



# Therapeutic effects of an alpha-casozepine and L-tryptophan supplemented diet on fear and anxiety in the cat

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## Abstract

**Objectives** This study assessed the anxiolytic effectiveness of a test diet (Royal Canin Feline Calm diet) supplemented with L-tryptophan and alpha-casozepine.

**Methods** Subjects were 24 cats that were classified as mildly or markedly fearful based on the presence of a person in their home room. Three different protocols were used to assess anxiety: (1) evaluation of the response to a human in the cat's home room (home room test); (2) analysis of the response to placement in an empty test room (open-field test); and (3) analysis of the response to an unfamiliar human (human interaction test). All three protocols were first run at baseline, and the results were used to assign the animals to control and test diet groups that showed equivalent fear and anxiety. Both groups were retested on the three protocols after 2 weeks (test 1) and again after 4 weeks (test 2).

**Results** The diet groups differed for two behavioral measures in the open-field test: inactivity duration and inactivity frequency. The control group showed statistically significant increases in inactivity duration between baseline and test 1 and baseline and test 2, while the group fed the test diet showed a marginally not significant decrease in inactivity duration between baseline and test 1 and a not significant decrease for test 2. There was also a significant increase in inactivity frequency between baseline and test 1 in the test diet group and marginally not significant decrease in the control group. There were no differences between groups in the approach of the cats toward people for the home room test and the human interaction test.

**Conclusions and relevance** These results suggest that the test diet reduced the anxiety response to placement in an unfamiliar location, but that fear in the presence of an unfamiliar person was not counteracted by the diet.

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## Introduction

Fear and anxiety are accompanied by similar and overlapping responses; however, there are important differences. Anxiety is a response to an anticipated threat, uncertainty, or lack of control, while fear is an emotional response to a specific situation or stimulus that is perceived as a danger.<sup>1–4</sup> Both fear and anxiety are adaptive responses, associated with autonomic activation, that can negatively affect the cat's health and welfare, weaken the human-animal bond and lead to behavioral issues such as aggression, avoidance and soiling, which are a major cause of relinquishment.<sup>1,5</sup> However, behavioral strategies differ depending on the individual, stimulus and environment. Fear triggers defensive behaviors including fight, flight and freeze, fearful facial

expressions and body postures, startle and displacement behaviors arising from conflict (eg, yawning, lip licking, circling).<sup>1,2,4</sup> In addition, fear suppresses distress vocalization.<sup>6</sup> The anxiety response is related to uncertainty

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and lack of control that might be expressed as hyper-vigilance, restlessness, escape or avoidance, circling, yawning, lip licking and distress vocalization.<sup>1,2,4</sup>

Pharmacological therapy, in combination with environmental and behavioral modification, is often indicated to deal with the problem and address the wellbeing of the pet. To date, however, no pharmacologic therapeutics have been approved for the treatment of fear and anxiety in cats. While a variety of natural products claim to be effective in reducing anxiety, these claims have not been comprehensively assessed.

Royal Canin Feline Calm is an alpha( $\alpha$ )-casozepine and L-tryptophan supplemented balanced diet. Both nutrients have been reported to have anxiolytic effects.<sup>7-9</sup> Tryptophan is an essential amino acid and the metabolic precursor to melatonin and serotonin (5HT) that has been implicated in the regulation of many behavioral processes, such as mood, aggression and susceptibility to stress.<sup>10-13</sup> L-tryptophan has been evaluated in the treatment of behavior disorders in cats including signs of repetitive behavior, vocalization and agonistic behaviors.<sup>8</sup> However, supplementation with L-tryptophan alone may not be effective, because of limitations in its ability to pass through the blood-brain barrier as it competes with other large neutral amino acids for a common transporter. Therefore a lowered level of large neutral amino acids in relationship to tryptophan may increase tryptophan availability, leading to increased 5HT synthesis in the brain.<sup>13</sup>

$\alpha$ -casozepine is a bioactive peptide originating from  $\alpha$ S1 casein, a protein in cow's milk, which has an affinity for gamma-aminobutyric acid (GABA) receptors in the brain. It is reported to have an anxiolytic effect similar to benzodiazepines, with none of the side effects such as incoordination and disinhibition of aggression.<sup>9,14,15</sup> Beata et al reported that fear of strangers, contact with familiars, general fears, fear-related aggressions and autonomic disorders were all significantly improved.<sup>9</sup> In a recent study, the 'Calm' diet was found to reduce urinary cortisol and increase the plasma tryptophan/large neutral amino acid ratio; however, no behavioral changes were observed. In that study, all enrolled cats were perceived to be normal, which limited the possibility of observing a reduction in stress.<sup>16</sup>

The purpose of the present study was to evaluate the effectiveness of the Royal Canin Feline Calm diet (test diet) in reducing fear and anxiety in cats in an open-field test (OFT) and a human interaction test (HIT). We have previously demonstrated that cats that exhibit fear of people show greater inactivity in an OFT than non-fearful cats, and that inactivity in cats with mild fear of people is intermediate.<sup>6</sup> In the HIT, non-fearful cats had significantly shorter and more frequent bouts of inactivity than either mildly fearful or highly fearful cats. Treatment with diazepam caused a reduction in inactivity in both the OFT and HIT.<sup>6</sup> Therefore, in this study we

selected cats that had been categorized as either fearful or mildly fearful.

