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Associations of behaviour with secretory immunoglobulin A and cortisol in domestic cats during their first week in an animal shelter

Nadine Gourkow[a], Alora LaVoy[b], Gregg A. Dean[b], Clive J.C. Phillips[a,*]

[a] Centre for Animal Welfare and Ethics, School of Veterinary Medicine, University of Queensland, QLD, Australia
[b] Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, CO, USA

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ABSTRACT

We tested the hypothesis that during their first week in an animal shelter, cats exhibit groups of behaviours that are connected to mucosal immune and adrenal responses. The behaviour of 34 cats was observed from admission to day 5 and immunoglobulin A (S-IgA) and cortisol were quantified from faeces. A multidimensional model constructed by Principal Component Analysis indicated the presence of three distinct behavioural dimensions. Behaviours forming dimension 1 were hiding, flat postures, freeze, startle, crawl and retreat from humans. These were significantly contrasted (R −0.6 to −0.4) to dimension 3 behaviours which included normal patterns of feeding, grooming, sleeping and locomotion, sitting at the front of the cage while calmly observing activities, sleeping or resting while lying on their side, rubbing on cage items and friendly behaviour towards humans. Dimension 2 behaviours included persistent meowing, scanning, pacing and pushing, together with bouts of destructive behaviour, attempts to escape and redirected aggression. Dimension 2 was not significantly contrasted to dimension 3 ($R < -0.4$ except for sleep = 0.6) or dimension 1 ($R \leq -0.2$). S-IgA values were greater ($P < 0.001$) for cats clustered in dimension 3 (mean 7.1 ± 0.5 $\log_e$ μg/g), compared to dimensions 1 and 2 which were not significantly different ((1) 5.6 ± 0.6; (2) 5.6 ± 0.7 $\log_e$ μg/g). Cortisol values were similar for the three dimensions. Despite the difficulty in generalising the results to the shelter cat population due to small sample size, our findings suggest that behaviour is a good indicator of mucosal immune function in shelter cats. This may be of clinical significance for the management of upper respiratory disease in animal shelters.

1. Introduction

Upper respiratory infections (URI) are pervasive in shelter cats worldwide, and represent the most common health problem that results in euthanasia of both cats and kittens (Dinnage et al., 2009). Entering an animal shelter represents a major change in routine and exposes cats to known stressors such as confinement, exposure to unfamiliar humans, animals and overcrowding. These conditions increase their susceptibility to respiratory pathogens (Pedersen et al., 2004) that are found in high concentrations in multi cat environments (Hurley, 2005). In humans, distress increases the likelihood of contracting respiratory infection and lengthens recovery time (Cohen, 2005; Corbett et al., 2010). Scientists have

* Corresponding author at: University of Queensland, School of Veterinary Sciences, Gatton Campus Gatton 150, Gatton, QLD 4343, Australia. Tel.: +61 7546 01251/+61 7 5460 1158, mob. 0406340133; fax: +617 5460 1444.
E-mail address: c.phillips@uq.edu.au (C.J.C. Phillips).

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argued that emotions compromising the health of animals in shelters must be identified and addressed (McMillan, 2002; Griffin, 2012). Several studies have shown that electrostimulation targeted at areas of the brain that elicit emotional responses in cats, such as anxiety, fear and stereotypic restlessness also alter physiological correlates, particularly cell-mediated immunity (Kojima et al., 2000; Mori et al., 2001; Kaname et al., 2002; Sumida et al., 2004).

Many of these studies utilised invasive neural stimulation, but non-invasive measures of affective states that relate to behaviour and immunity of cats following entry to an animal shelters are now needed. More specifically, and because it can be measured non-invasively, mucosal immunity, the first line of defence against upper respiratory infections (URI), warrants investigation as to how it is affected by the entry of cats into animal shelters.

Secretory immunoglobulin A (S-IgA), the major class of mucosal antibody, prevents respiratory pathogens that are inhaled or ingested from penetrating the epithelial walls at mucosal sites (Hannant, 2002; Stokes and Waly, 2006). Feline S-IgA has been quantified in saliva (Harley et al., 1998) and faeces (Yamada et al., 1984; Rodriguez et al., 2007), however the influence of affective states, inferred from behavioural responses, on secretory activity has not been examined. The effect of presumed affective states and psychological stress on S-IgA has been examined in some other species, including sheep, rats and mice (Napolitano et al., 1995; Eriksson et al., 2004; Rammal et al., 2010), but the feline mucosal immune system bears a stronger resemblance to that of pigs, dogs and humans (Stokes and Waly, 2006). In the latter species, acute and chronic stressors appear to increase and inhibit S-IgA secretion, respectively. A series of environmental and social stressors have been shown to inhibit S-IgA secretion in several species (pigs, Royo et al., 2005; dogs: Berteselli et al., 2005), although brief negative experiences may increase secretion (Muneta et al., 2010). Reduced secretion may be reversed by providing positive environmental and social interactions (e.g. Taniguchi et al., 2007; Noto et al., 2010).

Despite apparently confounding results due probably to variation in S-IgA levels across faecal (Carlsson et al., 2007) and salivary (Harley et al., 2003) samples, as well as fluctuation in the circadian rhythm (Kikkawa et al., 2005), affective states appear to be significant moderators of S-IgA. However, non-invasive measure of affect remains elusive in non-human animals. Nonetheless, it is generally accepted that affect can be inferred from behaviour (e.g. Reefmann et al., 2009; Mendl et al., 2010). Moreover, in cats, behavioural expressions of emotions elicited by hypothalamic or pharmacological stimulation are undistinguishable from the behavioural responses observed in response to threats, such as presentation of a dog, restraint, waving a stick and poking them (e.g. Brudzynski et al., 1990).

