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Research

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

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ABSTRACT

Rabbits are popular companion animals. There are numerous welfare issues affecting the majority of the population, including the fact that most rabbits are fearful when handled. Pet Remedy ™ (Unex Designs) is an herbal product containing valerian, marketed as a natural calming aid. Its efficacy for domestic rabbits is previously untested. We describe a randomized, double-blind, placebo-controlled trial into the effectiveness of Pet Remedy. Fifty rabbits underwent a baseline test followed by both a placebo and a Pet Remedy treatment. We measured rabbit's behavior by a novel arena test, responses to the experimenter when in the home enclosure and when being handled, and heart and respiratory rates during handling. Repeated measures analysis of variance tested differences between treatments, taking into account rabbits' individual baselines. Exposure to Pet Remedy was associated with a significant decrease in heart rate during handling ($F_{(1,42)} = 4.41$, $P = 0.042$) and a significant increase in the number of positive behaviors observed in the novel arena ($F_{(1,47)} = 4.52$, $P = 0.039$). Other variables which may have been predicted to change were unaffected. Rearing in the novel arena increased with day ($F_{(1,45)} = 6.91$, $P = 0.012$). Significant individual variation occurred throughout, and heart rates were universally high suggesting that handling is generally an aversion to rabbits. The results suggest that Pet Remedy may have potential value for rabbits during periods of acute stress, slowing heart rate, and allowing the performance of more positive, relaxed behaviors. It may be useful during veterinary visits and initial handling. However, given the high levels of physiological and behavioral stress exhibited by rabbits, optimal handling and appropriate habituation and desensitization and counter-conditioning protocols should also be simultaneously implemented.

Introduction

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

A study by Mullan and Main (2007) found that 20% of owners were not confident handling their rabbit(s). A larger survey found even higher levels of owner uncertainty (Rooney et al., 2014), with 27% of owners unwilling to classify themselves as "very confident". This lack of confidence is likely to lead to poor, possibly unsafe handling practices that can exacerbate the rabbit's fear response when handled. In fact, when observed in their own home, 61% of 221 rabbits were seen to show signs of fear when handled by their owners (Rooney et al., 2013b). Common fear behaviors include crouching or hunching, freezing with the ears held back, running away when approached and aggressive behavior toward handlers

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(Magnus, 2005). Thumping of the hind limbs and growling or grunting are also signs that a rabbit is fearful (McBride, 2014). However, owners' apparently often lack the ability to recognize that these behaviors can be symptomatic of fear; when asked in a questionnaire, 45.7% of owners reported their rabbit to be calm when handled (Rooney et al., 2014); however, an objective observer reported that only 33.8% were calm when handled by their owner (Rooney et al., 2013b).

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

Fear is an aversive emotion, and when prolonged, repeated, or intense, it can also have several long-term and potentially fatal consequences for rabbits. Cardiomyopathy, lymphopenia, reduced renal blood flow, and gut stasis, are all common conditions known to be caused or exacerbated by stress (Varga, 2014). Hence, fear of handling is a key area of welfare concern for which research is required to find methods of management and amelioration.

Use of products to reduce fear in companion animals

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

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

Valerian is an example of an herb which has the potential to improve animal welfare. It is derived from the root of the *Valeriana officinalis* plant (Hatteshol et al., 2008) and it has been shown to have anxiolytic effects on rodents and humans (Murphy et al., 2010, Becker et al., 2014). The suggested mode of action involves valerenic acid, the predominant anxiolytic root extract, interacting with gamma amino butyric acid (GABA) receptors (specifically $GABA_A$ receptors) in the central nervous system (Murphy et al., 2010). This interaction causes neural inhibition by increasing the uptake of chloride ions, thus magnifying the hyperpolarization effect of GABA on the neurons. Further neural inhibition could also be a result of other valerian root extracts inhibiting GABA transaminase activity, preventing the breakdown of GABA (Murphy et al., 2010).

