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Effect of casozepine administration on stress in dogs during a veterinary examination – A randomized placebo-controlled trial

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ABSTRACT

The aim of the study was to investigate the stress-reducing effect of a casozepine before a veterinary examination in dogs. It should be examined whether the dogs are less stressed during a standardized veterinary examination after an oral application of casozepine over 2 days and whether the administration has an influence on the salivary concentrations of the stress hormones vasopressin and cortisol. Across the study group (n=36), a significantly lower stress score (P=0.0026) and lower mean (P=0.01) and maximum (P=0.024) pulse rates were seen at follow-up after casozepine administration, in contrast to the placebo group (n=26). Salivary vasopressin concentrations increased during follow-up in the placebo group (P=0.04), whereas they remained the same in the casozepine group. Cortisol concentrations increased during follow-up in the casozepin group (P=0.01). The results indicate that although dogs in both groups remained excited at follow-up, short-term casozepine administration before a veterinary visit had a weak stress-reducing effect in dogs based on subjective stress scoring and pulse rate.

Introduction

Stress, which can be caused by an unfamiliar environment or separation from the owner, is a common problem in dogs and cats (Beerda et al., 1996; Beerda et al., 1999; Bowman et al., 2015; Juodzente et al., 2018). A visit to the veterinarian or veterinary clinic is a particularly stressful situation for a number of animals (Hekman et al., 2012), although it is often unavoidable. The opportunities to counteract stress in an unfamiliar situation in everyday life are limited. Studies have already been successfully carried out in dogs to reduce stress using sedatives such as the α2 agonist dexmedetomidine (Korpivaara et al., 2017) or an anticonvulsant such as the GABA analogue gabapentin (Stollar et al., 2022).

A therapeutic approach to counteract psychological stress in unfamiliar everyday situations is the peptide casozepine, which has been shown to have anxiolytic effects (Violle et al., 2006), as it has an affinity for the GABA receptor in the central nervous system (Miclo et al., 2001). Naturally, it is a component of breast milk and should calm the puppies (Miclo et al., 2001; Violle et al., 2006), on the market it exists as an available casozepine for dogs and cats in form of capsules or chews. In the case of a long-term stressful situation, such as a household move, the manufacturer recommends a long-term administration of casozepine over 1–2 months. In the case of a short-term stressful situation, such as a pet transport or a stay in a boarding kennel, an administration of casozepine over 1–2 days is recommended.

There are several ways to assess stress in dogs and cats. Often, known clinical vital signs such as the dog's heart rate, respiratory rate or body temperature are measured to assess stress (Smith et al., 1996; Smith et al., 1999). Furthermore, within various studies, objective ranking systems for the evaluation of behaviour have been developed to assess physical or psychological stress of an animal, so-called "Visual Analogue Scales" (VAS) (Srithunyarat et al., 2016; Korpivaara et al., 2017; Srithunyarat et al., 2018; Hauser et al., 2020).

Non-invasive salivary parameters such as oxytocin, vasopressin and cortisol have been used in various experimental studies of stress in dogs (Bowman et al., 2015; Srithunyarat et al., 2016; MacLean et al., 2018; Srithunyarat et al., 2018; Pirrone et al., 2019).

Cortisol is a hormone that is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Herman, 1993; Herman et al., 1995). It is a hormone used in human and veterinary medicine to study stress (Wykes, 1991; Dreschel and Granger, 2009; Cobb et al., 2016).

Vasopressin is a neuropeptide that is secreted in the hypothalamus and is also known as antidiuretic hormone due to its vasoconstrictor effect (Ginsburg, 1954; Stassen et al., 1985; Osterman et al., 1986;

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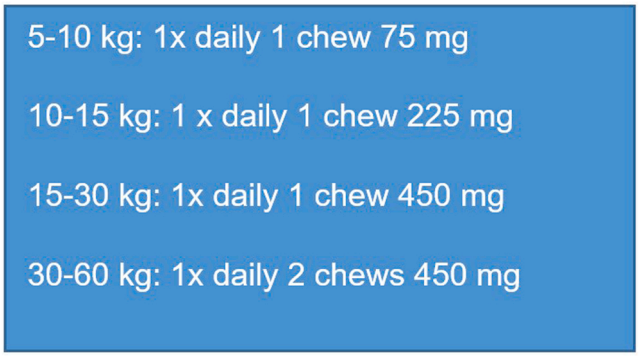

Fig. 1. Dosage of casozepine chew depending on the body weight of the dogs.

