Effect of a Pheromone on Stress-Associated Reactivation of Feline Herpesvirus-1 in Experimentally Inoculated Kittens

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Background: Stress contributes to reactivation of feline herpesvirus-1 (FHV-1). The usage of pheromones to decrease stress in FHV-1 experimentally inoculated kittens has not previously been investigated.

Hypothesis/Objectives: To determine whether a feline pheromone would lessen stress, resulting in decreased recurrence of FHV-1-associated illness in kittens.

Animals: Twelve 5-month-old, purpose-bred kittens.

Methods: Randomized, double-blind, placebo-controlled clinical trial. Kittens previously infected with the same dose of FHV-1 were randomized into 2 separate but identical group rooms. After a 2-week equilibration period, a diffuser containing either the pheromone or placebo was placed in each of the rooms, and the kittens acclimated for an additional 2 weeks. Every 2 weeks thereafter, for the 8-week study period, housing was alternated between kennel- and group housing. Blinded observers applied a standardized clinical and behavioral scoring rubric daily. After each 2-week period, serum cortisol concentrations and quantitative PCR for FHV-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) ratios were evaluated. Clinical, behavioral, and laboratory test results were compared between groups within individual and combined study periods.

Results: Sneezing occurred more frequently in the placebo group during individual ($P = 0.006$) and combined study periods ($P = 0.001$). Sleep at the end of observation periods occurred more frequently in the pheromone group during individual ($P = 0.006$) and combined study periods ($P < 0.001$).

Conclusions and Clinical Importance: The findings suggest that the pheromone decreased stress, and the decrease in stress response may have resulted in decreased sneezing associated with FHV-1.

Key words: Behavior; Cat; Feliway; FHV-1; Respiratory; Rhinotracheitis.

Feline herpesvirus-1 (FHV-1) is a common infectious disease of cats. Infection can be subclinical, or it may result in clinical signs of disease including pyrexia, conjunctivitis, keratitis, sneezing, cough, dyspnea, inappetence, lethargy, and occasionally, pneumonia and death.[1–5] Morbidity and mortality in crowded or stressful environments, such as shelters, can be high.[1,3,4]

After acute exposure, most cats develop persistent infection, with the trigeminal ganglia serving as the main site of viral latency.[5–8] Reactivation of FHV-1 then can recur, resulting in clinical signs, and FHV-1 shedding increases.[2,6,8,9] Stressful events may precipitate FHV-1 reactivation in some instances.[5,8,10,11] In a shelter study, cats with the highest stress scores during the first week in the shelter were more likely to develop

Abbreviations:

CI	confidence interval
DNA	deoxyribonucleic acid
FHV-1	feline herpesvirus-1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
OR	odds ratio
Period E	equilibration period
Period G#	group-housing period number
Period K#	kennel-housing period number
qPCR	quantitative polymerase chain reaction
SS#	snapshot time point number
URI	upper respiratory infection

upper respiratory infection (URI).[10] Unfamiliar handlers and environments, altered feeding schedules or husbandry activities, kennel confinement, impoverished or nonstimulating environments, aversive stimuli such as noise, odors, uncomfortable temperatures, and lack of hiding resources all can cause stress in cats.[8,12–18] Furthermore, changing housing from group housing to kennels also has been shown to trigger FHV-1-associated disease.[8,19] Stressful events are believed to result in FHV-1 reactivation within the first 3 weeks, with an approximate lag phase of 4–11 days after the stress.[5,8,10]

The mechanisms by which stress induces reactivation of FHV-1 are unclear. Although acute stress can be adaptive, allowing the animal to cope with and avoid or lessen the impact of the stressor, persistent distress can lead to a damaging pathophysiological reaction in the animal, leading to faulty immune response and disease susceptibility.[20,21] Activation of the stress response system, however, is dependent on individual history, the context in which the stressor occurs, and the

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expectation the individual has for the outcome of the event.[22–24]

Several strategies with variable outcomes have been employed in an attempt to mitigate FHV-1 reactivation in cats.[9,19,25–27] Lessening stress is a strategy that may decrease signs of URI and viral shedding in shelter cats.[11,28] In shelters, stress reduction methods have included gentle stroking and speaking to the cat, grooming, playing, and use of hiding enrichment and minimally invasive daily kennel cleaning.[11,18,28,29]

A feline facial pheromone fraction[a] contained in a commercial preparation has been assessed in a variety of studies as another potential stress reducing modality.[30–32] Use of this product has been evaluated in the management of feline behaviors sometimes associated with stress, such as urine spraying, as well as stress-associated diseases such as feline idiopathic cystitis. Use of the product also has been shown to decrease signs of stress during transportation or when visiting a veterinary clinic and to improve appetite in hospitalized patients.[30,31,33–36]

Our study was designed to determine whether experimentally induced FHV-1-infected kittens housed in equivalent rooms containing either a pheromone[a] diffuser or a placebo diffuser and subjected to housing change-induced stress would differ in behavioral scores, clinical scores, FHV-1 shedding, or serum cortisol concentrations.