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

Studies examining the effects of Valerian on vigilance in healthy human volunteers suggest it does not result in sedation (Kuhlmann et al., 1999; Hallam et al., 2003; Gutierrez et al., 2004;). Studies using animal models similarly found Valerian extracts induced pronounced anxiolytic effects in rats with no associated reduction in locomotor activity, nor prolongation of ether-induced anesthesia (Hattesohl et al., 2008). Valerenic acid has also produced anxiolytic effects on mice with no effects on motor behavior or coordination (Murphy et al., 2010) and pigs exposed to a Valerian-containing herbal product during transportation had lower heart rates compared with controls (Peeters et al., 2004). There are currently several valerian-containing products, marketed as stress reduction aids, primarily for cats and dogs, and available in varying forms including sprays, dermal spot-ons and products for oral administration.

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

These preliminary studies have produced contradictory and to our knowledge no such studies currently exist for rabbits. Fear of handling, as well as of novel environments and open spaces have been identified as key areas of welfare concern for this species (Rooney et al.,2014). Consequently, should Pet Remedy be effective at reducing fear in rabbits, it could have potential to significantly improve welfare. Therefore, this study seeks to test the efficacy of Pet Remedy on rabbits, while overcoming the limitations of previous studies on other species. Small group sizes (Barrington, 2014) and lack of observer blinding are two potential issues as, even in trials with placebo treatments, it is likely that experimenters could identify the active substance in Pet Remedy from its strong odor (Barrington, 2014; Taylor and Madden 2016). In the present study, we used a rehoming population of rabbits to provide large group sizes, and a scented mask and nose clip were worn by the experimenter to mask the smell of the Pet Remedy and hence improve observer blinding. Because the subjects were of diverse and unknown histories, we expected wide ranging initial responses. The experimental design is within subjects, with all rabbits undergoing both treatments (Pet Remedy and placebo) on successive days and responses compared with their own baseline levels.

The study tests the response of fifty rabbits when encountering an experimenter and a novel environment scented with Pet Remedy or a placebo substance of distilled water. The rabbits' responses toward a novel handler during an initial approach, when being picked up, handled and when placed in a novel arena, were recorded. We measure behavioral responses but also take physiological measurements, that is, heart and respiratory rate during handling. The test procedure utilized was adapted from that piloted by Rooney et al., (2013b). We use a novel arena (Prut and Blezung, 2003) to allow measurement of its general confidence and its response to standardized behavior from a person to be measured.

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

Methods and materials

Ethical approval

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

Subjects

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

Any rabbits with a pre-existing illness and those that were known to be aggressive toward handlers were excluded. All subjects had been at the location and in the same grouping for a minimum of one week. Where rabbits were kept in pairs, only one was used per cohort group. The second was tested at least a week later to avoid carryover effects from approaching and picking up the first rabbit. Where rabbits were housed in groups of three or more, two were used per cohort but both were subjected to the same treatment on the same day and there was a gap of at least one hour between rabbits. To avoid unconscious bias (e.g., where calmer animals were selected first), the rabbit from each pair or group with the darkest fur or markings was used first.

Experimental design

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

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

Experimental procedure

In each rehoming center, the testing arena was assembled in a quiet, contained area out of olfactory contact with any of the subject animals. The wooden, collapsible arena, measuring 2m x 2m with 1m high walls was erected. Its floor was divided into nine equal squares for measurement of locomotory activity. A video camera was set up on a tripod on one side with a full view of the floor, and all tests filmed (Sony® Handycam DCR-SR58) in case behaviors were missed and for interobserver testing.

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

Before the start of testing on days 1 and 2, the appropriate treatment spray was applied to the experimenter's coat and a towel which was initially placed in a wire carrier used to transport the rabbit and then placed into the novel arena with the rabbit. One spray (approximately

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

Cohort	Group	Day two product	Day three product
1	a) n = 4	Placebo	Pet Remedy
	b) n = 4	Pet Remedy	Placebo
	Total n = 8		
2	a) n = 4	Placebo	Pet Remedy
	b) n = 2	Pet Remedy	Placebo
	Total n = 6		
3	a) n = 4	Pet Remedy	Placebo
	b) n = 4	Placebo	Pet Remedy
	Total n = 8		
4	a) n = 5	Placebo	Pet Remedy
	b) n = 5	Pet Remedy	Placebo
	Total n = 10		
5	a) n = 5	Pet Remedy	Placebo
	b) n = 4	Placebo	Pet Remedy
	Total n = 9		
6	a) n = 5	Pet Remedy	Placebo
	b) n = 4	Placebo	Pet Remedy
	Total n = 9		

