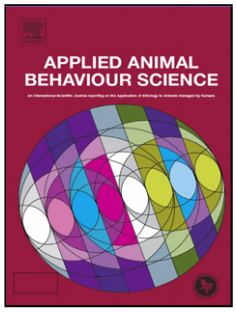

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Original Article

Baseline and postprandial concentrations of cortisol and ghrelin in companion dogs with chronic stress-related behavioural problems: a preliminary study.

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Highlights

- Stress response varied between two chronic stress-related behavior problems.
- Cortisol and ghrelin decreased in dogs with separation anxiety after food intake.
- Comfort foods may help alleviating stress in dogs with separation anxiety.
- Dogs with aggression towards family may fail to suppress ghrelin after eating.

Abstract

Ghrelin has been proposed as an essential element regulating the stress response in both humans and rodents. The aim of this work was to study the relation between cortisol and ghrelin in companion dogs showing chronic stress-related behaviour problems and the effect of the administration of high energy palatable food on these hormones. Baseline and post-prandial serum concentrations of both hormones were analyzed in a group of stressed companion dogs (n=16) showing social conflict-related aggression (SCA, n=10) or separation anxiety (SA, n=6), and in a group of non-stressed control companion dogs (n=16). Significant differences (p<0.05) between groups emerged for post-prandial levels, with SCA dogs showing higher cortisol than the control group. The change in cortisol levels (%) after eating in this group was positive, differing from the SA and control groups, which decreased their cortisol after food consumption. Ghrelin also decreased significantly in SA dogs after eating.

Taking together, these findings suggest that a parallel meal-induced decrease in both cortisol and ghrelin occurred in the SA group, but not in the SCA group, pointing towards a failure to suppress ghrelin (and cortisol) after intake in the latter. Thus, even though SCA and SA were considered chronic stress-related behaviour problems, their different nature may affect animals in their stress response to the administration of palatable food. The possible alleviatory effect of food consumption, particularly "comfort foods", after the owner is back home in SA dogs, as well as the changes in dogs' eating behaviour in response to emotional states or stress ("emotional eating") will require further studies.

Keywords: Aggression; Anxiety; Cortisol; Dog; Ghrelin; Stress.

1. Introduction

Numerous behavioural problems in dogs involve stress and anxiety. In fact, some forms of aggression, separation stress disorders and noise phobias are classified as anxiety-related problems by several authors (Overall, 2013; Ogata, 2016; Wormald et al., 2017). Usually, suffering from these problems occurs for long periods of time, even the whole life of the animal, which entails the dogs living under a permanent condition of chronic stress. Habitual exposure to anxiety-inducing stimuli for a certain period of time can negatively affect the physical, mental or social health of dogs (Dreschel, 2010; Mills et al., 2014) and thus reduce their quality of life. Daily stress, fears and anxieties have been clearly shown to be related to health problems (mainly skin diseases) and shortage of lifespan in dogs (Dreschel, 2010; Shihab et al., 2011; Snitcofsky and Mentzel, 2015). In addition, comorbidity of different anxiety-related problems is frequently observed in the same animal (Bamberger and Houpt, 2006; Fatjó, 2007; Yalcin and Batmaz, 2007; Tiira et al., 2016). Finally, the fact of having a dog with these problems may prompt owners to use aversive training methods or inappropriate behavioural modification techniques that can compromise welfare (Blackwell et al., 2008; Herron et al., 2009; Yeates, 2012).

Besides the just cited direct effects on the animal, it is important to mention that behavioural problems are one of the main causes for dog relinquishment as well as for returning to shelter after being adopted (Salman, 2000; Shore, 2005; Diesel et al., 2008 and 2010; Coe et al., 2014; Hill and Murphy, 2016), and also for euthanasia (RSPCA, 2007). Considering all of this, behavioural problems have been pointed out as an important canine welfare concern (Fatjó et al., 2007; Sonntag and Overall, 2014; Col et al., 2016; Luño et al., 2017).

