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

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## 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.

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## 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 was 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.

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## 1. Introduction

Despite our historic relationship with domestic dogs (*Canis familiaris*), today, many council-funded animal shelters and charitable rehoming 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 needs 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

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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 F<sub>2a</sub> (“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 the husbandry staff (such as physiological parameters) and those which could easily and robustly be used by the 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. 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.

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

Two dogs had arthritis, one related to an historical injury and one related to age deterioration. Another dog had a small hole in his heart, which was not reported to have caused any health issues. The data from these three dogs were examined closely (using the ‘Explore’ feature of SPSS, version 19). The dogs did not represent consistent outliers in home measurement data and, so, were not excluded from the analyses. No other health problems were reported. No dogs were reported to have shown aggressive behaviour towards humans in the past, where aggressive behaviour towards humans was defined as having bitten someone on at least one occasion. Two owners reported occasional destructive behaviour in their dog when left at home alone; however, these dogs were not home alone when measurements were taken, and destructive behaviours were not observed in either environment.

Of those dogs that came from multi-dog households (41% of total sample), eight (66.7%) were kennelled with all of their home companions, two (16.7%) were kennelled with one of the two ( $n = 1$ ) or three ( $n = 1$ ) dogs with which they shared their home, and two (16.7%) were housed individually in the boarding kennels. To avoid selection bias in homes with more than one dog, each dog in the household was assigned a number and the subject was randomly selected using the “true random number generator” on [www.random.org](http://www.random.org). In two 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 rehoming 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

t1.1 **Table 1**

t1.2 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:  
 t1.3 ISOP – 8-iso-prostaglandin F<sub>2α</sub>; TBARS – thiobarbituric acid reactive substances; MDA – malondialdehyde; DPPH – 2,2-diphenyl-1-picrylhydrazyl; FRAP – ferric reducing antioxidant  
 t1.4 power; VMA – vanillylmandelic acid; HVA – homovanillic acid; 5-HIAA – 5-hydroxyindole-3-acetic acid.

| t1.5  | Parameter     | Measurement  | Prediction and references  |
|-------|---------------|--|--|
| t1.6  | Physiological | Oxidative stress and damage as measured by:<br>ISOP:creatinine ratio (ISOP/Cr)<br>TBARS:creatinine ratio (TBARS/Cr)<br>MDA:creatinine ratio (MDA/Cr) | Oxidative stress and damage in kennels will be greater than baseline levels [23,25,33–35].   |
| t1.7  |               | Total antioxidant capacity as measured by:<br>DPPH assay<br>FRAP assay   | Total antioxidant capacity in kennels will be lower than baseline values [35,36].  |
| t1.8  |               | Cortisol:creatinine ratio (C/Cr)   | C/Cr in kennels will be higher than baseline values [5,9].   |
| t1.9  |               | Epinephrine and norepinephrine as measured by:<br>VMA: creatinine ratio (VMA/Cr)   | VMA/Cr in kennels will be higher than baseline values [37,38].   |
| t1.10 |               | Dopamine as measured by:<br>HVA:creatinine ratio (HVA/Cr)  | HVA/Cr in kennels will be higher than baseline values [39,40].   |
| t1.11 |               | Serotonin (5-HT) as measured by:<br>5-HIAA:creatinine ratio (5-HIAA/Cr)  | 5-HIAA/Cr in kennels will be higher than baseline values [41–44].  |
| t1.12 | Physical      | Whole body condition   | Body condition in kennels will be lower than baseline values [45].   |
| t1.13 |               | Eye redness  | Scleral blood vessels will be more visible (red) in the kennel than in the home environment [46].  |
| t1.14 |               | Skin dryness (scurf)   | Dogs will have more scurf in the kennel than in the home environment [46–48].  |
| t1.15 |               | Surface temperature  | Surface temperature in kennels will be lower than baseline values [49–51].   |
| t1.16 |               | Core body temperature  | Core body temperature in kennels will be higher than baseline values [52–54].  |
| t1.17 |               | Amount of food eaten   | Dogs will eat less food in the kennel than in the home environment [55,56].  |
| t1.18 | 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. |
| t1.19 |               | Behavioural diversity  | Dogs will show less behavioural diversity in the kennels than in the home environment [59].  |

195 dogs in their own homes first, and from the remaining 14 dogs in Section 2.4.1.1), and between 1630 h and 1830 h. The remaining dogs  
 196 boarding kennels first. were fed once per day, between 1630 h and 1830 h. 231

## 197 2.3. Housing 232

### 198 2.3.1. Boarding kennel environment 233

199 Dogs were kennelled in one of three private boarding kennel estab-  
 200 lishments in Northern Ireland (denoted BK1, BK2 and BK3) following  
 201 each establishment's standard procedures and practices. Fifteen dogs  
 202 (51.7% of total sample) were kennelled in BK1, ten dogs (34.5%) in BK2  
 203 and four (13.8%) in BK3, predominantly due to owners' prior bookings  
 204 with those establishments or recruitment of subjects through that partic-  
 205 ular establishment. All kennels in BK1 and BK2 were contained within  
 206 one building in a line block design, which prevented kennelled dogs  
 207 from visual, but not auditory, contact with all other kennelled dogs. All  
 208 kennels in BK2 and 90% of kennels in BK1 comprised an indoor (BK2:  
 209 112 cm × 180 cm; BK1: 144 cm × 179 cm) and covered outdoor area  
 210 (BK2: 160 cm × 180 cm; BK1: 144 cm × 306 cm), separated by a steel  
 211 guillotine door in brick wall. The remaining kennels in BK1 comprised  
 212 an indoor area only (154 cm × 300 cm). Dogs boarding in 'indoor only'  
 213 kennels ( $n = 2$ ) were given regular access to an enclosed, uncovered,  
 214 outdoor exercise area for toileting (dogs housed in indoor/outdoor  
 215 kennels were also given access to this area). All indoor kennels in BK3  
 216 were detached wooden chalets. Each chalet (213 cm × 213 cm) was set  
 217 in an individual, uncovered, approximately-circular outdoor area  
 218 (366 cm × 457 cm) enclosed with wire fencing. The wire fence and  
 219 semi-circular positioning of chalets on the site allowed dogs visual and  
 220 auditory contact with all other dogs when in their outdoor area.