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	0 - Freeze/out of sight 1 - No response, withdraw, out of sight 2 - Turn head, no other movement, come out of hiding 3 - Approach, sniff hand 4 - 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	0 - Freeze, out of sight 1 - No response, withdraw, out of sight 2 - Turn head, no other movement, come out of hiding 3 - Approach, sniff hand 4 - Contact hand
2. Pickup	Latency to pickup	Time taken (seconds) to capture rabbit in hutch or enclosure	*Grouped for analysis*: 1 - <10 secs 2 - <30 secs 3 - <60 secs 4 - <120 secs 5 - 120+ secs
	Negative responses to being picked up	Number of negative responses observed during capture of rabbit	*Negative responses*: Run away Thump Vocalize 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	*Grouped for analysis*: 1 - 0 secs 2 - 1-5 secs 3 - 6-10 secs 4 - 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	*Grouped for analysis*: 1 - 0 rears 2 - 1-5 rears 3 - 6-10 rears 4 - 11+ rears
	Number of different negative behaviors	Number of types of negative behaviors observed during two-minute novel arena test	*Negative behaviors*: 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	*Positive behaviors:* Explore Groom Rear up Lie stretched Sit and sniff
	Positivity of response	Most common behavior observed during the two-minute novel arena test	0 - Frantic to get out 1 - Freeze/scratch corners 2 - Sit and sniff 3 - Explore/rear up 4 - Groom 5 - 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	1 - Furthest corner away, two squares away 2 - One square away, same square 3 - 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	*Negative behaviors*: Freeze Attempt escape Thump Vocalize
	Number of different positive behaviors observed	Number of positive behaviors observed during 30 second period in which experimenter sat in arena with rabbit. Each behavior was counted only once	*Positive behaviors:* Approach Sniff or contact Sit but alert Carry on as before Climb on lap

Table 2 (*continued*)

Test component	Variable	Description	Scale
6. Experimenter handling	Positivity of response	Scale of positivity of behavioral responses to experimenter handling of rabbit.	0 - Can't be handled 1 - Bites/scratches/kicks, struggles intensely 2 - Freezes, struggles slightly 3 - Tense, no struggling 4 - 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	*4-point scale where*: 1 - Very calm 4 - 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	1 - Sits unheld 2 - Held loosely 3 - Held firmly 4 - 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
	Respiratory rate	Respiratory rate of rabbit at end of all tests	Measured over 15 second and calculated per minute 60 seconds

0.2 ml) was applied to each of the experimenter's cuffs, one to the body of the coat and three sprays to the towel (1.2 ml in total).

Testing protocol

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

The testing protocol had seven sub-tests

Hutch approach

The experimenter placed a small animal wire carrier close to the hutch or enclosure but out of sight of the rabbit. She approached and placed her hand against the hutch bars or opened the enclosure door (if there were no bars/wire) and held her hand out, at least one body length from the rabbit. She remained for thirty seconds and the rabbit's initial and maximum (most positive) responses as well as their position for most of the period were recorded (Table 2).

Pickup

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

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

Novel arena

The video camera was activated; the towel was removed from the wire carrier and the carrier lifted into the arena. The rabbit was lifted out of the carrier and placed in the central square of the arena, the carrier removed, and the towel placed close to the center of the arena. Latency to move, number of squares passed into and interactions with the towel, rearing frequency, and the number and types of positive (exploring, grooming, rearing, lying stretching out, sniffing) and negative behaviors exhibited (freeze, scratch corners, escape attempts) were then recorded over a 2-minute period.

Experimenter hand and carrot

The experimenter placed her hand, holding a piece of carrot in the arena corner, at least one square away from the rabbit. The closest the rabbit moved toward the carrot within a 30 second period was recorded.

Experimenter in novel arena

The experimenter quietly entered the arena and sat for 30 seconds, cross-legged. The rabbit's response, the frequency of positive and negative behaviors, was recorded.

Experimenter handling

The experimenter placed the towel on her crossed legs before moving to pick up the rabbit and place it on the towel. She then spent one minute gently stroking the rabbit from head to tail and assessing body condition score (Mullan and Main, 2006). During the second minute, respiratory rate and heart rate were measured, subjective ratings were recorded for overall level of reactivity during handling and level of restraint required to maintain hold of the rabbit. The number of escape attempts was also recorded.