Blood or salivary cortisol levels have been traditionally used to measure stress either acute or chronic in dogs (Beerda et al., 1996; Kobelt et al., 2003; Hellhammer et al., 2009; Hennessy, 2013). Regarding behavioural problems, a previous study showed concentrations of plasma cortisol in aggressive dogs, especially those showing aggression towards the family members, significantly higher than those found in a control group (Rosado et al., 2010 and 2011). In fact, dogs displaying owner-directed aggression frequently display ambivalent signals during conflict situations, which are believed to be indicative of high arousal and suggest the presence of fear or stress (Bamberger and Houpt, 2006; Reisner et al., 2007). In the case of dogs showing separation anxiety, to the authors' knowledge, basal cortisol has not been analyzed in comparison with a control population, but salivary cortisol has been used to measure stress during different test conditions related to separation from the owner (see Mongillo et al., 2013; Shin and Shin, 2016).

More recently, ghrelin has been suggested as an essential element regulating the hypothalamic pituitary adrenal (HPA) axis when the individual is exposed to both acute and chronic stress. Ghrelin is a hormone (28aa peptide) mainly synthesized in the stomach that acts as a hunger signal for stimulating food intake (Yokoyama et al., 2005). The ghrelin receptor (GHSR) is expressed throughout the brain, including in feeding and metabolism-associated areas, in the pituitary gland and in stress response-associated brain regions such as the amygdala. Ghrelin is increased in both acute and chronic stress situations (Perelló and Zigman, 2012) and this increase takes place together with a stress-induced rise in glucocorticoids (Spencer et al., 2015). Previous studies in humans and rodents have shown that abnormalities in the ghrelin system contribute to the development of stress response-related mood disorders (Perelló and Zigman, 2012).

Both cortisol and ghrelin are purported to be related to so called "emotional or stress-related eating", that is, the change in the eating behaviour in response to (negative) emotional states or stress as a way of coping with that situation (McMillan, 2013). Emotional eating especially involves the intake of food with specific characteristics, the so called "comfort foods", which are rich in energy, fat or sugar, and are consumed to obtain psychological comfort and emotional wellness (Dubé et al., 2005; la Fleur et al., 2005; Zellner et al., 2006). In rats, the intake of these foods has been demonstrated to stimulate the hypothalamic release of endogenous opioids (Dum et al., 1983; Mercer and Holder, 1997) and to decrease cortisol levels, so the intake of these comfort foods has been considered as a coping strategy for stressed individuals (Wiener et al., 1983; Foster et al., 2009; Ulrich-Lai et al., 2010). On the other hand, ghrelin has been suggested to increase the rewarding properties of certain foods by acting on the mesolimbic dopamine reward system in mice (Chuang and Zigman, 2010). Thus, intact ghrelin signaling would be required for normal eating behaviour and body weight responses, especially to hedonically rewarding high-fat diets (Perelló and Zigman, 2012).

It has been observed that the meal-induced fall in plasma cortisol occurs together with a fall in plasma ghrelin, which is consistent with reports showing that ghrelin promotes glucocorticoid secretion by stimulating ACTH release from the anterior pituitary (revised by Spencer et al., 2015). Raspopow and colleagues (2010) observed that stress-induced elevations in plasma ghrelin found in high "emotional eaters" (so-called due to their experienced food cravings and increased consumption of comfort foods in response to negative emotions and stress), fail to decline acutely following food consumption.

In canine species, ghrelin levels have been studied in relation to the metabolism of growth hormone (Yokoyama et al., 2005; Bhatti et al., 2006) and obesity (Jeusette et al., 2005a;b) as well as in relation to the effect of diet and ovariectomy (Jeusette et al., 2006; Lubbs et al., 2010; Schauf et al., 2016; Schauf et al., 2018). Only one study has analyzed differences in baseline ghrelin and cortisol in dogs with and without compulsive tail chasing (Yalcin et al., 2017). However, no studies exist that focus on the relation of this hormone with stress, either acute or chronic, or emotional eating in dogs. According to a previous survey, a great proportion of owners (82.7%) noticed that their dog showed

emotional eating at some level of intensity, and a high perception of emotional eating was related to some behavioural problems (Luño et al., 2018).

