Adaptation of the Animal Welfare assessment grid (AWAG) model to monitor laboratory rats and mice

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Abstract

The Animal Welfare assessment grid (AWAG) is a cloud based online tool designed to assess and monitor the welfare and cumulative lifetime experience of animals. It has been successfully used to monitor the wellbeing of animals from an experimental, zoological, farm or pet background. No previous studies have reported the use of this method for monitoring the welfare of rodent species housed in laboratory settings. This project was designed to evaluate the applicability of AWAG in rodent species undergoing procedures commonly performed in animal research facilities. AWAG was able to capture physical, psychological, environmental and procedural parameters of rodents in genetically modified mice in different strains of mice and rats and following catheterisation and self-administration as well as following headpost surgery. Overall, AWAG scores were relatively low in our studies, even following highly invasive surgery. Transient fluctuation in AWAG scores in certain parameters were detected in response to change in housing conditions (single/group), cage cleaning, water and food restriction. The results described in this paper demonstrate that the AWAG has been successfully applied for rodent species in a laboratory setting and is effective at capturing the welfare of animals undergoing experimental procedures, including genetically modified breeding and experimental procedures, in a structured, controlled and visually represented way. This paper describes how the AWAG system has been applied to studies involving food restriction, surgery and breeding in rodents and considers how the AWAG can be used more widely and effectively in larger laboratory facilities to assist with 3Rs (reduction, refinement and replacement) strategies and further improvements in Animal Welfare practices.

Introduction

The Animal Welfare assessment grid (AWAG) is a web-based programme which can be used to score and input data to help monitor the welfare and cumulative lifetime experience of animals. To date the model has been used to monitor the quality of life of macaques involved in infectious disease studies,[1] guinea pigs housed under different biocontainment strategies,[2] animals housed in zoos,[3,4,5,6] in veterinary clinics[7,8,9] and livestock research studies.[10] However, this is the first study to apply the AWAG model to small laboratory animals such as

rats and mice. Rodents (rats and mice) are the most frequently used animal model in biomedical research testing in the United Kingdom (UK), being used in 70% of all *in vivo* experimental procedures and encompass 86% of genetically altered animals created and bred for biomedical research purposes in 2023.[11] The use of these species is due to their similar physiology to humans, with rodents (rats and mice) sharing 95% of the human genome.[12] In addition, technological advances in genetics in the last 30 years have made genetic manipulation in these species relatively easy, especially in mice. When regulated procedures are performed on animals it is important for them to not to suffer poor welfare, since in addition to ethical and legal reasons, studies have shown that poor welfare can impact on the accuracy and reproducibility of the data of scientific experiments.[13,14,15] The application of the AWAG to monitor rodent laboratory species will help researchers and Animal Technologists in assessing and refining the impact of experimental procedures on different aspect of animals' welfare. It will provide key information which will help assess the harm/benefit analysis required by funders and by regulators such as the Home Office in the UK. In addition, it will help in refining the humane endpoints to be applied to minimise suffering.[16,17]

Protection of animals used for scientific purposes

As defined in UK legislation[18] (Animal (Scientific Procedures) Act 1986) (ASPA) and the EU Directive 2010/63, all adult vertebrates (excluding humans) are classed as protected species. Any scientific use of a protected species (including rats and mice) which causes any amount of distress, harm or suffering greater than a needle injection is classed as a regulated procedure. In the UK, those proposing the conduct of regulated procedures must apply the principles of replacement, reduction and refinement (3Rs) when applying for Home Office Project Licences (PPL) which allow the use of protected species in animal research studies and similar principles are applied in many other countries.

The 3Rs have been considered the pillar of laboratory Animal Welfare worldwide since Russell and Burch's publication in 1959.[19] Application of these principles includes providing suitable enrichment that enables and encourages the animals to display natural behaviours. Not only does this minimise suffering and help researchers comply with the legislation, the introduction of the 3Rs has helped influence public opinion on the use of animals in science[20] as well as improving the quality of research[21,22] by reducing baseline stress, hence improving reproducibility and reliability of data produced *in vivo*.