Physiological measures

The experimenter observed the rise and fall of the chest to count respiratory rate and placed a stethoscope to determine heart rate, counted over 15 seconds and converted to breaths/beats per minute.

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

Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics 23 for Windows. Initially 113 variables were recorded during testing and descriptive analysis and histograms determined the spread for

each variable. Variables describing very rare behaviors were eliminated, as were those showing very little variation. Where appropriate, frequencies were scaled to improve spread, and binary responses for specific subtests were grouped over the entire test, thereby producing 20 meaningful variables for subsequent analysis. Within each subtest, we compared the variables using a Spearman Rank Correlation test. For any correlations above 0.7 (i.e., over 50% of variation being explained), the more subjective of the measures was eliminated. Thus, from the experimenter handling subtest, we eliminated "Restraint required during handling" as this was highly correlated to "Number of escape attempts" (0.773) leaving 19 variables (Table 2).

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

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

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

Results

Of the seven variables tested for interobserver reliability, all showed significant agreement between the two observers ($P < 0.005$).

Three variables were significantly affected by treatment (Table 3); latency to pick up, number of different positive behaviors in the novel arena, and heart rate. Latency to pick up decreased with both placebo and Pet Remedy treatments, but the greatest reduction relative to the baseline ($M = 3.80 \pm 0.95$) was seen with placebo ($M = 3.46 \pm 0.91$) as compared with Pet Remedy treatment ($M = 3.78 \pm 0.97$; Figure 1A; Table 4).

The number of different positive behaviors observed in the novel arena was approximately the same with placebo ($M = 1.98 \pm 0.92$) as at the baseline ($M = 1.98 \pm 0.77$) but significantly higher after treatment with Pet Remedy (2.388 ± 1.07; Figure 1B).

Heart rate was marginally increased during placebo trials ($M = 265.23 \pm 29.56$) as compared with the baseline ($M = 263.67 \pm 27.77$), while treatment with Pet Remedy was accompanied by a decrease in heart rate ($M = 253.23 \pm 33.70$; Figure 1C).

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

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

The interaction between order and treatment was significant for two variables: number of escape attempts during experimenter handling ($F_{(1,47)} = 8.04$, $P = 007$) and reactivity during handling ($F_{(1,47)} = 7.93$, $P = 0.007$; Table 3). In those rabbits treated with placebo first, the number of escape attempts decreased from the baseline ($M = 1.12 \pm 1.35$) to day 2 ($M = 0.92 \pm 1.00$). When these rabbits were then exposed to Pet Remedy on day 3, the mean number of escapes decreased further ($M = 0.71 \pm 1.08$). By contrast, in those that received Pet Remedy first, the number of escapes attempts increased from the baseline to day 2 ($M = 1.60 \pm 1.12$), but decreased greatly in relation to both the baseline and day 2 data when treated with placebo on day 3 ($M = 0.92 \pm 1.00$; Figure 3A).

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

Table 3
Results of Repeated Measures General Linear Model analysis of all 19 variables, including F- and P-values for each

Test component	Variable name	ANOVA test results							
		Treatment		Baseline		Study day (order)		Treatment x order interaction	
		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. Pickup	Latency to pickup	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. Experimenter hand with carrot	Closeness to experimenter hand	0.44	0.511	3.60	0.001*	0.01	0.938	1.02	0.318
5. Experimenter sits	Number of negative behaviors 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. Experimenter handling	Positivity of response	2.09	0.155	2.44	0.019*	0.09	0.767	0.11	0.737
	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. Physiological measures	Heart rate	4.41	0.04*	3.37	0.002*	0.14	0.710	0.20	0.657
	Respiratory rate	0.81	0.373	6.87	<0.001*	0.97	0.329	0.02	0.900

$P < 0.05$ (*) was accepted as a significant result.