## Materials and methods

### Experimental design

A parallel group design was used to evaluate the anxiolytic effectiveness of the test diet. At baseline, physical examination, complete blood count, biochemical profile and urinalysis were all within normal limits. Cats were fed twice daily in separate feeding cages. Each cat was given a measured amount of the prescribed food calculated for its optimum weight. All cats were initially placed on the control diet (Purina Cat Chow) for 2 weeks of adaptation, after which they were tested in the home room, OFT and HIT over three consecutive days. These baseline data were used to place subjects into two groups that showed equivalent fear and anxiety. One group was then switched to test diet and the other maintained on control diet for the following 4 weeks. Subjects were retested on the three tasks after 2 and 4 weeks.

All procedures were approved by the animal use and care committee and were conducted in accordance with the guidelines set forth by the Canadian Council on Animal Care. The facility is a licensed animal research facility under the Ontario Ministry of Agriculture, Farms and Rural Affairs.

### Subjects

The subjects were 24 domestic shorthair cats (five males; 19 females) from the Vivocore colony, ranging in age from 4–12 years. Cats were selected from the population of fearful and mildly fearful cats as described in our initial study<sup>6</sup> (Table 1).

### Test protocols

Three different test protocols were conducted to assess measures of fear and anxiety. The first involved characterizing the cat's response to an unfamiliar person entering their home room (home room test).<sup>6</sup> The second (OFT) examined the cat's response to placement in an empty test room. The final protocol examined the cat's response to placement in the test room with an unfamiliar person (HIT). Both the OFT and HIT have been previously described.<sup>6</sup>

### Home room test

All cats had been categorized using a home room test as described in our model development study.<sup>6</sup> Home room assessment was performed by the first author (GL), a veterinary behaviorist with whom the cats had minimal familiarity. Briefly, the cats classified as fearful had been obtained approximately 6 months earlier from a research facility in which they had been identified as fearful. These cats were housed together and continued to actively avoid their caregivers. In the home room test, they

**Table 1** Subjects and allocation

Identification	Test group	Age	Sex (neuter status)	Anxiety status
Herman	Control	5.45	M (n)	Fearful
Katniss	Control	5.63	F (n)	Fearful
Larisa	Control	4.56	F	Mildly fearful
Lily	Control	9.70	F (n)	Mildly fearful
Maddox	Control	4.33	M (n)	Mildly fearful
Melanie	Control	4.25	F	Mildly fearful
Mimi	Control	4.42	F	Mildly fearful
Mina	Control	5.86	F (n)	Fearful
Moon	Control	4.25	F	Mildly fearful
Onca	Control	11.61	F (n)	Mildly fearful
Poppins	Control	6.18	F	Mildly fearful
Slash	Control	5.51	M (n)	Fearful
Andrew	Treatment	4.38	M (n)	Mildly fearful
Aya	Treatment	5.51	F (n)	Fearful
Delta	Treatment	4.30	F (n)	Mildly fearful
Gael	Treatment	5.45	M (n)	Fearful
Iris	Treatment	5.53	F (n)	Fearful
Jackie	Treatment	6.18	F (n)	Mildly fearful
Lacey	Treatment	6.17	F	Mildly fearful
Marissa	Treatment	4.42	F	Mildly fearful
Masha	Treatment	6.17	F	Mildly fearful
Milly	Treatment	4.42	F	Mildly fearful
Prudence	Treatment	5.51	F (n)	Fearful
Ruby	Treatment	4.42	F	Mildly fearful

n = neutered

actively avoided and displayed threats if approached. The other cats were intermixed in various housing rooms. The cats were assessed as non-fearful if they would voluntarily approach or did not withdraw when approached and would solicit or accept physical contact (touch, stroke) without signs of fear or avoidance. Those cats that would allow approach but would withdraw when contact was attempted were considered mildly fearful, and those cats that actively avoided approach or displayed signs of threats or aggression were considered fearful. Non-fearful cats were excluded from the study.