Thibonnier, 1987). In addition to regulating water balance in the body, vasopressin also stimulates the release of adrenocorticotropic hormone (ACTH), which is responsible for the release of the stress hormone cortisol (Bankir et al., 2017). The measurement of vasopressin concentrations in saliva has already been successfully used in dogs to estimate stress in clinical studies (MacLean et al., 2018; Pirrone et al., 2019; Jeong et al., 2020).

The purpose of the present study was to determine for the first time whether administration of a casozepine for 2 days prior to a veterinary visit can demonstrably reduce stress during a 10-min general examination on the treatment table. Stress was determined on the basis of the owner's evaluation, the clinical general examination including permanent measurement of the pulse rate, a stress scoring by the veterinarian and on the basis of cortisol and vasopressin concentrations in the saliva of the animals.

Materials and methods

Dogs

All studies were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ludwig-Maximilians-University Munich (reference number 282, approved 18 March 2021). The study included healthy dogs that were pre-reported to be stressed in everyday life, especially in unfamiliar situations such as at the vet, according to their owners. Owners could voluntarily apply to participate in the study with their dog and signed that they agreed to the examinations during the simulated veterinary visit. The prerequisite was that the dogs were not fearful dogs, for whom handling by the veterinarian is generally not feasible, but that it was possible to take a saliva sample from the dogs and perform a general examination on a treatment table. Thus, dogs were included that showed stress (grade 1–3/3) in the initial examination according to the visual analog scale (Srithunyarat et al., 2016). Dogs with obesity, diseases that could affect the function of the pituitary-adrenal axis, diseases of the oral cavity that could affect the quality of salivary samples or acute pain that could affect the stress assessment of the animals were also excluded. Since this was a pilot study, it was decided before the start of the study that at least 15 dogs per group (examination or control group) had to take part for each test value.

Study protocol

All dogs in the experimental and control groups underwent a standardized health check at 2-day intervals, which included a general veterinary examination (see below) on a treatment table. The waiting time before the examination was 10 min in front of the clinic without direct contact with other dogs. The examination time in the treatment room was 10 min.

Both the initial (day 1) and follow-up (day 3) examinations took place in the morning. After the morning examination (day 1) as well as the next morning (day 2) and the morning after (day 3), the owner fed a chew containing casozepine (Zylkene, Vetoquinol, Bayern) according to the corresponding weight class (Fig. 1).

The dogs in the control group received a similar-looking chew without ingredients (Milkdrop, Trixie) as a placebo. Whether a dog was assigned to the study or control group was determined by a random selection procedure. At the time of the examinations, the examiner and the owner were blinded to the grouping.

A saliva sample was taken immediately before and after each examination to measure the stress hormones cortisol and vasopressin. Sampling before the test was done in front of the clinic and contact with other dogs was avoided. A commercial salivette (Salimetrics LLC) was used for sample collection, which remained in the dog's mouth (under the tongue and laterally in the cheek pouches) for approximately 60 s. The saliva obtained was stored in an appropriate tube (Salimetrics LLC), centrifuged, transferred by pipette into an Eppendorf tube and stored at −20°C until evaluation. Vasopressin concentrations were analysed in the in-house laboratory using a commercial antibody-based ELISA kit (Nordic BioSite; Intra-Assay coefficient of variation (CV) < 8 %; Inter-Assay CV < 10 %). For the determination of cortisol, the saliva samples were sent refrigerated to the Faculty of Psychology at the Technical University of Dresden, Germany, where they were analysed using chemiluminescence immunoassay (CLIA) (IBL International; mean Intra-Assay CV=4.3 %; mean inter-assay CV=13.2 %). According to the manufacturer, both ELISA kits are suitable for the determination of the corresponding parameters in saliva across all animal species.

During the initial saliva sampling, the Stress Behaviour Visual Analogue Scale was used to assess whether the animal showed "0: no stress", "1: mild stress", "2: moderate stress" and "3: severe stress" by observed avoidance behaviors (Srithunyarat et al., 2016). Body posture and defence reactions of the animals were assessed.