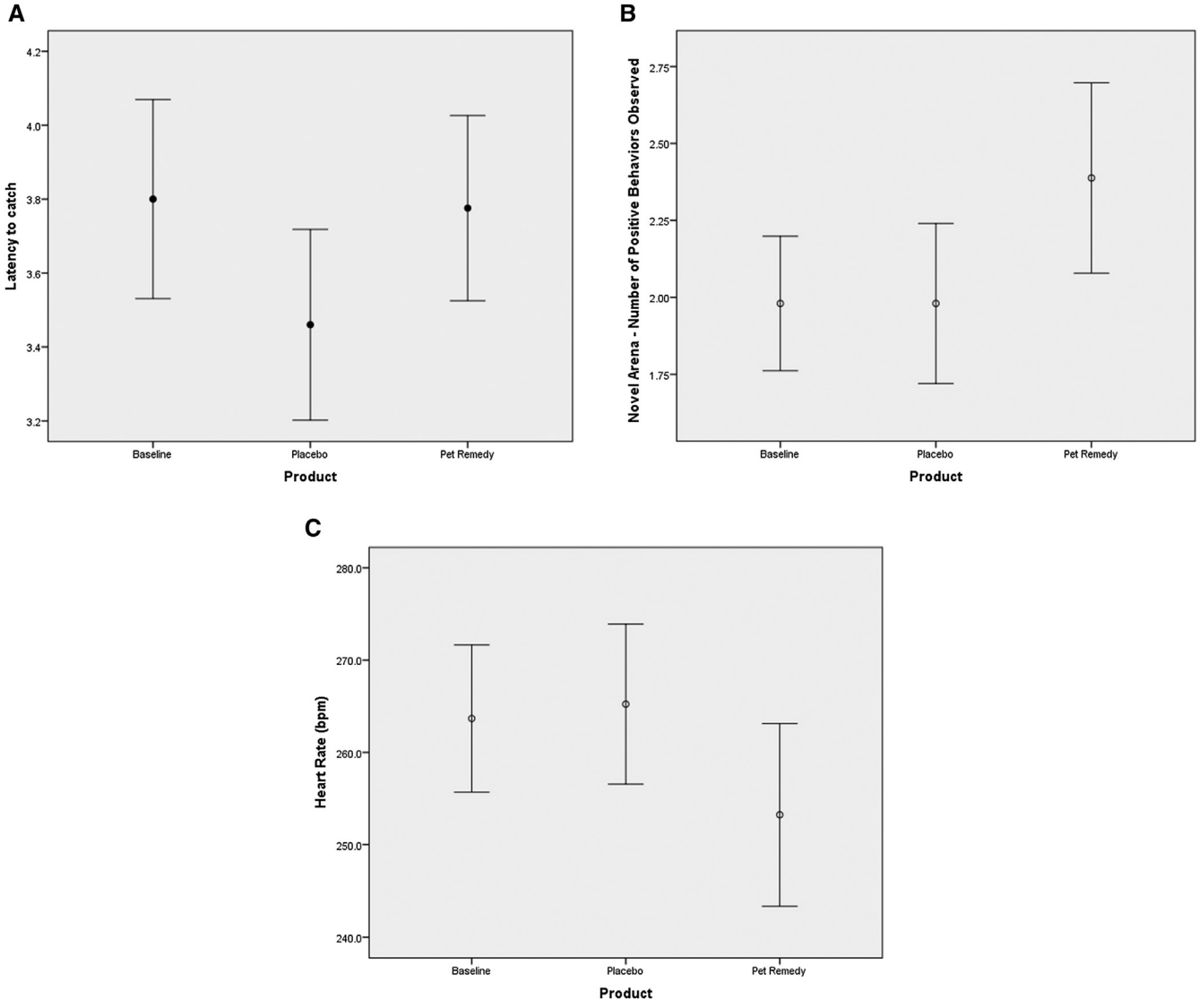

Figure 1. Graphs illustrating significant effects of treatment with Pet Remedy. (A) Graph showing the effect of treatment on latency to catch. (B) Graph showing the effect of treatment on the number of different positive responses observed in the novel arena. (C) Graph showing the effect of treatment on heart rate.

Discussion

This study, overall, saw few differences in behavior between trials in which the placebo and Pet Remedy were administered. Of the 19 variables analyzed, only three were significantly different according to treatment. Treatment with Pet Remedy was associated with a significant decrease in heart rate during handling and a significant increase in the number of different positive behaviors, observed in a novel environment compared with placebo. Although the latency to pick up the rabbit was lower in trials with placebo because this was the first encounter with the experimenter during the test and often followed a Pet Remedy trial, this too may support a calming effect. By contrast, other variables such as respiratory rate, reactivity to handling and number of different negative behaviors observed in a novel arena or during handling showed no significant differences with treatment. Two variables, number of escape attempts and the subjective rating for reactivity during handling showed complex treatment/order interactions which require further investigation.

