found in the sheltered housing environment such as lack of environmental control and behavioural expression, high levels of noise and exposure to dogs and unfamiliar people. Exposure to ginger, coconut, vanilla and valerian resulted in significantly lower levels of vocalisations ($p < 0.0001$) and movement ($p < 0.0001$) compared to the control conditions, while coconut ($p < 0.05$) and ginger ($p < 0.01$) additionally increased levels of sleeping behaviour. The results suggest these odours may have application in rescue shelters due to the reduction of behaviours such as barking and activity, which may be indicative of stress as well as being traits perceived as undesirable by adopters. Odours may have the potential to positively influence public perception of dog desirability and their subsequent likelihood of being rehomed.

3.4.2 Rationale

The odours chosen have been purported to enhance wellbeing in a wide range of species including lions, wombats, domestic cats and sea lions (e.g. Schuett and Frase, 2001; Hogan *et al.,* 2010; Bol *et al.,* 2017; Samuelson *et al.,* 2017). However, these odours are understudied in the canine field of research. A valerian-based stress relief product, Pet Remedy®, was found to have no discernible effect on pet dog behaviour (e.g. Taylor and Madden, 2016). Binks *et al.,* (2018) built on the Taylor and Madden (2016) study by further investigating any effects of valerian itself when combined in a single product, instead of part of the essential oils formulation in Pet Remedy®. The study also built on Taylor and Madden's (2016) research by seeking to identify the value of a wider range of odours such as coconut, ginger and vanilla in reducing stress in dogs. Understanding the efficacy of odours in reducing stress in sheltered dogs has the potential to inform those involved in the care of captive dogs, for example through improved management practices such as effective odour selection and use which may lead to stress reduction.

3.4.3 Contribution to the field of odour research

To the author's knowledge, this was the first study to test coconut, ginger and vanilla in sheltered dogs and developed knowledge of the efficacy of less conventional odours in reducing stress-related behaviour in shelters. As such, it offered a unique insight into how dogs respond to novel odours within a shelter setting. The study also built upon Taylor and Madden (2016) and addresses the lack of published clinical research on the effects of valerian alone on canine behaviour. Binks *et al.,* (2018) showed that all of the odours tested significantly reduced the frequency of vocalisations and activity levels, suggestive of a stress relieving effect. In addition, coconut and ginger increased sleeping behaviour indicative of relaxation. The study therefore provides support for such odours in reducing stress-related behaviour in dogs housed in rescue shelters. Habituation to repeat use of the same odours may occur, therefore, practical application of the study findings can be used to promote coconut, ginger, vanilla and valerian to expand the choice of odours currently used in shelters. The odours can also be rotated regularly to overcome the issue of habituation (Melfi *et al.,* 2007). Though how regularly odours are rotated to obtain maximum benefit for dogs is yet to be determined.

3.4.4 Research methodologies and limitations

The study employed a quasi-experimental, fixed treatment order, controlled design. A weakness of the study was the presentation of the odours which were presented simultaneously. This caused olfactory condition to be entangled with order of presentation. The fixed presentation of odours was chosen to avoid the odours interfering with one another. This was due to the open mesh design of the kennels that did not prevent odour transmission, and was appropriate to address limitations of the kennel design. Captive environments differ in enclosure design and size, and this will impact decisions on what method to disperse odours most effectively. Plug in, electronically heated diffusers vaporise the odour into the surrounding environment and are used in rescue shelters where wider coverage is required, and odour transmission is not an issue. However, odour efficacy is dependent on the number of diffusers and whether the odour fills the volume of the environment in sufficient quantities to be effective (Mills *et al.*, 2012). Location of diffuser is also important, as odours can be blocked by doors or obstructions which limit the odour from filling the environment (Mills *et al.,* 2012). It is key to consider these limitations when conducting research as animals are unable to retreat from potentially aversive odours when in captivity, which can cause stress. Delivery method limitations were taken into consideration, and it was decided to use odour impregnated cloths which sustain the odour and allow multiple odour points for even distribution, but which also provide the opportunity for animals to move away and retreat (Clark and King, 2008). Multiple cloths were used to help sustain the odour in the environment (Wells, 2009) but placed so dogs could retreat from them if desired, which addressed ethical concerns.

A control condition was implemented prior to the presentation of odours and consisted of a non-scented cloth to act as odour control. The presence of a cloth however, may have been construed by the dogs as a novel item in the kennel. Therefore, the control condition may not have been a true baseline measure (Buckley, 2019). However, using a visual non-olfactory stimulus as a control can be a valuable tool in determining whether the odour delivery method itself is likely to provoke a neophobic response (Clark and King, 2008). Since a control was also included at the end of the experimental conditions, any changes in behaviour were likely to reflect the effects of olfactory stimulation. Order effects are particularly problematic within the field of research, and highlight the importance of ensuring adequate randomisations of odours and time in between conditions to ensure odours are fully dissipated from the environment. Randomising the presentation of odours eliminates selection bias and ensures accidental bias does not occur (Suresh, 2011). Randomising odours also improves external validity e.g. effectiveness by generalising the study findings to a wider population (McLeod, 2013). Using "wash out" periods when testing the effects of multiple odours is particularly beneficial also (Clark and King, 2008). These can be aided by ventilation and fan systems to ensure any previous odours are fully dispersed. If wash out periods are not implemented, there is a risk that one odour may influence the perception of another odour, which induces a behavioural response that is not a result of the odour being tested (Clark and King, 2008). Similarly, there may be an accumulation or combining of odours that produces a response that is different to the presentation of a single odour in its original quantity. This can present results that are inaccurate. To overcome this issue in the study, and strengthen the research, new scent articles were provided daily to prevent accumulation of odours, a two day 'wash out' period was implemented to allow each odour to dissipate in between conditions and gloves were used when applying odours to avoid transmission of scents.

In the study, the sample size (N = 15) was relatively small compared to similar research (e.g. Graham *et al.,* 2005). However, due to the transient nature of a rescue shelter, it makes it inherently difficult to recruit large numbers of dogs as they are frequently rehomed part way through the study, leading to loss of data. The study was completed over several weeks, and this meant dogs were rehomed part way through data collection and were therefore removed from the study; thus, limiting sample size.

An ethogram used during the study was adapted from a previous study that investigated odour use in dogs (e.g. Graham *et al.,* 2005). However, the ethogram was limited in the number of behavioural categories, where more subtle behaviours were not recorded despite possibly indicating stress e.g. trembling, lip licking, low posture (Beerda *et al.*, 2000). The ethogram also did not allow positive or negative arousal effects to be disentangled. For example, vocalising is frequently used as an indicator of stress in dogs (Beerda *et al.,* 1997; Stephen and Ledger, 2006; Taylor and Mills, 2007) but can also indicate excitement, be used to attract human attention or to express content, such as during play (Yin, 2002; Yin and McCowan, 2004). Vocalising observed in the study could have been caused by the presence of visitors, which may have been exciting for the dogs. Although, vocalising was recorded both during visitor opening times and when the shelter was closed to visitors, so may have been caused by other environmental factors. However, changes in other behaviours suggestive of stress were observed e.g. decreased activity levels and an increase in sleeping. These were also supported by being observed within an aversive context e.g. a stressful rescue shelter environment, therefore supporting that the vocalisations were most likely due to stress.

3.4.5 Implications and questions generated

Knowledge of biological relevance and specific effects of odours at reducing stress-related behaviour in captive animals can help justify odour use, which is often absent in the literature (Clark and King, 2008). Binks *et al.,* (2018) findings can inform those that are responsible for caring for sheltered dogs, so they are able to integrate odours that are effective and omit non-effective odours. This will subsequently help create environments-such as rescue shelters- that enhance the welfare of captive dogs (Nielsen *et al.,* 2015). However, selecting an appropriate olfactory intervention needs to be considered with regard to temperament of the dog. There are individual differences in the behavioural expression of stress (Rooney, Gaines and Bradshaw, 2007), and these can be attributed to temperament (Jones and Gosling, 2005). Dogs that show hyperactive or stereotypic traits should not be exposed to odours that have stimulating properties, as these may actually increase stress (Graham, Wells and Hepper, 2005). It would be worthwhile for future research to incorporate the use of CBARQ to evaluate temperament in dogs to help inform the selection of appropriate odours and to avoid odours which may increase stress.

A wider application of odour use may influence adoption success in sheltered dogs. Excessive vocalisations and activity are indicative of stress and are deemed as undesirable traits by potential adopters (Wells and Hepper, 1992; Wells and Hepper, 2000; Protopopova and Wynne, 2014). Therefore, a reduction in barking and locomotory behaviour may be more desirable by adopters, leading to an increase in adoptions of dogs that display calm and relaxed behaviours. Given that shelters are often at maximum capacity, quicker adoptions are not only beneficial for the organisation in freeing up space, but also advantageous for the dogs by removing them from a potentially stressful environment.

The odours investigated in this study could offer shelters a tool to help reduce stress in shelter housed dogs. However, there are also wider implications of the study findings which could be applicable to human research. Given the reported beneficial effects of the odours on dogs in the study, it would be of interest for future research to investigate any impacts of odours used in shelters on visitors, considering odours have been found to influence the emotional state of a human (Diego *et al.*, 1998; Weber and Heuberger, 2008). Future research could focus on whether certain odour use influences human emotions and subsequently, adoption rates. Canine welfare could be enhanced if odours are found to increase adoption rates resulting in shorter kennel stays, an environment deemed to be significantly stressful for dogs.

Visitor related measures of shelter dog behaviour could also be quantified using a Qualitative Behaviour Assessment (QBA). Qualitative Behaviour Assessment is a tool which focuses on how an animal performs behaviour such as expression or demeanour (Wemelsfelder *et al.,* 2007). Expressions of behaviour are assessed live or via video assessment and scored using terms such as 'contentedness' or 'uneasiness' (Cooke *et al.*, 2022). Although QBA is reliant on human interpretation of body language (Dawkins, 2021), it has been found to be a quick, reliable and non-invasive tool to assess emotional expression across a range of animals such as pigs, sheep, calves, donkeys, dolphins, hens and sheltered dogs (Walker *et al.,* 2010; Wemelsfelder *et al.*, 2012; Phythian *et al.*, 2013; Ellingsen *et al.*, 2014; Walker *et al.,* 2016; Minero *et al.,* 2016; Warner, Brando and Wemelsfelder, 2022; Vasdal *et al.,* 2022). Qualitative Behaviour Assessment has also been found to be an effective learning tool for assessing behaviour (Duijvesteijn *et al.,* 2014), with Old and Spencer (2011) suggesting QBA can be utilised by students to improve their knowledge of behaviour prior to handling animals. Stubsjøen *et al.,* (2020) suggest QBA can be used to aid student learning of shelter dog body language, handling skills and safety. Qualitative Behaviour Assessment could therefore have potential to educate adopters on how to identify shelter dog body language.

This could lead to safer handling practices during pre and post adoption with potential to improve the human-animal bond and animal welfare, subsequently leading to a reduction in returns to shelters. Qualitative Behaviour Assessment has also been used to reliably assess the quality of the human-animal relationship in horses (Minero *et al.,* 2018) and cattle (Ellingsen *et al.,* 2014) so could be adapted to also be utilised by shelter staff to assess the adopter-dog relationship for suitability.

Qualitative Behaviour Assessment could have practical application when assessing the efficacy of odours. Adopters who are trained to identify body language pre-adoption, could utilise QBA to assess behavioural expression of dogs exposed to odours during the transition from shelter to home, to assess whether dogs are coping in their new home environment and whether the odours can be gradually reduced or stopped altogether. A fixed list of descriptors alongside post-adoptive support provided by shelter staff could be utilised by adopters, for a more objective measure of behavioural expression (Arena *et al.,* 2019). Qualitative Behaviour Assessment has been utilised by shelter staff to evaluate expression of behaviour in sheltered dogs (Menchetti *et al.,* 2019), but not when dogs are exposed to odours. Qualitative Behaviour Assessment could also be used to assess visitor related measures of stress, given QBA has been found to be a reliable assessment of reactivity to humans (e.g. Ebinghaus *et al.,* 2016; Minero *et al.,* 2016). This could be paired with the exposure of odours as a measure of whether visitor induced stress and reactivity are reduced when odours are present. Therefore, there is potential for QBA not only to be utilised alongside odour exposure post adoption when assessing behavioural expression but to also aid staff decisions on stress reduction and odour management practices. These are areas that have yet to be tested and need exploring in future research, given QBA's potential for odour assessment.

