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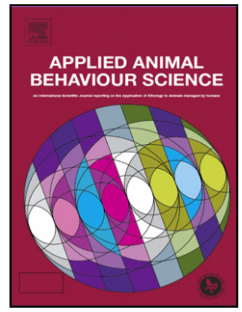

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The effect of conspecific removal on the behaviour and physiology of pair-housed shelter dogs.

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Abstract

Dogs (*Canis familiaris*) are a highly social species and within a shelter environment pair-housing is recommended to prevent the stress associated with social isolation. Separation of individuals which may have formed bonds in this environment is a usual occurrence, as a result of rehoming or euthanasia. To investigate the impact of separation, the behaviour, cognitive bias, faecal S-IgA and cortisol levels were examined in 12 adult pair-housed dogs, maintained in a private animal shelter. Prior to separation, dogs engaged in more affiliative than agonistic behaviour with conspecifics (means of 3 and 0.1% of time respectively). Following separation, increased activity was observed in the form of more running and grooming (P = 0.02), circling (P = 0.006), figure of 8 movement (P = 0.01), posture changes (P = 0.003) and stretching (P = 0.005), and less play behaviour was observed (P = 0.01). Secretory IgA increased (P = 0.02) after separation (mean = 443.7 ± 182.5 ng/mL; before separation mean = 370.1 ± 108.2 ng/mL). Cortisol concentrations were not affected by separation (P = 0.26, mean before separation = 792 ng/g; mean after separation = 874 ng/g). There was no indication from cognitive bias testing that the dogs' emotional valency was affected, as latencies to reach ambiguous cues before and after separation did not differ significantly (P = 0.33). These results demonstrate that separation of a dog from a conspecific negatively affected behaviour and stimulated the immune system, changes which could be indicative of stress.

Keywords: *Immunoglobulin A; Cognitive Bias; Conspecific Separation; Cortisol; Dog; Behaviour*

1.0 Introduction

For social animals, separation from conspecifics has negative physiological (Boissy and Le Neindre, 1997; Guesdon et al., 2012; Hennessy, 1997) and behavioural (Donaldson et al., 2002) effects on the animals' ensuing welfare states (Newberry and Swanson, 2008). Dogs (*Canis familiaris*) form strong social bonds with conspecifics, the function of which, from an evolutionary perspective, is to maintain relationships essential for survival (Archer, 1999; Topál et al., 2005). Attachment between mother and offspring is the most commonly documented social bond in animals (Newberry and Swanson, 2008; Mogi et al., 2011), however, separation of conspecifics is also documented to result in pronounced behavioural changes, suggestive of distress, in a range of species. For example primate species, including chimpanzees (Bard and Nadler, 1983) and bonnet macaques (Boccia et al., 1997), many farm animal species (Rault, 2012), including goats (Lyons et al., 1993), cattle (Boissy and Le Neindre, 1997; Flower and Weary, 2003) and sheep (Guesdon et al., 2012), and some companion animal species, including horses, donkeys (Murray et al., 2013) and dogs (Hepper, 1994; Ward et al., 2008) all show behaviour indicative of distress when separated. For dogs, these behavioural responses can include withdrawal, inactivity, stereotypic behaviours, increased vocalisations and increased cortisol measures (Beerda et al., 1999b; Hennessy et al., 2001; Wells, 2004).

Traditionally, the physiological impact of conspecific separation has been assessed by evaluating activation of the HPA axis, through the measurement of cortisol. Fluctuations of cortisol resulting from separation have been documented in numerous species, as well as across a range of social relationships (for a review see Hennessy, 1997). More recently, the response of an animal's immune system to acute and chronic stressors has also been considered. IgA is present in the mucosal membranes of the intestinal, respiratory, biliary and genital tracts and is the dominant immunoglobulin in mucosal secretions of dogs (Stokes and Waly, 2006). In dogs, IgA concentrations have been documented to increase as a result of experiencing acute stress (Kikkawa et al., 2003) and decrease as a result of chronic stress (Skandakumar et al., 1995).

The effect of emotional states on cognition is well documented in humans (Mathews and MacLeod, 1994; Mellers et al., 1999). Recently, in animals, the term ‘cognitive bias’ has been coined to describe the possible role played by emotions in an animal’s cognitive processing. It is based on the idea that when an animal evaluates a situation with ambiguous stimuli, its emotional valence affects its interpretation of the situation and possible outcomes (Broom, 2010; Mendl et al., 2009). Using this methodology, emotional valence has been investigated in a range of mammal species (Burman et al., 2008; Douglas et al., 2012; Doyle et al., 2010a; Svendsen et al., 2012), including the domestic dog (Mendl et al., 2010; Müller et al., 2012; Titulaer et al., 2013), as well as in birds (Brilot et al., 2009; Wichman et al., 2012) and insects (Bateson et al., 2011). This body of research has demonstrated that animals in a negative emotional state, comparative to animals in a positive emotional state, are more likely to display pessimistic behaviour and vice versa. For example, cognitive bias methodology has successfully been used to identify dogs suffering from separation anxiety (Mendl et al., 2010).

Pair-housing of dogs is recommended within a shelter environment (Wells, 2004) due to the stress of social isolation (Beerda et al., 1997; Bergamasco et al., 2010; Hennessy et al., 1997). However, eventual separation is inevitable for most dogs (due to rehoming or euthanasia), and due to the social nature of this species we hypothesised that this would result in a negative experience, evidenced by increases in behaviours indicative of stress, increased cortisol, reduced IgA levels and more pessimistic responses during cognitive bias testing.

2.0 Materials and methods

2.1 Subjects

Twenty-four shelter-housed dogs (four entire males, five desexed males, 11 entire females and four desexed females), ranging in estimated age from 0.75 – 7 years (mean 2.18 ± SD 1.38) were included in this study. Eight of the dogs were purebred (Greyhound n=5; Labrador n=1; Griffon Terrier n=1, German Sheppard n=1) and the remainder crossbred. The subjects had been maintained in the same companion animal facility for a mean of 126 ± 29.4 days prior to the present study.

2.2 Housing:

Only dogs involved in the study were housed across three allocated kennel blocks. Other dogs within the facility that were available for adoption were maintained in a different part of the facility with separate access. Each kennel had an indoor and outdoor section, both 2.9 x 1.5 m (Fig. 1). There were three guillotine doors, one between the two sections and one in the side, both indoors and out, to allow access to the adjoining kennel for paired housing at all times except during cleaning (08:00-10:00 h and 15:00-16:00 h) and feeding (08:00 h and 14:00 h) when the dogs were separated into their individual kennels . Kennels had painted concrete flooring, and walls were a combination of solid plastic and wire mesh both indoors and out. The solid plastic component comprised two-thirds of the wall from the ground and acted to prevent contact (both visual and physical) between dogs housed in adjoining kennels. Kennel access doors were made of wire mesh.

[Insert Fig. 1 Here]

2.3 Daily Husbandry

Dogs were provided with clean material bedding (Kennel Solutions PTY LTD, Queensland, Australia) after morning cleaning, and in the afternoon if it was soiled. Each pair of dogs was allocated two, 30-min sessions in a 100 m^2 outdoor exercise area with a play feature, sand pit, and water bath. Volunteers walked each subject for 45 min daily. The dogs did not receive any other interaction with humans for the duration of the study. Predetermined homes were established before the dogs entered the study. Enrichment was provided in the form of toys (balls, chew toys, boxes and soft toys), changed daily, and the random presentation of various feeding enrichments (a puzzle feeder [KONG company, Golden, Colorado, USA], scatter feeding or frozen meals). Each kennel block contained two Dog Appeasing Pheromone diffusers (CEVA Deivet PTY LTD, Seven Hills, New South Wales, Australia). Dogs were fed dried kibble pellets (Advance Adult Dog All Breed, Waltham, Wodonga, Australia), 275-465 g/day, depending on individual requirements, with water available ad libitum. Each dog was weighed weekly.

