Mosquito-borne Arboviruses:
Protecting UK Horses

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Introduction

- Climate change and globalisation have led to increased concerns about the spread of arboviruses to new geographic regions, particularly Northern Europe.
- A recent study has demonstrated that several species of mosquito, found in the UK, are capable of transmitting a number of important equine arboviruses; including VEEV and RRV.
- In the event of an equine arbovirus outbreak in the UK, particularly for diseases for which there are no vaccines available, individual horse protection will be of great importance to horse owners.
- It is therefore critical to know which protection methods are most effective against UK mosquitoes.
- Repellent sprays are thought to be the most common bite-reduction method used by UK horse owners. However, as repellent sprays are not considered medicinal under UK or EU legislation, there are currently no licensing requirements for repellents marketed for horses and their efficacy in preventing horses from being bitten by UK mosquito species is currently unknown.

Aims

- Investigate the efficacy of different repellent sprays at preventing horses from being bitten by UK mosquito species.
- Investigate whether there is a statistical significance between the repellency effect provided by each of the different repellent sprays.
- Aim to identify which repellent spray is likely to provide the most protection to horses.

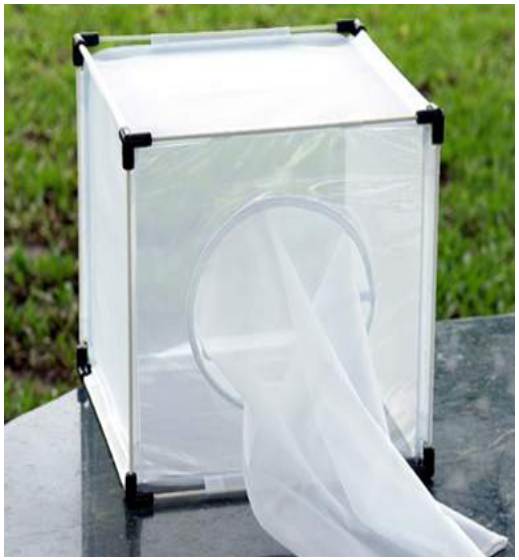
Materials: Repellents

- All of the repellents tested were commercially available spray products marketed in the UK for the purpose of repelling flies from horses.
- The repellents tested were:
  - NAF Off ® DEET Power Spray (NAF, Monmouth) containing 19.6% w/v N,N- Diethyl-m-toluamide (DEET)
  - NAF Off ® Extra Effect Spray (NAF, Monmouth) containing 1% w/v P-menthane- 3.8-diol (PMD)
  - Power Phaser (Leovet, Lahnau, Germany) containing 5.51% w/v DEET and 4.91% w/v IR3535 (Ethyl Butylacetylaminoproprionate)
  - Red Zone Super Spray (Red Horse Products, Henley-on-Thames) containing 20% Saltidin (icaridin) and undisclosed amounts of bog myrtle oil, cade oil, garlic oil and lemon eucalyptus oil

Materials: Mosquitoes

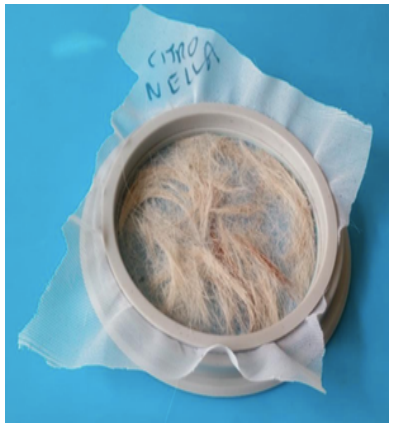
- *Oc. detritus* mosquitoes used for experiments were collected as larvae or pupae from salt marshes on the Wirral Peninsula, North West England, and reared in ambient conditions.