The objectives of this study were (1) to identify how the short-term behavioural expression of cats entering a shelter may allow them to be clustered into emotional profiles, and (2) to explore how such profiles may relate to cortisol and S-IgA secretion.

2. Materials and methods

This study was approved by the University of Queensland Animal Ethics Committee (CAWE/231/10).

2.1. Animals

Forty cats entering an animal shelter (British Columbia Society for the Prevention of Cruelty to Animals, Vancouver, Canada) during April and May, 2010, were enrolled in the study. Of these, 53% ($n = 18$) and 47% ($n = 16$) were designated by the relinquisher as strays and owned, respectively, and this classification was checked by the receiver. No cats were rejected and of the 40 cats, 34 remained to the end of the study. Twenty-two were adults (1 to 7 years of age), 7 seniors (>7 years of age) and 5 juveniles (<12 months); 8/18 females and 9/16 males were sexually intact during the study. All cats were examined at intake by a Veterinary Health Technician. Vaccination was delayed until after the study (day 7); however, cats were dewormed before starting (Strongid® T. Pfizer, Kirkland, Québec, Canada).

2.2. Housing

All cats were housed individually in a stainless steel cage ($76 \times 76 \times 71$ cm) furnished with a litter box and non-absorbent cat litter (Veterinary Concepts, Spring Valley, Wisconsin, USA). Stainless steel food and water bowls were fastened to the cage door, and a towel provided for bedding. Cages were either barren or equipped with a Hide, Perch & Go Box™ (Animal Behaviour Systems Australia Pty Ltd, Hoppers Crossing, Victoria, Australia) (Gourkow, 2007) of dimensions $46 \times 41 \times 31$ cm, to simulate housing provided in different shelters (Kry and Casey, 2007). Cats were randomly allocated to cages with and without a box, however the final ratio (20:14, respectively) was unequal because some cats from the 'no box' group were removed from the study after 4 days without defecating. Cages were cleaned daily by removing all waste, changing bedding and wiping walls with a clean cloth soaked in water, and cages were disinfected between cats with a 1% disinfectant solution (Virkon®, Du Pont, Mississauga, Ontario, Canada). Windows provided natural light and an ambient temperature of $20 \pm 2\,^{\circ}$C was maintained. Feed was provided twice daily (07:00 h and 17:00 h) (Science Diet, Hill's Pet Nutrition, Inc. ®/™, Vancouver, British Columbia, Canada) and fresh water was provided ad libitum.

2.3. Behavioural observations

Outside each cage, an infrared camera (Sony CCD25 M crystal-View Super Hi-Res ICR IR Camera SLED w/9–22 mm Vari-focal Lens, Microtech Advanced Technologies Ltd, Vancouver, British Columbia, Canada) was mounted at cage height on a rod suspended from the ceiling at 1 m from the cage door. Footage was both viewed in real-time in an adjacent room and stored for subsequent analysis. The starting point was an ethogram limited to 40 behaviours reported in observational studies of caged and household cats (Fangel and Kaada, 1960; Ursin, 1964; Kessler and Turner, 1997; Bernstein, 2006). To facilitate observations, the behaviours

Table 1
Ethogram used for the observation of cats, with behaviours recorded as durations (*D*) or frequencies (*F*).

Categories	Behaviour	Description	D/F
Location	Under towel	Whole or partial body under bedding	D
	In box	In box at the back and barely visible	D
	Front of cage	Whole body, near door of cage	D
	On perch	On top of box	D
	Behind Box	Body all or partially behind box	D
	Behind litter tray	Body all or partially behind litter tray	D
Posture	Lie		
	Rolled up	Body rolled up in a ball	D
		Back in contact with floor of cage and abdomen exposed	D
	On back		
	Ventral high	Sternally recumbent, relaxed body	D
	Ventral flat	Sternally recumbent, body flattened to floor, neck retracted, head held low, ears flattened	D
	Lie on side	Laterally recumbent, body stretched, neck and ventral area exposed, limbs and tail extended	D
	Stand		D
	Tall	Stand, legs straight, head up	D
	Flat	Stand, legs partially bent, low tail, head held low, flattened body	D
	On hind limbs	Hind paws on ground, front paws on wall or door, body upright	D
	Sit	Front paws and rump on the ground, head held high, ears up	D
	Crouch	Legs bent, paws on ground, body slightly raised off the ground	D
	Upside down	Hanging on cage door with body inverted	D
Self-maintenance	Eat/drink	Takes food or water into mouth, ingest or imbibe	D
	Groom	Licks fur or paws and rubs head with paws (without chewing or pulling)	D
Response to human	Neutral	No change in behaviour	F
	Friendly	Approaches human standing tall, tail erect, short high pitched vocalization and rubbing neck or forehead against bars of cage or hand during interaction with human	F
	Aggressive	Applying pressure on arm or hand of human with teeth in an attempt to bite or swiping with paw while remaining in high posture and close to human (not defensive)	F
	Retreat	Move away from human	F
Activities	Freeze	Tense, immobile, flattened body posture, eyes wide open	D
	Sleep	Relaxed body and neck, eyes closed	D
	Paw wall/floor	Scratching at wall or floor, but not as a precursor to elimination	F
	Paw through door	Pushing paw through bars of cage door	F
	Knead	Rhythmic motion of pushing paws into floor, claws alternately extended and retracted	D
	Rub	Part or the whole length of the body is rubbed along object	F
	Escape attempt	Pushing on door and door latch, hanging upside down on door, paws through door.	F
	Pushing	Hitting or throwing objects around the cage in a destructive manner (not associated with play) using head, body or paws	F
	Meow	Open mouth vocalisation emitting meow sound	F
	Hiss	Mouth held tensely open with teeth exposed whiskers retracted and emitting a "hiss" sound	F
	Startle	Sudden retreat to back of cage	F
	Scan	Slow visual investigative monitoring of cage	D
	Pace	Repetitive and fast walking back and forth following a similar path	D
	Circling	Repetitive walking in circle	D
	Crawl	Low body posture, slow locomotion, move from one area to another	D
	Walk	High body posture, normal locomotion, move from one area to another	D
	Play	Gentle rolling or lifting of objects. Attention focussed on interacting with object rather than getting it out of the way as in pushing	F

