



## Original Research

# Calming Benefit of Short-term Alpha-Casozepine Supplementation during Acclimation to Domestic Environment and Basic Ground Training of Adult Semi-Feral Ponies

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## ABSTRACT

To evaluate potential calming effects of alpha-casozepine on horses, we blindly compared behavior and training efficiency of adult semi-feral ponies treated with either alpha-casozepine or control supplement during transition to domestic management and handling. Six ponies (three matched pairs) aged 2 to 8 years that had been reared and kept since birth under semi-feral social and environmental conditions were given either alpha-casozepine (1000 mg orally once daily for ponies weighing 160 to 205 kg) or control supplement, beginning 5 days before being moved to a domestic facility for a 2-week introduction to stabling, haltering, leading, tethering, social separation, stall confinement, grooming, simulated girthing, lifting feet, health care treatments, and transportation. Objective quantitative behavior measures (latencies to complete tasks, avoidance responses, and nervous defecations) were derived from video-recorded handling sessions. For each of the 14 sessions, ponies were ranked 1 (best) to 6 (poorest) for calm, compliance, and acclimation/skill progress. All human–animal interactions, video analyses, and rankings were done blindly to supplement assignments. For most daily sessions across the 2-week training period, each of the three alpha-casozepine-treated ponies performed better than their matched control counterparts, and they also had the top three sums of daily session ranks, with a mean of 35.2 compared with 62.8 for control ponies ( $P < .05$ , dependent  $t$ -test). At 6 weeks after the 2-week training period, the alpha-casozepine-treated ponies retained the best three sum of ranks for the seven specific skills re-assessed at that time. These results provide evidence of the benefit of alpha-casozepine supplementation to horses undergoing potentially stressful situations inherent to domestic management.

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## 1. Introduction

The nutritional supplement alpha-casozepine, a decapeptide derived from bovine milk  $\alpha$ -<sub>S1</sub> casein, has been found to have calmativ e anxiolytic-like properties in

humans [1-5] as well as several animal species, and in a variety of stress models. In rats, alpha-casozepine was found to have behavioral effects comparable with those of the anxiolytic diazepam, both for the conditioned defensive burying and the elevated plus-maze models of stressful events [6,7]. In cats, in a randomized blind clinical trial across multiple practices, treatment with oral alpha-casozepine resulted in significantly greater improvement in their interaction with humans compared with placebo, including fear of strangers, contact with familiars, general fears, and fear-related aggression [8]. In dogs, in similar

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randomized blind clinical trials, treatment of anxiety-related disorders using alpha-casozepine resulted in improvement in rating on a standard inventory of emotional disorder symptoms similar to that obtained with the standard reference treatment with selegiline [9].

Domestic horses are particularly vulnerable to stress, often related to fear, during everyday handling and training, resulting in behavioral (eg avoidance, aggression, stereotypies) and physiologic (eg gastric ulcers, injury) responses impacting animal health and welfare as well as horse and human safety. Common potentially stressful experiences for horses include weaning, training for routine ground handling or performance, social regrouping or isolation, transportation, changes in housing and environment, as well as mildly aversive routine health care examination and treatment procedures and restraint, including dental and hoof care. Although appropriate pharmaceuticals are available and are practical for use in some circumstances, a nutritional supplement with anxiolytic-like calming effects would be more practical in many instances as an aid in reducing stress and its consequences.

The purpose of the current study was to evaluate the effects of alpha-casozepine on behavior and training efficiency of horses during potentially stressful experiences. The model used was acclimation of adult semi-feral ponies to domestic management, including movement from life-long family groups to new smaller enclosures and indoor environments, social separation and isolation, as well as grooming, haltering and leading, routine health care procedures, and transportation. Our assumption in this model is that more efficient acclimation/training progress with fewer stress and avoidance behaviors (such as stress vocalizations, nervous defecations, tail swishes, freezing, avoidance responses, or aggressive postures and responses to handling) reflects less fear or anxiety.

