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**Zytkéne to Load? The effects of alpha-casozepine on compliance and coping  
in horses during loading.**

Carrie Ijichi\*<sup>1,2</sup>, Sophie Green<sup>1</sup>, Keith Squibb<sup>1</sup>, Aisling Carroll<sup>1</sup> & Isobel Bannister<sup>1</sup>

*Hartpury University, Hartpury, Gloucester, UK, GL19 3BE*

<sup>2</sup>*Present address: School of Animal, Rural and Environmental Science, Nottingham*

*Trent University, Southwell, NG25 0QF*

[\\*carrie.ijichi@ntu.ac.uk](mailto:carrie.ijichi@ntu.ac.uk)

1 **Abstract**

2 Horses are routinely travelled for access to safe off-road riding, veterinary care,  
3 breeding, sale or moving to a new home environment. However, transport is a known  
4 stressor in horses. For this reason, problem behaviour when loading is a commonly  
5 reported issue which presents risks to handlers and horse welfare. Existing literature  
6 and manufacturers recommendations suggests that alpha-casozepine may be  
7 effective in improving the behaviour and welfare of horses during loading onto a  
8 vehicle for transport. The current paper aims to assess the behavioural and  
9 physiological effects of a commercially available alpha-casozepine feed supplement  
10 (Zylkéne Equine) in horses during loading and confinement on a transport lorry.  
11 Subjects (n = 10) were loaded once with the supplement and once without, in a  
12 balanced random order with each subject acted as their own control. The handler  
13 was blind to treatment. Time to load onto the lorry, and movement of feet, licking and  
14 chewing, and vocalising within the lorry, were recorded as behavioural indicators of  
15 compliance and coping. Heart rate, heart rate variability, salivary cortisol, and  
16 infrared thermography of both core temperature and the discrepancy between eyes,  
17 were measured as indicators of arousal. There were no significant differences in  
18 physiology between Treatment and Control ( $P > 0.05$ ). Treatment resulted in a  
19 significantly shorter Loading Time than control ( $P = 0.04$ ), however, the actual  
20 difference in median time was only 0.45 seconds. No other behavioural indicator  
21 differed between Treatment and Control ( $P > 0.05$ ). Power analysis revealed the  
22 sample was sufficient to detect a significant effect. Where modest effects were  
23 observed for a small number of variables, Treatment effect contradicted predictions.  
24 Taken together, this indicates that alpha-casozepine does not affect a horse's ability  
25 to cope with loading and confinement in a horse lorry. Further work is required to

26 ascertain whether the maximum dosage – twice that used here – might affect coping  
27 and behaviour in horses.

28

29 **Keywords:** alpha-casozepine; horse loading; infrared thermography; salivary  
30 cortisol; heart rate variability; stress

31

## 32 **Introduction**

33 Horses are routinely travelled for access to safe off-road riding, veterinary care,  
34 breeding, sale or moving to a new home environment. However, transport is a known  
35 stressor in horses (Schmidt et al., 2010) due to features such as confinement, novel  
36 noises, unstable flooring, the presence of unfamiliar conspecifics and sudden  
37 changes in light. For this reason, problem behaviour when loading is a commonly  
38 reported issue, which presents risks to handlers and horse welfare. During loading,  
39 the handler motivates an approximately 500kg animal with a highly evolved flight  
40 response into a confined space, which neither the horse nor handler can easily  
41 escape if an accident occurs. Sedation may be offered to improve behaviour but this  
42 may reduce the motor-control of the animal, increasing the risk of loss of balance  
43 during transport and subsequent injury. Further, sedatives are commonly banned in  
44 horses being transported for competition (FEI, 2018) and sedatives that mainly affect  
45 motor-control may make the horse more manageable and cause them to appear  
46 calmer, without addressing the underlying anxiety trigger by the environment.  
47 Ideally, correctly applied behaviour modification techniques aimed at habituating the  
48 horse and training them to respond to lead-rope pressure should be implemented,  
49 rather than the use of force (McGreevy and McLean, 2009). Such training aims to  
50 improve the horse's ability to tolerate a stressor, however, this process may still incur

51 risk to even experienced trainers. Additionally, it is possible that an animal may need  
52 to be transported at short notice, without the benefit of such training. Therefore, any  
53 practical solutions that may improve the efficacy of training, or limit the welfare  
54 impact of unavoidably stressful events, are warranted.