The studies would have been terminated if the animal showed stress or defensive reactions during saliva sampling. However, in the present study, all dogs tolerated saliva sampling and the casozepine was well accepted.

During the general examination, the general condition and the mucous membranes were evaluated, the capillary filling time was checked, the respiratory rate was counted and the heart was auscultated at the puncta maxima. The lymph nodes as well as the abdomen were palpated. The time on the treatment table was 10 min. During the examination, all dogs wore a belt (Polar H10) with which the pulse was measured continuously. The belt was attached to the dog after the initial saliva sample collection, cranially on the thorax using a little ultrasound gel so that no fur had to be shorn.

In the assessment of the effect by the owner, the owner could indicate "no effect" (0), "slight effect" (1) and "strong effect" (2) on day 1, 2 and 3 respectively. In case of refusal or intolerance (e.g. vomiting, diarrhoea, allergy) of the casozepine preparation, the owners were asked to stop the trial. Whether the owner could see an effect, he was asked during the follow-up examination on day 3, without knowing which group he was in.

The follow-up examination on day 3 was performed according to the standardised study protocol as on day 1. Finally, the owner was told whether his dog was in the casozepine or placebo group.

Statistical analysis

Cortisol and vasopressin data were both right skewed and were log-transformed to achieve normality for the subsequent analysis. The following model assumptions were always checked: (1) the normality of residuals was checked by the Shapiro–Wilk normality test, (2) the homogeneity of variances between groups was checked with Bartlett test, and (3) the heteroscedasticity (constancy of error variance) was checked with Breusch–Pagan test. Potential outliers were present in several models and a robust mixed effect model regression was applied to

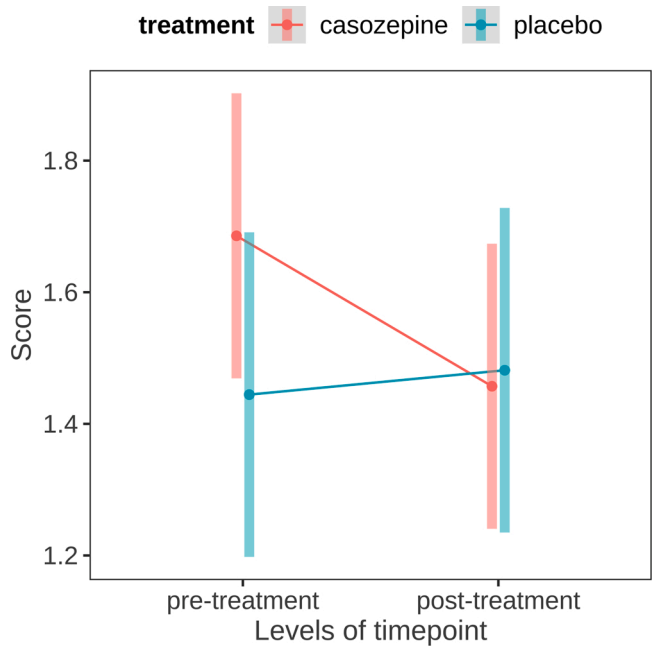

Fig. 2. Stress score of the 62 dogs in the clinic before and after the 2-day casozepine treatment in the casozepine (n=36) and placebo group (n=26).

address heteroscedasticity problem. Particularly, we studied stress score in the clinic, the average and maximum pulse rates, pre- and post treatment levels of cortisol and vasopressin before and after clinical examination and saline levels as response variable. The interaction between two predictors treatment and timepoint and the random effect of the individual animals were repeated in every model. All contrasts (differences) between particular groups were assessed after model-fitting by the estimated marginal means (R package - emmeans) with Tukey P-value correction for multiple comparisons. Results with a P-value < 0.05 were considered statistically significant. Data analysis was performed using R 4.3.1 (2023–06–16) statistical software.

The reason that the salivary hormones cortisol and vasopressin could not be measured in all dogs was that a predetermined amount of saliva is required for the measurement. As this could not be obtained from all dogs, these animals had to be excluded from the statistic analysis.