The aim of the present work was to analyze baseline and post-prandial cortisol and ghrelin levels in dogs showing chronic stress-related behavioural problems and a control group. We hypothesized that stressed dogs would show higher baseline cortisol and ghrelin levels than control dogs and that both hormones would decrease after eating a portion of high energy palatable food.

2. Materials and methods

The study was approved by the Ethic Advisory Committee for the Animal Experimentation of the University of Zaragoza with the authorisation reference number PI15/17, and an informed consent was obtained before enrolling any dog in the study.

2.1 Animals

Stressed companion dogs included in the present study were collected from two sources, including those cases attending the Behavioural Medicine Service of the Veterinary Hospital at the University of Zaragoza (HVUZ) and those recruited by means of a questionnaire disseminated via social media (Facebook). This questionnaire was the C-BARQ (Hsu and Serpell, 2003), translated to Spanish and published online using commercially available software (Google questionnaires, Google, USA). Those questionnaires from animals that suited the inclusion criteria (see below) were preselected and owners were invited to attend a face-to-face consultation at the Behavioural Medicine Service. Diagnosis of behavioural problems and final selection of the animals were made by means of a detailed standard questionnaire regarding the behaviour and daily routine of the dogs, made by a specialist in behavioural medicine following common criteria described in text books and clinical experience.

The common inclusion criteria for both the stressed and the control group were: being older than 1-year old, not suffering from illnesses nor consuming any medications (excepting antiparasitic drugs) and having lived with the owners for at least the previous 6 months. The control dogs were recruited

during routine veterinary consultations and they were all healthy individuals. A behavioural consultation was also performed to discard behavioural problems in these dogs.

The specific inclusion criteria for the problem group were: to suffer from at least one chronic stress-related problem such as social conflict-related aggression directed to the family members (SCA), or separation anxiety (SA), for at least the last 6 months, in a high intensity, and with a minimum frequency of presenting symptoms of twice a week. For instance, in the case of aggressive dogs, these symptoms included showing teeth, growling, attempting to bite or biting to the family members in contexts of resource guarding, approaching or manipulating the dog. In the case of dogs with separation anxiety, symptoms included vocalizations, inappropriate elimination, destructiveness and other anxiety signs (intense pacing, panting or hypersalivation), during the absence of the owner. For a reliable diagnosis of SA, a video recording of the dog alone at home was requested. When an animal showed various behavioural problems, including simultaneous SCA and SA, then the main diagnosis was established as the most long-lasting, severe (in the sense of symptoms intensity) and frequently observed problem. The remaining problems were then considered as secondary diagnoses. Interviewed owners of dogs that did not fulfill the inclusion criteria were excluded from the study, but they were advised in detail how to manage the behavioural problem as in a routine behaviour consultation.

Two groups of 16 dogs were recruited. The stressed group consisted of 10 males (8 neutered) and 6 females (3 spayed), with a mean age of 5.5 years old (ranging from 2.7 to 9.5 years), belonging to 11 different breeds and their crosses (Table 1). The control group was made up of 8 males (6 neutered) and 8 females (6 spayed), with a mean age of 5.1 years old (ranging from 1.8 to 8.8 years), and of 13 different breeds, similar in size (apart from breed) to those in the problem group.

2.2 Experimental procedures

During behavioural consultation, selected dogs were examined in order to detect any underlying causative or contributory medical condition to the behavioural problem. A more accurate and

definitive diagnosis was obtained by a complete anamnesis. After this, dogs were offered a small amount of the food that would be used on the test day to check their acceptance.