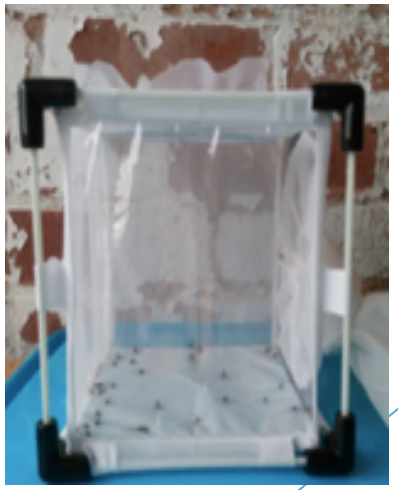
- Once emerged, *Oc. detritus* adults were reared in BugDorms and feed on 10% Sucrose Solution until the age of 7-10 days



Human-Bait Tests: Experimental Set-Up

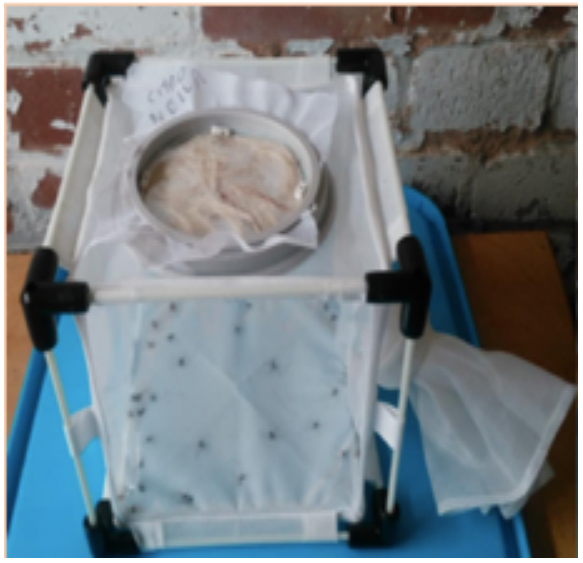
- Two adult volunteers (1 male and 1 female) were used as human bait to test the repellents.
- 1ml of repellent was added to 1g of horse hair in a petri dish and left to dry on a net for 6 hours. After this time, the horse hair was placed onto a new net and held in an up-turned Donut Lid.
- 30 female mosquitoes were placed into testing cages and moved to the testing facility at least 60 minutes before testing, to allow them to acclimatise. Testing cages are made of polyester netting and have a clear plastic side panel to enable the experiment to be filmed.
- The bites from *Oc. detritus* may cause severe localised reactions and wild-caught mosquitoes cannot be guaranteed to be pathogen-free, hence it was considered inappropriate to allow the mosquitoes to bite the humans and horses used for this research. Therefore, Testing cages that physically separate the mosquito from the human or horse bait were used.

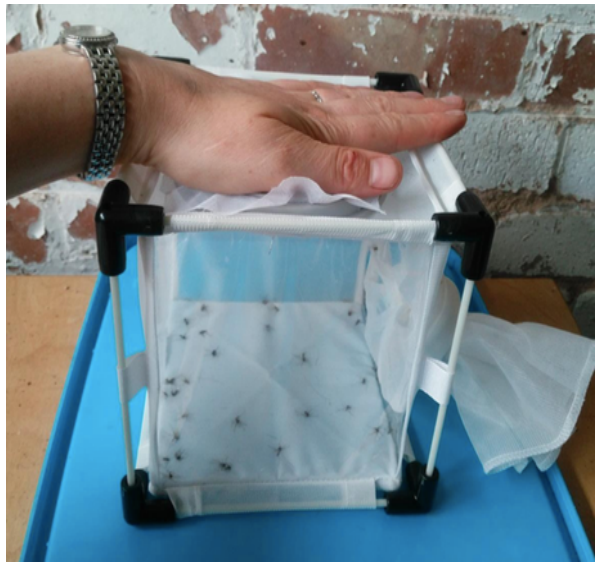




Human-Bait Tests: Method

1) Control test – Mosquitoes were encouraged to feed by using a carbon dioxide stimulus, which was provided to the mosquitoes by means of the researcher breathing on the cage for 15 seconds. Immediately after these 15 seconds, the volunteer's hand was placed on top of the Donut lid containing the untreated horse hair for a testing period of 60 seconds.

