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1 **A double-blind, placebo-controlled trial investigating the**  
2 **value of Pet Remedy in ameliorating fear of handling of**  
3 **companion rabbits**

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6  
7 Short Title: Investigating the value of Pet Remedy use in companion rabbits

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## 19 **Abstract**

20 The domestic rabbit is a popular companion animal in the UK with an estimated population of 0.9  
21 million. Research has highlighted a large number of welfare issues related to the way rabbits are  
22 commonly kept, affecting a majority of the population. One major welfare issue is the large  
23 proportion of pet rabbits that are fearful when handled. Pet Remedy™ (Unex Designs) is an herbal  
24 product containing valerian, marketed as a natural calming aid for reducing stress in all companion  
25 animal species. Its efficacy for domestic rabbits is previously untested. We describe a randomized,  
26 double-blind, placebo-controlled trial into the effectiveness of Pet Remedy when sprayed on an  
27 object in the environment and the clothing of a novel person handling the rabbit.

28

29 A rehoming population of 50 rabbits each underwent a baseline test followed by both a placebo and  
30 a Pet Remedy treatment on three consecutive days (following cross-over design). On each day, a  
31 novel arena test was conducted, and the rabbits' behavioral responses to the experimenter  
32 measured when in its home enclosure and when being handled. Heart and respiratory rates were  
33 measured during handling. Repeated measures ANOVAs tested within-subjects differences between  
34 Pet Remedy and placebo trials, while taking into account rabbits' individual baselines.

35

36 Treatment with Pet Remedy was associated with a significant decrease in heart rate during handling  
37 ( $F_{(1,42)}=4.41, P=0.042$ ) and a significant increase in the number of positive behaviors observed in a  
38 novel arena test ( $F_{(1,47)}= 4.52, P=0.039$ ). Rabbits took marginally longer to be picked up at the start of  
39 a Pet Remedy trial ( $F_{(1,47)}= 4.08, P=0.049$ ). Other variables which may have been predicted to change  
40 were unaffected. Overall the amount of rearing in the novel arena increased with day  
41 ( $F_{(1,45)}=6.91, P=0.012$ ), showing an increase in exploratory behavior with habituation. Significant  
42 individual variation occurred throughout, with almost all variables being markedly affected by an

43 individual's baseline data, and heart rates were universally high suggesting that handling is generally  
44 aversive to many rabbits.

45

46 The results of this study suggest that Pet Remedy may have some potential value for rabbits during  
47 periods of acute stress, slowing heart rate and allowing the performance of more positive, relaxed  
48 behaviors. It may thus be useful during veterinary visits and during initial handling. However, given  
49 the high levels of physiological and behavioral stress exhibited, effects were small and hence we  
50 suggest optimal handling and appropriate habituation, desensitisation and counter-conditioning  
51 protocols should also be simultaneously implemented. The efficacy of pet remedy for long-term use  
52 remains untested.

53

## 54 **Introduction**

55 The domestic rabbit (*Oryctolagus cuniculus*) is a popular choice of companion animal, with an  
56 estimated population of 0.9 (PFMA, 2018) to 1 million (PDSA, 2018 ), making it the third most  
57 commonly kept mammalian species in the United Kingdom (PFMA, 2018) . However, in their annual  
58 audit of welfare of companion animals in the UK in 2011, the People's Dispensary for Sick Animals  
59 (PDSA) identified the rabbit as one of the most neglected species (PDSA, 2011): Subsequent studies  
60 have confirmed a range of potential welfare issues (e.g. Rooney et al., 2013a) and a recent large-  
61 scale survey commissioned by the Royal Society for the Prevention of Cruelty to Animals (RSPCA)  
62 quantified (Rooney et al., 2014) and prioritised (Rooney et al., 2013a,) these, identifying fear of  
63 handling to be amongst the most important.

64

65 A study by Mullan and Main (2007) found that 20% of owners were not confident handling their  
66 rabbit(s). A larger survey found even higher levels of owner uncertainty (Rooney et al., 2014), with

67 27% of owners unwilling to classify themselves as “very confident”. This lack of confidence is likely  
68 to lead to poor, possibly unsafe handling practices that can exacerbate the rabbit’s fear response  
69 when handled. In fact, when observed in their own home, 61% of 221 rabbits were seen to show  
70 signs of fear when handled by their owners (Rooney et al., 2014). Common fear behaviors include  
71 crouching or hunching, freezing with the ears held back, running away when approached and  
72 aggressive behavior towards handlers (Magnus, 2005). Thumping of the hind limbs and growling or  
73 grunting are also signs that a rabbit is fearful (McBride, 2014). However, owners’ apparently often  
74 lack the ability to recognise that these behaviors can be symptomatic of fear; when asked in a  
75 questionnaire, 45.7% of owners reported their rabbit to be calm when handled, however an  
76 objective observer reported that only 33.8% were calm when handled by their owner (Rooney et al.,  
77 2014).

78

79 Despite the difficulty many owners have, handling is often necessary to facilitate veterinary visits, to  
80 perform routine health checks and to move rabbits between enclosures. Overall, 85.6% of rabbits  
81 are handled at least weekly and only a small number (2.2%) are never handled (Rooney et al 2014).  
82 In addition, traditionally, and even today, rabbits are often purchased as children’s pets, with  
83 surveys reporting 25% (PDSA, 2016), 39% (Edgar and Mullan, 2011), and even 45% (Mullan and  
84 Main, 2006) of rabbits being bought for a child. Young children are likely to handle rabbits  
85 frequently and sometimes inappropriately, hence it is unsurprising that 49% of rabbits are unable to  
86 be handled easily by children (Mullan and Main, 2007).

87

88 Fear is an aversive emotion, and when prolonged, repeated or intense, it can also have several long-  
89 term and potentially fatal consequences for rabbits. Cardiomyopathy, lymphopenia and reduced  
90 renal blood flow can all result, as well as gut stasis, a common condition for which stress is a known

91 cause (Varga, 2014). Hence fear of handling is a key area of welfare concern for which research is  
92 required to find methods of management and amelioration.