2.4 Pair Selection and Separation

Dogs were pair-housed, in line with usual kennel management practices, for a mean of 54 days (three pairs for 49 days and nine pairs for 56 days). They were allocated to matched pairs by senior shelter staff based on similar size, breed, age and sex and temperament. Three of the pairs were entire female/desexed male; two pairs were entire female/entire female; two pairs were entire female/desexed female; two pairs were entire female/entire male; one pair was entire male/entire male and the remaining pair was desexed female/desexed male.

The temperament of each dog was assessed by shelter staff using an in-house test. This assessment recorded demographics of the dogs (name, breed, approximate age and sex) and background information if known (e.g. origin and behavioural history). The remainder of the assessment was made up of 10 categories: (1) demeanour (‘happy to see you’, ‘calm’, ‘confident’, ‘anxious/cautious’, ‘disinterested’ ‘backing away’, ‘submissive/timid’, ‘frustrated’, ‘destructive’, ‘lunging at bars’, ‘barking’, ‘growling/snarling’); (2) general character (‘social’, ‘cautious’, ‘over-excited’, ‘unfriendly’); (3) response to basic commands (‘sit’, ‘down’, ‘stay’, ‘come’); (4) toy interaction (‘no interest’, ‘interactive’, ‘difficulty retrieving’); (5) food refusal (‘focused’, ‘displacing’, ‘demanding’, ‘uninterested’); (6) play preference (‘uninterested’, ‘chase games’, ‘plays alone’, ‘retrieve and relinquish’); (7) handling, which was divided into three sub-sections: (7a) ‘stroking along back’, (7b) ‘head parts’ and (7c) ‘muzzle tolerance’, (‘seeks affection’, ‘over excited’, ‘remained still’, ‘tolerant’, ‘mouthy’ ‘growl/snarl/snap’); (8) restraint (‘comfortable’, ‘freezes’, ‘struggles’, ‘mouths’, ‘growl/snarl/snap’); (9) food/feeding (‘comfortable’, ‘froze’, ‘ate fast’, ‘growls’, ‘snapped’); and (10) dog-to-dog interaction (‘polite social’, ‘play’, ‘barks’, ‘unsure/avoids interaction’, ‘pushy/rough’, ‘lunges forward/growls/snarls’, ‘fight’). The assessment ended with a recommendation for additional testing and a pass/fail verdict.

Within each pair, any dog that did not have a permanent home to go to at the end of the trial was designated to be the focal animal (n = 12). If both dogs in the pair had homes, the second dog to be assigned a home was designated the focal animal. If neither dog had a pre-selected home, the selection of the focal animal was at random. Separation of members of each pair occurred between day 49 - 56 (hereafter called day 0), dependent on when the dog could be received by the new caregivers. Those with homes to go to were relocated, otherwise they were installed in foster care homes.

2.5 Behavioural Observations

Sixteen colour infra-red tube cameras (Kobi, K-32HCVF, Video Security Products, Queensland, Australia) were installed above each pair of kennels to cover the indoor and outdoor areas. Each camera was connected to one of two 9-channel 1000 Gb High Definition Drive Digital Video recorders (Kobi, K9 XQ H.264, Video Security Products, Queensland, Australia). The behaviour of each focal animal was recorded continuously for a 24 h period, in 'real-time' mode at 100 frames/s on days −6, −3, −1 before separation and days +1, +3, +6 after separation. The resulting audio/visual data were analysed using the 'Observer XT' software package (version 7, Noldus Information Technology, 2007, Wageningen, The Netherlands). An ethogram (Table 1) was developed, based on those previously published (Adams and Johnson, 1993; Palestrini et al., 2010; Tod et al., 2005; Walker et al., 2010), which allowed interpretation of all recorded behaviours. A continuous recording method (Martin and Bateson, 1993) was used to describe each focal dog's behaviour for a 5-min period, at 30-min intervals, across each 24 h day.

[Insert Table 1 Here]

2.6 Cognitive Bias Training

Cognitive bias training was modified from that of Burman et al. (2008) and Mendl et al. (2010). The dogs were initially trained to discriminate the positioning of a clearly identifiable blue food bowl between two reference locations; a positive cue location (P, reinforced with a food reward) and a negative cue location (N, no food) placed at either side of the test area (Fig. 2). The type of food reward (Pedigree Casserole Mars Petcare, Victoria, Australia) was selected due to its highly palatable nature comparative to the dogs' standard dry food diet. As described in Mendl et al. (2010) and Müller et al. (2012), six of the dogs were randomly assigned to receive the positive cue on the right hand side, whilst for the remaining six dogs the positive cue was on the left hand side. At the start of each trial the researcher led the dog to the test arena and placed it into a wire crate covered with a blanket to prevent the dog having visual access to the cues. The researcher then baited the food bowl with a desert spoonful of the reward food and placed it in the relevant location. To avoid audible clues, the researcher walked between the positive and negative locations six times tapping the bowl with the spoon before actual baiting occurred (or did not occur for negative cues). The same bowl was used for both positive and negative cues and was deliberately not washed between trials, leaving odour of food present in both locations in an attempt to control for odour cues.

[Insert Fig. 2 Here]

Each dog initially received two positive cue trials then two negative cue trials, after which positive and negative cue trials were randomised, with no more than two of the same type occurring consecutively. After baiting occurred the dog was released and allowed to approach the food bowl, with the latency to place his/her head in the bowl recorded (maximum 30 s, otherwise a new trial was initiated). After a minimum of 15 training trials a dog was considered successfully trained when his/her longest latency to reach the positive cue location (in the three preceding trials) was shorter than any of the three preceding latencies to reach the negative cue location.

2.7 Cognitive Bias Testing

Cognitive bias testing was carried out on day -1 (prior to separation) and again on day +1 (post separation). For this the cue (empty food bowl) was located at one of three ambiguous locations equally spaced along a 3.5 m arc from the crate, between the positive and negative cue locations: near-positive cue (NP: one third of the distance from the positive cue), middle cue (M: half way between the positive cue and the negative cue) and near-negative cue (NN: one third of the distance from the negative location) (Fig. 2). Three test trials were undertaken in each location (nine test trials in total) in the following order: first test trial = M, NP, NN; second test trial = NP, NN, M; third test trial = NN, M, NP. Before the first test trial, and between subsequent test trials, four standard training trials were carried out; two P trials and two N trials (training trials = P, P, N, N). As with the training trials, each dogs latency to approach each of the ambiguous cues during test trials was recorded and provided a measurement of whether dogs ran fast (suggesting 'optimistic' judgement e.g. the anticipation of food) or slow (suggesting 'pessimistic' judgement e.g. no anticipation of food). By comparing each dogs approach latencies before and after separation we were able to investigate whether the dogs behaved more 'pessimistically' after separation from a conspecific which would be suggestive of an underlying negative affective state.

2.8 Physiological Sample Collection:

For each test subject a faecal sample was collected on days −3, −1, +1, +3, +6, coinciding with days for which behaviour was recorded. The first spontaneous defecation of the day was collected immediately upon elimination (between 08:00-09:00 h), separated into two sterile urine collection cups (Becton Dickenson, North Ryde New South Wales, Australia) and frozen at -70^{0}C.

2.9 Physiological Sample Analysis

2.9.1 IgA

Faecal samples were freeze dried (Ilshin Bio Base, Dongduchun City Kyunggi-do, Korea) for 96 h at 5 millitorr pressure and -82^{o}C, and the product homogenised. A mean of 0.75 ± 0.12 g of the sample was transferred to a 15 mL polystyrene conical tube (Becton Dickenson, North Ryde New South Wales, Australia) and extracted with 10 mL of phosphate buffered saline, 5% skim milk, 50 mmol EDTA, 0.2 mg/mL soybean trypsin inhibitor Sigma, St. Louis, Missouri, USA) and 2 mmol phenylmethylsulfonylfluoride (Sigma, St. Louis, Missouri, USA) per mg of faecal dry weight. This was suspended for 20 s prior to spinning at 4500 *g* for 10 min, after which a clear supernatant was removed and stored at -20^{o}C.

