The 3Hs Initiative – housing, handling, habituation

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Abstract

The 3Hs Initiative is a concept that arose from the research and management of laboratory mice and rats of Professor Emma Robinson's laboratory at the University of Bristol. Our research is predominantly concerned with the treatment of psychiatric disorders and particularly symptoms of depression and apathy. Due to the nature of our work, it is important that we are confident that the animals we are working with are not being put into a negative affective state by their environment or as an unintended consequence of our interactions with them. Our approach focussed on methods which provide the optimal control populations and ensure we can quantify specific and relevant behavioural changes arising for experimental manipulations.

As our research programme evolved, we have become more aware of the impact of stress caused to our animals by routine management approaches and physical restraint during procedures.

We have looked at our housing, handling and habituation approaches to identify areas to make improvements. This is critical for our research outputs and has the benefit of reducing the cumulative suffering the animals experience by reducing stress and negative affective experiences associated with their day-to-day lives. We identified some key changes which can be made which benefit both Animal Welfare and our scientific objectives and experimental outcomes.

Introduction

One of the principal areas of interest for our research is emotional disorders and investigating the mechanisms of action of antidepressants.[1,2] Until recently, objective methods to quantify the affective state of non-human animals has been limited. Methods such as measuring stress hormones or overt signs of distress lack both sensitivity and specificity.[3] Our approach builds from human experimental medicine and uses tasks designed to quantify affective state using cognitive tasks to quantify affective biases. Affective biases are observed when the emotional state of the subject biases their behaviour and affective biases have been shown to influence a number of different cognitive domains including attention, learning and memory and decision-making.[4] Just as is seen in humans, studies in non-human animals have found that similar affective biases can be quantified in rats and mice.[5] Using these methods we have been able to assess the refinements we have put in place and show that they are reducing the stress caused to the animals and having a positive impact on their affective state.

At least some aspects of the techniques and protocols that we use can be easily implemented by other researchers and animal care staff throughout the industry. To disseminate our work more widely, we have launched a website: www.3Hs-initiative.co.uk. Here we provide examples of our work, explain the rationale behind our methods and the research we have undertaken for validation. We provide guidance documents and videos so that others can replicate our methods at their facilities. The initiative is an ongoing project, and we plan to continue to develop our understanding of how to manage laboratory animals for scientific procedures while also reducing their cumulative suffering.

Housing

When considering how to house laboratory animals there is a need to balance scientific and husbandry requirements with Animal Welfare. Ideal housing from a welfare perspective would allow the animals to express their full range of natural behaviours, meet their needs

in terms of shelter and provide optimal social interaction opportunities. However we also must consider that all animals need to be welfare checked daily, soiled cages need changing on a regular basis and we need to be able to easily access animals for experimental procedures.

Basic shoebox cages and independently ventilated cages (IVCs) have benefits in terms of space requirements, reducing cross-contamination and making Animal Welfare checks and cage changing simpler but they are not ideal for allowing animals to express their natural behaviours. Adding cage furniture and enrichment items increases the available space in the cage as well as providing areas to hide or create nests. The right types of enrichment can lead to significantly lower stress levels in animals.[6,7] However the addition of too many items can make it difficult to perform Animal Welfare checks and may lead to competition for resources. When deciding on the best enrichment to provide, the principal factor is identifying items that provide the most opportunity for enhancing space and supporting the expression of natural behaviours. For example, increasing the surface area within the cage and incorporating nesting materials can enhance the home cage environment. We also largely overlook the fact that mice and rats explore their surroundings using touch and olfaction and so providing greater variety in terms of textures and scents can provide greater value.

There are other factors that we must consider when selecting enrichment materials. In group-housed, male mice, aggression can be a problem and can be exacerbated by high value resources and additional monitoring after the introduction of novel enrichment items may be useful. Also, aged animals, post-surgery animals and animals with reduced mobility may require modified enrichment to enable interaction without risking injury.