Materials and Methods

Cats

Six neutered male and 6 spayed female, 5-month-old, mixed breed kittens bred for use in research projects were used in this 12-week pilot study. Eight weeks before the study, each of the 12 kittens was infected with FHV-1 by intranasal instillation for a study in which the 12 kittens were the group-housed control group (Contreras et al., unpublished data, 2017). Using these control kittens from a prior study for the purpose of our study eliminated the need for experimental inoculation of additional kittens, an objective of both the investigators and the sponsor. In that previous study, FHV-1 infection was confirmed in all kittens by quantitative polymerase chain reaction (qPCR), and each developed clinical signs consisting of fever, sneezing, ocular discharge, nasal discharge, nasal congestion, conjunctivitis, blepharospasm, or some combination of these. At the time of the study, none were undergoing treatment for URI, and occasional sneezing, serous nasal discharge, or serous ocular discharge were the only potential manifestations of FHV-1 present intermittently in the kittens. Both the prior study and our study were approved by the Institutional Animal Care and Use Committee.

Housing

Kittens were randomized into a pheromone[a] group or a placebo group (placebo diffuser); each group consisted of 3 males and 3 females. The kittens were housed in 2 separate but similarly sized rooms (pheromone group = 8′6″ width × 10′ 3″ length × 9′ height; placebo group = 9′7″ width × 9′ length × 9′ height) within the research facility; each room had a separate air exchanger and 2 similarly sized litter boxes. During kennel housing, each room contained 3 sets of top and bottom individual wire kennels (36″ × 25.5″ × 22″); kittens spent 1 2-week kennel period in a top kennel and the other 2-week kennel period in a bottom kennel. During kennel housing, kittens were in visual contact with the other kittens in kennels, other than the 1 kitten in a respective top or bottom kennel. Some of the kittens also had physical contact with the other kittens through the bars of the kennels. All kittens were provided dry food and water ad libitum and were provided a table with 2 levels that were approximately 36″ height × 15″ width × 36″ length during group housing and a similarly sized kennel perch during kennel housing. Kittens were provided 2 white ping-pong balls during group housing and one white ping-pong ball per kennel during kennel housing. Between 2 and 4 cardboard boxes of various shapes and sizes also were provided during group housing; boxes were identical between rooms and were exchanged for new, different boxes every week. The litter boxes, table, and enrichment devices were movable and not always in the same position within the rooms. While observers were interacting with the kittens during group housing, novel objects such as paper balls, writing implements, and the observers' outer garments were used as toys and enrichment devices by the kittens; the novel objects were removed when the observers departed after scoring. Enrichment was kept to a minimum because the study was designed to evaluate the effect of the pheromone on stress.

Clinical scoring

A clinical score sheet adapted from other FHV-1 vaccination and treatment studies, including the previous study of which these kittens were a part, was used in our study (Table 1).[9,27,37] A total clinical score was calculated for each kitten each day by adding the individual clinical score variables recorded for that day. Body temperatures were estimated by microchip.[38] Increased body temperature was defined as >102.5°F. Heart rates were measured daily when auscultation was not obscured by purring. Body weights were measured weekly.

The protocol included a rescue clause for kittens that developed moderate to severe signs of FHV-1 infection and a loss of appetite for 48 hours. Supportive care and treatment that could be administered included SC administration of fluids, buprenorphine for discomfort, topical cidofovir, or PO famciclovir, as needed and determined by the investigators.

Behavioral scoring

Several different behavioral assessment scales used in previous shelter, and other studies were reviewed as tools to assess stress and behavior in the kittens.[12,13,18,39–42] Based on observations of the kittens during the study in which they were inoculated with FHV-1, and because these purpose-bred research kittens already were habituated to the research facility, housing, each other, and human interactions, a modified scale was designed (Table 2). The behavioral observation metrics also were designed to accommodate ease and efficiency and to avoid distracting from FHV-1 clinical scoring. The rubric contained lists of typical feline postures, vocalizations, and actions that represented either normal, relaxed calm, or stress-related behaviors that could be observed and objectively scored (Table 2). Because of the overall engaging personalities and temperaments of the kittens in the study, the rubric was further adapted before and during the equilibration period. The final rubric that was applied when the diffusers were introduced into the rooms contained 28 individual behaviors, recorded at five different specified time points during the 45-minute scoring period per room each morning (Table 2). Snapshot observations were performed for 15 seconds per kitten at the following 4 time points: upon entry into room (SS1 time point), during clinical scoring handling (SS2 time point), immediately after clinical scoring handling (SS3 time point), and at the 45-minute mark (SS4 time point).

Table 1. Clinical scoring rubric.