In the current study, it was problematic in fully determining whether responses to odours were due to arousal or stress (eustress/distress). Cognitive testing could be utilised in future odour research to help determine whether responses are perceived as pleasant or aversive rather than simply a reaction to stimuli. A recent area of interest is the use of cognitive testing and whether affective state is influenced by essential oils (Uccheddu *et al.*, 2018). In particular, whether oils reduce stress in sheltered dogs. Cognitive biases were not tested in the current study, but given they have been found to infer affective state in animals (Mendl *et al.*, 2009) alongside behavioural and cortisol testing (Uccheddu *et al.*, 2018), cognitive testing would be useful to incorporate into future odour research alongside behavioural and physiological parameters. Uccheddu *et al.*, (2018) used cognitive testing alongside behavioural observations and salivary cortisol to investigate whether essential oils influenced the affective states of sheltered dogs. The researchers found a blend of nine essential oils (*Cananga odorata, Cistus ladaniferus, Citrus aurantium, Cupressus sempervirens, Juniperus communis var. montana, Lavandula angustifolia, Laurus nobilis, Litsea citrata, Pelargonium graveolens*) reduced latency in response to an ambiguous cue suggesting an optimistic bias and proposed that this indicated improved welfare. *Lavandula angustifolia* was found to significantly reduce cortisol, suggestive of a stress relieving effect. *Cananga odorata* reduced time spent nosing, also suggestive of reduced stress (Beerda *et al.*, 1998). An enriched environment has been proposed to indicate a more optimistic cognitive bias that is suggestive of a more positive affective state in pigs (Douglas *et al.*, 2012) and this is supported by Uccheddu *et al.*, (2018) who suggest essential oils as a form of enrichment improves optimism in sheltered dogs. However, not all oils when tested individually, were effective at reducing behavioural or physiological indicators of stress. Therefore, further work is required to investigate the influence of individual essential oils on sheltered dog welfare. Future research should focus on testing the efficacy of oils at different concentrations (Uccheddu *et al.*, 2018), as it is known that dosage can influence effectiveness (Galindo *et al.*, 2010). Further research should also be directed towards a focus on determining minimum and maximum distances where olfactory effects are created to reduce potential olfactory confounding effects (Uccheddu *et al.*, 2018).

Research that focuses on longer duration of exposure to odours where any long term or accumulative effects can be evaluated in sheltered dogs is required to confirm the findings of the current study (Bombail, 2017). Nonetheless, the findings provide knowledge on less recognised odours and demonstrates their potential value in improving the welfare of sheltered dogs. Utilising cognitive testing and physiological parameters such as infrared thermography (IRT) alongside behavioural indicators will help obtain a more direct measure of stress in dogs, and their response to odours. Building a larger evidence base will increase knowledge and understanding of odour efficacy where findings, positive or negative, can be used to inform industry and influence decisions on odour implementation at an organisational level. Furthermore, investigating other olfactory stimuli such as pheromones may be beneficial, considering they are also purported to reduce stress-related behaviour in captive domestic dogs and widely used in industry.

CHAPTER FOUR

ADAPTIL®

4.1 Focus of the critical review

Pheromonatherapy is the use of man-made pheromones which act as a stress preventative and also as an adjunct to behavioural modification in animals (Hargrave, 2014; Mills, 2005). The synthetic pheromone Adaptil® is advertised on the market with putatively stress relieving properties for dogs. Beneficial effects of Adaptil® are equivocal in the literature and there is a lack of reported clinical efficacy (Riemer, 2020), in particular on the Adaptil® spray. Chapter 4 introduces Pheromonatherapy and assesses the efficacy of Adaptil® as a means of reducing stress-related behaviour in sheltered dogs.

4.2 Introduction to Pheromonatherapy

In nature, pheromones are released by an individual and received by another of the same species, and this transaction induces a change in the recipient's behaviour (Hargrave, 2014). In captivity, synthetic analogues of pheromones have been developed and are used in a therapeutic context when managing behavioural change in animals (Mills *et al.,* 2012). The clinical use of pheromones is known as Pheromonatherapy (Mills *et al.,* 2012). Pheromonatherapy is used to prompt behavioural changes to the environment by evoking familiarity and reassurance. This can act as a stress preventative in a challenging environment by providing the concept of security (Hargrave, 2014). Pheromonatherapy is also used as an adjunct to behavioural modification where behavioural problems and stress already exists, and can be used to modify pet behaviours that are deemed as undesirable by care givers, including stress and anxiety (Hargrave, 2014). Pheromonatherapy is commonly used in dogs with separation-related issues (Gaultier *et al.*, 2005; Kim *et al.,* 2010), noise aversion (Sheppard and Mills, 2003; Levine, Ramos and Mills, 2007; Landsberg *et al.,* 2015) and can help dogs adapt to stress inducing environments such as veterinary practices, kennels and laboratories (Mills *et al.,* 2012). Despite anecdotal accounts of positive effects of pheromonatherapy, clinical studies have reported equivocal effects of pheromones and their stress relieving properties in dogs (Hewson, 2014; Mooney, 2020). Currently, there is one pheromonal product marketed for dogs in the UK

(BSAVA, 2017), formerly known as Dog Appeasing Pheromone (DAP) and more recently adopting a new name: Adaptil® (Ceva Santé Animale) (Mills *et al.*, 2012).

4.3 Adaptil®

Adaptil® is marketed as a pheromonal calming product for dogs and is widely available over the counter as an alternative to drugs, which may have unwanted side effects. Adaptil® composition consists of canine appeasing pheromone analogue in different quantities, depending on the format of delivery (Table 6). Adaptil® can be delivered via a diffuser, collar or spray and works by altering an animal's perception of the environment and response to stimuli (Mills *et al.*, 2012). This in turn creates an environment that is perceived as calm and reassuring (Mills *et al.*, 2012).

Table 6: Adaptil® composition (Adaptil, 2021b and 2021c)

Delivery Format	**Ingredients**
Diffuser	2% canine appeasing pheromone analogue and Isoparaffinic Hydrocarbon 48ml diffuser (hydrocarbons, C14-C19, isoalkanes, cyclics, and <2% aromatics)
Collar	5% canine appeasing pheromone analogue, Excipients q.s. 1 collar
Spray	2% canine appeasing pheromone analogue and Isopropanol q.s. 20ml spray

Behavioural variation in dogs and how they respond to pheromones has been noted (Sheppard and Mills, 2003). Inflammation of the VNO and anatomical restrictions attributable to breed (e.g. brachycephalic dogs) have been suggested to impact how effectively pheromones are detected (Mills *et al.*, 2012). Nasal features are suggested to impact the functionality of the olfactory system (Lawson *et al.*, 2012) however, Hall *et al.*, (2015) found the brachycephalic Pug breed excelled at an odour discrimination task despite being proposed to have poor olfactory performance as a result of a rotated olfactory lobe or dysfunctional turbinate bones. Hall *et al.*, (2015) found behavioural differences in pugs compared to other breeds may have accounted for their success in the discrimination task. The authors suggest that pugs may alter their sniff pattern as a result of previous olfactory reinforcement leading to more intense sniffing, which potentially alters olfactory perception. Nostril laterality has also been suggested to determine whether dogs perceive an odour as non-aversive or aversive, similar to auditory and visual perception (Siniscalchi *et al.*, 2008; Siniscalchi *et al.*, 2010; Siniscalchi *et al.*, 2011). This is based on the Valence-Specific (VS) Hypothesis, which proposes negative emotions are associated with the right hemisphere, and positive emotions are associated with the left hemisphere (Davidson, 1992). Dogs have a prevalence of the right nostril (right hemisphere) that they first use to sniff stimuli, which is then shifted to the left nostril (left hemisphere) if the odour is perceived as familiar or non-aversive such as food. If the stimuli is perceived as novel, stressful or arousing (e.g. exposure to conspecific odour when isolated from owner in a unfamiliar environment) there is a right nostril (right hemisphere) preference use (Siniscalchi *et al.*, 2011; Siniscalchi *et al.*, 2016; Kokocińska-Kusiak *et al.*, 2021). As behavioural lateralisation has been found to reflect asymmetries of the brain (Siniscalchi *et al.*, 2016), it may be that technologies such as functional magnetic resonance imaging (fMRI) can confirm the influence of odours and pheromones in future studies. Given that the processing of odours and pheromones differ, future research is required to determine the full extent that breed and associated morphological and behavioural factors have upon pheromone detection. Behavioural research that focuses on the way dogs sniff (e.g. sniff patterns) and nostril laterality, which may influence olfactory perception, would be useful to further determine the relationship between breed specific morphological and behavioural differences and olfaction (Hall *et al.*, 2015).

Throughout the last two decades, Adaptil® has been tested in numerous studies of which most have focused on the use of diffusers and collars when assessing fear and anxiety related behaviour in dogs (Riemer, 2020).

4.3.1 Adaptil® diffuser

One of the earliest clinical studies evaluated Dog Appeasing Pheromones (DAPs) as a potential treatment for dogs that showed a noise fear response to fireworks. In the open label trial, dogs in a home environment were exposed to an Adaptil® diffuser and subsequently noise from fireworks (Sheppard and Mills, 2003). Nine out of 14 of the most common fear behavioural indicators were reported by dog owners as improved compared to a baseline measurement. This suggests that the intensity of fear was reduced. However, open label studies where both the researcher and the owner are aware of the treatment provided should be interpreted with caution (Riemer 2020). Unblinded studies can lead to placebo by proxy effects, where the owner perceives the product to be effective when there are no objectively measured benefits to the animal (Riemer 2020). Owner compliance can be problematic in studies where researchers are reliant upon dog owners to implement the treatment according to manufacturer instructions. To be effective, the pheromone diffuser should be kept switched on at all times (Adaptil, 2020) and owners should monitor and replace the diffuser when the pheromone runs out (Mills *et al.*, 2012). Owner compliance was not evaluated in Sheppard and Mills (2003), so it is unknown whether owners complied with treatment instructions. The study also relied heavily on owner reporting of behaviour, which is subjective (Frank *et al.*, 2010).

4.3.2 Adaptil® diffuser combined with behavioural modification

Pheromones dispersed via a diffuser combined with behavioural modifications, such as firework desensitisation, CD's and counterconditioning, have been investigated in dogs fearful of fireworks (Levine, Ramos and Mills, 2007). A mixture of owner reporting of behaviour and researchers scoring behavioural footage found an improvement in behaviour and fear severity scores in dogs post pheromone exposure. Although, there were no reported improvements for vigilance behaviour (Levine, Ramos and Mills, 2007), which is considered an anxiety related behaviour (Kim *et al.*, 2010). Similar to Sheppard and Mills (2003), the study was unblinded. It also lacked a placebo control; therefore, these findings may be somewhat limited by observer bias. A follow up study of longer duration also found improvements in dog behaviour when exposed to fireworks in combination with Adaptil® as well as desensitisation and counterconditioning using noise CD's, but lacked a placebo group, therefore restricting comparative data (Levine and Mills, 2008). A key limitation of these studies (and the field in general) is the inability to disentangle any effects of the pheromones and those produced by behavioural modification (Frank *et al.*, 2010), and whether one influences the other.

4.3.3 Adaptil® collar

The effects of dog appeasing pheromone collars on fear and anxiety in puppies during training have been studied (Denenberg and Landsberg, 2008). The authors focused on longer term impacts by assessing whether puppies exposed to pheromones were better socialised and adapted to novel situations quicker as they matured. Compared to previous studies (e.g. Levine, Ramos and Mills, 2007; Levine and Mills, 2008), a blinded placebo controlled experimental design was implemented which tested for any effects of the collar. Dogs exposed to the pheromone were found to have improved socialisation compared to a placebo group, and displayed reduced fear during puppy classes. However, it could not be determined what effect factors such as training had on the puppies. Any behavioural effects of pheromones may be affected by learning and experience (Coureaud *et al.*, 2010), which may occur during training in dogs. In rabbits *(Oryctolagus cuniculus)*, natural mammary pheromones have been found to aid acquisition of information from the surrounding environment that supports physical and social development and enhances learning of novel odourants and mixtures of odours (Coureaud *et al.,* 2010). Although this is poorly understood at the behavioural level (Mainen, 2006), and warrants further investigation in dogs to help understand effects of pheromones on behaviour. Synthetic pheromones also differ in complexity of compounds compared to the natural mammary pheromone and this may change the meaning of a message (Mills *et al.*, 2012), therefore potentially altering an animals response to stimuli and subsequent learning.

More recently, pheromone impregnated collars have been reported to reduce behaviour scores of fear and anxiety in laboratory housed Beagles when exposed to simulated thunderstorm recordings using a placebo-controlled study design (Landsberg *et al.*, 2015). However, the sample was small (N = 24) and may not be representative or generalisable to other populations (Polgar *et al.*, 2019), such as home environments where factors such as owner behaviour may influence how dogs respond to fireworks. Furthermore, laboratory housed dogs may respond differently to companion dogs due to differing management practices and interactions with caregivers (de Souza *et al.*, 2017). Many of the Beagles also spent a large proportion of the time hiding when exposed to pheromones. This is surprising, given hiding behaviour is an expression of fear in dogs (Beerda *et al.,* 1997) and pheromones are purported to reduce fear. Though the authors suggested increased hiding behaviour was counteracting noise-related increased reactivity (Landsberg *et al.,* 2015). Conversely, it may be that the pheromone collars simply had no effect on the individuals in reducing fear related behaviour.

