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## Is Alpha-casozepine Efficacious at Reducing Anxiety in Dogs?

A Knowledge Summary by

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### Clinical bottom line

There is currently no evidence to show that alpha-casozepine is effective as an anxiolytic when administered to dogs shortly (minutes to a few days) before exposure to an anxiety provoking stressor. There is limited and weak evidence to suggest that it may have a role to play in reducing anxiety in dogs over the medium to longer term but the available evidence is of low quality and / or high risk of bias, with confounding variables providing alternative explanations for the findings. More research is needed in this area.

### Question

Do anxious dogs administered alpha-casozepine show reduced signs of anxiety compared with dogs not administered alpha-casozepine?

### Clinical Scenario

The veterinary team are reviewing the products that they keep within the practice for use in supporting clients whose dogs are anxious for various reasons. They realise that they have never examined the evidence for the use of alpha-casozepine in relation to canine anxiety. However, they have been widely recommending products that contain it, and have had mixed reports back from clients who have used it, so are curious about its efficacy. Thus, they decide to find out how strong the research evidence base is for this biomolecule.

### The Evidence

The Three peer reviewed papers were identified that either partially or fully addressed the PICO. Two of these studies (Palestrini et al., 2010; Kato et al., 2012) investigated the use of diets with added alpha-casozepine, though, in the Kato et al. (2012) study the diet was also supplemented with another compound (tryptophan) thought to have anxiolytic properties. The remaining study (Beata et al., 2007) investigated the use of a daily capsule of alpha-casozepine. The two studies focused on dietary interventions (Palestrini et al., 2010; Kato et al., 2012) used a placebo-controlled study design, whereas Beata et al. (2007) compared alpha-casozepine to another intervention (selegiline) that was already used commercially as an anxiolytic.

There is no evidence that alpha-casozepine has any effect on canine anxiety in the short term (e.g. when exposed to fireworks or another short acting stressor) or when administered a few minutes to a few days before exposure to potential stressor. All of the studies focus on the potential anxiety-reducing effects of alpha-casozepine when administered in the medium to long term. There was some evidence that alpha-casozepine may reduce anxiety as, where effects exist, the direction of effect is always a reduction in the level of whatever parameter was used to measure anxiety. However, the evidence available is low quality. Despite being clinical trials, experimental design and / or data handling was weak, with confounding variables affecting interpretation of the findings. There is a need for better quality research that specifically investigates the use of alpha-casozepine as an anxiolytic in the scenarios that practices would commonly promote its use for.

## Summary of the evidence

Palestrini (2010)	
<b>Population:</b>	Healthy young adult female laboratory-based beagles
<b>Sample size:</b>	<p>32 dogs:            Anxious dogs: n = 16 (later reduced to 14, possibly 13<sup>1</sup>), split between two groups (experimental diet versus control diet).            Non-anxious dogs: n = 16, split between two groups. 1 group (n = 8) received the experimental diet; 1 group received the control diet (n = 8).</p> <p><sup>1</sup>The authors reporting of this aspect is weak and they report eliminating 3 anxious dogs, but then claim later in the same section to have reduced the anxious treatment group from 16 dogs to 14 dogs. They do not report how this affected numbers of dogs in each group, therefore, the minimum sample size for one of the diet groups could be as low as 6 (if 2 are missing) or 5 (if 3 are missing).</p>
<b>Intervention details:</b>	<p>Dogs were split into four groups:</p> <ol style="list-style-type: none"> <li>1. Anxious dogs fed the control diet</li> <li>2. Anxious dogs fed the experimental diet</li> <li>3. Non-anxious dogs fed the control diet</li> <li>4. Non-anxious dogs fed the experimental diet</li> </ol> <p>The two diet treatments compared were:</p> <ol style="list-style-type: none"> <li>1. Experimental diet: a complete dry dog food that was coated with caseinate hydrolysate (CH) (the milk component containing alpha-casozepine). The authors do not report the inclusion rate of CH.</li> <li>2. Control diet: the same diet as the experimental diet but without the CH.</li> </ol> <p>Researchers were blinded to which dogs were receiving which treatment.</p> <p>Experimental timeline:            Forty dogs were assessed for anxiety and either included in the study (n = 32) or rejected (n = 8).</p> <p>Anxiety assessment used two methods:</p> <ol style="list-style-type: none"> <li>1. The Evaluation Scale of Anxiety, and</li> <li>2. The Reactivity Evaluation Form (REF) (more details below).</li> </ol> <p>Based on the combined scores, dogs were ranked for level of anxiety. The top 16 dogs were then assigned to the anxious group and the bottom 16 dogs were assigned to the non-anxious group. The intermediary 8 dogs were not used.</p> <p>Within group, the dogs were paired-housed such that dogs with the</p>

most similar scores were housed together (e.g. the most anxious dog was housed with the second most anxious dog, etc).

A randomised block design was then used to allocate diet treatments to the dogs. Dogs were blocked according to whether they were anxious or non-anxious (based on the anxiety assessment methods outlined above), and then the 8 pairs of dogs within each block were randomly allocated to receive one of the two diet treatments. Both dogs within the pair received the same diet.

The dogs included were allocated as described above and then pair-housed. Dogs were introduced to their new kennel mate and given 3 days to adjust to this. Then, the study commenced.