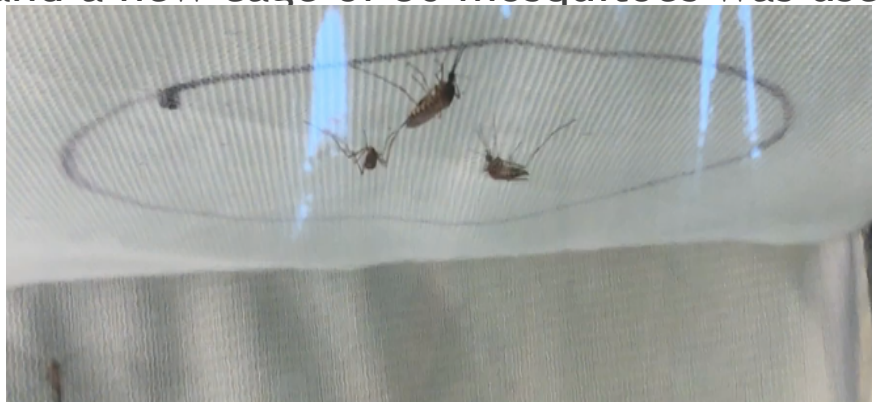




Human-Bait Tests: Method

2) An Apple IPhone 7+ camera, stabilised in a tripod, was used to film the testing area for the duration of the 60 second testing period whilst the volunteer's hand was placed over the horse hair. The testing area for analysis was defined by the black circle marked on the cage netting prior to testing.

If less than 10 mosquitoes were observed probing, the cage was discarded and a new cage of 30 mosquitoes was used.



Human-Bait Tests: Method

3) Treatment test – Immediately after the control test had taken place, the net with the untreated horse hair was removed and replaced with a net containing treated horse hair. Carbon dioxide stimulus was then provided again to the same cage of mosquitoes for another 15 seconds. Immediately after these 15 seconds of stimulus, the volunteer's hand was placed on top of the Donut lid containing the repellent treated horse hair for a testing period of 60 seconds. This was again filmed by the Apple IPhone 7+ camera.

This process was repeated using 3 different mosquito cages for each repellent, to provide 3 replicate tests for each repellent per volunteer.

Human-Bait Tests: Video Analysis

- For each replicate of a repellent test, two 60 second videos were analysed: the control test and the treatment test. For each 60 second video, 4 values were recorded: number of probing events (a mosquito beginning to probe in the test area for the first time), number of landing events (a mosquito landing in the test area), maximum number of mosquitoes probing in the testing area at any one time and maximum number of mosquitos resting in the testing area at any one time. The number of mosquitoes already resting on the testing area at the beginning of the test period were not counted as landing events but were counted for probing if this behaviour was observed.
- Blinding of video identity was required to prevent the order of alternate control and treatment videos from affecting the analysis. Blinding was achieved by renumbering videos, using random sampling of a numerical list produced by the sample function in R.
- Videos were then analysed in this random order. Repeatability was checked by repeating counts on all videos 2 days after they were originally analysed. Counts of all measures were identical during re-analysis. Results were de-coded once all videos had been analysed.

Human-Bait Tests: Results

- The results of the human bait tests are presented below:

Treatment	C: Probing events	C: Landing events	C: Max no. Probing	C: Max no. resting	T: Probing	T: Landing	T: Max no. Probing	T: Max no. resting
NAF Off® DEET Power Spray	22	18	6	6	17	13	5	5
	30	26	9	9	10	10	7	7
	32	27	11	11	8	2	5	5
	25	25	7	7	7	5	5	5
	31	28	9	9	10	6	6	6
	28	23	8	9	8	6	4	4
Power Phaser	25	20	7	7	4	4	2	2
	30	29	10	10	5	3	3	3
	28	28	8	8	3	4	2	2
	26	22	8	8	4	4	3	3
	21	18	8	8	2	3	2	2
	19	19	5	5	2	2	1	1
NAF Off® Extra Effect Spray	18	17	5	5	9	9	4	4
	22	17	7	7	10	13	6	6
	16	11	6	6	3	3	2	2
	18	16	8	8	8	8	4	4
	28	25	11	11	10	10	6	6
	30	25	9	9	14	14	6	6
Red Zone Super Spray	21	20	10	10	18	12	6	6
	21	21	9	9	2	3	1	1
	18	17	8	8	3	3	2	2
	28	26	11	11	4	4	2	2
	26	25	8	8	3	4	3	3
	30	25	10	10	5	5	2	3