#### *Open-field test*

The OFT was conducted over 10 mins in a dedicated room (2.74 m × 3.66 m and 2.98 m in height) that included three cameras for recording movement, with one of the cameras set up to also record audio. The behavioral measures were recorded with the use of a behavioral tracking system, EthoVision XT (Noldus). Behavioral measures included: distance traveled; duration and frequency of inactivity, defined as when an animal was sitting or lying down or not exhibiting any overt behavior; and vocalization frequency.

#### *Human interaction test*

In the HIT, a person unfamiliar to the cat was seated on the floor in the center of the room before entry of the cat.

For each test a different person was used but the same person was used for all cats at each test. The person was instructed not to wear any perfume or cologne. During the test the person was instructed not to move, look directly at the animals or make any attempt to elicit attention. The human interaction was conducted over 10 mins and we recorded the same behavioral measures that were assessed in the OFT. In addition, we quantified frequency and time spent in proximity to the person (within 0.5 m) and in contact with the person.

#### *Statistical analyses*

All statistical analyses were conducted using Statistica version 11.0 software package with significance set to  $P < 0.05$ . Analysis of data obtained during the treatment phase for each measure for measures that were common to both the OFT and HIT was based on a repeated measures analysis of variance (RMANOVA) with time of test (baseline, test 1 and test 2) and task (open field and human interaction) as within-subject variables and both group (control, treatment) and level of fear (mildly fearful, fearful) as the between-subject variables. For all analyses in which the tasks were compared, task (open field vs human interaction) was added as a second within-subject variable. For the home room test data, task was not included as a main effect in the RMANOVA.

**Table 2** Home room measures

Group	Time of testing		
	Baseline	Test 1	Test 2
Control	Mean	3.17	2.42
	SEM	0.41	0.36
Treatment	Mean	3.42	2.79
	SEM	0.39	0.31
			0.43

Post-hoc analyses were carried out using Fisher's least significant difference test. For the HIT the Mann-Whitney U-test was used for group comparisons due to the skewed nature of score distributions which violated the assumptions necessary for use of parametric statistics. As guidelines for statistical interpretation, we used the 0.05 level of significance to evaluate whether or not we could reject the null hypothesis. In cases where the level of significance was  $>0.05$  and  $<0.10$ , we a priori characterized the result as marginally not significant, which justified further examination.

## Results

### Home room test

The group means for the home room test are summarized in Table 2. The home room scores for each group were compared using an RMANOVA with time of test (baseline, 2 weeks and 4 weeks) as a within-subject variable and group as the between-subject variable. There were no differences between groups, but there was a change with time of test [ $F(2,44) = 6.186705, P <0.005$ ]. A post-hoc Fisher's test revealed that home room scores for both test 1 ( $M = 2.6042$ ; SEM = 0.2369;  $P <0.005$ ) and test 2 ( $M = 2.6875$ ; SEM = 0.2655;  $P <0.01$ ) were significantly lower than baseline ( $M = 3.2917$ ; SEM = 0.279).

### Behavioral measures of anxiety

Table 3 summarizes the grouped data for the OFT.

### Distance traveled

The RMANOVA revealed a statistically significant main effect of level of fear ( $F[1,21] = 10.5974, P <0.005$ ), which reflected less activity in the fearful animals [ $M = 6.809$ , SEM = 5.623] than in the mildly fearful [ $M = 29.229$ ; SEM = 3.98)]. The analysis also revealed a marginally not significant treatment by task by group effect ( $F[2,42] = 2.737, P <0.08$ ). To explore this further, we next looked at each group separately for both the OFT and HIT, using an RMANOVA on distance traveled, with time of test as a within-subject variable.