The study lasted either 68 or 69 days (not clear enough to say for certain).

1. Baseline period (6 days): baseline parameters were measured for serum cortisol, serum lysozyme, the neutrophil:lymphocyte ratio, and mean heart rate. Heart rate was measured telemetrically using a heart rate monitor strapped to the dog's chest and recorded at 5 second intervals. Dogs were fed their normal diet during this period.
2. Initial evaluation phase (T1) (2 or 3 days, this is unclear from the paper\*): on the first day, the REF was completed and a blood sample taken. On day two, the mean heart rate was determined for ten minutes while the dogs were video recorded for 10 minutes. This video was later viewed by an observer blinded to treatment and group and the frequency or proportion of time spent performing certain behaviours recorded.

\* In the experimental phase section of the paper, the authors report "after an initial 3 day evaluation phase (T1)" and "each evaluation (T1, T2, and T3) lasted 2 days".

3. Feeding phase 1 (28 days). Dogs were given ad libitum access to the food with food replenished once a day (PM), with both dogs in the pen fed the same diet.
4. Second evaluation phase (T2, lasted 2 days). Data collection as documented in T1.
5. Feed phase 2 (28 days). Dogs fed as documented in feeding phase 1.
6. Third evaluation phase (T3, lasted 2 days). Data collection as

	<p>documented in T1.</p> <p>Initial assessment of anxiety (to select dogs):</p> <ol style="list-style-type: none"> <li>1. Evaluation scale of anxiety: a subjective assessment of anxiety in which dogs were observed and scored from 1 (low) – 6 (high).</li> <li>2. REF. A two part objective behavioural assessment, comprising an ordinal scale of anxiety level (score 1 – 4) and the presence of various clinical signs of anxiety (e.g. lick lip: yes / no, score 1 point if yes) based on the dogs response to handler presence outside and inside the test pen.</li> </ol> <p>Both assessment tools were developed by the researchers of this study.</p> <p>Two experienced handlers familiar with the dogs independently assessed the dogs using these two assessment methods and then agreed the final score per dog.</p>
<p><b>Study design:</b></p>	<p>Randomised clinical trial</p>
<p><b>Outcome studied:</b></p>	<p>Behavioural outcome measures:</p> <ol style="list-style-type: none"> <li>1. REF</li> <li>2. Proportion of time spent performing various behaviours in the test pen over 10 minutes (exploration; locomotion, passive behaviour, orientated to the environment, scratching, oral behaviour, vocalisation, play, panting, grooming)</li> <li>3. Frequency of behaviours performed while in the test pen for 10 minutes (yawning, lip – licking, elimination, drinking, eating)</li> </ol> <p>Physiological parameters:</p> <ol style="list-style-type: none"> <li>1. Heart rate</li> <li>2. Cortisol</li> <li>3. Neutrophil: lymphocyte ratio</li> <li>4. Lysozyme</li> </ol> <p>It is not clear whether plasma or serum cortisol and lysozyme was measured as the authors report the terms interchangeably.</p>
<p><b>Main findings: (relevant to PICO question):</b></p>	<p>No significant differences were found between the baseline period data and the initial evaluation phase (T1) data, so the authors used T1 as their baseline data period.</p> <p>Behavioural outcome measures:</p> <ol style="list-style-type: none"> <li>1. REF: <ul style="list-style-type: none"> <li>- At the start (T1), anxious dogs had significantly higher REF scores than non-anxious dogs (Mann-Whitney U Test, <math>P &lt; 0.001</math>).</li> <li>- At the start (T1), there was no significant difference</li> </ul> </li> </ol>

	<p>between anxious dogs fed the control diet and anxious dogs fed the experimental diet.</p> <ul style="list-style-type: none"> <li>- Dogs on the experimental diet had significantly lower REF scores by T3 (end of the study) (Friedman Test, <math>P &lt; 0.01</math>).</li> <li>- No further formal statistical comparisons are reported. The figure associated with this outcome measure suggests that anxious dogs fed both the control and experiment diets always scored higher than non-anxious dogs fed either diet. It also suggests that there is no divergence in anxiety scores of non-anxious dogs fed either diet.</li> </ul> <p>2. Behaviour:</p> <ul style="list-style-type: none"> <li>- Anxious dogs and non-anxious dogs show no significant differences in behaviour at the start of the study (T1).</li> <li>- Anxious dogs showed less exploratory behaviour at the end of the study (T3) compared to the beginning (T1) (Friedman, <math>P &lt; 0.05</math>). There was no change in level of exploratory behaviour between time points in anxious dogs fed the control diet.</li> <li>- Non-anxious dogs fed the control diet showed less scratching behaviour over time (Friedman, <math>P &lt; 0.05</math>)</li> <li>- Anxious dogs fed the control diet showed increased lip licking over time (Friedman, <math>P &lt; 0.05</math>).</li> <li>- Anxious dogs showed a significant increase in orientation to the environment behaviour at T3, compared with T1 (Friedman, <math>P &lt; 0.05</math>).</li> </ul> <p>Physiological outcome measures:</p> <ol style="list-style-type: none"> <li>1. Heart rate: <ul style="list-style-type: none"> <li>- No significant effects were observed.</li> </ul> </li> <li>2. Cortisol: <ul style="list-style-type: none"> <li>- Remained within the normal physiological range for anxious and non-anxious dogs during the whole study</li> <li>- Cortisol level of anxious and non-anxious dogs did not differ at the start (T1).</li> <li>- Anxious dogs fed the experimental diet showed a significant decrease in cortisol over time (Friedman, <math>P &lt; 0.05</math>). Post hoc testing indicated that a significant reduction was observed between T1 and T2 (Wilcoxon, <math>P &lt; 0.05</math>).</li> <li>- Non-anxious dogs showed a tendency to have a reduction in cortisol over time (Friedman, <math>P &lt; 0.10</math>).</li> </ul> </li> <li>3. Neutrophil:lymphocyte ratio: <ul style="list-style-type: none"> <li>- A significant reduction in neutrophils (and increase in lymphocytes) was observed over time in anxious dogs (General Linear Model, <math>P &lt; 0.05</math>) but not non-anxious dogs. Diet treatment had no effect on the ratio for either group of dogs.</li> </ul> </li> <li>4. Lysozyme: <ul style="list-style-type: none"> <li>- No PICO relevant significant effects were observed.</li> </ul> </li> </ol>
<b>Limitations:</b>	The authors fail to report the inclusion rate for caseinate