were organized into five main categories (location in cage, posture, self-maintenance behaviour, response to humans and other activities) (Table 1). Behaviours were measured as frequency or duration and coded with the aid of Observer software (Noldus Information Technology, Wageningen, The Netherlands), using focal sampling of 5 min/h for days 1, 3 and 5 post-entry. Due to different arrival time of cats on day 1, short periods of camera malfunction and cats disappearing from view, not all cats could be observed for the same allotted time of 24 sessions, each of 300 s, per day. Hence, adjusted daily means were calculated for observation time (duration: s/number of observations) and frequency (frequency: counts × 24/number of observation sessions). Observation days not followed by a stool within 48 h were discarded. In total 1712 sessions, each of 300 s, were used for behaviour observation.

2.4. S-IgA and cortisol assays

Faecal samples were collected when available (mean three stools per cat), weighed and immediately frozen at −40 °C. To prepare faecal extracts for IgA ELISA analysis, 10 mL phosphate-buffered saline (PBS) containing 10% V/V goat serum (Equitech-Bio, Kerrville, Texas, USA) and 0.1% V/V Kathon (Sigma, St Louis, Missouri, USA) was added to 1 g wet faeces and then vortexed for 10 min or until fully homogenized. A 2 mL aliquot of homogenized sample was centrifuged at 10,000 × *g* for 10 min at 4 °C. Complete, EDTA-free Protease Inhibitor cocktail (Roche Diagnostics Corporation, Indianapolis, Indiana, USA) was added to aliquots of the supernatant before storage at −80 °C. ELISA plates (Lumitrac 600, Greiner Bio-One, Tokyo, Japan) were coated overnight at 4 °C with 100 μL/well

goat anti-cat IgA (Bethyl Laboratories, Montgomery, Texas, USA), diluted to 1 μg/mL in carbonate/bicarbonate coating buffer at pH 9.5. Plates were then blocked for 30 min at 37 °C with 200 μL/well coating buffer containing non-fat dried milk (5% W/V) and Kathon (0.1% V/V), then washed three times with PBS containing Tween 20 (Thermo Scientific, Rockford, Illinois, USA) (0.05% V/V) (PBST). Faecal extracts were diluted in PBS containing non-fat dried milk (5% W/V), normal goat serum (20% V/V), Tween 20 (0.05% V/V), and Kathon (0.1% V/V), starting at 1:1000, and extending to 1:128,000. Standards (Cat Reference Serum, Bethyl Laboratories, Montgomery, Texas, USA) were diluted to 20 ng/mL, then serially diluted two-fold to 0.31 ng/mL. Diluted samples and standards were added at 100 μL/well in triplicate and incubated at 37 °C for 1 h. Plates were washed five times with PBST, then goat anti-cat IgA peroxidase conjugate (Bethyl Laboratories, Montgomery, Texas, USA), diluted 1:100,000 in sample diluent, was added at 100 μL/well and incubated at 37 °C for 60 min. Plates were washed five times with PBST and developed with 100 μL/well of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, Massachusetts, USA) for 2 min. They were read immediately on a Wallac Victor 2 multilabel plate reader (Perkin Elmer Inc. Waltham, Massachusetts, USA). Prism software (GraphPad, La Jolla, California, USA) was used to calculate sample IgA concentrations from a standard curve generated by a four-parameter logistic equation.

For faecal cortisol extraction and quantification, 0.2 g faeces were placed into a 15 mL conical centrifuge tube and stored at −80 °C. Diluent (5 mL of 90% ethanol in distilled water) was added to thawed faecal samples and the resulting liquid vortexed for 30 min. Samples were heated to 98 °C for 20 min and then centrifuged at 2500 × *g* for 10 min. Supernatant was collected and analysed using a chemiluminescence assay to measure cortisol in the Immulite 1000 system (Siemens Diagnostics. Tarrytown, New York, USA). The assay was validated by spiking blank diluent with known amounts of feline cortisol (United States Pharmacopeia. Rockville, Maryland, USA) at low, medium and high concentrations. In each case spiked values were within the recommended tolerance range. Results are reported as concentrations in wet faeces. Intra-assay coefficients of variability (CVs) were 5.4% and 7.1% for s-IgA and cortisol, respectively. S-IgA and cortisol inter-assay CVs were 9.1% and 7.9%, respectively.

2.5. Statistical analyses

The main goals of our statistical analyses were to (1) extract behavioural groups from our dataset, (2) examine clusters of cats associated with each, (3) determine the relationships between individual behaviours, S-IgA and cortisol and differences between clusters for these two measures.