Clinical Sign	Score
Conjunctivitis[a]	0 = None
	1 = Mild
	2 = Moderate
	3 = Severe
Blepharospasm[a]	0 = None
	1 = Eye<25% closed
	2 = Eye 25–50% closed
	3 = Eye 50–75% closed
	4 = Eye completely closed
Ocular discharge[a]	0 = None
	1 = Mild serous (clear) discharge
	2 = Moderate mucoid (white) discharge
	3 = Severe mucopurulent (moist yellow-green) discharge
Body temperature (microchip)	0: ≤102.5
	1: >102.5
Cough	0 = None
	1 = Observed
Sneezing (yes/no)	0 = None
	1 = Observed
Nasal discharge[a]	0 = None
	1 = Mild serous (clear) discharge
	2 = Moderate mucoid (white) discharge
	3 = Severe mucopurulent (moist yellow-green) discharge or hemorrhagic (bloody/red) discharge
Nasal congestion[a] (if score varies during observation period, record highest score observed)	0 = None (no congestion present; able to breathe through both nares without difficulty)
	1 = Mild/Minor congestion (barely audible; audible on close listening, subtle snoring sounds on inhalation ANY time during the observation period
	2 = Moderate congestion (easily audible; consistently audible throughout observation period; audible snoring sounds on inhalation or expiration that are likely to originate from the nasal cavity)
	3 = Severe congestion (audible across the room, with or without open mouth breathing; minimal nasal air flow noted from1 or both nares after local debris is cleared away)

[a]Due to statistically low occurrence of scores >1, binomial analyses were performed using 0 or 1 to indicate presence or absence of clinical sign.

Behaviors also were recorded throughout the 20 to 30 minutes between SS3 and SS4 (Long time point; Table 2).

Experimental design

Kittens were housed in their respective group rooms on weeks 1, 2 (period E); 3, 4 (period G0); 7, 8 (period G1); and 11, 12 (period G2; Fig. 1). Kittens were housed in kennels in their respective rooms on weeks 5, 6 (period K1) and 9, 10 (period K2). Two trained scorers, blinded regarding treatment allocation, applied the standardized clinical and behavioral scoring system at approximately the same time and order every morning, for 45 minutes per room, throughout the study (Fig. 1).

Assays

At the beginning of the study and after each of the 6 periods (E, G0, K1, G1, K2, G2), kittens were sedated; proparacaine was applied to their corneas; and blood, caudal pharynx mucosal cells, and conjunctival swabs were collected. Sera, oropharyngeal, and conjunctival swabs were stored at −80°C until assayed in batches. Total DNA was extracted from the oropharyngeal and conjunctival samples and evaluated for DNA of FHV-1 and DNA of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, by quantitative PCR (qPCR) as previously described.[43] Results of the GAPDH assay were used as a positive control for sample adequacy because this house-keeping gene is present in all feline cells. Results of the FHV-1 qPCR assay were expressed as the ratio of FHV-1 DNA/GAPDH DNA to standardize specimens. Serum cortisol concentrations were measured at a commercial laboratory.[b]

Statistical evaluation

After randomization but before starting the study, the total clinical scores associated with FHV-1 that developed after primary infection in the previous study were compared between the pheromone group and the placebo group using the Wilcoxon rank sum test, and the groups were found to not have different median scores ($P = 0.9$).

Because there were no manipulations of the kittens in G0 other than adding the diffusers, this was not considered a stress period for the final comparisons between groups. Only the results from periods K1, G1, K2, and G2, in which the kittens' routines were disrupted by housing changes potentially associated with stress, were evaluated individually and in combinations. Descriptive statistics were calculated, and categorical data were expressed as frequencies, whereas continuous data were expressed as means, medians, and ranges. The Shapiro-Wilk test was used to assess normalcy of data. Because of non-normality of all variables, the Wilcoxon rank sum test was used to compare group median results for total clinical score, total stress score, FHV-1/GAPDH ratios, heart rate, and weekly body weight changes in the pheromone group as compared to the placebo group. The Wilcoxon rank sum test also was used to compare median heart rates in kenneled periods as compared to group-housed periods, median serum cortisol concentrations at the beginning of the study as compared to the end of the study, and median serum cortisol

Table 2.   Behavioral scoring rubric.

BEHAVIOR *(italics indicate only scored in EITHER group- OR kennel-housing)*	SCORE Y = yes or 1; N = no or 0
Snapshot "SS1" Time point: Upon entry into room	
(Group-housed only) Greeting: Greets me at door	*Y / N*
Vocalization: Meow	Y / N
Vocalization: Hiss/growl	Y / N
(Kennel-housed only) Pawing through kennel	*Y / N*
(Kennel-housed only) Pacing (repetitive walking back and forth)	*Y / N*
Snapshot "SS2" Time point: During clinical scoring	
Reaction to clinical scoring period, temp wand/handling	L: allows, leans in, purr, soft body posture, ears forward F: freeze, crouch, immobile, dilated pupils, ears not forward, tense, stiff R: mildly resistant, fidgets, some squirming, some displacement grooming V: very resistant, "obsessively" attempts escape, scratches; doesn't allow, won't stay still
Reaction to petting DURING CLINICAL SCORING PERIOD (scorers will pet a few times after temperature wand/clinical scoring in order to assess)	L: leans in, "enjoys" R: moves away, flinches, not interested, "done with you"
Vocalization: Meow	Y / N
Vocalization: Purring	Y / N
Kneading	Y / N
Vocalization: Hiss/growl	Y / N
Snapshots "SS3" and "SS4" Time points: approximately 15 seconds per each cat **SS3: After clinical scoring, in same order as when performed clinical scoring** **SS4: At 45-minute mark**	
Is cat "up" with 4 paws on floor? Standing, walking, running, pacing *(Okay to have "Up" AND "Not standing" categories if performed >1 in 15 seconds)*	Y: up, walking, climbing, standing N: sleeping, lying down, sitting
IF NOT "up", cat is: *(Okay to have >1 category if cat is positioned in >1 way during 15 seconds)*	ZZ: sleeping; S: sitting; LE: lying on side, legs extended; LA: Lying down, abdomen exposed; LVU: Lying down ventrally, head up and alert; LVD: Lying down ventrally, head down
Active / Passive: Is cat "Active?" doing something, acting, reacting, watching, ready to pounce, or is cat instead absorbing, relaxing, sleeping	Y: Active N: Passive
Urinating/Defecating	Y / N
Eating/drinking	Y / N
Vocalization: Meow	Y / N
Interacting/playing with objects or other cat(s) or human	Y / N
Vocalization: Hiss	Y / N
(Group-housed only) Climbing on objects or on object	*Y / N*