hydrolysate (containing alpha-casozepine) in the diet (g/kg/Dry matter).

The dogs were fed *ad libitum* during the study so the dose rate would have varied between dogs.

Laboratory population in which the breeding life experiences of the dogs were homogenised and tightly controlled. This contrasts to a pet population and may limit extrapolation of the findings to the wider population of dogs.

The authors undertook a discriminant analysis to retrospectively (after all the data was collected) eliminate dogs whose REF score did not accurately predict which dogs were identified as anxious through the Evaluation Scale of Anxiety (or vice versa, it is not clear which). As a consequence, 2 or 3 anxious dogs were removed. The authors fail to report how this affected the sample size of anxious dogs in the control diet and experimental diet groups respectively. Given the low sample sizes within each group (n = 8), this has implications for the interpretation of any findings or extrapolation to the wider population. This is compounded by the wide degree of variation observed (where standard deviations are reported). Retrospective removal of such a large proportion of the sample size for a group should also be questioned.

The authors fail to report the measure of central tendency used to report the REF and cortisol data, and do not provide any measure of variation around this central tendency.

The authors do not provide enough information to interpret the cortisol findings. They report that anxious and non-anxious dogs' cortisol levels did not differ at T1, but do not report the findings for the subgroups relevant to the treatments (especially anxious dogs fed the experimental diet versus anxious dogs fed the control diet). Eyeballing the figure presented suggests that these two sub-groups might vary at the start and combining this data would have increased the variation associated with the anxious group data and made it more difficult to pick out significant differences. However, the lack of error bars makes it difficult to draw many inferences here.

The authors report appears to use the convention of using P < 0.01 (tendency) P < 0.05 (significant), P < 0.01 and P < 0.001 (highly significant). It would have been useful to see the exact P value associated with each finding as most appear to be P < 0.05. The way that they have analysed the behavioural data that they have reported suggests that there may have been many statistical tests performed and this increases the likelihood of a type 1 error (finding a significant finding where one does not exist).

The ethogram derived behavioural data is very problematic to interpret:



	<ul style="list-style-type: none"> <li>- The authors report the findings using mean (<math>\pm</math> standard deviation) which implies the data is normally distributed. However, they analyse this data using non parametric tests (Friedman test, with post-hoc testing via the Wilcoxon test) which implies the data is not normally distributed. The error bars suggest that there was a large amount of variation associated with the performance of some behaviours.</li> <li>- The authors do not provide enough information for readers to be able to draw their own conclusions about what the behavioural data may indicate. It would have been useful to see the descriptive statistics reported for all the ethogram data, broken down by both treatment diet and anxiety group.</li> <li>- The authors appear to have cherry picked which behavioural outcome comparisons to report and key comparisons (e.g. between anxious dogs fed the control diet and anxious dogs fed the experimental diet) are largely missing.</li> <li>- It is not immediately clear that changes between some of the behaviours in the ethogram would represent a change in the level of anxiety.</li> </ul>
<b>Beata (2007)</b>	
<b>Population:</b>	<p>Pet dogs with anxiety-related disorders</p> <p>The inclusion criteria was as follows:</p> <ul style="list-style-type: none"> <li>• At least 3 months old</li> <li>• Weigh between 1.5 – 42kg</li> <li>• Diagnosed with a behavioural complaint related to anxiety</li> <li>• At the first visit, the dog scored above 19 (out of a maximum of 45) on the Emotional Disorders Evaluation in dogs scale (EDED) (as used by Pageat, 1995).</li> </ul> <p>Dogs were excluded if:</p> <ul style="list-style-type: none"> <li>• The problem had been present for less than four weeks</li> <li>• There was any evidence that the problem was caused by disease, illness or injury.</li> <li>• Any psychotropic medications had been administered in the previous two weeks</li> <li>• If the dog scored less than 20 on the EDED scale.</li> </ul>
<b>Sample size:</b>	<p>Forty dogs started the trial, 38 dogs completed it. Two dogs died (one per treatment) during the study for unrelated reasons (accidents).</p>
<b>Intervention details:</b>	<p>Two treatments (19 dogs per treatment) were compared:</p> <ol style="list-style-type: none"> <li>1. Behavioural modification therapy (BMP) <i>plus</i> oral alpha-casozepine (15mg/kg/24hrs)</li> <li>2. BMP plus oral selegiline hydrochloride (0.5mg/kg/24hrs)</li> </ol> <p>Study design:</p> <ul style="list-style-type: none"> <li>• Multi-centre trial that utilised 7 certified veterinary</li> </ul>