Results

In total, the stress of 36 study dogs and 21 control dogs could be estimated before and after casozepine or placebo in the clinic by stress scoring and pulse rate. In addition, vasopressin concentrations in saliva were measured in 22 dogs in the study group and 21 dogs in the control group, and cortisol concentrations were measured in 15 dogs in each group. In the study group, the average age was 5 years (range 1–12), the average weight was 20 kg (range 5–42) and the proportion of females was 67 % (24/36). In the control group, the average age was 6 years (range 1–13), the average weight was 19 kg (range 6–40) and the proportion of females was 73 % (19/26). The general examinations at the initial and follow-up examinations did not reveal any special findings, irrespective of the excitement of the dogs.

Based on the Visual Analogue Scale of the dogs in the clinic, it showed that the stress of the dogs was significantly higher in the casozepine group before casozepine administration than after treatment (P=0.0026), while it remained the same in the placebo group (P=0.6567) (Fig. 2).

With regard to the average and maximum pulse rate during the veterinary examination, it was shown that these were significantly lower in the study group at the follow-up after casozepine administration (P=0.01, P=0.024) than in the initial examination, while no difference was seen in the placebo group (Fig. 3).

When evaluating the vasopressin concentrations in saliva, it was striking that they remained the same in the study group before and after casozepine administration during veterinary examination, whereas they increased significantly in the placebo group at follow-up (P=0.04) (Fig. 4).

Salivary cortisol concentrations remained the same during initial examination, whereas they increased after administration at follow-up in both groups, although only in the casozepine group was this increase significant (P=0.01) (Fig. 5).

When owners in the casozepine group were interviewed, 22 % of owners on day 1, 28 % of owners on day 2 and 31 % of owners on day 3 subjectively saw a stress-reducing effect in their daily lives, compared with 15 % in each of the placebo groups (Fig. 6).

Overall, it was noticeable that the average pulse in the casozepine group before treatment was significantly higher than in the control group (P=0.0422), while the stress score in the clinic, the maximum pulse, the vasopressin and cortisol concentrations in saliva were not significantly increased (P=0.1472, P=0.2018, P=0.5585, P=0.3374).

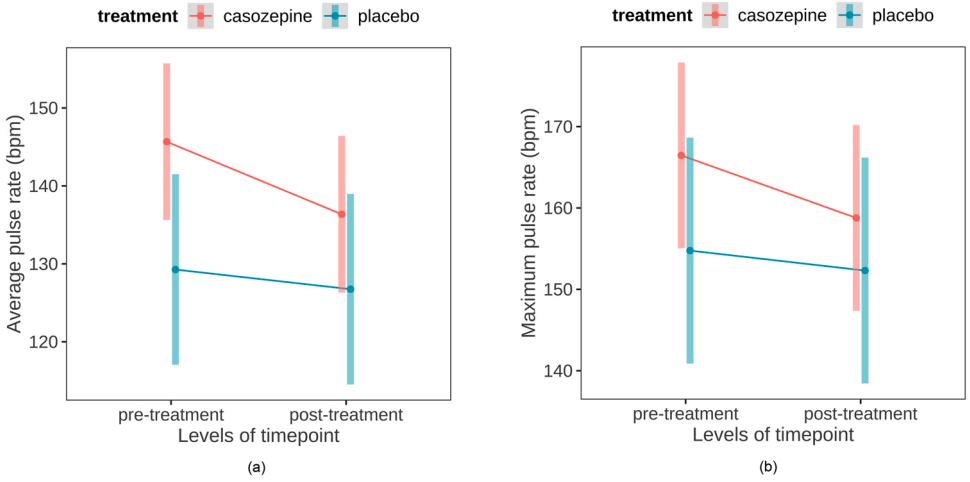

Fig. 3. Average (a) and maximum pulse (b) in beats per min. (bpm) before and after the veterinary examination at initial investigation and follow-up in the casozepine and control group.