Table 2.   Continued

(Group-housed only) Climbing on person; or currently on person	*Y / N*
Grooming self	Y / N
(Group-housed only) Grooming another cat	*Y / N*
(Group-housed only) Licking person	*Y / N*
(Kennel-housed only) Pawing through kennel	*Y / N*
(Kennel-housed only) Pacing (repetitive walking back and forth)	*Y / N*
"Long" time point: While sitting down in room, approximately 20-30 minutes, during time between SS3 and SS4	
Housing disarray: Litterbox overturned with litter, feces on floor during group-housing. During kennel-housing: kennel disarray - kibble, water, urine, litter scattered throughout kennel (litterboxes were permanently affixed to kennels so they could not easily be overturned by kitten)	Y / N (scored during Long time-point because more time allotted; however, this housing disarray score represents the condition of room or kennel upon scorers' entry into room)
Diarrhea present?	Y / N
Urinated/Defecated (did you SEE the kitten urinate/defecate)	Y / N
Eating/drinking observed? (did you SEE the kitten eat/drink)	Y / N
(Group-housed only) Vocalization: Purring	Y / N (N also includes unknown - if not close enough to hear)
Vocalization: Meow *(0, 1 during group-housing)* *(0, 1, 2 during kennel-housing)*	0: no meows 1: some occasional meows during period *2: excessive meowing during period (during kennel-housing)*
Vocalization: Hiss/growl	Y / N
Any fighting/spats/aggression toward other cats or humans - present?	Y / N
Hiding behavior	Y / N
Interacting/playing with objects or other cat(s) (or human during group-housing)	Y / N
Kneading	Y / N
(Group-housed only) Climbing on objects or on object	*Y / N*
(Group-housed only) Climbing on person; or currently on person	*Y / N*
Groomed self	Y / N
(Group-housed only) Groomed another cat	*Y / N*
(Group-housed only) Licked person	*Y / N*
(Kennel-housed only) Pawing through kennel	*Y / N*
(Kennel-housed only) Pacing (repetitive walking back and forth)	*Y / N*

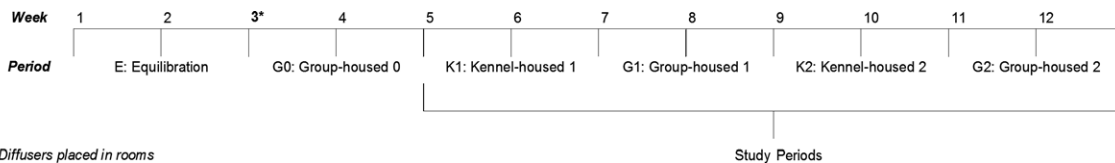| Week	1	2	3*	4	5	6	7	8	9	10	11	12
Period	E: Equilibration			G0: Group-housed 0	K1: Kennel-housed 1		G1: Group-housed 1		K2: Kennel-housed 2		G2: Group-housed 2	

* Diffusers placed in rooms

Study Periods

Fig 1.   Study timeline by week and corresponding group- or kennel-housing period number.

Table 3. Median, mean, and range for the total clinical score by group and study period.

Study Period Group	G0[a] Median, Mean (Range)	K1[a] Median, Mean (Range)	G1[a] Median, Mean (Range)	K2[a] Median, Mean (Range)	G2[a] Median, Mean (Range)	K1, G1, K2, G2 Median, Mean
Placebo	13, 13.8 (5–31)	14.5, 15.3 (2–29)	12.5, 16.8 (8–36)	14, 15.5 (3–33)	13.5, 15.3 (4–34)	58, 63
Pheromone	9.5, 12.8 (3–31)	15, 15 (2–28)	12, 12.3 (3–28)	16, 16 (5–29)	11, 13.2 (3–31)	55.5, 56.5
P value	0.58	0.92	0.06	0.66	0.32	0.23

G#, group-housed period number; K#, kennel-housed period number. G1, G2: the 2, 2-week group-housing periods after placement of the diffusers in G0; K1, K2: the 2, 2-week kenneled periods. The means and ranges are shown to demonstrate variation between the 6 kittens per group; the group medians are compared by Wilcoxon rank sum test. Statistical significance: $P < 0.05$.