221 The guillotine/chalet door was closed for the night between 1900 h  
 222 and 2300 h, which restricted dogs to the indoor area until data collection  
 223 began the following morning, between 0630 h and 0900 h. All dogs had  
 224 continuous access to water and bedding in their indoor kennel, and  
 225 were exercised for a minimum of 1 h each day on a lead walk/partially  
 226 off-lead walk and/or in an enclosed outdoor exercise area. In accordance  
 227 with the dogs' usual feeding routines in the home environment, the  
 228 majority of dogs (82.8%) were fed twice daily in kennels; between 0800  
 229 h and 1000 h, following collection of urine and saliva samples (see

### 2.3.2. Home environment 232

233 Owners were asked to keep the routine as normal as possible on the  
 234 day that home measurements were taken. Dogs had access to the room/  
 235 s and/or outdoor areas that they typically had access to on non-  
 236 measurement days. In the home environment, data collection began  
 237 between the hours of 0600 and 0930; at the time when dogs typically  
 238 awoke and passed their first urine of the day. 239

## 2.4. Data collection 239

240 Home measurements were taken a minimum of 7 full days (mean =  
 241 12.89; SD = 2.33) either before the dog entered the boarding kennel  
 242 establishment or after the dog returned home from the establishment.  
 243 This timing was considered sufficient to avoid potential changes in the  
 244 owners' normal routine, behaviour and/or mood (related to their time  
 245 away from home) having an effect on the dogs' physiology and behaviour  
 246 when measurements were taken in the dogs' home first [5], and for the  
 247 dog to readapt to the home environment when measurements were  
 248 taken in the kennel first. Kennel measurements were taken on the first  
 249 ( $n = 25$ ), second ( $n = 3$ ) or third ( $n = 1$ ) day after admission to the  
 250 establishment. The number of days dogs spent in boarding kennels  
 251 ranged from 1 to 21 (median = 1 day).

### 2.4.1. Measurements 252

253 The same physiological, physical and behavioural measurements  
 254 were recorded for each dog in both environments in the order that  
 255 they are described below.

#### 2.4.1.1. Physiological measurements 256

257 2.4.1.1.1. Urine collection and analysis. Dogs were walked outdoors  
 258 on-lead and a mid-stream sample of naturally voided urine was collected  
 259 in a disposable aluminium foil tray. Dogs were then returned to their  
 260 kennel/home. Urine was transferred to a disposable plastic beaker (Fisher  
 261 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 50 ml) was equally divided between six 2 ml and 5 ml Nunc Cryo Tubes (Fisher Scientific U.K. Ltd.) using a 5 ml syringe (BD Plastipak). The remaining 40% of total urine collected was stabilised with 1 M hydrochloric acid (HCl) within 40 min of sample collection. 1 M HCl was added to urine using a 0.5 µl–10 µl 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 1 M HCl used was recorded as volume per ml of urine. Stabilised urine was then divided equally between additional four Cryo Tubes. All samples were stored on ice for a maximum of 2.5 h 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<sub>2</sub> 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 (515 nm) 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 100 µl 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 5 ml syringe to release the saliva. The volume of saliva (up to 3 ml) was divided equally between two 1.5 ml Eppendorf snap-cap microcentrifuge tubes (Fisher Scientific U.K. Ltd.). Samples were stored on ice for a maximum of 2 h before being centrifuged and transferred to a –80 °C freezer. The samples were stored at –80 °C for a maximum of three months until packed in dry-ice and sent to the University of Lincoln for analysis.

Saliva samples were tested for antioxidant capacity using (i) the Ferric Reducing Antioxidant Power (FRAP) assay method of Benzie and Strain [64], as modified by Hayes et al. [65], and (ii) by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. In the former, the antioxidant capacity of saliva was determined at 4 min and 45 min of reaction time, and values were expressed as equivalent concentrations of ferrous ion (µmol/l). The DPPH assay was based on the decolourisation of a stable free radical (DPPH) in a buffered ethanolic/aqueous solution by antioxidants present in the saliva. The reaction with saliva was measured

after 60 min and compared with a standard antioxidant (uric acid). The antioxidant capacity of the saliva was expressed as the equivalent concentration of uric acid (nmol/ml) that would give the same decolourisation.

#### 2.4.1.2. Physical measurements.

- (i) Whole body condition was scored using the Purina "Understanding your Dog's Body Condition"<sup>1</sup> standard 9-point scale, by sight and running hands over the dog's body. The first 18 dogs were independently scored by two researchers, with an inter-rater reliability of 1.00 (95% CI = 1.00–1.00) assessed using the intraclass correlation coefficient. The last 11 dogs were scored by one of these researchers. To reduce the number of groups for between-subjects comparisons, whole body condition was categorised as 'ideal' (scores of 4 and 5) or 'not ideal' (scores of 1–3 and 6–9).
- (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 the video footage of each dog under each condition was analysed. In each case, the first 30 min and last 10 min of the video footage were discarded before random selection of a 30 min section (start time determined using 'true random number generator' – [www.random.org](http://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.

