Elsevier Editorial System(tm) for Physiology & Behavior
Manuscript Draft

Manuscript Number:

Title: Physiological, physical and behavioural changes in dogs (Canis familiaris) when kennelled: Testing the validity of stress parameters.

Article Type: Research paper

Keywords: Animal welfare; Domestic dog; Acute stress; Cortisol; Vanillylmandelic acid; Surface temperature

Corresponding Author: Ms. Cherie Elizabeth Part, B.Sc., M.Sc.

Corresponding Author's Institution: Queen's University Belfast

First Author: Cherie Elizabeth Part, B.Sc., M.Sc.

Order of Authors: Cherie Elizabeth Part, B.Sc., M.Sc.; Jenna L Kiddie; William Hayes; Daniel Mills; Rachel F Neville; David B Morton; Lisa M Collins

Abstract: Domestic dogs (Canis familiaris) housed in kennelling establishments are considered at risk of suffering poor welfare. Previous research supporting this hypothesis has typically used cortisol:creatinine ratios (C/Cr) to measure acute and chronic stress in kennelled dogs. However, the value of C/Cr as a welfare indicator has been questioned. This study aimed to test the validity of a range of physiological, physical and behavioural welfare indicators and to establish baseline values reflecting good dog welfare. Measurements were taken from 29 privately-owned dogs (14 males, 15 females), ranging in age and breed, in their own home and in a boarding kennel environment, following a within-subjects, counterbalanced design. Pairwise comparisons revealed that C/Cr and vanillylmandelic acid:creatinine ratios (VMA/Cr) were higher in the kennel than home environment ($P = 0.003$; $P = 0.01$, respectively) and were not associated with differences in movement/exercise between environments. Dogs' surface temperature was lower in kennels ($P = 0.001$) and was not associated with ambient temperature. No association with age, or effects of kennel establishment, kennelling experience, sex or source were found. Dogs were generally more active in kennels, but showed considerable individual variability. C/Cr and 5-HIAA:creatinine ratios (5-HIAA/Cr) were negatively correlated with lip licking in kennels. Baseline values for each parameter are presented. The emotional valence of responses was ambiguous and no definitive evidence was found to suggest that dogs were negatively stressed by kennelling. It was concluded that C/Cr and, particularly, VMA/Cr and surface temperature provide robust indicators of psychological arousal in dogs, while spontaneous behaviour might be better used to facilitate interpretation of physiological and physical data on an individual level.

Professor Randall Sakai
Editor-in-Chief
Physiology & Behavior

October 29, 2013

Dear Professor Sakai,

I am pleased to submit our original research article entitled "Physiological, physical and behavioural changes in dogs (*Canis familiaris*) when kennelled: Testing the validity of stress parameters" for consideration for publication in *Physiology & Behavior*.

Urinary cortisol:creatinine ratio (C/Cr) is currently the gold-standard physiological measure of stress and welfare in dogs. However, the value of this measure has been questioned, and rightly so. In this novel manuscript, we set out to test the validity of nine physiological, six physical, and 28 behavioural stress parameters in dogs. We show that activity levels, surface temperature, and concentrations of urinary cortisol and vanillylmandelic acid change when dogs are kennelled. However, we highlight the equivocal nature of such data, and call into question a widely held belief that kennelling is a psychologically stressful experience for dogs.

We believe that our manuscript will be of great interest to your readers because of its wide scope in terms of physiological and behavioural measures of stress in a relatively understudied species (i.e. pet dogs), our exploration of links between physiology and behaviour under different environmental conditions, and our cautious interpretation of data which challenges earlier conclusions. Our manuscript also contains baseline values for each parameter that reflect good canine welfare and which could greatly facilitate future research in this area.

This manuscript describes original work. It has not been published elsewhere and is not under consideration for publication in any other journal. All authors have approved the manuscript and this submission. We declare no conflicts of interest. If you decide to send our manuscript for peer-review, we suggest the following reviewers:

1. **Dr Nicola Rooney.** School of Veterinary Sciences, University of Bristol, Langford House, Langford, Bristol BS40 5DU, United Kingdom.
   Email: nicola.rooney@bristol.ac.uk

2. **Dr Nancy A. Dreschel.** College of Agricultural Sciences, Department of Animal Science, Penn State University, 312 Agricultural Sciences and Industries Building, University Park, Pennsylvania 16802, United States.
   Email: nad5@psu.edu

3. **Dr John Bradshaw.** School of Veterinary Sciences, University of Bristol, Langford House, Langford, Bristol BS40 5DU, United Kingdom.
   Email: j.w.s.bradshaw@bristol.ac.uk

4. **Dr Daniela Ramos.** Department of Medical Clinics, Faculty of Veterinary Medicine and Animal Sciences, University of São Paulo, Campus of São Paulo, Dr. Orlando Marques de Paiva, 87 - University City, São Paulo, Brazil CEP 05 508 270
   Email: daniela.ramos@usp.br

Thank you for receiving our manuscript and considering it for review. We appreciate your time and look forward to your response.

Sincerely,

Chérie Part, MSc
PhD student, School of Biological Sciences
Queen's University Belfast

Email: cpart01@qub.ac.uk

Highlights

- A range of stress parameters were compared within-dogs at home and in kennels.
- Baseline values reflecting good dog welfare are presented for each parameter.
- Dogs were generally more active in kennels but showed large individual variability.
- Cortisol, VMA and surface temperature offer robust measures of canine arousal.
- Short-term kennelling did not seem to represent a negative stressor for these dogs.

Physiological, physical and behavioural changes in dogs (*Canis familiaris*) when kennelled: Testing the validity of stress parameters

C.E. Part[a], J. L. Kiddie[b], W. Hayes[c], D. Mills[c], R.F. Neville[c], D.B. Morton[d], L.M. Collins[a]

[a] School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT7 9BL, UK.

[b] The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield AL9 7TA, UK.

[c] Animal Behaviour Cognition and Welfare Group, School of Life Sciences, University of Lincoln, Riseholme Park, Lincoln LN2 2LG, UK.

[d] School of Biosciences, Emeritus Professor of Biomedical Science & Ethics, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

Corresponding author: Chérie Part. School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT7 9BL, UK. Tel: +44 (0) 7545971748. Fax: +44 (0) 2890975877. Email: cpart01@qub.ac.uk

Abstract

Domestic dogs (*Canis familiaris*) housed in kennelling establishments are considered at risk of suffering poor welfare. Previous research supporting this hypothesis has typically used cortisol:creatinine ratios (C/Cr) to measure acute and chronic stress in kennelled dogs. However, the value of C/Cr as a welfare indicator has been questioned. This study aimed to test the validity of a range of physiological, physical and behavioural welfare indicators and to establish baseline values reflecting good dog welfare. Measurements were taken from 29 privately-owned dogs (14 males, 15 females), ranging in age and breed, in their own home

and in a boarding kennel environment, following a within-subjects, counterbalanced design. Pairwise comparisons revealed that C/Cr and vanillylmandelic acid:creatinine ratios (VMA/Cr) were higher in the kennel than home environment ($P = 0.003$; $P = 0.01$, respectively) and were not associated with differences in movement/exercise between environments. Dogs' surface temperature was lower in kennels ($P = 0.001$) and was not associated with ambient temperature. No association with age, or effects of kennel establishment, kennelling experience, sex or source were found. Dogs were generally more active in kennels, but showed considerable individual variability. C/Cr and 5-HIAA:creatinine ratios (5-HIAA/Cr) were negatively correlated with lip licking in kennels. Baseline values for each parameter are presented. The emotional valence of responses was ambiguous and no definitive evidence was found to suggest that dogs were negatively stressed by kennelling. It was concluded that C/Cr and, particularly, VMA/Cr and surface temperature provide robust indicators of psychological arousal in dogs, while spontaneous behaviour might be better used to facilitate interpretation of physiological and physical data on an individual level.

Keywords: Animal welfare; Domestic dog; Acute stress; Cortisol; Vanillylmandelic acid; Surface temperature.

1. Introduction

Despite our historic relationship with domestic dogs (*Canis familiaris*), today, many council-funded animal shelters and charitable re-homing centres across the United States (U.S.) and United Kingdom (U.K.) are often filled to capacity with stray, abandoned and unwanted dogs [1, 2]. The welfare of kennelled dogs is of concern, given that many experience minimal social contact, exercise and control over their environment [3] as well as unpredictable and

high levels of noise, novelty and disrupted routines [4]. Such concern need not only be directed towards dogs in rehoming centres, but also to kennelled working dogs [3, 5] and dogs kennelled for research purposes [6].

Previous research suggests that dogs experience acute stress following admission to kennels [5, 7] and chronic stress in response to prolonged kennelling [6]. Stress "implies a threat to which the body needs to adjust", resulting in physiological and behavioural changes [8, p.E260). For example, cortisol, which is secreted following activation of one of the major stress response systems – the hypothalamic-pituitary-adrenal (HPA) axis – [8], was found in significantly higher concentrations after one night in kennels than baseline levels measured both within- [5] and between-subjects in a home environment [7, 9].

Urinary cortisol:creatinine ratio (C/Cr) is perhaps the most widely used physiological indicator reported in published studies of canine welfare [10], and is considered a valid measure of both acute [5, 11] and chronic stress in dogs [6, 12]. However, recent research has found C/Cr to be less reliable and less informative than previously thought for kennelled dogs [13]. Individual variability in cortisol response to kennelling has been reported in several studies [9, 14]. Moreover, cortisol secretion lacks specificity as a stress-response, which greatly increases the potential for misinterpretation of data [15, 16]. For instance, cortisol levels have been found to increase after exercise [17, 18] and excitement [19], and appear to provide an indication of arousal [16] without specifying the emotional valence of that arousal [16, 20, 21]. Such findings have led researchers to question the value of glucocorticoid levels as a welfare indicator [e.g. 22].

Physiological indicators of stress and/or affect identified in other species might offer more reliable and specific welfare indicators in dogs than the classic stress hormones, and/or enable the valence or quality of arousal to be determined when measured alongside C/Cr. For example, the stress of immobilisation can lead to oxidative stress and damage in tissue by causing an imbalance of antioxidant status in rats [23]. Similarly, increased oxidative stress has been associated with chronic stress in humans [24], and may be implicated in the pathophysiology of depression [25]. Lipid peroxidation, of which 8-iso-prostaglandin F2a ("ISOP") [26] and thiobarbituric acid reactive substances (TBARS) [27] are products, provides a biomarker of oxidative stress [28]. Malondialdehyde (MDA) provides a further measure of lipid peroxidation [29] and has been used as a biomarker of oxidative stress in brain tissue of rabbits [27] and in plasma of dairy cows [30].

Although combining multiple physiological measures provides a means of triangulating the level and duration of an animal's stress response, husbandry staff in kennel establishments require quick, robust and economical measures of welfare. Therefore, in addition to testing nine physiological parameters in this study, we also recorded six physical and 28 behavioural measures.

Measurement of any parameter is difficult to interpret accurately without comparative baseline values and, with no single diagnostic test, an animal's welfare or quality of life should be judged on how far measurements deviate from 'normality' [31]. Nonetheless, few studies have examined the physiology and behaviour of dogs under normal home conditions [32]. To the authors' knowledge, only one published study has followed the same subjects from a home to kennel environment and only C/Cr was measured within-subjects under both conditions [5].

Therefore, the current study aimed to: (i) Test the validity of a range of physiological, physical and behavioural parameters as indicators of acute, kennelling-induced, stress in dogs using a within-subjects design; (ii) Establish baseline values for each parameter that reflect 'normality', as measured in dogs' normal home environment; and (iii) Test for relationships between welfare indicators that are informative but difficult to conduct cheaply or quickly by husbandry staff (such as physiological parameters) and those which could easily and robustly be used by husbandry staff on a regular basis.

It was assumed that dogs would show higher levels of stress in the kennel compared to home environment, and it was predicted that this would be reflected in physiological, physical and behavioural measurements deviating from normality (baseline values) when dogs entered boarding kennels. The predicted directions of deviation are presented in Table 1.

2. Material and Methods

2.1 Subjects

The subjects were 29 privately-owned dogs from 29 separate households in Northern Ireland. To test the robustness of each measurement as a general canine indicator of acute stress, we did not control for dogs' age, sex, breed or background; however, dogs with a history of aggressive incidents were excluded from participating in the study. Subject information (i.e. age, breed, sex, known health problems, behavioural problems, history of kennelling, source [purchased as puppy from breeder; rehomed], neuter status and number of dogs in the household) was gathered from the owners.