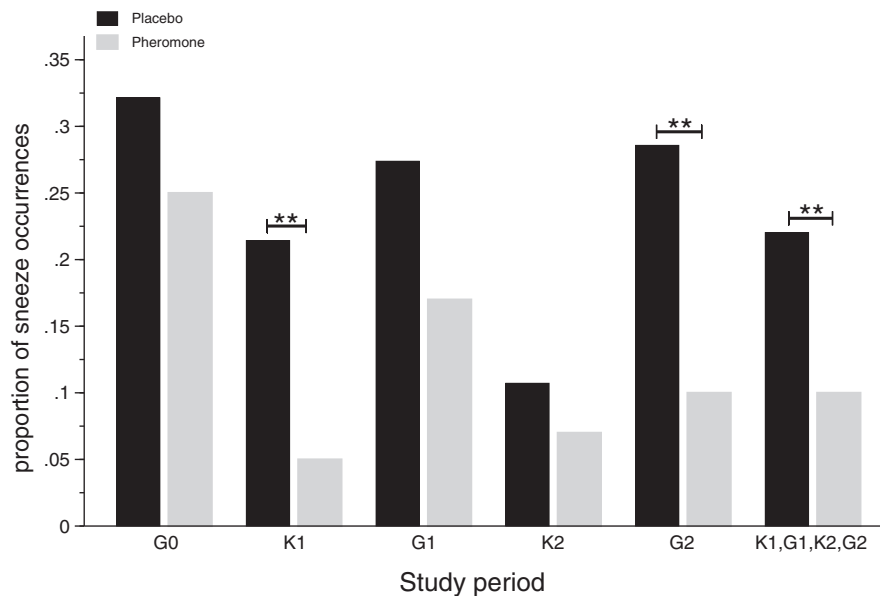[a]n = 84 observations: 14 observations per 6 kittens per 2-week study period per group.

Fig 2. Proportion of sneezing occurrences (presence) by group and individual and combined study periods. K#, kennel-housed period number; G#, group-housed period number. *Statistical significance of $P < 0.05$ for group comparisons using multivariate logistic regression, adjusted for lack of independence.

concentrations in kittens that shed FHV-1 during any study period as compared to serum cortisol concentrations in those kittens that did not shed FHV-1 during any study period. Individual clinical and behavioral variables were categorized into dichotomous variables of presence or absence of these variables each day. The proportions of observations of dichotomous clinical or behavioral variables were compared between the pheromone group and placebo group by use of the 2-tailed Fisher exact test. The 2-tailed Fisher exact test also was used to compare dichotomous variables at the beginning of the study with dichotomous variables at the end of the study. To control for lack of independence among observations because of repeated measurements on the same kitten over time, mixed model regression analyses were used, and odds ratios (OR) and 95% confidence intervals (CI) were calculated.

Commercially available software[c] was used for all comparisons. Significance was defined as $P < 0.05$.

Results

Clinical findings

At the end of the equilibration period, no significant differences were found between groups ($P = 0.58$). All

kittens had normal appetites, gained weight consistently, and had soft, groomed hair coats each day throughout the study. None of the cats required medical intervention for FHV-1 infection. Weight changes did not differ significantly between groups. Heart rates did not differ significantly between groups. Heart rates, however, were significantly higher during kennel-housed periods when compared to group-housed periods in the pheromone group ($P < 0.001$), placebo group ($P < 0.001$), and both groups combined ($P < 0.001$); significant differences were retained after adjustment for lack of independence, but no clinical sequelae were noted.

For 1 of the 12 kittens, the temperature sensing microchip malfunctioned, and body temperature in this kitten was measured in the axillary space. Median, mean, range, and group comparison results for the total clinical score listed by groups and study period are presented in Table 3.

Coughing was rarely heard during the study. Mild ocular discharge (score = 1) was recorded at many observation points for several kittens before and during

Table 4. Frequency of potential stress-related behaviors during time points within kenneled periods.

Time point	Behavior	Group	K1 *	K2 *	unadjusted P value [a]	adjusted P value [b]
SS1	Kennel Pacing	Placebo	52%	33%	.74	n/a
		Pheromone	45%	36%		
	Kennel Pawing	Placebo	21%	11%	.13	.19
		Pheromone	11%	7%		
SS3	Kennel Pacing	Placebo	7%	2%	.04**	.05,
		Pheromone	1%	0%		
	Kennel Pawing	Placebo	6%	1%	.60	n/a
		Pheromone	10%	1%		
LONG	Kennel disarray	Placebo	44%	48%	.005**	.44
		Pheromone	36%	25%		
	Kennel Pacing	Placebo	25%	6%	.14	.12
		Pheromone	17%	2%		
	Kennel Pawing	Placebo	35%	14%	.14	.12
		Pheromone	30%	5%		
	Kennel Meow excessive	Placebo	57%	37%	.046	.57
		Pheromone	43%	29%		
SS4	Kennel Pacing	Placebo	0%	0%	n/a	n/a
		Pheromone	2%	0%		
	Kennel Pawing	Placebo	5%	0%	.75	n/a
		Pheromone	7%	0%		