93

## 94 **Use of Products to Reduce Fear in Companion Animals**

95 Non-pharmacological products that are marketed for reducing fear and anxiety are becoming  
96 increasingly popular in cats and dogs, when used alongside improved handling and behavioral  
97 training (Cracknell and Mills, 2008). Synthetic pheromone treatments and herbal remedies are  
98 commonly used 'alternatives' to pharmacological products. Efficacy data are usually lacking, so it is  
99 essential that the efficacy of such alternative treatments is tested. If they are ineffective they are at  
100 best a waste of client money and trust, but could also exacerbate problems and delay the use of  
101 more effective treatments.

102

103 Nutraceuticals (products derived from food sources and believed to have health or medical benefits)  
104 are growing in popularity and trials suggest that some may have potential value when used on  
105 ponies (McDonnell et al., 2013), horses (McDonnell, 2014), dogs (Beata et al., 2007a) and cats (Beata  
106 et al., 2007b). Interest has also grown in the use of natural herbs for reducing stress and anxiety in  
107 companion animals, including rabbits.

108

109 Valerian is an example of an herb which has the potential to improve animal welfare. It is derived  
110 from the root of the *Valeriana officinalis* plant (Hatteshol et al., 2008) and it has been shown to have  
111 anxiolytic effects on rodents and humans (Murphy et al., 2010, Becker et al., 2010). The suggested  
112 mode of action involves valerenic acid, the predominant anxiolytic root extract, interacting with  
113 gamma amino butyric acid (GABA) receptors (specifically GABA<sub>A</sub> receptors) in the central nervous

114 system (Murphy et al., 2010). This interaction causes neural inhibition by increasing the uptake of  
115 chloride ions, thus magnifying the hyperpolarisation effect of GABA on the neurons. Further neural  
116 inhibition could also be a result of other valerian root extracts inhibiting GABA transaminase activity,  
117 preventing the breakdown of GABA (Murphy et al., 2010).

118

119 Valerian has been shown to have relaxant properties for humans and is sometimes used in  
120 treatment of insomnia (Donath et al., 2000). Apparent reduced behavioral responsiveness could  
121 potentially be due to either an anxiolytic or a sedative effect. The mode of action is critical as the use  
122 of agents which induce sedation, with no true anxiolytic properties, is contra-indicated in animals  
123 showing signs of fear or anxiety. Other than preventing the animal from injuring itself in cases of  
124 extreme behavioral response, sedation alone is of limited benefit and may even make the problem  
125 worse; if it renders the animal unable to escape or utilise its natural coping mechanisms, thus  
126 potentially increasing the risk of sensitisation to the stressor. The animal will likely then be more  
127 (not less) fearful of the stimuli when it is next encountered. It is therefore vital to determine  
128 whether a product calms and hence facilitates reduced fear to stimuli or simply sedates and thus  
129 reduces avoidance responses.

130

131 Studies examining the effects of Valerian on vigilance in healthy human volunteers, suggest it does  
132 not result in sedation (Kuhlmann et al., 1999; Hallam et al., 2003; Gutierrez et al., 2004;). Studies  
133 using animal models similarly found Valerian extracts induced pronounced anxiolytic effects in rats  
134 with no associated reduction in locomotor activity, nor prolongation of ether-induced anaesthesia  
135 (Hattesoehl et al., 2008). Valerenic acid has also produced anxiolytic effects on mice with no effects  
136 on motor behavior or coordination (Murphy et al., 2010) and pigs exposed to a Valerian-containing  
137 herbal product during transportation had lower heart rates compared to controls (Peeters et al.,  
138 2004). There are currently several valerian-containing products, marketed as stress reduction aids,

139 primarily for cats and dogs, and available in varying forms including sprays, dermal spot-ons and  
140 products for oral administration.

141

142 Pet Remedy® is a Valerian-based herbal product developed in the United Kingdom and  
143 manufactured and distributed through Unex Designs Ltd.. It is marketed as a safe, natural remedy  
144 for the treatment of stress and anxiety in all companion animals, including small mammals. The  
145 product range comprises a plug-in diffuser, battery-operated atomiser and a calming spray (Pet  
146 Remedy, 2017a). The spray is likely the most practical for rabbits, as many are kept outdoors. It is a  
147 pH-neutral, water-based formulation containing a blend of essential oils. Valerian oil is the principle  
148 component with smaller quantities of vetiver, basil and sage. Further constituents include  
149 polysorbate 20, a surfactant to facilitate mixing of the essential oils and the water base, and sodium  
150 benzoate and potassium sorbate as preservatives. Manufacturer guidelines suggest optimal results  
151 when applied to animal bedding, handler clothing or directly on to the animal's coat (Pet Remedy,  
152 2017a). Positive anecdotal reports abound, but to date, there have been a limited number of mainly  
153 unpublished trials into the efficacy of Pet Remedy for cats and dogs (Pet Remedy,2017b; Barrington,  
154 2014; Taylor and Madden, 2016).

155

156 These preliminary studies have produced contradictory and to our knowledge no such studies  
157 currently exist for rabbits. Fear of handling, as well as of novel environments and open spaces have  
158 been identified as key areas of welfare concern for this species (Rooney et al.,2014). Consequently,  
159 should Pet Remedy be effective at reducing fear in rabbits, it could have potential to significantly  
160 improve welfare. Therefore, this study seeks to test the efficacy of Pet Remedy on rabbits, whilst  
161 overcoming the limitations of previous studies on other species. Small group sizes (Barrington, 2014)  
162 and lack of observer blinding are two potential issues as, even in trials with placebo treatments, it is  
163 likely that experimenters could identify the active substance in Pet Remedy from its strong odour  
164 (Barrington, 2014; Taylor and Madden 2016). In the current study, we used a rehoming population



165 of rabbits to provide large group sizes, and a scented mask and nose clip were worn by the  
166 experimenter to mask the smell of the Pet Remedy and hence improve observer blinding. Since the  
167 subjects were of diverse and unknown histories, we expected wide ranging initial responses. The  
168 experimental design is within-subjects, with all rabbits undergoing both treatments (Pet Remedy and  
169 placebo) on successive days and responses compared to their own baseline levels.