55 Dietary supplementation with alpha-casozepine is thought to have anxiolytic  
56 properties. Alpha-casozepine originates from S1 casein, a protein in cow's milk and  
57 fits into a segment of GABA-B receptors which are responsible for anxiolytic activity  
58 (Landsberg et al., 2017). Whilst research into this supplement is limited, McDonnell  
59 et al., (2014) found a significant improvement in horses' compliance and comfort  
60 during twelve routine healthcare and treatment procedures when supplemented with  
61 Zylkène Equine, a commercially available alpha-casozepine. This supported  
62 previous findings (McDonnell et al., 2013) which showed that semi-feral ponies  
63 treated with Zylkène whilst undergoing the process of initial training were more calm,  
64 compliant and progressed better than those not having Zylkène in their feed. This  
65 anxiolytic effect is also noted in rats (Miclo et al., 2001) and cats (Beata et al.,  
66 2007). Zylkène Equine is suggested by the manufacturers for use in loading and  
67 transporting horses (Vetoquinol, 2018). Moreover, it is safe for use and not currently  
68 listed as banned for competition use (FEI, 2018). However, no studies to date have  
69 measured the physiological impact of such supplementation and compliance is not  
70 necessarily an appropriate indicator of coping in horses (Squibb et al., 2018), though  
71 it is highly desirable for handlers.

72 Physiological indicators of arousal can be measured non-invasively in a number of  
73 ways. Heart Rate Variability (HRV) is advantageous because it can be used to  
74 investigate the functioning of the autonomic nervous system, as variability decreases  
75 with an increase in stress (von Borell et al., 2007). Infrared thermography (IRT) on

76 ocular (eye) surface temperature has also been used in horses to monitor stress  
77 responses (Ijichi et al., 2018; Valera et al., 2012). It has been validated against  
78 cortisol (Valera et al., 2012) and can detect fear during novel object tests (Dai et al.,  
79 2015). Additionally, a discrepancy in temperature between the left and right eye may  
80 indicate hemispheric dominance indicative of affective state (Lush and Ijichi, 2018),  
81 though this requires further validation. Cortisol is released as a response to stressful  
82 events and can be measured from saliva samples (Yarnell et al., 2013). Studies  
83 based on blood plasma cortisol changes have repeatedly shown that transport is  
84 stressful for horses, however, blood sampling causes stress in itself (e.g. Fazio et al.,  
85 2008). As salivary cortisol is validated against blood samples (Peeters et al., 2011),  
86 salivary cortisol sampling is the best candidate for non-invasively sampling rapid  
87 changes in cortisol.

88 The current experiment aims to assess the effects of a commercially available feed  
89 supplement, Zylkéne Equine, on behaviour and physiology in horses during loading  
90 and confinement on a transport lorry. To this end, subjects were loaded once with  
91 the supplement and once without, in random treatment and subject order. Time to  
92 load onto the lorry, movement of feet, licking and chewing, and vocalising within the  
93 lorry were recorded as behavioural indicators of compliance and coping. Heart rate,  
94 heart rate variability, changes in salivary cortisol and infrared thermography of both  
95 core temperature and the temperature discrepancy between eyes, were measured  
96 as indicators of arousal. It was hypothesised that horses would load more quickly  
97 but move, vocalise, lick and chew less within the lorry in the treatment, compared to  
98 the control tests. It was also hypothesised that horses would have lower heart rate,  
99 higher heart rate variability, lower core temperature, more negative discrepancy  
100 scores and reduced cortisol changes in the treatment, compared to the control tests.

101

## 102 **Materials and methods**

### 103 *Subjects*

104 10 healthy horses (6 geldings and 4 mares) of mixed breeds and ages were tested  
105 between 26<sup>th</sup> March and 12<sup>th</sup> April 2018. Ages ranged from 8-25 years of age (mean  
106 = 12.6; IQR = 9.25-14.5). Horses were stabled at two private livery yards in  
107 Gloucestershire and were tested in their home environment to reduce the effect of  
108 environmental novelty. Subjects were travelled at least once a month as part of their  
109 normal management routine and had no known phobia to travelling. This restriction  
110 was imposed by Hartpury University's ethics committee to ensure high animal  
111 welfare standards were met. Horses were managed at the discretion of their owners  
112 which meant that workload, turnout and feeding varied according to age and current  
113 use, as well as owner preferences.

114

### 115 *Experimental Design*

116 This was a within-individual experimental design with each subject acting as its own  
117 control. Each subject was loaded once with Zylkéne Equine and once without. The  
118 order of the treatments were randomly allocated. To counterbalance the study there  
119 were equal numbers of supplemented and control horses in each trial. This limited  
120 the possibility of a false positive due to habituation through repeated exposure  
121 (Hawson et al., 2010). Subject order within the group was pseudo-randomised to  
122 account for owner availability. The handler was blind to treatment to prevent any  
123 sub-conscious bias affecting handling and therefore subject responses. Tests were  
124 repeated 2 weeks apart at the same time of day  $\pm$  30 minutes. With the exception of

125 the test itself, subjects were managed as per their normal daily routine reducing the  
126 impact of differing management of each testing day. A wash-out period has not been  
127 established for this supplement by the manufacturer and so two weeks was used as  
128 an estimated generous wash-out period for subjects receiving Zylkène in the first  
129 trial. This assumption was tested during data analysis (see 2.9 Statistical Analysis).