How does the AWAG work?

A key element of ensuring good welfare is to provide an environment that allows the animals to express their natural repertoire of behaviours as far as possible.[23] Welfare assessment should therefore include consideration of the environment and the behaviour of the animals and not simply their physical condition. The use of AWAG[1,24] offers a schematic of parameters reflecting the five domains of Animal Welfare:[25]

1. nutrition
2. environment
3. health
4. behaviour
5. mental state

By monitoring the physical condition of the animal, its psychological condition, the quality of the environment and the procedures being carried out whether for scientific, veterinary or husbandry management. Each AWAG parameter consists of a group of different factors for example, body condition score, use of nesting material, impact of experimental procedures on animals' behaviour and general wellbeing which contribute to a total score for each parameter (Table 1). Each of the factors is scored for individual animals or group of animals and factor profiles for each parameter can be adapted to make them more relevant for a given species or set of experimental circumstances. Scores are given from 1 to 10 (1 = good, 10 = bad) and each level of scoring is defined in the tool to reduce any inter-observer variability that may otherwise occur between individuals using AWAG (see Table 2).

The AWAG records change in the state of an animal over time, allowing for predictive, retrospective or scheduled event monitoring and illustrates the duration of the components of suffering. The lifetime experience graph records cumulative welfare assessment (CWAS) and can be investigated at any time point to produce a grid showing the effect of the specific components of the domains of Animal Welfare, allowing the effect of specific refinements to be clearly evaluated. The grid includes contingent as well as direct suffering to reflect cumulative harms and allows an evaluation of the animal's quality of life. It can demonstrate the welfare implications of research and the effect of refinements, both at the planning stage and when retrospectively assessing studies to ensure that the harm/benefit analysis remains to justify the work. This assessment method is a valuable tool for those tasked with ensuring ethical oversight as well as for those planning the use of, or monitoring of animals in research and facilitates dialogue between all parties. It is particularly applicable to animals used in long term studies. The use of a welfare assessment grid can be helpful in balancing some of the difficult decisions about the future of an animal, whether in research or other welfare context.

Behaviour of laboratory rodent species

Laboratory mice and rats can exhibit behaviour very differently to their wild counterparts due to the different environments in which they live. For instance, in the wild male mice do not naturally cohabitate and are usually territorial towards each other.[26] In study environments where littermates are usually housed in confined cages, aggression can occur. This can cause pain and injury which can lead to death as the mice cannot escape the confines of the cage.[27] Interventions involving isolating the aggressor or victim may be useful in decreasing these behaviours. However it may also decrease welfare as individuals may exhibit symptoms of anxiety and depression.[28,29] due to confinement in a cage on its own. Other behaviours which can be seen in laboratory species include abnormal repetitive behaviours which are also known as stereotypies.[30] These involve the animals taking part repeatedly in activities such as bar mouthing, climbing and circling. Whilst some of these behaviours such as climbing have been described as natural,[31] others have been linked to environmental conditions[32] and mental conditions such as repetitive grooming linked to obsessive compulsive disorder (OCD) in mice and circling with psychomotor behaviour in response to psychostimulants and opioids[33] and drug related disorders.[34] As a result environmental complexity should be considered when assessing Animal Welfare. Behaviour has also been seen to differ among strains[35] suggesting that abnormal behaviour presented by rodents is possibly genetic and not caused by its environment alone or may be an interaction of both. For the scenarios outlined above the AWAG can be used to identify and demonstrate this thus directing researchers to consider possibly different environments for different strains when assessing the welfare of these animals which can then be quantified for effectiveness.

Method

Four different studies/groups were chosen to give a wide range of applications for Animal Welfare assessment. All data was collected from animals on studies or in breeding colonies authorised by appropriate PPLs approved by the Animal Welfare and Review Body (AWERB) at the University of Sussex. All experimental procedures were conducted in accordance with ASPA.