	<p>behaviourists.</p> <ul style="list-style-type: none"> <li>• Double or triple blind (not clear which) methodology in which the owner, veterinary behaviourist and the supervisors (undefined, researchers?) were unaware which dog received which drug while the study was ongoing.</li> <li>• Dogs were allocated to treatments using a randomised block design (blocked according to a veterinary behaviourist treating the dog, then randomly allocated to receive a treatment based on a pre-defined randomisation list e.g. dog 1 was given drug 1, dog 2 was given drug 2, and so on).</li> <li>• The BMP was tailored to the dog, diagnosis given and how severe this problem was. Thus, it varied between dogs but BMP approach did not vary systematically between the two treatment groups.</li> <li>• The trial lasted 57 days.</li> </ul> <p>Experimental timeline:</p> <ul style="list-style-type: none"> <li>• Day 0: Inclusion visit, owners completed the EDED questionnaire. Dogs that scored greater than 19 were included.</li> <li>• Day 14: Follow up phone call.</li> <li>• Day 28: Follow up physical visit.</li> <li>• Day 42: Follow up phone call.</li> <li>• Day 56: Follow up physical visit.</li> </ul> <p>At each contact, the owner was asked to complete the EDED scale again AND provide a subjective assessment of improvement on a scale of -10 to +10, with 0 indicating no change, and values higher and lower than this indicating, respectively, an improvement or worsening of the dog's behavior.</p> <p>The EDED score was previously developed by one of the authors for use in investigating the ability of seligiline to reduce anxiety (Pageat, 1995, cited by the current authors), and uses a range of questions to assess the dog's emotional state on a scale from 9-45. A score of 9-13 is considered to be normal, 14-18 indicative of a dog with a phobia, with dogs that have a score that sits between 18-30 considered to have an anxiety-related disorder. The authors do not define what a score above 30 means in relation to the dog's emotional state. They do not state whether this scale is validated for this use.</p>
<p><b>Study design:</b></p>	<p>Randomised clinical trial</p>
<p><b>Outcome studied:</b></p>	<p>The outcome studied at each time point was:</p> <ul style="list-style-type: none"> <li>• EDED score</li> <li>• Owner subjective assessment of improvement</li> </ul>

	<p>The authors also introduced a binomial outcome (success / failure of treatment) “success” measurement. This was based upon the dog achieving two things:</p> <ol style="list-style-type: none"> <li>1. An EDED score of &lt; 20, and</li> <li>2. An owner subjective assessment of improvement score of ≥ +6.</li> </ol>
<p><b>Main findings: (relevant to PICO question):</b></p>	<p>Please note: in reporting the findings, I have included the phase “+ BMP” to each treatment group to improve reader understanding of the treatment group structure. The authors of this paper refer instead only the groups as ‘alpha-casozepine’ and ‘selegiline’ but it is felt that this is misleading.</p> <p>EDED scores:</p> <ul style="list-style-type: none"> <li>• The average EDED score for both treatment groups decreased between visits 1 (day 0) and visit 3 (day 56) (alpha-casozepine group + BMP: <math>F(4, 18) = 22.20</math>, <math>P &lt; 0.0001</math>; selegiline + BMP: <math>F(4,18) = 26.33</math>, <math>P &lt; 0.0001</math>).</li> <li>• At the start of the study (visit one), there was no significant difference between the two treatment groups. The average EDED scores on visit 1 were: alpha-casozepine + BMP: 25.0; selegiline + BMP: 24.2</li> <li>• At the end of the study (visit 3, day 56), there was no significant difference between the two treatment groups. The average EDED scores on visit 3 were: alpha-casozepine + BMP: 26.3; selegiline + BMP: 16.6.</li> </ul> <p>Owner subjective assessment of improvement:</p> <ul style="list-style-type: none"> <li>• The data analysed using an ANOVA statistical test are not reported in a clear enough way to precisely define what the researchers think they found.</li> <li>• The average improvement score of the apha-casozepine + BMP group increased from 2.7 on day 14 to 5.3 on day 56.</li> <li>• The average improvement score of the selegiline + BMP group increased from 3.0 on day 14 to 5.4 on day 56.</li> <li>• At the end of the trial (day 56) the improvement score did not significantly differ between the two treatment groups (Mann-whitney U test, <math>P = 0.73</math>)</li> </ul> <p>Success / failure measurement:</p> <ul style="list-style-type: none"> <li>• There was no significant association between treatment received and proportion of dogs that responded successfully to the treatment (<math>\chi^2</math> test, d.f. = 1, <math>P = 0.74</math>).</li> <li>• The number of successes (failures) in the two groups was: alpha-casozepine + BMP: 10 (9); selegiline + BMP: 9 (10).</li> </ul>
<p><b>Limitations:</b></p>	<p>The study was funded by Ingredia SA (Arras, France). Ingredia is a company producing milk products and value-added bioactive compounds related to milk. It is not stated whether the funder had input into the study design but its authors report that, during data collection, only the sponsor knew which dog received which treatment during the trial.</p>

There was no BMP only treatment group and / or BMP + placebo group.