Using playpens (Figure 1) can be a good compromise between the needs of the animals and the welfare and scientific requirements as the animals have access to a large area with lots of enrichment where they can run and explore for a set amount of time and they can be monitored throughout if required. Playpens have been shown to be rewarding for rats and mice and they can also be used to provide supervised social interaction for rats which may have limited opportunity in their home cage.[8,9]

A study conducted by our laboratory showed that when placed in a ball pit or a playpen, rats emitted a high number of 50kHz calls (Figure 2) which are associated with positive events.[8,10,11] These high numbers of calls were sustained over consecutive days which suggests that the animals are not just reacting to the novelty of the playpen and that the positive effect does not decline with repeated exposure.

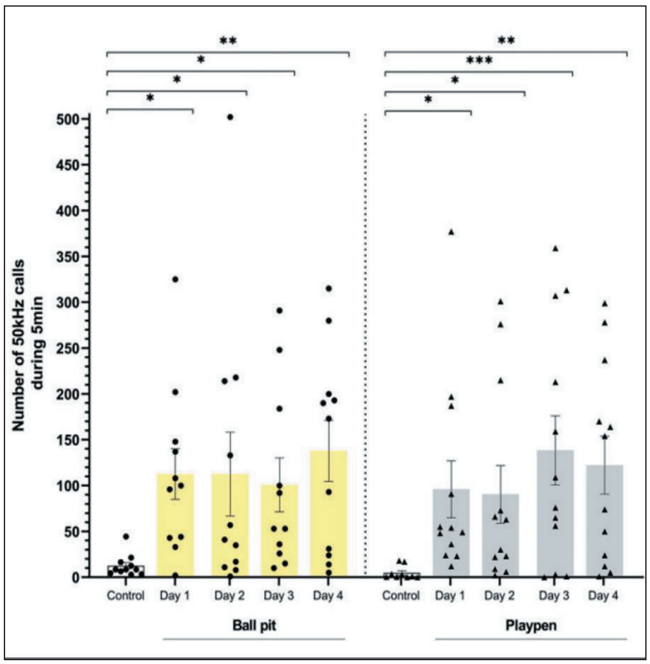

Figure 2. 50kHz calls emitted by rats exposed to ball pit or playpen over four consecutive days.

In a follow-up study we found that when the animals were injected with a drug that induces a strong anxiogenic effect, if they were subsequently given access to a playpen, the effect of the drug was attenuated. This suggests that,

Figure 1. Rat and mouse play pens.

if the animals must undergo an aversive intervention, allowing them playpen time after the negative event could mitigate against the negative affective experience caused by the procedure.

Habituation

When we talk about habituation, our aim is to gradually introduce animals to human interaction, experimental equipment and procedures in a way that promotes positive rather than negative associations. This is especially important for handling as this is something that the animals will encounter often throughout their lives from husbandry to regulated procedures and can result in high levels of stress.[12,13]

If we can reduce or remove the stress from these situations, we can have a significant impact on the cumulative stress that the animal experiences over its lifetime.[14,15,16] Put simply, if an animal forms a positive rather than negative association with human contact, the cumulative impacts of routine care and procedures can benefit their welfare rather than add to their suffering. The benefits of habituation may also be felt by the animal care staff and researchers as positive human-animal interactions may be linked to decreased compassion fatigue in laboratory animal workers and increased satisfaction in their work.[17]

Examples are shown in Figure 3 of the protocols we use for mouse and rat handling habituation.

The key things to note are that the first interactions are kept short. This reduces the chance that anything negative could happen during the interaction and reduces any stress the animal might experience from the novel situation. It is immediately followed by a reward to build the positive association. When progressing through the habituation protocol you should not move to a new stage before the animal is comfortable with the current one. Some animals and strains are more anxious than others, so it is important that you do not undo all your work building a positive association by jumping ahead before the animal is ready. It is much easier to build a positive association from scratch than it is to overcome a negative one. Keeping a record of overt measures of stress e.g. vocalisation, urination and faecal pellets may be useful to ensure the animal is not finding the current stage aversive. A habituated animal should be showing very few, if any, of the overt signs listed above.