- Animals were housed in conventional cages with temperatures and humidities being maintained between 21+/- 2°C and 40 to 70% humidity.
- Animals were provided with standard enrichment including chews, tunnels, forage mix (not in food restriction studies), nesting material and rodent houses (breeding animals).
- Light cycles were 12H:12H light/dark and animals were fed a commercially available rodent diet (BK001).

Clinically relevant strains and studies involving major interventions were used to assess wellbeing over time or the impact of interventions that potentially increase/decrease the AWAG score. Sample size varied for studies 1 and 2. These were mice from stock cages rather than scientific experimental studies. In study 4, with a small number of rodents undergoing surgery the animals were recorded individually. Data was collected usually on a weekly basis by observing and scoring each cage and recording data on the AWAG accordingly (Figure 1). The observations were recorded by the same people which helped minimise variation in observer bias. As the data was collected, the AWAG tool plotted the data entry producing a grid showing the mean welfare score of each parameter and line graphs showing the cumulative Animal Welfare score (CWAS) for each study. Through collaboration with the researchers of the original studies, some retrospective data was also obtained to help with analysis which is stated in the appropriate results. The reasoning for each of the factors is presented in Table 1. Each of the factors is part of one of the 4 parameters (physical, psychological/behavioural, environmental and procedural) and scores from 1 to 10. From 1 showing good welfare vs. 10 meaning a significant impact of welfare on the animal (in some cases the death of that animal). It would be highly unlikely for an animal to reach a score of 10 in most factors as it would have been euthanised after reaching a defined humane endpoint.[36] A score of 10 may be useful in some infectious agent studies where acute onset of debilitating clinical signs requires immediate euthanasia and AWAG can support the action taken. It will also indicate poor welfare of animals not covered by ASPA such as farm and domestic animals and can be used as a justification for euthanasia.[16] The factors were tested on experimental study animals and breeding colonies for a minimum of two months to improve the accuracy of scoring and to provide an opportunity to refine the definitions of each factor as appropriate.

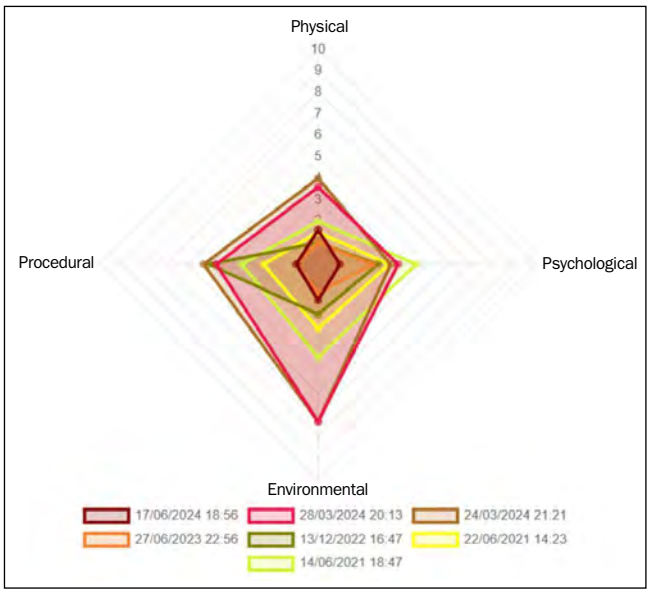

Figure 1. Welfare assessment grid at various time points.