170

171 The study tests the response of fifty rabbits when encountering an experimenter and a novel  
172 environment scented with Pet Remedy or a placebo substance of distilled water. The rabbits'  
173 responses towards a novel handler during an initial approach, when being picked up, handled and  
174 when placed in a novel arena were recorded. We measure behavioral responses but also take  
175 physiological measurements, i.e., heart and respiratory rate during handling. The test procedure  
176 utilised was adapted from that piloted by Rooney et al. (2014). We use a novel arena (Prut and  
177 Blezung, 2003) to allow measurement of its general confidence and its response to standardised  
178 behavior from a person to be measured.

179

180 We predict that if Pet Remedy has the effects claimed, individual rabbits will show calmer responses  
181 to handling, more positive, relaxed behaviors and fewer negative (fear and anxiety) behaviors after  
182 Pet Remedy as compared to placebo treatments. We hypothesise that significant decreases in heart  
183 and respiratory rates will be seen following Pet Remedy administration when compared to baseline  
184 and placebo levels. We also test whether Pet Remedy exerts a sedative effect on rabbits as  
185 locomotory behavior would be expected to decline if the action is sedative.

## 186 **Methods and Materials**

187

188 *Ethical Approval*

189 This study was approved by the Animal Welfare and Ethical Review Board (AWERB) of the University  
190 of Bristol; Veterinary Investigation Number: VIN/16/001.

191 *Subjects*

192 Fifty rabbits were sourced from two locations; 14 rabbits from a privately-owned rehomed group in  
193 Taunton, and 36 from a rehoming centre in Gloucestershire. All testing was carried out in January  
194 and February 2016. Rabbits were minimally handled outside of the test procedures and so sex was  
195 recorded as reported by previous owners; 32 female and 18 male. All except three were neutered.  
196 The rabbits were of varying breed and were classified according to what they most closely  
197 resembled. The most common were Lionhead and their Crosses (n=9), Mini/Dwarf Lops and their  
198 crosses (n=8) and Cross Breeds (n=8). Rabbits were housed in various hutch and enclosure types; 18  
199 were kept in wooden hutches with no attached runs and 32 were housed in either large wooden  
200 sheds with access to an outdoor enclosure or indoor runs. Most rabbits were housed individually  
201 (n=22) or in pairs (n=23), three were housed in a group and the remaining two housed within a  
202 group of four. All rabbits remained in the care of their owner or rehoming centre after completion of  
203 the study.

204

205 Any rabbits with a pre-existing illness and those that were known to be aggressive towards handlers  
206 were excluded. All subjects had been at the location and in the same grouping for a minimum of one  
207 week. Where rabbits were kept in pairs, only one was used per cohort group. The second was tested  
208 at least a week later to avoid carry-over effects from approaching and picking up first rabbit. Where  
209 rabbits were housed in groups of three or more, two were used per cohort but both were subjected  
210 to the same treatment on the same day and there was a gap of at least one hour between rabbits.

211 To avoid unconscious bias (e.g., where calmer animals were selected first), the rabbit from each pair  
212 or group with the darkest fur or markings was used first.

213

#### 214 *Experimental design*

215 The design of this study was within- subjects so each rabbit was compared to itself because the  
216 sample was a mix of sexes and breeds. Each subject rabbit was tested three times by the same  
217 experimenter (SU) over three consecutive days. Baseline measures were taken on day 1 with no  
218 product being applied and on day 2 half the rabbits were randomly assigned to be exposed to Pet  
219 Remedy and half to placebo, by the experimenter who was blinded to the treatment. On day 3, the  
220 other substance was applied. The total population (n=50) was divided into six cohorts, each  
221 containing between six and ten rabbits which were tested over the same three-day period. For each  
222 cohort, one Pet Remedy (Unex Designs Ltd Manufactured 12/12/2015) and one placebo bottle were  
223 used. Twelve spray bottles were labelled one to twelve, (six Pet Remedy and six placebo containing  
224 distilled water) by an assistant, and randomly allocated as the first or second treatments to ensure  
225 the experimenter remained blind.

226

227 Each rabbit cohort was divided into two groups. Half (Group A) were tested in the morning and half  
228 (Group B) in the afternoon (Table 1). To avoid cross-contamination of the arena with the product, all  
229 rabbits tested in the morning received the same treatment, whilst all rabbits tested in the afternoon  
230 received the other. When the morning group had been completed, the arena was cleaned (using  
231 Beaphar® Deep Clean) and testing was stopped for 45 minutes to allow the arena to air out and  
232 eliminate residual smell for the later tests. At this point, the experimenter changed laboratory coat  
233 and the towel used to cover the carrier in which rabbits were moved.

234

235 *Table 1: Order of treatments received after Day 1 baseline by each cohort of rabbits*

236	Cohort	Group	Day Two	Day Three Product
237			Product	
238	<b>1</b>	a) n=4	Placebo	Pet Remedy
		b) n=4	Pet Remedy	Placebo
		Total n=8		
	<b>2</b>	a) n=4	Placebo	Pet Remedy
		b) n=2	Pet Remedy	Placebo
		Total n=6		
	<b>3</b>	a) n=4	Pet Remedy	Placebo
		b) n=4	Placebo	Pet Remedy
		Total n=8		
	<b>4</b>	a) n=5	Placebo	Pet Remedy
		b) n=5	Pet Remedy	Placebo
		Total n=10		
	<b>5</b>	a) n=5	Pet Remedy	Placebo
		b) n=4	Placebo	Pet Remedy
		Total n=9		
	<b>6</b>	a) n=5	Pet Remedy	Placebo
		b) n=4	Placebo	Pet Remedy
		Total n=9		

239 *Experimental procedure*

240 In each rehoming centre the testing arena was assembled in a quiet, contained area out of olfactory  
 241 contact with any of the subject animals. The wooden, collapsible arena, measuring 2m x 2m with  
 242 1m high walls was erected. Its floor was divided into nine equal squares for measurement of  
 243 locomotory activity. A video camera was set up on a tripod on one side with a full view of the floor

244 and all tests filmed (Sony® Handycam DCR-SR58) in case behaviors were missed and for inter-  
245 observer testing.