Keep in mind that every time we interact with our animals, they are learning something from us and the first interaction they have might be the most important. If they have a negative experience the first time they meet you, then the next time you approach them they will be anticipating another negative experience and they will avoid you. This leads to them trying to evade handling which will increase their stress and increase

Mouse Habituation	Rat Habituation
Day 1 – Open cage and remove all furnishings accept a familiar tube. Keeping the tube within the cage, lift the mouse in the tube and gently encourage to step over your hand back into the cage. Repeat a few times and for each animal in the cage and then place reward into the cage and replace all furniture and lid.	**Day 1** – Cage lid off, furniture removed, remaining within the confines of the cage, pick up the rat around the shoulder then immediately put back in cage. Repeat with each cage mate then give reward in home cage.
Day 2 – Repeat day one but try to have the mouse pause on the hand with gentle cupping each time. With cup handling, the mouse should not feel restrained and should be released if it starts trying to evade handling. For animals which do not require physical handling for the procedures repeat tube handling habituation days 3 to 5.	**Day 2** – Cage lid off, furniture removed, pick up and transfer to a travel box containing rewards, move all rats from the cage to the travel box, give additional rewards then once consumed, pick up and return to cage, give reward in cage.
Day 3 – For animals requiring physical restraint for procedures progress to cup handling. Repeat day 2 but cup restrain the mouse for a brief period before release. Gradually increase the time restrained as the animal's tolerance increases.	**Day 3** – Open cage lid and wait for rat to approach then pick up, transfer to travel box containing reward, move all rats from the cage to the travel box, give additional rewards then once consumed, pick up and return to cage, give reward in cage.
Day 4-5 – repeat day 3 increasing time animal is cup restrained. Individual and strain specific differences will impact on the number of sessions required for the animals to accept each stage and it is important not to progress to a new stage before the animal is comfortable with the current one.	**Day 4-5** – Repeat day 3 and start introducing dosing positions and taking palatable solutions from a syringe. If using palatable dose training, this can also serve as the reward. Habituation to sitting on a piece of Vetbed can also be useful for certain procedures e.g. subcutaneous injections and administration or withdrawal of substances through surgically implanted devices.

Figure 3. Habituation protocols.

Reward Options		
Reward	Primary Macronutrient Group	Features
Sunflower seeds (whole or crushed)	Fat	Can be provided irradiated, autoclavable, contaminant screened
Yogurt Drops	Carbohydrate/ Fat	Can be provided irradiated, autoclavable, contaminant screened
Forage Mixes	Carbohydrate	Can be provided irradiated, autoclavable, contaminant screened
Cereal Grains	Carbohydrate	Can be provided irradiated, autoclavable, contaminant screened, low calorie
Dried Fruits/ Vegetables	Carbohydrate	Can be provided irradiated, autoclavable, contaminant screened
Dried Mealworms	Protein	Can be provided irradiated, autoclavable, contaminant screened
Precision Reward Pellets	20% Sucrose enriched food pellet	Can be provided irradiated, autoclavable, contaminant screened

Figure 4. Rewards.

their negative association with you. By starting with a positive experience paired with a reward you will make every subsequent interaction easier as they are anticipating the reward.

Once the animal is habituated to being handled you can start to introduce holding them in dosing positions and even sham injections with an empty syringe without a needle to get them used to the whole process before beginning a dosing study. If you wish to use a Vetbed as part of your handling or dosing procedures, then providing rewards whilst the animal is sitting on the Vetbed will help the animals form a positive association with the context which can mitigate the effects of subsequent mildly aversive procedures such as subcutaneous injection or procedures in animals with head mounted devices.

Even if time and management systems limit your ability to use the full handling habituation protocol, providing animals with a food reward after each human interaction can mitigate their negative experiences and lead them to anticipate human contact with a positive outcome.