Parameter	Factor	Reasoning
Physical	Activity Level	Inactive rodents may be injured or ill, whilst hyperactive creatures may be showing stereotypical activity or stressed.
	Body Condition Score (BCS)	Visual observation of animals based on the amount, or lack, of fat found in the different parts of an individual's body. Overweight animals may have an increased chance of heart disease or breathing problems, whilst underweight animals may die from malnutrition or infection.
	Clinical Assessment	Assessing the physical health of an animal. If they have a condition, it may impact on other factors or their welfare overall. Animals must not be suffering as part of the Five Domains (Animal Welfare Act 2006[37]).
	Faecal Changes	Changes in frequency may indicate bowel or diet problems, whilst other substances such as blood or mucus could be a sign of major health issues.
	Food/Water Intake	Part of the Five Domains. If food and water is measured routinely, it can be used to detect whether an animal is eating or drinking healthily and intervene if not. Can also be used to note restriction of these substances during studies. (Animal Welfare Act 2006[37])
Psychological/ Behavioural	Behaviour	As part of the Five Domains, animals should be able to express natural behaviours. Abnormal behaviours, such as stereotypies, could be a sign of stress or poor welfare. (Animal Welfare Act 2006[37])
	Fear/Aggression	If not acclimatised to handlers, aggressive and nervous behaviour may occur which can in turn cause stress. Long-term aggression towards conspecifics can cause stress or injury if not intervened.
	Grimace Score	A visual way of measuring pain through a rodent's facial expressions: orbital tightening, nose/cheek appearance, ear and whisker positioning. Previous studies have used this as an intervention tool.[38,39]
Environmental	Housing	Measuring whether the cages met the Home Office Code of Practice for the rodents. This includes the environment (temperature, air quality, humidity) being sufficient for individuals. NC3Rs criteria was used to score with different factor profiles for rats and mice. (Animal Welfare Act 2006[37])
	Nesting	Nesting is form of enrichment for rodents as they spend a large amount of their time foraging for materials and building nests in the wild. It is important for shelter as well as conserving heat, particularly for new-born pups.
	Noise Level	Rodents are very sensitive to noise, having a larger hearing range than humans and being able to hear ultrasound. Prolonged noise may have a physical or mental effect on individuals.
Procedural	Contingent Events	Events that have occurred which may have impacted on the other factors and caused stress. For instance, an individual moving to a new enclosure or construction work in the facility which may cause a complete change of environment for the animal. Can help pinpoint reasoning behind changes in scores over time.
	Husbandry Procedures	Measuring how an individual reacts during husbandry events, such as adding new food/water or cage cleans.
	Study Procedures	How invasive a study is and how an individual reacts to them. It can also be used to help refine studies by measuring welfare if factors are changed to a procedure.
	Surgical Procedures	How invasive a surgery is and how an individual reacts to them. Major surgeries with good recovery, can lead to good health, whilst those with poor recovery may lead to stress and other health problems.
	Veterinary Procedures	Medication administered may cause side effects in an animal, which can change behaviour or worsen health.

Table 1. The reasoning for choice of factor profiles for each rodent AWAG parameter.

An example of the scoring is shown in table 2, each one is defined to reduce any variance between observers.

Figure 1 is a graphical representation of the AWAG output outlining scores for physical, psychological, environmental and procedural parameters is shown as diamond plots at different days. AWAG scores were based on scoring definition illustrated in table 2, ranging from 1 (nothing observed) to 10 (very severe clinical signs). The factors profile and reasoning of choice of factor profiles based on which AWAG parameters are based are seen in table 1. Important events (e.g. single housing, cleaning) annotated in the relevant graphs.

Score	Definition
1	Nothing observed.
2	Mild clinical signs that show no impact on the animal's ability to perform normal behaviours. Full recovery expected. (e.g. pregnancy).
3	Mild clinical signs having a short-term impact on the animal's ability to perform normal behaviours. Full recovery expected.
4	Mild clinical signs having a longer-term impact on the animal's ability to perform normal behaviours. Full recovery expected.
5	Moderate clinical signs having limited impact on the animal's ability to perform normal behaviours. Full recovery expected.
6	Moderate clinical signs having limited impact on the animal's ability to perform normal behaviours. Recovery potential unknown.
7	Moderate clinical signs with medium to long term impact on animals' ability to perform normal behaviours. Recovery potential unknown.
8	Severe clinical signs but with short term impact and expected recovery OR moderate to severe signs with long term impact on animal's welfare and little chance of recovery.
9	Severe or chronic clinical signs that are having serious negative impact on the animal's ability to perform normal behaviours.
10	Severe clinical signs that are rendering the animal recumbent/unable to carry out any normal behaviour i.e. eating, drinking.