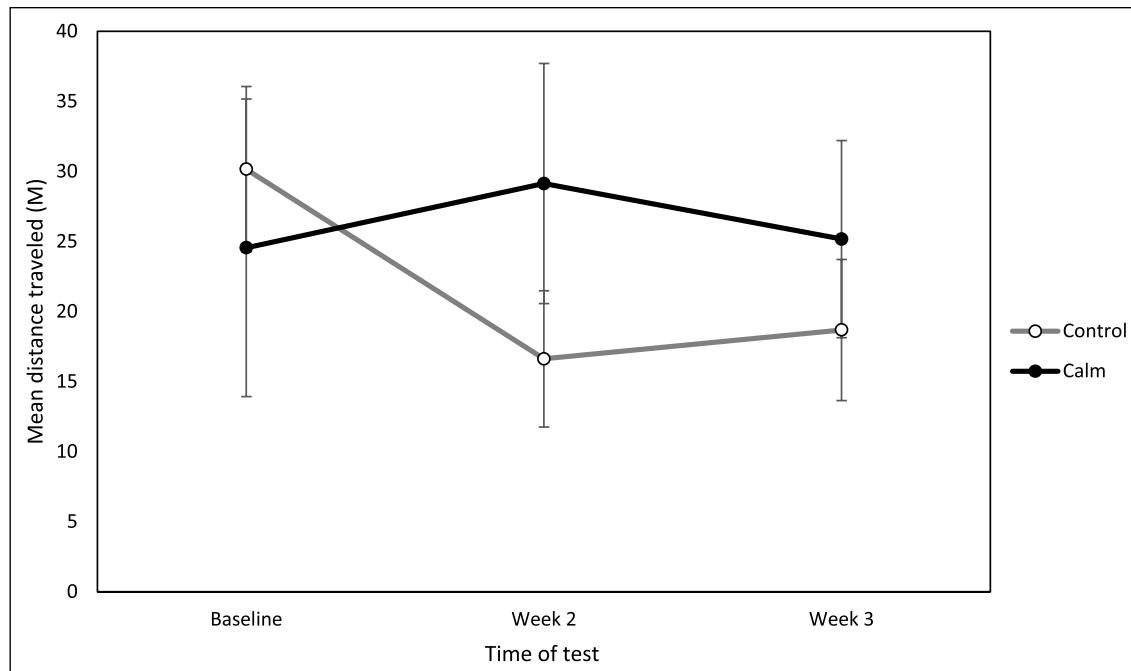
For the OFT (Table 3, raw data for which can be found in the supplementary material), the analysis for the control group revealed a significant effect of treatment day ( $F[2,22] = 3.504; P <0.05$ ). For the treatment group, by contrast, the effect was non-significant ( $P >0.1$ ). We then used the Dunnett test to compare baseline with test sessions for both the control and treatment group. The analysis revealed a statistically significant decrease between baseline and test 1 ( $P = 0.05$ ) and a marginally not significant decrease between baseline and test 2 ( $P <0.09$ ) for the animals on the control diet, and a non-significant increase for the animals on the test diet. Figure 1 shows that these results reflect reduced activity with repeated testing in control group, but not in the group with the test diet. In the HIT there were no significant changes.

### Inactivity duration

There were two significant effects. The first was a significant main effect of level of fear ( $F[1,21] = 8.325143, P <0.01$ ) and the second was a significant three-way interaction between time of test, task and group ( $F[2,42] = 5.349922, P <0.01$ ). The level of fear effect was due to a significantly ( $P <0.01$ ) lower overall inactivity

**Table 3** Open-field measures

Time of testing	Group	Distance		Inactivity		Vocalization
			(m)	Duration (s)	Frequency	Frequency
Baseline	Control	Mean	30.16	464.39	18.58	40.92
		SEM	5.91	27.24	3.14	13.86
	Treatment	Mean	24.56	501.76	13.58	42.92
		SEM	10.62	27.56	3.09	12.63
Test 1	Control	Mean	16.62	514.96	13.83	35
		SEM	4.87	24.30	3.23	13.02
	Treatment	Mean	29.14	456.44	20.08	58.5
		SEM	8.57	32.63	3.81	15.82
Test 2	Control	Mean	18.68	525.43	14.42	30.92
		SEM	5.05	16.68	3.05	10.56
	Treatment	Mean	25.17	490.51	17.17	40.58
		SEM	7.03	22.45	3.07	10.01



**Figure 1** Distance traveled in open-field task as a function of treatment and session. On the control diet there was a statistically significant decrease ( $P < 0.05$ ) between baseline and test 1 and a marginally not significant decrease ( $P < 0.09$ ) between baseline and test 2. By contrast, cats on the test diet showed a non-significant increase

duration in the mildly fearful animals ( $M = 474.11$ , SEM = 16.06) than in the fearful animals ( $M = 554.36$ , SEM = 22.707). The origins of the three-way interaction were due to open-field inactivity decreasing on the first test session in the test diet group, but increasing in the control group. In the HIT, inactivity went down in both groups on test session 1.

To further analyze the data we then looked at the OFT separately using an RMANOVA comparing the groups at each of the three time points. There was a significant level of fear effect ( $F[1,21] = 9.486518$ ,  $P < 0.05$ ) and a significant time of test by group interaction ( $F[2,42] = 4.28005$ ,  $P < 0.05$ ). For the fear effect, a post-hoc Dunnett test revealed that the anxiety effect in the open field was due to fearful cats having a greater inactivity duration compared with mildly fearful ( $P < 0.05$ ).