4.3.4 Adaptil® spray

In a rescue shelter environment, Adaptil® diffused into the environment has been reported to reduce mean barking amplitude and barking frequency in dogs, suggestive of reduced stress; though the findings are open to interpretation (Tod *et al.,* 2005). Barking can be expressed by different behavioural manifestations including socially facilitated, excitement and attention seeking (Sales *et al.,* 1997; Sheppard and Mills, 2003) therefore, gauging the underlying motivation for barking behaviour is problematic. Nonetheless, it is reasonable to assume that given shelters are inherently stressful environments due to being novel, uncontrollable and noisy environments, that barking is likely to stem from stress in these environments (Tod *et al.,* 2005). Previous studies in shelters have focused on Adaptil® diffusers, and collars have been tested in similar environments such as laboratory conditions but, to date, Adaptil® spray has not been tested in sheltered dogs. A disadvantage of the diffuser and collar format of delivery is the length of time it takes to disperse into the environment. The manufacturer states that these methods can take up to 24 hours to diffuse into the environment to become effective, primarily due to reliance on the product being heated through the electronic diffuser or through contact with the dog's skin on the collar (Adaptil, 2021). This means that there may be a delay in observing any effects. On the other hand, a benefit of the spray is its rapid application which may elicit a prompt and almost immediate effect in reducing stress-related behaviours. Its practical application may be useful in situations where dogs enter a kennel environment, which is an acute stressor (Rooney, Gaines and Bradshaw, 2007; Dudley *et al.,* 2015) and where dogs may benefit the most from reduced stress.

Further work is required to establish any acute effects of Adaptil® spray on stress-related behaviours in confined sheltered dogs. These findings may inform rescue shelters whether Adaptil® is an effective and cost-efficient method to reduce stress-related behaviour in sheltered dogs. If products are not effective, they may be wasting valuable funds which could be better spent elsewhere. For example, it may be more cost-efficient for shelters (where financial budgets are limited) to implement other stress reducing measures which are supported by a stronger evidentiary basis.

4.3.5 Alternative treatments

There is variable evidence in the field-and a lack of rigorous empirical evidence- that supports pheromone use, including similar calming products and their effectiveness at relaxing dogs (Riemer, 2020). 'Alternative' treatments purporting a calming effect have been evaluated using an owner completed online questionnaire when assessing dogs with a fear of fireworks (Riemer, 2020). The alternative treatments consisted of pheromones, herbal remedies, nutraceuticals, essential oils, homeopathic remedies and Back flowers (Riemer, 2020). Owner reported success rates of all evaluated treatments were in the range of 27-35%, similar to response rates to placebos which typically report success rates in the region of 35% (Muñana *et al.,* 2010). It was proposed that alternative treatments are no more effective than a placebo, suggesting that a placebo by proxy affect may have taken place instead of any treatment effect (Riemer, 2020). A key limitation of this study is that individual products were not evaluated, but instead grouped together into an 'alternative' category. Likewise, method of pheromone delivery (e.g. collar, diffuser, spray) was not indicated. This blanket approach meant no meaningful comparisons could be drawn between the individual products or the method of pheromone delivery, therefore limiting any conclusions.

4.3.6 Methodological limitations of Adaptil® research

Some studies suggest little effect of Adaptil® in reducing stress-related behaviour. In the last decade, a meta-analysis has been conducted on the efficacy of pheromone treatments for dogs (Frank *et al.,* 2010). Only one study reported a sufficient evidence base for a reduction in fear and anxiety in dogs, compared to six studies which were rendered as insufficient evidence bases due to a range of methodological issues (Frank *et al.*, 2010). These included poor inclusion criteria, unclear randomisation methods, and non-reporting of dogs with treatment failure (Frank *et al.,* 2010), which all limit any conclusions drawn on the efficacy of Adaptil®. Beyond a paucity of research, it is important to investigate the efficacy of Adaptil® using robust measures. Detailed inclusion criteria, clear reporting of randomisation methods and findings are key to conclusively determining what effect Adaptil® has in reducing stress in dogs. Studies which lack clear reporting of methods can weaken internal validity and whether any effects observed are due to the treatment or other factors within the environment (Frank *et al.,* 2010; McLeod, 2013). Similarly, a lack of randomised trials can introduce biases, which impact external validity and how generalisable the study findings are to the wider population (Frank *et al.*, 2010; McLeod, 2013). In some cases, study findings have also been prevented from publication due to being unfavourable to the pharmaceutical and pet product industry (Yaun *et al.*, 2011). This risks introducing bias (Mlinarić *et al.,* 2017), which can misinform canine welfare and veterinary industries and subsequently clinical practice. Therefore, it is important that studies are published where an effect is not found.

Within Evidence Source 3 and Appendix 1, the efficacy of Adaptil® spray in reducing vocalisation intensity and frequency of stress-related behaviours in sheltered dogs will be explored by gathering data using a counterbalanced repeated measures design. These will be taken forward to address the lack of research on Adaptil® spray in sheltered dogs. The study findings can contribute to understanding the efficacy of Adaptil® spray as a stress relief product through generating data which was previously unknown, and provides fundamental knowledge about the efficacy of Adaptil® spray and stress in sheltered dogs, which can be used as an evidence base for future research to build on. The impact of this research can then be used to inform veterinary professional decision making when recommending such products to clients or to help shelters assess whether the use of Adaptil® is an effective and cost-efficient method to reduce stress-related behaviour in dogs.

4.4 EVIDENCE SOURCE 3

Hermiston, C., Montrose, V.T. and **Taylor, S**. (2018) 'The effects of dog-appeasing pheromone spray upon canine vocalizations and stress-related behaviors in a rescue shelter', *Journal of Veterinary Behavior*, 26, pp.11-16.

4.4.1 Summary

Hermiston *et al.,* (2018) (Appendix 1) aimed to determine whether Dog Appeasing Pheromone (e.g. Adaptil®) spray reduced vocalisation intensity and the frequency of stress-related behaviours in 25 sheltered dogs upon exposure to an acute stressor (e.g. brief presence of an unfamiliar dog). Barking intensity, frequency of barking, and stress-related behaviours in the presence of a stressor were recorded over a 16-week period using a repeated measures design with and without the use of spray pheromones. There was a significant reduction in mean barking intensity (dB) ($P < 0.002$). Intensity of barking was reduced by 6.48 dB in dogs exposed to DAP spray, but there was no significant difference ($P > 0.05$) in the frequency of barking or occurrence of stress-related behaviours. It was difficult to interpret any clinical significance in a reduction in barking, and the results suggest a reduction in barking alone may not represent improved welfare as other stress indicators remained unchanged.

4.4.2 Rationale

This study built on Taylor and Madden (2016) and Binks' *et al.,* (2018) odour research by investigating pheromonal olfactory stimuli, which similarly to natural essential oils, have also been purported to reduce stress-related behaviour in captive dogs. Previous shelter studies have focused on administration of Adaptil® using diffuser or collar delivery methods however, to the author's knowledge, no previous study has investigated Adaptil® spray in a shelter environment. This was the first study to examine canine behavioural responses to Adaptil® spray in a shelter setting where treatment efficacy is unknown. The study also addressed the lack of efficacy data in the field and, unlike many previous studies, was not funded by the manufacturer which potentially introduces bias. Furthermore, the rapid application of Adaptil® spray may be particularly beneficial in reducing acute stress in the shelter environment, where dogs are admitted, and acute stress is prevalent (Rooney, Gaines and Bradshaw, 2007). If found to be effective, Adaptil® spray may improve the welfare of dogs admitted to shelters. Previous reports of beneficial effects of Adaptil® spray have been anecdotal, and several studies using other Adaptil® delivery methods have relied on subjective owner reports of efficacy (e.g. Sheppard and Mills, 2006, Levine, Ramos and Mills, 2007 and Riemer, 2020). Therefore, clinical trials are key to justify the use and efficacy of stress relief products. There are ethical complications if products are ineffective as, if acute and chronic stress is not resolved, it can cause immunosuppression and in turn increase disease from pathogens, leading to health implications (McEwen, 2005; Tanaka *et al.,* 2012); consequently, affecting life span. (McEwen, 2005).

4.4.3 Contribution to the field of synthetic pheromone research

To the author's knowledge, this was the first study to clinically test the efficacy of Adaptil® spray in reducing stress-related behaviour in sheltered dogs, and as such provides a unique scientific contribution to research in the field where the effects of Adaptil® spray are unknown. Adaptil® spray was not found to reduce behaviours indicative of stress in this study. It is plausible that given the small sample size (N = 25), behaviours indicative of stress reduction may not have been representative where behavioural variation was limited. It may be possible that an effect was not detected due to low power therefore, in an ideal scenario, future research should be conducted with a larger sample size, with a recommendation of N = 383 dogs (Survey System, 2022). However, given the high turnover of dogs in a shelter this may not be practically feasible, although dogs tested across multiple shelter sites could be utilised to help overcome this issue. Furthermore, the brief exposure to the stressor dog may not have been long enough to induce an effect, therefore exposure to different stressors needs to be investigated. The number of different breeds utilised in the study may also have influenced variation of pheromone detection and susceptibility (e.g. pharmacokinetics). Using a standardised breed in a shelter to reduce breed-specific variability is difficult given mixed breeds are most commonly relinquished to shelters (Carter and Martin, 2021), although there may be some scope to utilise Staffordshire Bull Terriers (see 2.3.4). Nonetheless, the study findings can be used as an evidence base for future research to build on, as well as addressing methodological limitations in the field simultaneously (see 4.4.4). The study findings contribute to understanding the efficacy of Adaptil® spray as a stress relief product through generating data which was previously unknown, and provides fundamental knowledge about the efficacy of Adaptil® spray and stress in sheltered dogs, which can be built upon. The study also addresses the need for publishing studies where an effect is not found to avoid misinforming canine welfare, veterinary industries and subsequently, clinical practice.

4.4.4 Research methodologies and limitations

The study was limited by only measuring behavioural parameters and did not include cognitive or physiological testing. Therefore, the emotional valence of dogs- and the underlying motivations for barking behaviour- was unable to be determined. It is important to note that behaviour can be context dependent, and the same behaviour can serve different functions. For example, the function of barking in dogs may serve as intraspecific communication, whether that is to exhibit signs of boredom or to alert owners of a potential threat (Protopopova *et al.,* 2016). As a result, this limits behavioural measures in determining overall welfare. Therefore, behaviour should be considered alongside the context the behaviour is occurring in, as well as cognitive and physiological indicators which may help determine the mental and physiological state of an animal and aide evaluation of emotional valence and stress. Context was considered in the study and given that novel laboratory environments are inherently stressful, it is reasonable to assume that the motivation for barking was likely due to stress. A validated quality of life assessment tool as proposed by Kiddie and Collins (2015) or Qualitative Behaviour Assessment (QBA) (Wemelsfelder, 2007) (see 3.4.5) to measure emotional expression could be used alongside these parameters, and would be cheap and simple to implement.

A further limitation is the lack of a fully blinded and placebo-controlled study design which, if incorporated, would have reduced potential observer bias. However, robust measures such as a repeated measures design and clear reporting of randomisation and findings were included, these addressing limitations of previous studies (highlighted by Frank *et al.,* 2010), strengthening reliability and validity. Nonetheless, further work needs to incorporate fully blinded and placebo-controlled study designs to reduce bias and ascertain treatment effects.

Quantity of pheromones dispersed into the environment relative to the kennel size may impact treatment efficacy. Although the amount of pheromones applied followed manufacturer guidelines, it is conceivable that the quantity of pheromones dispersed was not enough for the product to be effective in this instance. To increase the chances of synthetic pheromones being detected, large concentrations are required (Mills *et al.*, 2012). This is because when synthetic pheromones are used, there is an absence of an accessory signal and an individual's unique protein produced by the animal, with this naturally aiding engagement of the VNO in detecting the pheromone (Pageat and Gaultier, 2003; Mills *et al.*, 2012). It is possible that the volume of pheromones dispersed into the environment was not sufficient in activating the VNO, and therefore a behavioural change was not observed. Selecting the amount of pheromones to apply to the environment was guided by the manufacturer's instructions, which recommend 8-10 pumps for every application at least 15 minutes prior to dog exposure (Adaptil, 2020). Two pumps of Adaptil® spray were applied to each of the four corners of the kennels 30 minutes before dogs came into contact with them, in accordance with previous Adaptil® studies (e.g. Tod *et al.*, 2005; Levene *et al.*, 2007). Consequently, there should have been a sufficient volume of Adaptil® dispersed into the environment to reduce stress-related behaviour if it occurred. The manufacturers suggest one plug in Adaptil® diffuser is used for an area of up to $70m^2$ (Adaptil, 2020), however there is no guidance on area size and application for the spray. The kennels were situated in a row with a wire mesh fronted barrier; therefore, it is possible that the pheromones dissipated beyond the immediate kennel area and became less effective. Nevertheless, all dogs in the kennel block were tested in the Adaptil® condition at the same time, so there should have been a sufficient amount of Adaptil® in the surrounding environment to counteract any effects of the pheromone dispersing beyond the intentional area. To counteract the issues with an open kennel design, fully enclosed kennels could be used to test Adaptil® spray in the future.