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

388 2.4.1.3.3. *Behavioural analysis: activity budgets.* JWatcher version 1.0  
 389 was used to record the frequency or duration of 38 behaviours using  
 390 continuous sampling. Behaviours that were displayed by 10% or less of  
 391 dogs in both environments were excluded from analysis, as suggested  
 392 by Hiby et al. [14] (i.e. stretch; investigate object; startle; roll; urinate;  
 393 defecate; crouch; lean) as well as those behaviours that could not be  
 394 meaningfully compared between- or within-subjects (i.e. initiate  
 395 human contact; ignore human; jump; groom conspecific; look out [of  
 396 kennel]). Thus, 25 behaviours were analysed (see Table 2). Dogs were  
 397 not observable from the video footage at all times. Therefore, to ensure  
 398 meaningful comparisons were made both within- and between-  
 399 subjects, duration of behaviours was recorded as proportion of time  
 400 in-sight, and frequency of behaviours was analysed as frequency per  
 401 minute in-sight.

402 2.4.1.3.4. *Behavioural analysis: behavioural diversity.* The diversity of  
 403 behaviours performed was calculated for each dog within each environ-  
 404 ment using the Shannon Diversity Index ( $H$ ) [66,67]:

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

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

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

- 414 (i)  $H_{(Posture/Locomotion)}$  – sit; stand; lie; travel; circling before lying  
 415 down; and crouch.  
 416 (ii)  $H_{(Activity/Maintenance)}$  – scratch; object play; sniff object; autogroom;  
 417 drink; feed; and investigate object.

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

## 2.5. Data analysis

420

Data were analysed using IBM SPSS Statistics 19. Where parametric 421  
 tests were used, all test assumptions were met. Shapiro–Wilk tests 422  
 were used to determine the normality of data, on each level of the 423  
 independent variables where appropriate, before conducting statistical 424  
 comparative/correlational tests. Non-parametric tests were used where 425  
 data did not approximate a normal distribution. 426

### 2.5.1. Within-subjects comparisons between home and kennel environments

427 Within-subjects comparisons were made using paired  $t$ -tests or 428  
 Wilcoxon Signed Rank tests. Dichotomous categorical measurements 429  
 were compared using McNemar's Chi-squared tests. The association 430  
 between surface and ambient temperatures in the home environment 431  
 was analysed using Pearson's product moment correlation coefficient 432  
 (Pearson's  $r$ ) and in the kennel environment using Spearman's rank 433  
 correlation coefficient (Spearman's  $\rho$ ). 434

435 Before undertaking within-subject comparisons, we tested for an  
 436 interaction between order and condition in the cross-over design.  
 Here, a selection of measurements (3 of 9 physiological measurements, 437  
 2 of 6 physical measurements and 10 of 28 behavioural measures) were 438  
 chosen at random (using the 'true random number generator' – [www.random.org](http://www.random.org)) to reduce the probability of Type I errors. 'Deviation from 439  
 baseline' data were calculated by subtracting home values from kennel 440  
 values for each measurement taken from each dog. These data were 441  
 then used to compare dogs that were tested at home first ( $n = 15$ ) 442  
 with dogs that were tested in kennels first ( $n = 14$ ) using independent 443  
 $t$ -tests and Mann–Whitney  $U$  tests. 444  
 445

### 2.5.2. Between-subjects comparisons

446 To test the robustness of measurements as indicators of kennelling- 447  
 induced stress, those parameters that deviated significantly from baseline 448  
 (home values) following kennelling were compared between- 449  
 subjects. 'Deviation from baseline' data were used for all between- 450  
 subject comparisons. 451

452 One-way ANOVA and Kruskal–Wallis tests were used to compare:  
 453 (a) subjects housed at different boarding kennel establishments; (b)

t2.1 **Table 2**

t2.2 Behaviours recorded from video footage of dogs at home (30 min) and in kennels (30min), measured as frequency per minute in-sight (F) or duration as a proportion of time in-sight (D).

| t2.3  | Behavioural category | Behaviour                  | Definition   | Measurement  |
|-------|----------------------|----------------------------|--|--|
| t2.4  | Arousal              | Alert                      | Eyes open and head and ears moving. Dog can be lying down, sitting, standing or moving.  | D  |
| t2.5  |                      | Sleep/rest                 | Lying motionless with eyes closed. Might occasionally open eyes to scan area or move ears.   | D  |
| t2.6  | Posture              | Sit                        | Hindquarters in contact with the ground and front legs extended.   | D  |
| t2.7  |                      | Stand                      | Four feet in contact with the ground and legs fully, or almost fully, extended.  | D  |
| t2.8  |                      | Lie                        | Part of both the upper and lower body in contact with the ground.  | D  |
| t2.9  | Tail position        | High tail                  | Standing or moving with tail held higher than the plane of the back.   | D  |
| t2.10 |                      | Level tail                 | Standing or moving with tail on the same plane as the back, or sitting/lying with tail extended.   | D  |
| t2.11 |                      | Low tail                   | Standing or moving with tail held lower than the plane of the back, or sitting/lying with tail curled around body.   | D  |
| t2.12 | Maintenance          | Drink                      | Laps water.  | D  |
| t2.13 |                      | Feed                       | Consumes food.   | D  |
| t2.14 |                      | Autogroom                  | Licks or chews own body.   | D  |
| t2.15 | Locomotion           | Travel                     | Ambulates at any speed.  | D  |
| t2.16 |                      | Kennel rear                | Stands up on hind legs with forelegs against front of kennel, or jumps up and down at front of kennel. Forepaws may scabble on the vertical surface.                           | D  |
| t2.17 |                      | Circling before lying down | Walking in tight circles, with diameter of path approximating length of dog's body, before lying down.   | D  |
| t2.18 | Investigation        | Sniff object               | Orientates nose to within 5 cm of an object, wall or ground and twitches nose.   | D  |
| t2.19 |                      | Vocalisations              | Bark   | Short loud sound with mouth open. Slight movement of ears and shoulders with each bout of sound. |
| t2.20 | Activity             | Whine                      | Prolonged high-pitched sound. Mouth may be open or closed.   | D  |
| t2.21 |                      | Panting                    | Breathes deeply and quickly with mouth open and tongue hanging out.  | D  |
| t2.22 |                      | 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  |
| t2.23 |                      | Scratch                    | Scratches body with hind leg.  | D  |
| t2.24 |                      | Yawn                       | Opens mouth wide and closes eyes without vocalising.   | F  |
| t2.25 |                      | Lick lips                  | Tongue protrudes and licks own lips or snout.  | F  |
| t2.26 |                      | Body shake                 | Shakes whole body, including head, rapidly from side-to-side.  | F  |
| t2.27 |                      | Paw lifting                | Raises single forepaw while sitting or standing and holds it above the ground.   | D  |
| t2.28 |                      | Wag tail                   | Tail moves repetitively from side-to-side.   | D  |