On the day selected to run the test, the dogs were presented to the hospital at 9:00 AM, after 12-hour fasting. In the consultation room, a 5 ml-blood sample from the jugular vein was obtained, and introduced into an EDTA tube (0.5 ml, for hematology), a heparin tube (1 ml, for biochemistry) and an anticoagulant-free tube with a separator gel (3.5 ml, for serum for pre-prandial cortisol and ghrelin). Just after the first venipuncture, dogs were taken to the test room and, in the presence of their owners, they were offered a portion (1/4 of their daily needs according to the manufacturer's recommendations) of a high-in-energy and palatable food (a/d Hills®). Dogs were offered the food by putting the bowl on the floor. Looking at or disturbing the dog while eating was not allowed, especially for SCA dogs. The eating latency (time to start eating after the food bowl was placed in the floor) and duration (time to finish the food after starting to eat) were registered by a researcher using a chronometer. Once the dog had finished the food, both the owner and the dog were to wait in the test room for 45 minutes until the second blood extraction. The postprandial extraction occurred 45 minutes after eating according to previous studies that have observed that blood concentration of ghrelin decreased from 15 to 60 min postprandial (Schauf, 2015). During this time, the treatment for the behavioural problems of the dog was explained to the owner. Then, again in the consultation room, a second 3.5 ml blood sample was obtained by using the same technique to obtain serum for post-prandial cortisol and ghrelin.

2.3 Laboratory analysis

Hematology and biochemistry were performed immediately at the HVUZ laboratory to confirm the absence of medical problems (ABC Hematological Counter® and IDEXX Catalyst Dx®). Serum was obtained after keeping the tubes at room temperature for 1h to allow clot formation and being centrifuged at 3500 rpm at 4ºC for 10 minutes. Aliquots of serum were then frozen at -80ºC (optimal storage conditions according to Tvarijonaviciute et al., 2013a) and sent to Interlab-UMU (University of Murcia, Spain) for cortisol and ghrelin analyses. All samples were measured in one batch. Serum

cortisol was measured by chemiluminescence (CanineTSH, Immulite, Siemens) and serum ghrelin was determined by a double-antibody sandwich ELISA (Human unacylated Ghrelin ELISA; BioVendor-Labaratorni Medicina a.s., Modrice, Czech Republic). The two methods were previously validated for use with canine serum samples showing the intra-assay coefficients of variation below 10 in all cases (Tvarijonaviciute et al., 2010; Higgs et al., 2014). Cortisol and ghrelin concentrations were expressed in nmol/l and pmol/l, respectively. The change (%) in cortisol and ghrelin concentrations after eating was expressed as a ratio between the difference in the concentrations [post-prandial – baseline] and the baseline concentrations.

2.4 Statistical analysis

Two main types of factors were considered for statistical analysis, the inter-individual factors “group”, “subgroup”, “sex” and “reproductive status” and the intra-individual factor “food”. The inter-individual factor “group” included the categories “control” and “stressed” and the factor “subgroup” included SCA and SA subcategories. The intra-individual factor “food” comprised the “baseline” and “post-prandial” conditions.

The distribution of the variables was studied by the Saphiro-Wilk test which is indicated for small sample sizes, as most of them were found to have a non-normal distribution. Mann-Whitney test was used to evaluate the effect of the factors "group", “sex” and “reproductive status” in baseline levels of ghrelin and cortisol. Kruskal-Wallis test was used to assess the effect of the factor “subgroup” in baseline levels of these hormones. In the case of repeated measures, the Wilcoxon test was applied to evaluate the effect of the factors "group", "subgroup" and "food". Finally, a correlation study was performed among the analyzed parameters using the Spearman correlation test.

Calculations were carried out using the statistical program SPSS 17.0 for Windows (SPSS, Inc.). $p < 0.05$ was considered to denote statistical significance.

3. Results

Demographic information of the group of dogs diagnosed with SCA and SA are described in Table 1. Thus, SCA was diagnosed as the main diagnostic category in the 62.5% (n=10) of the cases, and 31.3% (n=5) of the dogs suffered from SCA and SA at the same time. In addition to these main diagnoses, comorbidity with other behavioural problems was observed in the totally of recruited cases, following this order: aggression towards dogs (75.0%), noise phobia (43.8%), inappropriate elimination (43.8%), aggression towards strangers (31.3%), hyperexcitability (12.5%), compulsive disorders (12.5%), fear or phobia towards strangers (12.5%), and abnormal attention seeking behaviours (6.3%).