A previous study of oral administration of Valerian, the main active component of Pet Remedy, produced a decrease in heart rate in pigs during simulated transport (Peeters et al., 2004). Despite the difference in method of administration in the current trial (olfactory rather than oral), Pet Remedy was again associated with a significant decrease in heart rate when compared with the baseline and placebo in rabbits. However, heart rates were relatively high throughout this study averaging 260 compared with normal resting levels, which are 154-300 (Varga, 2014). These high heart rates may partly be explained by the exercise during capture, which occurred several minutes before testing, but we suggest it predominantly shows that handling, and in particular handling by a novel person, is particularly stressful to rabbits. Heart rate is reduced by Pet Remedy administration, and it is possible that this decrease was associated with a concurrent reduction in stress (the concurrent increase in positive behaviors suggests that this was not a reduction in positive arousal), but the Pet Remedy treatment alone is insufficient to reduce heart rate to the low end of the normal range. It is interesting to note that no concurrent significant change in respiratory rate was seen. As respiratory and heart rates are usually closely linked, a change would have been expected. However, respiratory rates were very high, averaging 146, compared with normal 30-60 breaths per minute (Varga, 2014), suggesting an

Table 4
Mean and standard deviation (Sd) for all variables shaded are the variables significantly affected by treatment

Test Component	Variable name	Condition					
		Baseline		Placebo		Pet Remedy	
		Mean	Sd	Mean	Sd	Mean	Sd
Hutch approach	Positivity of response (at 0 seconds)	1.80	0.904	1.68	0.819	1.55	0.792
	Maximum positivity of response	2.22	0.932	2.04	1.009	1.94	0.944
Pickup	**Latency to pick up**	**3.80**	**0.948**	**3.46**	**0.908**	**3.78**	**0.872**
	Negative responses to being picked up	1.90	1.129	1.98	1.000	1.94	1.162
Novel arena	Latency to move	1.92	0.853	1.58	0.810	1.61	0.812
	Number of squares entered	16.80	10.882	11.12	9.506	11.04	7.681
	Number of interactions with towel	3.38	1.999	2.41	1.645	2.49	1.781
	Number of rears	1.90	0.707	2.08	1.017	2.27	1.036
	Number of different negative behaviors	1.34	0.982	1.20	1.06	1.55	1.308
	Number of different positive responses	1.98	0.769	1.98	0.92	2.39	1.077
	Positivity of response	2.39	0.45	2.31	0.56	2.30	0.506
Experimenter hand and carrot	Closeness to experimenter hand	2.04	0.781	1.62	0.81	1.71	0.842
Experimenter in novel arena	Number of different negative 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**
Experimenter handling	Positivity of response	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
Physiological measures	**Heart rate**	**263.67**	**27.77**	**265.23**	**29.56**	**253.23**	**33.70**
	Respiratory rate	144.76	32.63	144.20	36.74	149.06	39.84

extreme physiological response that may have been insensitive to relatively minor treatment differences.

Treatment with Pet Remedy was also associated with a significant increase in the number of different positive behaviors observed in the novel arena test. Positive behaviors such as exploring, grooming, rearing up, lying down, sitting, and sniffing the environment are unlikely to be seen when rabbits are distressed and their increase after Pet Remedy administration supports the hypothesis that Pet Remedy has a calming effect. Other behaviors measured during handling and hutch approaches which may also be indicators of relaxation or distress were not significantly affected. However, the increase in the variety of positive behaviors, combined with no significant difference in the number of squares entered, suggests that Pet Remedy did not cause sedation.