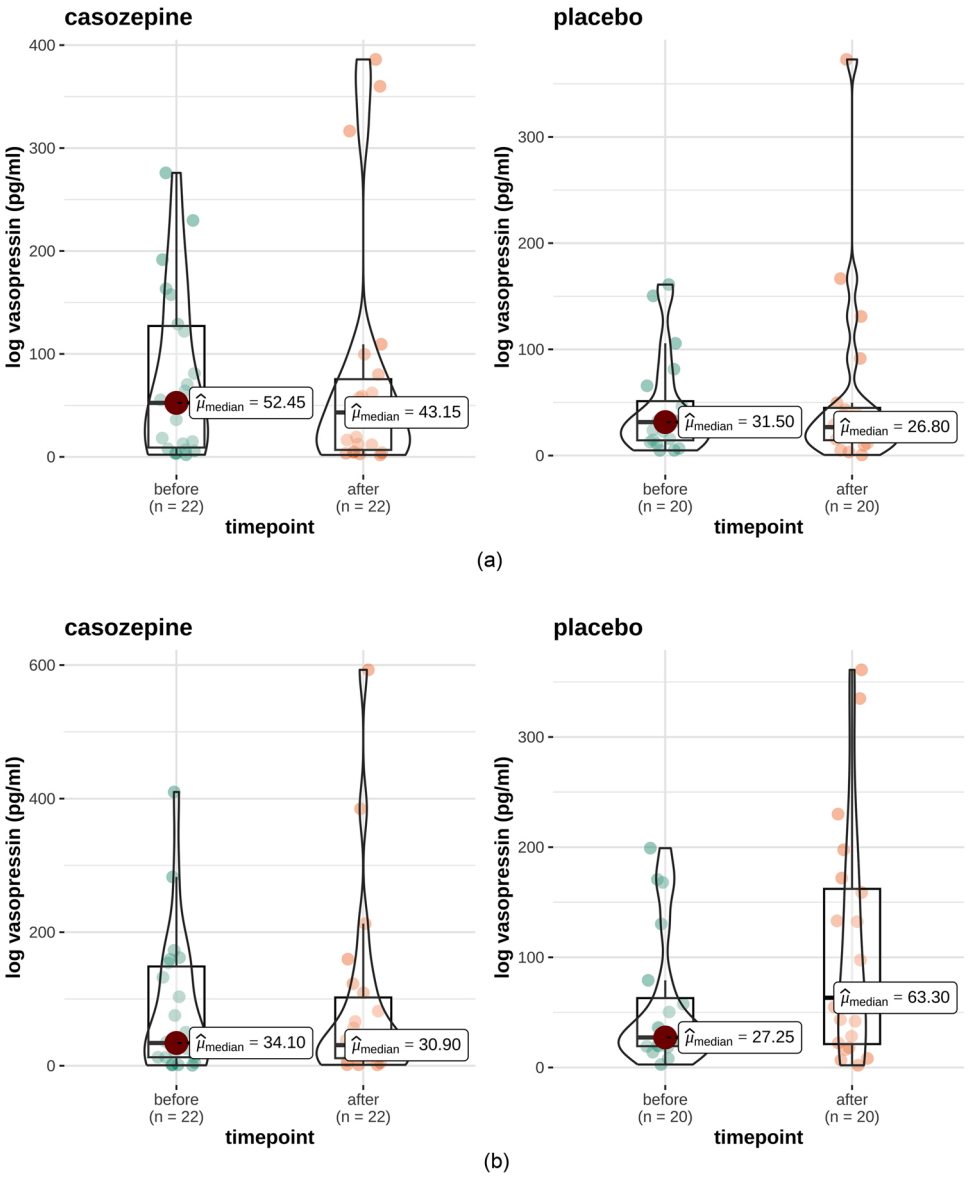

Fig. 4. Log_{10} vasopressin concentrations (nmol/L) before (a) and after (b) the veterinary visit on the day of the initial examination (left) and at follow-up in the casozepine (n=22) and placebo (n=21) groups.

Discussion

In the present study, the influence of casozepine administration on the stress of dogs during a general veterinary examination was investigated. It was shown that the dogs in the study group had a significantly lower stress score and a significantly lower pulse rate after 2 days of administration, while no effect was seen in the placebo group. This could suggest that casozepine administration may have a weak stress-reducing effect. Comparable studies on the use of casozepine in dogs do not yet exist. However, other authors also advocate a positive effect on stress behaviour through casozepine administration in cats. In a clinical study, stress behaviour was assessed in 24 cats over a period of up to 4 weeks using behavioural observations. For this purpose, half of the cats received a diet with L-trypthophan and α-casozepine (Royal Canin Feline Calm diet) for a period of up to 4 weeks (Landsberg et al., 2017). It was demonstrated that the diet had a positive effect on the animals in an unfamiliar environment but that in an acute situation with an unfamiliar person, the therapy had no effect.