To calculate IgA concentrations, the optical density of samples was compared to the optical density of a standard with a known concentration of IgA, using the Dog IgA ELISA Quantitation Kit (BETE40-104, Bethyl Laboratories, Quantum Scientific, Brisbane, Australia). Seven dilutions of the standard (1:31.25, 1:62.5, 1:125, 1:250, 1:500, 1:1000, 1:2000) were prepared to develop a standard curve between optical density and IgA concentration. Optimal dilution was determined from the optical density that was within the concentration range (standard curve) of the standards (1:30,000). All assays were performed in duplicate. Each well was coated with 100 μL of diluted anti-dog IgA antibody (BETE40-104, Bethyl Laboratories, supplied by Quantum Scientific, Australia) and incubated at room temperature for 60 min. Anti-dog IgA antibody was diluted with carbonate-bicarbonate buffer (0.05mol/L, pH 9.6). The plates were then washed five times with wash solution (50mmol/L tris(hydroxymethyl)aminomethane, 0.14mol/L NaCl, 0.5mL/L TWEEN® 20 [Sigma-Aldrich, New South Wales, Australia], distilled water) and then blocked. Then 200 μL/well of blocking solution (50mmol/L tris(hydroxymethyl)aminomethane, 0.14mol/L NaCl, distilled water, 1% bovine serum albumin) was added to each well, and plates were incubated at room temperature for 30 min. Plates were washed five times and 100μL/well of diluted standards or samples was added (sample dilute: 50mmol/L tris(hydroxymethyl)aminomethane, 0.14mol/L NaCl, 0.5mL/L TWEEN® 20 [Sigma-Aldrich, New South Wales, Australia], distilled water, 1% bovine serum albumin). Plates were incubated at room temperature for a further 60 min and then washed five times, followed by the addition of 100μL/well of diluted anti-dog IgA horseradish peroxidase antibody (BETE40-104, Bethyl Laboratories, Quantum Scientific, Brisbane, Australia) and incubation at room temperature for a further 60 min. Plates were washed a further five times and 100 μL/well of tetramethylbenzidine substrate solution was added, with a stop solution (0.18mol H_2SO_4) 100μL/well added after 5 min. Optical density was read at 450 nm with a microplate reader. The concentration of IgA in each sample was calculated using a logistic equation calculated from a linear regression of the known standard concentrations. Results are reported per ng dry weight of faeces.

2.9.2 Cortisol

For faecal cortisol extraction and quantification, 200 ± 1 mg of dry faecal powder was weighed into a 16 x 100 glass test tube. Borate buffer, 2mL (pH 6.5, 0.1mol), was added to the dry powder, vortexed and then 50 µL of beta glucuronidase (b-D-Glucuronoside glucuronosohydrolase, EC 3.2.1.31, Sigma, St. Louis, Missouri, USA) containing approximately 4,000 Units was added to each test tube. Test tubes were incubated for 4 h at 37°C in an orbital mixer. Then 3 mL redistilled diethyl ether was added to each tube and it was vortexed for 2 min, and allowed to stand for 2 min. The lower aqueous phase was frozen in liquid nitrogen and the supernatant ether was decanted into 12 x 75 mm glass test tubes and evaporated to dryness at 40°C in a hot block evaporator in a fume hood. The residue containing extracted steroid was re-dissolved in 200 µL of diluted zero cortisol calibration solution (Saliva Free Cortisol Kit, Demeditec Diagnositics, Kiel-Wellsee, Germany), diluted 1: 10 and placed on an orbital mixer at 37°C for 60 min, followed by short, high-speed vortex (20 s). Then 100 µL of test samples, standards and controls was pipetted into wells of the Saliva Free Cortisol Kit (Demeditec Diagnositics, Kiel-Wellsee, Germany). The efficiency of the extraction process was progressively tracked by addition of 30,000 dpm 3H-cortisol (1,2,6,7 3H cortisol 160curie/mmol Perkin-Elmer Life Sciences, Waltham, USA), and the final assay concentration for cortisol was corrected for this efficiency. Serial dilutions of glucuronidase-treated canine faecal extracts run against Demeditec assay kit calibrator standards gave a satisfactory degree of parallelism for the assay. Assay data were analysed employing a four parameter logistic fit using MyAssays Analysis Software Solutions (www.myassays.com). All analyses were reported per g dry weight of faeces.

2.10.0 Ethics

Ethical approval was obtained from the University of Queensland Animal Ethics Committee Approval number CAWE/139/10.

2.11.0 Statistical Analysis:

All statistical analysis was carried out using Minitab (version 16). After an initial descriptive analysis of the recorded behaviours, the behaviours ‘exit rear’, ‘lip lick’, ‘scratching’, ‘paw lift’, ‘bar-pawing’, ‘roll’, ‘human interaction’, ‘neighbour interaction’, ‘eat’, ‘defecate’, ‘urinate’ and ‘shake’ were removed due to low frequency ($n < 3$). 'Affiliative' and 'agonistic' behaviour was not included in statistical analysis as the remaining focal dogs were not able to engage in these behaviours after the removal of their kennel-mate. This left 18 categories of behaviour (Table 1). Due to differences in the total number of 5-min observations for each focal dog, the total time (s) engaged in each behaviour was converted to a proportion by dividing the total duration of each behaviour by the total number of 5- min observation session per day. This was calculated separately for data obtained before and after separation. For each focal dog, the frequency of occurrence of each behaviour was also calculated and converted to a proportion of the total frequency per day, by dividing the total count of each behaviour by the total count of 5-min observation sessions per day. Residuals did not follow a normal distribution pattern and data were not able to be mathematically transformed to achieve normal distribution, hence the non-parametric Wilcoxon (matched-pairs) Signed Rank Test was used to investigate differences in the performance of behaviours before and after separation. Friedman's Rank Test, with post hoc Wilcoxon (matched-pairs) Signed Rank Test was used to investigate differences in behaviour across the 6 days of observations.

Individual dog's mean latencies to reach the food bowl were calculated for each cognitive bias trial, as described by Mendl et al. (2010). The latency to reach the bowl in the three ambiguous locations (NP, M, NN) was adjusted for differences in the running speeds of each dog (Mendl, 2010), but as this did not affect the significance of results and they are presented here in unadjusted form. A Friedman's Rank Test with post hoc Wilcoxon (matched-pairs) Signed Rank Test was used to investigate differences in latencies to approach cues before and after separation.

The difference in mean values before and after separation, of both IgA and cortisol, was investigated with a Wilcoxon (matched-pairs) Signed Rank Test.

As data analysis in this study included multiple comparisons of related data, a sequential Bonferroni correction was applied to control for type one errors (Holm, 1979). Variables that met this criterion are indicated in Tables and described within the text as significant effects. Cohen's *r* was used to test the effect size.

3.0 Results

3.1 Behavioural Observations

Prior to separation, observations of interactive behaviour between dogs indicated that the focal dogs spent a mean of 3.2 ± 0.68% of total time engaged in affiliative behaviour (Fig. 3). Dogs spent a mean of 0.1 ± 0.05% of total time in agonistic behaviour, but only two of the 12 dogs contributed to this.

