



Research

Assessment of noise-induced fear and anxiety in dogs: Modification by a novel fish hydrolysate supplemented diet



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ABSTRACT

This study examined the effectiveness of 2 different dosage levels of a fish hydrolysate, a natural supplement derived from fish protein, in reducing fear and anxiety in beagle dogs. A thunderstorm model was used, which entailed playing a recorded track of a thunderstorm to elicit measures in an open field test. Fear and anxiety were assessed with behavioral measures, which included noise induced activity and inactivity and an observational behavioral assessment, and blood cortisol levels. The test compound showed some effectiveness in reducing a hyperactivity response to thunder and in reducing the cortisol response. The results of this study provide initial support for considering the use of fish hydrolysate as a dietary supplement to reduce fear and anxiety.

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Introduction

Anxiety can be defined as a response to the anticipation of prospective or imagined danger or uncertainty, whereas fear is a state of alarm and agitation caused by present or threatened danger (Sherman and Mills, 2008). Individual differences in fear and anxiety are underlying factors in many canine behavior problems. An estimated 29% of pet dogs show signs of anxiety-related behaviors (Denenberg et al., 2013), and 17% to 49% of all dogs have been estimated to demonstrate an aversion to noise (Blackwell et al., 2013). Resultant behavior issues may adversely affect the human-pet relationship leading to a decreased commitment to pet care, relinquishment, or euthanasia (Bamberger and Houpt, 2006; Casey, 2002). In addition, the stress associated with fear and anxiety can adversely affect health and lifespan (Dreschel, 2010).

Psychoactive drugs, including benzodiazepines, azapirones, tricyclic antidepressants, and selective serotonin reuptake inhibitors, are commonly prescribed in the treatment of behavioral disorders, particularly those associated with fear and anxiety (Overall, 2013; Landsberg et al., 2013a). However, only fluoxetine (a selective

serotonin reuptake inhibitors) and clomipramine (a tricyclic antidepressant) have been licensed for anxiety treatment in dogs and both have limitations. They can take a month or longer to achieve their full therapeutic effects, and some owners are hesitant or unwilling to use these medications because of the potential for side effects or personal bias against psychotropic drug use (Sheppard and Mills, 2003).

There are also numerous natural products marketed for behavior therapy, but very few have demonstrated any evidence of efficacy. Although there may be a lay belief that natural products cause minimal undesirable effects, the possibility of adverse effects, interactions with concurrent drugs and supplements, and lack of standardization must be considered (Fugh-Berman and Cott, 1999; Landsberg et al., 2013a). To date, studies support a potential anxiolytic effect in dogs with alpha-casozepine, a tryptic hydrolysate of milk protein (Beata et al., 2007; Palestini et al., 2010), L-theanine (Araujo et al., 2010), dog appeasing pheromone (Denenberg and Landsberg, 2008; Siracusa et al., 2010), a blend of phellodendron and magnolia extracts (DePorter et al., 2012), and a combination of tryptophan and alpha-casozepine (Kato et al., 2012).

The objective of the present study was to evaluate the anxiolytic effectiveness of 2 dosages of a novel therapeutic, fish hydrolysate, intended as a supplement in dog food. Fish hydrolysate is easily digestible and high in polyunsaturated acids fatty acids, which confer numerous benefits (Dorman et al., 1995; Poncin et al., 1996a;

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Poncin et al., 1996b). The study product is produced from a sustainable white fish species (Gadidae type, by-products), which is obtained from a single fishing area in the North Atlantic. The production process uses enzymatic hydrolysis to release the peptides and amino acids assumed to be responsible for its therapeutic properties. Analytic testing ensures inter-batch standardization. The PC60 fish hydrolysate (cod and mackerel) has been found to have benzodiazepine-like effects on the hypothalamic-pituitary-adrenal axis, sympathoadrenal activity, and levels of gamma-aminobutyric acid in the hippocampus and the hypothalamus (Bernet et al., 2000). Stabium 200, a derivative of PC60, was found to reduce anxiety in humans (Dorman et al., 1995) and to improve memory and learning performances in rats (Le Poncin, 1996a) and humans (Le Poncin, 1996b).

Methods and materials

Subjects and housing

Subjects were 45 beagle dogs of both sexes ranging from 2.7 to 17 years of age and housed in the Vivocore Inc. colony. Before the start of the study, all dogs had veterinary examinations, a complete blood count, biochemistry profile, and urinalysis. Dogs were excluded from the study if they had observable health or mobility issues, impaired hearing, or clinically significant abnormalities on laboratory tests. Dogs were housed in groups of 4 in pens measuring approximately 5.2 × 1.5 m. All pens were located in an animal containment room measuring approximately 24.4 × 12.2 m, consisting of a cement floor and a 3-meter ceiling. Dogs were provided free access to water and were fed Royal Canin Medium Adult Dry Diet once daily. Dogs were provided with music, interactive toys, raised platforms, and a rigid plastic open topped cage in each pen and monitored multiple times daily for general health, behavior, and feeding by the trained facility animal care staff. The facility is a registered Research Facility by the Ontario Ministry of Agriculture, Food and Rural Affairs (Canada). The studies were approved by the local animal care and use committee without reservation and were designed under the guidelines set forth by the Canadian Council on Animal Care in accordance with the “Guide for the Care and Use of Experimental Animals.”

Study design

We used a thunderstorm model of noise-induced fear and anxiety to assess the effectiveness of the test compound. Dogs were placed in a relatively unfamiliar room. Research with other species (Crawley, 1985) and with dogs (Araujo et al., 2013; DePorter et al., 2012) has shown that both inactivity and hyperactive behaviors (Landsberg et al., 2013b; Milgram et al., 2014) may occur in response to thunder.