There is a wide variety of options for food reward available from commercial suppliers. All can be provided irradiated or certified contaminant free and they can be autoclaved for further biosecurity. Depending on the dietary requirements of your animal and study, there will be a suitable reward to meet your needs.

Handling

Once you have built up positive associations with the handler, the animals should be quite easy to handle for basic husbandry and health checks but there are still going to be some occasions when we need to physically restrain the animals for procedures.

The instinct a lot of the time is to restrain the animal as securely as possible as this is perceived to be better for the safety of the animal and the handler and the accuracy of the procedure but physical restraint is highly aversive for animals and is widely used to model depression.[18,19,20]

The negative impacts of restraint do not seem to decrease with repeated exposure although animals may exhibit passive coping strategies such as learned helplessness when overt signs of distress decline but the affective experience does not.[21] It has even been shown that, when comparing animals that were restrained and underwent an intraperitoneal (I.P) injection with animals that were just restrained, the stress response was the same across both groups.[22] This suggests that the primary source of stress during this procedure was the restraint and not the injection. The stress caused to the animal will have an impact on the results of your study as well as the welfare of the animal handler.[23] Instead, maybe we should be asking, what is the least amount of restraint we can use to achieve the outcome we need, or can we even remove the need for restraint altogether?

Oral dosing

Oral dosing of rats and mice is commonly conducted using an oesophageal cannula. This procedure is distressing for the animals requiring physical restraint.[24] There are also risks of adverse events including incorrect placement and tracheal dosing and oesophageal

trauma.[25,26] This method also requires the experimenter to have a high level of skill and may result in bite or scratch injuries and stress.

An alternative approach is to use voluntary ingestion of test substances in palatable solutions. Although this approach has previously been reported by several research groups it is still not widely used and it is not clear if this is due to a lack of awareness or whether researchers have encountered problems using this approach.[27,28,29,30] Our own experience has been that rats and mice readily take palatable solutions from a syringe but can quickly form negative associations with ingestion of some drug solutions which we hypothesise arises if drugs have a bitter taste or from conditioned aversion i.e. where any aversive effects of the drug become associated with the palatable solution in which it was administered. To reduce the potential for this to develop, we have developed a protocol to increase the reliability of this dosing method.

The animals are introduced to the palatable substance a week before the start of the dosing study so that they can become familiar with the novel taste and drinking from a syringe. The palatable solution can even be used as a reward during their initial habituation.[31] The animals develop a positive association with the syringe and will quickly approach when it is presented. Once the animals are reliably drinking the palatable solution from the syringe, the required drug can be added and the animals should readily consume the drug without the need for gavage. By forming the strong positive association with the palatable solution in the syringe before combining it with a drug we reduce the likelihood that the animals will make a connection between any aversive effects of the drug and the palatable solution thus decreasing the risk of conditioned aversion. We can further mitigate against this by providing a second syringe of palatable solution without drug on dosing days at a later timepoint. By doing this, the animals will have drunk the palatable solution twice that day but only felt the effects of the drug on one occasion so again, we are reducing the likelihood of an association forming between the aversive effects and the solution.

Using this approach, we have administered a wide range of psychiatric drugs without issue[31] (Figure 5). This refined method takes what would previously have been a stressful and risky procedure and replaces it with a positive experience for both the animal and the person carrying out the procedure. Although the approach will not be compatible with all test substances, this method offers both welfare and scientific benefits and could reduce variability between subjects and improve the quality and reproducibility of scientific studies. We are continuing to refine this dosing method by exploring methods of masking the bitter taste of certain drugs to increase the range of drugs that can be reliably dosed by voluntary ingestion.

Dosing by injection

There will be occasions where we still need to inject our animals and in these cases we try to use very low restraint methods. We have modified our handling techniques for rats and mice to eliminate the need for scruffing in rats or tail handling in mice prior to injecting the animal. We have been able to use objective measures of affective state and the stress response to show that these methods offer welfare benefits.[32,33] Importantly, these techniques have been taught to and used by researchers with a range of prior experience levels and all have demonstrated competency within a short period of time. These methods offer both welfare and scientific benefits and could reduce variability between subjects and improve the quality and reproducibility of scientific studies.