2.5.1. Initial reduction of behavioural data

We first reduced and transformed our dataset to *Z* scores to meet the assumptions of Principal Component Analysis. Spearman Rank analysis was used to combine behaviours with apparently similar motivations and with statistically significant correlations (Spearman's Rank Order Correlation (r_s) between 0.3 and 0.9, $P \leq 0.05$) into new behavioural categories. We eliminated behaviours with large numbers of zero values (>10%) when aggregated over the 3 days. We determined the appropriateness of combining the samples with and without the box using Student's *t*-test, with the combined dataset tested for normal distribution by the Anderson-Darling Test.

Generalized estimated equation (GEE) and a Repeated Measures ANOVA were performed to determine if behaviours were consistent over time. The mean, s.d., s.e. and N for the duration of each behaviour were examined for each day. As some differences were found, we used *Z* scores for each cat on each day.

2.6. Principal component analysis

To determine the structure of our data set, the Matrix (63 observation days by 24 variables) was subjected to PCA (without rotation). Sampling adequacy was determined by the Bartlett test of sphericity and the Kaiser–Meyer–Olkin test.

2.6.1. Biplot analysis

The relationships between variables and between cases (cats) and variables within each dimension were examined by Classical Biplot Analysis. According to this methodology (Gabriel, 1971; Kohler and Luniak, 2005; Salinas et al., 2013), the projection (length) of each vector (line) in the biplot approximates the variances of the variable. Vectors with endpoints close to the perimeter of the biplot have high variance and are therefore better represented within the dimension, whereas very short vectors are considered multivariate outliers. The angle between vectors (the cosine) is equal to the Pearson coefficient and therefore indicates the direction and strength of association between variables. A 90° angle indicates no correlation (cos = 0). An acute angle indicates a positive correlation, reaching a perfect correlation as the angle declines (cos = 1). Obtuse angles signify a negative correlation, reaching a perfect correlation at 180° (cos = −1).

Further, to establish the clusters, the coordinates of the symbol (representing cats) and their characteristics (source, sex, age, sterilization status) within each biplot were used to determine their representation within the dimensions, and the distance between these symbols was used to approximate the similarity between cats.

2.6.2. Cortisol and S-IgA

GEE was used to determine change in cortisol and S-IgA over time and to examine the association between cortisol and S-IgA.

2.6.3. Behaviour, cortisol and S-IgA

We performed a GEE comparing means for cortisol and S-IgA in the presence or absence of each behaviour.

2.6.4. Clusters of cats, cortisol and S-IgA

Differences in mean values of S-IgA and cortisol for each cluster of cats were examined using GEE, thereby accounting for the correlation between days for each cat. A GEE was

performed to determine if there was a significant difference in the $\log_e$ IgA and $\log_e$ cortisol levels for sex, source, sterilization status and age variates. Analyses were conducted in XLSTAT-Pro 2010 (Microsoft Corp, USA) and R (version 2.14.11), using the function geeglm from geepack (Halekoh et al., 2006). Results were considered significant at alpha ≤ 0.05.

3. Results

3.1. Initial reduction of behavioural variables

The Spearman Rank analysis using behaviours with apparently similar motivations and statistically significant correlations (Spearman's Rank Order Correlation (r_s) between 0.3 and 0.9, $P \leq 0.05$) resulted in three new behavioural categories (Hiding, Flat and Escape bouts). 'Hiding' represented the sum of times spent behind the box, in the box but not visible, behind or in the litter and under the towel. 'Flat' represented the sum of time spent in two similar postures (ventral flat and stand flat). 'Escape bouts' was constructed by adding frequencies of standing on hind limbs while pawing at the wall/floor or cage door, upside down posture and attempts to open the door latch. In addition, we constructed two separate categories (Front escape and Front sit) to distinguish cats spending time at the front of the cage while trying to escape or while sitting quietly. Seven behaviours indicated in Table 1 were discarded due to the large number of zero values (>90%) over the 3 days of recording.

The set of behaviours used for further analyses included the new categories: hiding, flat, escape bouts, front escape and front sit, along with those retained from the original ethogram (Table 1).

3.1.1. Combining samples of cats with and without the box

After transformation of the data to Z scores ($n-1$), samples (box/no box) were compared by Student's t-test for the 24 variables (behaviours). No significant differences between samples were found (box: mean = -0.012 ± 0.15; no box: mean = -0.001 ± 0.26, difference 0.011, 95% CI: -0.11 to 0.14, $P > 0.05$) and normality was established by Anderson-Darling ($A^2 = 0.415$, $P = 0.31$).

3.1.2. Exploring the amalgamation of behaviours over time

Based on Generalized estimated equation (GEE) and repeated measures ANOVA, most behaviours did not change over days 1, 3 and 5 ($P \geq 0.17$), with the exception of walk, sleep, friendly to human, and eat/drink, which all increased significantly over time ($P < 0.05$).