Two levels of supplementation were tested in a blinded, placebo control study. A matched group procedure was used for assigning each dog into 1 of 3 dose groups: a high dose group (1500 mg), a low dose group (750 mg), and a control group (maltodextrin 750 mg). For scheduling purposes, the dogs were divided into 2 cohorts, which were tested on successive days. As outlined in Table 1, after entrance into the study, all dogs were placed on a control diet and given 2 adaptation sessions of 9 minutes each in the open field activity room. At baseline (days –8 to –5) each dog received a 9 minute open field test, which was followed 2 days later by a 9 minute thunder test. In the thunder test (described as follows), dogs are placed in the open field activity room for 3 minutes. The thunder recording is played over the next 3 minutes, which is followed by 3 minutes without thunder (postthunder interval). A blood sample for serum cortisol level was collected 1 hour before

Table 1
Schedule of events

Study day	Key event
–17 to –16	CBC/clinical chemistry/veterinary examinations
–15 to –12	Adaptation days to open field
–11	Control diet wash-in begins
–8	Open field test (cohort 1)
–7	Open field test (cohort 2)
–6	Thunderstorm test (cohort 1) Cortisol sampling
–5	Thunderstorm test (cohort 2) Cortisol sampling
–2 and –1	Allocation and group selection
1	Start of treatment
7	Adaptation cohort 1
8	Adaptation cohort 2
14	Thunderstorm test (cohort 1) Cortisol sampling
15	Thunderstorm test (cohort 2) Cortisol sampling
21	Adaptation cohort 1
22	Adaptation cohort 2
26	Thunderstorm test (cohort 1) Cortisol sampling
27	Thunderstorm test (cohort 2) Cortisol sampling

CBC, complete blood count.

testing and again at 5 minutes and at 1 hour after the end of the test session. Grouping of subjects into the 3 treatment groups was based on sex, the cortisol response to thunder, and on the dogs' global fear and anxiety response to the thunder recording as described below. The subjects were then started on treatment (day 1 through day 27). Each group of 15 dogs was divided into 1 of 2 cohorts to enable equal dogs from each group to be tested on any given day. Twenty-one dogs were in cohort 1 (7 dogs per group) and 24 dogs (8 dogs per group) were in cohort 2. Each dog was then given a day of readaption with no thunder (day 7 or 8), a thunder test with cortisol levels taken (day 14 or 15), another day of readaption (day 21 or 22), and a final thunder test with cortisol levels (day 26 or 27).

Allocation of subjects

Grouping of subjects was based on sex, the cortisol response to thunder, and on the dog's global anxiety response to the presence of thunder. The subjects were first ranked on the magnitude of the cortisol response and on their subjective global anxiety response to thunder, which were summed together. The subjects were then separated by sex and within each sex ranked from high to low. Within each sex, the overall ranking was used to assign animals into 3 groups using the protocol illustrated in Table 2.

Each group was then assigned randomly to 1 of the 3 treatments, which insured that all staff involved in testing, data collection, and data analysis remained blinded with respect to treatment until the completion of the study.

Table 2
Sum of ranks: example of procedure used in group placement of males that showed a cortisol response

Ranking	Group	Sex
1	1	M
2	2	M
3	3	M
4	3	M
5	2	M
6	1	M
7	1	M

M, male.

Dosing

Subjects were dosed orally using weighed quantities of the test and control compounds, which had been placed into size 000 capsules. The test compound was GABOCEAN 3D PTP55 which was available in a powder form and provided by Diana Pet Food, VIT2BE, Elven, France, Batch No. 86. The control material maltodextrin was obtained from Sigma-Aldrich Co., St. Louis, MO.

The treatment administered to each of the groups was

- (1) Control: maltodextrin, 750 mg (1 capsule)
- (2) Test compound 1: functional fish hydrolysate, 750 mg (1 capsule)
- (3) Test compound 2: functional fish hydrolysate, 1500 mg (2 capsules)

Testing room equipment and data acquisition

The open field arena was an empty room 2.74 × 3.66 m, containing 3 cameras that were used to record behavior. One camera was secured to the ceiling of the room, providing a view of the entire room from above. Two cameras were secured on opposite walls of the room. One of the cameras was also set up to record audio, which was used for collection of vocalization data. For the behavioral analyses, the videos were played simultaneously, giving the observers 3 different perspectives for assessing behavior. Before each test, the room was cleaned with a peroxide based cleaner (Accelerated Hydrogen Peroxide) and a mop.

Open field activity testing

All subjects received 2 9-minute adaptation sessions in the open field 3 to 7 days before the baseline open field test (see Table 1). The behavior was monitored and taped during these sessions but was not scored. The initial baseline open field test was administered either 7 or 8 days before the start of treatment. This test was divided in 3 3-minute blocks, and each block was scored separately. Dogs received a second adaptation session on days 21 and 22, between the 2 thunder tests. A final open field test was conducted at the conclusion of the trial (2 days after the final thunder test) to evaluate conditioned fear or anxiety measures.

Thunderstorm activity testing

Subjects were tested on the thunderstorm activity test on 3 occasions. The first was at baseline, the second (thunder test 1) after 2 weeks of treatment, and the third (thunder test 2) after 4 weeks of treatment using the thunderstorm model previously described by Araujo et al. (2013). At the start of the test, the subjects were placed in the open field arena and were allowed to freely explore the room over the first 3 minutes (prethunder interval). Over the next 3 minutes, the dogs were exposed to a thunder track, consisting of segments from the Sound Scary Thunder Therapy CD (Sound Therapy 4 Pets Ltd, Chester, England) played over a stereo speaker system mounted in the ceiling, with sound peaks at around 90 decibels. Over the last 3 minutes (postthunder) the thunder recording ceased. Recorded footage was analyzed for each 3 minute period both in real time and from saved files.