Mean concentrations of the studied parameters according to the group are shown in Table 2. Post-prandial cortisol concentrations in the stressed dogs were significantly higher than in the control group ($p<0.05$). When the diagnosis category was considered (Table 3), the SCA group was found to show higher post-pandrial cortisol in comparison with the control group. Non-significant differences according to sex or reproductive status were found (mean values not shown).

Wilcoxon test revealed various significant differences ($p<0.05$) between baseline and post-prandial concentrations (Tables 2 and 3). Thus, the control group showed significantly lower cortisol concentrations after the meal, whereas the stressed group showed significantly lower ghrelin post-prandial concentrations in comparison with the baseline condition. The SA subgroup showed significantly lower cortisol and ghrelin concentrations after eating food.

Mean values for cortisol and ghrelin change (%) after eating food, as well as for eating latency and duration are shown in Table 4. The SCA group showed a positive (increase) mean change (%) both in cortisol and ghrelin concentrations after the meal, whereas in the case of the SA and control groups, this change was negative (decrease). In fact, the cortisol change was significantly different in SCA dogs in comparison with the rest of groups (SA and control). Regarding eating latency, it was observed that the SA group showed a significantly higher latency in comparison with both the control and the SCA groups.

Spearman test revealed several significant correlations which are listed below. Baseline and post-prandial ghrelin were positively correlated (rho=0.945; $p<0.001$) as a whole, and when the studied

dogs were classified according to the group, diagnosis, sex and reproductive status. The cortisol change (%) was negatively correlated with the eating latency (rho=-0.444; p=0.014). In the control group, a positive correlation it was found between eating latency and eating duration (rho=0.645; p=0.007), as well as a negative correlation between baseline cortisol and the cortisol change (%) (rho=-0.718; p=0.002). In the case of the SA group, a negative correlation between eating latency and cortisol change (%) (rho=-0.928; p=0.008), a negative correlation between eating duration and baseline cortisol (rho= -0.943; p=0.005), and a negative correlation between baseline ghrelin and the cortisol change (%) (rho=-0.829; p=0.042) were found. No correlations were found in the SCA group, apart from that between baseline and post-prandial ghrelin.

4. Discussion

In the present study, baseline and post-prandial serum levels of cortisol and ghrelin were measured in a group of dogs showing behavioural problems believed to be associated with chronic stress, in particular social conflict-related aggression (SCA) and separation anxiety (SA), in comparison with a group of normally behaved control dogs. Both entire and neutered dogs were included in the study, and there were not statistical differences in either ghrelin or cortisol levels according to sex or neuter status. Sterilization has been demonstrated not to change ghrelin levels (Jeusette et al., 2006), although increased ghrelin concentrations have been observed after LH peak in intact females (Tvarijonaviciute et al., 2013b). Cortisol and ghrelin mean values in the control group were within the normal range for canine species according to previous studies using the same laboratory methodology (Tvarijonaviciute et al., 2010; Higgs et al., 2014).

Post-prandial cortisol concentrations in the stressed dogs were significantly higher than in the control group ($p<0.05$), and when the diagnosis category was considered, only SCA dogs differed from the control group in this measure. Previous studies have reported higher plasma cortisol levels in dogs showing aggression, especially SCA, than in non-aggressive dogs (Rosado et al., 2010 and 2011), suggesting that these animals may suffer from (chronic) stress. Moreover, the change of cortisol (%) in this group was shown to be positive (i.e., increased), differing from the SA and control group, which

decreased their cortisol values after eating. In contrast with our hypothesis, it seems therefore that the fact of eating high-in-energy and palatable food did not affect the cortisol levels in the SCA dogs, that is, did not act as a comfort food to decrease stress, as previously observed in laboratory animals, pigs and humans (Dubé et al., 2005; la Fleur et al., 2005; Zellner et al., 2006; Figueroa and Salazar, 2017). It is possible however that the quantity of food administered (¼ of daily needs) was not enough to decrease cortisol in SCA dogs and, therefore, it did not act as an effective comfort food to alleviate stress. In this respect, a recent study revealed that pigs under social stress or restraint stress did not prefer sweet solutions (0.5% sucrose solution) over tap water as non-stressed animals significantly did. However, when sucrose concentration was increased (1%), stressed animals preferred this over tap water at the same or to an even greater magnitude than non-stressed animals (Figueroa and Salazar, 2017). Moreover, in women and ewes, it has been reported that high cortisol-responders are more prone to eat more in response to stress than low-cortisol responders (Hewagalamulage et al., 2016).