[Insert Fig. 3 Here]

Following separation, dogs increased their duration of running (H = 8, P = 0.02, r = 0.49), and grooming (H = 8, P = 0.02, r = 0.49), and the frequencies of circling (H = 1.5, P = 0.006, r = 0.57), figure of 8 (H = 2, P = 0.01, r = 0.52), posture change (H = 1, P = 0.003, r = 0.61) and stretching (H = 3, P = 0.005, r = 0.58) (Table 2). A decrease in play was observed after separation (H = 53, P = 0.01, r = -0.52) (Table 2).

[Insert Table 2 Here]

Friedman's Rank Test revealed differences between the 6 observation days for 'grooming'(S = 17.4, df=5, P = 0.004), 'playing' (S = 25.8, df=5, P < 0.0001), 'interaction with the environment' (S = 12.9, df=5, P =0.024), 'circling'(S = 26.40, df = 5, P < 0.0001), 'stretch'(S = 23.70, df = 5, P < 0.0001), 'posture changes' (S = 33.43, df = 5, P < 0.0001) and 'rest' (S = 13.06, df = 5, P = 0.023). Post hoc Wilcoxon (matched-pairs) Signed Rank Test, Bonferroni Correction applied at a 0.006 level revealed that 'grooming' behaviour significantly increased (H = 65, P = 0.004, r = 0.58) between days -1 and +3 (Fig. 4), 'circling' behaviour significantly increased (H = 1.5, P = 0.005, r = -0.57) between days -6, -1 and +3 and -6 and +1 and posture changes significantly increased (H = 1, P = 0.004, r = -0.58) between days -6, -1 and +1 and -3, -1 and +3 (Fig. 5). This suggests that the increase in grooming following separation declined between day 3-6, whilst increases in posture change, interaction with the environment, resting and circling declined between days 1 and 6 following separation, but that the reduction in play was maintained until day 6 post-separation.

[Insert Fig. 4 and 5 Here]

3.2 Cognitive Bias Testing

The number of training trials required to reach the pre-determined threshold at which dogs learned to discriminate between the positive and negative cues varied from 17 to 31 (mean 22.3 ± se 1.2). During subsequent testing sessions, bowl position influenced latency both before (S = 37.33, $n = 12$, $P < 0.0001$) and after (S = 30.13, $n = 12$, $P < 0.0001$) separation (Fig. 6). Dogs ran fastest to the bowl when it was presented near the positive cue location and became progressively slower as the bowl was placed in locations nearer to the negative cue. However, there was no difference in latencies to reach the bowl before and after separation (H = 26, $n = 12$, $P = 0.33$, $r = 0.03$), nor in latencies to reach individual bowl locations (P_{before} vs P_{after} [$P = 0.39$, $r = -0.18$], NP_{before} vs NP_{after} [$P = 0.785$, $r = 0.05$], M_{before} vs M_{after} [$P = 0.15$, $r = 0.23$], NN_{before} vs NN_{after} [$P = 0.67$, $r = -0.1$] and N_{before} vs N_{after} [$P = 1$, $r = 0.00$]). Latencies to reach the bowl tended to increase after separation for one dog ($P = 0.059$), however, for the remaining 11 dogs latencies before and after separation did not differ significantly ($P = 0.2$-1).

[Insert Fig. 6]

3.3 IgA and Cortisol

Intra-assay coefficients of variability (CVs) were 2.3% and 5.5% for IgA and cortisol, respectively. Inter-assay CVs were 2.3% and 5.4% for IgA and cortisol, respectively. There was a significant increase (H = 10, *P* = 0.02, *r* = 0.47) in IgA after separation (mean before separation = 370 ng/mL; mean after separation = 444 ng/mL; SED = 58.0 ng/mL). Cortisol concentration in faeces was not significantly different before and after separation (mean before separation = 792 ng/g; mean after separation = 874 ng/g; SED = 108.2 ng/g; *P* = 0.26, *r* = 0.24).

4.0 Discussion

The separation of conspecifics within a shelter environment was hypothesised to produce a stress response. We found an increase in activity after separation, including running, posture changes and stretching, suggesting the dogs were more restless following separation. An increase in some behaviours that are recognised as stereotypic and indicative of stress and decreased welfare in kennel-housed dogs (Beerda et al., 1999b) were observed, including an increase in the stereotypic tracing of a continual circle and a figure of 8 pattern. However, other well recognised indicators of stress such as yawning, panting, wall bouncing, lip licking and paw lifting either did not change significantly, or occurred with insufficient frequency to warrant inclusion in statistical analysis. It is possible that we did not observe some of these traditional indicators of stress because the dogs did not share a strong bond, and consequently the experience of separation might have been less stressful than for those with a stronger bond. The variability between pairs in affiliative behaviour may indicate variation in the strength of the bond between individuals, indeed some dogs that showed little affiliative behaviour may have been tolerating each other's presence. Agonistic behaviour was however rare, suggesting that the pairs were settled in their social relationship.

Grooming behaviour also increased after separation, and although it is considered a normal behaviour performed to maintain the healthy integument of the animal, in many species it is documented to increase above normal levels when animals are exposed to stress (e.g. Audet et al., 2006; Beaver, 2003; Wittig et al., 2008). Play behaviour decreased following separation. This behavioural change could be a reflection of a change in inner state (e.g. a reflection of stress), or alternatively could be a reflection of the absence of social facilitation previously caused by presence of a conspecific.

Collectively these behavioural changes indicate arousal and describe a trend in behavioural changes similar to those evidenced in dogs experiencing acute or chronic stress in a kennel environment (Beerda et al., 1997; Hennessy et al., 1997; Rooney et al., 2009).

We used cognitive bias testing to investigate the emotional valence of the dogs prior to and post separation. As the dogs ran significantly faster towards the rewarded 'positive' cue in comparison to the unrewarded 'negative' cue both before and after separation, they were able to discriminate between these reference cues. The absence of differences in latencies to reach the ambiguous cues before and after separation suggests that the dogs did not experience major negative emotional valence post separation. Cognitive bias testing may not be sensitive enough to detect minor changes in emotional valence, as demonstrated when short-term owner absence did not induce a negative cognitive bias in a sample of 24 pet dogs (Müller et al., 2012). Non-affective explanations such as motivation, learning and/or activity could also be implicated (Burman et al., 2011; Mendl et al., 2009). Each dog acted as his/her own control and experienced the ambiguous cues several times, which previous research in sheep found to result in increased latency to approach ambiguous cues due to learning that they were unrewarded (Doyle et al., 2010a). Continued approaches to ambiguous cues probably derive from the lack of negative consequences and/or the motivation to gather information about potential food sources (Burman et al., 2011). It is possible the dogs may have learned simple associations between approaching the ambiguous cues and subsequent interaction with the human handler (when confining the dog in the crate for the subsequent round), which could have inadvertently induced positive emotional valence through human engagement with the dogs in the test room environment. Human-dog interactions within a shelter environment have previously been evidenced to result in a positive effect on behaviour (Hennessy et al., 2002; Hennessy et al., 2006). Discrepancies between the predicted and observed outcomes in cognitive bias measures of emotional valence (both positive and negative) have been described in dogs (Burman et al., 2011; Müller et al., 2012) and sheep (Doyle et al., 2010b), with the suggestion that performance in the cognitive bias task itself, the experience of the rewarding event and human contact could all initiate an unanticipated positive emotional valence (Burman et al., 2011). Consequently, future research utilising cognitive bias to measure emotional valence in dogs must first address the variables that might elicit affective states before it can be applied to differentiate between treatment groups.