Target measures

Three protocols were used to assess fear and anxiety. The first, an objective behavioral analysis, used a behavioral tracking system (EthoVision) to monitor behavioral activity before, during, and after the presentation of thunder. The second protocol used a trained

observer to provided ratings using a fear and anxiety scale developed by CanCog Technologies (discussed as follows). The third protocol measured changes in serum levels of cortisol, which were obtained both before and after the presentation of thunder.

Measurement of behavioral activity

The behavioral tracking system used analysis utility EthoVision XT (Noldus, Leesburg, VA) software which was calibrated to the measurements of the room to automatically provide a measure of distance. Inactivity was recorded by a trained observer via key presses. Two outcome measures were used: locomotion, defined as total movement, and inactivity, defined as time spent in an inactive state (sitting or lying down and not engaging in any other behaviors).

Observational assessment

Using the recordings from the open field activity and thunderstorm activity testing, the dogs were assessed for each 3-minute block using observational measures of fearful and anxious behaviors (Landsberg et al., 2013b, Milgram et al., 2014). The behavioral scores were developed from a compilation of signs described in the veterinary literature for the assessment of dogs with fear of noises (Dreschel and Granger, 2005; Levine et al., 2007; Cracknell and Mills, 2011; Sheppard and Mills, 2003; Mills et al., 2013). As indicated in Table 3, the positive score was based on behaviors associated with increased activity, and the negative scores were based on behaviors associated with suppression of activity and triggering of an autonomic response. Global scores were based on frequency (bouts of behavior) and intensity (severity) of anxious behavior. Intensity of anxious behavior was classified as none, mild, moderate, or severe, and frequency of behavior was classified as occasional (approximately 20% or less), some of the time (approximately 50%), and most of the time (approximately 80% or higher). A score of 1 equaled none, 2 equaled mild and occasional, 3 equaled either mild and some of the time or moderate and occasional, 4 equaled moderate and some of the time, 5 was either severe and some of the time or moderate and most of the time, and 6 equaled severe and most of the time. Positive and negative scores were based on the intensity of the signs (mild, moderate, or severe) as they related to the global score.

Each of the behavioral measures was assigned during the pre-thunder interval (minutes 1-3 of each test session), the thunder interval (minutes 4-6), and the postthunder interval (minutes 7-9). Before starting the study, each evaluator was trained on the model. Inter-observer reliability was measured.

Cortisol

Blood samples were collected from each subject 60 minutes (±5 minutes) before the thunderstorm test, at 11 minutes

Table 3
Behavioral observational assessment

Positive/active score (increased activity)
(1) Startle, scan (orient), bolt
(2) Active responses including aimless, repetitive or stereotypic pacing, running, or circling; digging, climbing, jumping, barking
Negative/passive score (decreased activity)
(1) Decrease activity: freeze-against wall-at door
(2) Lowered body postures: crouch (cower), tail between legs, ears back
(3) Autonomic/conflict: pant, shake (tremble), alert/tense/vigilant, salivate, yawn, lip lick, fore leg lift, whine
Global score takes all signs into consideration (both positive and negative) over each 3 minute interval

(± 1 minute) following the onset of the thunder recording (5 minutes ± 1 minute after the conclusion of the test session), and at 60 minutes after the presentation of thunder. Blood samples were obtained with a syringe and needle, placed into 4 mL serum separator tubes, centrifuged at 2800 rpm, refrigerated, and shipped on ice by the same day courier to Antech laboratories. Because only a subgroup of dogs show a significant cortisol elevation in response to thunder, the initial baseline data were used to classify individual subjects as responders. Cortisol measures were only obtained for animals that had been deemed to be responders.

Statistical analyses

A baseline analysis was done to test for a behavioral change in response to the presentation of thunder and in the postthunder interval, compared to the prethunder interval. We used distance traveled as the primary activity measure.

Each of the measures of fear and anxiety (global, positive, negative) was analyzed with a repeated measures analysis of variance (ANOVA) using the time-interval (prethunder, thunder, and postthunder) as within subject variables and group as between subject variables.

The analyses of the treatment data initially used a repeated measures ANOVA to compare the baseline and 2 treatment sessions, with time interval (prethunder, thunder, and postthunder) as a second within subject variable. Subsequent analyses examined performance restricted to single sessions, when appropriate, using the Fisher least significant difference test to compare means.

Results

Group allocation

Of the 45 total dogs, 30 showed an increase that was $>25\%$ of baseline in the postthunder cortisol measurement, as measured approximately 11 minutes after the onset of thunder (5 minutes posttesting). In the final group allocation, 2 animals had their groups switched so that we had an equal number of responders (10) in each group (Table 4). The control group consisted of 7 neutered males and 8 spayed females with a mean age of 7.9 (2.8–14) years. The low dose group included 7 neutered males and 8 spayed females with a mean age of 8.22 (3.3–11.8) years. The high dose group had 5 neutered males and 10 spayed females and a mean age of 10.7 (6.9–17.3) years.

Objective data

To establish the response to thunder at baseline and to confirm equivalency between groups, the distance traveled measure was first analyzed with a repeated measures ANOVA, with time interval and task (open field vs. thunderstorm test) as within-subject variables and group as a between-subject variable. The results revealed a statistically significant main effect of time interval ($P = 0.0000$) and a statistically significant interaction between task and time interval ($P = 0.00078$). The main effect of time interval was

due to a decrease on distance traveled from the first (prethunder) to the second (thunder) time interval, and a further decrease during the postthunder interval (Figure 1). There was a greater decrease in activity in the open field test during the second time interval than there was during the presentation of thunder. Equivalent results were seen in all 3 groups. Activity was increased during thunder, relative to the change seen in the open field alone session.