SS#, snapshot time point number; K#, kennel-housed period number.
* n=84 observations: 14 observations per 6 kittens per 2-week study period per group; n/a = not applicable due to too few observations.
[a] Fisher 2-tailed exact test comparing groups.
[b] Multivariate logistic regression, comparing groups, adjusted for lack of independence due to repeated kitten observations.
**Statistical significance: $P < .05$.

the study periods and potentially associated with stress, but it was never associated with conjunctivitis. Mild nasal discharge (score = 1) was recorded frequently, but moderate mucoid discharge (score = 2) associated with nasal congestion was not detected during the study. Thus, coughing, ocular discharge, and nasal discharge were not evaluated further. Mild nasal congestion (score = 1) was reported commonly, but moderate congestion was rarely reported, and this variable was not evaluated further. Sneezing was the most common

Table 5. Median and range for the total stress score by group and kennel study period.

Study Period Group	K1[a] Median (Range)	K2[a] Median (Range)	K1 and K2 Combined Median (Range)
Placebo	36 (19–52)	19 (6–45)	55 (26–97)
Pheromone	25 (19–46)	16 (4–27)	42 (23–73)
P value*	0.04**	0.04**	0.004**

K#, kennel-housed period number. Median represents the 6 kittens' total stress scores in each group, per kennel period and per both kennel periods combined. Range represents the lowest (minimum) and highest (maximum) among the 6 kittens' total stress scores in each group.

[a]n = 84 observations: 14 observations per 6 kittens per 2-week study period per group.

*Unadjusted P values from Wilcoxon rank sum test are displayed for the comparison between groups.

**Statistical significance: P < 0.05.

finding that likely was associated with FHV-1 infection. After the diffusers were placed during G0 but before the induction of stress, no differences were observed (P = 0.39) between the placebo group (32%; n = 27/84 observations) and the pheromone group (25%; n = 21/84 observations) in occurrence of sneezing (Fig. 2).

After adjusting for lack of independence, sneezing (presence) occurred less frequently in kittens in the pheromone group when compared to the placebo group during period K1 (P = 0.006), period G2 (P = 0.005), and when the study periods potentially associated with stress were combined (P < 0.001, Fig. 2). In the combined study periods, kittens in the placebo group were 2.7 (95% confidence interval [CI], 1.7–4.6) times more likely to have a sneezing occurrence than kittens in the pheromone group (Fig. 2). Kittens in the pheromone group also were 3.3 (95% CI, 1.3–8.0; P = 0.009) times more likely to have a sneezing occurrence at the beginning of the study (G0) when compared to the end of the study (G2; Fig. 2). In contrast, no significant differences were identified in sneezing occurrence among these study periods for the placebo group (P = 0.61).

Behavioral findings

Based on review of the literature and agreement by the clinical scorers at the end of the study, four behaviors were believed to be objective indicators of stress in this cohort of cats: kennel pacing, kennel pawing, kennel disarray, and excessive vocalization. Kennel pacing and kennel pawing were evaluated in the SS1, SS3, SS4, and Long time points (Table 2), but there were too few occurrences of kennel pacing in SS4 to be evaluated. Kennel disarray and excessive vocalization were evaluated in the Long time point (Table 2).

Differences were detected between the 2 groups during the kenneled periods (Table 4). More frequent kennel pacing in placebo group kittens was borderline significant during the SS3 time point (P = 0.05). A total stress score for each kitten was calculated by adding the four stress-related behavior scores within the K1 and K2 study periods; the total stress score then was compared between groups. The pheromone group had significantly lower total stress scores as compared to the placebo group in univariate analyses (Table 5). However, after controlling for lack of independence between kitten observations over repeated measures, the total kitten stress scores were not significantly different between groups.

Sleep was used as a correlate for calm/relaxed behavior, as it was observed and recorded at the SS4 time point, after the room gradually quieted after the previous 45 minutes of activity. At that last time point, if the kitten had a relaxed body posture and closed eyes, sleep was recorded.[13,16,18,41] During the equilibration period, none of the kittens in either group had any occurrences of sleeping (Table 6).

During study period G0, there were only two occurrences of sleeping in the pheromone group and no occurrences of sleeping in the placebo group. In univariate analyses, the pheromone group had significantly more sleeping events during the K1, K2, and G2 study periods (Table 6), when K1 and K2 were combined (P < 0.001) and when all 4 of the study periods (K1, G1, K2, and G2) potentially associated with stress were combined (P < 0.001). After controlling for lack of independence between kitten observations over repeated measures, the pheromone group had significantly more sleeping occurrences during the final G2 study period (P = 0.006) and when all 4 of the study periods potentially associated with stress were combined (P = 0.002). In the combined study periods, kittens in the pheromone group were 3.9 (95% CI, 1.4–6.4) times more likely to sleep than kittens in the placebo group (Table 6).

Table 6. Frequency of observations of kittens sleeping during SS4 time point, by group and study period.