Table 1. Predicted direction in which measurements would deviate from baseline values when dogs were kennelled, with reference to previous research that led to these predictions Abbreviations: ISOP - 8-iso-Prostaglandin $F_{2\alpha}$; TBARS - thiobarbituric acid reactive substances; MDA – malondialdehyde; DPPH - 2,2-diphenyl-1-picrahydrazyl; FRAP – ferric reducing antioxidant power; VMA - vanillylmandelic acid; HVA - homovanillic acid; 5-HIAA - 5-hydroxyindole-3-acetic acid.

Parameter	**Measurement**	**Prediction and references**
Physiological	Oxidative stress and damage as measured by: ISOP:creatinine ratio (ISOP/Cr) TBARS:creatinine ratio (TBARS/Cr) MDA:creatinine ratio (MDA/Cr)	Oxidative stress and damage in kennels will be greater than baseline levels [23, 25, 33, 34, 35].
	Total antioxidant capacity as measured by: DPPH assay FRAP assay	Total antioxidant capacity in kennels will be lower than baseline values [35, 36].
	Cortisol:creatinine ratio (C/Cr)	C/Cr in kennels will be higher than baseline values [5, 9].
	Epinephrine and norepinephrine as measured by: VMA: creatinine ratio (VMA/Cr)	VMA/Cr in kennels will be higher than baseline values [37, 38].
	Dopamine as measured by: HVA:creatinine ratio (HVA/Cr)	HVA/Cr in kennels will be higher than baseline values [39, 40].
	Serotonin (5-HT) as measured by: 5-HIAA:creatinine ratio (5-HIAA/Cr)	5-HIAA/Cr in kennels will be higher than baseline values [41, 42, 43, 44].
Physical	Whole body condition	Body condition in kennels will be lower than baseline values [45].
	Eye redness	Scleral blood vessels will be more visible (red) in the kennel than in the home

		environment [46].
	Skin dryness (scurf)	Dogs will have more scurf in the kennel than in the home environment [46, 47, 48].
	Surface temperature	Surface temperature in kennels will be lower than baseline values [49, 50, 51].
	Core body temperature	Core body temperature in kennels will be higher than baseline values [52, 53, 54].
	Amount of food eaten	Dogs will eat less food in the kennel than in the home environment [55, 56].
Behavioural	Spontaneous behaviour	Dogs will show increased lip licking, paw lifting [57], yawning, bodyshaking and restlessness [58] – as indicated by less time spent lying down and sleeping/resting and by more time spent travelling – in the kennel than in the home environment.
	Behavioural diversity	Dogs will show less behavioural diversity in the kennels than in the home environment [59].

123

124 Dogs (14 males, 15 females) were aged between 1 and 10 years (mean = 4.43 years; SD =
125 2.69). The neutering status of three dogs (1 male, 2 females) was unknown. Of the remainder,
126 65.4% (8 males, 9 females; 58.6% of total sample) were entire and 34.6% (5 males, 4
127 females; 31.0% of total sample) were neutered. Purebred dogs constituted 82.8% of the
128 sample and represented 21 different breeds. Crossbreeds (offspring of purebred parents of
129 two different breeds) and mixed-breeds (unknown parentage, or offspring of non-purebred
130 parents) were also represented in 10.3% and 6.9% of the sample, respectively.

131

132 Two dogs had arthritis, one related to an historical injury and one related to age deterioration.
133 Another dog had a small hole in his heart, which was not reported to have caused any health
134 issues. The data from these three dogs were examined closely (using the 'Explore' feature of
135 SPSS, version 19). The dogs did not represent consistent outliers in home measurement data
136 and, so, were not excluded from the analyses. No other health problems were reported. Two
137 owners reported occasional destructive behaviour in their dog when left at home alone;
138 however, these dogs were not home alone when measurements were taken, and destructive
139 behaviours were not observed in either environment.

140

141 Of those dogs that came from multi-dog households (41% of total sample), eight (66.7%)
142 were kennelled with all of their home companions, two (16.7%) were kennelled with one of
143 the two (n = 1) or three (n = 1) dogs with which they shared their home, and two (16.7%)
144 were housed individually in the boarding kennels. To avoid selection bias in homes with
145 more than one dog, each dog in the household was assigned a number and the subject was
146 randomly selected using the "true random number generator" on www.random.org. In two
147 out of 12 multi-dog households, the owners chose the focal dog because the alternative dogs

showed signs of nervousness in the presence of strangers or suffered from long-term ill health.

2.1.1 Recruitment of subjects

Dog owners were recruited through future bookings at the participating boarding kennel establishments, from the staff and student population at Queen's University Belfast, and by advertisements in the monthly newsletter of one boarding kennel, a local newspaper, a pet supply store, and a veterinary clinic. All dog owners consented to all measurements being taken from their dog and no personal information about the owners was requested.

2.2. Research design

A within-subjects design was employed where measurements (see section 2.4.1) were taken from all subjects in two different environments: (i) dogs' own homes and (ii) boarding kennels. Boarding kennels were chosen over re-homing centres to obtain true baseline (non-stressed) levels in subjects that were, presumably, already experiencing a stable home environment. Using boarding kennels also enabled feasible counterbalancing of the design: Measurements were taken from 15 dogs in their own homes first, and from the remaining 14 dogs in boarding kennels first.

2.3 Housing

2.3.1 Boarding kennel environment

Dogs were kennelled in one of three private boarding kennel establishments in Northern Ireland (denoted BK1, BK2 and BK3) following each establishment's standard procedures and practices. Fifteen dogs (51.7% of total sample) were kennelled in BK1, ten dogs (34.5%) in BK2 and four (13.8%) in BK3, predominantly due to owners' prior bookings with those

establishments or recruitment of subjects through that particular establishment. All kennels in BK1 and BK2 were contained within one building in a line block design, which prevented kennelled dogs from visual, but not auditory, contact with all other kennelled dogs. All kennels in BK2 and 90% of kennels in BK1 comprised an indoor (BK2: 112cm x 180cm; BK1: 144cm x 179cm) and covered outdoor area (BK2: 160cm x 180cm; BK1: 144cm x 306cm), separated by a steel guillotine door in brick wall. The remaining kennels in BK1 comprised an indoor area only (154cm x 300cm). Dogs boarding in 'indoor only' kennels (*n* = 2) were given regular access to an enclosed, uncovered, outdoor exercise area for toileting (dogs housed in indoor/outdoor kennels were also given access to this area). All indoor kennels in BK3 were detached wooden chalets. Each chalet (213cm x 213cm) was set in an individual, uncovered, approximately-circular outdoor area (366cm x 457cm) enclosed with wire fencing. The wire fence and semi-circular positioning of chalets on the site allowed dogs visual and auditory contact with all other dogs when in their outdoor area.

The guillotine/chalet door was closed for the night between 1900h and 2300h, which restricted dogs to the indoor area until data collection began the following morning, between 0630h and 0900h. All dogs had continuous access to water and bedding in their indoor kennel, and were exercised for a minimum of one hour each day on a lead walk/partially off-lead walk and/or in an enclosed outdoor exercise area. In accordance with the dogs' usual feeding routines in the home environment, the majority of dogs (82.8%) were fed twice daily in kennels; between 0800h and 1000h, following collection of urine and saliva samples (see section 2.4.1.1), and between 1630h and 1830h. The remaining dogs were fed once per day, between 1630h and 1830h.

2.3.2 Home environment

198 Owners were asked to keep the routine as normal as possible on the day that home
199 measurements were taken. Dogs had access to the room/s and/or outdoor areas that they
200 typically had access to on non-measurement days. In the home environment, data collection
201 began between the hours of 0600 and 0930; at the time when dogs typically awoke and
202 passed their first urine of the day.
203
204 *2.4 Data Collection*
205 Home measurements were taken a minimum of 7 full days (*mean* = 12.89; SD = 2.33) either
206 before the dog entered the boarding kennel establishment or after the dog returned home from
207 the establishment. This timing was considered sufficient to avoid potential changes in the
208 owners' normal routine, behaviour and/or mood (related to their time away from home)
209 having an effect on the dogs' physiology and behaviour when measurements were taken in
210 the dogs' home first [5], and for the dog to readapt to the home environment when
211 measurements were taken in the kennel first. Kennel measurements were taken on the first (*n*
212 = 25), second (*n* = 3) or third (*n* = 1) day after admission to the establishment. The number of
213 days dogs spent in boarding kennels ranged from 1 to 21 (median = 1 day).
214
215 *2.4.1 Measurements*
216 The same physiological, physical and behavioural measurements were recorded for each dog
217 in both environments in the order that they are described below.
218
219 *2.4.1.1 Physiological measurements*
220 *2.4.1.1.1 Urine collection and analysis*
221 Dogs were walked outdoors on-lead and a mid-stream sample of naturally voided urine was
222 collected in a disposable aluminium foil tray. Dogs were then returned to their kennel/home.

Urine was transferred to a disposable plastic beaker (Fisher Scientific U.K. Ltd.) and urine pH was recorded using a pH-ORP Test Kit. Sixty per cent of the total volume of urine collected for each dog in each environment (up to 50ml) was equally divided between six 2ml or 5ml Nunc Cryo Tubes (Fisher Scientific U.K. Ltd.) using a 5ml syringe (BD Plastipak). The remaining 40% of total urine collected was stabilised with 1M hydrochloric acid (HCl) within 40 min of sample collection. 1M HCl was added to urine using a 0.5 µL - 10µL variable volume Fisherbrand pipettor (Fisher Scientific U.K. Ltd.), set to 10 µL, until urine pH reached between 2.0 and 4.0. The total volume of 1M HCl used was recorded as volume per ml of urine. Stabilised urine was then divided equally between an additional four Cryo Tubes. All samples were stored on ice for a maximum of 2.5hrs before being transferred to a -80°C freezer. The samples were stored at -80°C for a maximum of three months and then packed in dry-ice and sent to the University of Lincoln, U.K. for analysis.

Non-acidified urine samples were analysed for: urinary free cortisol, creatinine, 8-iso-Prostaglandin $F_{2\alpha}$ ("ISOP"), malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS). Urine acidified to pH 2-4 was analysed for vanillylmandelic acid (VMA), 5-hydroxyindole-3-acetic acid (5-HIAA) and homovanillic acid (HVA).

Urinary free cortisol was measured using an Assay Designs Correlate-EIA Cortisol Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI). Creatinine content was determined by UV-Spectrophotometer, following the Jaffe reaction method. ISOP was analysed using an Assay Designs 8-iso-Prostaglandin $F_{2\alpha}$ Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI). MDA was determined using the HPLC-Fluorescence method of Agarwal and Chase [60] using MDA-TBA_2 chromagen peak height for calibration, and an aliquot of the same butan-1-ol extract used for MDA was analysed simultaneously for TBARS by

Spectrofluorophotometer (Shimadzu RF-1501 Spectrofluorophotometer, Shimadzu U.K. Ltd.) using fluorescence intensity at the same excitation (515nm) and emission (553) wavelengths.

VMA, 5-HIAA and HVA were determined using liquid-liquid extraction and gradient elution HPLC with fluorescence detection. The method for canine urine was based on the method for human urine [61] with four modifications: (1) The gradient elution was modified so that VMA could be separated from interference peaks. (2) The modification to the gradient elution made the usual internal standard, iso-VMA, difficult to quantify accurately. Therefore the internal standard was replaced by 5-HICA (5-hydroxyindole-2-carboxylic acid). (3) The efficiency of the extraction was improved by adding ammonium sulphate to the urine samples during preparation and extracting twice with diethyl ether, as suggested by Manickum [62]. (4) The extraction procedure was scaled down to handle 100µL urine sample volumes.

All urinary measurements were standardised for variations in urine concentration, body weight and dilution by calculating (measurement):creatinine ratios [5].