Potential effects of dietary supplementation were analyzed with respect to movement and inactivity using a repeated measures ANOVA with test (baseline, test 1 and test 2) and time interval (prethunder, thunder, postthunder) as within-subject variables and group as a between-subject variable. The analysis of the distance travelled measure showed statistically significant effects of test ($P = 0.031$) and time interval ($P = 0.000$), but there were no significant effects related to group.

Analysis of the inactivity data showed a statistically significant effect of test ($P = 0.0067$), time interval ($P = 0.000$), and a test by time-interval interaction ($P = 0.0015$). The test by time-interval interaction reflected increased inactivity over repeated testing, largely driven by the low dose treatment group (Figure 2).

Observational data

Each of the observational behavioral measurements was analyzed using a repeated measures ANOVA with test (Baseline, Test 1 and Test 2) and time interval as within-subject variables and group as a between-subject variable.

Global behavior

Analysis of the global behavior score showed statistically significant effects of test ($P = 0.000$), time interval ($P = 0.000$), and for the interaction between test and time interval ($P = 0.000$). However, there were no significant effects relating to differences between groups. Figure 3 shows that the time interval by test interaction reflects increases in fear and anxiety during the thunder and postthunder intervals, when compared to pre-thunder.

Negative behavior

The ANOVA for the negative behavioral scores revealed statistically significant main effects of test session ($P = 0.000$), time interval ($P = 0.000$), and a significant interaction between time interval and test session ($P = 0.0000$). There were no effects related to groups.

Each treatment period was examined separately. The results from the first treatment session revealed a significant effect of time interval ($P = 0.000$) on increased fear or anxiety during the thunder presentation interval compared to the prethunder period (Figure 4). The results on the second treatment period were similar, with a significant main effect of time interval ($P = 0.000$) but not of group.

Positive behavior

The initial analysis of the positive behavioral data revealed significant main effects of test ($P = 0.0015$) and time interval ($P = 0.000$). There were significant interactions between test and time interval ($P = 0.0003$) and between test, time interval, and group ($P = 0.0344$). Figure 5 shows that the primary origin of the 3-way interaction is a difference in the test score and baseline score. The control animals showed increased positive fear or anxiety at test 1, whereas both treatment groups showed a decrease.

Cortisol

Thirty-two dogs showed an elevated cortisol response during the baseline thunder test when assessed 5 minutes after leaving the

Table 4
Baseline comparisons between 3 groups on grouping variables

Group	Males/ females	Cortisol responders	Mean cortisol (nmol/L) + SD		Mean global anxiety response
			Baseline	Postthunder	
1	5/10	10	38.98 \pm 10.69	162.8 \pm 62.78	4.33 \pm 0.379
2	7/8	10	47.9 \pm 17.39	160.8 \pm 68.65	4.375 \pm 0.297
3	7/8	10	48.16 \pm 28.81	163.0 \pm 68.213	4.417 \pm 0.343

SD, standard deviation.

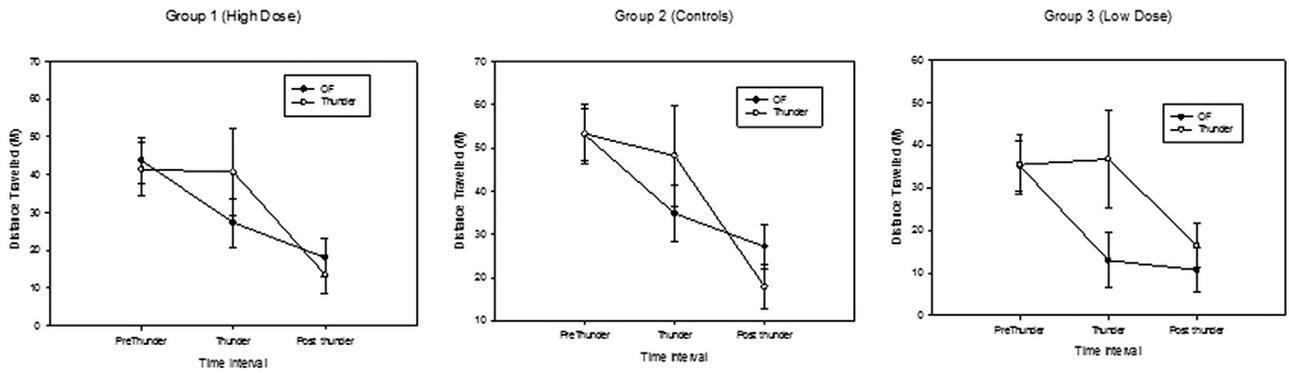


Figure 1. Baseline activity as a function of task (open field vs. thunder), group, and time interval.

room (11 minutes from the start of the thunder track), 3 of the dogs showed cortisol readings that were virtually unchanged, and 10 of the dogs showed a decrease in cortisol. Of the 32 dogs with increased cortisol, 2 dogs showed a very small increase of less than or equal to 5 nmol/L (12.5%) and so were not included as cortisol responders. The remaining 30 dogs all had increases of 19 nmol/L (39%) and higher. Ten cortisol responders were initially allocated into each of the 3 treatment groups, but one subject was excluded from the final cortisol analysis due to illness during the test phase. The cortisol data were first analyzed with a repeated measures ANOVA with time of sample (pre, post, and 1 hour post) and test as within subject variables, and group as a between subject variable (Table 5). The ANOVA revealed a significant effect of test session ($P = 0.00022$) and time of sample ($P = 0.000$). The significant test effect was caused by a decrease overall in the postthunder cortisol (5 minutes posttesting) (Figure 6).