The paper cited by the authors (Pageat, 1995) is in French so it is not possible to use this paper to better understand the usage of the EDED scale in relation to assessment of selegiline.

The authors do not make clear what they mean by average. Were they presenting medians or means? They do not report any measures of variation (e.g. standard deviation, inter-quartile range, standard error of the mean).

The authors switch between using parametric and non-parametric tests to study aspects of the same data. They do not report any information to indicate that they considered the underlying distribution of the data.

Statistical output reported for the between treatment group pairwise comparisons at visit 1 (day 0) and visit 3 (day 56) is unclear as only one P value is reported (P = 0.79) for both results.

Technically, the authors cannot make the claim reported in the EDED scores section (that scores decreased between visits one and three for each treatment group) because they only appear to have carried out an ANOVA. The degrees of freedom indicate that they fitted 5 time points (presumably physical visits 1-3, and telephone calls 1-2). However, no post hoc test has been carried out to identify between which two (or more) pairs of time points the significant difference(s) arises between. Eyeballing the graphical data suggests that a significant difference was most likely between visit 1 and 3, but this is complicated by the lack of variation reported. This criticism also applies the ANOVA analysed aspect of the owner subjective assessment of improvement scores.

The reporting of the ANOVA analysis for the owner subjective assessment of improvement scores is not clear enough. It is assumed that they are comparing four time points (physical visits 2-3, telephone visits 1 -2 because of the degrees of freedom reported) but they do not include enough information.

The claims that are made throughout the research paper are misleading and introduce a potential interpretation bias on the part of the reader with limited research methodology knowledge. The authors consistently refer to the two groups as alpha-casozepine and selegiline, but make limited mention of the fact that these dogs all also received a behaviour modification programme, with no mention at all in the results or conclusion (there is no discussion section). Despite this major confounding variable, the authors make statements like:

“both compounds were equally efficacious” (results), “both products were efficient to decrease the EDED score” (abstract), “due to this efficacy.... Alpha-casozepine (Zylkene) should be considered an

	<p>option by the veterinary surgeon for the biological management of anxiety” (abstract), etc.</p> <p>These claims cannot be made given the lack of an adequate control group as each compound could be equally efficacious at having no impact on anxiety (i.e. the BMP was the successful component).</p>
<b>Kato (2012)</b>	
<b>Population:</b>	<p>Healthy adult small-medium (1.4 – 12.6kg) pet dogs that scored as ‘anxious’ on the Canine Behavioural Assessment &amp; Research Questionnaire (C – BARQ). Both genders were included and a variety of ages and breeds.</p> <p>Dogs were excluded if they:</p> <ul style="list-style-type: none"> <li>• Were pregnant</li> <li>• Less than one year of age</li> <li>• Had had a medical condition (i.e. not necessarily current)</li> <li>• Were a recipient of behavioural therapy or medications aimed at treating behavioural problems (i.e. psycho-active drugs)</li> </ul>
<b>Sample size:</b>	<p>44 dogs were recruited to the study. 28 dogs completed the study.</p> <ul style="list-style-type: none"> <li>• 4 dogs were lost to follow up during the study; the others withdrew for reasons unconnected to the trial (health issues of the dogs or personal circumstances of the owner).</li> </ul>
<b>Intervention details:</b>	<p>The two diet treatments compared for their effects on indices of canine anxiety in a crossover, single-blind, single order trial were:</p> <ol style="list-style-type: none"> <li>1. Control diet (Royal Canin Select Skin Care Vets Plan)</li> <li>2. Experimental diet containing elevated levels of the amino acid tryptophan and alpha-casozepine (Royal Canin Calm Canine) <ul style="list-style-type: none"> <li>• The alpha-casozepine inclusion rate was 1.35g/kg dry matter (DM), to provide dogs with a dose of approximately 20mg/kg/day (the recommended dose for dogs under 15kg).</li> <li>• The tryptophan content of the experimental diet was 3.04g/kg DM (the control diet contained 2.83g/kg DM).</li> <li>• The diets were not identical in all other respects. All the nutritional components reported differed in inclusion rate.</li> <li>• The dogs were retained in their home environment throughout the study and fed by their owner (who was blinded to the treatment they were feeding their dog).</li> </ul> </li> </ol> <p>The experimental timeline is as follows:</p> <ul style="list-style-type: none"> <li>• The owners of potential canine participants completed a C-BARQ questionnaire. Dogs that scored as anxious (n = 44) were included in the trial.</li> <li>• Dogs received the control diet for 8 weeks. They were fed twice a day. Data collection took place in week 7 (see below for outcome measures) and comprised completion of the C - BARQ questionnaire to assess behavioural indices of anxiety and two urine samples collected to assess urinary cortisol</li> </ul>