3.2. Principal component analysis

A PCA analysis of the reduced data set (Matrix: 63 by 24) extracted 4 axes with Eigen values >1. The first axis contrasted two groups of six behaviours (negative loadings > −0.7) and 10 behaviours (positive loadings >0.5). The second axis included eight behaviours with positive loadings >0.7. Aggression and meow (Axis 2) also had loadings

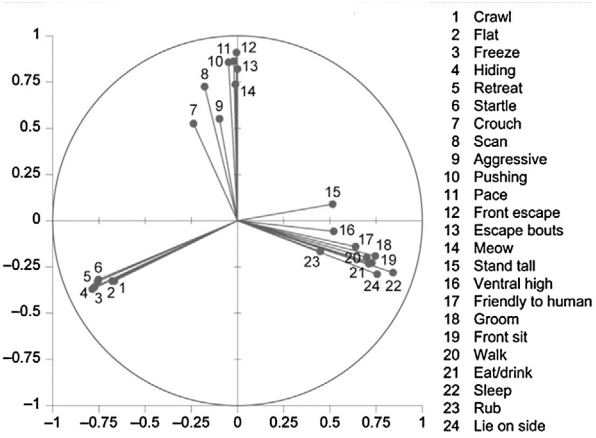

Fig. 1. Grouping of behaviours into a three dimensional model extracted by Principal Component Analysis (without rotation) of the matrix (63 observation days × 24 variables). Groups were labelled dimension 1 (bottom left quadrant), dimension 2 (top quadrant) and dimension 3 (bottom right quadrant).

≥0.5 on axes 3 and 4, respectively; however their associated squared cosine values were <0.4. Thus only Axes 1 and 2 were retained which together explained 67.8% of the variability in the data. Adequacy of the sample was established by Bartlett's sphericity test ($\chi^2 = 315.8$, d.f. 276, $P < 0.0001$, alpha 0.05) and the Kaiser–Meyer–Olkin test (KMO = 0.76). The three groups of behaviour were labelled dimension 1 (bottom left quadrant), dimension 2 (top quadrant) and dimension 3 (bottom right quadrant) (Fig. 1).

3.3. Biplots

3.3.1. Relationship among variables

Dimension 1 included the behaviours with loadings >−0.7 on Axis 1 (hiding, freeze, flat, startle, crawl and retreat from humans, Fig. 1, bottom left quadrant). The PCA for the matrix (63 × 6) extracted one component with Eigen value >1 explaining 80.5% of the variability in the data. Sampling adequacy was determined by Bartlett's sphericity test ($\chi^2 = 24.9$, d.f. 15, $P < 0.0001$) and Kaiser–Meyer–Olkin measure of sampling adequacy (KMO = 0.85). The projection of the vectors indicated that all were well represented within this dimension. Extreme vectors forming an acute angle ($\llcorner 39^\circ$, cos = 0.8) indicated a strong positive correlation ($P \leq 0.01$) among all D1 behaviours. Representation in order of importance (based on coordinates of vector endpoints) was flat > freeze > hiding > crawl > startle > retreat from human.

Dimension 3 included the behaviours with loadings >0.5 on Axis 1 (lie on side, sleep, ventral high, friendly to humans, walk, eat, groom, rub, stand tall and front sit, Fig. 1). PCA extracted two components with Eigen values >1 that explained 63.8% of the variability in the data. Sampling adequacy was good according to the Bartlett's sphericity test (χ^2 critical = 61.7, d.f. 45, $P < 0.0001$) and the Kaiser–Meyer–Olkin measure of sampling adequacy was 0.84. As illustrated in Fig. 2b, ventral high was poorly represented (very short vector), suggesting that it may be a multivariate outlier. Representation of other behaviours in

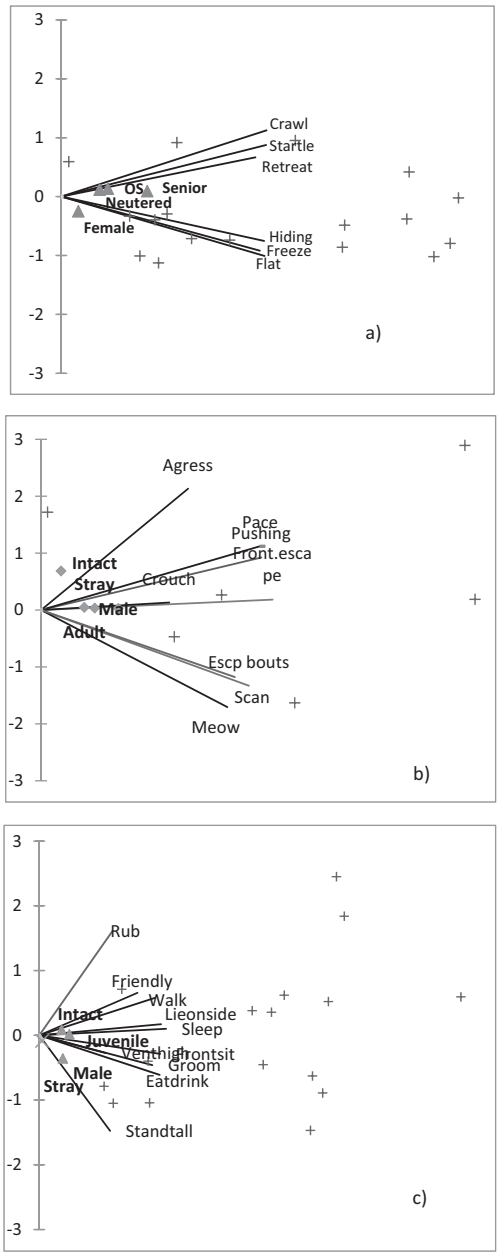

Fig. 2. (a) Biplot of dimension 1 (matrix of 63 observation days by 6 behaviours) Bartlett's sphericity test ($\chi^2 = 24.9$, d.f. 15, $P < 0.0001$) and Kaiser–Meyer–Olkin measure of sampling adequacy (KMO = 0.85). Owner surrendered (OS). (+) represents all cats with Z scores above 0 for this dimension. (b) PCA biplot of dimension 2 (matrix of 63 observation days by 8 behaviours). Bartlett's sphericity test ($\chi^2 = 41.3$, d.f. 28, $P < 0.0001$) and Kaiser–Meyer–Olkin measure of sampling adequacy (KMO = 0.74). Agress (Aggression to humans), (+) represents all cats with Z scores above 0 in this dimension. (c) PCA biplot of dimension 3 (matrix of 63 observation days by 10 behaviours). Bartlett's sphericity test ($\chi^2 = 61.7$, d.f. 45, $P < 0.0001$) and Kaiser–Meyer–Olkin measure of sampling adequacy (KMO = 0.84). Friendly (Friendly to humans), (+) represents each cat with a Z score above 0 in this dimension.