We further analyzed group differences for cortisol levels and the postthunder interval, only. We found a significant effect of test ($P = 0.000$) and nonsignificant interaction between test session and group ($P = 0.06992$) (Figure 7). The Fisher multiple comparisons test was used to further analyze these data and compare each of the groups with baseline. For the low dose group, both test 1 ($P = 0.008$) and test 2 ($P = 0.001$) were significantly different from baseline, as they were for the high dose group ($P = 0.0004$ and $P = 0.000$, respectively). There were no significant differences in the responses of the control group.

If we defined cortisol responders as those experiencing an increase of at least 100% greater than their baseline response, by the last test session, 7/9 control animals remained as cortisol responders, but only 3/10 animals in the low-dose group were responders, and 5/10 animals in the high dose group were responders.

Discussion

These results provide initial data on the anxiolytic effectiveness of a novel compound, a fish hydrolysate, in reducing noise-induced fear and anxiety.

We used 3 different protocols to characterize the anxiolytic effect. The first entailed an automated tracking system to quantify movement and inactivity. The second involved fear and anxiety rating scales, developed specifically for use in this thunderstorm model. The final protocol involved analysis of serum levels of cortisol taken 5 minutes after the end of the testing (11 minutes after the initial presentation of thunder).

Earlier work reported that the typical response using this testing model was one of decreased locomotion and inactivity (Araujo et al., 2013). This experiment confirmed that activity decreased, on average, when the prethunder interval was compared to the thunder interval. However, when the response during the thunder interval was compared to the response during the second 3-minute block of an open field test, locomotion was actually higher in

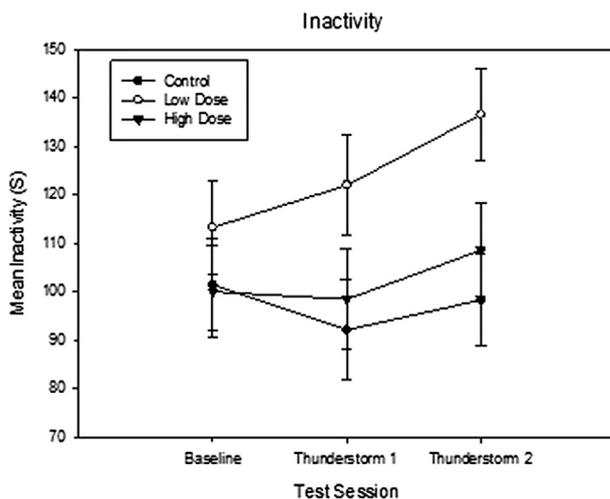


Figure 2. Inactivity as a function of test session and treatment group. Each score is the mean over each 3 minute test block on each of the three test days.

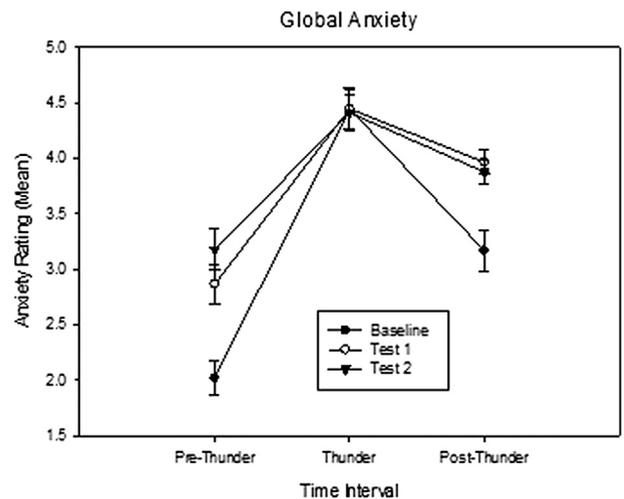


Figure 3. Global anxiety as a function of time interval. The data are for all the groups combined.

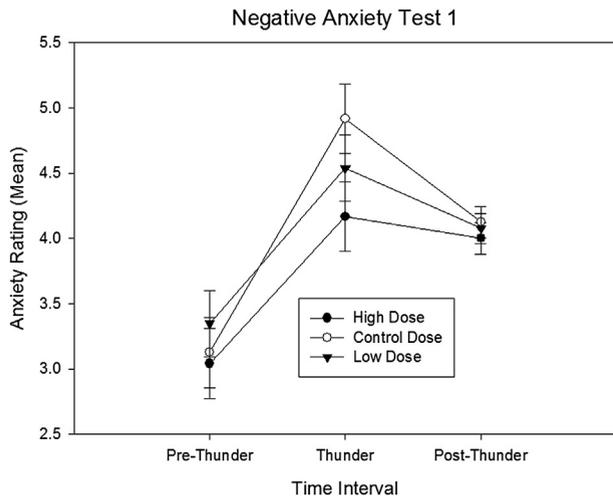


Figure 4. Anxiety as a function of time interval with respect to presentation of thunder and group on first treatment test.

response to thunder and inactivity was lower in this study. These data reflect the fact that a high percentage of the dogs used in this study responded to the presentation of thunder by increasing activity, whereas in previous studies the more typical response was one of a decrease in activity. One key feature of the observational scale was the distinction between active and inactive signs of fear of anxiety. Some animals showed hyperactivity in response to thunder, which would score high on active measure of fear and anxiety, whereas others showed freezing, which would score high on the inactive measure of fear and anxiety. Fearful responses can be both active (fight, flight) and passive (freeze, conflict), which is consistent with previous studies assessing noise aversion in dogs (Dreschel and Granger, 2005; Levine et al., 2007; Cracknell and Mills, 2011; Sheppard and Mills, 2003; Mills et al., 2013). A recent study identified 2 categories of dog responses; those with active responses (extrovert) and those with passive responses (introvert) (Mariti et al., 2013).