IP injection in rats

The conventional handling methods for I.P. dosing all involve the rat being tightly held with its abdomen stretched out.[34]

Figure 5. Mice and rats voluntarily drinking palatable solutions from a syringe.

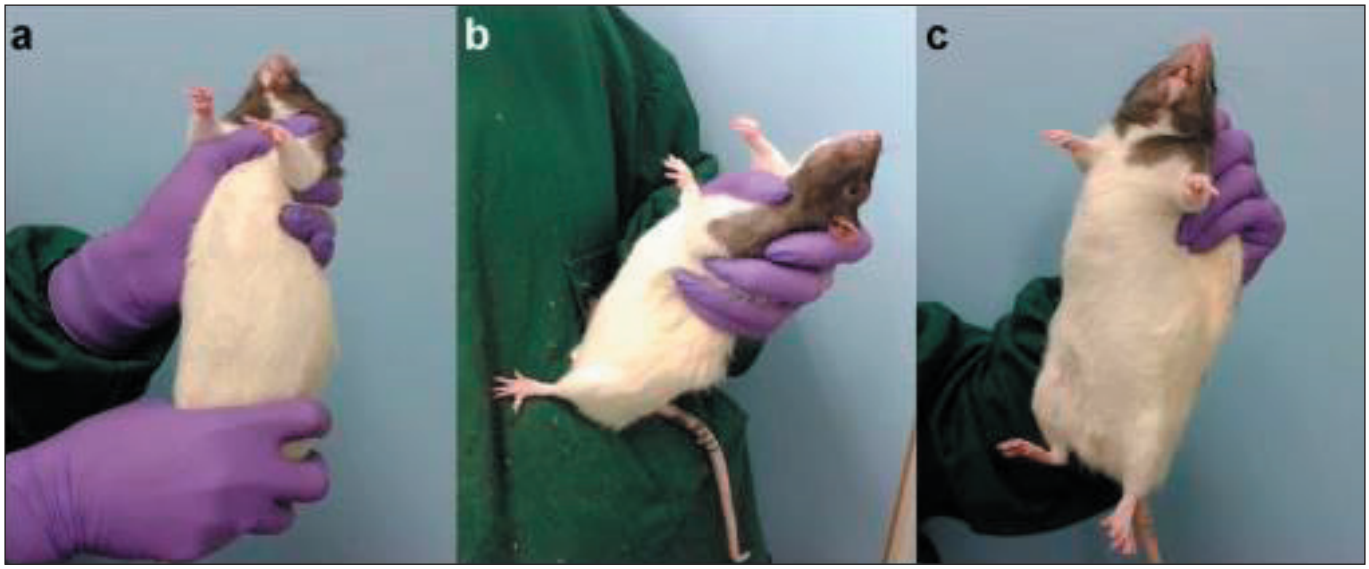

Figure 6. Conventional restraint methods for IP injections in rats.

To avoid the stress caused by this firm restraint we use a method where the animal is held lightly, in a relaxed position, with a rounded abdomen to decrease the pain of the injection.[31] The rats are habituated to being held in this position and sham injected with an empty syringe with no needle attached. By getting them used to the hold and pairing it with reward, the rats have a positive association with being held in this way and this leads to significantly lower overt signs of stress as well as physiological and behavioural indicators of stress and aversion[35] (Figure 7).

Subcutaneous dosing in rats

Once a rat is habituated to being handled, subcutaneous (S.C.) dosing can be carried out with almost no restraint at all. The rat can be habituated to being held against the handler's body, on the handler's lap or on a piece of Vetbed.[31] (Figure 8) Once the animal is comfortable sitting in the required position you can introduce sham injections with an empty syringe with no needle attached and go through the motions of tenting the skin and injecting in the appropriate place. By pairing this scenario with positive rewards, when the actual injection occurs,

Figure 7. Refined handling method for IP injection in rats.