130

### 131 *Feeding Protocol*

132 Zylkène Equine was fed once daily for four days prior to testing, as per minimum  
133 dosage in the manufacturer's instructions (Vetoquinol, 2018). Horses weighing up to  
134 500kg were fed 1000mg daily, while horses over 500kg were fed 2000mg of Zylkène  
135 Equine. Mean subject weight was 492.9kg ( $\pm 70.34$ ). The researcher met with the  
136 owners of the horses a week before the test was due to take place to provide the  
137 correct amount of Zylkène Equine supplement and ensure that the owner was clear  
138 about how the supplement must be fed. The supplement needed to be fed to the  
139 horses in their morning feeds to ensure that they received their final dose on the  
140 morning that the test took place. Prior to testing on treatment trials, the same  
141 researcher (S.G.) confirmed that the subject had been fed the supplement. Aside  
142 from the addition of the supplement for one trial, feeding was kept as per the  
143 subject's normal routine.

144

145

### 146 *Handling and Loading*

147 The current study used the same Equi-trek rear-facing 3.5t lorry for all tests. The  
148 internal divider was removed to allow the handler to move safely in the lorry with the



149 subject and to provide the subjects with more room to express behaviour (Figure 1).  
150 Subjects wore protective equipment such as rugs, travel boots or poll-guards at the  
151 discretion of their owner. All subjects were handled by the same individual (C.I.) who  
152 is experienced in loading horses and experimental handling and was blind to  
153 treatment. Horses were led to a marker 3.5m from the ramp of the lorry and halted.  
154 Horses were handled using appropriate pressure and release (McGreevy and  
155 McLean, 2009). Forward pressure on the leadrope was used to indicate the horse  
156 should step forward. This was immediately released when the horse complied. If the  
157 horse did not respond to leadrope pressure, they were rhythmically tapped on the  
158 rib-cage first with increasing speed and then increasing intensity if required, until  
159 they took a forward step. Soft vocal cues were also used to indicate correct  
160 responses and tactile positive reinforcement, including wither scratching  
161 (Thorbergson et al., 2016). This was used on loaded horses to encourage them to  
162 stand while the ramps were closed. Once inside the closed lorry, subjects were  
163 cross-tied in the center of the lorry with elasticated safety lines (Figure 1). The  
164 handler then took the post-loading IRT images before stepping through the internal  
165 door and sitting out of the subject's vision on a stool placed in the equipment  
166 compartment. Each horse remained within the lorry for 5 minutes, the doors were  
167 then re-opened and the subject unloaded.

168

169

### 170 *Infrared Thermography*

171 Using a FLIR E4 thermal imaging camera (FLIR Systems, USA.), the researcher  
172 took an image of both of the subject's eyes. The camera was held at approximately a

173 ninety-degree angle and 1m distance from the eye as accurately as possible within  
174 the confines of the space available. IRT readings were taken in the stable before  
175 testing (S.G.), once loaded onto the lorry when the ramp had been closed and before  
176 the ramp was opened and the horse was unloaded from the lorry (C.I.). The  
177 temperature was analysed for each horse retrospectively using FLIR tools (ver.  
178 5.9.16284.1001). The maximum temperature within the palpebral fissure from the  
179 lateral commissure to the lacrimal caruncle (Yarnell et al., 2013) was used and the  
180 discrepancy between the temperatures for each eye was calculated by subtracting  
181 the temperature of the left eye from the right eye (Lush and Ijichi, 2018). C.I. and  
182 S.G. analysed the images independently and, on the rare instance where they  
183 varied, the highest recorded temperature for each image was used for analysis. The  
184 average of both eyes is referred to as Core Temperature. The difference in  
185 temperature between the eyes is referred to as Temperature Discrepancy.

186

### 187 *HRV Readings*

188 A Polar Equine V800 heart rate monitor (Polar Electro Oy, Kempele, Finland) was  
189 paired to an elasticated adjustable surcingle. This was fitted to each horse after IRT  
190 images were taken but prior to leaving the stable, by wetting the girth area and then  
191 ensuring close contact to ensure conductivity (S.G.). The paired watch was looped  
192 onto the surcingle to ensure that it remained within connectivity boundaries at all  
193 times. Subjects had a minimum of 5 minutes to habituate to the surcingle which was  
194 deemed to be sufficient as all subjects had previously worn girths and/or lunging  
195 rollers. Recordings began at a marker 3.5m meters from the ramp of the lorry and  
196 recorded continuously during loading, confinement and unloading. Recording was  
197 stopped when the horse returned to the marker after unloading.