2.4.1.1.2 Saliva collection and analysis

Saliva samples were collected by placing one large veterinary cotton bud (Millpledge Veterinary) in the cheek of the dog for 1-2 min [63]. Salivation was encouraged by holding a piece of cheddar cheese in front of the dog's nose. The cotton buds were then compressed in a 5ml syringe to release the saliva. The volume of saliva (up to 3ml) was divided equally between two 1.5ml Eppendorf snap-cap microcentrifuge tubes (Fisher Scientific U.K. Ltd.). Samples were stored on ice for a maximum of 2hrs before being centrifuged and transferred

272 to a -80°C freezer. The samples were stored at -80°C for a maximum of three months until
273 packed in dry-ice and sent to the University of Lincoln for analysis.
274
275 Saliva samples were tested for antioxidant capacity using (i) the Ferric Reducing Antioxidant
276 Power (FRAP) assay method of Benzie and Strain [64], as modified by Hayes et al. [65], and
277 (ii) by 2,2-diphenyl-1-picrahydrazyl (DPPH) assay. In the former, the antioxidant capacity of
278 saliva was determined at 4 min and 45 min of reaction time, and values were expressed as
279 equivalent concentrations of ferrous ion (µmol/L). The DPPH assay was based on the
280 decolourisation of a stable free radical (DPPH) in a buffered ethanolic/aqueous solution by
281 antioxidants present in the saliva. The reaction with saliva was measured after 60 minutes and
282 compared with a standard antioxidant (uric acid). The antioxidant capacity of the saliva was
283 expressed as the equivalent concentration of uric acid (nmol/mL) that would give the same
284 decolourisation.
285
286 *2.4.1.2 Physical measurements*
287 (i) Whole body condition was scored using the Purina "Understanding your Dog's Body
288 Condition"[1] standard 9-point scale, by sight and running hands over the dog's body. The first
289 18 dogs were independently scored by two researchers, with an inter-rater reliability of 1.00
290 (95% CI = 1.00-1.00) assessed using the intraclass correlation coefficient. The last 11 dogs
291 were scored by one of these researchers. To reduce the number of groups for between-
292 subjects comparisons, whole body condition was categorised as 'ideal' (scores of 4 and 5) or
293 'not ideal' (scores of 1-3 and 6-9).

[1] http://www.purina.com/dog/weight-and-exercise/bodycondition.aspx Last accessed on 21st June 2012.

(ii) The sclera of the right eye was scored for the presence of redness (a visible meshwork of blood vessels) as 'white' or 'red'. There were no cases where the sclera of dogs' right and left eyes differed in colour.

(iii) Skin dryness was measured by the presence or absence of scurf in the coat and scored as 'absent' (less than 10 flakes of scurf in the coat) or 'present' (10 or more flakes of scurf in the coat).

(iv) Surface temperature (°C) was measured from the nose using a Standard ST-8861 non-contact dual laser InfraRed Thermometer (Intech Calibration Ltd.). The mean of three consecutive measurements was recorded. Test-retest reliability was very good (0.92 – 0.96) as assessed in kennel conditions using Pearson's product moment correlation. Ambient temperature (°C) was also recorded to account for variations in surface temperature using a plastic wall thermometer (Faithfull).

(v) Core body temperature (°C) was measured from the inner ear canal using the Vet-Temp Instant Ear Thermometer, VT-150 (Advanced Monitors Corporation).

(vi) Amount of food eaten. Normal breakfast was given to those dogs that typically ate breakfast (82.8% of total sample) and the amount of food eaten was recorded as 'less than half' or 'more than half'.

2.4.1.3 Behavioural measurements

2.4.1.3.1 Ease of measurement: The researcher's success in taking physical measurements from each dog within each environment was recorded as 'successful data collection' or 'difficult to handle'

2.4.1.3.2 Behavioural recording

The dogs' behaviour was recorded using one or more of the following video cameras: Sony Handycam DCR-SX33E digital video camera recorder; JVC Everio G-Series GZ-MG365 hard disk camcorder; Panasonic SDR-H40 SD/HDD Video Camera. In the kennel environment, cameras were positioned to record the dogs' behaviour in the indoor area. In the home environment, video cameras were positioned in the room or rooms that the owners believed the dogs spent the majority of time. For those dogs kept outdoors, video cameras were positioned indoors to record as much of the outdoor area as possible. Cameras were left unattended during the recording period to minimise disruption to the dog's activities.

Recording started immediately after the physical measurements were taken, usually between the hours of 0800 and 1030, and typically ended between 1600 and 1800. A 30 min section of video footage of each dog under each condition was analysed. In each case, the first 30 min and last 10 min of video footage were discarded before random selection of a 30 min section (start time determined using 'true random number generator' - www.random.org) to allow the dogs time to settle after having the above measurements taken and to ensure behaviour was not affected by the return of the researcher, respectively.

2.4.1.3.3 Behavioural analysis: Activity budgets

JWatcher version 1.0 was used to record the frequency or duration of 38 behaviours using continuous sampling. Behaviours that were displayed by 10% or less of dogs in both environments were excluded from analysis, as suggested by Hiby et al. [14] (i.e. stretch; investigate object; startle; roll; urinate; defecate; crouch; lean) as well as those behaviours that could not be meaningfully compared between- or within-subjects (i.e. initiate human contact; ignore human; jump; groom conspecific; look out [of kennel]). Thus, 25 behaviours were analysed (see Table 2). Dogs were not observable from the video footage at all times.

Therefore, to ensure meaningful comparisons were made both within- and between-subjects, duration of behaviours was recorded as proportion of time in-sight, and frequency of behaviours was analysed as frequency per minute in-sight.

2.4.1.3.4 Behavioural analysis: Behavioural diversity

The diversity of behaviours performed was calculated for each dog within each environment using the Shannon Diversity Index (*H*) [66, 67]:

$$H = -\sum (p_i * \ln p_i)$$

Where p_i is the proportion of time engaged in the *i*-th behaviour. The value of *H* increases with the number of behaviours performed and with equality of time spent engaged in each behaviour. Lower values represent less behavioural diversity [68].

The index requires that behaviours are mutually exclusive. However, recorded behaviours were often not mutually exclusive. Therefore, behavioural diversity was calculated for two categories of mutually exclusive behaviours:

(i) $H_{(\text{Posture/Locomotion})}$ - sit; stand; lie; travel; circling before lying down; and crouch.

(ii) $H_{(\text{Activity/Maintenance})}$ - scratch; object play; sniff object; autogroom; drink; feed; and investigate object.

Here, p_i represented duration of time engaged in *i*-th behaviour as a proportion of time engaged in all behaviours within that category, where total time spent on all behaviours within each category = 1.0.

Table 2. Behaviours recorded from video footage of dogs at home (30-minutes) and in kennels (30-minutes), measured as frequency per minute in-sight (F) or duration as a proportion of time in-sight (D).

Behavioural category	**Behavioural variable**	**Definition**	**Measurement**
Arousal	Alert	Eyes open and head and ears moving. Dog can be lying down, sitting, standing or moving.	D
	Sleep/rest	Lying motionless with eyes closed. Might occasionally open eyes to scan area or move ears.	D
Posture	Sit	Hindquarters in contact with the ground and front legs extended.	D
	Stand	Four feet in contact with the ground and legs fully, or almost fully, extended.	D
	Lie	Part of both the upper and lower body in contact with the ground.	D
Tail Position	High tail	Standing or moving with tail held higher than the plane of the back.	D
	Level tail	Standing or moving with tail on the same plane as the back, or sitting / lying with tail extended.	D
	Low tail	Standing or moving with tail held lower than the plane of the back, or sitting / lying with tail curled around body.	D
Maintenance	Drink	Laps water.	D
	Feed	Consumes food.	D
	Autogroom	Licks or chews own body.	D
Locomotion	Travel	Ambulates at any speed.	D
	Kennel rear	Stands up on hind legs with forelegs against front of kennel, or jumps up and down at front of kennel. Forepaws may scrabble on	D

		the vertical surface.	
	Circling before lying down	Walking in tight circles, with diameter of path approximating length of dog's body, before lying down.	D
Investigation	Sniff object	Orientates nose to within 5cm of an object, wall or ground and twitches nose.	D
Vocalisations	Bark	Short loud sound with mouth open. Slight movement of ears and shoulders with each bout of sound.	F
	Whine	Prolonged high-pitched sound. Mouth may be open or closed.	D
Activity	Panting	Breathes deeply and quickly with mouth open and tongue hanging out.	D
	Object play	Manipulates toy or other object with paws and/or mouth. Dog may pat at the object with paws, throw object into air, pounce on it, wrestle with it, chew it, or play bow to it.	D
	Scratch	**S**cratches body with hind leg.	D
	Yawn	Opens mouth wide and closes eyes without vocalising.	F
	Lick lips	Tongue protrudes and licks own lips or snout.	F
	Body shake	Shakes whole body, including head, rapidly from side-to-side.	F
	Paw lifting	Raises single forepaw while sitting or standing and holds it above the ground.	D
	Wag tail	Tail moves repetitively from side-to-side.	D

364

365 *2.5 Data analysis*

366 Data were analysed using IBM SPSS Statistics 19. Where parametric tests were used, all test
367 assumptions were met. Shapiro-Wilk tests were used to determine the normality of data, on
368 each level of the independent variables where appropriate, before conducting statistical

comparative/correlational tests. Non-parametric tests were used where data did not approximate a normal distribution.

2.5.1 Within-subjects comparisons between home and kennel environments

Within-subjects comparisons were made using paired *t*-tests or Wilcoxon Signed Rank tests. Dichotomous categorical measurements were compared using McNemar's Chi-squared tests. The association between surface and ambient temperatures in the home environment was analysed using Pearson's product-moment correlation coefficient (Pearson's r) and in the kennel environment using Spearman's rank correlation coefficient (Spearman's rho).

Before undertaking within-subject comparisons, we tested for an interaction between order and condition in the cross-over design. Here, a selection of measurements (3 of 9 physiological measurements, 2 of 6 physical measurements and 10 of 28 behavioural measures) were chosen at random (using the 'true random number generator' - www.random.org) to reduce the probability of Type I errors. 'Deviation from baseline' data were calculated by subtracting home values from kennel values for each measurement taken from each dog. These data were then used to compare dogs that were tested at home first (n = 15) with dogs that were tested in kennels first (n = 14) using independent *t*-tests and Mann-Whitney *U* tests.

2.5.2 Between-subjects comparisons

To test the robustness of measurements as indicators of kennelling-induced stress, those parameters that deviated significantly from baseline (home values) following kennelling were compared between-subjects. 'Deviation from baseline' data were used for all between-subject comparisons.

One-way ANOVA and Kruskal-Wallis tests were used to compare: (a) subjects housed at different boarding kennel establishments; (b) subjects with different levels of kennelling experience; and (c) subjects of different sex/neuter status. Where significance levels (<0.05) were reached for one-way ANOVA and Kruskal Wallis comparisons, Tukey *post-hoc* and Mann-Whitney *U* tests were conducted, respectively.

Independent *t*-tests and Mann-Whitney *U* tests were used to compare two independent groups: (c) males and females; and (d) rehomed dogs and dogs purchased as puppies. In order to test for associations between age and stress responses, correlational analyses (Pearson's r and Spearman's rho) were conducted between age and deviation from baseline values on each parameter that differed significantly within-subjects.

2.5.3 Relationships between parameters

2.5.3.1 Movement/exercise and physiological responses to kennelling

Using 'deviation from baseline' data, Pearson's r and Spearman's rho were used to test for relationships between each physiological measurement that differed significantly between environments and each behavioural indicator that reflected movement/exercise (i.e. travelling, object playing and diversity of posture/locomotion behaviours) to determine if changes in physiology were associated with changes in physical activity.

2.5.3.2 'Difficult to measure' and 'easy to measure' parameters

Spearman's rho was used to test for associations between the physiological measurements that differed within-subjects and behavioural and interval-scale physical variables. These relationships were examined in the home and kennel environments separately. Independent *t*-

tests and Mann-Whitney *U* tests were used to compare physiological measurements between groups that differed in their categorical physical measurements.

2.5.4 Note on multiple testing

Multiple testing was necessary to assess the validity and robustness of a wide range of behavioural, physiological and physical parameters as indicators of acute stress. No correction was made for this. Within-subject comparisons (section 2.5.1) were hypothesis-driven, and all other statistical analyses were used to either test the robustness and generality of stress parameters that were identified through within-subject comparisons (sections 2.5.2 and 2.5.3.1) or identify practical measures of acute stress (section 2.5.3.2). Rather than reducing the number of tests performed or increasing the likelihood of a Type II error though correction for multiple testing, all statistical output was interpreted with caution – like previous research in this field [5] – bearing in mind the possibility of significant findings having resulted from Type I errors.

2.6 Ethical note

Before commencing, this study was approved by the Research Ethics Committee at Queen's University Belfast. Data collection was designed to be minimally invasive. Kennelling is a normally occurring stressor for dogs and, where possible, kennel measurements were taken during a previously organised stay at the boarding kennel establishment. Where this was not possible, dogs stayed in kennels for the minimum time required to collect meaningful data (typically 24-30 hours).