Considering the vasopressin concentrations in the saliva of the dogs, it was noticeable that these remained the same in the study group during veterinary examination at both appointments, whereas they increased in the placebo group during the follow-up study on day 3. This could be due to the fact that the dogs in the placebo group may have been slightly more stressed during the follow-up examination, but this is contrary to the evaluations of the stress score and the pulse rates, which were not higher during the follow-up examination.

In a study on separation pain, dogs were left alone for ten min and immediately afterwards vasopressin concentrations were measured in saliva (MacLean et al., 2017). It was shown that the dogs separated from humans had higher vasopressin concentrations in their saliva than the control animals that continued to interact with humans. In another study on separation pain in dogs, increased vasopressin concentrations were found in the saliva of the dogs immediately after 3 min after separation from the owner (Pirrone et al., 2019). As two saliva samples were taken 10 min apart in the present study, it can be assumed that a vasopressin increase in saliva can be indicative of a stress response.

The cortisol concentrations in the saliva of the dogs at the time of the follow-up examination also showed an increase during the 10-min veterinary examination, whereby the increase was statistically significant only in the casozepine group and only in the follow-up examination.

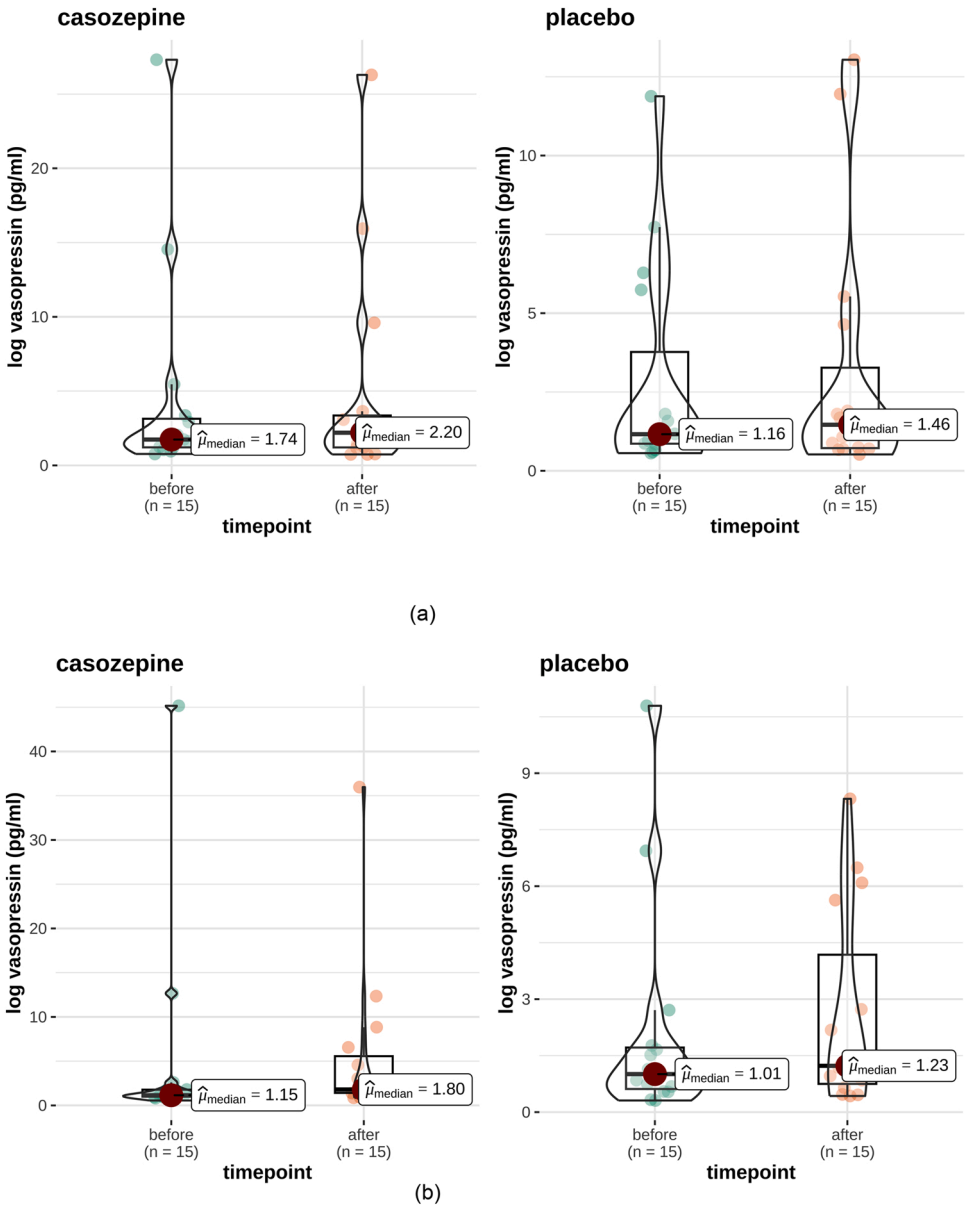

Fig. 5. Log_{10} cortisol concentrations (nmol/L) before and after the veterinary visit on the day of the initial examination (left) and in the follow-up examination (right) in the casozepine (n=15) and placebo group (n=15).

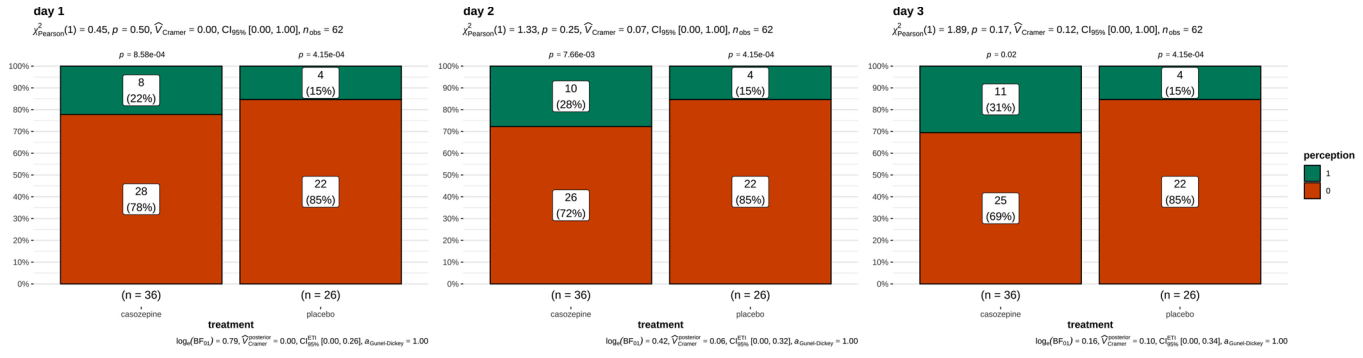

Fig. 6. Owners' perception of a stress-reducing effect on the respective days 1–3 in the casozepine (n=36) and placebo (n=26) groups.

This is contrary to the results of the stress score and pulse rate, which were overall lower in the follow-up than in the initial investigation.

In a study on the stress of separation in dogs, it was demonstrated that after only five min of separation from the owner, the stress reaction could be traced by an increase in cortisol in the dogs' saliva (Shin and Shin, 2016). It can be assumed in the present study that the animals in