The synthetic pheromone may also not have been effective because it may not support the rapid promotion of learning, which occurs naturally on exposure to the maternal pheromone in neonatal mammals thus allowing behavioural adaptation to the environment (Gottlieb, 1976; Alberts, 1987). Maternal pheromones act as olfactory reinforcers, where learned odourants help orient new-borns to safe areas of the environment (Coureaud *et al.,* 2010). In rabbits *(Oryctolagus cuniculus),* olfactory cues located on the doe's abdomen exposed during nursing act as reinforcers that turn a neutral odour into a meaningful olfactory cue (Coureaud *et al.*, 2010). The omission of this process through the use of synthetic pheromones may mean that this process does not occur. The application of the natural pheromone versus the synthetic version differs (e.g. dispersed from mother versus dispersed into the air via a diffuser or spray). Therefore, the perception of the pheromone may be different given the different contexts associated with application (Mills *et al.,* 2012).

It is also important to note that the study was underpowered by the small sample (N=25) and so may not have detected important therapeutic effects of Adaptil® (Mills *et al.,* 2012). Furthermore, the sample size may have restricted the broad behavioural repertoire that can be seen in a larger number of dogs, and so may not have been representative of shelter dog behaviour. However, the sample size is consistent with other research in the field (e.g. Sheppard and Mills, 2003; Mills *et al.,* 2006; Landsberg *et al.,* 2015). Recruiting a large sample of dogs for shelter-based research is problematic, given the nature of rescue shelters where dogs are frequently being adopted and leaving the shelter at short notice. The length of the research period (1 hour per dog) was primarily chosen as it was feasible for the research team as well as rescue shelter staff. Mills *et al.,* (2012) recommend trials of at least 4 weeks in length to see any effects of Adaptil®, so it was feasible that dogs may not have been exposed long enough to observe any effects. However, given the spray is designed to elicit an almost immediate effect, a rapid stress reducing effect should have been observed. Nonetheless, future research should focus on using a longer exposure time and any long-term effects of Adaptil®, given that many dogs admitted to shelters stay for an average of 42 days where chronic stress may prevail (Brown *et al.,* 2013). A longer study duration would also reflect the reality of prolonged shelter stays for dogs, and may provide a more detailed assessment of the impacts of pheromones in shelters. Therefore, a study duration of at least 4 weeks is recommended for future research.

4.4.5 Implications and questions generated

A useful wider application of Adaptil® spray beyond the shelter environment may be when adopted dogs enter new homes and encounter short term stressors, such as existing pets within the household. Entering a new household can be a stressful environment for dogs and behavioural problems can arise as a result, therefore there is merit in testing the efficacy of Adaptil® spray in this scenario; which has yet to be tested.

It would also be interesting to conduct neurological studies on the effects of pheromones in dogs as, to date, studies have only been carried out in pigs (Anderson *et al.*, 2001). Functional magnetic resonance imaging (fMRI) could be used to provide a detailed evaluation of how blood flow in regions of the brain react to pheromones, including whether there is a positive association, which has been evidenced through maximum activation of the caudate nucleus in canine brain responses to familiar human odours (Berns, Brooks and Spivak, 2015). Functional magnetic resonance imaging has been validated for deciphering olfaction driven brain activations and is a reliable method for testing olfactory neuroimaging in dogs (Jia *et al.*, 2014; Jia *et al.*, 2015; Berns, Brooks and Spivak, 2015). This would negate some of the difficulties associated with subjective behavioural observation used as a standalone measure and false negatives in the future. However, while dogs used in fMRI studies are awake and unrestrained, they are trained to keep their heads still in a behaviour laboratory setting and are limited to being stationary which does not replicate real-life scenarios where changes in the brain may be more representative of stress and what would occur in a more 'natural' setting. As technology advances, it may be feasible to combine both behavioural and brain activity observation (Jia *et al.*, 2015) while the dog is ambulatory and in a real world setting (e.g. kennel environment). This would provide a more detailed assessment of underlying cognitive processes and pheromone efficacy in 'real life' stressful environments (Thompkins *et al.*, 2016; Kokocińska-Kusiak *et al.*, 2021), but this has yet to be developed and tested. Jia *et al.*, (2014) utilised an external infrared camera alongside fMRI to track head motion and retrospectively corrected motion-related artefacts in the data, but this technology needs to be adapted for a full body range of motion in dogs as currently rapid movement is unable to be analysed due to poor fMRI temporal resolution (Kokocińska-Kusiak *et al.*, 2021). In the interim, Kokocińska-Kusiak *et al.*, (2021) suggest prospective motion correction using an external camera or image based tracking in stationary dogs (Thesen *et al.*, 2012; Maclaren *et al.*, 2012; Todd *et al.*, 2015), which could be used when dogs are exposed to pheromones. Research incorporating fMRI should be carried out in accordance with the Guideline for the Use of Animals in Research described by the Association for the Study of Animal Behaviour (ASAB) and experimental protocols reviewed and approved by the institutions Ethics Committee for full scrutiny of ethical concerns (Gábor *et al.*, 2020).

Another future area of interest is sniffing duration and nostril laterality, which have been used to determine perception of odours in dogs (Siniscalchi *et al.*, 2008; Siniscalchi *et al.*, 2010; Siniscalchi *et al.*, 2011; Hall *et al.*, 2015). These measures could be recorded in conditions with and without odours and pheromones to determine whether dogs have detected the scent and whether this correlates with a decrease in stress-related behaviours. Sniffing duration in detection dogs has been found to be shorter for true negatives than false negatives, true positives, and false positives (Concha *et al.*, 2014). Furthermore, left nostril lateralisation indicates familiar and non-aversive odours, while right lateralisation suggests odours are novel, threatening, or arousing (Kokocińska-Kusiak *et al.*, 2021). A focus on sniffing behaviour and nostril lateralisation could be used to further assess odour and pheromone efficacy and help avoid ambiguous results and potential false negatives in the future.

Similarly, it would be interesting to incorporate physiological parameters when testing Adaptil® (addressed by Evidence Source 4). Heart rate (HR), heart rate variability (HRV), cortisol and infrared thermography (IRT) measurements of ear and eye temperature have been reliably used alongside behavioural parameters as indicators of stress in dogs (Brugarolas *et al.*, 2015; Travain *et* *al.*, 2015; Riemer *et al.*, 2016; Cobb *et al.*, 2016), but few studies exist using physiological measures of stress when testing Adaptil®. Neurological study findings could indicate whether pheromones change brain functioning in response to stress, while physiological parameters and cognitive testing could help assess physiological and cognitive changes in response to pheromones.

CHAPTER FIVE

BEHAVIOURAL AND PHYSIOLOGICAL PARAMETERS OF STRESS AND ADAPTIL®

5.1 Focus of the critical review

Behavioural responses of dogs to Adaptil® have been widely studied as a single measure of stress though physiological indicators of stress are not widely reported in Adaptil® studies. Combining physiological and behavioural assessments can triangulate stress level and duration to obtain a more accurate assessment of stress (Part *et al.*, 2014; Hekman, Karas and Sharp, 2014; Ott *et al.*, 2014). As a result, the true efficacy of Adaptil® may be determined. Chapter 5 evaluates behavioural and physiological parameters as measures of stress including: heart rate (HR), heart rate variability (HRV) and infrared thermography (IRT) using eye and ear pinna temperature. The chapter also explores the importance of combining parameters, as well as reviewing the use of behavioural and physiological parameters as tools to measure the effects of Adaptil® in reducing canine stress.

5.2 Behavioural parameters

A common way to measure an animal's response to stress is through behavioural observations and recording responses to the environment (Bergamasco *et al.*, 2010). An obvious benefit of using behavioural observations is the method's non-invasive nature (Pastore *et al.*, 2011), which reduces confounding factors relating to handling stress. Observing and recording behaviour also allows researchers to consider individual behavioural responses of animals (Pastore *et al.*, 2011), which can then be conveyed to groups of individuals on a wider scale. However, behavioural observations are reliant on observer interpretation of behaviour, and are therefore subjective (Pastore *et al.*, 2011). Ethograms have been developed in an attempt to standardise methods to describe behaviour (Bateson and Martin, 2021), with these helping researchers to record behaviour more objectively; though they do not consider individual variability between animals. Further, the context stress occurs in, the intensity of the threat perceived by the animal and how well the animal copes will differ between individuals (Appleby and Hughes, 1998). How an animal copes with stress is influenced by previous experiences of stress (Horowitz, 2004), but is also influenced by genetic factors such as genetic predisposition (Horowitz, 2004; Stephen and Ledger, 2006) including breed and sex (Serpell and Hsu, 2005). Environmental factors including experience (Appleby, Bradshaw and Casey, 2002; Horowitz, 2004), rearing environment (Harvey *et al.*, 2016), temperament (Jones and Gosling, 2005) and neuter status (Serpell and Hsu, 2005) can also impact coping strategies. Similarly, whether a dog's coping style is reactive (negative) or proactive (positive) will influence how they cope with stress (Blackwell *et al.*, 2010; Rooney *et al.*, 2016). These factors should be taken into consideration and controlled for when quantifying stress in individuals.

5.3 Physiological parameters

An animal's internal response to stress can be measured using physiological parameters that record fluctuations in the cardiovascular, endocrine and integumentary systems. These fluctuations are measured on how far they deviate from normal parameters when assessing stress. However, they are influenced by factors that make their interpretation problematic. These include circadian rhythms (Kikkawa *et al.*, 2003), health (Lucassen *et al.*, 2014), level of locomotor activity (Hiby, Rooney and Bradshaw, 2006), stress intensity (Beerda *et al.*, 1998) and life experiences (Haverbeke *et al.*, 2008). These factors can impact the reliability of findings, so is therefore recommended that several physiological parameters are evaluated simultaneously. Using multiple parameters will triangulate stress level and duration to obtain a more accurate assessment of stress (Part *et al.*, 2014; Hekman, Karas and Sharp, 2014; Ott *et al.*, 2014). A typical combination of physiological parameters used to measure stress include: heart rate (HR), heart rate variability (HRV) and Infrared Thermography (IRT) via measurements of core eye and ear temperature.

5.3.1 Heart Rate (HR) and Heart Rate Variability (HRV)

An increasingly popular methodology employed by researchers to obtain cardiovascular data is the use of non-invasive human heart rate monitors, which have been approved for use in animals (Vincent and Leahy, 1997; Kuruvilla and Frankel, 2003; Palestrini *et al.,* 2005; Ogata *et al.,* 2006; Fallani *et al.,* 2007; Rehn and Keeling, 2011; Jonckheer-Sheehy *et al.,* 2012). Compared to traditional electrocardiogram (ECG) use in animals, heart rate monitors are portable and cheap to purchase (Jonckheer-Sheehy *et al.,* 2012). Heart rate is measured using the number of times the heart beats per minute (BPM). When used in isolation, interpreting HR can be difficult (Bombail, 2017). This is because HR reflects intensity of arousal rather than whether the arousal is positively or negatively induced. Moreover, increases in HR are also influenced by locomotor activity, posture and breed (Palestrini *et al.,* 2005; Maros *et al.,* 2008; Varshney, 2020), limiting its use as a single measure when attempting to determine stress linked with emotional state (von Borell *et al.,* 2007).

A more useful tool to assess stress is heart rate variability (HRV) (von Borell *et al.,* 2007; Brugarolas *et al.,* 2015; Katayama *et al.,* 2016; Maccariello *et al.,* 2018). Heart rate variability is considered a sensitive assessment of stress as it takes into account the functioning of the autonomic nervous system (ANS) in response to stressors, rather than being influenced by factors that alter heart rate e.g. arousal (Palazzolo, Estafanous and Murray, 1998; Maccariello *et al.,* 2018). Heart rate variability is the length of time between beats, also known as R-R interval, and is typically calculated using standard deviation of normal-to-normal R-R intervals (SDNN) (Katayama *et al.,* 2016). When stress is encountered, SDNN will decrease alongside an elevated HR (Mohr *et al.,* 2002; Gacsi *et al.,* 2013). Heart rate variability has been validated to assess stress across numerous veterinary and behavioural research settings in a variety of species and contexts (von Borell *et al.,* 2007) including pigs (Jong *et al.,* 2000), cows (Mohr *et al.,* 2002), chickens (Korte *et al.,* 1999) and horses (Reitmann *et al.,* 2004). Heart rate variability in dogs has been assessed alongside endocrine (serum cortisol) and behavioural responses to stress (Beerda *et al.,* 1997; Beerda *et al.,* 1998; Beerda *et al.,* 1999; Hydbring-Sandberg *et al.,* 2004;

Bergamasco *et al.,* 2010; de Souza *et al.,* 2017), although compared to other species, there are fewer studies that use HRV when trying to establish emotional state in dogs.