order of importance (based on coordinates of vector endpoints) was sleep > lie on side > front sit > groom > walk > eat/drink > friendly to humans > stand tall > rub. Behaviours sleep to front sit ($\angle \leq 40^\circ$, cos = 0.8) were strongly correlated with each other, whereas rub and stand tall showed weaker association to the group.

Dimension 2 included the eight behaviours with loadings >0.7 on axis 2 (meow, scan, escape bouts, front escape, pacing and pushing, crouch and redirected aggression, Fig. 1). PCA of the matrix (63×8) extracted two components with Eigenvalues >1 explaining 82.1% (dimension 1 = 61.9% and dimension 2 = 20.3%) of the variability in the data. Sampling adequacy was determined by Bartlett's sphericity test ($\chi^2 = 41.4$, d.f. 28, $P < 0.0001$). Kaiser–Meyer–Olkin measure of sampling adequacy (KMO) was 0.732.

Meow, escape bout and scan showed loadings >0.8 (cos > 0.7). Front escape showed a slightly weaker loading on dimension 1 (loading, 0.65 cos = 0.4) than dimension 2 (loading, 0.71 cos = 0.5). Crouch had a very weak loading on both Axes (<0.2, cos < 0.1).

The biplot (Fig. 2c) confirmed that projection of the vectors revealed a good representation of all variables, with the exception of crouch (very short vector) which was thus considered an outlier. Behaviours meow, scan and escape bouts were strongly associated to each other (vector $\angle 10^\circ$, cos = 0.98), as were aggressive to humans, pace, and pushing (vector $\angle 30^\circ$, cos = 0.86). However, extreme vectors of each substructure ($\angle 80^\circ$, cos = 0.1) showed a very weak association between these two behaviours (meow and aggression), whereas both substructures were strongly associated to front escape ($\angle \leq 50^\circ$, cos $\geq$ 0.6).

3.3.2. *Characteristics of cats within each dimension*

Clusters of cats were labelled according to their association to each dimension (Clusters D1, D2 and D3). Of the 63 observation days, 44%, 19% and 37% clustered in dimensions 1 to 3, respectively. Cluster D1 (Fig. 2a) was best represented by the characteristics owned, senior, neutered and female. Cluster D2 (Fig. 2b) was best represented by the characteristics adult, male and intact, and Cluster D3 (Fig. 2c) was best represented by the characteristics juvenile, female and intact.

3.4. *S-IgA and cortisol*

There was no correlation between S-IgA and cortisol (GEE, $P = 0.37$), and no change over time for either S-IgA (GEE, $P = 0.80$) or cortisol (GEE, $P = 0.57$). When we compared the first time point to the other time points using a t-test there was no significant difference for S-IgA ($P = 0.11$) or cortisol ($P = 0.15$).

3.4.1. *Physiological measures in the presence or absence of behaviour*

The GEE analysis performed to compare cortisol and S-IgA when the behaviours were present versus absent showed that cortisol means were significantly greater when the behaviours pace, pushing, aggression (D2) and walking and friendly to humans (D3) were present rather than absent ($P < 0.05$). S-IgA means were significantly

Table 2
Comparison of mean cortisol and S-IgA (μg/g wet faeces) values according to whether behaviours were absent or present. Front.escp = Front escape, Aggres = Aggressive to human, Ventr.high = Ventral high.

Behaviours	Cortisol					S-IgA				
	Absent		Present		*P* value	Absent		Present		*P* value
	Mean	*N*[1]	Mean	*N*[1]		Mean	*N*[1]	Mean	*N*[1]	
Hiding	3.83	32	3.10	31	0.21	1017	32	356	31	<0.001
Flat	3.9	28	3.13	35	0.11	1065	28	393	35	<0.001
Freeze	4.15	22	3.11	41	0.04	1304	22	363	41	<0.001
Crawl	3.70	46	2.87	17	0.44	851	46	260	17	0.01
Startle	3.92	34	2.94	29	0.46	997	34	334	29	<0.001
Retreat	3.87	34	3.00	29	0.44	1001	34	329	29	<0.001
Crouch	3.5	13	3.47	50	0.93	1056	13	597	50	<0.001
Front.escp	3.35	46	3.79	17	0.06	795	46	412	17	<0.001
Pace	3.29	51	4.23	12	<0.001	778	51	324	12	<0.001
Escape bouts	3.34	43	3.75	20	0.20	726	43	619	20	0.02
Pushing	3.16	41	4.05	22	<0.001	832	41	430	22	<0.001
Meow	3.08	29	3.81	34	0.34	688	29	695	34	0.01
Scan	3.78	29	3.21	34	0.82	987	29	440	34	0.07
Aggres	3.37	57	4.43	6	0.05	749	57	144	6	<0.001
Front.sit	3.31	17	3.53	46	0.80	230	17	863	46	<0.001
Ventr.high	3.10	4	3.50	59	0.38	309	4	718	59	0.05
Stand tall	2.89	10	3.58	53	0.22	196	10	785	53	0.01
Lie on side	3.46	39	3.5	24	0.58	354	39	1241	24	<0.001
Walk	3.48	19	3.47	44	0.02	233	19	890	44	<0.001
Sleep	3.57	13	3.45	50	0.43	251	13	806	50	<0.001
Rub	3.30	47	3.97	16	0.48	475	47	1328	16	<0.001
Friendly	3.16	36	3.89	27	0.02	314	36	1195	27	<0.001
Eat/drink	3.23	24	3.62	39	0.08	301	24	932	39	<0.001
Groom	3.34	21	3.54	42	0.90	260	21	908	42	<0.001