S-IgA increased in dogs after separation from conspecifics. The primary function of secretory IgA is part of a localised immune response that serves to prevent bacteria and viruses from attaching and invading enterocytes (Flickinger et al., 2004). IgA is the dominant immunoglobulin in mucosal secretions of both dogs and cats (Stokes and Waly, 2006). Immune functioning is likely to be influenced by emotional valence, and in humans negative emotional valence has been correlated with decreased immunocompetence (e.g. Herbert and Cohen, 1993; Segerstrom and Miller, 2004). Studies of the relationship between emotions and immune system functioning, specifically measures of S-IgA, are scarce and sometimes contradictory. For example in rats, IgA levels are reduced as a result of social stress (Guhad and Hau, 1996), and in dogs salivary IgA has been demonstrated to decrease after exposure to a short-term acute noise stressor (Kikkawa et al., 2003). Conversely, other research reports increasing IgA levels in dogs after the acute stress of entry to a kennel environment and a decrease as a result of continued confinement in that environment (Skandakumar et al., 1995). In other species, a similar response has been reported, for example in pigs IgA rises as a result of acute stress resulting from social isolation and restraint (Muneta et al., 2010; Royo et al., 2005) and decreases as a result of chronic stress (Royo et al., 2005). More recently, enhanced IgA mediated immunity has been correlated with positive emotional states in shelter-housed cats (Gourkow et al., 2014). Based on the findings in the present study it appears likely that positive emotional valence is reflected in increased IgA secretions and negative emotional valence with decreased IgA secretions, with the exception that significant acute stressors (such as conspecific separation in the present study) result in temporary increases in IgA secretions.

The absence of an effect of separation on cortisol might be explained by the extensive duration of time, on average 182 days, the dogs had been maintained in the shelter. This duration of kennelling may have increased negative feedback or diminished sensitivity of the components of the HPA axis, reducing the possibility of a cortisol response**.** During the first 3 days that a dog is confined within a welfare shelter, cortisol levels rise dramatically and decline thereafter (Beerda et al., 1999a; Hennessy et al., 1997; Stephen and Ledger, 2007). This decline may be explained by the adaptation of the HPA axis to the stressor (Beerda et al., 1998; Hennessy et al., 2001). Prolonged stressors (such as long-term kennelling) result in increased negative feedback of cortisol on brain structures controlling HPA activity and/or reduce the sensitivity of the pituitary or adrenal glands to these stimulating hormones (Beerda et al., 1998; Hennessy et al., 2001). Although evaluation of cortisol has traditionally been used as a measure of the impact of social separation, it is worth considering that variations in cortisol may not be sensitive enough to detect the distress responses that occur during the separation of non-kin conspecifics. Future research could include measurement of other hormones, in particular those involved in the control of affiliative behaviour, e.g. oxytocin and vasopressin.

4.1 Conclusions

We describe increases in active behaviours and stereotypic behaviours indicative of stress in dogs, reductions in play behaviour and increases in S-IgA as a result of separation from conspecifics. The results suggest that separation from conspecifics within a shelter environment results in stimulation that may be indicative of an acute stress response. Consequently, shelters should consider giving special care to individual dogs separated from a conspecific. Future research could investigate the health and welfare of separated dogs over a longer period, as well as the influence of length and strength of attachment on separation effects.

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Figure Captions:

Fig. 1: A pair of kennel enclosures, showing guillotine doors to allow shared housing by the pair of dogs

Fig. 2: Cognitive bias experimental facility, showing the five possible food bowl locations.

Fig. 3: Affiliative and agonistic behaviours (% total time) performed by focal dogs prior to separation from a conspecific

Fig. 4: Mean duration of behaviours for all 12 dogs per day. Post hoc Wilcoxon (matched-pairs) Signed Rank Test, Bonferroni Correction applied at a 0.006 level; 'grooming' (^ = P = 0.004).

Fig. 5: Mean occurrence of behaviours for all 12 dogs per day. 'Post hoc Wilcoxon (matched-pairs) Signed Rank Test, Bonferroni Correction applied at a 0.006 level; 'circling' (^, $^{\#}$ *= P = 0.005), 'posture change' (^,* $^{\#}$ *= P = 0.004).*

Fig. 6: Mean latency for dogs to reach the food bowl in each location both before and after separation during cognitive bias testing.

Table 1:Ethogram used for the observation of dogs before and after separation from a conspecific.

Locomotive Behaviour	
Walk	Forward movement with legs resulting in shift of whole body to new postion in enclosure.
Run	As walking but faster paced where multiple paws leave the ground at the same time.
Stand	All four paws on ground and legs upright and extended supporting body.
Sit	Hind quarters on ground with front two legs being used for support.
Rest	Ventral/lateral lying on ground with all four legs resting and in contact with ground. Dog may also be curled up in a tight ball. Head is either resting on ground or held up in air. Eyes are either open or closed.
Roll*	Dog lies on back and rotates body laterally.
Circle	A circular motion in one location and traced in one direction repeatedly.
Figure of 8	A figure of 8 motion traced around the kennel (both inside and outside) in a repeated fashion.
Paw Lift*	Front limb raised.
Stretch	Moves body into playbow position by extending front legs and lowering chest and head towards the ground.
Shake*	Rapid lateral rotation of the body in the standing position.
Posture Low*	Head lower than shoulders, tail low, ears lowered.
Posture Change	Changes postural position during rest or sleep e.g. from sternal to lateral recumbancy.
Interact with Environment	Any vigorous behaviour directed toward the environment/cage that does not involve oral manipulation (e.g. digging/manipulating bedding, flooring, walls or water/food containers)
Oral*	Any vigorous behaviour directed toward the environment/cage using the mouth (including chewing, biting, shaking and pulling with the mouth).
Maintanence Behaviour	
Eat*	Ingests food provided by kennel attendent.
Drink*	Drinks from automated water system.
Defecate*	Passes a faecal motion in standing or squatting position.
Urinate*	Eliminates urine in standing or squatting position. May also lift one rear leg during standing.
Vocal Behaviour	
Bark	Release sound with mouth opened and closed rapidly.
Oral Behaviour	
Lip Lick*	Tongue is protruded and moved along the upper lip.
Yawn	Mouth open wide then closed with prolonged inhalation and expiration.
Pant	Mouth open with tongue extended accompanied by rapid breathing.
Sniff	Air sampling through the nose to detect odours.
Social Interaction	
Agonostic#	Any form of intraspecific behaviour relating to aggression or fear (e.g. raised hackles, submissive body posture, teeth baring, biting)
Affiliative#	Any form of intraspecific positive behaviours (e.g. allo-grooming, touching, play bow)
Neighbour Interaction*	Sniffs neighbouring dog through small opening at the corner of the kennel or jumps up to reach neighbouring dog over kennel top.
Escape Behaviours	
Exit ‘rear’*	Stands on hind legs with front legs resting against exit.
Wall bounce	Stands on hind legs with front legs rebounding off wall, usually repetitive.
Bar pawing*	Using paws to reach through cage bars in a digging motion.
Exit Stare	Dogs gaze is focused on exit points or things outside of kennel.
Other	
Play	Any vigorous or galloping gaited behaviour directed towards a toy, including chewing, biting, shaking from side to side, scratching or batting with the paw, chasing rolling balls and tossing using mouth. Destruction not included.
Chew Bone/Toy	Gnaw bone/toy with mouth.
Groom	Behaviours directed towards the subjects on body, including licking, self-biting and scratching.
Human Interaction*	Physical contact with human.