Human-Bait Tests: Results

- Paired T-tests were carried out to identify whether there was a statistically significant difference between the number of probing events in the control and treatment tests. The results are presented below:

Product	Paired T-test Results
	Df=5
NAF Off® DEET Power Spray	P = 0.0012** t = 6.6169 SED = 2.720
Power Phaser (DEET + IR3535)	P = 0.0001*** t = 16.4095 SED = 1.310
NAF Off® Extra Effect Spray (PMD)	P = 0.0003*** t = 9.1924 SED = 1.414
Red Zone Super Spray (Icaridin)	P = 0.0030** t = 5.3584 SED = 3.390

* = statistically significant difference

** = very statistically significant difference

*** = extremely statistically significant difference

Human-Bait Tests: Results

- The percentage repellency was calculated using Abott's formula:

$$\%\ repellency = 100\ x \frac{(C - T)}{C}$$

Where C = control probing events count, T = treatment probing event count

Product	Percentage Repellency (95% CI)
NAF Off® DEET Power Spray	64.29 (56.80 - 71.14)
Power Phaser (DEET + IR3535)	86.58 (80.18 - 91.14)
NAF Off® Extra Effect Spray (PMD)	59.09 (50.56-67.11)
Red Zone Super Spray (Icaridin)	75.69 (68.06 - 81.97)

- Confidence intervals were were calculated using the Wilson method.

Human-Bait Tests: Results

- T-test were completed using the probing data to identify whether there is a statistical difference between efficacy of each repellent.

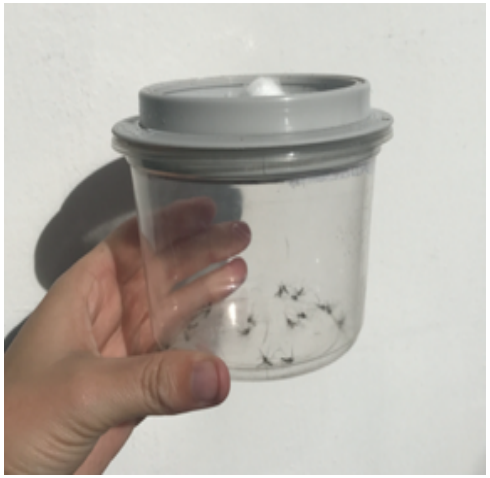
Product	NAF Off® DEET Power Spray	Power Phaser (DEET + IR3535)	NAF Off® Extra Effect Spray (PMD)	Red Zone Super Spray (Icaridin)
NAF Off® DEET				
Power Spray	X	X	X	X
Power Phaser	t = 1.1592			
(DEET + IR3535)	SED = 3.019	X	X	X
	p = 0.2733			
NAF Off® Extra	t = 1.6308	t = 4.4090		
Effect Spray (PMD)	SED = 3.066	SED = 1.928	X	X
	p = 0.1340	p = 0.0013**		
Red Zone Super	t = 0.0383	t = 0.9171	t = 1.4065	
Spray (Icaridin)	SED = 4.347	SED = 3.635	SED = 3.673	X
	p = 0.9702	p = 0.3807	p = 0.1899	
df = 10				

Human-Bait Tests: Results

- In this study, Power Phaser gave the highest percentage repellency with 86.6%. Whilst NAF Off® Extra Effect Spray gave the lowest percentage repellency with 59.1%.
- Confidence levels for all products ranged from 10-17%.
- The paired T-test identified that all the products tested had a statistically significant repellency effect.
- The results of the unpaired T-tests revealed that there was only a statistically significant difference in the efficacy of Power Phaser and NAF Off® Extra Effect, which were the best and worst performing products. This result signifies that Power Phaser should provide significantly greater protection against Oc. Detritus than NAF Off® Extra Effect.