Table 2. Scoring definitions for clinical assessment factor, part of physical parameter.

Study 1: HSOD1-G93A vs. HSOD1-WT strains

The HSOD1-G93A (GA) strain of mice contains the SOD1 gene mutation. The SOD1 is a 153 amino acid cytosolic antioxidant protein and transgenic mice with human SOD1 gene mutation exhibits high degree of neuroinflammation which usually presents as paralysis of one or both hind legs and decreased life expectancy. In humans this is linked to the formation of amyotrophic lateral sclerosis (ALS).[40] Mice were housed in breeding pairs/trios and group housed after weaning with mice occasionally being individually housed due to aggression between cage mates. Generally cleaning activities were performed early in the week and in February 2022 there was some disruption due to general painting works in the facility corridors. Mice of both sexes were observed in breeding cages and stock cages from three weeks old. After weaning, mice with certain genotypes were given a mashed supplementation diet (soaked BK001 food pellets), to ensure that they were not dehydrated with muscle wasting and paralysis potentially starting in later in life at around four months of age.[41] The AWAG was recorded for HSOD1-G93A and compared to control HSOD1-WT mice (wild type).

Study 2: C57BL/6J vs. CRL:CD1(ICR) (inbred vs. outbred) strains

C57BL/6J mice are the most used inbred genetically modified mice strain due to its robustness and well-known genetic history;[42] being the first mouse strain to have its genome fully sequenced.[43] CRL:CD1(ICR) is an outbred strain; it is commonly used in obesity, ageing and toxicology studies. Individuals tend to be larger and more robust than average strains.[44,45] Mice were housed in breeding pairs/trios and group housed after weaning with mice occasionally being individually housed due to aggression between cage mates. Generally cleaning activities were performed early in the week. Mice of both sexes were observed in breeding cages and stock cages from three weeks old.

Study 3: Observation of rats undergoing catheterisation and drug self-administration addiction study

Lister hooded rats are commonly used in addiction studies for their increased executive function for multi-step tasks and more sociable nature.[12] In this study, 12 male Lister hooded rats (Crl:LIS) were used for self-administration studies to test a comparison of rats' motivation between cocaine and heroin, with their vocalisations being recorded, as described by Avvisati.[46] A group of 12 male rats were housed in groups of 4 in a reverse-light room in the facility and handled daily by the researcher for at least one week before surgery. Surgeries were performed between 28th February and 10th March 2022 and the rats were fed 1.5mg/ml meloxicam via syringe before they were anaesthetised with 100mg/kg

ketamine and underwent surgery. The right jugular vein was isolated via blunt dissection before a sterile, saline filled catheter (Instech Laboratories) was inserted and then sutured into the blood vessel. The catheter was tunnelled around the right shoulder subcutaneously and attached to a vascular access button (VAB Instech Laboratories) for easy administration of drugs. Saline was flushed through the VAB and the rats were placed in pairs for recovery. Meloxicam 0.5g was administered orally and Enrofloxacin through the VAB for 2 days and 5 days after surgery, respectively. Rats were housed individually for three days following surgery before being pair housed. After a week of recovery their food was restricted and the rats began being acclimatised to the test chamber. Rats underwent 35 sessions divided into several consecutive phases with testing occurring daily except for a two-day break after the self-administration training phase. The rats were observed on an approximately weekly basis in their home cage, during which the AWAG plotted the scores from the study.

Study 4: Observation of headpost surgery mice

Three male VIP-Cre mice (N 61, 62, 63) were observed after cranial window and headpost surgery was performed. Animals received an administration of Metacam for three days post-surgery and were then transferred back into an environmentally enriched cage in the reverse-light room of the facility with mice numbers 61 and 62 being singly housed and mouse 63 pair housed. The mice underwent behavioural training, with mouse number 63 also undergoing water restriction.