	<p>levels. One sample was taken in the home environment (baseline, unstressed measurement) and one sample was taken 2hr post vet clinic visit (the 'stressed' measurement).</p> <ul style="list-style-type: none"> <li>• Dogs then received a 1 week wash out period. The diet fed during this period is not stated and is assumed to be the dog's normal diet.</li> <li>• Dogs then received the experimental diet for 8 weeks. All other details are the same as for the control diet phase.</li> </ul> <p>Further details:</p> <ul style="list-style-type: none"> <li>• The wash out period length was determined by a prior preliminary experiment using high-pressure liquid chromatography which demonstrated that the dietary additives would be entirely removed from the dogs systems within one week.</li> <li>• Cortisol analysis was carried out in duplicate.</li> <li>• Urine collection was carried out at the same point of day to reduce the risk of time of day effects on cortisol production.</li> </ul>
<p><b>Study design:</b></p>	<p>Experimental cross-over trial</p>
<p><b>Outcome studied:</b></p>	<p>The outcome measures studied were all potentially relevant to answering this PICO. The outcome measures included:</p> <ul style="list-style-type: none"> <li>• Urinary cortisol:creatinine ratio (UCCR) <ul style="list-style-type: none"> <li>- Baseline measurement: taken in the dog's home environment</li> <li>- Post-stressor measurement: taken 2hrs post vet clinic visit</li> </ul> </li> <li>• Severity of stranger – directed aggression</li> <li>• Severity of owner – directed aggression</li> <li>• Severity of stranger – directed fear</li> <li>• Severity of non-social fear</li> <li>• Level of touch sensitivity</li> </ul> <p>All, bar UCCR, were assessed using specific questions within the C-BARQ questionnaire. This questionnaire asks owners a series of questions to assess each parameter, and owners are asked to score their dog on a scale from 0 (no signs of the behavior) to 4 (signs are severe). The scores for each parameter's questions (e.g. for stranger-directed aggression there are 10 related questions) are then averaged, to provide a mean score for that parameter.</p> <p>The C-BARQ questionnaire is a widely used, validated method of assessing canine behavior in a range of ways (not specifically anxiety).</p>
<p><b>Main findings: (relevant to PICO question):</b></p>	<p>UCCR:</p> <ul style="list-style-type: none"> <li>• Baseline UCCR of the dogs was not significantly different</li> </ul>

	<p>between treatments.</p> <ul style="list-style-type: none"> <li>• Post stressor UCCR was significantly different to baseline measurement, irrespective of treatment (i.e. the dogs were stressed by the veterinary visit) (<math>P &lt; 0.01</math>).</li> <li>• The Mean (<math>\pm</math> standard error of the mean, S.E.M.) UCCR of the dogs post-stressor was significantly lower (<math>P = 0.04</math>) when they were being fed the experimental diet (<math>32.17 \pm 0.21 \times 10^{-6}</math>) than when they were being fed the control diet (<math>39.61 \pm 0.3^{-6}</math>).</li> </ul> <p>Questionnaire assessment of the dogs' anxiety-related behaviours:</p> <ul style="list-style-type: none"> <li>• There was a significant reduction in stranger-directed aggression when the dogs were being fed the experimental diet (<math>t = 2.44</math>, d.f. = 15, <math>P = 0.014</math>). The mean (<math>\pm</math> S.E.M.) scores were: control: <math>1.25 (\pm 0.22)</math>; experimental: <math>0.85 (\pm 0.20)</math>.</li> <li>• There was no effect of diet on owner-directed aggression. The mean (<math>\pm</math> S.E.M.) scores were: control: <math>0.47 (\pm 0.07)</math>; experimental: <math>0.43 (\pm 0.07)</math>.</li> <li>• There was a significant reduction in stranger-directed fear when the dogs were being fed the experimental diet (<math>t = 3.08</math>, d.f. = 22, <math>P = 0.014</math>). The mean (<math>\pm</math> S.E.M.) scores were: control: <math>1.51 (\pm 0.22)</math>; experimental: <math>1.15 (\pm 0.14)</math>.</li> <li>• There was a significant reduction in non-social fear when the dogs were being fed the experimental diet (<math>t = 2.00</math>, d.f. = 17, <math>P = 0.031</math>). The mean (<math>\pm</math> S.E.M.) scores were: control: <math>1.44 (\pm 0.22)</math>; experimental: <math>1.20 (\pm 0.23)</math>.</li> <li>• There was a significant reduction in touch sensitivity when the dogs were fed the experimental diet (<math>t = 3.56</math>, d.f. = 25, <math>P &lt; 0.001</math>). The mean (<math>\pm</math> S.E.M.) scores were: control: <math>1.34 (\pm 0.14)</math>; experimental: <math>1.01 (\pm 0.12)</math>.</li> </ul>
<p><b>Limitations:</b></p>	<p>The study was partially funded by Royal Canin. The authors do not state whether Royal Canin had any input into the experimental design.</p> <p>The study was not specifically designed to study the effects of alpha-casozepine on anxiety. Rather, it was set up to study the effects of a commercial diet that contained alpha-casozepine and increased levels of tryptophan. Thus, there was a complete confounding variable when using this study to address the PICO question.</p> <p>The diets used were not identical in all other respects, other than the dietary additives. This was sufficient for a commercial aim (to show the diet was beneficial) but limited interpretation of the effects of any specific additive.</p> <p>The study did not randomise the order in which the dogs received the treatments. Therefore, the study is at risk of order effect biases. It is not clear why this was not done as the order that the diets was presented to the dogs was under the control of the experimenters and the experimenters provide no explanation for why this approach was taken.</p>

The authors refer to preliminary data to establish that dietary additives to the diet would be fully eliminated from the body within one week, in order to justify the one week washout period. However, it is unclear what dietary additives they are referring to as the experimental diet containing the additional dietary additive (alpha-casozepine) or increased levels of the dietary additive tryptophan was presented to all dogs in the second (post wash out period) phase of the study.