3. Results

3.1 Population statistics

444 The majority of dogs (72.4%) had a history of kennelling: 34.5% of dogs stayed in boarding
445 kennels a maximum of once or twice per year (Group1/2); 37.9% boarded at least three times
446 per year (Group3); and 27.6% had no known history of kennelling (Group0). Thirty-one per
447 cent of dogs had been rehomed a minimum of 12 months before the study began, and 69% of
448 dogs had been purchased as puppies. Forty-one per cent of dogs shared their home with at
449 least one dog (median = 1 dog, range = 1 - 10). In the home environment, the majority of
450 dogs (n = 23) lived indoors and the others (n = 6) lived outdoors with continuous access to
451 shelter (wooden kennel: n = 4; garage: n = 2).
452
453 *3.2 Within-subjects comparisons between home and kennel environments*
454 There was no evidence of an interaction between condition and order of condition in the
455 cross-over design: Deviation from baseline values did not differ significantly between dogs
456 tested in their own home first and dogs tested in kennels first (independent t –tests and Mann
457 Whitney U tests, $P > 0.05$).
458
459 *3.2.1 Physiological indicators*
460 Pairwise comparisons revealed that C/Cr (mmol/L:mmol/L x 10^6) was significantly higher in
461 the kennel compared to the home environment (Z = -2.984, n = 17, P = 0.003). VMA/Cr
462 (µmol/mmol) was also higher in kennels than at home ($t_{(18)}$ = 2.898, P = 0.01) (medians and
463 IQRs presented in table 3). No other physiological measurement differed significantly
464 between home and kennel environments ($P > 0.05$; see table 3).
465
466 *3.2.2 Physical indicators*
467 Dogs' surface temperature (^oC) was significantly lower in the kennel compared to the home
468 environment ($t_{(27)}$ = -3.950, P = 0.001). Surface temperature was not associated with ambient

Table 3. Mean ± standard deviation (S.D.) or median and interquartile range (IQR) of physiological and interval-scale physical parameters measured in dogs' normal home environment (baseline values) and in boarding kennels, with *P* values for within-subjects comparisons between environments.

Measurement	**Home environment**	**Kennel environment**	**Statistical test**	***P* value**
MDA/Cr (µmol/g)	6.71 (IQR 5.33 – 12.46)	5.900 (IQR 3.79 – 11.10)	*Z*	0.943
TBARS/Cr (µmol/mmol)	1.00 (IQR 0.76 – 1.72)	0.955 (IQR 0.643 - 1.468)	*Z*	0.906
ISOP/Cr (ng/mg)	5.30 (IQR 4.40 – 7.20)	6.10 (IQR 4.15 – 8.60)	*Z*	0.795
C/Cr (mmol/L:mmol/L x 10^6)	1.53 (IQR 1.23 – 2.42)	3.335 (IQR 2.55 – 4.515)	*Z*	0.003**
5-HIAA/Cr (µmol/mmol)	1.456 (IQR 1.123 - 1.882)	1.431 (IQR 1.136 – 1.786)	*Z*	0.872
HVA/Cr (µmol/mmol)	1.932 (IQR 1.477 – 2.546)	2.012 (IQR 1.615 – 2.673)	*Z*	0.277
VMA/Cr (µmol/mmol)	0.082 ± 0.024	0.104 ± 0.037	*T*	0.01**
DPPH (nmol/mL equivalents [as uric acid])	83.95 (IQR 41.70 –164.25)	66.00 (IQR 32.60 –106.73)	*Z*	0.983
FRAP 4min 45min (µmol/L)	271.50 (IQR 170.50–590.50) 517.50 (IQR 371.25 –965.75)	295.00 (IQR 160.00–518.50) 532.00 (IQR 337.50 –790.50)	*Z* *Z*	0.476 0.903
Surface Temp. (^{o}C)	25.233 ± 4.275	22.105 ± 3.306	*T*	0.001***
Core Temp. (^{o}C)	36.739 ± 0.976	36.631 ± 0.752	*T*	0.748

*Mean ± S.D. are presented where data approximated normal distribution as determined by Shapiro-Wilk tests. Median and IQR are presented where data were not normally distributed in home and kennel environments. Z = Wilcoxon Signed Rank test; t = Paired t-test. **significant at the 0.01 level; ***significant at the 0.001 level.*

469 temperature in the home ($r = 0.226$, $n = 29$, $P > 0.05$) or kennel environment ($r_s = 0.243$, $n =$
470 28, $P > 0.05$). No other physical measurement differed significantly within-subjects (all $P >$
471 0.05). (Data for interval scale measurements summarised in Table 3; data for ordinal scale
472 and categorical measurements not shown).
473
474 *3.2.3 Behavioural indicators*
475 Dogs spent significantly less time (milliseconds as proportion of time in-sight) lying down (Z
476 = -2.920, $n = 27$, $P = 0.004$) and sleeping/resting ($Z = -2.349$, $n = 27$, $P = 0.019$) and a greater
477 proportion of time alert ($Z = -2.337$, $n = 27$, $P = 0.019$), sitting ($Z = -2.172$, $n = 27$, $P = 0.03$),
478 standing ($Z = -2.372$, $n = 27$, $P = 0.018$), travelling ($Z = -1.971$, $n = 27$, $P = 0.049$) and
479 panting ($Z = -2.023$, $n = 27$, $P = 0.043$) when kennelled compared to when at home. Dogs
480 also showed a significantly greater diversity of posture/locomotion behaviours (H) in kennels
481 than at home ($Z = -2.057$, $n = 27$, $P = 0.04$) (medians and IQRs presented in Table 4).

Table 4. Median and interquartile range (IQR) of behaviours measured in dogs' normal home environment (baseline values) and in boarding kennels, with *P* values from Wilcoxon Signed Rank tests for within-subjects differences between environments.

Behavioural measurement		**Home environment**	**Kennel environment**	***P* value**
Alert	D	0.273 (IQR 0.085 – 0.619)	0.690 (IQR 0.261 – 0.994)	0.019*
Sleep/rest	D	0.718 (IQR 0.381 – 0.915)	0.310 (IQR 0.000 – 0.739)	0.019*
Sit	D	0.000 (IQR 0.000 – 0.004)	0.008 (IQR 0.000 – 0.105)	0.030*
Stand	D	0.009 (IQR 0.000 – 0.089)	0.057 (IQR 0.028 – 0.558)	0.018*
Lie	D	0.964 (IQR 0.725 – 1.000)	0.513 (IQR 0.062 – 0.894)	0.004**
High tail	D	0.000 (IQR 0.000 – 0.069)	0.002 (IQR 0.000 – 0.041)	0.583
Level tail	D	0.011 (IQR 0.000 – 0.184)	0.012 (IQR 0.000 – 0.097)	0.309

Low tail	D	0.956 (IQR 0.670 – 1.000)	0.975 (IQR 0.772 – 1.000)	0.647
Drink	D	0.000 (IQR 0.000 – 0.000)	0.000 (IQR 0.000 – 0.001)	0.441
Feed	D	0.000 (IQR 0.000 – 0.000)	0.000 (IQR 0.000 – 0.000)	0.273
Autogroom.	D	0.000 (IQR 0.000 – 0.005)	0.000 (IQR 0.000 – 0.000)	0.470
Travel	D	0.010 (IQR 0.000 – 0.044)	0.076 (IQR 0.012 – 0.136)	0.049*
Circling before lying down	D	0.000 (IQR 0.000 – 0.000)	0.000 (IQR 0.000 – 0.000)	0.263
Sniff object	D	0.000 (IQR 0.000 – 0.007)	0.006 (IQR 0.000 – 0.016)	0.194
Bark	F	0.000 (IQR 0.000 – 0.000)	0.000 (IQR 0.000 – 0.804)	0.388
Whine	D	0.000 (IQR 0.000 – 0.000)	0.000 (IQR 0.000 – 0.000)	0.128
Panting	D	0.000 (IQR 0.000 – 0.000)	0.000 (IQR 0.000 – 0.000)	0.043*
Object play	D	0.000 (IQR 0.000 – 0.000)	0.000 (IQR 0.000 – 0.000)	0.686
Scratch	D	0.000 (IQR 0.000 – 0.000)	0.000 (IQR 0.000 – 0.000)	0.465
Yawn	F	0.000 (IQR 0.000 – 0.034)	0.000 (IQR 0.000 – 0.000)	0.442
Lick lips	F	0.097 (IQR 0.000 – 0.358)	0.017 (IQR 0.000 – 0.204)	0.601
Body shake	F	0.000 (IQR 0.000 – 0.000)	0.000 (IQR 0.000 – 0.038)	0.374
Paw lifting	D	0.000 (IQR 0.000 – 0.000)	0.000 (IQR 0.000 – 0.000)	0.161
Wag tail	D	0.000 (IQR 0.000 – 0.016)	0.000 (IQR 0.000 – 0.014)	0.875
Diversity – posture	*H*	0.153 (IQR 0.000 – 0.610)	0.584 (IQR 0.273 – 0.928)	0.040*
Diversity – activity	*H*	0.000 (IQR 0.000 – 0.430)	0.248 (IQR 0.000 – 0.665)	0.594

Behaviours measured as: D = duration (milliseconds) as a proportion of time in sight.
F = frequency per minute in sight. H = Shannon diversity index.
**significant at the 0.05 level; **significant at the 0.01 level.*

482

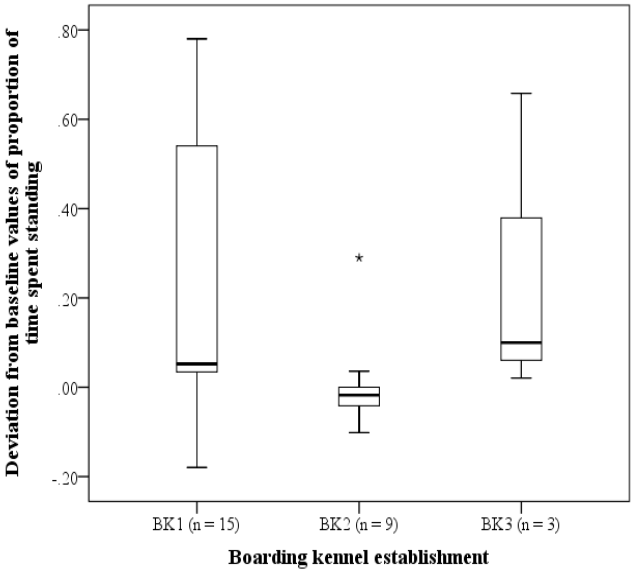
483 As can be seen from the IQRs in Table 4, considerable individual variability was observed,

484 particularly in proportion of time spent alert and sleeping/resting both at home and in

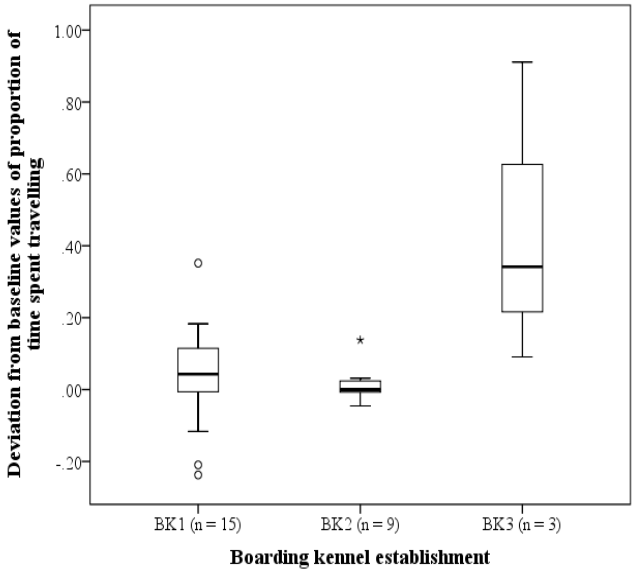
485 kennels. Time spent standing and lying down when kennelled also varied substantially
486 between subjects, as did the diversity of posture/locomotion behaviours observed both in the
487 home and in kennel environments. It should be noted that only 5 individuals were observed
488 panting during the study; therefore, the majority of subjects did not demonstrate this
489 behaviour in either environment. No other behaviours differed in frequency or duration
490 between environments ($P > 0.05$). Ease of measurement (EOM) also did not differ between
491 environments as determined by McNemar's test ($n = 28$, $P > 0.05$), which suggested that
492 dogs were not more averse to handling in the kennels than at home.
493
494 *3.3 Between-subjects comparisons*
495 All results presented in section 3.3 are based on 'deviation from baseline' data (within-
496 subjects, 'kennel minus home' values per measurement, per dog).
497
498 *3.3.1 Boarding kennel establishment*
499 The rise in dogs' C/Cr and VMA/Cr, and decline in surface temperature, following
500 kennelling did not differ significantly between groups of dogs kennelled at different
501 establishments (denoted as BK1, BK2 and BK3) (one-way ANOVA, $P > 0.05$). Of those
502 behavioural variables that differed significantly between environments (see table 4), within-
503 subjects differences in 'time spent standing' ($H_{(2)} = 7.064$, $n = 27$, $P = 0.029$), 'time spent
504 travelling' ($H_{(2)} = 6.156$, $n = 27$, $P = 0.046$) and 'time spent lying down' ($F_{(2, 24)} = 3.829$, $P =$
505 0.036) were significantly different between the three groups kennelled at different
506 establishments (see Figure 1).
507

Figure 1. Boxplots illustrating deviation from baseline comparisons between groups of dogs kennelled at different boarding kennel establishments.