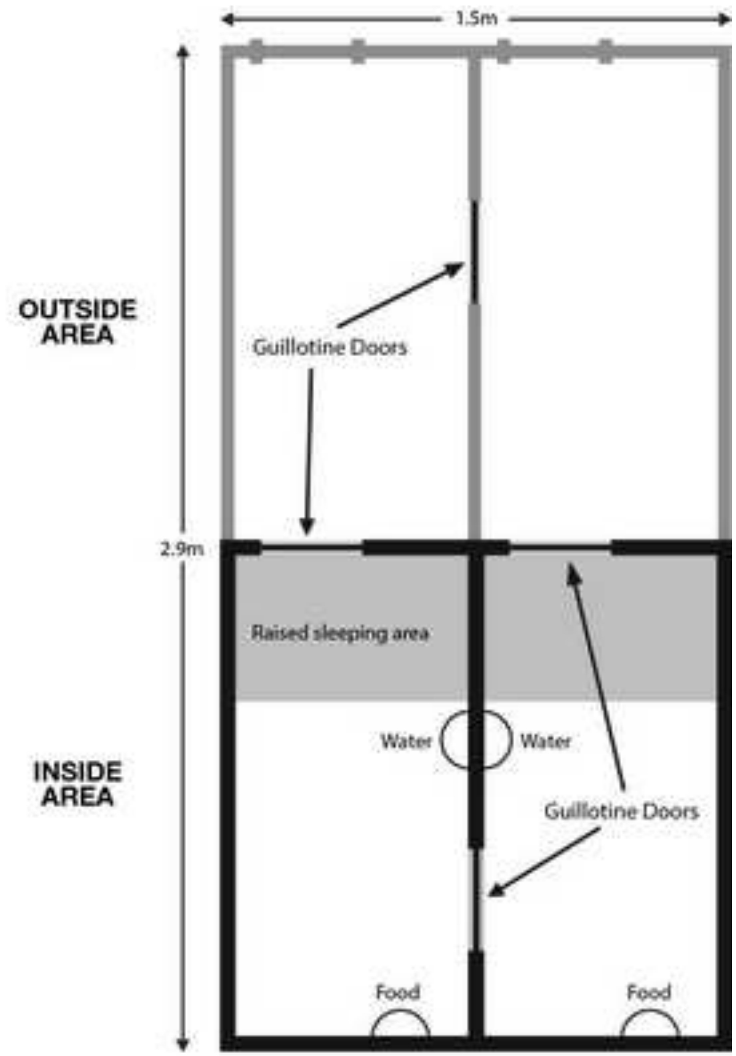
* Behaviours excluded from statistical analysis due to low frequency of occurrence ($n < 3$)
\# Behaviours excluded from statistical analysis because they could not be performed after separation.

Table 2: Mean (±SED) proportion of time (s) or frequency (count) spent in each behavioural category both before and after separation.

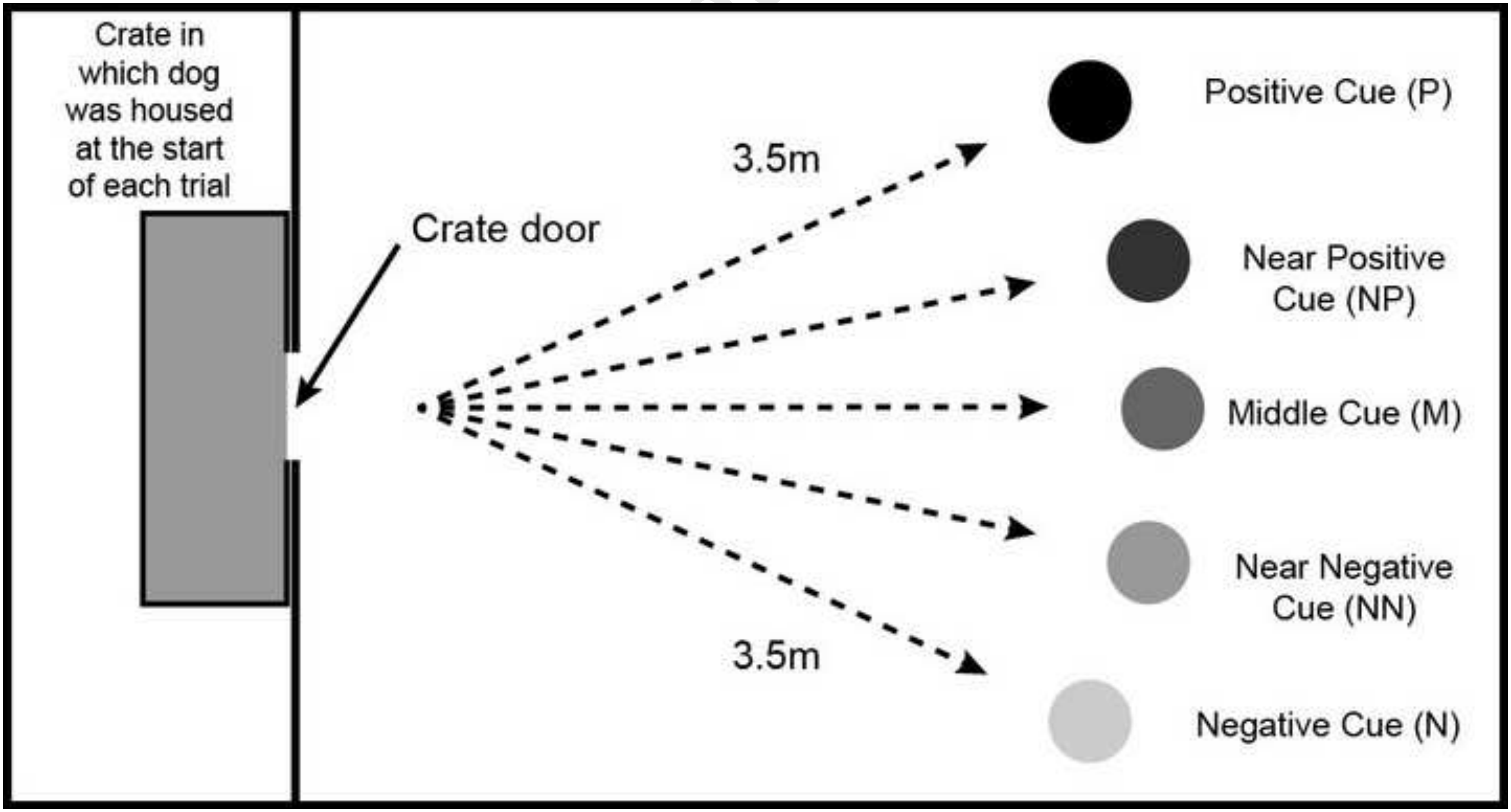
Behaviour	Before Separation	After Separation	SED	P-value
States (s/5 min)				
Stand	15.7	9.5	5.15	0.11
Walk	5.1	4.6	1.20	0.61
Run	0.5	0.9	0.29	0.02* Bonferroni Correction Value P = 0.04
Sit	4.1	2.9	1.71	0.61
Rest	95.1	69.6	30.10	0.09
Pant	3.2	3.8	0.89	0.61
Groom	1.0	2.5	0.76	0.02* Bonferroni Correction Value P = 0.04
Play	2.9	0.2	1.67	0.01* Bonferroni Correction Value P = 0.03
Interact with environment	0.1	0.5	0.31	0.08
Sniff	0.5	0.4	0.11	0.37
Exit stare	16.2	13.3	6.73	0.85
Chew bone/toy	0.9	0.4	0.78	0.26
Events (count/5 min)				
Circle	11.1	23.2	7.96	0.006* Bonferroni Correction Value P = 0.03
Bark	87.1	110.1	42.06	1
Yawn	0.6	1.1	0.33	0.12
Figure of 8	5.2	14.7	5.94	0.01* Bonferroni Correction Value P = 0.03
Wall bounce	5.8	9.3	3.57	0.17
Posture change	1.7	16.1	4.37	0.003* Bonferroni Correction Value P = 0.03
Stretch	1.1	5.5	1.73	0.005* Bonferroni Correction Value P = 0.03

*Sequential Bonferroni correction criteria applied (Holm, 1979)