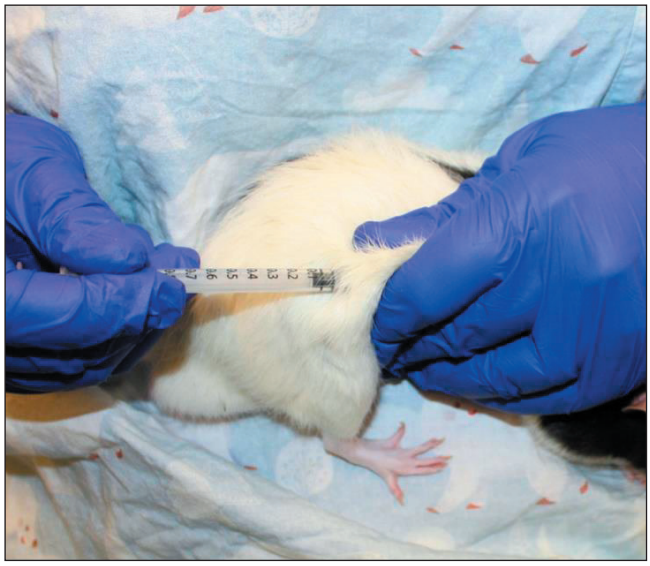

Figure 8. SC dosing of rats.

the rat will easily tolerate the transitory pain of the needle insertion without struggling or attempting to evade the needle. This results in a virtually zero restraint procedure.

Alternative scruffing methods in mice

It has been very well established that tail handling is aversive for mice and that experimental outcomes can be improved by using cup or tunnel handling instead of tail handling.[36] The majority of United Kingdom (UK) establishments have moved away from tail handling for their husbandry and basic handling but when it comes to procedures, common practice still involves using the base of the tail to facilitate scruffing the mouse. This can be replaced by placing the mouse onto your arm or a piece of Vetbed[31] (Figure 9), cupping a hand on top of them, letting them squeeze their head out between your thumb and forefinger and then scruffing them from there. This technique produces the same level of control as the conventional method but causes significantly lower overt signs of stress such as struggling, vocalisation and aversion on release.[33] (Figure 10)

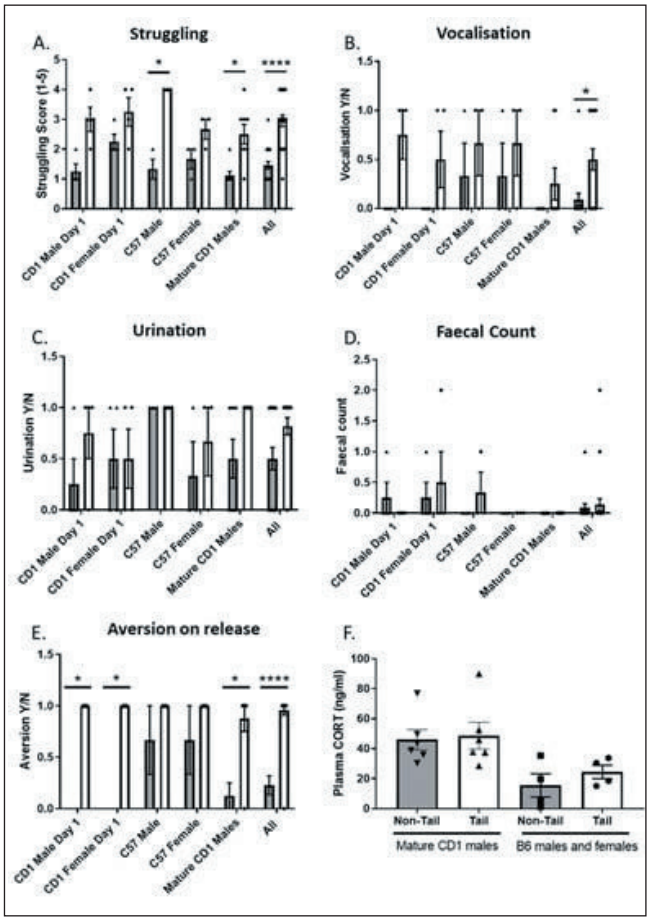

Figure 10. Overt measures of stress showing significantly decreased struggling ($p<0.0001$), vocalisation ($p<0.0121$), and aversion on release ($p<0.0001$).