Study Period Group	Equilibration	G0[a]	K1[a]	G1[a]	K2[a]	G2[a]
Placebo	0%	0%	0%	0%	0%	1%
Pheromone	0%	2%	10%	4%	13%	20%
P value*	n/a	0.5	0.007**	0.25	0.0007**	0.0001**

G#, group-housed period number; K#, kennel-housed period number; SS4, snapshot-4 time point. Bold G0: diffusers placed at the start of G0.

[a]n = 84 observations: 14 observations per 6 kittens per 2-week study period per group; n/a=not applicable due to too few observations.

*Unadjusted P values from Fisher 2-tailed exact test are displayed for the comparison between groups.

**Statistical significance achieved at P < 0.05.

Other potential behavioral indicators of stress occurred infrequently in this kitten cohort. Isolated hissing events occurred between kittens during group play with novel objects on 4 occasions in both groups during period E, twice in both groups during period G0, and twice in the pheromone group and on 6 occasions in the placebo group during G1 and G2 combined. Similarly, fighting was observed during group play with novel objects, twice during period E and once during period G0 in the pheromone group, and it occurred on four occasions during period G1 in the placebo group. During the kenneled periods when fecal character could be ascribed to an individual kitten, diarrhea was reported on one occasion in 3 placebo group kittens and 2 pheromone group kittens and on four occasions in 1 placebo group kitten. When group-housed, all kittens greeted scorers at the door daily. Hiding was not observed among the kittens during the study.

Cortisol results

All serum cortisol concentrations were within the reference range reported by the laboratory. The results for the pheromone group and the placebo group did not differ over the course of the study. However, the median cortisol results were higher at the end of the last study period (G2) when compared with the start of the first study period (K1) in the pheromone group ($P = 0.02$), placebo group ($P = 0.05$), and the two groups combined ($P = 0.001$).

FHV-1/GAPDH ratios

GAPDH DNA, as an indicator of viable feline cells (GAPDH-positive), was amplified from almost all swabs collected from the kittens, and thus, adequate sample collection was obtained. However, FHV-1 DNA was only amplified from 2 kittens in the placebo group and 4 kittens in the pheromone group after starting the first study period (K1) potentially associated with stress. Because of the low number of samples with detectable FHV-1 DNA, comparisons of numbers of positive or negative samples between groups or comparison of the FHV-1/GAPDH ratios as a measure of viral shedding magnitude between groups were considered inaccurate, and thus, results are not presented. Serum cortisol concentrations at the end of the study, after period G2, did not differ significantly when comparing kittens that did shed FHV-1 (n = 6) to kittens that did not shed FHV-1 (n = 6; $P = 0.09$).

Discussion

After controlling for lack of independence between kitten observations over repeated measures, kittens in the room with the pheromone[a] diffuser still had increased sleeping and less sneezing when compared to kittens in the room with the placebo diffuser; findings support a treatment effect. Even when a standardized dose of FHV-1 is used to inoculate kittens born to FHV-1 naïve queens, variations occur in the clinical signs of disease.[43,44] In addition, collection of objective data to assess behavior in cats can be difficult, and individual cats respond to stress differently.[13,14,39] Reactivation of FHV-1-associated illness in response to stress may not occur in some cats, and when reactivation does occur, time until recognition of clinical signs can vary.[5,8,10] Thus, combining the results from different study periods likely to be associated with stress (K1, G1, K2, G2) seemed the most accurate way to evaluate for differences between groups in our study. We believe the most important limitation to this pilot study was the inclusion of only 6 cats per group, which may have lessened the chances of detecting significant differences between groups.[45]

This stress model to attempt to reactivate FHV-1 was used in another 12-cat study that evaluated a probiotic with presumed immunomodulating activity.[19] Similar to the results described in the current study, evidence for reactivation of FHV-1 varied among cats, and a treatment effect was documented.[19] In the previous study, conjunctivitis was common, and sneezing was rare, whereas in the study described here, conjunctivitis was rare, but sneezing was common. These differences between studies likely arose from the use of 2 different FHV-1 strains and inoculation methods. In the previous study, the field strain of FHV-1 was administered into the conjunctiva fornix, whereas the different FHV-1 strain used in the study described here was administered by nasal inoculation.

In our study, although mild, subjective clinical signs could not be evaluated individually, the objective clinical sign of sneezing was reliably scored and measured by two scorers present at the same time during the observation periods. All kittens still had intermittent sneezing from the primary FHV-1 infection at the time they entered our study, and sneezing still occurred in 32.1% of the observations for the placebo group and in 25.0% of the observations for the pheromone group during G0 when the diffusers were first introduced (Fig. 2). However, over time in the four study periods that may have been associated with stress (K1, G1, K2, G2), the pheromone group had decreased proportions of observation points with sneezing, whereas the placebo group did not (Fig. 2). These findings could indicate reactivation or maintenance of FHV-1-associated sneezing in the placebo group, presumably because of greater receptivity to stress exposure. If a similar study were to be performed again in the future, splitting the FHV-1 inoculum between the nose and eyes may better mimic a natural infection, potentially resulting in clearer reactivation of disease.