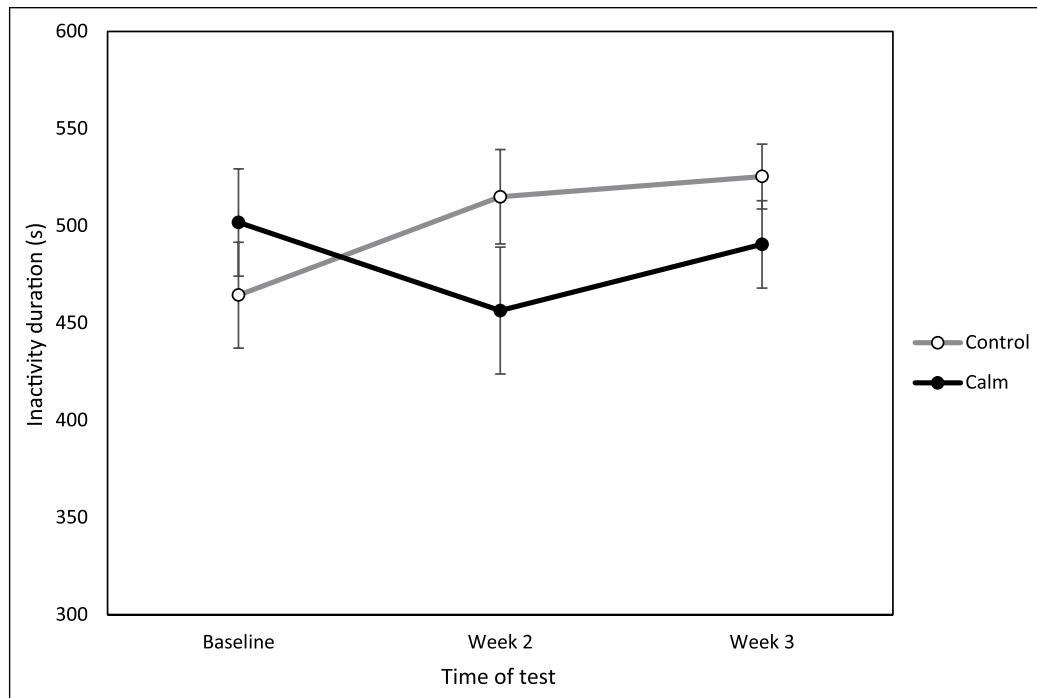
To assess the origins of the interaction we first looked at the Tukey HSD test and compared each score with every other score. None of the differences achieved statistical significance. In order to assess potential trends, we then examined the data using the Fisher multiple comparison test. This revealed statistically significant increases in inactivity duration between baseline and test 1 for the control animals ( $P < 0.05$ ). By contrast, the test diet group showed a marginally not significant decrease between baseline and test 1 ( $P < 0.07$ ). For test 2 there was a significant increase from baseline in inactivity in the control group ( $P < 0.05$ ) while the test diet group maintained a non-significant decrease in inactivity (Figure 2).