5.3.2 Infrared Thermography (IRT) – eye and ear pinna temperature

When an animal experiences a stressor, the HPA axis is activated, altering blood flow patterns and increasing core body temperature (Bouwknecht *et al.,* 2007; Mazzotti and Boere, 2009). This increase in body temperature can be measured using a non-contact Infrared Thermal Imaging (IRT) device, which detects radiation in the long-infrared range of the electromagnetic spectrum from the surface of an animal (Stewart *et al.,* 2008). This is then transformed into images (Stewart *et al.,* 2008) known as thermograms. Eye temperature has been reliably correlated with core body temperature through rectal measurement (Johnson *et al.,* 2011). Consequently, eye temperature has been used to measure fluctuations in body temperature in a number of animals, including dogs (Travain *et al.,* 2015; Travain *et al.,* 2016). Direction of temperature though, appears to relate to body area with decreases in temperature having been linked to body extremities. For example, decreases in surface temperature observed on the ear pinnae have been reported in dogs exposed to separation stress (Riemer *et al.,* 2016). It is thought that when the sympathetic nervous system is triggered, peripheral vasoconstriction causes a decrease in surface temperature, this having been recorded using IRT in human fingertips (Kistler *et al.,* 1998). The human fingertip is classed as an extremity, as is the ear pinnae area. Infrared Thermography is advantageous when measuring stress because it is a no contact device, though the animal is required to be restrained briefly to capture the image (Travain *et al.,* 2015). This has been found to produce an avoidance response in dogs when the camera is close to the muzzle. However, whether this is perceived as a stressor needs further investigation (Travain *et al.,* 2015). Changes in eye temperature can mirror changes in arousal although do not reflect emotional valance (Travain *et al.,* 2016). If IRT is used in combination with other physiological parameters such as HR and HRV, it can provide supplementary physiological data to further support behavioural indicators of stress.

5.4 Using behavioural and physiological parameters as tools to test the efficacy of Adaptil® in reducing canine stress

Historically, canine research has concentrated on reporting behavioural responses as a single measure of stress in dogs when investigating the effects of Adaptil®. This is potentially due to attempts to reduce confounding effects of handling, which is required to obtain physiological measurements of stress and may influence the effectiveness of the treatment. However, relying on behavioural states alone when interpreting stress can be ambivalent and difficult to interpret because they are reliant on context (Horwitz and Pike, 2014). Incorporating physiological measures and therefore using multiple parameters will triangulate stress level and duration to obtain a more accurate assessment of stress (Part *et al.,* 2014; Hekman, Karas and Sharp, 2014; Ott *et al.,* 2014). Physiological stress responses are largely unreported in pheromone studies. Consequently, there is an opportunity to combine both physiological and behavioural parameters to obtain a more objective and broader measure of stress in dogs (Maccariello *et al.,* 2018), and their responses to Adaptil®. Heart rate and HRV can be paired with IRT as indicators of psychological stress (Squibb *et al.,* 2018). Combining these measures can provide supplementary physiological data to further support behavioural indicators of stress. This may help verify stress responses in dogs subjected to pheromonal application as well as whether these applications are effective at reducing stress. Testing the efficacy of pheromone products is important. As Adaptil® is widely used in the clinical setting (BSAVA 2017), study findings are applicable to canine welfare and veterinary industries. Study findings can be used to inform clinical practice in relation to the efficacy of synthetic pheromones in reducing stress behaviours of dogs. In particular, research can inform whether pheromonal products are a worthwhile investment in reducing behavioural and physiological indicators of stress.

Within Evidence Source 4 and Appendix 1, the efficacy of the Adaptil® diffuser in reducing behavioural and physiological stress responses in dogs will be explored. Data will be gathered from confined pet dogs. The dogs will be tested in a 'laboratory like' standardised condition using both behavioural and physiological measurements of stress, using a blinded, placebo-controlled, repeated measures design. These will be taken forward to address limitations of current research by combining behavioural and physiological parameters for a more objective and broader measure of stress, and to test the efficacy of Adaptil®. The impact of this research can then be used to inform clinical practice regarding whether pheromone applications are a worthwhile investment.

5.5 EVIDENCE SOURCE 4

Taylor, S., Webb, L., Montrose, V.T. and Williams, J. (2020) 'The behavioral and physiological effects of dog appeasing pheromone upon canine behavior during separation from owner', *Journal of Veterinary Behavior,* 40, pp.36-42.

5.5.1 Summary

Taylor *et al.,* (2020) (Appendix 1) aimed to determine whether Adaptil® (formerly known as dog appeasing pheromone) dispersed using a diffuser reduced behavioural and physiological stress responses in 10 pet dogs when separated from their owners in a 'laboratory like' standardised environment. Acute stress responses were recorded, induced by brief exposure to a novel environment and short term separation from the owner. A repeated measures design with and without the use of Adaptil® and in the presence and absence of the owner was used. Behavioural responses were recorded in real time and video recorded using a focal instantaneous sampling technique. To control for potential bias, 10% of the videos were scored using a second blinded scorer to assess interrater reliability. Heart rate (HR), heart rate variability (HRV) using standard deviation of normal-to-normal beats (SDNN), and eye and ear temperature (ºC) were also collected to assess the dog's physiological state. When owner separated, oriented behaviour and eye temperature increased with Adaptil®. Eye temperature also increased when the owners were present after exposure to Adaptil®, which suggests arousal due to owner presence or absence rather than any effect of Adaptil®. There was no significant effect of Adaptil® on HR or ear temperature. Overall, the results suggested that the application of a Adaptil® diffuser did not markedly influence the behaviour, HR, and eye or ear temperature of pet dogs.

5.5.2 Rationale

Behavioural studies (e.g. Sheppard and Mills, 2003; Levine, Ramos and Mills, 2007; Levine and Mills, 2008) had highlighted that the use of Adaptil® resulted in calm behaviours suggestive of decreased stress in dogs. However, all of these studies lacked a placebo control which restricted comparative data and all but one lacked blinding, meaning that findings may have been limited by observer bias. Taylor *et al.,* (2020) addressed the limitations of Sheppard and Mills

(2003), Levine, Ramos and Mills (2007) and Levine and Mills (2008) and strengthened the field of research by incorporating a placebo controlled and blinded study, reducing potential detection bias. Taylor *et al.,* (2020) also addressed the need for the recording of physiological stress responses alongside behavioural parameters. Combining physiological and behavioural assessments can gauge underlying motivations for the behaviour, which can help provide a broader picture of stress (Mason and Mendl, 1993) and have historically been successfully validated to measure fear and anxiety in other canine studies (e.g. (Brugarolas *et al.*, 2015; Mariti *et al.,* 2018).

5.5.3 Contribution to the field of synthetic pheromone research

This study provided new insights into the efficacy of Adaptil® on stress physiology and behaviour in dogs. Historically, physiological parameters have rarely been used in pheromonal studies of stress in dogs, with behaviour often being used as a single measure of stress. Incorporating physiological measures and therefore using multiple parameters helps triangulate stress level and duration and obtains a more accurate assessment of stress (Part *et al.,* 2014; Hekman, Karas and Sharp, 2014; Ott *et al.,* 2014). The study provided HR, HRV and IRT data to supplement behavioural indicators of stress, and provided a more robust assessment of stress responses in dogs exposed to Adaptil® which had been lacking in previous studies. The study findings suggest that the application of Adaptil® did not markedly influence the behaviour or physiological parameters of dogs in a laboratory environment. When owner separated, oriented behaviour and eye temperature increased with Adaptil®. Eye temperature also increased when the owners were present after exposure to Adaptil®, which suggests arousal due to owner presence or absence rather than any effect of Adaptil®. Therefore, it is likely that owner presence and absence influenced changes in behaviour and some physiological parameters rather than Adaptil® having any discernible effect. The study also supports Hermiston *et al.,* (2018), Grigg and Piehler (2015) and Broach and Dunham (2016) who reported no effects of Adaptil® in reducing stress-related behaviour in confined dogs.

5.5.4 Research methodologies and limitations

Heart rate and HRV was recorded using a human Polar® RS800CX heart rate monitor, which has been validated for use in dogs (Jonckheer-Sheehy *et al.*, 2012; Essner *et al.*, 2013; Essner *et al.*, 2015). A limitation of recording HR and HRV is poor conductivity, which may be influenced by incorrectly located or fastened electrodes paired with locomotor activity (Jonckheer-Sheehy *et al.*, 2012; Lensen *et al.*, 2017). This can result in introducing noise in the data and invalid traces or artefacts, and it is recommended data are corrected during analysis to improve result reliability (Salo *et al.*, 2001; Peltola, 2012; Aranda *et al.*, 2017). To overcome issues with movement, early studies suggested using standardised conditions in order to identify treatment effects e.g. non-ambulatory conditions using stationary dogs (von Borell *et al.*, 2007; Jonckheer-Sheehy *et al.*, 2012). However, more recent studies have validated the Polar® RS800CX heart rate monitor for use in dogs that are locomotory (e.g. Essner *et al.*, 2013; Essner *et al.*, 2015), and so dogs were not restricted in the study. Our restricted study design also largely limited the motor activity of dogs (e.g. small laboratory size). However, there was one case where the signal dropped out, which has been reported as an issue in previous research, including Taylor and Madden (2016).

A pilot study was conducted to ensure the HR equipment fitted properly. There was some slipping of the sensor due to movement of the dogs, as such the strap designed for humans was adapted. To ensure a good contact was maintained, additional Vetrap 3M™ was secured around the monitor and strap to hold the sensors in place. This worked well for the pilot study but contact with the sensor was lost in one dog during one of the conditions during the main study. Anomalies were removed when selecting data for analysis, but this did consequently reduce the number of samples. Future research could use interpolation methods instead which replace the non-normal R–R intervals with new interpolated R–R intervals (Aranda *et al.*, 2017). A benefit of this method as opposed to deletion is that the number of samples remains the same (Aranda *et al.*, 2017). Dogs were also habituated to wearing the heart rate monitor prior to being observed to ensure novel effects and any influence on HR and HRV were reduced (Rehn and Keeling, 2011; Polgar *et al.*, 2019). Breed can also confound HR, with HR in toy and small breeds higher than larger breeds (Varshney, 2020). However, the repeated measures design minimised this by allowing comparisons of HR during conditions.

Due to limited control over access to the laboratory facilities and time constraints, the study design was not counterbalanced, and dogs were not able to visit the facilities at the same time for both conditions. As such, learning may have occurred, and non-randomisation and time effects are potential confounding factors. Nonetheless, a washout period was incorporated in an attempt to reduce potential confounding effects and ensure behaviours observed were related to the conditions. A 1 day "washout period" was implemented after the Adaptil® condition which allowed the product to fully dissipate from the room, aided by a built-in air conditioning system. The study was limited by the small sample size (N = 10), which may have contributed to the results where no effect of Adaptil® was found. As such, a larger sample to increase statistical power and level of evidence may be beneficial. A sample size calculation was conducted for future studies, with a recommended sample size of 383 dogs (Survey System, 2022).

5.5.5 Implications and questions generated

Findings from the study, supported by Hermiston *et al.,* (2018), could be used to inform clinical practice regarding whether pheromone applications are a worthwhile investment. For example, it may be more cost-efficient for vets to recommend, and for owners to purchase, products where a stronger evidentiary base exists for reducing stress-related behaviour. Furthermore, taken together the practical application of the findings from Hermiston *et al.,* (2018) and Taylor *et al.,* (2020) could be used by rescue shelters to evaluate the efficacy of pheromone products to help them assess whether the use of Adaptil® is an effective and cost-efficient method to reduce stress-related behaviour in shelter dogs. It may be financially more prudent to focus finances on addressing welfare issues using products which are supported by a strong evidentiary base that showcases their effectiveness. However, longitudinal studies that expose a larger number of dogs to Adaptil® for longer periods of time are required to further examine the findings of Taylor *et al.,* (2020). Building a larger evidence base that utilises both behavioural and a wider range of physiological parameters when testing Adaptil® will increase knowledge and understanding of the efficacy of stress relief products. Going forward, research is required that includes the addition of cortisol alongside these parameters, as well as further validating IRT methodology to include correlation of HR, cortisol levels and behaviour in response to Adaptil®.

Further building on the findings from Taylor *et al.,* (2020), it would be interesting to assess the efficacy of Adaptil® in pet dogs in the home environment. There were only brief owner absences and returns in the current study which was conducted in 'laboratory like' conditions that may not have replicated the home environment, in which longer periods of confinement and separation often occur (Rehn and Keeling, 2011) and where Adaptil® is frequently used. The owners also made repeated returns to their dog, therefore, the conditions may not have provided enough time to observe a full range of separation-related behaviour. Owner reporting of separation related behaviour is also subjective, with subtle behaviours often missed and anxiety related behaviours going unnoticed, particularly when not video recorded (Ogata, 2016; van Rooy *et al.,* 2018). This may lead to under reporting of the behavioural problem. Therefore, consideration of the separation behaviour phenotype beyond owner reporting in the home environment would be beneficial in future Adaptil® research.