198 Heart rate analysis was carried out by C.I. using Kubios HRV software (ver. 3.0.2  
199 Biomedical Signal Analysis and Medical Imaging Group, Department of Applied  
200 Physics, University of Eastern Finland, Kuopio, Finland.). Kubios settings were  
201 adjusted in line with previous equine studies (Ille et al., 2014; Squibb et al., 2018).  
202 Specifically, artefact correction was set to custom level 0.3, thus removing RR levels  
203 varying by more than 30% from the previous interval. Therefore, where a single RR  
204 interval was more than 30% different from the preceding interval, it was deemed to  
205 be an incorrect reading. Trend components were adjusted using the concept of  
206 smoothness priors set at 500ms, to avoid the effect of outlying intervals. The STD  
207 RR value, being the standard deviation of RR intervals, was used as the HRV figure  
208 to reflect both short-term and long-term variation with the series of RR intervals. The  
209 root mean square of successive RR intervals (RMSSD value) was recorded as an  
210 indicator of vagal tone (Schmidt et al., 2010).

211

### 212 *Cortisol Samples*

213 Cortisol samples were taken using an Equisal saliva collection kit. The swab was  
214 removed from its packaging and inserted into the side of the horse's mouth through  
215 the interdental space, between the front and back teeth and above the tongue. The  
216 swab was moved gently around the top of the tongue until enough saliva was  
217 collected. This was judged using the colour change indicator, which turned from  
218 white to pink when sufficiently saturated. Once the sample collection was complete  
219 the swab was placed into a tube and chilled until it could be frozen, awaiting  
220 analysis.

221 Two saliva samples were taken, per horse, for each condition. The first sample was  
222 taken in the stable to determine a baseline level of cortisol for each horse by the  
223 same experimenter (S.G.). This was done after IRT readings – to ensure that the  
224 swabbing did not elevate core temperature - but before the heart rate monitor was  
225 fitted – which might affect cortisol in sensitive horses. The second saliva cortisol  
226 sample was taken after 5 minutes within the lorry, after the final IRT images were  
227 taken and before the subject was unloaded. The researcher (C.I) re-entered the  
228 horse compartment through the internal door and took the second sample in the  
229 same method described above. Pre-test cortisol values were subtracted from post-  
230 test values to indicate the change in cortisol as a result of loading and confinement  
231 (Table 1). This was to account for any variation in cortisol that was not the result of  
232 testing, such as slight diurnal differences or uncontrollable extraneous sources of  
233 stress. Baseline cortisol, post-test cortisol and changes in cortisol were included in  
234 further analysis.

235

236 **Table 1.** Baseline, post-test and change in salivary cortisol levels ( $\mu\text{g}/\text{dL}$ ) for each  
237 subject in treatment and control trials.

Subject	Treatment			Control		
	Baseline	Post-test	Change	Baseline	Post-test	Change
1	0.3	0.4	0.1	0.19	0.26	0.07
2	0.14	0.09	-0.05	0.19	0.17	-0.02
3	0.08	0.101	0.021	0.15	0.13	-0.02
4	0.24	0.13	-0.11	0.12	0.11	-0.01
5	0.11	0.16	0.05	0.16	0.07	-0.09
6	0.05	0.07	0.02	0.12	0.09	-0.03
7	0.05	0.06	0.01	0.2	0.22	0.02
8	0.06	0.04	-0.02	0.08	0.04	-0.04
9	na	na	na	na	na	na
10	0.02	0.04	0.02	0.06	0.04	-0.02

238

239 Samples were analysed by S.G., K.S., A.C. and I.B. Saliva samples were kept within  
240 an ice cooler until transported to the laboratory where they were stored at -20  
241 degrees until analysed. Samples were frozen on the day of sampling within  
242 approximately 4 hours of the saliva collection. To defrost swabs, all samples were  
243 stored at 4 °C Samples were spun down using a centrifuge for approximately 5  
244 minutes at full speed to extract the liquid.