The third assessment measure was that of serum cortisol. Numerous studies have demonstrated an increase of salivary and plasma cortisol in response to noise exposure to gunshots, air blasts or a thunder recording over 1 to 6 minutes, and to a vacuum cleaner

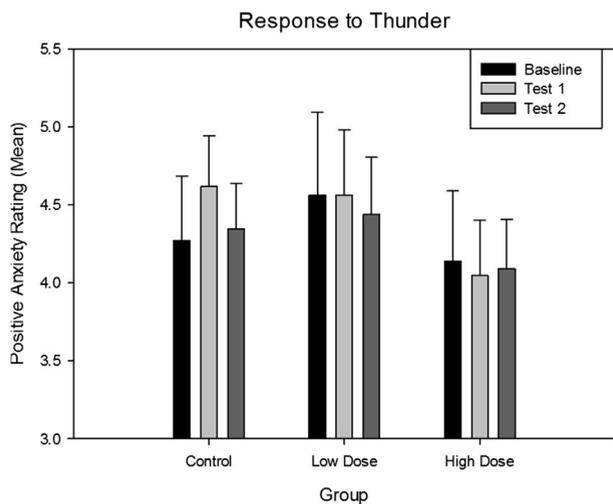


Figure 5. Positive anxiety response to thunder as a function of test session.

Table 5
Cortisol data by group and treatment phase

Baseline							
Group	N	Prethunder		Postthunder (5 minutes ^a)		Postthunder (1 hour)	
		Mean (nmol/L)	SD	Mean (nmol/L)	SD	Mean (nmol/L)	SD
High dose	10	38.98	10.686835	162.8	62.79561	72.46	65.15643
Control	9	47.9	17.387416	160.8	68.65178	52.06	23.66977
Low dose	10	48.16	26.80776	163	68.21372	52.76	15.90801
Test 1							
High dose	10	48.46	35.148959	118.6	57.12987	42.6	11.76813
Control	9	52.46	24.418217	127.2	84.5469	47.36	23.64568
Low dose	10	37.82	10.060903	102.5	58.41661	41.86	16.49971
Test 2							
High dose	10	43.48	14.742893	107.1	70.0832	51.76	15.35789
Control	9	47.96	26.242764	137.15	79.85336	51.22	26.17385
Low dose	10	61.62	48.201286	80.8	45.70874	66.96	76.73283

SD = standard deviation.

^a 11 minutes after the start of thunder.

over 30 minutes, with peak levels occurring between 5–20 minutes, and declines occurring after 30–40 minutes postexposure (Hydbring-Sandberg et al., 2004; Dreschel and Granger, 2005; Beerda et al., 1997; Beerda et al., 1998; Vincent and Michell, 1992). Cortisol elevations have been reported in response to sound blasts ranging from 95 decibels over 6 minutes to 110–120 decibels over 1 minute but not 70 to 87 decibels for up to 18 minutes (Beerda et al., 1997; Beerda et al., 1998). Industrial noise over 80 decibels has been demonstrated to have a significant effect on salivary cortisol elevation in humans (Fouladi et al., 2012). Thunderstorms are a common cause of noise-induced anxiety in dogs, with aversion to thunder reported in 65% to 86% of dogs with noise phobias (Blackwell et al., 2013; Denenberg et al., 2013). Storm-related phenomena like rain, dark clouds, lightning, wind, or even changes in barometric pressure can also cause fear and anxiety (Crowell-Davis et al., 2003). The heterogeneity of storm-related events underscores the necessity of using a representative reproduction of a range of thunder stimuli (Overall et al., 2001). Our model evaluates only a recorded stimulus, which is a limitation in predicting clinical efficacy of any test product. The results of the analysis of the observational anxiety data provided further evidence of anxiolytic effectiveness of the test compound. Positive behavioral scores were reduced in both treatment groups over the 2

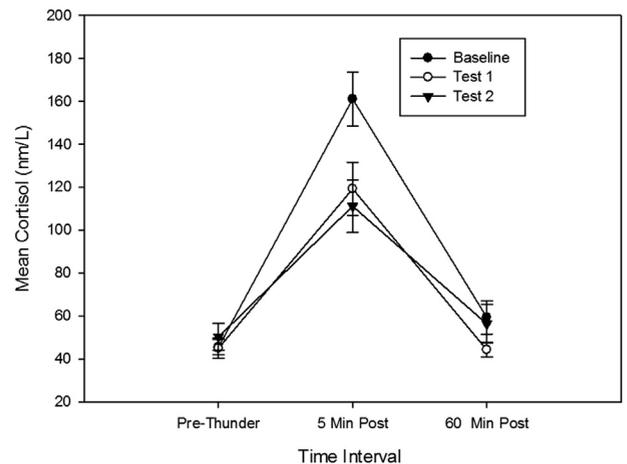


Figure 6. Cortisol response to thunder as a function of test (baseline, test 1 and test 2) and time between presentation of thunder and collection of blood samples.

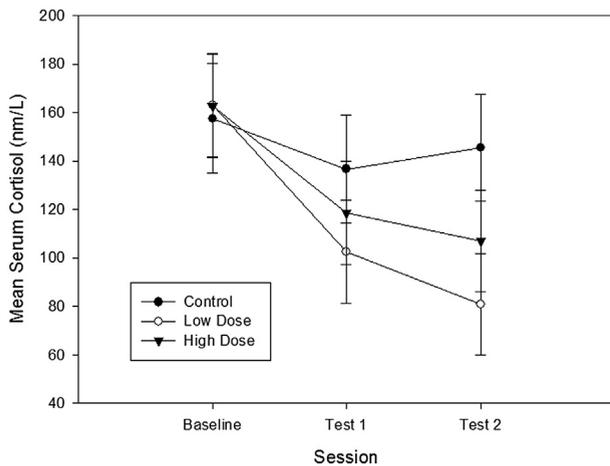


Figure 7. Immediate postthunder cortisol (11 minutes from onset) as a function of test session and group.

test sessions. By contrast, positive behavioral scores increased in the control group.