## 2. Methods

### 2.1. General Design

Six ponies, consisting of three matched pairs, with one of each pair randomly assigned to alpha-casozepine treatment and the other to control supplementation, were evaluated during standard procedures used routinely in this laboratory to transition previously untrained semi-feral animals to domestic housing, feeding, interaction with humans for basic ground handling, transportation, routine health care, and veterinary procedures in a stable setting. Handling was conducted over a 2-week period using positive reinforcement-based behavior modification protocols. Fear-response behavior, training progress, and retention of learned skills of alpha-casozepine-treated ponies and control ponies were compared. All animal handling, behavior assessments, data entry, and group comparisons were done blindly to group assignments.

### 2.2. Subjects

The subjects included six small Shetland-type (160-205 kg) ponies that had been born and kept continuously since birth under semi-feral herd social and environmental conditions. These included three pairs matched for gender,

age, and known temperament. Pair 1 was a 2-year-old filly and her 3-year-old full sister; pair 2 included two 2-year-old colts; and pair 3 included two mature stallions, aged 6 and 8 years. The ponies were relatively naïve to interaction with humans compared with domestically reared adult horses. In their semi-feral management, the limited interactions had been based on all-positive reinforcement behavior-modification techniques similar to what was used in this study, where the goal is to minimize anxiety and fear. None of the ponies had shown indication of acquired aversions to interaction with humans in general or to any of the specific procedures to which they had been exposed during their previous semi-feral management (observation, height and weight tape measure estimation, blood sampling two to four times per year, annual vaccination, occasional oral deworming, testicular palpation and measure).

Animal procedures were approved by the University of Pennsylvania Animal Care and Use Committee.

### 2.3. Treatments

Alpha-casozepine supplement consisted of one standard, commercially prepared, daily equine dose of 1000 mg alpha-casozepine (Zylkene®, ORSCO, 14 Porte du Grand Lyon 01700 Neyron, France) mixed with ¼ cup Equine Senior pellets (Purina Mills, LLC, Gray Summit, MO, USA) for horses up to 500-kg body weight, hand-fed daily at 07:00 hour starting 5 days before separation from family bands and continuing through the subsequent 11 days of acclimation/training procedures. Control treatment consisted of a similar volume of oat flour ground to a visual consistency similar to that of the alpha-casozepine mixed with Equine Senior and fed on a similar schedule.

### 2.4. Management and Handling Procedures and Associated Behavior Measures

Acclimation/training sessions were conducted once or twice daily Monday through Friday over a period of 2 weeks for a total of 14 sessions. All handling procedures and acclimation to stabling were video recorded for subsequent review to derive quantitative measures of compliance and comfort with the procedures. Table 1 summarizes the handling and management schedule, with details of the procedures and associated objective measures recorded for analysis. All animal care and handling was done by two handlers experienced with the specific procedures and with acclimation/basic ground handling of semi-feral ponies in transition to domestic management. One handler served as the primary handler, whereas the other was available for handling assistance as judged necessary by the primary handler to avoid prolonged delay or negative experience.

In addition to the measures listed in Table 1, at the completion of each training procedure session and at the end of the training period, the two handlers independently and subjectively ranked the ponies based on clinical impressions of each animal's level of comfort (vs. stress) with the experience and overall ease and progress of training or acclimation. Also, at the end of each week of training, the two handlers independently ranked the ponies specifically for learning efficiency based on the rate

of simple associative learning for specific new skills and acclimation procedures.

Six weeks after completion of the 2-week training period, with no interim handling, retention of seven specific skills introduced during the acclimation/training period was assessed. Handling for this assessment was done by a third trainer skilled with the all-positive behavior-modification techniques, but with no previous interactions with these ponies or knowledge of the nature of the study or treatment assignments.

### 2.5. Data Analysis

For each handling session, considering all objective measures, the ponies were ranked from 1 (best) to 6 (poorest) for calm, compliance, and training/acclimation progress. In addition, for each subject, the ranks for 14 sessions were summed to derive an overall 2-week program score for statistical comparison of groups using dependent *t*-test procedures [10]. For the retention assessment, based on quantitative measures, ponies were ranked for each of the seven skills indicated in Table 1, and an overall retention score based on the sums of those ranks was derived. The handler also subjectively similarly ranked the ponies from 1 (best) to 6 (poorest) for performance on each procedure and overall for the seven skills.