454 subjects with different levels of kennelling experience; and (c) subjects  
455 of different sex/neuter status. Where significance levels ( $<0.05$ ) were  
456 reached for one-way ANOVA and Kruskal–Wallis comparisons, Tukey  
457 post-hoc and Mann–Whitney  $U$  tests were conducted, respectively.

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

### 465 2.5.3. Relationships between parameters

466 2.5.3.1. *Movement/exercise and physiological responses to kennelling.* Using  
467 'deviation from baseline' data, Pearson's  $r$  and Spearman's  $\rho$  were used  
468 to test for relationships between each physiological measurement that  
469 differed significantly between environments and each behavioural indi-  
470 cator that reflected movement/exercise (i.e. travelling, object playing  
471 and diversity of posture/locomotion behaviours) to determine if changes  
472 in physiology were associated with changes in physical activity.

473 2.5.3.2. *'Difficult to measure' and 'easy to measure' parameters.* Spearman's  
474  $\rho$  was used to test for associations between the physiological  
475 measurements that differed within-subjects and behavioural and interval  
476 scale physical variables. These relationships were examined in the home  
477 and kennel environments separately. Independent  $t$ -tests and Mann–  
478 Whitney  $U$  tests were used to compare physiological measurements  
479 between groups that differed in their categorical physical measurements.

### 480 2.5.4. Note on multiple testing

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

### 494 2.6. Ethical note

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

## 502 3. Results

### 503 3.1. Population statistics

504 The majority of dogs (72.4%) had a history of kennelling: 34.5% of  
505 dogs stayed in boarding kennels a maximum of once or twice per year  
506 (Group1/2); 37.9% boarded at least three times per year (Group3);  
507 and 27.6% had no known history of kennelling (Group0). Thirty-one  
508 per cent of dogs had been rehomed a minimum of 12 months before  
509 the study began, and 69% of dogs had been purchased as puppies.  
510 Forty-one per cent of dogs shared their home with at least one dog

(median = 1 dog, range = 1–10). In the home environment, the major- 511  
ity of dogs ( $n = 23$ ) lived indoors and the others ( $n = 6$ ) lived outdoors 512  
with continuous access to shelter (wooden kennel:  $n = 4$ ; garage:  $n = 2$ ). 513

### 514 3.2. Within-subjects comparisons between home and kennel environments

515 There was no evidence of an interaction between condition and 515  
order of condition in the cross-over design: Deviation from baseline 516  
values did not differ significantly between dogs tested in their own 517  
home first and dogs tested in kennels first (independent  $t$ -tests and 518  
Mann–Whitney  $U$  tests,  $P > 0.05$ ). 519

#### 520 3.2.1. Physiological indicators

521 Pairwise comparisons revealed that C/Cr (mmol/l: mmol/l  $\times 10^6$ ) 521  
was significantly higher in the kennel compared to the home environ- 522  
ment ( $Z = -2.984$ ,  $n = 17$ ,  $P = 0.003$ ). VMA/Cr ( $\mu\text{mol}/\text{mmol}$ ) was 523  
also higher in kennels than at home ( $t_{(18)} = 2.898$ ,  $P = 0.01$ ) (medians 524  
and IQRs presented in Table 3). No other physiological measurement dif- 525  
fered significantly between home and kennel environments ( $P > 0.05$ ; 526  
see Table 3). 527

#### 528 3.2.2. Physical indicators

529 Dogs' surface temperature ( $^{\circ}\text{C}$ ) was significantly lower in the kennel 529  
compared to the home environment ( $t_{(27)} = -3.950$ ,  $P = 0.001$ ). 530  
Surface temperature was not associated with ambient temperature in 531  
the home ( $r = 0.226$ ,  $n = 29$ ,  $P > 0.05$ ) or kennel environment ( $r_s = 532$   
 $0.243$ ,  $n = 28$ ,  $P > 0.05$ ). No other physical measurement differed 533  
significantly within-subjects (all  $P > 0.05$ ). (Data for interval scale 534  
measurements are summarised in Table 3; data for ordinal scale and 535  
categorical measurements are not shown). 536

#### 537 3.2.3. Behavioural indicators

538 Dogs spent significantly less time (milliseconds as proportion of 538  
time in-sight) lying down ( $Z = -2.920$ ,  $n = 27$ ,  $P = 0.004$ ) and 539  
sleeping/resting ( $Z = -2.349$ ,  $n = 27$ ,  $P = 0.019$ ) and a greater 540  
proportion of time alert ( $Z = -2.337$ ,  $n = 27$ ,  $P = 0.019$ ), sitting 541  
( $Z = -2.172$ ,  $n = 27$ ,  $P = 0.03$ ), standing ( $Z = -2.372$ ,  $n = 27$ , 542  
 $P = 0.018$ ), travelling ( $Z = -1.971$ ,  $n = 27$ ,  $P = 0.049$ ) and 543  
panting ( $Z = -2.023$ ,  $n = 27$ ,  $P = 0.043$ ) when kennelled 544  
compared to when at home. Dogs also showed a significantly greater 545  
diversity of posture/locomotion behaviours ( $H$ ) in kennels than at home 546  
( $Z = -2.057$ ,  $n = 27$ ,  $P = 0.04$ ) (medians and IQRs presented in 547  
Table 4). 548