Differences in ghrelin levels between stressed and control group in both conditions (baseline and post-prandial) were not significant, possibly due to individual variability, as reflected by high standard deviation values (see limitations). In humans, inconsistent results regarding the association between high ghrelin levels and different psyquiatric conditions have been reported, with some authors finding an association with depression (Ozsoy et al., 2013; Akter et al., 2014), and others not observing a relationship with either depression and anxiety (Lawson et al., 2012) or obsessive-compulsive disorders (Emül et al., 2007). In the case of dogs, Yalcin and colleagues (2017) found lower levels of baseline ghrelin in dogs with compulsive tail chasing, pointing to the possible intervention of this hormone in the pathophysiological mechanisms associated with this behavior problem. Nevertheless, ghrelin levels significantly decreased after eating in the SA dogs. Taking together this and the previous findings, they all suggest that a parallel decrease in both ghrelin and cortisol levels occurred in this group, but not in the SCA group. This agrees with previous studies suggesting that the meal-induced fall in plasma cortisol may occur together with a fall in plasma ghrelin (revised by Spencer et al., 2015), as it seemed to occur in the SA and the control groups, whereas a failure to suppress ghrelin (and cortisol) after food consumption may have occurred in SCA dogs. In this sense, a failure to

suppress ghrelin after meal has been associated with a low perceived ability to cope with a stressor and subjective feelings of anxiety and stress in obese woman (Sarker et al., 2013). Moreover, Raspopow and colleagues (2010) observed that stress-induced elevations in plasma ghrelin found in high emotional eaters did not acutely decrease following food consumption in comparison with low emotional eaters. Whether or not the dogs with higher basal ghrelin levels were (high) emotional eaters remains unexplored. Another possible explanation is that 45 minutes were not enough to show differences in this subgroup, even if previous studies have observed a decrease in ghrelin concentrations from 15 to 60 min postprandial (Schauf, 2015). It is also interesting to note that SCA dogs showed a significantly lower eating latency compared to SA group. This might be explained by the fact that these dogs may be in heightened state of arousal when provided with a high-value meal, as in this experiment. In this sense, resources guarding aggression, one of the symptoms of SCA dogs, has been related to rapid ingestion (Jacobs, 2017). Nevertheless, not all SCA dogs in this study showed food protection but aggression when being approached or manipulated by the owners in their daily routines. In addition, the administration of food during the test was performed in a standardized non-threating way. In fact, none of the dogs showed any sign of aggression during the performance of the test. The lower eating latency of SCA dogs may be alternatively explained by an emotional eating motivation, although this hypothesis may deserve future research.

Despite simultaneous decrease in cortisol and ghrelin levels after meal in the SA (and the control group), a correlation between their post-prandial values was not observed. Only a negative correlation between baseline ghrelin and the cortisol change (%) was found in the SA group, suggesting that the higher the baseline ghrelin levels, the more difficult to decrease the cortisol levels after eating. Eating latency was also negatively correlated with the cortisol change (%) in this subgroup, suggesting that the more time to start eating, the lower change in cortisol levels. In general, the SA dogs tended to present longer eating latencies in comparison with the control and the SCA groups, despite the presumed resulting “positive effect” (i.e., a decrease) of food consumption in cortisol and ghrelin levels. This subgroup also showed a negative correlation between baseline cortisol and eating duration, meaning that the higher the baseline cortisol, the faster the consumption of food. This may

support the idea that craving for comfort food might increase when the stress levels are higher, as previously found in other species (Adam and Epel, 2007; Torres and Nowson, 2007; Tomiyama et al., 2011).