246

247 The experimenter obtained basic demographics from the center owner, but did not make contact  
248 with any animals prior to the start of testing. For testing the experimenter wore a white long-  
249 sleeved, knee-length laboratory coat, white face mask scented with lemon and a nose clip to prevent  
250 distinction of the scent of Pet Remedy.

251

252 Prior to the start of testing on days 1 and 2, the appropriate treatment spray was applied to the  
253 experimenter's coat and a towel which was initially placed in a wire carrier used to transport the  
254 rabbit and then placed into the novel arena with the rabbit. One spray (approximately 0.2 ml) was  
255 applied to each of the experimenter's cuffs, one to the body of the coat and three sprays to the  
256 towel (1.2ml in total).

257

## 258 ***Testing Protocol***

259 Rabbits were tested in the same order and at approximately the same time of day on all three days.  
260 If any rabbit showed extreme negative behaviors at any point during a test (e.g., open-mouth  
261 breathing), the test would be stopped immediately, and the rabbit returned to its home enclosure.  
262 This never occurred.

263 The testing protocol had seven sub elements:

264 **1. Hutch approach:** The experimenter placed a small animal wire carrier close to the hutch or  
265 enclosure but out of sight of the rabbit. She approached and placed her hand against the hutch bars  
266 or opened the enclosure door (if there were no bars/wire) and held her hand out, at least one body

267 length from the rabbit. She remained for thirty seconds and the rabbit's initial and maximum (most  
268 positive) responses were recorded as well as their position for the majority of the time period (Table  
269 2).

270 **2. Pick up:** The experimenter opened the hutch door or entered the enclosure and started the  
271 stopwatch. She allowed the rabbit to approach before moving to capture it calmly. The rabbit was  
272 carried close to the ground and placed in the wire carrier, lined with the towel. The time taken to  
273 pick up and the rabbit's behavioral responses (positive and negative) were recorded.

274 Once the rabbit was placed in the wire carrier, a large towel was placed over it and it was moved to  
275 the novel arena. To standardise exposure to the product, all rabbits remained in the carrier between  
276 one and two minutes.

277 **3. Novel Arena** The video camera was activated; the towel was removed from the wire carrier and  
278 the carrier lifted into the arena. The rabbit was lifted out of the carrier and placed in the central  
279 square of the arena, the carrier removed and the towel placed close to the centre of the arena.  
280 Latency to move, number of squares passed into and interactions with the towel, rearing frequency  
281 and the number and types of positive (exploring, grooming, rearing, lying stretching out, sniffing)  
282 and negative behaviors exhibited (freeze, scratch corners, escape attempts) were then recorded  
283 over a two minute period.

284 **4. Experimenter hand and carrot:** The experimenter placed her hand, holding a piece of carrot in  
285 the arena corner, at least one square away from the rabbit. The closest the rabbit moved towards  
286 the carrot within a 30 second period was recorded.

287 **5. Experimenter in novel arena:** The experimenter quietly entered the arena and sat for 30 seconds,  
288 cross-legged. The rabbit's response, the frequency of positive and negative behaviors was recorded.

289 **6. Experimenter handling:** The experimenter placed the towel on her crossed legs before moving to  
290 picked up the rabbit and placed it on the towel. She then spent one minute gently stroking the rabbit

291 from head to tail and assessing Body Condition Score (BCS; Mullan and Main, 2006). During the  
292 second minute, respiratory rate and heart rate were measured, subjective ratings were recorded for  
293 overall level of reactivity during handling and level of restraint required to maintain hold of the  
294 rabbit. Number of escape attempts were also recorded.

295 **7. Physiological measures:** The experimenter observed the rise and fall of the chest to count  
296 respiratory rate, and placed a stethoscope to determine heart rate, counted over 15 seconds and  
297 converted to breaths/beats per minute.

298

299 After completion of the test, recording was stopped, and the rabbit returned to the wire carrier and  
300 moved back to their home enclosure. The walls and floor of the novel arena and the wire carrier  
301 were sprayed with low-odour disinfectant (Beaphar® Deep Clean) and wiped down. The  
302 experimenter disinfected her hands with hand gel of minimal scent (Cuticura® Original) before  
303 testing the next rabbit.

304

### 305 *Statistical Analysis*

306 Statistical analysis was performed using IBM® SPSS® Statistics 23 for Windows. Initially 113 variables  
307 were recorded during testing and descriptive analysis and histograms determined the spread for  
308 each variable. Variables describing very rare behaviors were eliminated, as were those showing very  
309 little variation. Where appropriate, frequencies were scaled to improve spread, and binary  
310 responses for specific subtests were grouped over the entire test, thereby producing 20 meaningful  
311 variables for subsequent analysis. Within each subtest, we compared the variables using a Spearman  
312 Rank Correlation test. For any correlations above 0.7 (i.e., over 50% of variation being explained),  
313 the more subjective of the measures was eliminated. Thus, from the Experimenter Handling sub-test

314 we eliminated 'Restraint required during handling' as this was highly correlated to 'Number of  
315 escape attempts' (0.773) leaving 19 variables (Table 2).

316

317 A second trained observer blindly analysed 18 of the testing sessions. Her measures for seven  
318 variables were chosen including at least one from each subtest, the variable showing the most  
319 variability between rabbits. Observers were compared for inter-observer reliability using Kendall's  
320 Index of Concordance and Chi squared tests for continuous and binary variables respectively.