245 When analysing, all reagents and the microtitre plate were brought to room  
246 temperature before starting the protocol. A 1X wash buffer, enough for the current  
247 day's requirement, was prepared.. Plate layout was determined with standards,  
248 controls and saliva samples assayed in duplicate. The protocol followed Salimetrics  
249 Assay (Salivary Cortisol ELISA kit) and was as follows:

250 24 mL of Assay Diluent was pipetted into the disposable tube. 25  $\mu\text{L}$  of standards,  
251 controls, and saliva samples were pipetted into the appropriate wells. 25  $\mu\text{L}$  of Assay  
252 Diluent was pipetted into 2 wells to serve as the zero. 25  $\mu\text{L}$  of Assay Diluent was  
253 pipetted into each non-specific binding well. The Enzyme Conjugate was diluted

254 1:1600 by adding 15  $\mu$ L of the conjugate to the 24 mL tube of Assay Diluent  
255 prepared earlier. The conjugate tube was centrifuged for approximately 5 minutes to  
256 bring the liquid down to the tube bottom. The diluted conjugate solution was mixed  
257 and 200  $\mu$ L was added to each well. The plate was mixed on a plate rotator for 5  
258 minutes at 500 rpm and incubated at room temperature for a total of 1 hour. The  
259 plate was washed 4 times with the 1X wash buffer. After each wash, the plate was  
260 thoroughly blotted on paper towel before it was turned upright. The plate was mixed  
261 again on a plate rotator for 5 minutes at 500 rpm and incubated in the dark (covered)  
262 at room temperature for an additional 25 minutes. 50  $\mu$ L of Stop Solution was added  
263 to each well. The plate was mixed on a plate rotator for 3 minutes at 500 rpm. This  
264 was continued until all wells showed a yellow colour. The plate was read in a plate  
265 reader at 450 nm within 10 minutes of adding the Stop Solution.

266

### 267 *Behavioural Observations*

268 Researchers recording behaviour were blind to treatment. The time taken to load  
269 was measured by the same researcher (K.S.) using a stopwatch. Time was started  
270 when the handler stepped past the marker 3.5m from the ramp and ended when the  
271 final hind foot of the subject entered the lorry. Once inside the lorry, horse behaviour  
272 was recorded by a camera mounted on a tripod within the equipment compartment  
273 (C.I.). This recorded through the interior door between the equipment and horse  
274 compartments, which was secured in the open position (Figure 1). The recording  
275 began after the second IRT reading was taken and captured the 5 minute  
276 confinement period that followed.

277 Behaviour was recorded by the same researcher (C.I.) as individual instances for  
278 each variable. The number of times the subject moved their feet was recorded as an  
279 indication of frustration causing displaced locomotive behaviour and a failure to  
280 remain immobile (McGreevy and McLean, 2010). This included any instance where  
281 the foot was raised off the ground and included kicking, pawing and steps. The  
282 number of times the horse expressed licking and chewing behaviour was recorded.  
283 This included sideways movement of the jaw, accompanied by audible grinding, with  
284 or without the protrusion of the tongue. Although the ethological significance of  
285 licking and chewing is not yet fully understood, it is observed during potentially  
286 stressful circumstances (Krueger, 2007). Therefore, it was measured as a  
287 supplementary behavioural indicator. The number of vocalisations was recorded and  
288 characterised by audible neighing, separated by silence. Such vocalisations are  
289 used to regain contact with conspecifics (Houpt, 2001) and may indicate arousal  
290 caused by isolation within the lorry.

291

### 292 *Statistical Analysis*

293 Statistical analysis was carried out using R (R Development Core Team, 2017). The  
294 normality of the sampling distribution was tested using a Shapiro-Wilks test prior to  
295 tests of difference (Field et al., 2012). Paired T-tests or Wilcoxon ranked-sum tests  
296 were used to detect differences between Treatment and Control as appropriate for  
297 normality. Post-hoc effect sizes were calculated (Field et al., 2012; pp 393 & 665) to  
298 determine how meaningful changes in behaviour and physiology were. Power  
299 analysis was conducted on T-tests to determine whether non-significant differences  
300 were due to a lack of effect or insufficient sampling (Field et al., 2012).

301 Post-hoc tests of difference were conducted to determine whether an inadequate  
302 wash-out period may have confounded results, limiting the ability to detect a truly  
303 significant effect. If subjects were treated for the first trial, and the supplement had  
304 not completely washed out by the time they were tested for the Control trial, this may  
305 cause an insignificant effect in the whole sample, when in fact, the supplement is  
306 effective. Therefore, for variables that were not significantly affected by treatment in  
307 the whole sample, a subset of subjects who were tested with the control first ( $n = 5$ )  
308 were tested for differences between Treatment and Control. Subjects who were  
309 tested with the control first could not have had control trials affected by residual  
310 substance. Therefore, if this test of difference is significant, it indicates that non-  
311 significant findings in the whole sample were the result of residual supplementation  
312 and insufficient wash-out period. If the test is insignificant, it confirms non-significant  
313 results seen in the whole cohort.