Moving beyond owner reporting of separation behaviour, the use of accelerometers (e.g. SNIF tags, SNIF Labs, LLC) in combination with video recording to monitor activity levels in pet dogs when exposed to conditions with and without Adaptil® could be utilised. These could be used to assess whether there are changes in activity in the home environment and monitored remotely to reduce observer effect. Activity monitoring using accelerometers has been reliably recorded as a measure of stress in sheltered dogs (Jones *et al.,* 2014) and is accurate when compared with video recordings (Hansen *et al.,* 2007), but has yet to be tested in pet dogs in the home environment when exposed to Adaptil®. Orientation and head movement in the home environment could also be observed using global positioning system (GPS) and an inertial measurement unit (IMU) attached to a harness, to record whether there are changes in the dogs location, angular rate and orientation within the home environment when exposed to Adaptil®. For example, orientation towards entry and exit points, exploratory behaviour, orientation towards diffuser. Persistent orientation towards entry and exit points in the house and destruction of doors is frequently observed in dogs with separation-related behaviour (Lund and Jørgensen, 2011). A data logger connected to a GPS device and tilt ball sensor (e.g. Adafruit) could be utilised and has been successfully trialled in search and rescue dogs when assessing olfactory orientation (Jinn, Connor and Jacobs, 2020).

CHAPTER SIX

DISCUSSION

6.1 Focus of the critical review

Chapter Six critically reviews the results of the evidence sources found in Appendix 1 to assess odours and synthetic pheromones in reducing stress-related behaviour in dogs. A reflection on personal development is provided in Appendix 7.

6.2 Assessment of odours and synthetic pheromones in reducing stress-related behaviour in dogs

The goal of odours and pheromones is the same: they are used to target stress-related behaviour and are purported to alleviate stress and promote behaviours indicative of relaxation (Wells, 2009). However, there is a lack of evidence base in the use of odours and equivocal findings relating to the use of synthetic pheromones as effective strategies to reduce stress-related behaviour and thus improve captive canine welfare. The research presented aimed to address these limitations and investigate the efficacy of odours and pheromones in relieving stress-related behaviour in dogs. In particular, dogs housed in rescue shelters and pet dogs confined in novel 'laboratory like' conditions, which are challenging environments for dogs.

Odour assessment in pet dogs tested in a 'laboratory like' standardised condition (e.g. Taylor and Madden, 2016) found that Pet Remedy®, purported as a natural de-stress and calming product, did not reduce behavioural indicators indicative of a stress response. Although it is important to note that limited comparative data exists to validate the Taylor and Madden (2016) study findings. Existing published research only focuses on domestic rabbits *(Oryctolagus cuniculus)* and is not generalisable to dogs given species-specific behavioural differences. Interpretation of odour effects on behaviour can also be problematic. Behavioural indicators are inherently defined by the context they occur in, as well as being influenced by a large number of factors such as temperament (Jones and Gosling, 2005) and coping styles in response to confinement (Blackwell *et al.,* 2010; Rooney *et al.,* 2016). These can be influenced by genetic predisposition (Stephen and Ledger, 2006) including breed and sex (Serpell and Hsu, 2005) but also environmental factors including experience (Appleby, Bradshaw and Casey, 2002), rearing environment (Harvey *et al.,* 2016) and neuter status (Serpell and Hsu, 2005). It is possible that the large degree of individual differences that exist between dogs could propose explanations for the lack of differences observed, as behavioural responses have been found to differ between individuals (Beerda *et al.,* 1999; Hennessy *et al.,* 2001; Hiby *et al.,* 2006; Jones *et al.,* 2014; Part *et al.,* 2014), species and odour type (Bombail, 2017). Similarly, size and weight of dog, which will differ depending on breed, may have influenced how susceptible the dogs were to Pet Remedy® given that these factors can alter pharmacokinetics (Nair and Jacob, 2016). Given that there are individual differences in the behavioural expression of stress (Rooney, Gaines and Bradshaw, 2007), it may be that the product works for some dogs, but not others and may be dependent on previous experiences and temperament (Sheppard and Mills, 2003). Further research is warranted that accounts for individual variations. The study by Taylor and Madden (2016) was a first step in understanding the efficacy of Pet Remedy® in reducing stress-related behaviour in pet dogs. Until evidence-based research is available that supports the efficacy of Pet Remedy® as a standalone treatment for stress, dog owners and veterinarians could consider seeking other approaches that are supported by peer reviewed studies, for example behavioural modification. This could be alongside the use of Pet Remedy® in the interim, considering no harmful effects have been reported.

Although Taylor and Madden (2016) did not report Pet Remedy® reduced stress, some odours may be of value in enhancing wellbeing of confined dogs. Binks *et al.,* (2018) demonstrated that when applied individually, coconut, ginger, vanilla and valerian provide stress reducing effects on the behaviour of sheltered dogs, and is comparable to research on odour use in other kennelled dog studies (e.g. Wells *et al.,* 2002; Graham *et al.,* 2005). All of the odours tested reduced the frequency of vocalisations and activity levels. Excessive vocalisation and locomotory activity are expressions of stress in captive dogs (Wells and Hepper, 1992; Wells and Hepper, 2000; Beerda *et al.,* 2000), therefore a reduction in these indicators is suggestive of a stress relieving effect. In addition, coconut and ginger increased sleeping behaviour; indicative of relaxation. The information obtained could make a valuable contribution to the management of rescue shelters when implementing strategies to improve canine wellbeing. Some odours are widely used in shelters to promote calm behaviour (e.g. lavender). However, animals can become habituated to the repeated use of the same odours (Melfi *et al.,* 2007), whereby any beneficial effects eventually may become extinct. This is when an odour may no longer produce positive consequences and over time any beneficial behaviour associated with the odour decreases. Olfactory receptors can also become saturated with odours, leading to olfactory fatigue (Arasaradnam *et al.,* 2011). A practical application of the study findings by Binks *et al.,* (2018) could offer shelters a wider choice of odours, which can be regularly rotated to overcome any issues of habituation. An appropriate period of rotation has been determined in other species (e.g. big cats), however it is unknown how frequently odours would need to be rotated to maximise the benefits of any effects in dogs. Therefore, specific knowledge of effective odour rotation periods for dogs is an area which warrants further research. Using odours such as coconut, vanilla, ginger and valerian to enhance wellbeing in sheltered dogs is recommended given that behaviours indicative of relaxation and reduced stress were reported, supporting similar results in other species housed in captivity (e.g. Mezzacappa *et al.,* 2010; Meghani *et al.,* 2017; Samuelson *et al.,* 2017). However, odours should be tailored to individuals and effects carefully monitored for any increases in abnormal stress-related behaviours where alternative therapies may be more suitable. Odours that produce behaviours that are deemed as more desirable to adopters may have value in increasing adoptions. This is crucial, given that shelters are often at capacity. High adoption rates are beneficial for the organisation in freeing up space to allow the intake of further dogs requiring shelter. There are also important animal welfare benefits from high levels of adoptions, as dogs are removed from a potentially stressful environment where stress can manifest even during a short stay.

Pheromone assessment using Adaptil® dispersed in a spray and diffuser format (e.g. Hermiston *et al.,* 2018; Taylor *et al.,* 2020) found that Adaptil® did not influence behaviour in sheltered dogs or pet dogs tested in a ‘laboratory like’ novel condition, both of which are considered as stressful environments for dogs. The findings are comparable to other studies that also report no reduction in stress-related behaviours in dogs housed in confined novel environments and exposed to Adaptil® (e.g. Grigg and Piehler, 2015; Broach and Dunham, 2016). Again, interpretation of behaviour can be challenging, but when analysed with physiological parameters, such as those obtained by Taylor *et al.,* (2020), the information obtained made a valuable contribution to pheromone research by offering supplementary physiological data to further support behavioural indicators of stress, which had not been published in the field before. It was found that when behavioural responses were paired with physiological indicators of stress, Adaptil® did not influence heart rate, eye, or ear temperature of dogs (Taylor *et al.,* 2020). The findings may help verify stress responses in dogs subjected to pheromonal application and suggest that in the context used in this study at least, pheromone applications were not effective at reducing stress. However, it is important to consider explanations why a reduction in stress was not observed. Pheromones are only one factor in the environment that is perceived by an animal (Prior and Mills, 2020). Other factors such as significant stressors may also exist and, if they are deemed a threat by the animal, alterations in behaviour may supersede any effects of pheromones (Prior and Mills, 2020). It is plausible that the shelter combined with the additional stressor was perceived by the dogs as an overtly stressful environment in the Hermiston *et al.,* (2018) study, and as such the pheromones were not effective in this instance. Similarly, stress caused by the novel laboratory environment and separation from owners (Taylor *et al.*, 2020) may have been too great for Adaptil® to have a marked effect, especially if pheromonal analogue products produce only mild effects. This is especially relevant as the process of pheromone processing is currently not entirely understood (Broach and Dunham, 2016). Both the kennel and the novel laboratory environment were likely stress inducing, considering they are uncontrollable and unpredictable environments which can be a stressor for dogs (Tuber *et al.*, 1999). Similarly, in the Taylor *et al.,* (2020) study, owners left their dogs on multiple occasions. Owner separation can be a stressor for many dogs (Topál *et al.*, 1998; Prato-Previde *et al.*, 2003).

In extreme cases of stress and anxiety, it may be more effective to use pharmacological products (Mills *et al.,* 2012) or behavioural modification alongside Adaptil® (Prior and Mills, 2020). Adaptil® may not be effective where frustration is the underlying motive for the expression of stress-related behaviours (Mills *et al.,* 2012). The kennel and confined ‘laboratory like’ environment are likely to induce frustration through a lack of control and the inability to escape stressors, therefore Adaptil® may not have been effective in these instances. As such, identifying the cause of stress-related behaviour first and then assigning a treatment is important if Adaptil® is only effective for some behavioural problems. Another explanation why a reduction in stress-related behaviours was not observed may relate to the compounds in Adaptil®. The natural pheromone produced by the bitch consists of complex compounds in comparison to the man-made version, which is simpler in form. This may result in slightly different messages and may not have the same effects as the natural version (Mills *et al.,* 2012).

It is also important to consider that there are methodological issues in pheromone research. However, these was addressed by Taylor *et al.,* (2020) who also built on previous research by incorporating physiological indices for a more objective measurement of stress. Hermiston et al. (2018) was the first study to test the efficacy of Adaptil® spray in a canine shelter setting where treatment efficacy in reducing stress-related behaviours was unknown and based on anecdotal reports. Clinical trials, such as those conducted by Hermiston *et al.,* (2018) and Taylor *et al.,* (2020), are crucial to justify the use of stress relief products considering there are ethical complications if products are ineffective. However, there is a need for further research to investigate placebo controlled, blinded studies that test different stressors over a prolonged period to investigate whether Adaptil® is warranted or is only useful in certain contexts. If future research considers previous methodological limitations and also finds Adaptil® spray is not effective at reducing stress-related behaviours in sheltered dogs, it may be more cost-efficient to implement other measures (e.g. noise abatement measures). However, further work is required to confirm this. In the interim, veterinary professionals should be cautious about recommending such products to clients until there is a stronger evidentiary basis supporting the use of Adaptil®.

6.3 Limitations and challenges within odour and synthetic pheromone research

All the studies presented were underpowered by small sample sizes, and therefore effects of odours and pheromones may not have been detected (e.g. Taylor and Madden, 2016; Hermiston *et al.,* 2018; Taylor *et al.,* 2020). However, most of the research presented was comparable to previous odour and pheromone research in the field. Ideally, larger sample sizes would be used to substantiate conclusions drawn. The lack of an effect may be due to individual variation, as has been found previously (e.g. Sheppard and Mills, 2003). Equally, the difference in housing environments (e.g. kennels versus ‘laboratory-like’ conditions) and the level of interaction with humans may influence behaviour (Polgar *et al.,* 2019). Standardisation between subjects in shelter dog research samples is difficult to achieve due to the high turnover of dogs entering and leaving the shelter, often at short notice. A repeated measures experimental design was employed across all research presented in a bid to reduce individual variances, but implementing further controls such as standardised breed and weight (e.g. same breed and similar weights) would be beneficial to lower variance.

A major limitation in odour and pheromone research is the lack of randomised controlled clinical trials caused by restricted financial budgets and feasibility constraints (Frank *et al.*, 2010). A lack of blinding is also a weakness when testing odours, but is often unavoidable given the nature of research on odours and sense of smell. Non-blinded studies can introduce detection bias, which affects internal validity (Frank *et al.*, 2010). Similarly, selection bias due to inappropriate randomisation or inclusion/exclusion criteria will impact validity (Frank *et al.,* 2010). However, several pieces of the work presented addressed these concerns by incorporating blinded, randomised and controlled study designs. Nevertheless, there is still scope for further long-term investigation of odours and pheromones using placebo controlled, blinded studies to investigate any accumulative effects of pheromones (Bombail, 2017), which may be beneficial in addressing welfare concerns in long term shelter housed dogs.