321



323 *Table 2: Description of the 20 variables derived from data recorded during seven test components*

Test component	Variable	Description	Scale
1. Hutch Approach	Positivity of Response (At 0 secs)	Rabbit's initial response to experimenter's hand at front of hutch or in doorway of enclosure	<b>0</b> - Freeze/Out of sight <b>1</b> - No Response, Withdraw, Out of sight <b>2</b> - Turn head, no other movement, Come out of hiding <b>3</b> - Approach, Sniff hand <b>4</b> - Contact hand
	Maximum Positivity of Response	Rabbit's maximum positive response over 30 seconds to experimenter's hand at front of hutch or in doorway of enclosure	<b>0</b> - Freeze, Out of sight <b>1</b> - No Response, Withdraw, Out of sight <b>2</b> - Turn head, no other movement, Come out of hiding <b>3</b> - Approach, Sniff hand <b>4</b> - Contact hand
2. Pick up	Latency to Pick Up	Time taken (seconds) to capture rabbit in hutch or enclosure	<i>Grouped for analysis:</i> <b>1</b> - <10 secs <b>2</b> - <30 secs <b>3</b> - <60 secs <b>4</b> - <120 secs <b>5</b> - 120+ secs

	Negative Responses to Being Picked up	Number of negative responses observed during capture of rabbit	<b>Negative responses:</b> Run Away Thump Vocalise Freeze Struggle Slightly Struggle Intensely Bite/Scratch/Kick
3. Novel Arena	Latency to Move	Time taken (seconds) to move after being placed into novel arena	<i>Grouped for analysis:</i> <b>1</b> - 0 secs <b>2</b> - 1-5 secs <b>3</b> - 6-10 secs <b>4</b> - 11+ secs
	Number of Squares Entered	Number of squares moved into with all feet during two-minute test period	
	Number of Interactions with Towel	Number of interactions with the towel during two-minute test period	
	Number of Rears	Number of rears observed during two-minute test period	<i>Grouped for analysis:</i> <b>1</b> - 0 rears <b>2</b> - 1-5 rears <b>3</b> - 6-10 rears <b>4</b> - 11+ rears
	Number of Different Negative Behaviors	Number of types of negative behaviors observed during two-minute novel arena test	<b>Negative behaviors:</b> Freeze Scratch Corners Frantic to Get Out

	Number of Different Positive Behaviors	Number of types of positive behaviors observed during two-minute novel arena test	<b>Positive behaviors:</b> Explore Groom Rear Up Lie Stretched Sit and Sniff
	Positivity of Response	Most common behavior observed during the two-minute novel arena test	<b>0</b> - Frantic to get out <b>1</b> - Freeze/Scratch Corners <b>2</b> - Sit and Sniff <b>3</b> - Explore/Rear Up <b>4</b> - Groom <b>5</b> - Lie Stretched
4.	Experimenter hand and carrot	Closeness to Experimenter Hand	A 3-point scale of minimum distance between experimenter's hand and the rabbit over 30 seconds
			<b>1</b> - Furthest corner away, Two squares away <b>2</b> - One square away, Same square <b>3</b> - Contact, Eats Carrot
5.	Experimenter in novel arena	Number of Different Negative Behaviors Observed	Number of different negative responses observed after experimenter sat in novel arena with rabbit. Each behavior was counted only once
			<b>Negative Behaviors:</b> Freeze Attempt Escape Thump Vocalise
	Number of Different Positive Behaviors Observed	Number of positive behaviors observed during 30 second period in which	<b>Positive Behaviors:</b> Approach Sniff or Contact

		experimenter sat in arena with rabbit. Each behavior was counted only once	Sit but alert Carry on as before Climb on lap
6. Experimenter handling	Positivity of Response	Scale of positivity of behavioral responses to experimenter handling of rabbit.	<b>0</b> - Can't be handled <b>1</b> - Bites/Scratches/Kicks, Struggles Intensely <b>2</b> - Freezes, Struggles Slightly <b>3</b> - Tense, no struggling <b>4</b> - Calm
	Number of Escape Attempts	Number of attempts to escape during two-minute experimenter handling	
	Reactivity during Handling	Subjective scale of how reactive experimenter perceived rabbit to be during two-minute handling period	<i>4-point scale where:</i> <b>1</b> - Very calm <b>4</b> - Not calm at all
	Restraint Required During Handling	Scale of the level of restraint required by experimenter to maintain hold on rabbit during two-minute handling test	<b>1</b> - Sits unheld <b>2</b> - Held loosely <b>3</b> - Held firmly <b>4</b> - Held tightly
7. Physiological Measures	Heart Rate	Heart rate of rabbit at end of all tests	Measured over 15 second and calculated per minute 60 seconds

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Respiratory Rate	Respiratory rate of rabbit at end of all tests	Measured over 15 second and calculated per minute 60 seconds
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324

325 Data Reduction using Principal Components Analysis was trialled on the data but failed to reduce the  
326 variables to a smaller number of meaningful factors. Therefore, the raw variables were used in  
327 subsequent analysis and results interpreted with caution due to the dangers of multiple testing.

328

329 We examined the effect of treatment (Pet Remedy or Placebo), the order in which treatments were  
330 administered and the individual rabbit's baseline response (included as a covariate), and the  
331 interaction between order and treatment on each variable (Table 3).

## 332 **Results**

333 Of the seven variables tested for inter-observer reliability, all showed significant agreement between  
334 the two observers ( $p < 0.005$ ).

335

336

337 Table 3: Results of Repeated Measures General Linear Model analysis of all 19 variables, including F-  
 338 and P- values for each. P<0.05 (\*) was accepted as a significant result.