## 3. Results

### 3.1. Rankings Based on Objective Measures

For 13 of 14 sessions over the 2-week training period, the average rank among the six ponies (derived from daily objective quantitative measures reflecting training progress and comfort) was better (1 = best of the 6 ponies, 6 = poorest) for alpha-casozepine-treated ponies as compared with that for control ponies. For 7 of 14 training sessions, each of the three alpha-casozepine-treated ponies ranked better than their matched control counterparts. For 4 of the remaining 7 sessions, two of the three alpha-casozepine-treated ponies ranked better than their matched control counterparts.

The final 2-week program scores (sum of 14 session ranks) were 35, 44.5, and 26 for the three alpha-casozepine-treated ponies, with a mean of 35.2 compared with 57.5, 63, and 68, respectively, for corresponding matched control ponies with a mean of 62.8 (lower sums represent better performance). The difference is significant ( $P < .05$ , dependent *t*-test, 2df).

For the 6-week assessment of retention of seven specific skills, three of the three alpha-casozepine-treated ponies and two of the three control ponies performed at or above levels reached during the 2-week training. For four of the seven specific skills compared (approach and catch at pasture, leading, lifting/picking out feet, intranasal application), as well as for overall performance, each of the three alpha-casozepine-treated ponies scored better than their matched control counterparts. For two of the remaining three specific skills compared (clipper and cross-tie), two of the three alpha-casozepine-treated ponies ranked better than their matched control counterparts.

### 3.2. Handlers' Subjective Rankings

The two handlers' subjective daily training session rankings of level of comfort (vs. stress) with the experience and overall ease and progress of training or acclimation that were independently recorded after each handling session were consistently in agreement with each other as well as with the rankings derived from objective quantitative measures. Similarly, the two handlers' independently recorded rankings of ponies' learning efficiency consistently placed the three alpha-casozepine-treated ponies as the best three learners. Also, for the retention assessment, the handlers placed the three alpha-casozepine-treated ponies as best overall for comfort, compliance, and skill level.

## 4. Discussion

In this model of transition of adult ponies from life-long semi-feral social and environmental conditions to domestic management and introduction to basic ground handling using primarily positive reinforcement-based methods, all three ponies fed alpha-casozepine performed better overall as compared with their matched control counterparts. These results provide evidence that the nutritional supplement alpha-casozepine holds promise as a valuable aid to improving efficiency of handling and training horses. The procedures introduced to these ponies represent a reasonable sample of the various ground handling and management experiences of domestic horses associated with fear- and stress-related behavior. Consistently more compliant behavior and greater learning efficiency across the sample of handling experiences common to domestic horses suggest that alpha-casozepine supplementation may be of benefit generally to horses in potentially stressful situations. Further investigation is warranted.

Although nonconfrontational positive reinforcement-based behavior modification methods of animal handling and training are gradually gaining favor, traditional handling styles based on negative reinforcement and punishment likely remain more common throughout the equine industry. Although we would advocate more widespread use of positive reinforcement-based handling of horses, further work should address effectiveness of alpha-casozepine with different handling styles.

In this study, daily treatment commenced 5 days before the ponies were moved from semi-feral to domestic management and continued through the 2 weeks of acclimation and training. Alpha-casozepine has recently been marketed in Europe as a feed supplement for horses and ponies; it is available in 1000-mg dose packets, with the label instruction to feed one packet daily per horse or pony weighing up to 500 kg, and two packets for horses weighing more than 500 kg, starting 2 days before anticipated stressful experience. Daily milligram/kilogram dose would vary considerably across the range of body weights. These ponies, weighing 160 to 201 kg, were fed approximately 4.9 to 6.2 mg/kg daily. In clinical trials with cats [8], dogs [9], and humans [1-4], once daily oral dosing was found effective. In rat experiments, oral dosing 1 hour before the test procedures was also found effective [7]. Further work is needed to investigate effective dose rates and regimens for horses and ponies, particularly exploring