the study group may have been less stressed on the basis of stress score and pulse rate than at baseline, but continued to be highly excited during the follow-up, as indicated by the slight increase in salivary cortisol concentrations.

It was striking that the proportion of owners who saw an effect after casozepine administration in everyday life was higher than in the placebo group (15 %), but overall was comparatively low at 22 % on day 1, 28 % on day 2 and 31 % on day 3. This could be related to the fact that the casozepine either had no effect on the animals or that the animals were generally not highly stressed in their usual environment and in the presence of their owners, and hence the effect of casozepine in everyday life could not be well assessed. All in all, the owners' assessments were purely subjective, which also limits the results.

The short-term administration of a casozepine in connection with cortisol concentrations in the saliva has not yet been investigated in dogs. However, in a study with cats, the stress behaviour of the animals was tested depending on long-term casozepine administration. In contrast to the control group (11 animals), the study group (10 animals) received an appropriate diet with α-casozepine for up to 8 weeks. To estimate stress, cortisol concentrations in urine and plasma as well as the ratio of tryptophan to long-chain amino acids in plasma were determined. Here, as well, a positive effect on the stress of the animals could be demonstrated in the long term, although after acute stress situations, such as a visit to the vet or a blood sample, no differences could be seen based on the plasma concentration of cortisol. Therefore, the authors also advocate further studies on the effect of a casozepine diet (Miyaji et al., 2015). The administration time in the present study was 2 days including the day of the follow-up examination. In the case of long-term changes in the environment, e.g. in the case of a move, casozepine can also be administered over several months according to the manufacturer's instructions. The time of administration in the present study was thus comparatively short. However, it should be specifically examined in the context of the present study whether the administration of casozepine over a period of 2 days before a visit to the vet makes sense. The results show that owners should try out individually with their animal whether their animal benefits from the supplement in the short term.