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

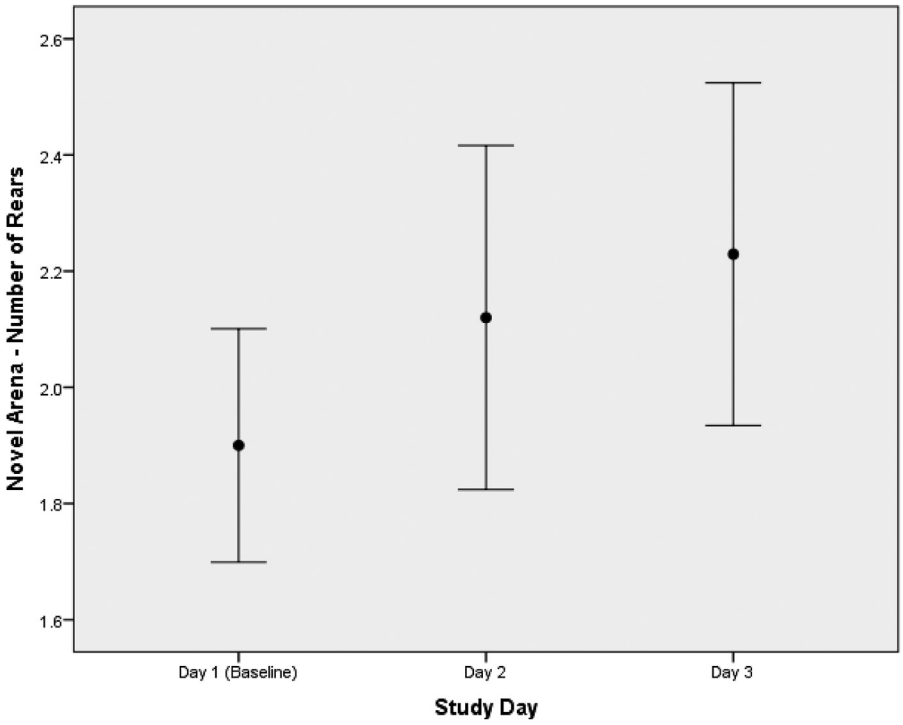

Figure 2. Line chart showing the effect of study day on the number of rears observed during the two-minute novel arena test.