GAPDH was amplified from almost all of the oropharyngeal swabs and conjunctival swabs, suggesting that sample collections were adequate. However, FHV-1 DNA was rarely amplified. Because FHV-1 is not eliminated after inoculation, it is likely that many of the FHV-1 PCR assay results were falsely negative. Several studies have shown that FHV-1 PCR assay results can be negative even in the presence of disease, because numbers of infectious viral particles are suppressed by the immune responses.[1,46,47] Use of small

biopsy specimens of the conjunctiva may be more sensitive than use of swabs, and this approach may be considered for use in future studies.[48]

Another limitation was the use of kittens that were socialized, affectionate, attention-seeking of people, and habituated to the research environment, each other, and the observers. All kittens accepted and often sought gentle handling and human contact, petting, and play with each other and the observers. Therefore, the typical indicators of stress and fear such as hiding, freezing, stiffness, crouching, hissing (unrelated to guarding of novel objects), dilated pupils, and holding ears back could not be evaluated in our study because those behaviors were not displayed by these particular kittens.[13,14,16,40–42] Although data were collected for 28 behaviors recorded during multiple time points, only kennel pacing, kennel pawing, kennel disarray, and excessive vocalization (Tables 4 and 5) could be evaluated as stress indicators for statistical comparisons between groups. Although these stress indicative scores and total stress scores usually were numerically higher in the placebo group, statistical differences were lost when analyses were adjusted for individual kitten variations. This failure to find differences between groups in the multivariate analyses could have been a result of limited sample size.[45] Individuals experience wide variations in stress responses, even when exposed to the same stressor, or they differ in their immune response to stress.[22,24,49] Thus, individual variations in our kittens' responses to stress also could have resulted in individual variations in reactivation of FHV-1 and outward clinical signs, because coping mechanisms and coping efficiencies differ among individuals.

Our study attempted to induce stress by housing change, minimal interaction during kenneled periods, and venipuncture. However, stress amount, type, frequency, and duration necessary to reactivate FHV-1 still are unclear. The kenneled study periods resulted in acute stress in the kittens, reflected in the analyzed behaviors as well as increased body temperature, and significantly increased heart rates during kenneled observation periods. However, the study only evaluated 45 minutes; behaviors occurring in the other 23 hours of the day were unknown. Because all kittens retained well-groomed hair coats, ate, and gained weight appropriately, and no clinically relevant diarrhea or vomiting was observed, it is possible that kittens did not exhibit stress behaviors or experience prolonged stress during the rest of the days when kenneled, without the observers present.[16,18,39,41]

Although serum cortisol concentrations remained in the reference range of the reference laboratory, a significant difference between samples collected before attempting to induce housing change stress and the end of study was observed in both groups of kittens, which may suggest that stress occurred over time. In addition, other events in the study beyond housing changes likely were associated with stress including blood collection, witnessing other kittens in distress during blood collection, and occasional variations in feeding and husbandry times.[12,17,50–53] In addition, the placebo group always was scored first, and the pheromone group kittens may have heard the kitten-human interactions, potentially leading to an additional 45-minute anticipatory state for the pheromone group. These potential confounding factors should be addressed in future studies. Also, the changes in serum cortisol concentration within the normal reference range may have been merely related to aging of the kittens over the course of the study.

In the kittens of our study, the best indicator of a relaxed state was sleeping at the end of the 45-minute observation period (SS4). We believe this behavior differed from feigning sleep, which has been used as an indicator of stress in cats, particularly shelter and kenneled cats and described as a defensive sleeping posture in captive felids in zoos, for example.[16] In contrast, the kittens recorded as sleeping in our study did not have a tense, immobile, or defensive posture, but rather a relaxed posture with closed eyes, and the kittens responded positively if awakened. At the end of the 45-minute observation time when the observers rose to depart the room, the sleeping kittens awakened, occasionally stretched, and actively sought engagement again from the observers. This finding supported our assessment of this behavior as an indicator of a relaxed state and not a fear or stress state. Furthermore, the SS4 time point recorded behaviors after the kittens were accustomed to the observers' presence in the room for 45 minutes, after the room gradually quieted subsequent to other activities. Sleeping observations did not occur during the equilibration period, during times of activity such as clinical scoring times, or when observers first entered the room or if facility noise was audible outside of the room. Sleeping observations were recorded during the quietest time, the SS4 time point. Sleeping also was observed with gradually increasing frequency in the room with the pheromone diffuser after the diffusers were in place (Table 6).

Conclusions

We believe our data support reactivation or maintenance of sneezing associated with FHV-1 in the kittens in the placebo group when compared to the kittens in the pheromone group. This difference may have resulted from a response to stress associated with the study design. The evidence for increased relaxing sleep and decreased sneezing in the pheromone group compared to the placebo group, supports the hypothesis that exposure to the pheromone lessened stress and consequently clinical signs associated with FHV-1.

Footnotes

[a] Feliway®; Ceva Santé Animale, Libourne, France.
[b] Endocrinology Laboratory, Michigan State University, Lansing, Michigan.

c StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX, StataCorp LP.

Acknowledgments

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