#### Inactivity frequency

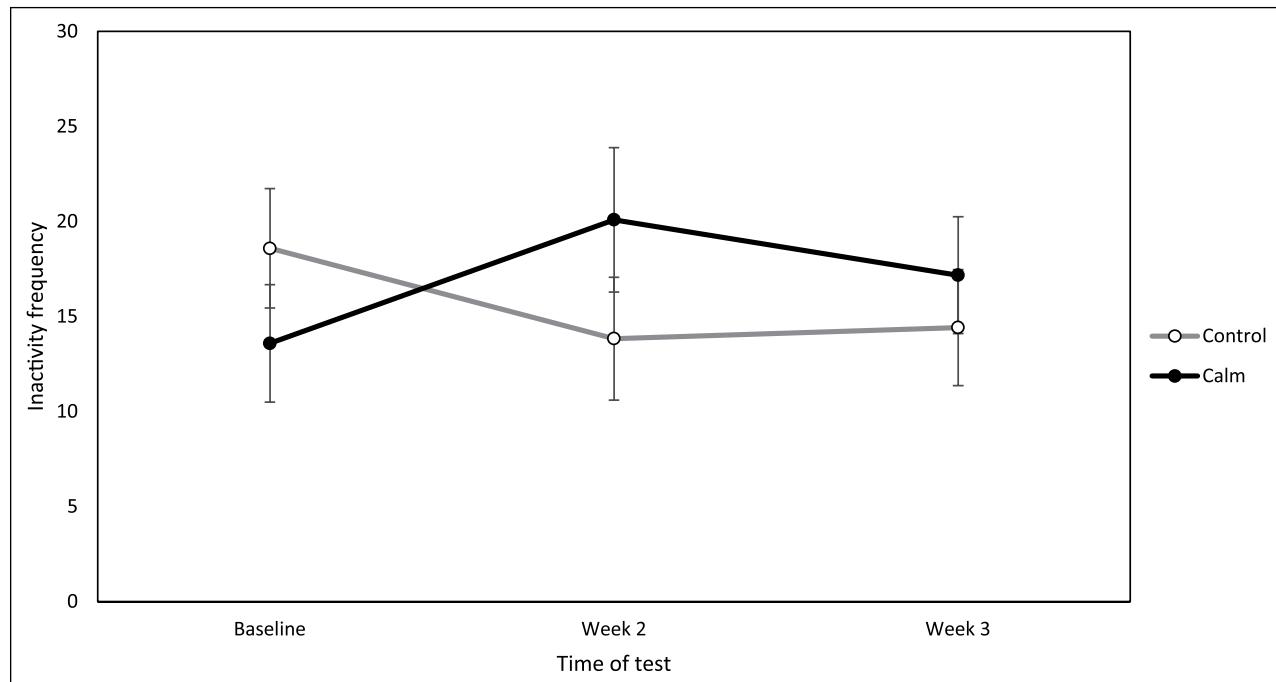
The results of the analysis of inactivity frequency revealed a statistically significant effect of level of fear ( $F[1, 21] = 6.812399$ ,  $P < 0.05$ ) and a statistically significant three-way interaction between time of test, task and group ( $F[2,42] = 3.859167$ ,  $P < 0.05$ ). To determine the origins of the interaction we compared inactivity frequencies separately in the OFT and HIT using an RMANOVA. The results of the ANOVA in the OFT demonstrated a significant effect of level of fear ( $F[1,21] = 7.899711$ ,  $P < 0.05$ ) and a significant interaction between time of test and group ( $F[2,42] = 5.053722$ ,  $P < 0.05$ ). The post-hoc Dunnett test revealed that the anxiety effect in the open field was due to mildly fearful cats having a greater inactivity frequency compared with fearful cats ( $P < 0.05$ ). For post-hoc analysis of group effects, we first compared means for each of the groups at each time point using the Tukey HSD test, which did not reveal any statistically significant differences. Next, to determine whether there were any clear trends we used the Fisher LSD test; the group effect due to an increase in inactivity frequency between baseline and test 1 for the test diet group was  $P < 0.05$  and was non-significant for test 2, while the control group showed a marginally not significant decrease at test 1  $P < 0.08$  and non-significant decrease at test 2 (Figure 3). For the HIT, there were no significant differences.

#### Vocalization frequency

The results revealed a significant main effect of task ( $F[1,21] = 11.25315$ ,  $P < 0.005$ ) and no other significant



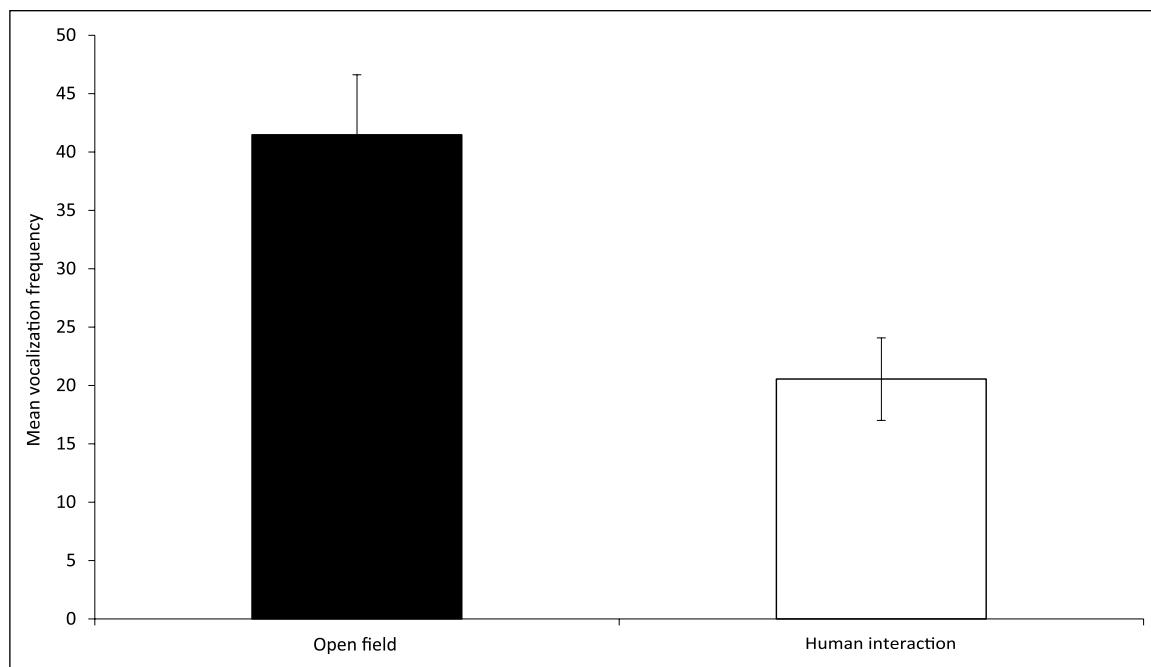
**Figure 2** Inactivity duration in open-field task as a function of treatment and session. Inactivity duration significantly ( $P < 0.05$ ) increased from baseline to test 1 in control cats while test diet cats showed a marginally not significant decrease ( $P = 0.07$ ). For test 2 there was a significant increase ( $P < 0.05$ ) from baseline in the control group and a non-significant decrease in the test diet group