549 As can be seen from the IQRs in Table 4, considerable individual 549  
variability was observed, particularly in proportion of time spent alert 550  
and sleeping/resting both at home and in kennels. Time spent standing 551  
and lying down when kennelled also varied substantially between-sub- 552  
jects, as did the diversity of posture/locomotion behaviours observed 553  
both in the home and in kennel environments. It should be noted that 554  
only 5 individuals were observed panting during the study; therefore, 555  
the majority of subjects did not demonstrate this behaviour in either 556  
environment. No other behaviours differed in frequency or duration 557  
between environments ( $P > 0.05$ ). Ease of measurement (EOM) also 558  
did not differ between environments as determined by McNemar's 559  
test ( $n = 28$ ,  $P > 0.05$ ), which suggested that dogs were not more averse 560  
to handling in the kennels than at home. 561

### 562 3.3. Between-subjects comparisons

563 All results presented in Section 3.3 are based on 'deviation from 563  
baseline' data (within-subjects, 'kennel minus home' values per 564  
measurement, per dog). 565

#### 566 3.3.1. Boarding kennel establishment

567 The rise in dogs' C/Cr and VMA/Cr, and decline in surface temperature, 567  
following kennelling did not differ significantly between groups of dogs 568

t3.1 **Table 3**

t3.2 Mean ± standard deviation (SD) or median and interquartile range (IQR) of physiological and interval scale physical parameters measured in dogs' normal home environment (baseline  
t3.3 values) and in boarding kennels, with *P* values for within-subjects comparisons between environments.

| t3.4  | Measurement   | Home environment  | Kennel environment                                      | Statistical test | <i>P</i> value |
|-------|---|---|---|------------------|----------------|
| t3.5  | MDA/Cr (µmol/g)   | 6.71 (IQR 5.33–12.46)                                   | 5.900 (IQR 3.79–11.10)                                  | Z                | 0.943          |
| t3.6  | TBARS/Cr (µmol/mmol)  | 1.00 (IQR 0.76–1.72)                                    | 0.955 (IQR 0.643–1.468)                                 | Z                | 0.906          |
| t3.7  | ISOP/Cr (ng/mg)   | 5.30 (IQR 4.40–7.20)                                    | 6.10 (IQR 4.15–8.60)                                    | Z                | 0.795          |
| t3.8  | C/Cr (mmol/l:mmol/l × 10 <sup>6</sup> )                                 | 1.53 (IQR 1.23–2.42)                                    | 3.335 (IQR 2.55 – 4.515)                                | Z                | 0.003**        |
| t3.9  | 5-HIAA/Cr (µmol/mmol)   | 1.456 (IQR 1.123–1.882)                                 | 1.431 (IQR 1.136 – 1.786)                               | Z                | 0.872          |
| t3.10 | HVA/Cr (µmol/mmol)  | 1.932 (IQR 1.477–2.546)                                 | 2.012 (IQR 1.615 – 2.673)                               | Z                | 0.277          |
| t3.11 | VMA/Cr (µmol/mmol)  | 0.082 ± 0.024   | 0.104 ± 0.037   | T                | 0.01**         |
| t3.12 | DPPH (nmol/mL equivalents [as uric acid])<br>FRAP 4 min 45 min (µmol/l) | 83.95 (IQR 41.70 –164.25)<br>271.50 (IQR 170.50–590.50) | 66.00 (IQR 32.60 –106.73)<br>295.00 (IQR 160.00–518.50) | Z                | 0.983<br>0.476 |
| t3.13 |   | 517.50 (IQR 371.25–965.75)                              | 532.00 (IQR 337.50 –790.50)                             | Z                | 0.903          |
| t3.14 | Surface Temp. (°C)  | 25.233 ± 4.275  | 22.105 ± 3.306  | T                | 0.001***       |
| t3.15 | Core Temp. (°C)   | 36.739 ± 0.976  | 36.631 ± 0.752  | T                | 0.748          |

t3.16 Mean ± SD are presented where data were approximated normal distribution as determined by Shapiro–Wilk tests. Median and IQR are presented where data were not normally  
t3.17 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.

569 kennelled at different establishments (denoted as BK1, BK2 and BK3)  
570 (one-way ANOVA, *P* > 0.05). Of those behavioural variables that differed  
571 significantly between environments (see Table 4), within-subjects  
572 differences in 'time spent standing' ( $H_{(2)} = 7.064, n = 27, P = 0.029$ ),  
573 'time spent travelling' ( $H_{(2)} = 6.156, n = 27, P = 0.046$ ) and 'time  
574 spent lying down' ( $F_{(2, 24)} = 3.829, P = 0.036$ ) were significantly  
575 different between the three groups kennelled at different establishments  
576 (see Fig. 1).

577 BK1 dogs generally showed a greater increase ( $U = 26.50, n =$   
578  $24, P = 0.014$ ) in time spent standing following kennelling (median =  
579  $0.052, IQR 0.033–0.554, n = 15$ ) than BK2 dogs (median =  $-0.018,$   
580  $IQR -0.058–0.018, n = 9$ ), while BK1 and BK3 dogs ( $n = 3$ ), and BK2  
581 and BK3 dogs, did not differ (Mann–Whitney *U* tests:  $P > 0.05$ ). When  
582 kennelled, dogs housed at BK3 showed a greater decrease (Tukey  
583 post-hoc test:  $P = 0.048$ ) in time spent lying down ( $-0.684 ± 0.484,$   
584  $n = 3$ ), and a greater increase ( $U = 1.00, n = 12, P = 0.021$ ) in time  
585 spent travelling (median =  $0.342, n = 3$ ), than dogs housed at BK2

( $-0.042 ± 0.282$ ; median =  $0.00, n = 9$ ; respectively). There were  
586 no significant differences between BK1 and BK3 dogs, or between BK1  
587 and BK2 dogs, in deviation from baseline lying or travelling behaviour  
588 ( $P > 0.05$ ). Furthermore, dogs kennelled at BK2 showed less individual  
589 variation than dogs kennelled at BK1 and BK3 in the amount that they  
590 deviated from baseline values of time spent standing (Fig. 1a), travelling  
591 (see Fig. 1b) and lying down (Fig. 1c).  
592

593 **3.3.2. Kennelling experience**

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

598 **3.3.3. 'Demographic' attributes**

599 No sex or source (rehomed/purchased as puppy) differences were  
600 found on any variable that differed within-subjects ( $P > 0.05$ ). However,

t4.1 **Table 4**

t4.2 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  
t4.3 tests for within-subjects differences between environments.