6.4 The future of odour and synthetic pheromone research

The research presented provides an evidence base for future odour and pheromone research to build on. Some odours may have value in reducing stress-related behaviour in some individuals, however, further research is required to confirm this. The value of pheromones and whether Adaptil® is warranted or is only useful in certain contexts has yet to be determined.

Incorporating cortisol as a measure of stress alongside robust measures of HRV, IRT cognitive and behavioural indicators may add to the established odour and pheromone knowledge base to help determine the underlying emotional state of dogs and whether responses are a result of eustress or distress. Breed has been found to influence the frequency of behaviours such as barking (Stephen and Ledger, 2006), therefore utilising a standardised breed type may reduce breed-specific variability in behavioural data when responding to odours and pheromones. For example, scent hounds and pugs, which have been found to excel in odour detection tasks, could be used to investigate whether an affect is more prominent in these breeds. It would also be worthwhile incorporating CBARQ into future odour research to evaluate temperament and compare lateralised behavioural responses to different odours to ascertain whether different temperaments are linked with whether dogs respond to odours or not. The Canine Behaviour Assessment and Research Questionnaire could also be used to evaluate temperament in dogs to help inform the selection of appropriate odours and to avoid those which may increase stress.

A focus on sniff patterns and nostril laterality in different breeds and how these factors may influence olfactory perception is required to determine the relationship between breed specific morphological and behavioural differences and olfaction (Hall *et al.*, 2015). Sniffing duration and nostril laterality could be recorded in conditions with and without odours and pheromones to determine whether dogs have detected the scent and whether this correlates with a decrease in stress-related behaviours. Sniffing duration in detection dogs has been found to be shorter for true negatives than false negatives, true positives, and false positives (Concha *et al.*, 2014). A focus on sniffing behaviour and nostril laterality could be used to further assess odour and pheromone efficacy and help avoid ambiguous results and potential false negatives in the future.

Cognitive testing such as affect-driven attention biases (ADABs) could also be utilised in future odour research, complimenting behavioural and physiological parameters. Attention was found to be modulated in sheep and cattle exposed to stress inducing stimuli and a high and low anxiety inducing pharmacological intervention, suggesting that attention bias can assess different levels of anxiety (Lee *et al.*, 2016; Lee *et al.*, 2018). In future research, olfactory driven stimuli used as a non visual attention bias task could be paired with the tracking of an individuals gaze, ear position and reaction time (Crump, Arnott and Bethell, 2018), to help determine whether attentional responses are influenced by different odours. This method would be biologically relevant for animals where olfaction is their primary sense, such as dogs. Through monitoring attentional responses according to odour, ADABs may help determine whether different odours influence stress responses such as anxiety in dogs. This could also be applied to pheromone research. However, cognitive bias testing including ADABs need careful interpretation as they are influenced by humans providing the stimuli, rather than the animals choosing what they want or do not want to interact with, which can influence biases (Dawkins, 2021). Therefore, cognitive bias testing should be used as a complementary approach alongside other more direct measures (Dawkins, 2021), such as behaviour, physiology, preference and motivation testing to improve validity and reliability (Burani, Pelosi and Valsecchi, 2022).

There is also potential to explore neurological studies on the effects of odours and pheromones in dogs; to date studies have only been carried out using pheromones in pigs (Anderson *et al.*, 2001). Neurological study findings could indicate whether pheromones change brain functioning, for example areas influenced by stress such as the amygdala, hippocampus, and prefrontal cortex (Bremner, 2006), which may provide a broader measure of stress. Functional magnetic resonance imaging (fMRI) could be used to provide a detailed evaluation of how blood flow in regions of the brain react to pheromones, including whether there is a positive association, which has been evidenced through maximum activation of the caudate nucleus in canine brain responses to familiar human odours (Berns, Brooks and Spivak, 2015). Due to limitations in technological advancements, fMRI is currently used only in stationary dogs in laboratory conditions which limits real world application (e.g. ambulatory conditions in a shelter environment). Until technology advances, pheromones could be tested in stationary dogs using an external infrared camera alongside fMRI to track head motion alongside prospective motion correction using an external camera or image based tracking (Thesen *et al.,* 2012; Maclaren *et al.*, 2012; Todd *et al.*, 2015) to reduce motion-related artefacts in the data.

6.5 Conclusions

The research presented demonstrated no evidence to suggest that Pet Remedy®, a valerian based de-stress and calming odour, reduced behavioural indicators suggestive of a stress response in confined pet dogs. Although other odours such as coconut, ginger, vanilla and valerian on their own may have value in reducing stress-related behaviour in sheltered dogs. These odours could be used to improve management practices by widening the choice of odours currently used in shelters. Using a wider range of odours could reduce habituation to repeat odour use, thus maximising odour effectiveness. The findings presented can also help inform shelter decision making when deciding on the type of odours to use which can be tailored to individuals as effective strategies to reduce stress. Furthermore, these odours may have value in increasing adoptions, if they encourage behaviour perceived more desirable by potential adopters. It is important to note that there is limited comparative data that focuses on Pet Remedy®. Further research utilising larger sample sizes that account for individual variation in dogs such as breed, previous experience and temperament is required. In the interim, it is recommended that Pet Remedy® is used alongside behavioural modification, which is an effective solution for managing manifestations of stress alongside alternative treatments until further clinical trials supports its use as a single treatment of stress. Alternatively, odours applied individually such as coconut, ginger, vanilla and valerian could be considered in reducing stress-related behaviour in kennelled dogs and may have some value as tools to improve canine welfare.

The value of pheromones and whether Adaptil® is warranted or is only useful in certain contexts has yet to be determined. There is a need for more research that utilises blinded, placebo controlled clinical trials performed over a prolonged period to determine the value of pheromone use in reducing stress-related behaviour in confined dogs. Incorporating cortisol, neurological and cognitive measurements to further supplement physiological and behavioural data and different stressors may aid in a broader assessment of stress reducing properties of pheromonal products and odours. Until there is stronger evidentiary basis substantiating pheromone use as a stress relief product, veterinarians should be cautious in recommending the use of such products.

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APPENDICES

APPENDIX 1: Evidence sources and links to a collection of published journal articles

Evidence Source 1. Taylor, S. and Madden, J. (2016) 'The effect of pet remedy on the behaviour of the domestic dog (Canis familiaris)', *Animals*, 6(11), p.64.

https://www.mdpi.com/2076-2615/6/11/64

Evidence Source 2. Binks, J., **Taylor, S**., Wills, A. and Montrose, V.T. (2018) 'The behavioural effects of olfactory stimulation on dogs at a rescue shelter', *Applied Animal Behaviour Science*, 202, pp.69-76.

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Evidence Source 3. Hermiston, C., Montrose, V.T. and **Taylor, S**. (2018) 'The effects of dog-appeasing pheromone spray upon canine vocalizations and stress-related behaviors in a rescue shelter', *Journal of Veterinary Behavior*, 26, pp.11-16.

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Evidence Source Taylor, S., Webb, L., Montrose, V.T. and Williams, J. (2020) 'The behavioral and physiological effects of dog appeasing pheromone upon canine behavior during separation from owner', *Journal of Veterinary Behavior*, 40, pp.36-42.

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APPENDIX 2: Summary evidence of meeting the UWE Bristol Doctoral descriptors

This summary discusses how the works submitted addresses the Doctoral criteria in conjunction with appendix 3 'Attainment of Doctoral learning criteria' below:

1. have conducted enquiry leading to the creation and interpretation of new knowledge through original research or other advanced scholarship, shown by satisfying scholarly review by accomplished and recognised scholars in the field:

I was lead author for three publications (the author supervised one of the projects in their capacity as dissertation supervisor), and co-authored the other. All works have been published in a wide range of peer reviewed journals by accomplished and recognised scholars in the field with high impact factors (e.g. Applied Animal Behaviour Science, Journal of Veterinary Behavior, Animals). Publications have led to generation of new knowledge in the field and can be used to inform industry.

2. can demonstrate a critical understanding of the current state of knowledge in that field of theory and/or practice:

A literature review was conducted for each publication to examine current theory and evidence base for the efficacy of stress relief products for dogs. Methodological weaknesses were examined which involved in-depth exploration and critique of literature surrounding natural odours and pheromones (evidenced in the critical commentary and also in the introduction and discussion sections of the articles published). I attended the UWE workshop 'Guide to Searching and Organising the Literatures' in November 2020 which provided me with guidance on how to effectively search literature databases. This helped me to define relevant search terms and field key information when searching for the most current literature to inform my critical commentary.

3. show the ability to conceptualise, design and implement a project for the generation of new knowledge at the forefront of the discipline or field of practice

including the capacity to adjust the project design in the light of emergent issues and understandings:

The research presented examined gaps in knowledge base. For example, evidence source 1 was the first study to test Pet Remedy®. Before the publication of this study, any measurable effects of Pet Remedy® on canine behaviour were unknown in the field. Similarly, historically there has been a lack of research on novel approaches to olfactory stimulation in kennelled dogs including Adaptil spray®. Evidence sources 2, 3 and 4 addressed these issues, generated new knowledge and created a unique insight into the efficacy of stress relief products. I had a leading role in conceptualisation, design and implementation of the research articles presented (e.g. development, data collection, analysis, write up, dissemination). All of the research articles outputs have been disseminated at Conferences (internal and external) and through lay press articles. Aspects of project design were adjusted throughout the period of research, in particular attempts to overcome issues with poor conductivity when using heart rate monitors (e.g. evidence sources 1 and 4) and these decisions were made in respect of current recommendations in literature (evidenced in critical commentary). Throughout all of the research articles, adjustments were made to the manuscript as a result of peer feedback from colleagues, the journal Editor and reviewers.

4. can demonstrate a critical understanding of the methodology of enquiry:

Rigorous methodological approaches and research methods were largely used. The critical commentary notes the strengths of these approaches but also where approaches could be improved. All of the research articles employed a repeated measures design and some were double blind, placebo controlled and counter-balanced, where feasible. Varied analysis was conducted, for example multivariate regression, principle component analysis (PCA), Friedman, Wilcoxon and Bonferroni adjustments. A wide range of measures were also employed such as HR, HRV, salivary cortisol, eye/ear temperature and behaviour. The best suited approaches were based on previous literature to address the aim and objectives. The knowledge and ability to apply a range of methods used to examine the efficacy of stress relief products for dogs has been evidenced throughout the critical commentary.

5. have developed independent judgement of issues and ideas in the field of research and / or practice and are able to communicate and justify that judgement to appropriate audiences:

Independent judgement (independent thought) has been evidenced in the critical commentary. The research presented has been dissemination to a wide range of audiences (e.g. academics, vets, shelter organisations, general public) through peer reviewed publications, conferences including internal, external, national and international, lay press articles (blogs/magazine articles) and also a national radio interview.

6. can critically reflect on their work and evaluate its strengths and weaknesses including understanding validation procedures:

Strengths and weaknesses of the research have been discussed in the critical commentary but have also been demonstrated in the published articles throughout the discussion sections, as well as the peer review process. Peer review enabled critical reflection and constructive discussion on limitations of the research, which have been incorporated into publications. Research procedures and validity have been discussed and justified in publications and have been discussed in more depth throughout the critical commentary.