**Figure 3** Inactivity frequency in open-field task as a function of treatment and session. Inactivity frequency was significantly increased ( $P < 0.05$ ) in test 1 and non-significantly in test 2 for the test diet, while the control diet had a marginally not significant decrease ( $P < 0.08$ ) at test 1 and a non-significant decrease at test 2

main effects or interactions. Figure 4 shows that the task effect reflected greater vocalization in the OFT than in

the HIT, which is consistent with the findings in our model development study.<sup>6</sup>



**Figure 4** Vocalization frequency in open-field and human interaction tests. Vocalization frequency was significantly greater ( $P < 0.005$ ) in the open-field test than the human interaction test

#### *Additional measures for the human interaction test*

For each of the HIT sessions, there were four measures related to frequency and duration of approach and contact. For each of the measures, the data were negatively skewed because a large percentage of animals in both groups showed no contact and no approach (4/12 control and 6/12 treatment) (Table 4). Since the score distributions violated the assumptions necessary for use of parametric statistics, the group comparisons were made with the Mann–Whitney U-Test, which did not reveal any statistically significant effects ( $0.40 \leq P \leq 0.887$ ).

## Discussion

The purpose of this study was to compare the effectiveness of a diet supplemented with L-tryptophan and  $\alpha$ -casozepine (Royal Canin Feline Calm diet) with a control diet in reducing fear and anxiety in cats that showed fearfulness to people.<sup>6</sup> We looked at three different test protocols: (1) response to an unfamiliar environment (OFT); (2) response to an unfamiliar person (HIT); and (3) assessment of fear in the home room (home room test).

Behavioral measures of fear and anxiety were based on activity, inactivity and vocalization, which were quantified using a behavioral tracking system. In the HIT, we also monitored time spent close to and in contact with the person.

For the inactivity measures in the OFT, the group on the control diet showed increases in inactivity compared with baseline while the group on the test diet showed decreases, which resulted in significant interactions

between group and time of test. Although the paired comparisons using the more conservative Tukey HSD test did not reveal statistically significant differences from baseline, the Fisher multiple comparison test found a statistically significant increase over baseline on both the first and second treatment test in the control group. By contrast, the group fed the test diet showed a marginally not significant decrease between baseline and the first OFT and a non-significant decrease in test 2. Similarly, for distance traveled there was a significant decrease in distance traveled at test 1 in the control group and a non-significant increase in the test diet group.

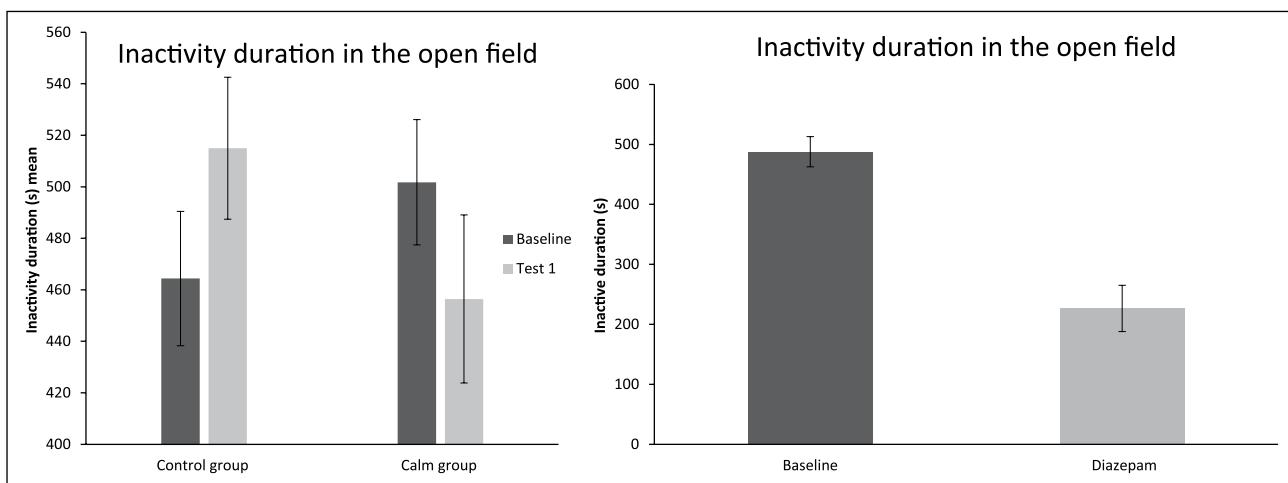
The other measure that showed sensitivity to the test diet in the OFT was inactivity frequency. This is a measure of the number of times that an animal stops moving and then restarts; the results parallel the inactivity duration data, with the test diet group showing a significant increase in inactivity frequency over baseline at test 1 and the control group a marginally not significant decrease.