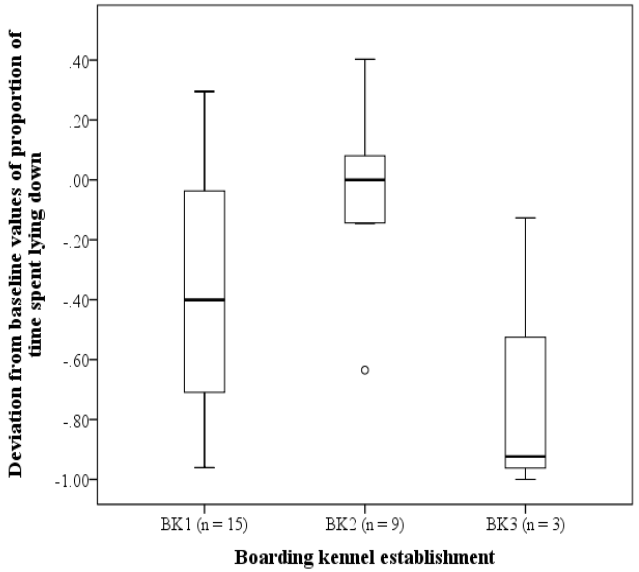
a) Deviation from baseline values of time spent standing when kennelled



b) Deviation from baseline values of time spent travelling when kennelled



c) Deviation from baseline values of time spent lying down when kennelled



No within-subjects change in measurement between environments (i.e. no deviation from baseline when kennelled) is represented by 0.00 on the y-axes. Positive values (above 0.00) indicate that values measured in the kennel were higher than within-subjects values measured at home. Negative values (below 0.00) indicate that values measured in the kennel were lower than within-subjects values measured at home.

BK1 dogs generally showed a greater increase (U = 26.50, n = 24, P = 0.014) in time spent standing following kennelling (median = 0.052, IQR 0.033 – 0.554, n = 15) than BK2 dogs (median = -0.018, IQR -0.058 – 0.018, n = 9), while BK1 and BK3 dogs (n = 3), and BK2 and BK3 dogs, did not differ (Mann-Whitney *U* tests: $P > 0.05$). When kennelled, dogs housed at BK3 showed a greater decrease (Tukey *post-hoc* test: $P = 0.048$) in time spent lying down (-0.684 ± 0.484, $n = 3$), and a greater increase ($U = 1.00$, $n = 12$, $P = 0.021$) in time spent travelling (median = 0.342, $n = 3$), than dogs housed at BK2 (-0.042 ± 0.282; median = 0.00, $n = 9$; respectively). There were no significant differences between BK1 and BK3 dogs, or between BK1 and BK2 dogs, in deviation from baseline lying or travelling behaviour ($P > 0.05$). Furthermore, dogs kennelled at BK2 showed less individual variation than dogs kennelled at BK1 and BK3 in the amount that they deviated from baseline values of time spent standing (figure 1a), travelling (see figure 1b) and lying down (figure 1c).

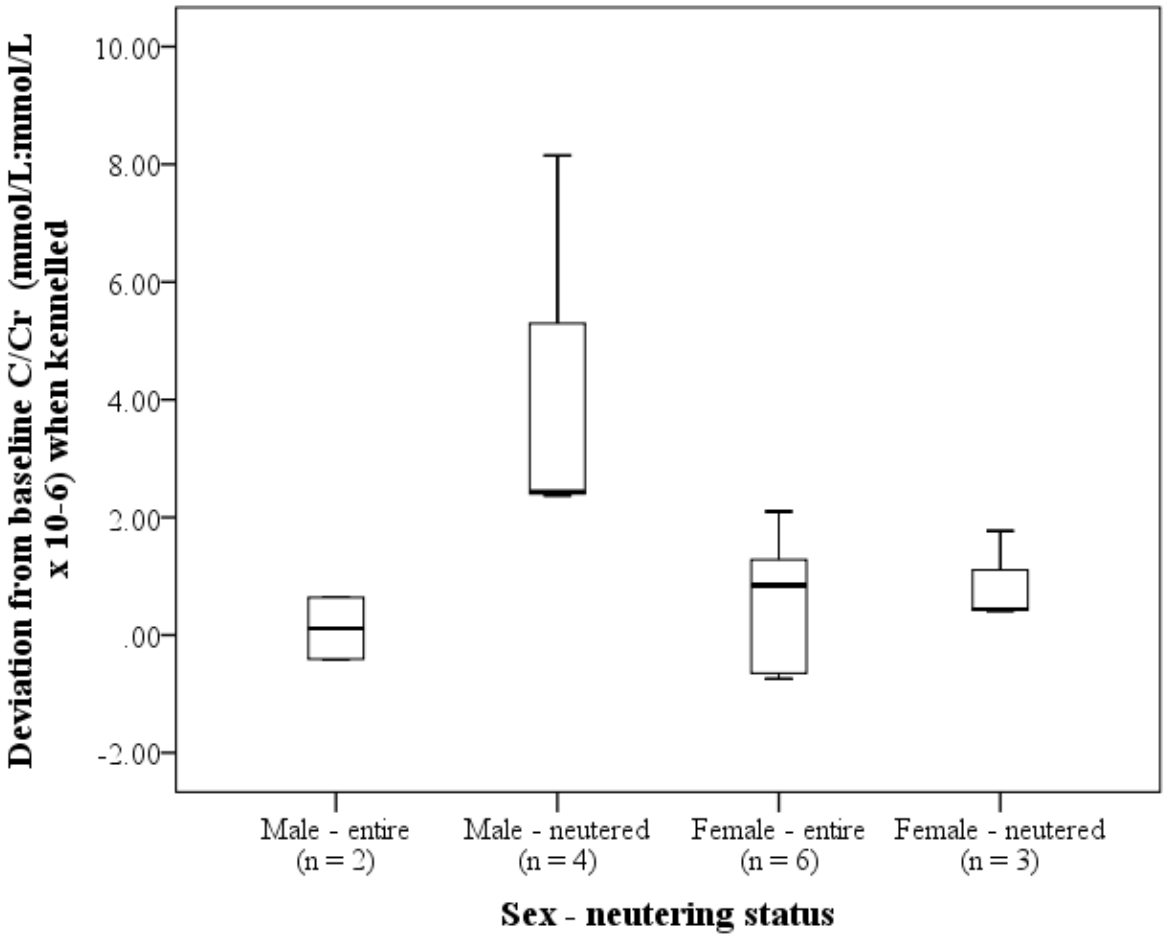
3.3.2 Kennelling experience

No significant differences ($P > 0.05$) were found between the 3 groups of dogs distinguished by their previous kennelling experience (i.e. Group0; Group1/2; Group3) on any parameter that differed significantly within-subjects (see section 3.2).

3.3.3 'Demographic' attributes

No sex or source (rehomed/purchased as puppy) differences were found on any variable that differed within-subjects ($P > 0.05$). However, the increase in C/Cr in males (2.639 ± 2.704, $n = 7$) compared to females (0.704 ± 0.918, $n = 10$) following kennelling almost reached significance ($t_{(15)} = 2.120$, $P = 0.051$).

Figure 2. Boxplot illustrating comparisons between male and female, entire and neutered dogs in C/Cr response to kennelling



No within-subjects change between environments (i.e. no deviation from baseline when kennelled) is represented by 0.00 on the y-axes. Positive values (above 0.00) indicate that values measured in the kennel were higher than within-subjects values measured at home. Negative values (below 0.00) indicate that values measured in the kennel were lower than within-subjects values measured at home.

When neutering status was incorporated into male/female comparisons, only C/Cr response to kennelling differed significantly between groups ($H_{(3)} = 8.525$, $n = 15$, $P = 0.036$). As shown in Figure 2, neutered males showed a greater cortisol response to kennelling (median = 2.435, IQR 2.40 – 5.295, $n = 4$) than neutered (median = 0.44, IQR 0.425 – 1.105, $n = 3$) and entire females (median = 0.845, IQR -0.65 – 1.28, $n = 6$) ($U = 0.00$, $n = 7$, $P = 0.034$; $U = 0.00$, $n = 10$, $P = 0.011$, respectively). Neutered males also appeared to show a greater C/Cr response

than entire males (see Figure 2), although there was not a sufficient number of entire males (n = 2) to determine significance between these groups. The small number of subjects in other groups must also be noted.

Age showed no significant relationship with surface temperature, C/Cr or VMA/Cr response to kennelling (Pearson's r [surface temp. and VMA/Cr] and Spearman's rho [C/Cr]: P > 0.05). Of those behavioural variables that differed within-subjects (section 3.2.3), only deviation from baseline 'time spent travelling' was associated with age (r_s = 0.443, n = 26, P = 0.024), with older dogs showing a greater increase in time spent travelling when kennelled. However, this relationship was fairly weak.

3.4 Relationships between parameters

3.4.1 Movement/exercise and physiological responses to kennelling

Deviation from baseline C/Cr and VMA/Cr were not significantly related to deviation from baseline values of travelling, object playing or diversity of posture/locomotion, as determined by Spearman's rho (P > 0.05) and Pearson's r (VMA/Cr and diversity of posture/locomotion behaviours only: P > 0.05).

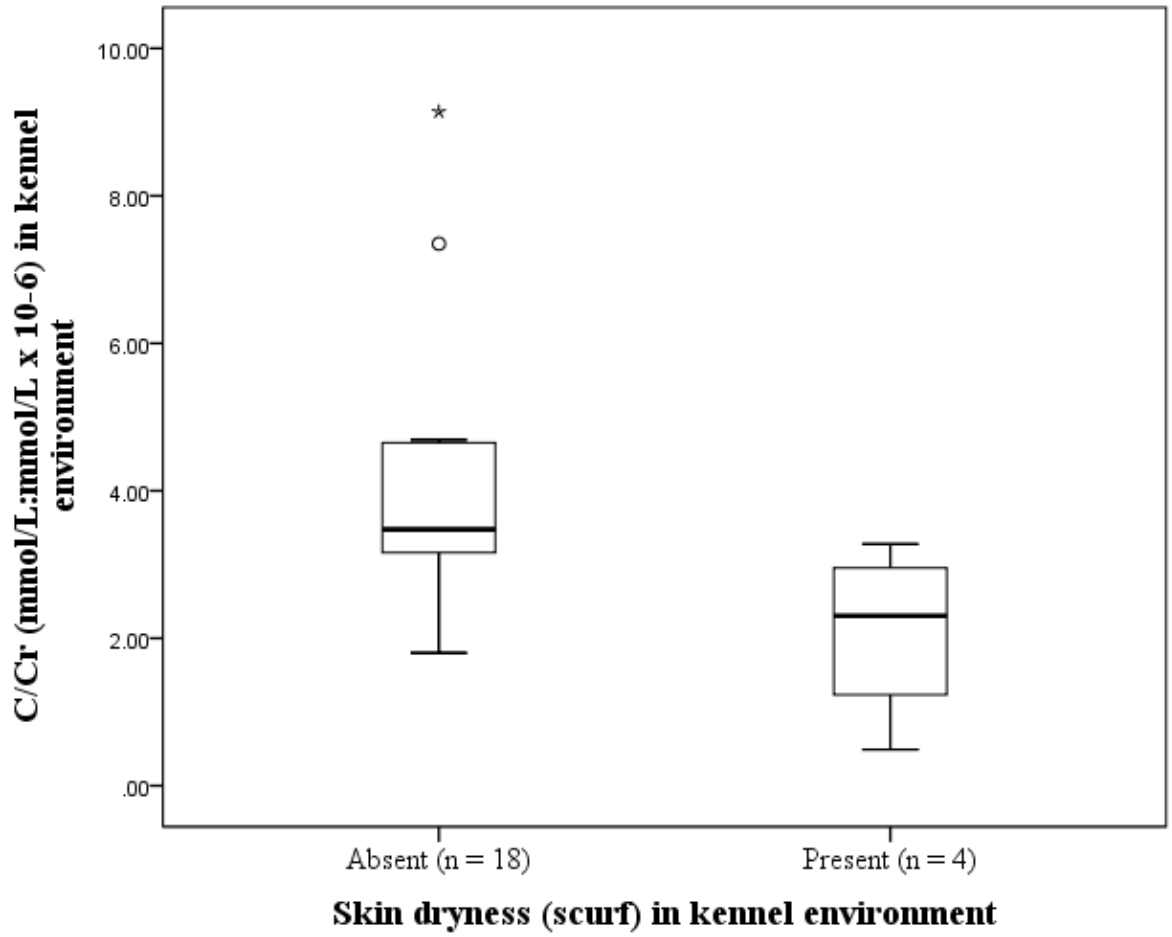
3.4.2 'Difficult to measure' and 'easy to measure' parameters

C/Cr did not correlate with any behavioural or interval scale physical indicator in either the home or kennel environment. However, higher VMA/Cr was associated with less lip licking in the kennel environment (r_s = -0.601, n = 20, P = 0.005). As this was the only significant relationship found, correlational analyses were conducted between lip licking and all other physiological parameters measured in the kennel environment to further explore the potential relationship between lip licking and physiological stress. These analyses revealed that higher

5-HIAA/Cr (µmol/mmol) was also associated with less lip licking (r_s = -0.502, n = 20, P = 0.024) in the kennel environment.