[1] *N* = number of cats exhibiting this behaviour.

different for all behaviours (dependent on whether the behaviour was absent or present) ($P < 0.02$), with the exception of scan ($P < 0.07$) (Table 2).

3.4.2. Clusters, S-IgA, cortisol

S-IgA values were significantly greater ($P < 0.001$) for Cluster D3 cats (mean $7.1 \pm 0.5 \log_e$ μg/g), than D1 ($5.6 \pm 0.6 \log_e$ μg/g) and D2 ($5.6 \pm 0.7 \log_e$ μg/g) cats. S-IgA concentrations of Clusters D1 and D2 cats did not differ ($P > 0.05$). Cortisol values were not significantly different for Clusters D1 ($3.0 \pm 1.0 \log_e$ μg/g), D2 ($4.2 \pm 2.0 \log_e$ μg/g) and D3 ($3.7 \pm 1.6 \log_e$ μg/g) (Estimate 0.13, SD 0.31, Wald 1.67E − 01, $P = 0.68$) cats. There was no significant effects of sex, source, neuter status or age on cortisol or S-IgA ($P > 0.05$, GEE analysis).

4. Discussion

The objectives of this study were to explore how the behavioural expression of cats entering a shelter may be clustered into emotional profiles and how such profiles may relate to faecal cortisol levels and secretion of S-IgA.

Our findings indicated the presence of three behavioural dimensions, each with varying effects on cortisol and S-IgA. Due to the paucity of information on emotional behaviour of cats in shelter environments, we discuss our findings in the light of current knowledge on the emotions of cats in response to various experimental procedures and with observational studies in various settings. We then explore the behavioural dimensions and their biomarkers as possible indicators of emotionality for shelter cats. Finally, we discuss the advantages and caveats of our chosen methodology for the construction of indices of emotionality in cats.

One group of highly correlated behaviours ($R > 0.7$) was formed by flat (while lying and standing), freeze (immobile), hiding, crawl, startle and retreat from humans during routine cleaning of the cage. These behaviours are characteristic of the responses observed in cats when faced with a threat or stressful conditions. Cats freeze when presented with an unfamiliar dog (Tsyrlin et al., 1983), startle before a blow or predatory attack (Yeomans and Frankland, 1995) and hide in response to hospitalization, laboratory or animal shelter conditions (Carlstead et al., 1993; Griffith et al., 2000; Kry and Casey, 2007).

Brain stimulation studies have shown that cats have two types of defensive responses, attack and retreat, each with distinct neural (Maeda and Maki, 1989; Ursin, 1964) and immunological correlates (Mori et al., 2001; Kaname et al., 2002). Both include defensive postures, however defensive attack is differentiated from defensive retreat by the expression of behaviours such as paw strike, growling and attack. Although aggression was observed in the study (dimension 2), it was not associated with defensive behaviours. Cluster D1 did not show aggressive responses even when provoked (approached by a human and cleaning of the cage), suggesting defensive retreat.

If the behaviours in dimension 1 were motivated by anxiety, our S-IgA findings coincide with the inhibition of S-IgA reported for anxious mice (Rammal et al., 2010), humans (Graham et al., 1988; Groer et al., 1994) and dogs (Skandakumar et al., 1995). However, the lack of correlation to cortisol is not supported by findings in humans, for which chronic anxiety has been associated with hypocortisolism

(Steudte et al., 2011), nor in a study of laboratory cats in which hiding caused a reduction in cortisol (Carlstead et al., 1993).

The group of behaviours contrasting with defensive retreat on the first dimension (Fig. 1) included sleep, eat, groom, walk, sitting at the front of the cage observing activities, rub, friendly response to human and also postures such as lie on side and stand tall. This dimension is in agreement with the Cat Stress Score's (CSS) lowest level of stress (fully relaxed) (Kessler and Turner, 1997). Thus the elevated cortisol in association with some of the behaviours included in this dimension (friendly, walk and eat) may be surprising. However, stress can be regarded as a physiological response to any change or demand on the body that is psychologically positive (eustress), negative (distress) or neutral (Selye, 1976; Paul et al., 2005). For example, the behaviour 'friendly response to human', which described the behaviour of cats during the routine cleaning of the cage, commonly involved the cat getting up and approaching the human, rubbing and eating. Similarly, the process of awakening and getting out of bed increases cortisol in humans (Pruessner et al., 1997). The anticipation of food (related in this study to human approach) can increase cortisol, with the levels remaining high during feeding (Saul et al., 2011). Thus the positive relationship between cortisol and these behaviours may have been due to increased activity and anticipation of food, as well as conceivably pleasure and excitement at the arrival of the human (Balcombe, 2009; Panksepp, 2011).

According to motivational theory the coordinates of these behaviours (in the opposite quadrant from defensive) should represent low arousal of the defence system (calm/relaxed), rather than pleasure/excitement related to the appetitive (reward) system (Mendl et al., 2010). The elevated S-IgA in this dimension may demonstrate a positive coping style (Boissy et al., 2007). In humans a positive mood stimulates S-IgA (Watanuki and Kim, 2005; Barak, 2006; Marsland et al., 2007).