Given the link between inactivity in an unfamiliar environment and anxiety, these results provide potential evidence of the anxiolytic effectiveness of the test diet. These findings are consistent with the effects seen with diazepam treatment in our previous study, in which inactivity duration was significantly decreased and inactivity frequency increased<sup>6</sup> (Figure 5).

There were no significant effects of diet in the HIT. Given the results from the OFT, this absence of significant effects in the HIT may reflect a selective benefit of the test diet in reducing anxiety, but not fear. Similarly,

**Table 4** Human interaction test measures

Time of testing	Group	Proximity to person		Contact with person	
		Duration (s)	Frequency	Duration (s)	Frequency
Baseline	Control	Mean	28.59	1.08	3.09
		SEM	16.17	0.61	1.37
	Treatment	Mean	47.32	1.33	19.34
		SEM	28.22	0.67	12.19
Test 1	Control	Mean	23.67	2.92	4.59
		SEM	12.13	1.42	2.42
	Treatment	Mean	12.29	1.50	7.59
		SEM	8.28	0.97	6.18
Test 2	Control	Mean	18.21	3.00	1.07
		SEM	12.65	1.96	0.75
	Treatment	Mean	16.67	2.17	5.58
		SEM	10.92	1.35	4.52

**Figure 5** Comparison of effects of diazepam and Royal Canin Feline Calm diet on open-field inactivity duration. Inactivity duration showed a marginally not significant decrease ( $P = 0.07$ ) after 2-week treatment with test diet while the control group significantly increased ( $P < 0.05$ ) (left). Similarly, inactivity duration decreased significantly after diazepam therapy (right) compared with baseline ( $P < 0.001$ ).

we have previously shown that in the HIT, the anxiolytic drug diazepam did not promote approach to humans in fearful cats.<sup>6</sup>

The decline in vocalization frequency from the OFT to the HIT in both groups is consistent with our previous study, in which much higher levels of vocalization were seen in the OFT than the HIT. One possible interpretation is that the presence of the person increased fear, which reduced vocalization.<sup>6</sup>

We also found differences in fear level with mildly fearful cats when compared with fearful cats, showing a decrease in distance traveled and inactivity duration, and greater inactivity frequency. This result replicates our previous observation in which fearful cats were more inactive than non-fearful cats, with mildly fearful cats intermediate.<sup>6</sup>

We interpret these results to indicate that the effectiveness of the test diet in reducing overall anxiety is probably limited to moderate anxiety-provoking situations. The absence of an effect on the fear response in the presence of an unfamiliar person may indicate a dissociation between reducing anxiety associated with an unfamiliar location but not in reducing the fear toward an animate stimulus. Another possibility is that the cats used in this study, which were pre-selected based on a fearful response to people, may have been too fearful for the diet to effect any improvement. Similarly, in our previous study fearful cats given diazepam spent the greatest time away from an unfamiliar person. Therefore a future study might be designed to evaluate a greater number of cats using cats that are minimally fearful or non-fearful in the home room.

assessment. In addition, as a previous study with  $\alpha$ -casozepine found that some cats improved in the second month of treatment, further studies might consider a longer course of therapy.<sup>8</sup>

As the test diet is supplemented with both  $\alpha$ -casozepine and L-tryptophan, the present results do not distinguish whether the beneficial effects are a result of either of these alone, or the two in combination.

## Conclusions

These results are suggestive of an anxiolytic effect of the Royal Canin Feline Calm diet to placement in an unfamiliar location based on a reduction in inactivity duration and increased inactivity frequency in the Calm diet compared with an increase in inactivity duration and decreased inactivity frequency in the control diet. Future studies of longer duration might further confirm the effect of the diet on fear and anxiety.

**Supplementary material** Raw data for the open-field testing from which Table 3 was compiled.

**Conflict of interest** Gary Landsberg, Dr Milgram, Christina de Rivera and Stephanie Kelly do not have any potential conflicts of interest to declare other than providing the research for this study as contracted by Royal Canin. Isabelle Mugeot is an employee of Royal Canin.

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