Cortisol is a non-specific measure of arousal, rather than a specific biomarker for stress. Therefore, it is possible that the dogs were excited or stimulated by a trip to the vets, rather than fearful. The authors fail to determine what the dogs' normal behavioural response is to a trip to the vets.

The C-BARQ questionnaire assessment of the dogs to determine which dogs were included in the study provides insufficient detail.

1. The C-BARQ questionnaire used is not a questionnaire that has been validated to anxiety per se. Several of the categories are specifically designed to assess fear, but some are not. Of particular relevance here is the aggression-related categories. Not all aggression is derived from anxiety.
2. The definition of an 'anxious dog' (i.e. that meets the inclusion criteria) was very loosely defined. Dogs were only excluded if, on *all* the questions asked ( $n = 32^1$ ), the owner scored the dog as a 0 (no sign) to 1 (mild signs observed). Thus, a dog could be included if the owner scored it as a 2 (somewhere between mild and moderate) on just one of the questions.

<sup>1</sup>Assumes mutual exclusivity of the questions. The authors do not clarify whether one question assesses more than one parameter simultaneously.

## Appraisal, application and reflection

Three peer reviewed papers were identified that either partially or fully addressed the PICO. Two of these studies (Palestrini et al., 2010; Kato et al., 2012) investigated the use of diets with added alpha-casozepine, though, in the Kato et al. (2012) study the diet was also supplemented with another compound (tryptophan) thought to have anxiolytic properties. The remaining study (Beata et al., 2007) investigated the use of a daily capsule of alpha-casozepine. The two studies focused on dietary interventions (Palestrini et al, 2010; Kato et al., 2012) used a placebo-controlled study design, whereas Beata et al. (2007) compared alpha-casozepine to another intervention (selegiline) that was already used commercially as an anxiolytic.

All of the studies focus on the potential anxiety-reducing effects of alpha-casozepine when administered in the medium to long term. Time from baseline measurement to the first and last assessment of anxiety ranged from 14-56 days (Beata et al., 2007), c.30-68/69 days (Palestrini et al. 2012) or 7 weeks (one defined timepoint) (Kato et al. 2012). The Palestrini et al. (2010) study also undertook an assessment of the dogs at the start of administering the diet (separate to an additional set of baseline measurements taken immediately before starting the diet). However, there is insufficient study detail to define the exact details



here and the number of days that comprised this phase is uncertain. Furthermore, as no significant differences were identified between this and the pre-diet baseline measurements, it is clear that there are no studies that found a significant effect of alpha-casozepine on indices of anxiety in the short term. Further, no studies were identified that looked at the effects of alpha-casozepine to a dog shortly before (e.g. minutes to a few days) exposure to a novel, or intermittent stressor (e.g. fireworks). Thus, there is currently no research evidence to support recommending the use of alpha-casozepine to clients seeking a quick acting anxiolytic product to alleviate short term stressors in the dog's immediate or short term future.

All three of the studies that examined the effect of alpha-casozepine administration in the medium-long-term of indices of anxiety were problematic in terms of experimental design. Of the studies that looked at the effect of alpha-casozepine, the industry-sponsored study by Beata et al. (2007) offered the weakest experimental design and the authors appear unaware of this limitation as the conclusions that they draw are misleading. Equivalence and / or non-inferiority type studies in which a new treatment is compared to an existing treatment are increasingly commonplace in medical research and justified as removing the ethical issues of not treating some patients at all (or giving a placebo). Beata et al. (2007) adopt this approach, but their study design also includes a behavioural modification programme (BMP) alongside each of the anxiolytic products compared. This represents a confounding variable. As a consequence, even though their findings appear to indicate that alpha-casozepine has a similar efficacy as selegiline, based on their study design, the level of efficacy (from no effect to very effective) cannot be determined for either product as the effect of BMP is also unknown. This problem is compounded by their exclusion criteria as they systematically exclude any dogs that have already received BMP. Thus, it is not possible for the authors to justify their conclusions by the claim that, prior to starting anxiolytic therapy, these dogs had failed to respond to the BMP. Though even this appeal would be sensitive to the effects of time spent implementing a BMP on indices of anxiety reduction. Any appeal to the efficacy of alpha-casozepine would require appeal to the wider literature demonstrating an effect of selegiline per se on indices of anxiety in the dog and the inherent limitations and problems that entails. Thus, this study fails to address adequately the PICO posed in the Knowledge Summary.