APPENDIX 3: Attainment of Doctoral learning criteria

Criteria	Evidence	Page Number
1. have conducted enquiry leading to the creation and interpretation of new knowledge through original research or other advanced scholarship, shown by satisfying scholarly review by accomplished and recognised scholars in the field;	2.3 Evidence source 1 3.4 Evidence source 2 4.4 Evidence source 3 5.5 Evidence source 4 6 Discussion Appendix 1	37 54 72 86 92 141
2. can demonstrate a critical understanding of the current state of knowledge in that field of theory and/or practice;	1 An introduction to stress, odours and synthetic pheromone use in dogs 2 Valerian and Pet Remedy® 3 Coconut, Ginger and Vanilla 4 Adaptil® 5 Behavioural and physiological parameters of stress and Adaptil® 6 Discussion	1 32 49 63 80 92
3. show the ability to conceptualise, design and implement a project for the generation of new knowledge at the forefront of the discipline or field of practice including the capacity to adjust the project design in the light of emergent issues and understandings;	2.3 Evidence source 1 3.4 Evidence source 2 4.4 Evidence source 3 5.5 Evidence source 4 6 Discussion Appendix 1	37 54 72 86 92 141
4. can demonstrate a critical understanding of the methodology of enquiry;	1 An introduction to stress, odours and synthetic pheromone use in dogs 2.3 Evidence source 1 3.4 Evidence source 2 4.4 Evidence source 3 5.5 Evidence source 4 6 Discussion	1 37 54 72 86 92

5. have developed independent judgement of issues and ideas in the field of research and / or practice and are able to communicate and justify that judgement to appropriate audiences;	2 Valerian and Pet Remedy® 3 Coconut, Ginger and Vanilla 4 Adaptil® 5 Behavioural and physiological parameters of stress and Adaptil® 6 Discussion Appendix 6 Appendix 7	32 49 63 80 92 152 156
6. can critically reflect on their work and evaluate its strengths and weaknesses including understanding validation procedures.	2.3 Evidence source 1 3.4 Evidence source 2 4.4 Evidence source 3 5.5 Evidence source 4 6 Discussion	37 54 72 86 92

APPENDIX 4: Training and continuing professional development (CPD)

The training requirement for the Doctoral award was met through completion of 60 credits of prior learning achieved through completion of a Master of Animal Behaviour at the University of Exeter:

Evidence of completion of prior learning

Module Name	Credits	Module Code	Date Achieved
Behavioural Science Research Skills	30	PSYM202	07/2013
Advances and Methods in Animal Behaviour	30	PSYM205	07/2013

Throughout the completion of my DPhil, I have actively engaged in CPD to develop my research skills (continued on page 117):

Evidence of CPD 2015 - 2021

Event	Date
Association of Pet Behaviour Counsellor (APBC) Conference	09/2015
Hartpury Research Seminar	2015, 2016, 2017, 2018, 2019, 2020, 2021
International Society of Applied Ethology (ISAE) Conference	07/2016
Elsevier webinars: Writing Journal Articles and How to Peer Review Articles	07/2017
UFAW Conference	03/2018
ScentWork Seminar	02/2019
Infrared Thermography (IRT) Training Session	06/2019
UFAW Symposium	07/2019
ScentWork Solutions for Behaviour Changes – APBC Webinar	01/2020
Effects of Pain in Dogs and Cats – CAHE Webinar	05/2020
SHAPE Enrichment – APBC Webinar	06/2020

Quality of Life in Cat Shelters – APBC Webinar	06/2020
UFAW Conference	07/2020
An Update on Increasing Welfare through Enrichment – A look at the science – APBC Webinar	11/2020
Guide to Searching and Organising the Literatures – UWE Workshop	11/2020
The Final Viva – UWE Workshop	11/2020
Introduction to Qualitative Research Methods – UWE Workshop	12/2020
Separation Anxiety: Life After Lockdown - APBC Webinar	02/2021
The Role and Value of C-BARQ Assessments in Clinical Animal Behaviour – APBC Webinar	11/2021

APPENDIX 5: Glossary of terms and abbreviations

Abnormal behaviour = an untypical reaction to a particular combination of motivational factors and stimuli.

Anxiolytic effect = reduction in anxiety

Behavioural modification = techniques to modify behaviour, can include systematic desensitisation, counterconditioning and habituation (see terms below).

Blinded study = the patients or subjects do not know (is blinded as to) what treatment they are receiving.

Chemosensory = relating to organs or receptors responsive to chemical stimuli.

Counterconditioning = a technique which involves conditioning an animal to alter its emotional response to a stimulus (i.e. a response that is independent of voluntary control). When a behaviour problem is associated with an aversive or negative emotional component, the goal is to pair the stimulus or event with a strong opposite emotional response (i.e. something highly positive). Counterconditioning is often used to modify the behaviour of fearful pets, and is used in combination with systematic desensitisation (see term below).

Distress = negative stress

Double blind study = neither the participants nor the experimenters know who is receiving a particular treatment.

Emotional valence = the value associated with a stimulus which is expressed on a continuum from pleasant to unpleasant.

Enrichment = any technique that implements environmental stimuli which optimises both an animal's physiological and psychological function. This can include: the provision of a more stimulating environment and incorporating measures to increase behavioural diversity, a reduction in abnormal and stereotypic behaviours, an increase in an animal's normal behavioural repertoire, more positive utilisation of an environment and an improved ability to cope with challenges.

Ethogram = catalogue of descriptions of discrete species-specific behaviour patterns that make up the basic behavioural repertoire of the species.

Eustress = positive stress

Habituation = the gradual loss of responsiveness to a stimulus due to repeated exposure which is dependent on the absence of consequences (positive/negative) due to stimulus exposure.

Neophobic = an extreme fear of anything new or unfamiliar

Non-pharmacological = treatment that does not use medication or drugs

Nutraceuticals = products derived from food sources which may have health benefits in addition to the basic nutritional value found in foods.

Olfactory epithelia = specialised epithelial tissue located in the nasal cavity that aids smell.

Placebo-controlled = an inactive substance (a placebo) is given to one group of participants while another group receive an active treatment.

Quasi-experimental = a study that does not use randomisation

Stereotypic behaviour = behaviour that is repetitive, invariant and has no obvious function.

Systematic desensitisation = a technique which exposes an animal repeatedly to stimuli that cause fear, anxiety or aggression in sufficiently small doses so as not to cause the response. The stimuli are then gradually increased at increments that do not lead to a recurrence of the response. The stimuli are repeated so many times with no effect that they become inconsequential.

ABM = awake but motionless

ACTH = adrenocorticotrophic hormone (ACTH)

ADAB = affect-driven attention biases

ANS = autonomic nervous system

ASAB = Association for the Study of Animal Behaviour

BPM = beats per minute

CNS = central nervous system

CRH = corticotrophin releasing hormone (CRH)

DAP = dog appeasing pheromone

fMRI = functional magnetic resonance imaging

GABA = gamma amino butyric acid

GPS = global positioning system

HR = heart rate

HRV = heart rate variability

IMU = inertial measurement unit

IRT = Infrared Thermography

ml = millilitres

º = degrees of temperature

R-R intervals = length of time between beats

SDNN = standard deviation of normal-to-normal

SNS = sympathetic nervous system

µl = microliter, often used as the measurement of salivary cortisol

VNO = vomeronasal organ (VNO), also known as the Jacobson's organ

VS = Valence-Specific Hypothesis

APPENDIX 6: Academic Curriculum Vitae (CV)

Qualifications:

- MSc Animal Behaviour (Merit), University of Exeter, completed 2013
- BSc Animal Behaviour and Welfare (First Class), UWE Hartpury, completed 2012
- FdSc Animal Behaviour and Welfare (Distinction), UWE Hartpury, completed 2011

Employment History:

- HE Lecturer in Animal Behaviour and Welfare (P/T), Hartpury University – January 2016. Undergraduate and Postgraduate Dissertation Supervisor, Dissertation Lead and Level 6 Academic Personal Tutor – ongoing.
- HE Lecturer and Programme Manager for FdSc Animal Behaviour and Welfare (F/T), Hartpury University - February 2015 – January 2016

Teaching (undergraduate modules taught):

Level 4 (first year)

- Animal Behaviour
- Introduction to Animal Welfare
- Animal Practice

Level 5 (second year)

- Behavioural Measurement
- Companion Animal Behaviour and Training
- Animal-Assisted Interventions
- Applied-Animal Assisted Interventions
- The Canine Sector

Level 6 (third year)

- Pet Behaviour Counselling
- Anthrozoology
- Developments in Animal Science

Publication Record:

Journal Articles:

- Powdrill-Wells, N., **Taylor, S.** and Melfi, V. (2021) 'Reducing Dog Relinquishment to Rescue Centres Due to Behaviour Problems: Identifying Cases to Target with an Advice Intervention at the Point of Relinquishment Request', *Animals*, 11(10), p.2766.
- **Taylor, S.**, Webb, L., Montrose, V.T. and Williams, J. (2020) 'The behavioral and physiological effects of dog appeasing pheromone upon canine behavior during separation from owner', *Journal of Veterinary Behavior*, 40, pp.36-42.
- Hermiston, C., Montrose, V.T. and **Taylor, S**. (2018) 'The effects of dog-appeasing pheromone spray upon canine vocalizations and stress-related behaviors in a rescue shelter', *Journal of Veterinary Behavior*, 26, pp.11-16.
- Binks, J., **Taylor, S**., Wills, A. and Montrose, V.T. (2018) 'The behavioural effects of olfactory stimulation on dogs at a rescue shelter', *Applied Animal Behaviour Science*, 202, pp.69-76.
- Baker, B., **Taylor, S**. and Montrose, V.T. (2018) 'The effects of olfactory stimulation on the behavior of captive ring‐tailed lemurs (Lemur catta)', *Zoo Biology*, 37(1), pp.16-22.
- **Taylor, S**. and Madden, J. (2016) 'The effect of pet remedy on the behaviour of the domestic dog (Canis familiaris)', *Animals*, 6(11), p.64.

Conference Presentations:

- Rood, A., **Taylor, S.** and Williams, J. September 2020: Can equine-assisted activities benefit people living with dementia: Two case study examples. Poster Presentation at: International Society for Anthrozoology (ISAZ) Conference 2020
- Lloyd, K., Wills, A. and **Taylor, S**. July 2019: The Anxiolytic Effects of Valerian *(Valeriana officinalis)* on the Domestic Dog *(Canis familiaris)*. Poster Presentation at UFAW Symposium, Bruges, 2019
- **Taylor, S.** and Madden, J. 2016: The effect of Pet Remedy on the behaviour of the domestic dog. Poster Presentation at: International Society for Applied Ethology (ISAE), 2016
- Hermiston, C. and **Taylor, S**. 2016: The effect of dog appeasing pheromones in regard to intensity of vocalisations and frequency of stress related behaviours

in a rescue shelter. Poster Presentation at: ASAB Easter Conference 2016, 30th March - 1st April 2016.

Lay Publications:

- **Taylor, S**. September 2020: The behavioural and physiological effects of dog appeasing pheromone on canine behaviour during separation from the owner. Animal Therapy Magazine.
- Carroll, A. and **Taylor, S.** July 2020: What is Separation Anxiety and tips on how to manage it. Animal Therapy Magazine.
- **Taylor, S.** and Binks, J. April 2020: Beating the Boredom Blues: Sniffing Out New Opportunities for Dogs. Companion Animal Psychology Blog Article.
- Dorma, L. and **Taylor, S.** September 2019: The benefits of nose work for ‘naughty’ and ‘reactive’ dogs. Companion Animal Psychology Blog Article
- **Taylor, S**. January 2019: Go Guinea Pigs! A Guide to Training Guinea Pigs. Guinea Pig Magazine.
- **Taylor, S**. November 2018: Dogs under Pressure – Are pressure vests beneficial at reducing signs of stress and anxiety in dogs? Animal Therapy Magazine.
- **Taylor, S**. August 2018: Wake up and smell the pheromones! The use of dog appeasing pheromones to reduce stress in dogs. Animal Therapy Magazine.
- **Taylor, S**. February 2018: To gesture or not to gesture? Are visual cues more effective than verbal cues in dog training? Companion Animal Psychology Blog Article.

Other:

- **Taylor, S**. Interview with BBC Gloucestershire discussing the effect of Pet Remedy on the behaviour of the domestic dog. 2016.

Reviewer Contributions:

I am currently a peer reviewer for the following journals:

- Animals
- Veterinary Evidence
- Journal of Veterinary Behavior

Professional Body Membership:

I am a member of the following institutions:

- Senior Fellow of the HEA (Advance HE) – 2021 – current
- Fellow of the HEA (Advance HE) – 2016-2021
- Association of Pet Behaviour Counsellors (APBC): Academic Membership – 2016 – current

APPENDIX 7: Reflection on personal development

Throughout the research process I have gained skills relating to all aspects of planning, design and implementation of research. This included taking a critical approach to evaluating the research process and making adjustments accordingly. The peer review process has been an integral part of the evaluative aspect of my research journey. At first, I found the process quite overwhelming. In hindsight, I was not fully prepared for the amount of time and work required to develop a manuscript fit for publication, most likely due to my lack of experience writing journal articles. There were major revisions to implement after submitting my first manuscript draft, including re-analysing the dataset. However, with the support from colleagues, I addressed the feedback while also gaining the confidence to rebut some of the reviewers concerns which led to the article being accepted for publication. Reflecting on the feedback received for my first manuscript, it really helped develop and improve the quality of my work and moving forwards I have used the feedback when writing subsequent manuscripts prior to submitting to journals. This has resulted in minor revisions and a much smoother review process. Rather than being daunted by the peer review process, I now find it a valuable and thought-provoking experience which has made me develop a more self-critical approach when conducting research. As a result, I am now a peer reviewer for three journals which I find an enjoyable experience and appreciate the amount of time reviewers take to provide in-depth feedback.

Since publishing the first couple of journal articles, I have developed a passion for writing lay press articles and now regularly contribute to blogs and magazines by writing about my research. Initially, I found it difficult to switch from writing a journal article to lay press as the style of writing is very different. However, as I gained more experience and received positive feedback, I have grown in confidence and find writing lay press articles a useful way to engage with members of the public, industry experts and also other academics. This enables knowledge transfer that is accessible to everyone, especially those that use odours and pheromones. Lay articles can be used by those to make an informed judgement which is based on science on the efficacy of stress relief products. Going forwards, I would like to continue my research journey by writing a chapter for a book on olfactory enrichment and, eventually, a book on sensory enrichment.