The final measure, serum cortisol, provided the most robust evidence of anxiolytic effectiveness. In both of the treatment groups, the postthunder response to cortisol decreased under the treatment condition, and the differences between the baseline conditions were statistically significant. For the control animals, by contrast, the postthunder cortisol response remained essentially unchanged. The differences between groups were increased on the second test session, raising the possibility that the anxiolytic effectiveness may be best if animals are maintained on the treatment for a relatively long time.

Conclusion

The results from the current study highlight the importance of individual differences in fear and anxiety behaviors in dogs. We have previously reported that dogs show decreased activity in response to fear evoking stimuli. In the current study, however, we noted that some dogs responded by increasing activity and others by decreasing activity. On average, dogs actually showed greater activity during the presentation period than they did in a comparable 3-minute period during an open field test. Overall, the results of this study indicate that fish hydrolysate has anxiolytic properties, which appear to be manifested by decreased hyperactivity and a reduced cortisol response to stress.

Ethical considerations

The facility is a registered Research Facility by the Ontario Ministry of Agriculture, Food and Rural Affairs (Canada). The studies were approved by the local animal care and use committee without reservation and were designed under the guidelines set forth by the Canadian Council on Animal Care in accordance with the Guide for the Care and Use of Experimental Animals. There was additional approval from the Royal Canin ethics committee, and the research protocol was registered by France Ministry of Research and Education.

Conflict of interest

Dr. Landsberg, Dr. Milgram, and Stephanie Kelly provided the research for this study as contracted by Royal Canin. Dr. Mougeot is an employee of Royal Canin.

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References

- Araujo, J.A., de Rivera, C., Ethier, J., Landsberg, G.M., Denenberg, S., Arnold, S., Milgram, N.W., 2010. ANXITANE® tablets reduce fear of human beings in a laboratory model of anxiety-related behavior. *J. Vet. Behav.: Clin. Appl. Res.* 5, 268–275.
- Araujo, J.A., de Rivera, C., Landsberg, G.M., Adams, P.E., Milgram, N.W., 2013. Development and validation of a novel laboratory model of sound-induced fear and anxiety in beagle dogs. *J. Vet. Behav.: Clin. Appl. Res.* 8, 204–212.
- Bamberger, M., Houpt, K.A., 2006. Signalment factors, co-morbidity, and trends in behavior diagnoses in dogs: 1,644 cases (1991–2001). *J. Am. Vet. Med. Assoc.* 229, 1591–1601.
- Beata, C., Beaumont-Graff, E., Diaz, C., Marion, M., Massal, N., Marlois, N., Muller, G., Lefranc, C., 2007. Effects of alpha-casozepine (Zylkene) versus selegiline hydrochloride (Selgian, Anipryl) on anxiety disorders in dogs. *J. Vet. Behav.: Clin. Appl. Res.* 2, 175–183.
- Beerda, B., Schilder, M.B., van Hooff, J.A., de Vries, H.W., Mol, J.A., 1998. Behavioural, saliva cortisol and heart rate responses to different types of stimuli in dogs. *Appl. Anim. Behav. Sci.* 58, 365–381.
- Beerda, B., Schilder, M.H., van Hooff, J.A., de Vries, H.J., 1997. Manifestations of chronic and acute stress in dogs. *Appl. Anim. Behav. Sci.* 52, 307–319.
- Bernet, F., Montel, V., Noël, B., Dupouy, J.P., 2000. Diazepam-like effects of a fish protein hydrolysate (Gabolysat PC60) on stress responsiveness of the rat pituitary-adrenal system and sympathoadrenal activity. *Psychopharmacol. (Berl)* 149 (1), 34–40.
- Blackwell, E.J., Bradshaw, J.W., Casey, R.A., 2013. Fear responses to noises in domestic dogs: prevalence, risk factors and co-occurrence with other fear related behaviors. *Appl. Anim. Behav. Sci.* 145, 15–25.
- Casey, R., 2002. Fear and stress. In: Horwitz, D., Mills, D., Heath, S. (Eds.), *BSAVA Manual of Canine and Feline Behavioral Medicine*. BSAVA, Gloucester, UK, pp. 144–153.
- Cracknell, N.R., Mills, D.S., 2011. An evaluation of owner expectation on apparent treatment effect in a blinded comparison of 2 homeopathic remedies for firework noise sensitivity in dogs. *J. Vet. Behav.: Clin. Appl. Res.* 6, 21–30.
- Crawley, J.N., 1985. Exploratory behavior models of anxiety in mice. *Neurosci. Biobehav. Rev.* 9, 37–44.
- Crowell-Davis, S.L., Seibert, L.M., Sung, W., Parthasarathy, V., Curtis, T.M., 2003. Use of clomipramine, alprazolam, and behavior modification for treatment of storm phobia in dogs. *J. Am. Vet. Med. Assoc.* 222, 744–748.
- Denenberg, S., Landsberg, G.M., Blizzard, P., 2013. Prevalence of fearful and anxious behaviors in dogs in the United States. *Proc. 2013 ACVB/AVSAB Veterinary Behavior Symposium*. Chicago, 50–51.
- Denenberg, S., Landsberg, G.M., 2008. Effect of dog-appeasing pheromones on anxiety and fear in puppies during training and on long term socialization. *J. Am. Vet. Med. Assoc.* 233, 1874–1882.
- DePorter, T., Landsberg, G.M., Araujo, J.A., Ethier, J.L., Bledsoe, D.L., 2012. Hemo-nese chewable tablets reduces noise-induced fear and anxiety in a laboratory canine thunderstorm simulation; a blinded and placebo-controlled study. *J. Vet. Behav.: Clin. Appl. Res.* 7, 225–232.
- Dorman, T., Bernard, L., Glaze, P., Hogan, J., Skinner, R., Nelson, D., Bowker, L., Head, D., 1995. The effectiveness of Garum armoricum (stabium) in reducing anxiety in college students. *J. Adv. Med.* 8, 193–200.
- Dreschel, N.A., 2010. The effects of fear and anxiety on health and lifespan in pet dogs. *Appl. Anim. Behav. Sci.* 125, 157–162.
- Dreschel, N.A., Granger, D.A., 2005. Physiological and behavioral reactivity to stress in thunderstorm-phobic dogs and their caregivers. *Appl. Anim. Behav. Sci.* 95, 153–168.
- Fouladi, D.B., Nassiri, P., Monazzam, E.M., Farahani, S., Hassanzadeh, G., Hoseini, M., 2012. Industrial noise exposure and salivary cortisol in blue collar industrial workers. *Noise Health* 14, 184–189.