Of the three studies, the Palestirini et al. (2010) most successfully set up their treatment groups to address the PICO. The two diets compared are identical other than the addition of caseinate hydrolysate (the milk protein isolate containing alpha-casozepine) and the dogs are not undergoing a behavioural modification programme. However, they fail to state the inclusion rate of the caseinate hydrolysate and the dogs were allowed to consume the diet on an ad libitum basis so individual dog exposure to the active ingredient was variable. One of the strengths of this study is that they also include a non-anxious group of dogs that are also randomly allocated to receive either the experimental or control diets. This potentially allowed them to identify whether any behavioural changes observed were due to other, non-anxiety based, properties of alpha-casozepine. However, the sample sizes utilised in this study were small to start with (n = 8 per subgroup), and they took the further, questionable, step at the end of data collection, of retrospectively removing from the main analysis, three of the dogs in the anxious group. They fail to report which of the treatment groups (experimental versus control diet) these dogs were allocated to and this means that one of these subgroups (crucial to answering the PICO) may have had as few as 5 dogs in it. Frequently, the paper moves from comparing diet groups (the aim of the study) to comparing the responses of anxious and non-anxious dogs and, therefore, fails to address the PICO at many points. There is some evidence (cortisol, reactivity evaluation form) to suggest the experimental diet reduced anxiety in anxious dogs by the end of the study, but most other physiological or behavioural parameters were either not affected by treatment or the findings are unreported. Poor data handling and reporting (see limitations) further limit the ability of the reader to draw meaningful inferences from the findings of this study. External validity is also questionable: with a captive-bred, born, reared and studied population of laboratory beagles and a maximum sample size per group of eight dogs (or lower), it is questionable how well any findings could be extrapolated to the pet population, with their varying genetic history and life time experiences.

Unfortunately, the final study (Kato et al. 2012) is also problematic in terms of addressing the PICO. In its

defence, this industry sponsored study was not set up specifically to study alpha-casozepine but rather the effects of feeding a diet containing both alpha-casozepine and tryptophan on longer term indices of anxiety in the pet dog. Thus, the confounding variable of these additives to the diet exists for the purpose of the PICO but not for the purpose of the original study. However, the treatment diets were still not tightly controlled with the two diets varying across many dimensions other than the additives thought to reduce anxiety. Dogs were used as their own control, with an eventual sample of 28 dogs. Data handling was better within this study with relative homogeneity of the treatment effects observed: of the six outcome measures used, only one parameter showed no effect of treatment (owner – directed aggression). The other five parameters (urinary cortisol: creatinine ratio, stranger directed aggression, stranger-directed fear, non-social fear, and touch sensitivity) were all significantly improved when the dogs were fed the experimental diet. However, inclusion criteria for the study was relatively lax. If a dog scored above 1 on any one question out of circa 28 questions selected from the pre-validated C-BARQ questionnaire for being thought (by the researchers) to be potential measures of anxiety in the dog, they were included in the study. The mean values for each dog on the control diet suggests that dogs were generally scoring low across categories anyway which suggests that, in general these were not necessarily a particularly anxious population of dogs that were studied. Furthermore, the experimental design put the findings at a relatively high risk of bias. Despite the authors having experimental control over the study population, they chose to undertake an experimental design in which all dogs first received the control diet, followed by all dogs receiving the experimental diet. This makes the outcome particularly sensitive to order effects as dogs may simply have improved over time. For example, the cortisol of the dogs was lower at the second visit to the veterinary clinic. This could be because the dog had habituated to the veterinary practice (though the authors point out these dogs visited the vets regularly). Alternatively, if owners chose to participate in a trial testing diets aimed at reducing anxiety, this might alert them to the need to other information that might be used to reduce anxiety in their dogs. Furthermore, the researchers refer to a wash out period of 1 week between trials to ensure that additives have been removed, yet it is not clear what additives they are referring to as the ones in question are only included in the second phase.

In summary, whilst there is some evidence (through a shared direction of effect where a significant effect exists) across the studies that alpha-casozepine may have a clinically beneficial effect in reducing canine anxiety in the medium to longer term, the current evidence is weak and there is a need for good quality, placebo-controlled, randomised clinical trials that specifically address this compound for common anxiety-causing scenarios that this biomolecule may be promoted as a potential solution for.

## Methodology Section

Search Strategy	
Databases searched and dates covered:	Pubmed (1900 – 2017); Web of Science (1970 –2017); CAB Abstracts (1983 – 2016)
Search terms:	(dog OR dogs OR canine OR bitch* OR canid OR puppy OR puppies) AND (“alpha casozepine” OR alfa1-casozepine OR alpha-casozepine OR casein OR “milk protein” OR zylkene OR milk) AND (anxiety OR anxious OR fear OR fearful OR stress OR stressed OR stressful OR panic OR phobi*)
Dates searches performed:	11 <sup>th</sup> July 2017

Exclusion / Inclusion Criteria	
Exclusion:	Pre-defined exclusion criteria: non English language, popular press articles
Inclusion:	Any comparative (control group utilised) study in which the effect of alpha-casozepine on anxiety (or similar) in dogs was studied.

Search Outcome						
Database	Number of results	Excluded – did not answer PICO question	Excluded – not English language	Excluded – conference abstract only	Excluded – duplicates	Total relevant papers
NCBI PubMed	22	22	0	0	0	0
Thomson Reuters Web of Science	33	30	1	0	0	2
CAB Abstracts	46	42	1	1	1	2
Total relevant papers when duplicates removed						3

## CONFLICT OF INTEREST

The author declares no conflict of interest.

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