314



315 **Results**

316 There were no significant differences in physiology between Treatment and Control  
 317 (Table 2).

318 **Table 2.** Differences in physiological measures between Treatment (T) and Control  
 319 (C). Paired T-tests (PT) state the mean, standard deviation (S.D.) and t-value (T)  
 320 and statistical power. Wilcoxon Signed-Rank (W) tests state the median, inter-  
 321 quartile range (IQR) and v-value (V). N = 10 for all tests, except cortisol (N = 9).

Variable	Test	Mean/ Median	S.D./IQR	Test	V/ T	P	Effect Size	Power
Heart Rate (bpm)	T	78.02	21.42	W	39	0.28	-0.35	NA
	C	75.36	20.43					
Heart Rate Variability (ms)	T	73.08	±32.97	PT	0.65	0.53	0.21	0.79
	C	66.19	±32.37					
RMSSD (ms)	T	47.25	34.62	W	25	0.77	-0.09	NA
	C	44.65	30.38					
Baseline Core Temp. (°C)	T	34.66	±1.17	PT	0.36	0.73	0.12	0.79
	C	34.37	±1.64					
Core Temp. Post-Loading (°C)	T	34.86	±0.64	PT	0.39	0.7	0.02	0.79
	C	34.65	±1.48					
Core Temp. Post-Confinement (°C)	T	34.95	±0.72	PT	0.8	0.45	0.11	0.82
	C	34.56	±1.06					
Baseline Temp. Discrepancy (°C)	T	0.24	±0.8	PT	-1.32	0.22	0.24	0.92
	C	0.51	±1.1					
Temp Discrepancy Post-Loading (°C)	T	-0.04	±0.58	PT	-1	0.34	0.15	0.87
	C	0.19	±0.55					
Temp. Discrepancy Post-Confinement (°C)	T	0.3	0.33	W	30.5	0.76	-0.1	NA
	C	0.1	0.46					
Baseline Cortisol (µg/dL)	T	0.12	±0.1	PT	-0.84	0.42	0.28	0.71
	C	0.14	±0.05					
Post-Test Cortisol (µg/dL)	T	0.12	±0.11	PT	-0.14	0.89	0.05	0.89
	C	0.13	±0.08					
Change in Cortisol (µg/dL)	T	0.005	±0.06	PT	0.93	0.38	0.31	0.86
	C	-0.016	±0.04					

322

323 There was a significant difference in Loading Time, but no other behavioural  
 324 indicator differed between Treatment and Control (Table 3).

325

326 **Table 3.** Differences in behavioural measures between Treatment (T) and Control  
 327 (C). Paired T-tests (PT) state the mean, standard deviation (S.D.) and t-value (T)  
 328 and statistical power. Wilcoxon Signed-Rank (W) tests state the median, inter-  
 329 quartile range (IQR) and v-value (V). N = 10 for all tests

Variable	Test	Mean/Median	S.D./IRQ	Test	V/ T	P	Effect Size	Power
Loading Time (secs)	T	8.5	1.7	W	7	<b>0.04</b>	-0.66	NA
	C	8.95	7.83					
Licking & Chewing	T	8.5	8.73	W	23	0.65	-0.15	NA
	C	11	10.25					
Feet Movement	T	31.5	±34.56	PT	-0.92	0.38	0.29	0.85
	C	44.5	±44.67					
Vocalising	T	2.8	±2.8	PT	0.61	0.56	0.2	0.78
	C	2.3	±2.5					

330

331

332 There were no significant differences between Treatment and Control in subjects  
 333 tested with Control before Treatment, with the exception of Core Temperature Post-  
 334 Confinement (Table 4). Power was sufficient in all tests (Tables 2, 3 & 4).

335

336 **Table 4.** Differences in measures between horses tested under Control (C)  
 337 conditions first and Treatment (T) second (n = 5). Paired T-tests (PT) state the  
 338 mean, standard deviation (S.D.) and t-value (T) and statistical power. Wilcoxon  
 339 Signed-Rank (W) tests state the median, inter-quartile range (IQR) and v-value (V).