Dogs with no skin dryness (scurf) had higher C/Cr (median = 3.475, IQR = 3.015 – 4.660, n = 18) than dogs with scurf (median = 2.305, IQR = 0.863 – 3.118, n = 4) (U = 12.00, n = 22, P = 0.041) in kennels, as shown in Figure 3. However, this difference was not observed in the home environment (Mann-Whitney U test: $P > 0.05$). No other differences in C/Cr or VMA/Cr were found between groups that differed in categorical measurements ($P > 0.05$).

Figure 3. Boxplot illustrating comparison of C/Cr between dogs with no scurf and dogs with scurf as measured in the kennel environment.



4. Discussion

This study set out to test the potential value and validity of a range of physiological, physical and behavioural parameters as indicators of kennelling-induced stress in dogs, to establish baseline values for each indicator as measured in dogs' normal home environments, and to test for relationships between 'difficult to measure' physiological parameters and 'easy to measure' behavioural and physical parameters.

4.1 Validity of indicators

As predicted, both cortisol:creatinine ratio (C/Cr) and vanillylmandelic acid:creatinine ratio (VMA/Cr) were elevated above baseline levels when dogs were kennelled. This indicated that both major stress-response systems – the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) system [8] – were activated in response to kennelling. The within-subjects rise in C/Cr and VMA/Cr was not associated with age or with differences in behavioural indicators of movement/exercise following kennelling and, on average, was observed in all dogs regardless of previous kennelling experience, sex or source and the boarding kennel establishment in which they were housed; although, sex/neuter status appeared to have some effect on dogs' C/Cr response to kennelling. Thus, assuming that kennelling was a stressful experience for the dogs, C/Cr and, particularly, VMA/Cr appear to provide robust physiological indicators of acute, kennelling-induced, stress.

However, in contrast to the predictions set out in Section 1, no other physiological measurement reliably deviated from baseline levels when dogs were kennelled, which could lead to one of two conclusions. Firstly, of those physiological indicators tested in this study, C/Cr and VMA/Cr may be the most sensitive and valid measures of acute distress in the domestic dog. In this context, the term distress is unqualified, and it may be that if the form

of stress could be further qualified, e.g. frustration versus anxiety, that other measures would show more specific relationships. However, this was outside the scope of this study. Secondly, dogs may not have perceived kennelling as a threat to their wellbeing, and the higher concentrations of urinary cortisol and VMA in kennels than in dogs' own homes may have reflected increased arousal of a positive nature induced by, for example, the potentially exciting new sounds and smells encountered in the unfamiliar kennel environment. It has long been recognised that urinary epinephrine levels (of which VMA is a metabolite) rise in response to emotional arousal of both positive and negative valence [69]. Similarly, increased cortisol levels indicate emotional arousal, but of non-specific valence [16, 20, 70].

Current findings did, however, concur with previous reports of higher C/Cr following one night in kennels than C/Cr measured in a home environment [5, 9], and contrast with recent research that found C/Cr to be less reliable than previously thought for kennelled dogs [13]. Nonetheless, individual variability in dogs' cortisol response to kennelling was evident in the current study, and was comparable to that found in dogs of various breeds, age, and sex following one night in a rehoming centre [9, 14]. Less between-subject variation was measured in dogs' VMA response to kennelling, which suggests that VMA/Cr may be a more reliable indicator of arousal than C/Cr.

Interestingly, a previous study suggested that urinary norepinephrine:creatinine (NE/Cr) and epinephrine:creatinine ratios (E/Cr) do not offer valid physiological measures of acute canine stress [11]. As a metabolite of epinephrine and norepinephrine, VMA is found in much higher levels in urine than the hormones themselves [71, 72] and, unlike urinary levels of epinephrine and norepinephrine [73], urinary VMA levels do not appear to be affected by exercise [74]. Thus, VMA/Cr may provide a more reliable indicator of acute psychological

622 arousal, and a more sensitive urinary measurement of SAM system response, in dogs than
623 epinephrine:creatinine (E/Cr) or norepinephrine:creatinine ratios (NE/Cr).
624
625 Consistent with current findings, previous research found no association between age and
626 cortisol response to kennelling [7, 9]. The tendency for males in our study to show a greater
627 cortisol response than females was not detected in earlier research [7, 9], which may be due
628 to our use of 'deviation from baseline' data rather than data collected only in kennels. Indeed,
629 when only using the data that we collected in kennels, sex differences in C/Cr did not come
630 close to reaching significance, indicating that both sexes have similar levels of urinary
631 cortisol when kennelled but that males tend to experience a greater rise in C/Cr than females
632 in order to reach that level. However, no sex difference was detected in baseline C/Cr, which
633 suggests the near-significant *p*-value occurred by chance. Moreover, Beerda et al. [12, 75]
634 found that females showed greater behavioural and HPA axis response to acute stressors (a
635 sound blast and corticotrophin-releasing hormone challenge). Although, the discrepancies
636 between current and Beerda et al.'s [12] findings may be explained by admission to boarding
637 kennels not representing a negative stressor for the dogs in this study. Unexpectedly, it was
638 the neutered males in our study that accounted for the near-significant sex difference in C/Cr
639 response to kennelling. There is no obvious explanation for this finding and, as sex, or
640 sex/neuter status, differences were not detected in any other parameter that reflected
641 increased arousal, we suggest that this was a Type I error, arising from a combination of the
642 small sample and multiple testing.
643
644 No differences in kennelling-induced cortisol response were found between dogs with, and
645 dogs without, previous experience of a kennel environment, which is in line with Hiby et al.'s
646 [14] findings after one night in a rehoming centre but contrasts with Rooney et al.'s [5]

findings after one night in a military training establishment. Individuality in early cortisol response may have masked the effects of past experience, as suggested by Hiby et al. [14]. However, the discrepancy in findings was more likely (or additionally) accounted for by the direct manipulation of kennelling experience in Rooney et al.'s [5] study, where kennel-experienced dogs were gradually habituated to a kennel environment using positive reinforcement before transfer to the training establishment.

Perhaps the most promising finding, in terms of identifying 'easy to measure' indicators of canine stress, was the drop in dogs' facial surface temperature that was observed following kennelling. Like C/Cr and VMA/Cr, no effects of kennel establishment, kennelling experience, sex, neuter status, source or age were found. Most surprisingly, surface temperature was not associated with ambient temperature in either the home or kennel environment. However, again, emotional valence cannot be determined as previous research in humans has found a decrease in facial skin temperature to be associated with both pleasant [e.g. 77, 78] and unpleasant emotions [e.g. 49]. Similarly, a drop in surface temperature has been shown to be associated with both positive and negative events in chickens [50, 51, 79, 80].

In contrast to our predictions, no other physical measurement differed between home and kennel environments. Although an increase in core body temperature appears to be a consistent response to unpleasant stimuli in all mammal species tested thus far [79], no significant rise in core body temperature was observed in dogs following kennelling, which suggested that the rise in C/Cr and VMA/Cr and drop in surface temperature following kennelling reflected increased arousal of a positive nature.

As predicted, within-subjects differences in behaviours revealed that dogs were generally more active in the boarding kennels than in their normal home environment, which supports Tuber et al.'s findings [81]. Nonetheless, increased activity levels might be considered to be a normal response to a relatively unfamiliar environment as opposed to indicating stress *per se*. Indeed, other behaviours that were predicted to increase in response to an acutely stressful situation (i.e. paw lifting, lip licking, yawning and bodyshaking) did not consistently differ in frequency or duration between home and kennel environments, further supporting the conclusion that admission to boarding kennels did not represent a stressful experience for the dogs in this study.

It has been suggested that behavioural indicators of welfare status may be difficult to establish in dogs due to years of selective breeding for specific behaviours, which has resulted in numerous breed types that exhibit distinct behavioural repertoires [10]. However, considerable variability in behavioural stress response has also been found in a sample of dogs of the same breed, age and sex and, thus, also appears to be influenced by individual experience [5]. As found in the current study, between-subject differences in spontaneous behaviours (i.e. time spent standing, travelling and lying down) may also be explainable by differences in kennel structure. With such between-subject variability and with observed behaviours often lacking specificity as a stress-response, spontaneous behaviour may be easily misinterpreted [6]. Therefore, it has been suggested that, in the absence of pronounced behavioural abnormalities, observations of spontaneous behaviour may be better used to facilitate interpretation of physiological data rather than as welfare indicators *per se* [6].

The final behavioural variable tested in this study was behavioural diversity, which has been found to increase following feeding enrichment in captive red foxes (*Vulpes vulpes*) [68] and

small cats (*Prionailurus viverrinus*, *Prionailurus bengalensis*) [67] and with environmental enrichment in fattening pigs [59]. However, unlike previous reports of greater behavioural diversity within more enriched environments [e.g. 59], dogs in our study showed greater diversity of posture and locomotion behaviours in kennels than at home. This conflict in findings is likely accounted for by the novelty of the kennel environment and familiarity with the home environment when measurements were taken. That is, the dogs had likely habituated to the stimuli within their home environment; whereas, the novel kennel environment provided greater stimulation in terms of new smells, sounds, etc. As the novelty of any environment will fade with time, comparisons of behavioural diversity observed within different environments might only offer an indication of the quality of those environments following equal exposure lengths.

4.2 Dogs at home: Baseline values

The average C/Cr of 1.53 x 10^{-6} (mmol/l:mmol/l) measured in dogs' home environment was somewhat lower than the mean ratios of 2.9 x 10^{-6} [82] and 4.8 x 10^{-6} [6] reported in previous studies. However, the difference between current and Van Vonderen et al.'s [82] findings could largely be accounted for by the different descriptive statistics used (median and mean, respectively) as, otherwise, the values were very similar. The higher C/Cr reported by Beerda et al. [6] may reflect differences in home environments between studies: In Beerda et al's [6] research, dogs were housed in outdoor kennels from 0800 to 1700h on working days; whereas the majority of dogs in the current study remained indoors when owners were not at home and, so, did not experience a regular change of housing conditions. Much larger differences were apparent between average baseline C/Cr reported here and those reported by Rooney et al. [5] of 14.25×10^{-6} (nmol/l:nmol/l) and Stephen and Ledger [9] of 17.8×10^{-6}. Reports of urinary C/Cr ratios in dogs vary between studies because the gold standard

gas chromatography-mass spectrometry (GC-MS) method with derivatisation for assays of urinary free cortisol is not used because it is too time-consuming. Instead, different assay kits (ELISA and radioimmunoassay), originally designed for human urine and which have not been properly validated against canine urine by GC-MS, are used for this task with variable cross-reactivities to other (mostly unknown) urinary steroids. These kits may be reliable for assessing changes within-subjects but the values should not be considered valid as absolute measures.

Average baseline levels of urinary 5-HIAA in males and females (30 and 22 µmol/L) were comparable to those previously reported in Labradors (12.5 and 24 µmol/L) and German Shepherd Dogs (17 and 31 µmol/L) [83]. However, in contrast to Venturi Rose et al. [83], we found slightly higher levels in females than males. Baseline HVA levels (5.3 mg/L) were, on average, lower than levels reported in a control group of Alaskan sled dogs (10.1 µg/mL) [84], which may be due to the extensive physical training and high fitness of the latter (working dog) group and the, non-working, pet role of the dogs in our study. Durocher et al. [84] did not detect VMA in urine samples taken from any dogs in their study. This is not surprising given the lower detection limit of 5µg/mL in Durocher et al.'s [84] assay method and the mean baseline concentration of 0.27mg/L VMA in undiluted urine found in our study.