Results

Study 1: HSOD1-G93A vs. HSOD1-WT strains

Observation of physical, psychological, environmental and procedural parameters, revealed no significant difference between the two genotypes (Figure 2). As can be seen, there were some occasions during the recording period where there was an increase in the environmental, psychological and procedural scores for both strains suggesting no general difference between the two strains. Environmental changes in both strains were due to occasions where there were poor quality nests and mice being singly housed which impacted on the overall scores. The minor procedural increases related to animals being observed after cage cleaning (suggesting a short-term impact as mice become accustomed to their clean cages) and some facility refurbishment taking place when the animals were observed on 22nd February 2022 (painting of corridors) although interestingly there did not seem to be an impact on the HSOD1-G93A mice from the painting. Generally, the increase in psychological scores was related to the impact of single housing and relatively more singly housed mice in the wild type colony. No obvious signs of muscle weakness were detected in the G93A mice, as indicated by the low physical score throughout the duration of the study. It is not clear if the mash supplementation may have helped in that respect. Although this may suggest the mash was beneficial most mice were culled within a few weeks of being weaned so it is plausible that the mice may not have reached the age when most physical signs of neuroinflammation are seen.

Mice from both genotypes were housed in breeding pairs/trios and group housed after weaning. Physical, psychological, environmental and procedural AWAG scores were recorded for each genotype weekly for a period of 4 months. Each data point represents the mean score for each AWAG parameter from all mice cages observed for each genotype. AWAG scores were based on scoring definition illustrated in table 2, ranging from 1 (nothing observed) to 10 (very severe clinical signs). Table 1 represents the factor profiles and the rationale behind their selection for each AWAG parameter. Key events, such as single housing and cleaning are annotated in the relevant graphs.

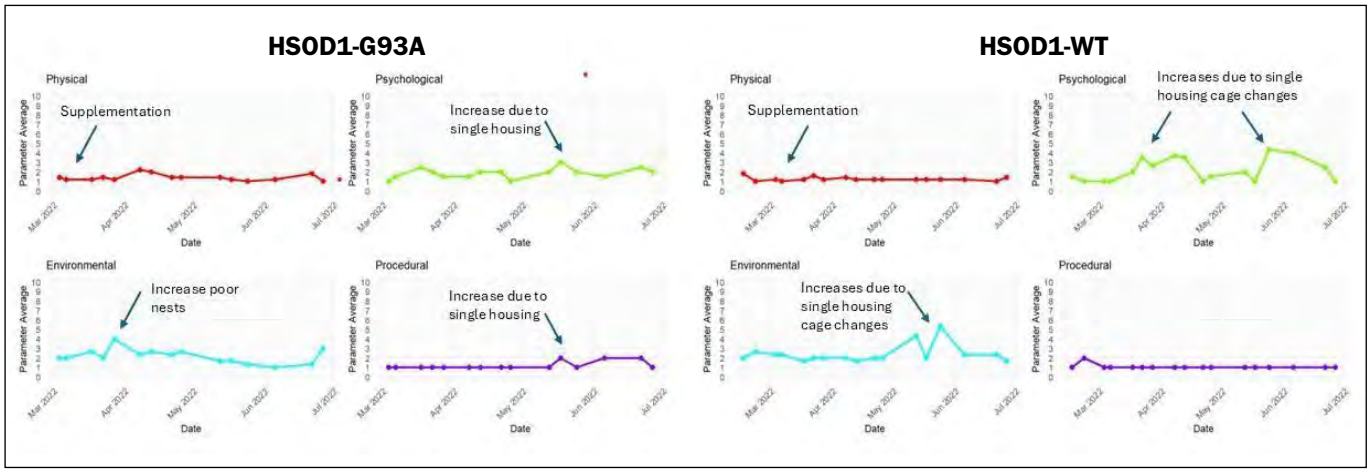

Figure 2. Study 1: AWAG scores plotted for each parameter in HSOD1-G93A and HSOD1 WT mice.