Figure

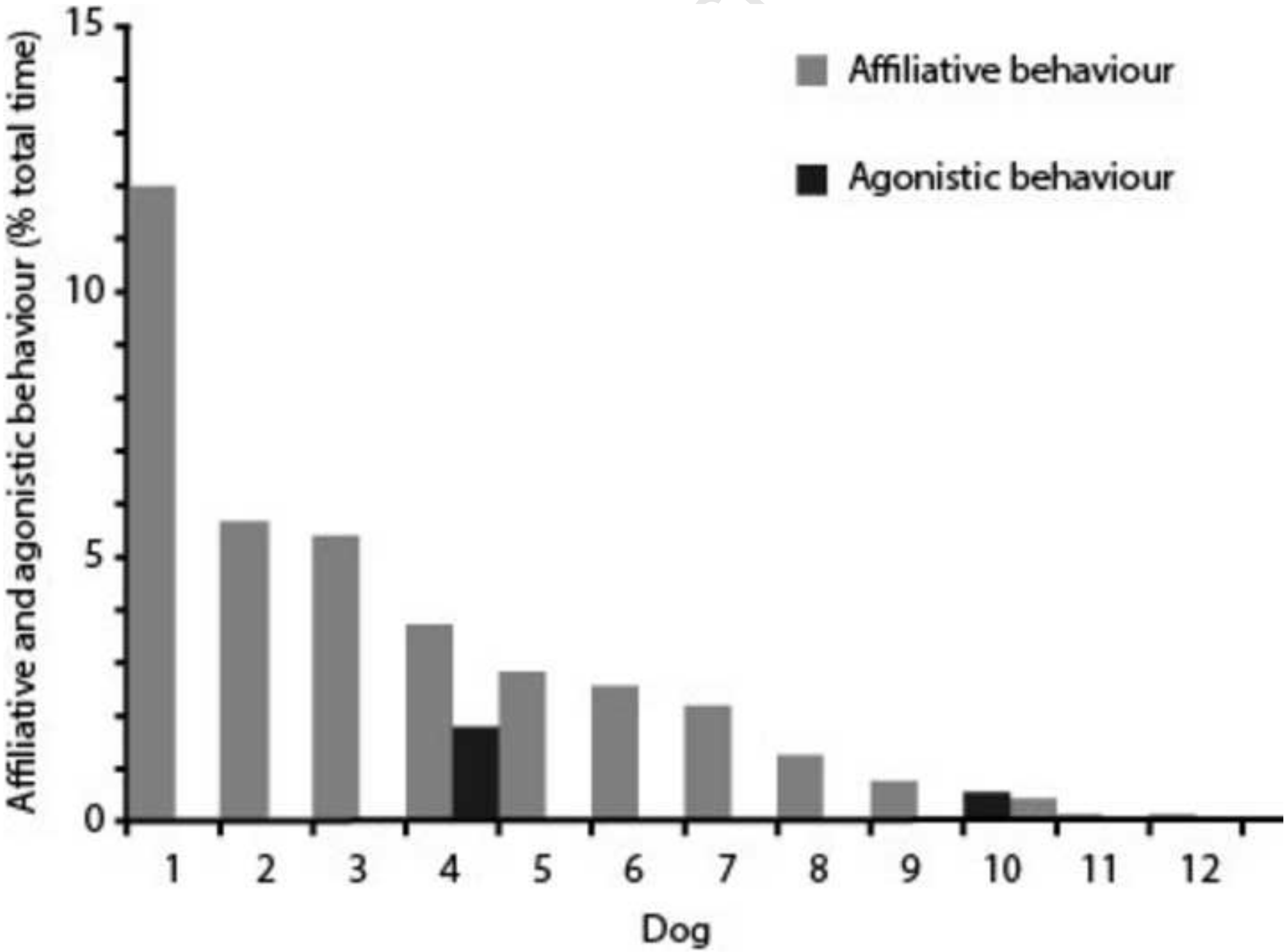

1.5m
OUTSIDE AREA
Guillotine Doors
2.9m
Raised sleeping area
Water
Water
INSIDE AREA
Guillotine Doors
Food
Food