The present study shows a number of limitations. The main one is the relatively low number of animals included within each group, especially when diagnostic subcategories were considered. The difficulty for researchers in recruiting dogs that fulfilled the strict inclusion (and exclusion) criteria to participate in the study limited the number of participants. A larger number of animals may have allowed finding more significant differences between groups as the variance in data for the biological parameters (cortisol and ghrelin) would have been reduced. Another limitation is that SCA and SA subgroups were not "pure" since comorbidity with other problems was present, including simultaneous SCA and SA problems. Nevertheless, one of the problems (i.e., SCA or SA) was always clearly more marked than the other one in the sense of severity and frequency of symptoms. Also, the fact that the study was carried out in a possibly stressful environment for the dogs, could have had some effect on the results, even if low stress handling was performed all the time. Performing studies with clinical cases instead of using experimental procedures with laboratory animals might account for these methodological limitations.

5. Conclusions

The present study shows that SCA dogs but not SA were more stressed than a group of control dogs in basal conditions and that their cortisol and ghrelin levels did not decrease after eating, suggesting the possibility of a failure to suppress ghrelin after meals in these dogs and, consequently, to decrease cortisol. On the contrary, both cortisol and ghrelin decreased in the SA dogs after the intake of food, suggesting a positive effect of eating in decreasing stress. Thus, the results suggest that even grouped together as "stressed" dogs, the different nature of SCA and SA problems may affect animals in their HPA response to the administration of a particular food, intentionally selected as a possible comfort food for dogs. This may open the possibility of exploring, among other alternatives, the alleviatory effect of comfort food consuming after the owner is back home to help recovering homeostasis in dogs

with SA, as a part of the therapy. This strategy should be better studied before being recommended as the expectation of a high-value food treat on the owners' return could potentially exacerbate the original behavior problem and lead to long-term problems with canine obesity.

This is the first study that has analyzed the serum levels of an orexigenic hormone, ghrelin, in relation with chronic stress-related behavioural problems in dogs, together with the cortisol measurement. Further studies including a larger number of animals should be performed to explore the role of ghrelin in the regulation of the HPA axis and emotional eating in dogs.

Conflict of interest statement

The authors declare that no conflicts of interest exist in any financial, personal or other relationships with other people or organizations within the years of beginning the submitted work that could inappropriately influence, or be perceived to influence, the work.

Declaration of interests: none.

Authorship

The idea for the paper was conceived by Belén Rosado. The experiments were designed by Sylvia García-Belenguer, Jorge Palacio, Belén Rosado and Isabel Luño. The experiments were performed by Belén Rosado and Isabel Luño. The data were analyzed by Sylvia García-Belenguer, Jorge Palacio, Belén Rosado and Isabel Luño. The paper was written by Belén Rosado and Isabel Luño. The paper was revised by Sylvia García-Belenguer, Jorge Palacio, Belén Rosado and Isabel Luño.

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Table 1.

Demographic information and diagnosis in the group of dogs with chronic stress-related behavioural problems.

Id	Breed	Age	Sex	Weight	BCS	Main diagnosis	Secondary diagnoses
1	Labrador	4yo	iF	28kg	3/5	SCA	Dogs aggression/ Hiperexcitability
2	German Shepherd crossbred	6yo	nM	48kg	3/5	SCA	Dogs and strangers aggression/ Compulsive disorder/ Noise phobia
3	Border collie	5yo	nM	15kg	3/5	SCA	Dogs aggression/ Noise phobia
4	Yorkshire terrier	4yo	sF	3kg	3/5	SA	SCA/ Noise phobia/ Dog aggression/Inappropriate elimination
5	Yorkshire terrier crossbred	8yo	iM	6kg	3/5	SCA	SA/ Dogs aggression/ Noise phobia/ Inappropriate elimination
6	Yorkshire terrier crossbred	8yo	iF	6kg	3/5	SCA	SA/ Dogs and strangers aggression/ Inappropriate elimination
7	Crossbred	5yo	nM	12kg	3/5	SA	SCA/Dogs aggression/Strangers fear
8	Yorkshire terrier	8yo	nM	3kg	3/5	SCA	Dogs and strangers aggression/ Inappropriate elimination / Noise phobia
9	Chihuahua	3yo	iM	2kg	3/5	SCA	Inappropriate elimination/ Attention seeking behavior/ Dogs aggression
10	French Bulldog	9yo	sF	12kg	3/5	SA	Dogs aggression
11	Belgian Shepherd crossbred	3yo	nM	32kg	3/5	SA	Dogs and strangers aggression
12	Pointer crossbred	5yo	nM	18kg	3/5	SA	Strangers fear/ Compulsive disorder
13	Andalusian Hound crossbred	5yo	sF	13kg	3/5	SCA	Strangers aggression
14	Labrador retriever	3yo	iF	28kg	4/5	SCA	SA/ Dogs aggression
15	Labrador retriever	5yo	nM	40kg	3/5	SCA	Hiperexcitability/ Noise phobia/ Inappropriate elimination
16	Maltese	5yo	nM	4kg	3/5	SA	Inappropriate