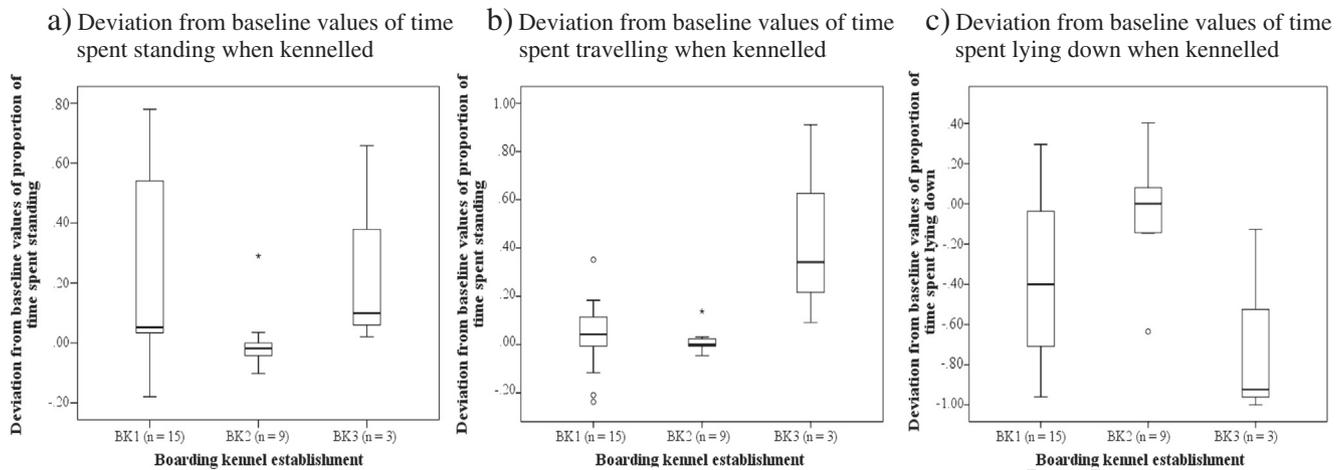
| t4.4  | Behaviour                  | Measurement | Home environment        | Kennel environment      | <i>P</i> value     |
|-------|----------------------------|-------------|-------------------------|-------------------------|--------------------|
| t4.5  | Alert                      | D           | 0.273 (IQR 0.085–0.619) | 0.690 (IQR 0.261–0.994) | 0.019 <sup>a</sup> |
| t4.6  | Sleep/rest                 | D           | 0.718 (IQR 0.381–0.915) | 0.310 (IQR 0.000–0.739) | 0.019 <sup>a</sup> |
| t4.7  | Sit                        | D           | 0.000 (IQR 0.000–0.004) | 0.008 (IQR 0.000–0.105) | 0.030 <sup>a</sup> |
| t4.8  | Stand                      | D           | 0.009 (IQR 0.000–0.089) | 0.057 (IQR 0.028–0.558) | 0.018 <sup>a</sup> |
| t4.9  | Lie                        | D           | 0.964 (IQR 0.725–1.000) | 0.513 (IQR 0.062–0.894) | 0.004 <sup>b</sup> |
| t4.10 | High tail                  | D           | 0.000 (IQR 0.000–0.069) | 0.002 (IQR 0.000–0.041) | 0.583              |
| t4.11 | Level tail                 | D           | 0.011 (IQR 0.000–0.184) | 0.012 (IQR 0.000–0.097) | 0.309              |
| t4.12 | Low tail                   | D           | 0.956 (IQR 0.670–1.000) | 0.975 (IQR 0.772–1.000) | 0.647              |
| t4.13 | Drink                      | D           | 0.000 (IQR 0.000–0.000) | 0.000 (IQR 0.000–0.001) | 0.441              |
| t4.14 | Feed                       | D           | 0.000 (IQR 0.000–0.000) | 0.000 (IQR 0.000–0.000) | 0.273              |
| t4.15 | Autogroom                  | D           | 0.000 (IQR 0.000–0.005) | 0.000 (IQR 0.000–0.000) | 0.470              |
| t4.16 | Travel                     | D           | 0.010 (IQR 0.000–0.044) | 0.076 (IQR 0.012–0.136) | 0.049 <sup>a</sup> |
| t4.17 | Circling before lying down | D           | 0.000 (IQR 0.000–0.000) | 0.000 (IQR 0.000–0.000) | 0.263              |
| t4.18 | Sniff object               | D           | 0.000 (IQR 0.000–0.007) | 0.006 (IQR 0.000–0.016) | 0.194              |
| t4.19 | Bark                       | F           | 0.000 (IQR 0.000–0.000) | 0.000 (IQR 0.000–0.804) | 0.388              |
| t4.20 | Whine                      | D           | 0.000 (IQR 0.000–0.000) | 0.000 (IQR 0.000–0.000) | 0.128              |
| t4.21 | Panting                    | D           | 0.000 (IQR 0.000–0.000) | 0.000 (IQR 0.000–0.000) | 0.043 <sup>a</sup> |
| t4.22 | Object play                | D           | 0.000 (IQR 0.000–0.000) | 0.000 (IQR 0.000–0.000) | 0.686              |
| t4.23 | Scratch                    | D           | 0.000 (IQR 0.000–0.000) | 0.000 (IQR 0.000–0.000) | 0.465              |
| t4.24 | Yawn                       | F           | 0.000 (IQR 0.000–0.034) | 0.000 (IQR 0.000–0.000) | 0.442              |
| t4.25 | Lick lips                  | F           | 0.097 (IQR 0.000–0.358) | 0.017 (IQR 0.000–0.204) | 0.601              |
| t4.26 | Body shake                 | F           | 0.000 (IQR 0.000–0.000) | 0.000 (IQR 0.000–0.038) | 0.374              |
| t4.27 | Paw lifting                | D           | 0.000 (IQR 0.000–0.000) | 0.000 (IQR 0.000–0.000) | 0.161              |
| t4.28 | Wag tail                   | D           | 0.000 (IQR 0.000–0.016) | 0.000 (IQR 0.000–0.014) | 0.875              |
| t4.29 | Diversity – posture        | H           | 0.153 (IQR 0.000–0.610) | 0.584 (IQR 0.273–0.928) | 0.040 <sup>a</sup> |
| t4.30 | Diversity – activity       | H           | 0.000 (IQR 0.000–0.430) | 0.248 (IQR 0.000–0.665) | 0.594              |