Variable		ANOVA Test Results								
		Treatment		Baseline		Study Day (Order)		Treatment x Order Interaction		
Name		F	P	F	P	F	P	F	P	
1.	Hutch Approach	Positivity of Response (at 0 seconds)	0.98	0.327	2.85	0.007*	1.05	0.311	0.00	0.984
		Maximum Positivity of Response	0.38	0.539	2.33	0.024*	1.00	0.324	0.14	0.706
2.	Pick up	Latency to Pick up	4.08	0.049	4.43	<0.001*	0.01	0.906	0.69	0.410
		Negative Responses to being picked up	0.14	0.706	4.38	<0.001*	0.67	0.417	0.38	0.539
3.	Novel Arena	Latency to Move	0.08	0.782	2.88	0.006*	0.00	0.961	2.39	0.129
		Number of Squares Entered	0.02	0.902	5.54	<0.001*	0.34	0.560	3.63	0.063
		Number of Interactions with Towel	0.10	0.748	3.40	0.001*	0.03	0.859	2.60	0.114

	Number of Rears	2.67	0.109	2.68	0.001*	6.91	0.012	0.30	0.589
							*		
	Number of Different Negative Behaviors	0.00	0.983	2.59	<0.013*	1.50	0.226	1.12	0.295
	Number of Different Positive Behaviors	4.52	0.039	2.63	0.012*	1.42	0.240	0.79	0.380
			*						
	Positivity of Response	0.12	0.729	4.49	<0.001*	0.01	0.938	2.92	0.094
4.	Closeness to Experimenter Hand with Carrot	0.44	0.511	3.60	0.001*	0.01	0.938	1.02	0.318
5.	Number of Experimenters Sits Observed	2.97	0.092	3.67	<0.001*	2.71	0.107	0.00	1.000
	Number of Positive Behaviors Observed	1.11	0.296	2.69	0.010*	1.68	0.201	1.95	0.169
6.	Positivity of Experimenters Response	2.09	0.155	2.44	0.019*	0.09	0.767	0.11	0.737

Handling	Number of escape attempts	1.72	0.196	2.73	0.009*	2.57	0.116	8.04	0.007*
	Reactivity during handling	0.05	0.825	2.44	0.019*	0.52	0.473	7.93	0.007*
7.	Heart Rate	4.41	0.04*	3.37	0.002*	0.14	0.710	0.20	0.657
Physiological Measures	Respiratory Rate	0.81	0.373	6.87	<0.001*	0.97	0.329	0.02	0.900

339

340 Three variables were significantly affected by treatment (Table 3); Latency to Pick up), Number of  
341 Different Positive Behaviors in the Novel Arena and Heart Rate Latency to Pick up decreased with  
342 both Placebo and Pet Remedy treatments, but the greatest reduction relative to baseline  
343 (M=3.80±0.95) was seen with Placebo (M=3.46±0.91) as compared to Pet Remedy Treatment  
344 (M=3.78±0.97; Figure 1A; Table 4).

345

346 The Number of Different Positive Behaviors observed in the Novel Arena was approximately the  
347 same with Placebo (M=1.98±0.92) as at Baseline (M=1.98±0.77) but significantly higher following  
348 treatment with Pet Remedy (2.388±1.07; Figure 1B).

349

350 Heart Rate was marginally increased during Placebo trials (M=265.23±29.56) as compared to  
351 baseline (M=263.67±27.77) whilst Treatment with Pet Remedy was accompanied by a decrease in  
352 heart rate (M=253.23±33.70; Figure 1C). *Table 4: Mean and Standard Deviation for all variables*  
353 *shaded are the variables significantly affected by treated*

<u>Variable Name</u>	<u>Condition</u>
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		<u>Baseline</u>		<u>Placebo</u>		<u>Pet Remedy</u>	
		<b>Mean</b>	<b>Sd</b>	<b>Mean</b>	<b>Sd</b>	<b>Mean</b>	<b>Sd</b>
1.	Positivity of	1.80	0.904	1.68	0.819	1.55	0.792
Hutch	Response (at 0						
Approach	seconds)						
	Maximum	2.22	0.932	2.04	1.009	1.94	0.944
	Positivity of						
	Response						
2.	Latency to Pick up	3.80	0.948	3.46	0.908	3.78	0.872
Pick up	Negative	1.90	1.129	1.98	1.000	1.94	1.162
	Responses to						
	being picked up						
3.	Latency to Move	1.92	0.853	1.58	0.810	1.61	0.812
Novel Arena	Number of	16.80	10.882	11.12	9.506	11.04	7.681
	Squares Entered						
	Number of	3.38	1.999	2.41	1.645	2.49	1.781
	Interactions with						
	Towel						
	Number of Rears	1.90	0.707	2.08	1.017	2.27	1.036
	Number of	1.34	0.982	1.20	1.06	1.55	1.308
	Different						
	Negative						
	Behaviors						
	Number of	1.98	0.769	1.98	0.92	2.39	1.077
	Different Positive						
	Responses						
	Positivity of	2.39	0.45	2.31	0.56	2.30	0.506
	Response						

4.	Closeness to Experimenter Hand and Carrot	2.04	0.781	1.62	0.81	1.71	0.842
5.	Number of Experimenter in Novel Arena Behaviors Observed	0.84	1.595	1.02	1.65	0.59	1.606
	Number of Different Positive Behaviors Observed	1.38	1.123	1.56	1.18	1.265	1.186
6.	Positivity of Response Experimenter Handling	2.38	0.780	2.54	0.91	2.27	0.818
	Number of Escape Attempts	1.12	1.35	0.92	0.99	1.16	1.179
	Reactivity during handling	3.82	0.77	3.74	0.78	3.73	0.758
7.	Heart Rate	263.67	27.77	265.23	29.56	253.23	33.70
Physiological Measures	Respiratory Rate	144.76	32.63	144.20	36.74	149.06	39.84

354

355 [ FIGURE 1 (A,B,C) ]

356 All variables analysed were significantly affected by an individual rabbit's baseline data, seven  
357 variables with P-values of less than 0.001 (Table 3), indicating an individual's response during the  
358 baseline test was highly predictive of how they would respond in subsequent tests.

359

360 Study Day (Day 2 or Day 3) had a significant effect on only one variable, the number of rears  
361 observed during the novel arena test ( $F_{(1,45)}=6.91, P=0.012$ ). There was a significant increase in the  
362 number of rears observed from Day 2 ( $M=2.12\pm 1.04$ ) to Day 3 ( $M=2.30\pm 1.02$ ) (Figure 2).