Regarding dogs' behaviour, previous research has shown that dogs spend most of their time lying down resting when at home alone [e.g. 85, 86], which was consistent with current findings. Again, this is difficult to interpret from a welfare perspective as, while increased resting/sleeping might signify learned helplessness [87], or apathy, in dogs, it may also indicate relaxation [88]. Due to habituation, dogs may no longer find the home environment stimulating, in which case long durations of inactivity may reflect a welfare concern [86]. On

747 the other hand, the considerable time spent sleeping/resting that has been observed in
748 privately owned dogs may be a consequence of the greater activity, exercise and stimulation
749 that dogs experience when their owners are home. These vastly different potential
750 interpretations of sleeping/resting behaviour further highlight the difficulties in accurately
751 interpreting snap-shots of spontaneous behaviour alone.
752
753 *4.3 Relationships between 'difficult to measure' and 'easy to measure' indicators*
754 Ultimately, research into animal welfare indicators should aim to identify valid, reliable and
755 specific measures that are practical for use 'on the ground', and on a regular basis, by animal
756 caregivers. Here, two associations were found between more 'difficult to measure' (in the
757 sense of cost, procedure and equipment required) indicators that were identified as valid
758 measures of acute canine arousal and 'easy to measure' spontaneous behaviour and physical
759 indicators. Firstly, dogs with no skin dryness were found to have higher C/Cr in the kennels
760 than dogs with scurf. However, as cause-and-effect was not explicitly tested, other
761 differences between groups may explain this relationship. For example, the majority of dogs
762 with no skin dryness in the kennels typically lived indoors at home; whereas, 75% of dogs (3
763 out of 4 dogs) with scurf lived outside in the home environment with access to a wooden
764 kennel for shelter. Given that a dog's cortisol response to its current environment appears to
765 be influenced by its appraisal of previous housing conditions [12], the difference between
766 dogs with, and dogs without, scurf may have been accounted for by differences in dogs'
767 home environments, or dogs' appraisal thereof. Of course, this interpretation requires further
768 investigation before any conclusions can be drawn as the difference may have reflected a
769 Type I error due to the number of tests performed and/or the large difference in sample size
770 between groups.
771

The negative correlations between lip licking and urinary VMA (epinephrine and norepinephrine metabolite) and 5-HIAA (5-HT metabolite) levels were unexpected as increased frequency of lip licking has previously been associated with stress in dogs [57]. As positive correlations between plasma VMA levels and measures of psychological stress have been found in humans [37] and higher plasma levels of 5-HT and urinary 5-HIAA have previously been associated with anxiety [41] and nervous behaviour [83] in dogs, the negative correlations between lip licking and VMA and 5-HIAA found here appear to suggest that decreased frequency of lip licking is associated with increased stress, which seemingly contradicts previous research. However, urinary epinephrine levels also increase in response to pleasant emotional arousal [69] and increased 5-HIAA has been associated with relaxation [89], which further complicates interpretation. One possible explanation is that lip licking is associated with derousal (calming signal) and is shown in some stressful situations because the dog is trying not to increase arousal. Clearly, additional research is required before any valid conclusions can be drawn. Nonetheless, as lip licking was observed in almost 50% of dogs (14 out of 29 dogs) in the kennel environment, this behaviour, and its relationship with emotional arousal, does warrant further investigation.

In the current study, urinary physiological and behavioural measurements represented two different time points: Overnight physiology and next-day behaviour [14]. Therefore, future research that synchronises measurements more accurately might identify relationships between parameters that were not picked up here.

Although admission to boarding kennels did not appear to be the aversive stressor for dogs that was required to thoroughly test the validity of each stress parameter, this study did highlight the difficulties in interpreting physiological, physical and behavioural data and also

called into question the presumption that short-term kennelling represents a negative psychological stressor for dogs. Furthermore, this study emphasises how important it is to examine a range of welfare indicators, as opposed to drawing conclusions on dogs' emotional state and/or welfare status from C/Cr and spontaneous behavioural data alone.

4.4 Conclusions

In conclusion, our findings strongly suggested that C/Cr and, particularly, VMA/Cr and surface (nose) temperature provide robust measures of psychological arousal in dogs. Surface temperature may provide a practical alternative to physiological measures that can be used by kennel staff. Nonetheless, these measures can be easily misinterpreted and do not provide unequivocal indicators of psychological stress. Therefore, validated and direct measures of emotional valence must be used in conjunction with C/Cr, VMA/Cr and surface temperature to minimise misinterpretation of data and increase their usefulness as measures of canine arousal from a welfare perspective.

Spontaneous behaviours are also difficult to interpret accurately and show considerable between-subject variability and, so, may be better used to facilitate interpretation of physiological and/or physical data on an individual level, as opposed to providing measures of stress *per se* [6]. However, the inconclusive relationship between lip licking and emotional arousal merits further investigation. Overall, findings appear to suggest that the dogs in this study did not perceive admission to boarding kennels as an aversive stressor and perhaps, instead, perceived kennelling as an exciting change of scene, at least in the short-term. This was not expected and, thus, further studies are required to determine the validity of measurements tested herein as indicators of acute and chronic stress in domestic dogs. The baseline values presented in this paper should facilitate such research.

Conflict of interest

The authors declare that there were no conflicts of interest.

Role of the funding source

This research was funded by Dogs Trust. Dogs Trust had no involvement in the study design, in the collection, analysis or interpretation of data, in the writing of the report, or in the decision to publish this article.

Acknowledgements

The authors would like to thank Daniel Annett for his help in collecting the data, the three boarding kennel proprietors for permitting use of their establishments and for promoting the study through their contacts. We are also extremely grateful to Dogs Trust for funding this research and to all of the dog owners (and dogs) who participated.

References

[1] RSPCA. The welfare state: Five years measuring animal welfare in the UK 2005-2009. URL: http://content.www.rspca.org.uk/cmsprd/Satellite?blobcol=urldata&blobheader=application%2Fpdf&blobkey=id&blobnocache=false&blobtable=MungoBlobs&blobwhere=1232997399246&ssbinary=true Last accessed on 25 March 2013.

[2] The Humane Society of the United States. HSUS Pet Overpopulation Estimates. 2009. URL: http://www.humanesociety.org/issues/pet_overpopulation/facts/overpopulation_estimates.html Last accessed on 24 March 2013.

[3] Rooney N, Gaines S, Hiby E. A practitioner's guide to working dog welfare. J Vet Behav 2009;4:127-34.

847 [4] Tuber DS, Miller DD, Caris KA, Halter R, Linden F, Hennessy MB. Dogs in animal
848 shelters: problems, suggestions, and needed expertise. Psychol Sci 1999;10:379-86.
849 [5] Rooney NJ, Gaines SA, Bradshaw JWS. Behavioural and glucocorticoid responses of
850 dogs (*Canis familiaris*) to kennelling: Investigating mitigation of stress by prior habituation.
851 Physiol Behav 2007;92:847–54.
852 [6] Beerda B, Schilder MBH, van Hooff JARAM, de Vries HW, Mol JA. Behavioural and
853 hormonal indicators of enduring environmental stress in dogs. Anim Welf 2000;9:49-62.
854 [7] Hennessy MB, Davis HN, Williams MT, Mellott C, Douglas CW. Plasma cortisol levels
855 of dogs at a county animal shelter. Physiol Behav 1997;62:485–90.
856 [8] von Borell EH. The biology of stress and its application to livestock housing and
857 transportation assessment. J Anim Sci 2001;79:E260–7.
858 [9] Stephen JM, Ledger RA. A longitudinal evaluation of urinary cortisol in kennelled dogs,
859 *Canis familiaris*. Physiol Behav 2006;87:911-6.
860 [10] Hewson CJ, Hiby EF, Bradshaw JWS. Assessing quality of life in companion and
861 kennelled dogs: A critical review. Anim Welf 2007;16:89-95.
862 [11] Beerda B, Schilder MBH, Janssen NSCRM, Mol JA. The use of saliva cortisol, urinary
863 cortisol, and catecholamine measurements for a noninvasive assessment of stress responses in
864 dogs. Horm Behav 1996;30:272–9.
865 [12] Beerda B, Schilder MBH, van Hooff JARAM, de Vries HW, Mol JA. Chronic stress in
866 dogs subjected to social and spatial restriction. II. Hormonal and immunological responses.
867 Physiol Behav 1999;66:243–54.
868 [13] Kiddie J, Hayes W, Mills D, Morton D, Neville R, Pfeiffer DU, et al. Assessing quality
869 of life of dogs in rehoming centres I: Physiological parameters. In prep.
870 [14] Hiby EF, Rooney NJ, Bradshaw JWS. Behavioural and physiological responses of dogs
871 entering re-homing kennels. Physiol Behav 2006;89:385-91.

872 [15] Mormède P, Andanson S, Aupérin B, Beerda B, Guémené D, Malmkvist J, et al.
873 Exploration of the hypothalamic–pituitary–adrenal function as a tool to evaluate animal
874 welfare. Physiol Behav 2007;92:317–39.
875 [16] Mason G, Mendl M. Why is there no simple way of measuring animal welfare? Anim
876 Welf 1993;2:310–9.
877 [17] Angle CT, Wakshlag JJ, Gillette RL, Stokol T, Geske S, Adkins TO, et al. Hematologic,
878 serum biochemical, and cortisol changes associated with anticipation of exercise and short
879 duration high-intensity exercise in sled dogs. Vet Clin Pathol 2009;38:370-4.
880 [18] Colborn DR, Thompson Jr DL, Roth TL, Capehart JS, White KL. Responses of cortisol
881 and prolactin to sexual excitement and stress in stallions and geldings. J Anim Sci
882 1991;69:2556-62.
883 [19] Fox SM, Mellor DJ, Firth EC, Hodge H, Lawoko CRO. Changes in plasma cortisol
884 concentrations before, during and after analgesia, anaesthesia and anaesthesia plus
885 ovariohysterectomy in bitches. Res Vet Sci 1994;57:110-8.
886 [20] Boissy A, Manteuffel G, Jensen MB, Moe RO, Spruijt B, Keeling LJ, et al. Assessment
887 of positive emotions in animals to improve their welfare. Physiol Behav 2007;92:375–97.
888 [21] Burman O, McGowan R, Mendl M, Norling Y, Paul E, Rehn T, et al. Using judgement
889 bias to measure positive affective state in dogs. Appl Anim Behav Sci 2011;132:160–8.
890 [22] Dawkins MS. Behaviour as a tool in the assessment of animal welfare. Zool
891 2003;106:383-7.
892 [23] Atif F, Yousuf S, Agrawal SK. Restraint stress-induced oxidative damage and its
893 amelioration with selenium. Eur J Pharmacol 2008;600:59–63.
894 [24] Djuric Z, Bird CE, Furumoto-Dawson A, Rauscher GH, Ruffin IV MT, Stowe RP, et al.
895 Biomarkers of psychological stress in health disparities research. Open Biomark J 2008;1:7–
896 19.

897 [25] Lucca G, Comim CM, Valvassori SS, Réus GZ, Vuolo F, Petronilho F, et al. Effects of
898 chronic mild stress on the oxidative parameters in the rat brain. Neurochem Int 2009;54:358–
899 62.
900 [26] Wong YT, Gruber J, Jenner AM, Ng MP-E, Ruan R, Tay FEH. Elevation of oxidative-
901 damage biomarkers during aging in F2 hybrid mice: Protection by chronic oral intake of
902 resveratrol. Free Radic Biol Med 2009;46:799–809.
903 [27] Kowalczuk K, Stryjecka-Zimmer M. The influence of oxidative stress on the level of
904 malondialdehyde (MDA) in different areas of the rabbit brain. Ann Univ Mariae Curie
905 Sklodowska Med 2002;57:160-4.
906 [28] Almroth BC, Sturve J, Berglund A, Förlin L. Oxidative damage in eelpout (*Zoarces*
907 *viviparus*), measured as protein carbonyls and TBARS, as biomarkers. Aquat Toxicol
908 2005;73:171-180.
909 [29] Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as
910 toxic molecule and biological marker of oxidative stress. Nutr Metab Cardiovasc Dis
911 2005;15:316-28.
912 [30] Turk R, Juretić D, Gereš D, Svetina A, Turk N, Flegar-Meštrić Z. Influence of oxidative
913 stress and metabolic adaptation on PON1 activity and MDA level in transition dairy cows.
914 Anim Reprod Sci 2008;108:98–106.
915 [31] Morton DB. A hypothetical strategy for the objective evaluation of animal well-being
916 and quality of life using a dog model. Anim Welf 2007;16:75-81.
917 [32] Timmins RP, Cliff KD, Day CT, Hart BL, Hart LA, Hubrecht RC, et al. Enhancing
918 quality of life for dogs and cats in confined situations. Anim Welf 2007;16:83-7.
919 [33] Akpinar D, Yargiçoğlu P, Derin N, Alicigüzel Y, Ağar A. The effect of lipoic acid on
920 antioxidant status and lipid peroxidation in rats exposed to chronic restraint stress. Physiol
921 Res 2008;57:893-901.