The second dimension included behaviours such as persistent meowing and visual scanning of the cage, pacing, pushing items around the cage, escape bouts and short bursts of aggression, that are consistent with irritability or redirected aggression (Beaver, 2004). However, the biplot analysis revealed two subgroups that were qualitatively different and with different association to cortisol: (1) persistent meowing, scanning and short bouts of escape behaviour (on hind limbs, pawing at floor/walls) and (2) pacing, pushing and aggression within the same PCA subspace (Fig. 2b).

This dimension has similarities to a group of behaviours elicited during hypothalamic stimulation of stereotypic restlessness (Tashiro et al., 1985; Mori et al., 2001). The authors describe restlessness as "arousal followed by walking restlessly in the cage, meowing, peeping and inserting a paw into a crevice of the cage and escaping if the door was opened, together with indicators of stress such as pupil dilation and increased heart rate" (p. 1088 and p. 326, respectively). Such restless behaviours represent low arousal of the reward system (frustration) normally occurring in uncontrollable conditions, such as delay or omission of an expected reward (e.g. food) (Arnone and Dantzer, 1980), and thwarting of ethological needs, (Duncan, 1970). Although aggression is classified as a fear behaviour in the CSS (Kessler and Turner, 1997), aggression observed in this study was congruent with irritable (or redirected) aggression in cats (Beaver, 2004). Persistent meowing and pacing have been observed in laboratory cats in response to an unpredictable feeding schedule (Carlstead et al., 1993). Evidence of frustration in shelter cats is sparse, but persistent meowing has been correlated with destructive behaviour (pushing in our study) in shelter cats following confinement (McCune, 1992). In accordance with studies of laboratory cats in captivity (Carlstead et al., 1992), cortisol was higher in cats engaged in pacing, pushing and aggression (Table 2).

While the link between restlessness or frustration and cortisol has not been previously examined in shelter cats, elevated cortisol has been reported in aggressive, stray female cats (Finkler and Terkel, 2010) and laboratory cats subjected to unpredictable feeding schedules (Carlstead et al., 1993). Similarly to the defensive dimension, this group of behaviours was negatively correlated with S-IgA. In humans it has been shown that lack of control in the work place inhibits S-IgA and increases incidence of upper respiratory infections (Schaubroeck et al., 2001). Furthermore, if we accept the notion that this group of cats were frustrated, representing low arousal of the appetitive/reward system, our findings are in accordance with those showing that pleasure/happiness (high arousal of the appetitive/reward system) increase secretion of S-IgA, e.g. during sexual activity (Charnetski and Brennan, 2004).

Our model appears consistent with the motivational framework model of emotions proposed by Mendl et al. (2010). A key difference, however, is the inclusion of specific behaviours as indicators of the emotions and the supporting evidence provided by immune parameters.

Adult stray intact males were more likely to engage in restless behaviour and redirected aggression than juvenile stray males and owned senior females. Adult males, whether stray or confined to a home, tend to have a wider territory than females and juveniles (Bradshaw, 1992; Bernstein and Strack, 1996). We hypothesized that these cats may have more difficulty adapting to confinement particularly when in close proximity to other cats. Our findings also showed that owned senior females tended to show more defensive behaviour than juveniles or adult cats of either sex. The presence of unfamiliar cats can be distressing for cats not socialized to other cats (Kessler and Turner, 1999). We hypothesized that owned cats may have had less experience with other cats and were therefore more distressed by their presence. Furthermore, seniors may be less adaptive to changes in lifestyle and routine (Landsberg et al., 2011). Stray juveniles on the other hand tend to live in colonies with other kittens and with several females (McDonald et al., 2000) and adapt well to change compared to adults (Lord et al., 2008)

4.1. Limitations of the study

The small sample size of this study limits application to a wider cat population. Furthermore, using multiple measures from the same individuals for the correlation

matrix is considered pseudoreplication by some (e.g. Budaev, 2010), but our GEE and repeated measures ANOVA accounted for correlation between days.

Some of the cats did not produce a stool for several days following admission to the shelter, which led to the removal of many observation days. In addition, our decision to quantify S-IgA from stools produced within 24–48 h post observation was based on the known time course between a stress event and the appearance of cortisol in cat faeces (Graham and Brown, 1996; Schatz and Palme, 2001), which may not be appropriate for S-IgA. It may not be a co-incidence that six cats were removed from the No box treatment and none from the box treatment for failure to defecate, as stress levels may have been greater without the box (Gourkow and Fraser, 2006). Similarly the absence of behavioural differences between the two groups may have been due to this selective removal. Moreover, whilst the S-IgA inter-intra assay variability ($\leq$10%) may have had clinical relevance (Murray et al., 1993), further research is needed to determine whether the S-IgA differences observed between dimensions are of clinical relevance to URI. In addition methods to quantify the production of antigen-specific IgA at mucosal surfaces are unclear, in particular faecal sampling method and reference to dry faecal weight.

5. Conclusions

We identified three dimensions of behaviours and their physiological correlates in a group of shelter cats. S-IgA was significantly lower in the first two dimensions, compared with the third. Although it was correlated to specific behaviours, faecal cortisol was not significantly different in the three dimensions. Determining the underlying affective states motivating the behaviour of animals in this way may be useful in the management of URI in shelter cats and contribute to the development of more targeted interventions for their mental well-being.

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