The dosage of casozepine was based on the body weight of the dogs and the manufacturer's instructions for the preparation approved in Germany. As these were weight ranges, the amount administered (mg/kg) varied only slightly from animal to animal, which is a limitation of the study. However, the form of administration of the chews was deliberately chosen so that the supplement could be given as stress-free as possible, especially to stressed dogs. However, it would be more precise to administer the casozepine in mg/kg, as it was done in a clinical study on the administration of casozepine in conjunction with behavioural therapy in dogs with separation anxiety. Nonetheless, the authors could not find any significant differences between the study group and the placebo group in their study and advocated further long-term studies (Werner, 2013).

The slight stress-reducing effect on the dogs can also be explained by the caregiver-placebo effect (Munana et al., 2010; Conzemius and Evans, 2012). However, the dogs were separated from their owners during the veterinary examination and the veterinary examination was carried out as part of a standardized examination process. the fact that only one examiner carried out all the appointments meant that the study design was intentionally kept uniform. The animals were given at least 10 min to relax before the visit to the vet and were not allowed any contact with other dogs in the waiting room prior to the examination, so that only the 10 min examination on the treatment table was considered to be a stress factor. However, it is likely that a number of day-to-day factors, such as transport to and from the clinic and the noise and odours inside and outside the clinic, will affect a dog's individual stress levels and therefore the results. In follow-up studies, saliva samples could also be taken at home before the dogs are taken to the veterinary clinic although studies on stress during the transport of animals have been carried out so far (Jeong et al., 2020; Hunt et al., 2023). As both pulse and cortisol and vasopressin concentrations can rise and fall within seconds, stress levels should be explicitly assessed during the 10-min veterinary examination on the treatment table. As potential changes in cortisol and vasopressin before and after the clinical examination may have been missed, additional samples could have been taken both before and after the experimental test to assess stress hormone release patterns, although this may have been more stressful for the animals.

The number of animals chosen is similar to those used in previous clinical studies examining cortisol and vasopressin concentrations in saliva in dogs to assess stress (Pirrone et al., 2019; Jeong et al., 2020). A major disadvantage of saliva analysis was that not all parameters could be measured in all dogs, as it is not always possible to obtain sufficient saliva from all dogs. Nevertheless, this analysis was preferred to blood sampling in this pilot study in order to minimise stress to the animals. Studies with a larger number of animals could be helpful in the future to assess the effect of casozepine on stress hormones in a more differentiated way.

In general, dogs with obesity, endocrine diseases that could influence the function of the pituitary-adrenal axis, diseases of the oral cavity that could influence the quality of the saliva samples or acute pain that could have influenced the stress assessment of the animals were excluded. Even though all animals in the present study had no previous reported diseases and were unremarkable in the general examination, it is possible that unknown diseases may have been missed, which is also a limitation of the study.

Conclusions

The options for acute stress reduction at the vet are often limited. Medications such as serotonin reuptake inhibitors (e.g. clomipramine) can be associated with gastrointestinal (e.g. vomiting and diarrhoea) as well as neurological side effects (e.g. lethargy, fatigue) (King et al., 2000). Moreover, in the studies on fluoxetine, for example, an effect is often only detectable in the long term (Dodman et al., 1996; Odore et al., 2020). To reduce stress for dogs in a veterinary practice as much as possible, various factors can contribute. Avoiding a crowded waiting room can help, but so can careful handling of the dog during the examination, rewarding with food and a calm environment (Glardon et al., 2010). The present study highlights that a 2-day administration of a casozepine before a veterinary visit had a weak stress-reducing effect during a veterinary visit based on subjective stress scoring and pulse rate. A higher dose recommendation might be useful to achieve a stronger stress-reducing effect.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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