Study 2: C57BL/6J vs. CRL:CD1(ICR) (inbred vs. outbred) strains

Figure 3 shows the parameter averages of the C57BL/6J and CRL:CD1(ICR) colonies. The recording was over a 7-month period with normal colony management resulting in different breeding pairs being observed over the duration of the project as breeding pairs were retired and new ones set up. Most of the cages housed breeding pairs with the females at various stages of pregnancy or with litters. The average number of cages measured each day was approximately 6 for CRL:CD1(ICR) and 8 for C57BL/6J colonies. As can be seen in August 2022, CRL:CD1(ICR) had a higher score on the physical parameter compared to the C57BL/6J. This is possibly due to the higher body condition scores (BCS) of pregnant or lactating CRL:CD1(ICR) mice (CRL:CD1(ICR) mice can become very large and relatively immobile in the later stages of gestation) whereas the C57BL/6J tend to have lower BCS. Environmental scores for both colonies were similar with the higher scores being related to single housing. In July and August 2022 the psychological scores were quite high and this was related to cage cleaning taking place and the increased activity seen within the cages when observations were being recorded. Cleaning days on average led to an increase in these factors for CRL:CD1(ICR) whilst C57BL/6J did not show as high activity. Overall on average, C57BL/6J had more disorganised nests whilst lactating and the pregnant CRL:CD1(ICR) mice nests were larger in size as reflected by the higher environmental scores on occasions between July and September 2022 (C57BL/6J) and on occasions between March 2022 through to July 2022 (CRL:CD1(ICR)). CRL:CD1(ICR) mice tended to climb more often than the C57BL/6J, with males in both stock and breeding cages showing high activity on certain occasions as reflected by the high psychological parameter detected in certain days during the study from June through to August 2022. The C57BL/6J colony also had more isolated male mice, as C57BL/6J has a larger colony size and the strain is prone to barbering in grouped males.[47]

Mice from both strains were housed in breeding pairs/trios and group housed after weaning. Physical, psychological, environmental and procedural AWAG scores were recorded for each strain weekly for a period of 9 months. Each data point represents the mean score for each AWAG score parameter from all mice cages observed for each genotype. AWAG scores were based on scoring definition illustrated in table 2, ranging from 1 (nothing observed) to 10 (very severe clinical signs). The factors profile and reasoning of choice of factor profiles based on which AWAG parameters are based are seen in table 1. Important events (e.g. single housing, cleaning, pregnancy) are annotated in the relevant graphs.

Study 3: Observation of rats undergoing catheterisation and self-administration addiction study

The rats were observed on an approximately weekly basis, during which the AWAG plotted the scores from the study as shown below in Figure 4. The CWAS (environmental and procedural) was higher whilst the rats were acclimatising (21st February 2022), due to inactivity (average score of 3) and fearfulness (average score of 3). These scores were likely due to the rats acclimatising into the facility, scoring a 9 in contingent/procedural events. After a week the rats were less fearful and more curious with one rat showing some inactivity (3) on one occasion. This individual was singly housed at that time as its cage mate was undergoing surgery so it is possible they may have shown negative behaviour due to isolation and this may have led to the dip in the environmental parameter on AWAG. Despite the addition of a catheter, the rats showed no visible signs of suffering and were still alert and active as previously seen. As they began food restriction in March, there was a small dip in the physical parameter after food restriction which may be due to the rats becoming accustomed to the switch from ad-libitum to being food restricted and the activity of the rats increasing, with some food-aggression between cage mates being displayed and increased climbing