Horse-Bait Tests: Experimental Set-Up

- Testing took place inside a treatment barn at the Phillip Leverhulme Equine Hospital, Leahurst. The treatment barn provided a sheltered, indoor location which eliminated the effects of wind and weather conditions from the tests, thereby reducing variables.
- Horses were restrained in the stocks, which they had been previously familiarised with, to ensure the safety of the researchers and the ease of testing. Testing was to be immediately abandoned if a horse became distressed and a replacement horse was to be used. Fortunately, this was not necessary at any point during testing.
- 20 female mosquitoes were placed into testing cages and were moved to the testing facility at least 60 minutes before testing to acclimatise. These cages are made of clear plastic and have a Donut Lid with mesh nylon netting.



Horse-Bait Tests: Horse Selection

The horses used in this study adhered to the selection criteria outlined below:

The same 3 horses were used to test each product and one week was left between testing each repellent.

- All horses must be healthy, with no previous history of any skin conditions.
- Horses must not have been treated with any topical insecticide within the last two months.
- Horses must not have been treated with any fly repellent or worming treatment within the previous month.

Horse-Bait Tests: Repellent Application

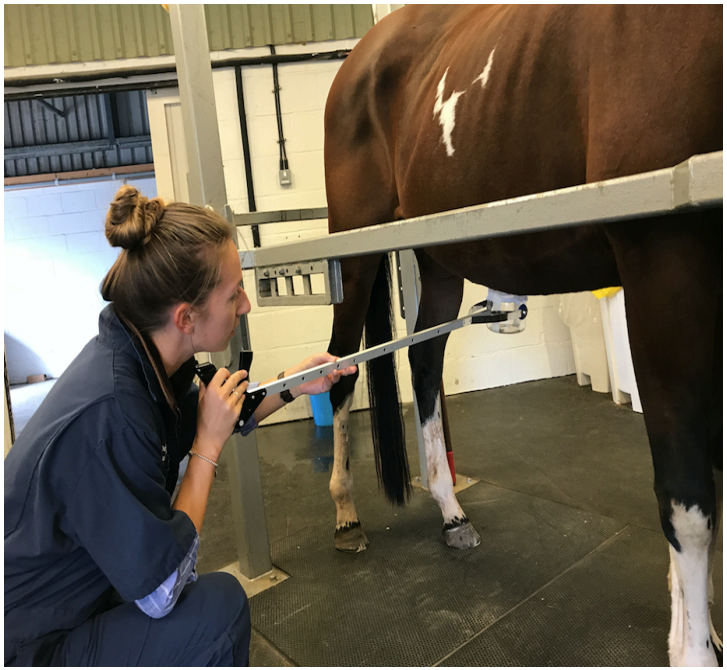
Horses were prepared for repellency testing by drawing around the Donut Lid of a BugDorm with white chalk at both the cranial and caudal aspect of the ventrum (belly). Resulting in two white circles that would become the 'testing areas'.

Care was taken to try and ensure that these circles were as far away from each other as possible, whilst still ensuring that the top and bottom of the BugDorm were parallel to the floor.

The repellent was applied to the caudal circle 4.5 hours before the test was due to take place. A small sponge was used to apply the repellent to the circle. 5ml of each repellent was used to dampen the circular patch of hair.

Horse-Bait Tests: Method

1) Control test – carbon dioxide stimulus was provided to the mosquitoes by means of the researcher breathing on the cage for 15 seconds. Immediately after these 15 seconds, the mosquito cage was placed onto the untreated cranial testing area on the horses ventrum and the number of probing events in the testing area was recorded for a 30 second testing period.