363

364 **[FIGURE 2]**

365

366 The interaction between order and treatment was significant for two variables; Number of Escape  
367 Attempts during Experimenter Handling ( $F_{(1,47)}=8.04, P=0.007$ ) and Reactivity during Handling  
368 ( $F_{(1,47)}=7.93, P=0.007$ ; Table 3). In those rabbits treated with Placebo first, the Number of Escape  
369 Attempts decreased from Baseline ( $M=1.12\pm 1.35$ ) to Day 2 ( $M=0.92\pm 1.00$ ). When these rabbits were  
370 then exposed to Pet Remedy on Day 3, mean number of escapes decreased further ( $M=0.71\pm 1.08$ ).  
371 In contrast in those that received Pet Remedy first, the Number of Escapes Attempts increased from  
372 Baseline to Day 2 ( $M=1.60\pm 1.12$ ), but decreased greatly in relation to both baseline and Day 2 data  
373 when treated with placebo on Day 3 ( $M=0.92\pm 1.00$ ; Figure 3A).

374

375 Both groups of rabbits were deemed more Reactive on Day 2 than Day 1, irrespective of product  
376 used. Those that received Pet Remedy on Day 2 were more reactive during both treatment tests  
377 than those who received Placebo first. A decrease in Reactivity from Day 2 to 3 was demonstrated by  
378 both groups, both groups falling below baseline data on this day (Figure 3B).

379 [FIGURE 3 A,B]

380

## 381 Discussion

382

383 This study, overall, saw few differences in behavior between trials in which placebo and Pet Remedy  
384 were administered. Of the 19 variables analysed, only three were significantly different according to  
385 treatment. Treatment with Pet Remedy was associated with a significant decrease in Heart Rate  
386 during handling and a significant increase in the Number of Different Positive Behaviors, observed in  
387 a novel environment compared to placebo. Although the Latency to Pick up the rabbit was lower in  
388 trials with placebo, since this was the first encounter with the experimenter during the test and  
389 often followed a Pet Remedy trial, this too may support a calming effect. In contrast, other variables  
390 such as Respiratory Rate, Reactivity to handling and Number of Different Negative Behaviors  
391 Observed in a Novel Arena or during Handling showed no significant differences with treatment.  
392 Two variables, Number of Escape Attempts and the subjective rating for Reactivity During Handling  
393 showed complex treatment/order interactions which require further investigation.

394

395 A previous study of oral administration of valerian, the main active component of Pet Remedy,  
396 produced a decrease in heart rate in pigs during simulated transport (Peeters et al., 2004). Despite  
397 the difference in method of administration in the current trial (olfactory rather than oral), Pet  
398 Remedy was again associated with a significant decrease in heart rate when compared with baseline  
399 and placebo in rabbits. However, heart rates were relatively high throughout this study averaging  
400 260 compared to normal resting levels, which are 154-300 (Varga, 2014). These high heart rates may  
401 partly be explained by the exercise during capture, which occurred several minutes before testing,

402 but we suggest it predominantly shows that handling, and in particular handling by a novel person, is  
403 particularly stressful to rabbits. Heart rate is reduced by Pet Remedy administration and it is possible  
404 that this decrease was associated with a concurrent reduction in stress, (the concurrent increase in  
405 positive behaviors suggests that this was not a reduction in positive arousal), but the Pet Remedy  
406 treatment alone is insufficient to reduce heart rate to the low end of the normal range. It is  
407 interesting to note that no concurrent significant change in respiratory rate was seen. As respiratory  
408 and heart rates are usually closely linked, a change would have been expected. However, respiratory  
409 rates were very high, averaging 146, compared to normal 30-60 breaths per minute (Varga, 2014),  
410 suggesting an extreme physiological response that may have been insensitive to relatively minor  
411 treatment differences.

412

413 Treatment with Pet Remedy was also associated with a significant increase in the Number of  
414 Different Positive Behaviors observed in the Novel Arena test. Positive behaviors such as exploring,  
415 grooming, rearing up, lying down, sitting and sniffing the environment are unlikely to be seen when  
416 rabbits are distressed and their increase after Pet Remedy administration supports the hypothesis  
417 that Pet Remedy has a calming effect. Other behaviors measured during handling and hutch  
418 approaches which may also be indicators of relaxation or distress were not significantly affected.  
419 However, the increase in the variety of positive behaviors, combined with no significant difference in  
420 the Number of Squares Entered, suggests that Pet Remedy did not cause sedation.

421

422 The time taken to pick up the rabbits from their home enclosure decreased from baseline with both  
423 treatments, but a greater effect was seen with the placebo than with Pet Remedy, although this is  
424 only marginally significant ( $P=0.049$ ). This may seem unexpected if the Pet Remedy is calming, one  
425 may expect the rabbit to be easier to pick up. However, one has to consider the order of the test  
426 components. Capture occurred immediately after the initial hutch approach when the rabbits had

427 had very little exposure to the odour. Hence, they were likely more affected by their previous  
428 interaction with the experimenter than the current trial. The rabbits when treated with Pet Remedy  
429 had experienced either a baseline or a placebo treatment on the previous day, (whilst placebo  
430 followed baseline or Pet Remedy) , so differences in capture time may have a carry-over from the  
431 last handling session. This points to the need to investigate the effect of Pet Remedy administered  
432 over successive handling and testing sessions to test whether it aids habituation or systematic  
433 desensitization and avoid sensitization in the animals.

434

435 The Number of Escape Attempts and the subjective rating of Reactivity during Handling were both  
436 significantly affected by the order in which the two treatments were given over the three days.  
437 Rabbits that were treated with Pet Remedy on Day 2 were rated as more Reactive to the  
438 experimenter handling, than those who received placebo on that day. This same group were again  
439 later classified as more Reactive on Day 3 when they were treated with the placebo spray. Escape  
440 attempts increased on Day 2 in rabbits given Pet Remedy first and subsequently decreased while  
441 those getting placebo first experienced a gradual increase in escape attempts. It is possible that  
442 escape attempts only happen when the animal is not freezing with fear and hence sufficiently  
443 relaxed to explore its environment, or they may be indicative of fear of the arena. The exact cause of  
444 this order dependent, behavioral change is unclear, but it does support the conclusion that rabbits  
445 were not sedated when exposed to Pet Remedy.