		elimination/ phobia	Noise

iM/iF: intact male/intact female; nM/sF: neutered male/spayed female.

SCA: social conflict-related aggression towards the family members; SA: separation anxiety.

Table 2.

Mean (standard deviation) concentrations of baseline and post-prandial cortisol and ghrelin according to the group of study.

Parameter	Group	Baseline			Post-prandial			
		Mean	(SD)	p^a	Mean	(SD)	p^a	p^b
Cortisol (nmol/l)	Stressed	88.29	(52.42)	NS	74.49	(49.66)	0.050	NS
	Control	71.73	(38.63)		41.39	(13.80)		0.017
Ghrelin (pmol/l)	Stressed	272.82	(194.09)	NS	261.46	(265.51)	NS	0.031
	Control	227.30	(130.12)		200.10	(144.74)		NS

[a]Mann-Whitney test (differences according to the inter-individual factor "group");

[b]Wilcoxon test (differences according to the intra-individual factor "food"). NS: non-significant difference.

Table 3.

Mean (standard deviation) concentrations of baseline and post-prandial cortisol and ghrelin according to the diagnostic category.

Parameter	Group	Baseline			Post-prandial			
		Mean	(SD)	p^a	Mean	(SD)	p^a	p^b
Cortisol (nmol/l)	SCA	77.25	(44.14)	NS	93.81	(57.94)^C	0.050	NS
	SA	107.60	(63.46)		46.90	(16.55)		0.046
	Control	71.73	(38.63)		41.93	(13.80)^{SCA}		0.017
Ghrelin (pmol/l)	SCA	273.27	(204.95)	NS	299.82	(319.44)	NS	NS
	SA	272.05	(193.44)		197.58	(141.25)		0.043
	Control	227.30	(130.12)		200.10	(144.74)		NS

[a]Kruskal-Wallis test (differences according to the inter-individual factor "sub-group" including the control group); [b]Wilcoxon test (differences according to the intra-individual factor "food").

NS: non-significant difference. Different letters in each line indicate significant differences between groups (capital letters: $p<0.05$).

SCA: social conflict-related aggression towards the family members; SA: separation anxiety. C: control.

Table 4.

Mean (standard deviation) values for cortisol and ghrelin change (%) after eating food, and for eating latency and duration.

Group	Cortisol change (%)	Ghrelin change (%)	Eating latency (seconds)	Eating duration (seconds)
SCA	36.1 (88.3)$^{SA, C}$	0.5 (38.9)	1.6 (4.6)^{SA}	66.11 (75.6)
SA	-42.7 (43.0)^{SCA}	-28.1 (23.8)	102.8 (160.2)^{C,SCA}	81.17 (63.7)
Control	-18.4 (59.8)^{SCA}	-12.8 (26.5)	8.6 (18.8)^{SA}	75.8 (88.3)

Kruskal-Wallis test (differences according to the inter-individual factor "sub-group" including the control group). Different letters in each line indicate significant differences between groups ($p<0.05$).

SCA: social conflict-related aggression towards the family members; SA: separation anxiety; C: control.