Conclusion

By implementing the 3Hs you do need to put in a little bit more work at the beginning of your study but it will make everything that you do throughout the study easier and possibly save time overall. This will prevent stress to you and your animals. This is so important when we think about the cumulative suffering that an animal will experience throughout its lifetime. Moreover, the effects stress to the animals and to you will have on the outcomes of your experiments.

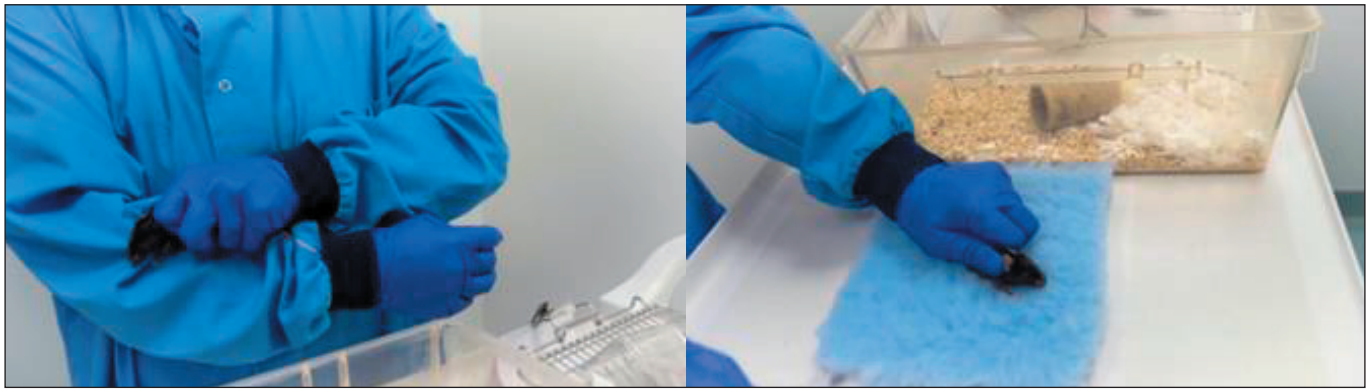

Figure 9. Refined scruffing methods in mice.

Whilst the examples shown in this article and on our website are taken specifically from the laboratory environment that we work in; the principles of the 3Hs can be translated into any other laboratory environment. Animals housed in higher biosecurity containment such as IVCs and isolators will still benefit from positive reinforcement after human interaction and all animals will benefit from habituation protocols that introduce new interactions with objects or personnel in a gradual way that establishes positive associations.

If you do not have the capability to provide a large playpen due to space or biosecurity concerns then you could provide a smaller space with enhanced enrichment that would not be practical within the home cage but could provide greater opportunities for natural behaviour such as digging, foraging, novel smells and textures. If biosecurity or cross contamination are a concern, you could consider using items that can be easily cleaned between animals or have separate labelled items for different groups to minimise these risks. If there are methods already in place in your facilities for decontamination e.g. fogging chambers, autoclaves, ethylene oxide, it is likely that you will have a suitable method already available for any novel enrichment or positive reinforcement aid you wish to use.

By creating the 3Hs Initiative website, we hope to provide technicians and researchers throughout the industry with evidence-based protocols that can be easily implemented in their own facilities. We have videos and images of all the techniques mentioned as well as downloadable guidance documents. We can improve our animals' lives and our experimental outcomes by enhancing housing, refining handling and introducing habituation protocols.

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