Horse-Bait Tests: Method

2) Treatment test – Immediately after the control test had taken place, carbon dioxide stimulus was provided to the same cage of mosquitoes for another 15 seconds. The same cage was then placed onto the caudal testing circle on the horses ventrum and again, the number of probing events in the testing area was recorded for a 30 second testing period.

This process was repeated using 3 different mosquito cages, to provide 3 replicate tests for each horse.

After testing, the repellent was washed from each horse's ventrum before the horses were returned to their fields.

Horse-Bait Tests: Results

The Results of the horse bait tests are presented below:

	Control	Treatment
Product	Probing Events	Probing Events
	18	16
NAF Off® DEET	16	12
Power Spray	16	14
	17	17
	13	12
	17	15
	17	19
	20	18
	19	17
Power Phaser	19	15
(DEET + IR3535)	20	14
	19	14
	19	17
	16	14
	17	14
	14	10
	13	9
	16	11

	Control	Treatment
Product	Probing Events	Probing Events
NAF Off® Extra	18	15
Effect Spray	18	16
(PMD)	19	19
	19	18
	19	15
	20	15
	20	15
	19	14
	20	16
Red Zone Super	17	17
Spray	18	15
(Icaridin)	18	14
	19	17
	19	16
	20	20
	17	17
	17	13
	16	13

Horse-Bait Tests: Results

- Paired T-tests were carried out to identify whether there was a statistically significant difference between the number of probing events in the control and treatment tests. The results are presented below:

Product	Paired T-test Results
	Df=8
NAF Off® DEET Power Spray	P = 0.0316* t = 2.6000 SED = 0.556
Power Phaser (DEET + IR3535)	P = 0.0001*** t = 8.5519 SED = 0.455
NAF Off® Extra Effect Spray (PMD)	P = 0.0008*** t = 5.2086 SED = 0.619
Red Zone Super Spray (Icaridin)	P = 0.0057** t = 3.7443 SED = 0.564

* = statistically significant difference

** = very statistically significant difference

*** = extremely statistically significant difference

Horse Bait Test – Results

- The percentage repellency was calculated using Abott's formula:

$$\%\ repellency = 100\ x \frac{(C - T)}{C}$$

Where C = control probing events count, T = treatment probing events count

Product	Percentage Repellancy (95% CI)
NAF Off® DEET Power Spray	8.50 (5.04 - 14.00)
Power Phaser (DEET + IR3535)	22.88 (16.94 - 30.15)
NAF Off® Extra Effect Spray (PMD)	16.86 (12.00 - 23.16)
Red Zone Super Spray (Icaridin)	11.80 (7.69 - 17.69)

- Confidence intervals were were calculated using the Wilson method.

Horse Bait Tests – Results

- T-test were completed using the probing data to identify whether there is a statistical difference between efficacy of each repellent.

Product	NAF Off® DEET Power Spray	Power Phaser (DEET + IR3535)	NAF Off® Extra Effect Spray (PMD)	Red Zone Super Spray (Icaridin)
NAF Off® DEET Power Spray	X	X	X	X
Power Phaser (DEET + IR3535)	t = 3.4048 SED = 0.718 p = 0.0036**	X	X	X
NAF Off® Extra Effect Spray (PMD)	t = 2.1381 SED = 0.831 p = 0.0483*	t = 0.8683 SED = 0.768 p = 0.3981	X	X
Red Zone Super Spray (Icaridin)	t = 0.8422 SED = 0.792 p = 0.4121	t = 2.4543 SED = 0.724 p = 0.0259*	t = 1.3274 SED = 0.837 p = 0.2030	X
df = 16				

*= significant difference in efficacy
**= very significant difference in efficacy