446

447 Although heart rate was reduced none of the behavioral results support the possibility that rabbits  
448 exposed to Pet Remedy were sedated. There was no significant difference in locomotory behavior  
449 recorded between Pet Remedy and placebo trials, nor was there any evidence that rabbits were  
450 more wary or fearful of the experimenter on the trials following Pet Remedy administration. In fact,  
451 the number of rears observed in the novel arena increased significantly from Day 1 through to Day 3,

452 in Pet Remedy as well as placebo conditions. This is likely due to habituation to the arena; with  
453 repeated exposure, the rabbits became more familiar and reared up to survey their surroundings.  
454 This would not be expected if the rabbits were sedated. This study however was only conducted  
455 over three days and we suggest that longitudinal studies involving repetitive handling of rabbits over  
456 longer periods of time are required with an emphasis on monitoring the animals for any signs of  
457 sedation as this could result in an inability to physically retreat from aversive and threatening  
458 stimuli.

459

460 The current study had several limitations. Although, we aimed to mask the smell of the Pet Remedy  
461 spray using a nose clip and lemon oil, and inter-observer reliability checks confirmed consistent,  
462 unbiased recording, there remains the possibility that the measures were insufficient to fully blind  
463 the tester or that the rabbits responded to the smell. It would therefore be valuable in future trials  
464 to be able to produce a placebo which smells similar but lacks the active ingredients of Pet Remedy.

465

466

467 Initial data reduction efforts failed to reduce the variables to meaningful underlying factors,  
468 therefore analysis was done on 19 raw variables. This raises the issue of repeat testing and plausible  
469 Type II errors and so individual results need to be interpreted with caution. The number of  
470 significant results is few, given the number of variables tested, although more than would be  
471 expected by chance alone. The results suggest that Pet Remedy may have some positive effect at  
472 reducing acute stress in rabbits and shows no evidence of sedating the rabbits, suggesting it may be  
473 of use in veterinary practice where visits are often short but can be particularly stressful, although  
474 application in the environment prior to handling is likely required. As this trial was only carried out  
475 over three days, the effectiveness of long-term usage remains to be tested.

476

477 For all the variables tested, an individual's response in the preliminary baseline test had a very  
478 significant effect on how they reacted in later tests. Variation in individual responses to stress within  
479 a population is widely acknowledged (Koolhaas et al., 1999) and would be expected to be a  
480 significant factor especially in a rehoming population where most subjects' histories are unknown  
481 and likely vary greatly. The within-subjects design and the use of baseline data as a covariate in the  
482 analysis meant that such inter-rabbit differences were taken into consideration. However, it is  
483 possible that rabbits of initially different reactivity, respond differently to Pet Remedy which may  
484 make significant effects more difficult to detect than in a more standardised population of animals.  
485 A rehoming population, however represents an important part of the pet population at which this  
486 product is aimed and hence this is a meaningful first study.

487

488

489 Apparent throughout this study, was the fact that within a population of fifty rabbits, the vast  
490 majority showed aversion to human approaches and handling. Very few voluntarily approached a  
491 person, and most struggled and attempted to escape from handling. Fear of handling is very  
492 common in the general population of pet rabbits (Mullan and Main, 2006). Mitigation via early  
493 socialisation, appropriate introduction to handling and optimal handling techniques are vital.

494

495 Early handling of rabbits is widely recognised as an effective method of preventing fear during  
496 handling later in life (Magnus, 2005; McBride, 2014). Research on laboratory populations shows  
497 handling kittens within the first week of life significantly affects their later behavioral responses  
498 (Bilko et al., 2000; Zucca et al., 2012) and handling around the time of nursing reduces fearfulness as  
499 an adult (Bilko et al., 2000; Pongrácz and Altbäcker, 2003). The technique used to handle rabbits is



500 also important. Wild rabbits are prey species, for whom being lifted off the ground signifies likely risk  
501 of death by predation. Since domestication has changed rabbits' natural behavior very little  
502 (Lehman, 1991). Lifting is also aversive to domestic rabbits. Handling them on the ground is generally  
503 acknowledged to be less aversive.

504

505 For those rabbits with established fears, behavior modification, incorporating techniques such as  
506 systematic desensitisation (DS), and counter-conditioning (CC) is recommended as an effective  
507 method by which to reduce fearful behaviors (Magnus, 2005). These techniques involve changing  
508 the animal's perception of the fear-eliciting stimulus, using a controlled gradual exposure (DS),  
509 whilst associating it with something positive, such as food (CC) and are well established in a number  
510 of species (e.g. Levine et al., 2007). Future validation and publication of optimal handling protocols  
511 (for adults and kittens), and behavior modification techniques have the potential to improve rabbit  
512 welfare further.

513

#### 514 Conclusions

515 We used a standardized test of rabbit-human behavior and analyzed rabbits' responses to a novel  
516 standardized handling test. This testing protocol produced a variety of measures which showed wide  
517 variation and high inter-observer reliability. Individual variation was great across the population,  
518 with baseline levels exerting a very pronounced effect on all aspects of behavior and physiology in  
519 later tests. This finding suggests the test was successful in measuring individual differences in  
520 behavioral responses.

521

522 The study also highlighted the high level of fear of handling within the rabbit population. Pet  
523 Remedy used on a handler's clothing and in a novel environment was associated with a significant

524 decrease in heart rate during handling and an increase in positive behaviors observed in a novel  
525 environment but produced no noticeable change in the rabbit's behavior towards the experimenter  
526 during a single administration. Use of Pet Remedy was also associated with a longer latency to pick  
527 up the rabbit, although this was possibly due to carryover effects from the previous testing session.  
528 Although there were no differences for most of the behavior measures, the reduction in Heart Rate  
529 and increase in positive behaviours suggest that Pet Remedy may have some potential value for  
530 rabbits during periods of acute stress and may thus be useful during veterinary visits and during  
531 initial handling and is worthy of further investigation.

532

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541

### 542 **Competing interests:**

543 We declare that none of the authors have any competing interests

544

545

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