Figure

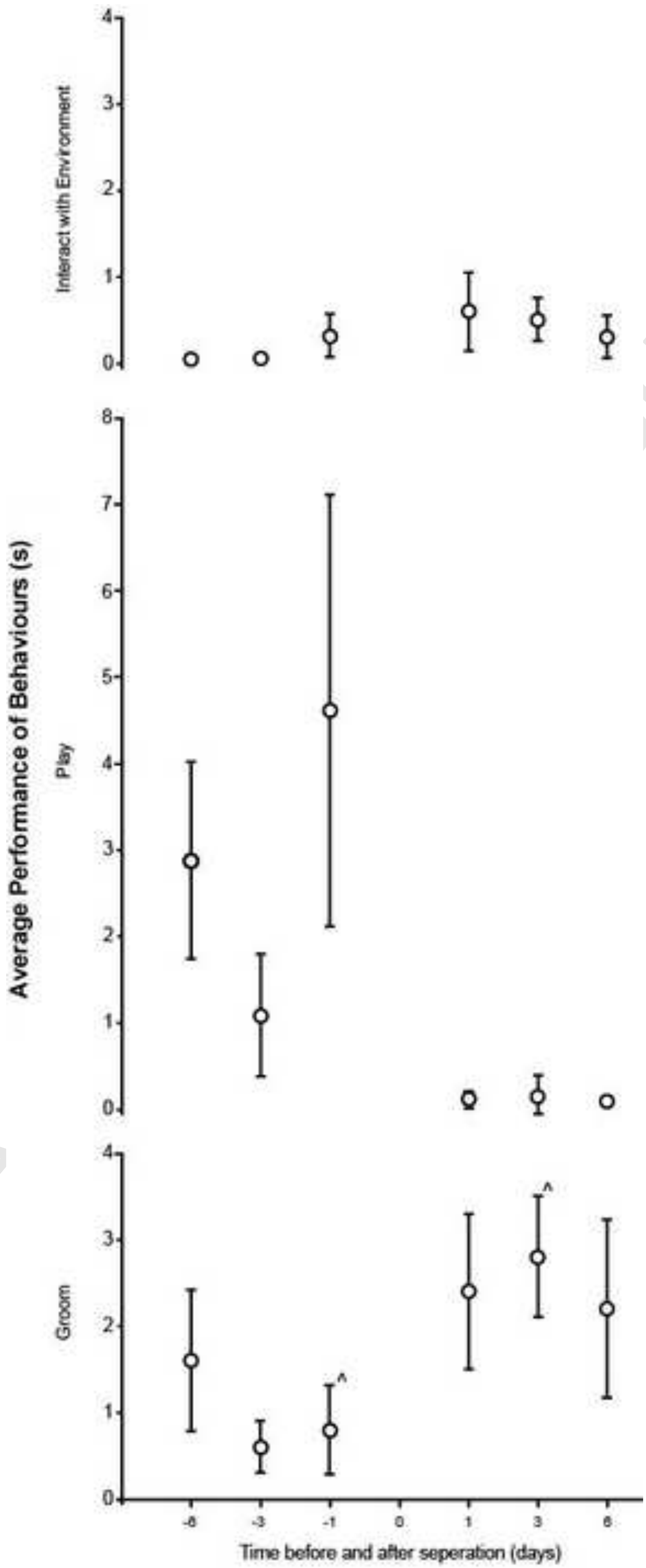


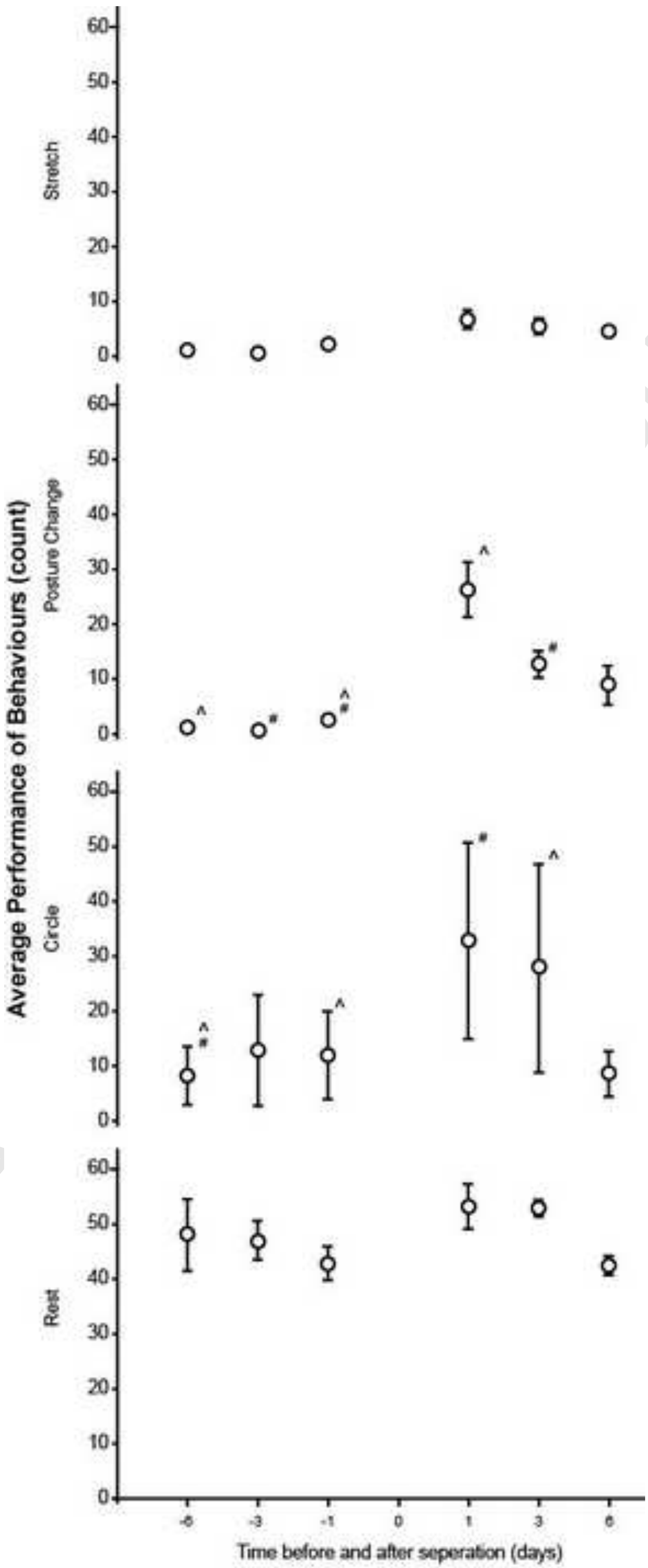
Crate in which dog was housed at the start of each trial
Crate door
3.5m
3.5m
Positive Cue (P)
Near Positive Cue (NP)
Middle Cue (M)
Near Negative Cue (NN)
Negative Cue (N)

Figure

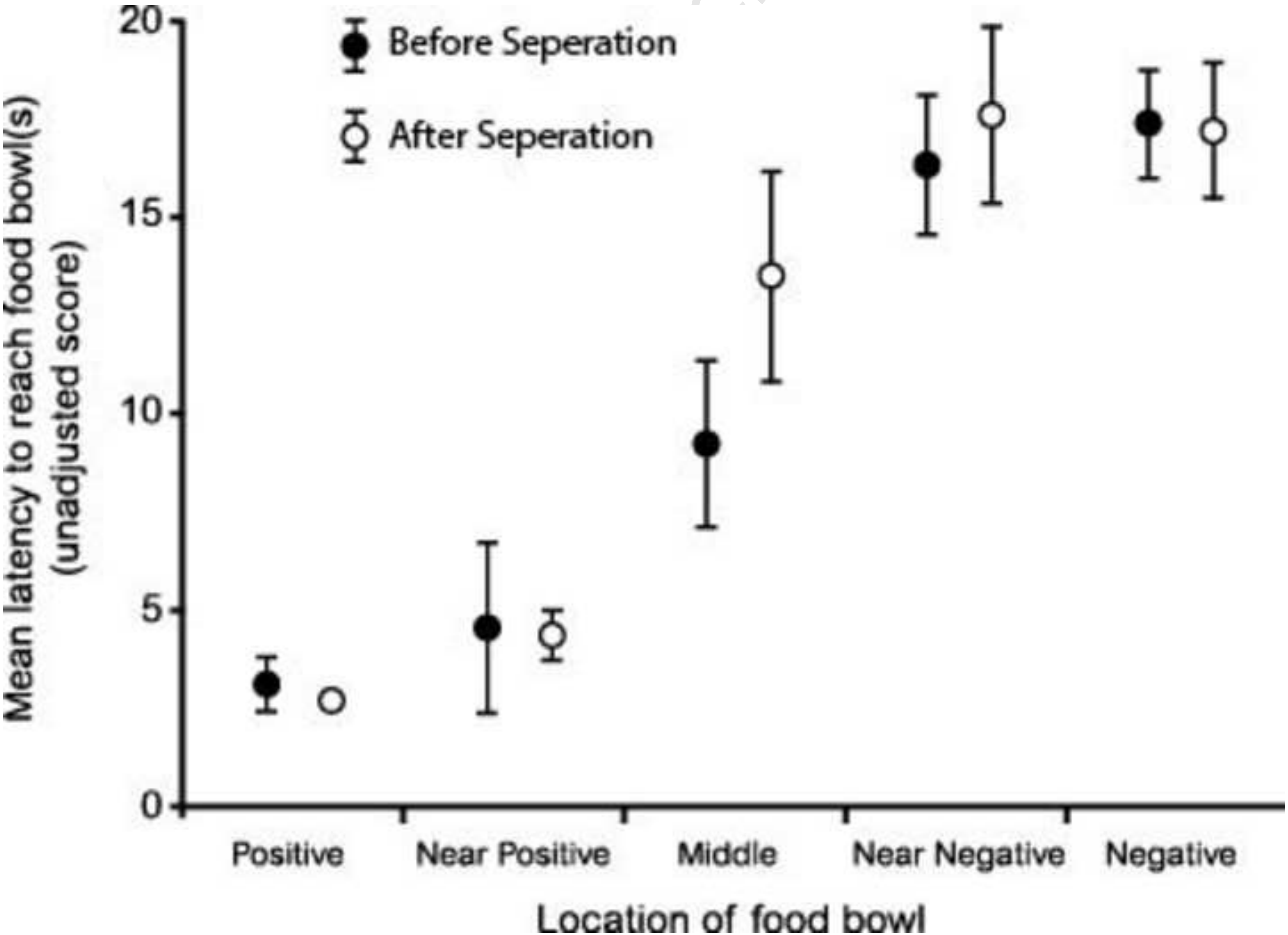


Affiliative behaviour
Agonistic behaviour
Affiliative and agonistic behaviour (% total time)
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
Dog


Average Performance of Behaviours (s)
Interact with Environment
Play
Groom
Time before and after seperation (days)
-6
-3
-1
0
1
3
6


Average Performance of Behaviours (count)
Stretch
Posture Change
Circle
Rest
Time before and after seperation (days)
-6
-3
-1
0
1
3
6
0
10
20
30
40
50
60

Figure



Before Seperation
After Seperation
Mean latency to reach food bowl(s) (unadjusted score)
20
15
10
5
0
Positive
Near Positive
Middle
Near Negative
Negative
Location of food bowl

Highlights

- We investigate the effect of conspecific separation in pair-housed shelter dogs.
- Increases in active behaviours, grooming, posture change and stretching occurred after separation.
- Secretory IgA increased after separation whilst cortisol levels remained unchanged.
- No major effect of separation on emotional valence was evident.
- Results demonstrate separation of a dog from a conspecific negatively affected behaviour and stimulated the immune system.