t4.31 Behaviours measured as: D = duration (milliseconds) as a proportion of time in-sight.

t4.32 F = frequency per minute in-sight. H = Shannon diversity index.

t4.33 <sup>a</sup> Significant at the 0.05 level.

t4.34 <sup>b</sup> Significant at the 0.01 level.



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

the increase in C/Cr in males ( $2.639 \pm 2.704$ ,  $n = 7$ ) compared to females ( $0.704 \pm 0.918$ ,  $n = 10$ ) following kennelling almost reached significance ( $t_{(15)} = 2.120$ ,  $P = 0.051$ ).

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 Fig. 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 Fig. 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:  $P > 0.05$ ).

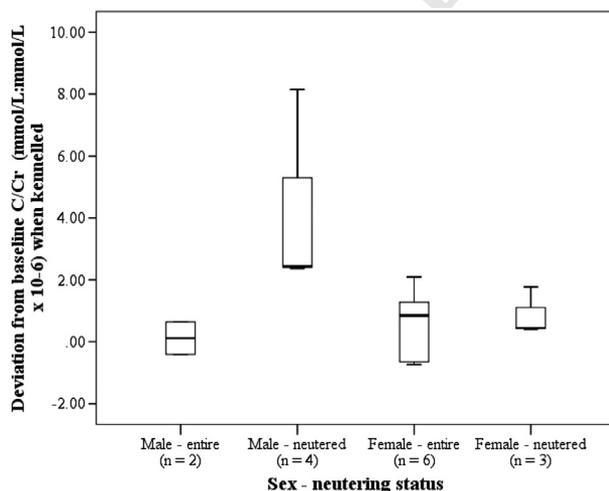
##### 3.4.2. 'Difficult to measure' and 'easy to measure' parameters

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 ( $\mu\text{mol}/\text{mmol}$ ) was also associated with less lip licking ( $r_s = -0.502$ ,  $n = 20$ ,  $P = 0.024$ ) in the kennel environment.

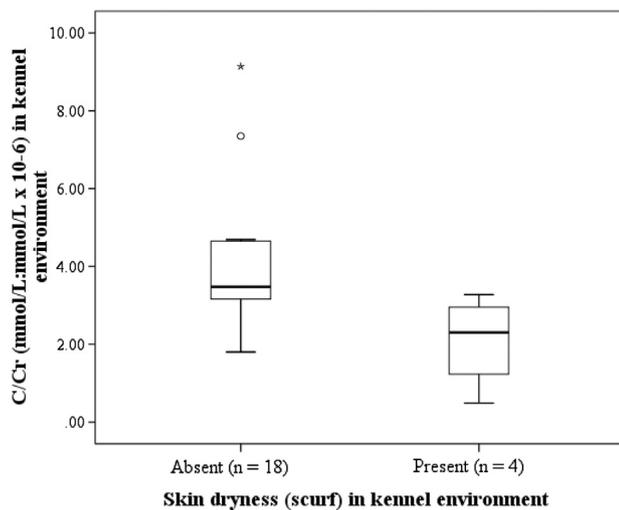
Dogs with no skin dryness (scurf) had higher C/Cr (median = 3.475, IQR = 3.015–4.660,  $n = 18$ ) than dogs with scurf (median = 2.305, IQR = 0.863–3.118,  $n = 4$ ) ( $U = 12.00$ ,  $n = 22$ ,  $P = 0.041$ ) in kennels, as shown in Fig. 3. However, this difference was not observed in the home environment (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 ( $P > 0.05$ ).

## 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.



**Fig. 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.



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

#### 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 arousal, and a more sensitive urinary measurement of SAM system response, in dogs than epinephrine:creatinine (E/Cr) or norepinephrine:creatinine ratios (NE/Cr).

Consistent with current findings, previous research found no association between age and cortisol response to kennelling [7,9]. The tendency for males in our study to show a greater cortisol response than females was not detected in earlier research [7,9], which may be due to our use of 'deviation from baseline' data rather than data collected only in kennels. Indeed, when only using the data that we collected in kennels, sex differences in C/Cr did not come close to reaching significance, indicating that both sexes have similar levels of urinary cortisol when kennelled but that males tend to experience a greater rise in C/Cr than females in order to reach that level. However, no sex difference was detected in baseline C/Cr, which suggests the near-significant *p* value occurred by chance. Moreover, Beerda et al. [12,75] found that females showed greater behavioural and HPA axis response to acute stressors (a sound blast and corticotrophin-releasing hormone challenge). Although, the discrepancies between current and Beerda et al.'s [12] findings may be explained by admission to boarding kennels not representing a negative stressor for the dogs in this study. Unexpectedly, it was the neutered males in our study that accounted for the near-significant sex difference in C/Cr response to kennelling. There is no obvious explanation for this finding and, as sex, or sex/neuter status, differences were not detected in any other parameter that reflected increased arousal, we suggest that this was a Type I error, arising from a combination of the small sample and multiple testing.