**Table 1**  
Daily acclimation/training sessions and specific objective measures

Session	Procedures	Measures
Week 1		
Day 1	Separation from family bands, loading into stock trailer, 1-mile transport followed by unloading into paddock with run-in shelter together with other subjects	Defecations (number and consistency), assistance required loading, latency to load (minute), assistance required unloading
Day 2	Catch and halter at pasture, lead into training barn for first experiences with multiple types of man-made surfaces (wet and dry), enclosed space with ceilings, walls, artificial lighting, rubber mats, metal floor drain grates, windows, shadows, barn furnishings. Introduction to grooming, introduction to tethering and cross-ties as judged ready	Latency to approach and halter in paddock, hesitations or stops during leading, latency to enter barn (minute), leading assists requested, defecations, percent time tolerating grooming, avoidance behaviors (pull back on lead, rear, spin, stamp, kick, bite, lunge forward)
Day 3 (a)	Repeat of day 2 leading and grooming plus start of training for foot lifting and acclimation to electric clipper	As day 2, plus foot lifting attempts and successes, percentage of time tolerating clipper and progress with clipper touch to body
Day 3 (b)	Obstacle course 2 m wide by 9 m long with wet concrete footing, stainless lab cart, empty feed bag on floor, floor drain grate running through the center of the course, video tripod, clear plastic bag on floor, brown paper bag hanging from ceiling to pony height moving in breeze, a 0.6-m high red plastic trash bin, 2 m × 3 m green fabric tarp on floor, and 0.6 m × 0.6 m bright blue wobbly metal floor plate	Latency (seconds) to complete obstacle course, hesitations, stops, leading assists requested, defecations, vocalizations
Day 4	Obstacle course as day 2 with different obstacles expected to present greater challenge, including a black hose on the floor dripping condensation, a water sprinkler fountain with run-off stream and puddle, the floor drain grate median, a snow shovel on floor, three black rubber feeding pans mounted on a board on the floor, a 20-L insulated water cooler, a hanging blinker hood, and the blue wobbly metal floor plate as above	As above
Day 5	Initial social isolation and stabling in a box stall (three solid walls with open grill front) within the training barn for 1.5 hours, with no other animals in the barn	Defecations, latency to become calm enough to eat hay, pawing bouts, vocalizations, escape attempts (climb, push, lunge at walls or stall front doors)
Week 2		
Day 6 (a)	Obstacle course in new 7 m × 7 m room brightly lit with both incandescent and fluorescent, with cluttered set of new obstacles: up steep ramp entry with textured rubber flooring, through narrow examination chute with hanging tarp curtain, AM radio with static, camera and monitor on cart, open door with breeze blowing curtain and 0.6-m square plastic bag on floor, wheeled muck cart, metal step ladder, tripod, 0.6 m × 6 m mirror, stool with tote box and sprinkler (not running) on top, leather dummy mount, hanging red fabric bag, red plastic trash bin 0.6-m high, followed by continued handling acclimation as above at the cross-ties in the following standard order: rectal temperature, height and weight tape measurement, sweep broom near pony on cross-ties, brush all over, drop plastic shovel to floor nearby, run clippers on body, saddle pad and girth, walk a 30-m distance with saddle pad and girth	Latency to complete obstacle course (minute), foot lifting attempts and successes, percentage time tolerating grooming, percentage time tolerating clipper and progress with clipper, defecations, avoidance behaviors as above
Day 6 (b)	Continued stall acclimation with six ponies in individual adjacent stalls together in the same barn for 2.5 hours	As on day 5 stall acclimation
Day 7	15-Minute battery of management, health care and veterinary procedures at cross-ties: jugular stick 1, IM needle stick, simulated intranasal vaccination, oral dewormer, spray bottle misting of body and legs, cold water hosing of body and legs, jugular stick 2	Latencies to complete each procedure, ease of introduction to spray misting, ease of introduction to hosing, avoidance behaviors as above
Day 8	Leading assessment over standard ¼ mile course over new terrain and in new buildings including through water, over hard and soft surfaces, new indoor spaces, variable natural and artificial lighting, narrow chutes, a hollow sounding wood floor, and near pastured groups of horses	Latency to complete ¼ mile course, nervous tail swishes, hesitations, stops, vocalizations, defecation, avoidance behaviors as above
Day 9 (a)	Load and stand in examination stocks for 10-minute veterinary examination per rectum with U/S imaging	Latency to load into stocks (second), avoidance behaviors as above, latency to relaxation during per rectum examination
Day 9 (b)	Leading across series of rubber floor mats of three different colors followed by introduction to ear clipping at cross-ties	Latency to be led cross series of red, white, and black rubber floor mats, hesitations, stops; latency (minute) to tolerate clippers applied to the poll and ears, avoidance behaviors as above