Variable	Test	Mean/ Median	S.D./IRQ	Test	V/ T	P	Effect Size	Power
Heart Rate (bpm)	T	100.59	±33.3	PT	-1.24	0.28	0.53	0.93
	C	85.06	±15.59					
Heart Rate Variability (ms)	T	54.97	59.62	W	7	1	0	NA
	C	52.68	29.19					
RMSSD (ms)	T	72.34	±61.31	PT	-0.77	0.48	0.36	0.84
	C	55.5	±36.31					
Baseline Core Temp. (°C)	T	35.0	0.3	W	2	0.19	-0.59	NA
	C	33.95	0.3					
Core Temp. Post- Loading (°C)	T	34.95	0.85	W	1	0.13	-0.69	NA
	C	33.75	1.6					
Core Temp. Post- Confinement (°C)	T	35.35	±0.33	PT	-2.76	<b>0.05</b>	0.81	0.99
	C	34.53	±0.35					
Baseline Temp. Discrepancy (°C)	T	0.64	±0.95	PT	0.85	0.44	0.31	0.86
	C	0.96	±1.45					
Temp Discrepancy Post-Loading (°C)	T	-0.1	0.3	W	3.5	0.58	-0.25	NA
	C	-0.1	0.3					
Temp. Discrepancy Post-Confinement (°C)	T	0.22	±0.2	PT	-0.99	0.38	0.44	0.89
	C	0.02	±0.27					
Change in Cortisol (µg/dL)	T	0.04	±0.05	PT	2.07	0.13	0.78	0.98
	C	-0.02	±0.07					
Feet Movement	T	16	2	W	12	0.31	-0.45	NA
	C	10	14					
Licking & Chewing	T	17.6	±10.78	PT	-.057	0.6	0.27	0.80
	C	17.6	±20.04					
Vocalisation	T	3	3	W	3	0.18	-0.6	NA
	C	4	4					

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## 345 **Discussion**

346 Problem behaviour is commonly seen during loading onto vehicles and anticipatory  
347 stress responses are seen in some horses in advance of transport (Schmidt et al.,  
348 2010). Supplementation with anti-anxiolytic substances may alleviate stress in this  
349 context and improve both horse welfare and handler safety due to improved  
350 behaviour (McDonnell et al., 2014). The current study aimed to determine the effects  
351 of alpha-casozepine supplementation on the behaviour and physiology of horses  
352 during loading and confinement in a horse lorry. Results indicate limited effects on  
353 behaviour at minimum dosage.

354 Supplementation with Zylkéne Equine had no significant effects on the physiological  
355 indicators examined. There was no difference in heart rate, heart rate variability,  
356 RMSSD, core temperature, discrepancy in eye temperature or salivary cortisol  
357 between Treatment and Control. Power analysis for all tests indicate that the sample  
358 size was adequate to detect an effect and therefore these results cannot be  
359 explained by limited sample size. The consistent lack of significant difference across  
360 all variables indicates that, at minimum recommended dosages, Zylkéne Equine was  
361 not effective in reducing anxiety or arousal in the current experiment. It is possible  
362 that the subjects in this experiment were not sufficiently aroused by the tests to  
363 differentiate between treatment and control as they had no known aversion to  
364 loading. On the contrary, if the substance has limited effect, efficacy may be further  
365 reduced in horses with a very pronounced anxiety response. Therefore, further  
366 testing on horses with known anxiety response to loading is required.

367 Interestingly, supplementation with Zylkéne Equine did have a significant and  
368 positive effect on time to load. Horses treated with alpha-casozepine loaded  
369 significantly faster into the lorry than when under control conditions. This result

370 cannot be explained by the handler biasing the loading procedure as this individual  
371 was blind to the randomised treatment order. Whilst this is a positive indicator that  
372 many horse owners would value, the actual difference in median time was only 0.45  
373 of a second. This is arguably not a meaningful difference that handlers would value.  
374 However, difference in loading time had a statistically strong effect. Therefore, a  
375 more pronounced differentiation between the two treatments in horses that have  
376 known reluctance to enter a transport vehicle may be possible. However, since the  
377 supplement had no significant effect on physiology, this cannot be assumed. Without  
378 altering the horse's affective state of arousal or stress, it is not clear how behaviour  
379 would be meaningfully altered. In addition, McDonnell et al., (2014) noted little to no  
380 effect of this supplement on loading time in their study. Within the current sample of  
381 10 horses, it is possible that uncontrollable variations in mood or the environment  
382 account for this difference. No behavioural variable other than time to load was  
383 affected by supplementation. Instances of licking and chewing, vocalising, and  
384 movement within the lorry were not significantly different between treatment and  
385 control. Previous studies noted modest differences in behavioural indicators of stress  
386 and compliance (McDonnell et al., 2014, 2013). However, these studies did not  
387 utilise within individual differences and had small sample sizes, leaving them  
388 vulnerable to the effects of individual differences.