- Fugh-Berman, A., Cott, J.M., 1999. Dietary supplements and natural products as Psychotherapeutic agents. *Psychosom. Med.* 61, 712–728.
- Hydbring-Sandberg, E., Walter, L.W., Hoglund, K., Svartberg, K., Swenson, L., Forkman, B., 2004. Physiological reactions to fear provocation in dogs. *J. Endocrinol.* 180, 439–448.
- Kato, M., Miyaji, K., Ohtani, N., Ohta, M., 2012. Effects of prescription diet on dealing with stressful situations and performance of anxiety-related behaviors in privately owned anxious dogs. *J. Vet. Behav.: Clin. Appl. Res.* 7, 21–26.
- Landsberg, G., Hunthausen, W., Ackerman, L., 2013a. Behavior Problems of the Dog and Cat (3rd Ed.). Saunders Elsevier, Edinburgh, UK, pp. 139–149, 181–186.
- Landsberg, G., Beck, A., Araujo, J., Milgram, N.W., 2013b. Effect of dog appeasing pheromone (DAP®) collar on a model of sound-induced fear and anxiety. In: Proc. 9th International Veterinary Behavior Meeting; PSI, Lisbon, Portugal, pp. 45–46.
- Le Poncin, M., 1996a. Experimental study: stress and memory. *Eur. Neuro-psychopharmacol.* 6, 110. P10-2.
- Le Poncin, M., 1996b. Nutrient presentation of cognitive and memory performances. *Eur. Neuro-psychopharmacol.* 6, 187. P19-4.
- Levine, E.D., Ramos, D., Mills, D.S., 2007. A prospective study of two self-help CD based desensitization and counter-conditioning programmes with the use of dog appeasing pheromone for the treatment of firework fears in dogs (*Canis familiaris*). *Appl. Anim. Behav. Sci.* 105, 311–329.
- Mariti, C., Tancini, V., Gazzano, A., Calvo, P., Fatjo, J., Bowen, J., 2013. Fear of thunder in dogs and owner related behavior; an Italian survey. In: Proceedings of the 9th International Veterinary Behavior Meeting. PSI, Lisbon, Portugal, pp. 60.
- Milgram, N.W., Landsberg, G.M., Snow, B., 2014. Anxiety Reducing Effectiveness of Calmz® Anxiety Relief System in Beagle Dogs in a Modified Thunderstorm Model. ACVB/AVSAB Veterinary Behavior Symposium, Denver, CO, 29–30.
- Mills, D., Braem Dube, M., Zulch, H., 2013. Appendix B. The Lincoln Sound-sensitivity Scale. In: Stress and Pheromonotherapy in Small Animal Clinical Behavior. John Wiley and Sons, West Sussex, UK, pp. 259–263.
- Overall, K.L., 2013. Manual of Clinical Behavioral Medicine for Dogs and Cats. Elsevier, St. Louis, pp. 231–261.
- Overall, K.L., Dunham, A.E., Frank, D., 2001. Frequency of nonspecific clinical signs in dogs with separation anxiety, thunderstorm phobia, and noise phobia, alone or in combination. *J. Am. Vet. Med. Assoc.* 219, 467–473.
- Palestrini, C., Minero, M., Cannas, S., Berteselli, G., Scaglia, E., Barbieri, S., Cavallone, E., Puricelli, M., Servida, F., Dall'Ara, P., 2010. Efficacy of a diet containing caseinate hydrolysate on signs of stress in dogs. *J. Vet. Behav.: Clin. Appl. Res.* 5, 309–317.
- Sherman, B.L., Mills, D.S., 2008. Canine anxieties and phobias: an update on separation anxiety and noise aversions. *Vet. Clin. N. Am.: Small Anim. Pract.* 38, 1081–1106.
- Sheppard, G., Mills, D.S., 2003. Evaluation of dog-appeasing pheromone as a potential treatment for dogs fearful of fireworks. *Vet. Rec.* 152, 432–436.
- Siracusa, C., Manteca, X., Cuenca, R., del Mar Acuela, M., Alba, A., Lavin, S., Pastor, J., 2010. Effect of a synthetic appeasing pheromone on behavioral, neuroendocrine, immune, and acute-phase perioperative stress responses in dogs. *J. Am. Vet. Med. Assoc.* 237, 673–681.
- Vincent, I.C., Michell, A.R., 1992. Comparison of cortisol concentrations in saliva and plasma of dogs. *Res. Vet. Sci.* 53, 342–345.