Day 10 (a) Day 10 (b)	Feet lifting assessment, holding each foot up for $\geq 3$ seconds Loading into stock trailer as on Day 1 for 2.6-mile transport alone followed by unloading	Latency (minute) to complete all 4 feet, avoidance behaviors as above Latency to load, defecations, vocalizations, avoidance behaviors as above, latency to unload
Assessment of skill retention at 6 weeks		
Catch and halter at pasture, lead $\frac{1}{4}$ mile previously untraveled route past multiple social challenges to cross-ties in training facility, lift and briefly pick out 4 feet, apply clipper to body, poll, and ears, simulated intranasal vaccination, hosing of legs and body and stand at cross-ties unattended for 5 minutes, and return $\frac{1}{4}$ mile route, all with a novel handler		Latency to approach and halter at pasture, latency to complete novel route, hesitations, stops, defecations, assists needed, latencies and avoidance responses and defecations to lift and pick feet, apply clipper, complete intranasal treatment, hosing of legs and body, and stand unattended at cross-ties, and return leading $\frac{1}{4}$ mile

acute treatment as reported for rats, that would be practical in many instances where a problematic aversion develops amidst the need to complete a procedure in a timely fashion, for example during veterinary care.

Alpha-casozepine and other milk proteins and metabolites with demonstrated anxiolytic-like effects are currently believed to work through the GABA neurotransmitter system, the same system mediating effects of the benzodiazepine anxiolytic agents [11,12]. The benzodiazepines were among the first generation of tranquilizers with anxiolytic effects at dose levels that did not impair physical coordination and mental alertness. Nonetheless, benzodiazepines are known for certain adverse effects on memory as well as reduced threshold (or disinhibition) for aggression. The benzodiazepine diazepam has been used as an anxiolytic in horses, within the specific context of releasing inhibited sexual behavior in stallions [13-15]. In this context in stallions, simultaneous disinhibition of aggressive behavior is a common undesirable side effect, which often becomes counterproductive if not anticipated by skillful handlers or tolerated by the stimulus mare. This conspicuous disinhibition of aggression in stallions, along with purported adverse effects on memory in other species, is likely the main reason that this class of calmatives agents has not been explored for more general application in horses. A promising feature of the milk protein-derivative supplements is that, although apparently GABA-mediated, their effects appear to be specific to anxiety, without the adverse effects on memory or disinhibition of aggression typical of benzodiazepine anxiolytics [11,12]. The alpha-casozepine-treated ponies in this study appeared normally alert. Learning ability, based on acclimation and training efficiency across a broad range of experiences, was greater for the alpha-casozepine-treated ponies. Learning progressed from day-to-day during the training/acclimation period, and skill and acclimation were well retained when assessed at 6 weeks. Similarly, although there was a generally low level of aggression elicited in this nonconfrontational handling method, there was no indication of disinhibition of aggression in the alpha-casozepine-treated ponies. Because styles of horsemanship vary within the equine industry such that many horses are handled and trained in a more confrontational style using principally negative reinforcement and punishment that tends to inadvertently elicit dangerously fear-responsive reactions that threaten animal welfare and human safety, further study specifically addressing effects of milk protein-derived calmatives on aggression is warranted.

In conclusion, the results of this blindly conducted study indicate the benefit of alpha-casozepine dietary supplement to ponies undergoing transition from semi-feral management to domestic management and handling. These findings in horses support a growing body of research and clinical evidence for a calmativ anxiolytic-like effect of alpha-casozepine in mammals.

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