Horse Bait Tests –Results

- In this study, Power Phaser gave the highest percentage repellency with 22.9%. Whilst NAF Off® DEET Power Spray gave the lowest percentage repellency with 8.50%.
- Confidence levels for all products ranged from 8-14%.
- The paired T-test identified that all the products tested had a statistically significant repellency effect.
- The results of the unpaired T-tests revealed that there was a statistically significant difference in the efficacy of a number of products:
  - Power Phaser and NAF Off® DEET Power Spray, which were the best and worst performing products respectively.
  - NAF Off® Extra Effect Spray and NAF Off® DEET power Effect, with NAF Off® Extra Effect Spray having the larger repellency effect of the two products.
  - Power Phaser and Red Zone Super Spray, with Power Phaser having the largest repellency effect.
- The results of the unpaired T-test suggest that Power Phaser should provide significantly greater protection against Oc. Detritus than NAF Off® DEET Power Spray and Red Zone Super Spray. Additionally, NAF Off® Extra Effect Spray should also provide a significantly more protection against Oc. Detritus than NAF Off® DEET Power Spray.

Discussion

- In the present study, none of the products tested provided 100% repellency.
- Power Phaser was identified as the repellent likely to provide the most protection to horses.
- The agreement between human and horse-bait test results suggests that human-bait experiments provide a useful screening technique to reduce testing on horses, although they do not represent a substitute for field testing repellents on horses.
- The results of the human-baited experiments are in broad agreement with those of human trials in previous studies, in which low concentrations of IR3535 where shown to provide comparable protection to DEET (Lupi et al, 2013)
- Whilst each of the repellents was found to have a statistically significant repellency effect in both the human and horse-bait experiments, the percentage repellency found in the horse-bait experiments was dramatically lower than that found in human-bait experiments. This difference is thought to be associated with a number of factors associated with both host and repellent chemical characteristics which may affect the performance of repellents on different hosts.

Study Limitations

- The main limitations of this study were due to a lack of mosquitoes available for testing.
- Ideally, three human volunteers and three horses would have been used for experiments. However, the abundance of pupae and larvae in the salt marshes on the Wirral Peninsula were uncharacteristically low for August and September, resulting in there being too few mosquitos collected to complete the experiments with three human volunteers.
- For the human-bait experiments, the use of hair from different horses in a cross-over design would have also improved the experimental design. The ability to carry out such a cross-over design was again limited by the availability of mosquito larvae and pupae.
- Repeatability of horse-bait experiments would be improved if the experiments could be filmed. However, due to the logistical challenges of stabilising the camera on a tripod in close proximity to the hind-legs of a horse and providing enough lighting to get a good quality image for analysis, this was not possible.

Further Research Recommendations

- Future research to validate or discount the horse-bait test method as a useful screening technique for repellency efficacy should attempt to find a way to film such experiments in order to improve experiment repeatability, as well as using larger sample sizes.
- Further improvements to the experimental design of horse-bait tests could involve testing the repellents efficacy intermittently for a period of time. For example, carrying out the horse-bait experiments hourly for a total period of 6 hours after application of the repellent. This would give an insight into how the repellents efficacy varies over time.
- Future research should carry out field studies using Power Phaser repellent spray, as well as other repellents containing Icaridin and PMD, to confirm or discount their efficacy in reducing mosquito bites in open field conditions.

Conclusions

- The results of this study revealed that all of the products tested had a statistically significant repellency effect and are potentially useful repellents for the protection of horses from nuisance mosquito bites, particularly those products containing combinations of IR3535 and DEET.
- Although this study has shown that all of the repellents have a statistically significant repellency effect, they are unlikely to provide significant protection against an outbreak of a mosquito-borne arbovirus, particularly if infection pressure is high, due to their low repellency percentages. Therefore, the licensing of vaccines that are widely used to protect horses in affected countries should be considered the main priority in the event of increased risk of equine arbovirus transmission in the UK.
- The true practicality of using repellents to protect horses from mosquito bites seems unclear. Given the timescales of protection derived from the results of this study, it seems likely that re-application may be necessary every few hours. During this study, 25ml was applied to each horse with a sponge at each application, this method is likely to prove time-consuming and potentially difficult due to equine temperaments, and therefore impractical for many horse owners, if not prohibitively expensive.