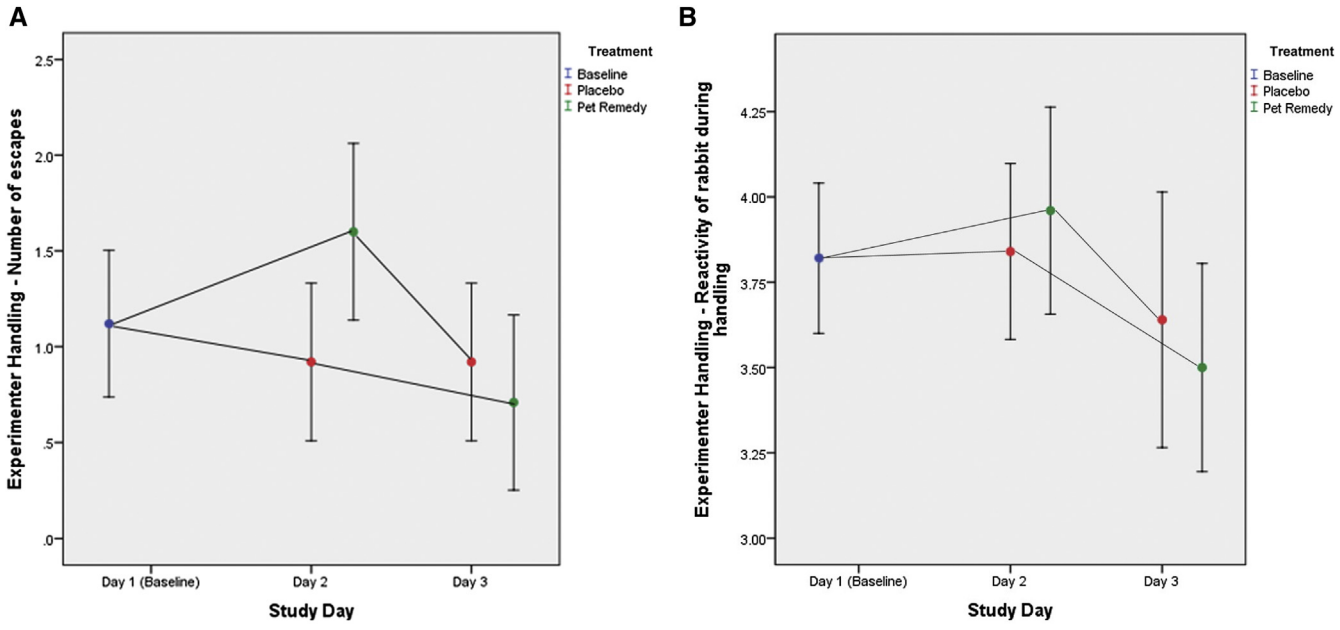

Figure 3. In both A and B, rabbits that received placebo first are represented by red point on day 2 and green on day 3. Those that received Pet Remedy first are represented by green point on day 2 and red on day 3. (A): Error bar graph to show effect of treatment and study day on the number of escape attempts during the experimenter handling test. (B): Error bar graph to show the effect of study day and treatment on level of reactivity of rabbits during handling. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

odor. Hence, they were likely more affected by their previous interaction with the experimenter than the current trial. When treated with Pet Remedy, rabbits experienced either a baseline or a placebo treatment on the previous day, so differences in capture time may have a carryover from the last handling session. This points to the need to investigate the effect of Pet Remedy administered over successive handling and testing sessions to test whether it aids habituation or systematic desensitization and avoid sensitization in the animals.

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

Although heart rate was reduced, none of the behavioral results support the possibility that rabbits exposed to Pet Remedy were sedated. There was no significant difference in locomotory behavior recorded between Pet Remedy and placebo trials, nor was there any evidence that rabbits were more wary or fearful of the experimenter on the trials after Pet Remedy administration. In fact, the number of rears observed in the novel arena increased significantly from day 1 through to day 3, in Pet Remedy as well as placebo conditions. This is likely due to habituation to the arena; with repeated exposure, the rabbits became more familiar and reared up to survey their surroundings. This would not be expected if the rabbits were sedated. This study, however, was only conducted over three days and we suggest that longitudinal studies involving repetitive handling of rabbits over longer periods of time are required with an emphasis on monitoring the animals for any signs of sedation as this could result in an inability to physically retreat from aversive and threatening stimuli.

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

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

For all the variables tested, an individual's response in the preliminary baseline test had a very significant effect on how they reacted in later tests. Variation in individual responses to stress within a population is widely acknowledged (Koolhaas et al., 1999) and would be expected to be a significant factor especially in a rehoming population where most subjects' histories are unknown and likely vary greatly. The within-subjects design and the use of baseline data as a covariate in the analysis meant that such inter-rabbit differences were taken into consideration. However, it is

possible that rabbits of initially different reactivity respond differently to Pet Remedy which may make significant effects more difficult to detect than in a more standardized population of animals. A rehoming population, however, represents an important part of the pet population at which this product is aimed, and hence, this is a meaningful first study.

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

Early handling of rabbits is widely recognized as an effective method of preventing fear during handling later in life (Magnus, 2005; McBride, 2014). Research on laboratory populations shows handling kittens within the first week of life significantly affects their later behavioral responses (Bilko et al., 2000; Zucca et al., 2012) and handling around the time of nursing reduces fearfulness as an adult (Bilko et al., 2000; Pongrácz and Altbäcker, 2003). The technique used to handle rabbits is also important. Wild rabbits are prey species, for which being lifted off the ground signifies likely risk of death by predation. Because domestication has changed rabbits' natural behavior very little (Lehman, 1991), lifting is also aversive to domestic rabbits. Handling them on the ground is generally acknowledged to be less aversive.

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

Conclusions

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

The study also highlighted the high level of fear of handling within the rabbit population. Pet Remedy used on a handler's clothing and in a novel environment was associated with a significant decrease in heart rate during handling and an increase in positive behaviors observed in a novel environment but produced no noticeable change in the rabbit's behavior toward the experimenter during a single administration. Use of Pet Remedy was also associated with a longer latency to pick up the rabbit, although this was possibly due to carryover effects from the previous testing session. Although there were no differences for most of the behavior measures, the reduction in heart rate and increase in positive behaviors suggest that Pet Remedy may have some potential value for rabbits during periods of acute stress and may thus be useful during veterinary visits and during initial handling and is worthy of further investigation.

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Conflict of interest

The authors declare that none of them have any competing interest.

Ethical considerations

None.

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