No differences in kennelling-induced cortisol response were found between dogs with, and dogs without, previous experience of a kennel environment, which is in line with Hiby et al.'s [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]. Further, our findings suggest that dogs' spontaneous behavioural response to a seemingly stressful situation (i.e. an unfamiliar environment) might also be influenced by the structure of that environment. For example, the greater increases in time spent standing when dogs were kennelled at BK1 compared to BK2 were largely explainable by the mesh kennel front at BK1 and solid steel kennel front at BK2; where the former allowed dogs to stand and look out of their kennel, and the latter prevented them from doing so. Moreover, dogs spent less time lying down and more time travelling when kennelled at BK3 compared to BK2, which was likely accounted for by the greater stimulation provided at BK3 in terms of visual contact with other kennelled dogs. Constraints of the BK2 kennel environment also appeared to reduce the potential for individual variability in dogs' behavioural response, evident from Fig. 1. 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 those 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  $1.53 \times 10^{-6}$  (mmol/l:mmol/l) measured in dogs' home environment was somewhat lower than the mean ratios of  $2.9 \times 10^{-6}$  [82] and  $4.8 \times 10^{-6}$  [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 1700 h 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  $14.25 \times 10^{-6}$  (nmol/l:nmol/l) and Stephen and Ledger [9] of  $17.8 \times 10^{-6}$ . 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  $\mu\text{mol/l}$ ) were comparable to those previously reported in Labradors (12.5 and 24  $\mu\text{mol/l}$ ) and German Shepherd Dogs (17 and 31  $\mu\text{mol/l}$ ) [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  $\mu\text{g/ml}$ ) [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 5  $\mu\text{g/ml}$  in Durocher et al.'s [84] assay method and the mean baseline concentration of 0.27 mg/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 the other hand, the considerable time spent sleeping/resting that has been observed in privately-owned dogs may be a consequence of the greater activity, exercise and stimulation that dogs experience when their owners are home. These vastly different potential interpretations of sleeping/resting behaviour further highlight the difficulties in accurately interpreting snap-shots of spontaneous behaviour alone.

#### 4.3. Relationships between 'difficult to measure' and 'easy to measure' indicators

Ultimately, research into animal welfare indicators should aim to identify valid, reliable and specific measures that are practical for use 'on the ground', and on a regular basis, by animal caregivers. Here, two associations were found between more 'difficult to measure' (in the sense of cost, procedure and equipment required) indicators that were identified as valid measures of acute canine arousal and 'easy to measure' spontaneous behaviour and physical indicators. Firstly, dogs with no skin dryness were found to have higher C/Cr in the kennels than dogs with scurf. However, as cause-and-effect was not explicitly tested, other differences between groups may explain this relationship. For example, the majority of dogs with no skin dryness in the kennels typically lived indoors at home; whereas, 75% of dogs (3 out of 4 dogs) with scurf lived outside in the home environment with access to a wooden kennel for shelter. Given that a dog's cortisol response to its current environment appears to be influenced by its appraisal of previous housing conditions [12], the difference between dogs with, and dogs without, scurf may have been accounted for by differences in

891 dogs' home environments, or dogs' appraisal thereof. Of course, this  
892 interpretation requires further investigation before any conclusions  
893 can be drawn as the difference may have reflected a Type I error due  
894 to the number of tests performed and/or the large difference in sample  
895 size between groups.

896 The negative correlations between lip licking and urinary VMA  
897 (epinephrine and norepinephrine metabolite) and 5-HIAA (5-HT  
898 metabolite) levels were unexpected as increased frequency of lip licking  
899 has previously been associated with stress in dogs [57]. As positive  
900 correlations between plasma VMA levels and measures of psychological  
901 stress have been found in humans [37] and higher plasma levels of 5-HT  
902 and urinary 5-HIAA have previously been associated with anxiety [41]  
903 and nervous behaviour [83] in dogs, the negative correlations between  
904 lip licking and VMA and 5-HIAA found here appear to suggest that  
905 decreased frequency of lip licking is associated with increased stress,  
906 which seemingly contradicts previous research. However, urinary epi-  
907 nephrine levels also increase in response to pleasant emotional arousal  
908 [69] and increased 5-HIAA has been associated with relaxation [89],  
909 which further complicates interpretation. One possible explanation is  
910 that lip licking is associated with derousal (calming signal) and is  
911 shown in some stressful situations because the dog is trying not to  
912 increase arousal. Clearly, additional research is required before any  
913 valid conclusions can be drawn. Nonetheless, as lip licking was observed  
914 in almost 50% of dogs (14 out of 29 dogs) in the kennel environment,  
915 this behaviour, and its relationship with emotional arousal, do warrant  
916 further investigation.

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

922 Although admission to boarding kennels did not appear to be the  
923 aversive stressor for dogs that was required to thoroughly test the validity  
924 of each stress parameter, this study did highlight the difficulties in  
925 interpreting physiological, physical and behavioural data and also called  
926 into question the presumption that short-term kennelling represents a  
927 negative psychological stressor for dogs. Furthermore, this study empha-  
928 sises how important it is to examine a range of welfare indicators, as  
929 opposed to drawing conclusions on dogs' emotional state and/or welfare  
930 status from C/Cr and spontaneous behavioural data alone.

#### 931 4.4. Conclusions

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

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

#### Conflict of interest statement

The authors declare that there were no conflicts of interest. 956

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[76] 962

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