922 [34] Chakraborti A, Gulati K, Ray A. Age related differences in stress-induced
923 neurobehavioral responses in rats: Modulation by antioxidants and nitrergic agents. Behav
924 Brain Res 2008;194:86–91.
925 [35] Madhyastha S, Chauhan R, Rao GM, Umesh H. Neuroprotective effects of Mucuna
926 pruriens against stress-induced oxidative damage. J Physiol Sci 2011;24:28-33.
927 [36] Nadeem A, Masood A, Masood N, Gilani RA, Shah ZA. Immobilization stress causes
928 extra-cellular oxidant— antioxidant imbalance in rats: Restoration by L-NAME and vitamin
929 E. Eur Neuropsychopharmacol 2006;16:260-7.
930 [37] Fukuda M, Hata A, Niwa S-I, Hiramatsu K-I, Honda H, Nakagome K, et al. Plasma
931 vanillylmandelic acid level as an index of psychological stress response in normal subjects.
932 Psychiatry Res 1996;63:7–16.
933 [38] Lefcourt AM, Elsasser TH. Adrenal responses of Angus x Hereford cattle to the stress of
934 weaning. J Anim Sci 1995;73:2669-76.
935 [39] Ahmad A, Rasheed N, Banu N, Palit G. Alterations in monoamine levels
936 and oxidative systems in frontal cortex, striatum, and hippocampus of the rat brain during
937 chronic unpredictable stress. Stress 2010;13:355-64.
938 [40] Yoshioka M, Matsumoto M, Togashi H, Saito H. Effect of conditioned fear stress on
939 dopamine release in the rat prefrontal cortex. Neurosci Lett 1996;209:201-3.
940 [41] Riva J, Bondiolotti G, Michelazzi M, Verga M, Carenzi C. Anxiety related behavioural
941 disorders and neurotransmitters in dogs. Appl Anim Behav Sci 2008;114:168–81.
942 [42] Summers CH, Winberg S. Interactions between the neural regulation of stress and
943 aggression. J Exp Biol 2006;209:4581–9.
944 [43] Winberg S, Nilsson GE. Roles of brain monoamine neurotransmitters in agonistic
945 behavior and stress reactions, with particular reference to fish. Comp Biochem Physiol C
946 Pharmacol Toxicol Endocrinol 1993;106:597–614.

947 [44] Yoshioka M, Matsumoto M, Togashi H, Saito H. Effects of conditioned fear stress on 5-
948 HT release in the rat prefrontal cortex. Pharmacol Biochem Behav 1995;51:515-9.
949 [45] Rich EL, Romero LM. Exposure to chronic stress downregulates corticosterone
950 responses to acute stressors. Am J Physiol Regul Integr Comp Physiol 2005;288:R1628–36.
951 [46] Haubenhofer D. Signs of physiological stress in dogs performing AAA/T work. In:
952 Helton, WS, editor. Canine Ergonomics: The Science of Working Dogs, Florida: CRC Press;
953 2009, p. 281-299.
954 [47] Ravichandran G, Bharadwaj VS, Kolhapure SA. Evaluation of the clinical efficacy and
955 safety of "Anti-Dandruff Shampoo" in the treatment of dandruff. Antiseptic 2004;201:5-8.
956 [48] Vyjayanthi G, Kulkarni C, Abraham A, Kolhapure SA. Evaluation of anti-dandruff
957 activity and safety of polyherbal hair oil: An open pilot clinical trial. Antiseptic 2004;101,
958 368-72.
959 [49] Mizukami K, Kobayashi N, Iwata H, Ishii T. Telethermography in infant's emotional
960 behavioral research. Lancet 1987;11:38–9.
961 [50] Cabanac M, Aizawa S. Fever and tachycardia in a bird (*Gallus domesticus*) after simple
962 handling. Physiol Behav 2000;69:541–5.
963 [51] Edgar JL, Lowe JC, Paul ES, Nicol CJ. Avian maternal response to chick distress. Proc
964 Biol Sci 2011;278:3129-34.
965 [52] Hennessy MB, Deak T, Schiml-Webb PA, Wilson SE, Greenlee TM, McCall E.
966 Responses of guinea pig pups during isolation in a novel environment may represent stress-
967 induced sickness behaviors. Physiol Behav 2004;81:5–13.

968 [53] Kuhnen G. The effect of cage size and enrichment on core temperature and febrile
969 response of the golden hamster. Lab Anim 1999;33:221-7.
970 [54] Parrott RF, Lloyd DM. Restraint, but not frustration, induces prostaglandin-mediated
971 hyperthermia in pigs. Physiol Behav 1995;57:1051–5.
972 [55] Hotta M, Shibasaki T, Arai K, Demura H. Corticotropin-releasing factor receptor type 1
973 mediates emotional stress-induced inhibition of food intake and behavioral changes in rats.
974 Brain Res 1999;823:221–5.
975 [56] Vallès A, Martí O, García A, Armario A. Single exposure to stressors causes long-
976 lasting, stress-dependent reduction of food intake in rats. Am J Physiol Regul Integr Comp
977 Physiol 2000;279:R1138–44.
978 [57] Beerda B, Schilder MBH, van Hooff JARAM, de Vries HW. Manifestations of chronic
979 and acute stress in dogs. Appl Anim Behav Sci 1997;52:307-19.
980 [58] Beerda B, Schilder MBH, van Hooff JARAM, de Vries HW, Mol JA. Behavioural,
981 saliva cortisol and heart rate responses to different types of stimuli in dogs. Appl Anim Behav
982 Sci 1998;58:365–81.
983 [59] Hirt H, Wechsler B. Behavioural diversity as a measure of welfare: a study in pigs. Appl
984 Anim Behav Sci 1994;40:82–3.
985 [60] Agarwal R, Chase SD. 2002. Rapid fluorimetric-liquid chromatographic determination
986 of malondialdehyde in biological samples. J Chromatogr B Analyt Technol Biomed Life Sci
987 2002;775:121-6.
988 [61] Gironi A, Seghieri G, Niccolai M, Mammini P. Simultaneous liquid-chromatographic
989 determination of urinary vanillylmandelic acid, homovanillic acid, and 5-hydroxyindoleacetic
990 acid. Clin Chem 1988;34:2504-6.

991 [62] Manickum T. Simultaneous analysis of neuroendocrine tumour markers by HPLC-
992 electrochemical detection. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877:4140-
993 6.
994 [63] Dreschel NA, Granger DA. Methods of collection for salivary cortisol measurement in
995 dogs. Horm Behav 2009;55:163–8.
996 [64] Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of
997 "Antioxidant Power": The FRAP assay. Anal Biochem 1996;239:70-86.
998 [65] Hayes WA, Mills DS, Neville RF, Kiddie J, Collins LM. Determination of the molar
999 extinction coefficient for the ferric reducing/antioxidant power assay. Anal Biochem
1000 2011;416:202-5.
1001 [66] Shannon CE, Weaver W. The Mathematical Theory of Communication. Urbana:
1002 University of Illinois Press; 1949.
1003 [67] Shepherdson DJ, Carlstead K, Mellen JD, Seidensticker J. The influence of food
1004 presentation on the behavior of small cats in confined environments. Zoo Biol
1005 1993;12:203-16.
1006 [68] Kistler C, Hegglin D, Würbel H, König B. Feeding enrichment in an opportunistic
1007 carnivore: The red fox. Appl Anim Behav Sci 2009;116:260-5.
1008 [69] Levi L. The urinary output of adrenalin and noradrenalin during pleasant and unpleasant
1009 emotional states: A preliminary report. Psychosom Med 1965;27:80-5.
1010 [70] D'Eath RB, Tolkamp BJ, Kyriazakis I, Lawrence AB. 2009. 'Freedom from hunger' and
1011 preventing obesity: the animal welfare implications of reducing food quantity or quality.
1012 Anim Behav 2009;77:275-88.
1013 [71] von Euler US. Pathophysiological aspects of catecholamine production. Clin Chem
1014 1972;18:1445-8.

[72] Roth CL, Hunneman DH, Gebhardt U, Stoffel-Wagner B, Reinehr T, Müller HL. Reduced sympathetic metabolites in urine of obese patients with craniopharyngioma. Pediatr Res 2007;61:496-501.

[73] Guilland JC, Moreau D, Genet JM, Klepping J. Role of catecholamines in regulation by feeding of energy balance following chronic exercise in rats. Physiol Behav 1988;42:365–9.

[74] Tang SW, Stancer HC, Takahashi S, Shephard RJ, Warsh JJ. Controlled exercise elevates plasma but not urinary MHPG and VMA. Psychiatry Res 1981;4:13–20.

[75] Beerda B, Schilder MBH, van Hooff JARAM, de Vries HW, Mol JA. Chronic stress in dogs subjected to social and spatial restriction. I. Behavioral responses. Physiol Behav 1999;66:233–42.

[76] Rabb MH, Thompson DL, Barry BE, Colborn DR, Garza F, Hehnke KE. Effects of sexual stimulation, with and without ejaculation, on serum concentrations of LH, FSH, testosterone, cortisol and prolactin in stallions. J Anim Sci 1989;67:2724-9.

[77] Nakanishi R, Imai-Matsumura K. Facial skin temperature decreases in infants with joyful expression. Infant Behav Dev 2008;31:137–44.

[78] Zajonc RB, Murphy ST, Inglehart M. Feeling and facial efference: Implications of the vascular theory of emotion. Psychol Rev 1989;96:395–416.

[79] Moe RO, Stubsjøen SM, Bohlin J, Flø A, Bakken M. Peripheral temperature drop in response to anticipation and consumption of a signaled palatable reward in laying hens (*Gallus domesticus*). Physiol Behav 2012;106:527-33.

[80] Nicol CJ, Caplen G, Edgar J, Browne WJ. Associations between welfare indicators and environmental choice in laying hens. Anim Behav 2009;78:413-24.

[81] Tuber DS, Sanders S, Hennessy MB, Miller JA. Behavioral and glucocorticoid responses of adult domestic dogs (*Canis familiaris*) to companionship and social separation. J Comp Psychol 1996;110:103-8.

[82] Van Vonderen IK, Kooistra HS, Rijnberk A. Intra- and inter-individual variation in urine osmolarity and urine specific gravity in healthy pet dogs of various ages. J Vet Intern Med 1997;11:30–5.

[83] Venturi Rose J, King S, Raymond C. Differences in the levels of canine urinary 5-hydroxyindoleacetic acid between sexes, breeds and in relation to some behavioural traits. Anim Welf 2004;13:S237-59.

[84] Durocher LL, Hinchcliff KW, Williamson KK, McKenzie EC, Holbrook TC, Willard M, Royer CM, Davis MS. Effect of strenuous exercise on urine concentrations of homovanillic acid, cortisol, and vanillylmandelic acid in sled dogs. Am J Vet Res 2007;68:107-11.

[85] Frank D, Minero M, Cannas S, Palestrini C. Puppy behaviour when left home alone: a pilot study. Appl Anim Behav Sci 2007;104:61–70.

[86] Rehn T, Keeling LJ. The effect of time left alone at home on dog welfare. Appl Anim Behav Sci 2011;129:129–35.

[87] Wells DL, Graham L, Hepper PG. The influence of length of time in a rescue shelter on the behaviour of kennelled dogs. Anim Welf 2002;11:317-25.

[88] Graham L, Wells DL, Hepper PG. The influence of olfactory stimulation on the behaviour of dogs housed in a rescue shelter. Appl Anim Behav Sci 2005;91:143–53.

[89] Bujatti M, Biederer P. Serotonin, noradrenaline, dopamine metabolites in transcendental meditation-technique. J Neural Transm 1976;39:257-67.