389 The current study used a paired design which limits the confounding effects of  
390 individual differences on results. One possible limitation of this approach is that  
391 subjects who are tested with the Treatment first may have confounded Control tests  
392 if a complete wash-out is not achieved. However, a sub-sample of subjects that  
393 received Control before Treatment were analysed and most tests of difference were  
394 not significant in this group. The only exception was core temperature post-

395 confinement which was significantly different in this sub-group. However,  
396 temperatures were significantly hotter in the treatment group, which does not support  
397 reduced arousal indicative of increased coping in subjects supplemented with  
398 Zylkéne Equine (Valera et al., 2012). Taken as a whole, this suggests that  
399 inadequate wash-out of the supplement does not explain the lack of effect noted in  
400 the current study.

401 The current study is not without limitations. For ethical considerations, only horses  
402 that were experienced travellers with no known aversion to loading were used and  
403 this may not reflect how the substance would act when used in anxious individuals.  
404 In particular, time taken to load may differ in subjects who find this aversive and  
405 increased arousal may differentiate between treatment and control. Further, the  
406 dosage was the minimum recommended by the manufacturer and manufacturer's  
407 guidelines are not sensitive to the body weight (Vetoquinol, 2018). Future work  
408 should test the substance at maximum recommended dosages which is  
409 approximately twice what was administered here. In addition, investigating the  
410 effects in subjects with a known aversion to loading is warranted.

411

## 412 **Conclusions**

413 In the current experiment, Zylkéne Equine had no significant effect on heart rate,  
414 heart rate variability, core temperature, discrepancy between eye temperatures or  
415 salivary cortisol. This indicates that this supplement does not affect a horse's ability  
416 to cope with loading and confinement in a horse lorry at the dosage used. These  
417 physiological indicators are supported by the behavioural indicators licking and  
418 chewing, feet movement, and vocalising when confined, which also did not differ

419 between treatments. However, horses did load significantly more quickly when  
420 supplemented with alpha-casozepine. Though it is important to note that the median  
421 difference was only 0.45 seconds and is therefore irrelevant. Further work is  
422 required to ascertain whether the maximum dosage – twice that used here – might  
423 affect coping and behaviour in horses. In addition, it is not clear whether the  
424 difference between Control and Treatment would be differentiated or attenuated by  
425 testing subjects with known anxiety responses during loading.

426

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432

### 433 **Ethical Statement**

434 Subjects took place following the informed written consent of the owner. The horses  
435 used in the sample were free from known injury or illness that would compromise  
436 welfare during testing and had transport experience. Subjects needed to have been  
437 transported at least once a month as part of their normal management routine.  
438 Subjects did not have any known phobia to travelling or the process of being  
439 transported (such as loading or unloading) that it would have been detrimental to  
440 their welfare.

441 After selection, horses were withdrawn from testing if a) the owner chose to withdraw  
442 the subject; b) C.I. deemed the horse physically or mentally unfit to continue, for

443 example, due to significantly increased HR on approaching the lorry; c) subjects took  
444 longer than 5 minutes to load. Horses were monitored constantly throughout the test  
445 via camcorder display screen by a researcher (C.I.) who remained in the lorry  
446 throughout the test. The test would be stopped immediately if a problem occurred or  
447 if the horse became overly stressed. If this situation occurred, the subject would be  
448 immediately removed and returned to their stable, though this did not occur.

449 Zylkène Equine is an extremely palatable, apple flavoured supplement which can be  
450 added to an existing diet. This ensured that there was no change to feeding or  
451 management practices. Additionally, there are no known side effects of Zylkène  
452 Equine and it is a product which is available 'over the counter' without a veterinary  
453 prescription. This supplement is safe to feed in conjunction with other therapies and  
454 in pregnant or lactating mares (Vetoquinol, 2018). There is no long term risk to the  
455 horse as this supplement is used short term, for the current study each horse  
456 required only four doses (the last day being the day of testing).

457 Although no side effects were expected to occur, horses were removed from the  
458 study if any adverse changes in behaviour were observed by the driver of the lorry,  
459 the owner or the researchers. Furthermore, the horses used in this study only took  
460 part with informed consent from their owners. The owners had the right to withdraw  
461 the horses from the trial at any point.

462 All data recorded during the experiment was solely for the purpose of the research  
463 described within the consent form and is only available to the researcher team. Any  
464 information personal to the subjects and their owners were kept discrete in  
465 compliance with The Data Protection Act 1998.

466 The authors of the current paper have no conflict of interest to declare.



467

468 **Authorship Statement**

469 The idea for this paper was conceived by Carrie Ijichi & Sophie Green; the study was  
470 designed by Carrie Ijichi and Sophie Green; the study was performed by Carrie Ijichi,  
471 Sophie Green, Keith Squibb, Aisling Carroll and Isobel Bannister; the data was  
472 analysed by Carrie Ijichi; the paper was written by Carrie Ijichi, Sophie Green and  
473 Aisling Carroll, the paper was edited by Keith Squibb and Isobel Bannister.

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