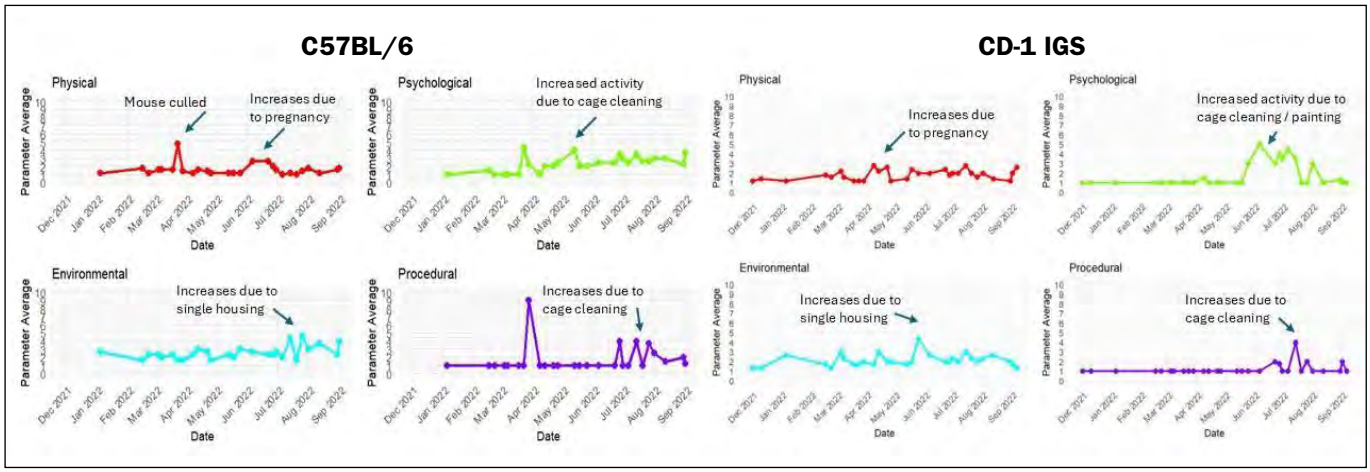

Figure 3. Study 2: AWAG scores plotted for each parameter in C57BL/6J and CD1 mice.

(psychological 2.5). Some aggression occurred towards researchers with one rat biting a researcher and was repeatedly aggressive towards them after being injected that was likely due to fear. One rat was euthanised earlier in the study due to a seroma forming around the VAB as a complication of the surgery. Another was euthanised later due to a leak in the catheter which could have increased the risk of becoming infected. Seromas are one of the more common complications of vascular catheterisation surgeries however they do not necessarily impact on the welfare of the rat as supported by the scores of the rat affected which did not change. Improved surgical technique would assist with prevention of seromas and leaks and minimisation of dead space when moving the catheter and implanting the VAB.[48]

Rats were singly housed for 3 days following surgery and were then pair housed. Physical, psychological, environmental and procedural AWAG scores were recorded for each rat weekly for a period of 2 months. Each data point represents the mean score for each AWAG score parameter from all rats observed. AWAG scores were based on scoring definition illustrated in table 2 ranging from 1 (nothing observed) to 10 (very severe clinical signs). The factors profile and reasoning of choice of factor profiles based on which AWAG parameters are based are seen in table 1. Important events (e.g. single/pair housing, cleaning, surgery, food restriction and acclimatisation) are annotated in the relevant graphs.

Study 4: Observation of headpost surgery mice

The observations of the three mice were recorded and displayed in figure 5. As can be seen, the AWAG score is high for each mouse in response to the surgical intervention but decreases over time following recovery and move into the reverse-light room (surgeries were performed between 11th May and 16th May 2022).

Mouse number 61's score begins to increase over the following weeks with the mouse losing its littermate and becoming singly housed. Its fearful behaviour begins to increase over time before it becomes inactive and refuses to eat and is euthanised on 10th July 2022. The mouse could have begun to show timidity due to being singly housed,[49] however mouse 63 was also singly housed around the same time but it did not behave in a similar way. Another theory is that negative interactions with humans and the moving of its cage created a conditional fear response. Common handling methods such as picking mice up by the tail can increase anxiety and would likely cause a fear response in the presence of humans.[50]

Mouse 63 was put onto water restriction around 22nd June 2022 and after an initial increase in the CWAS score this reduced shortly afterwards suggesting the mouse became accustomed to the water restriction before increasing again in mid-July which appears to be attributable to a cage move.

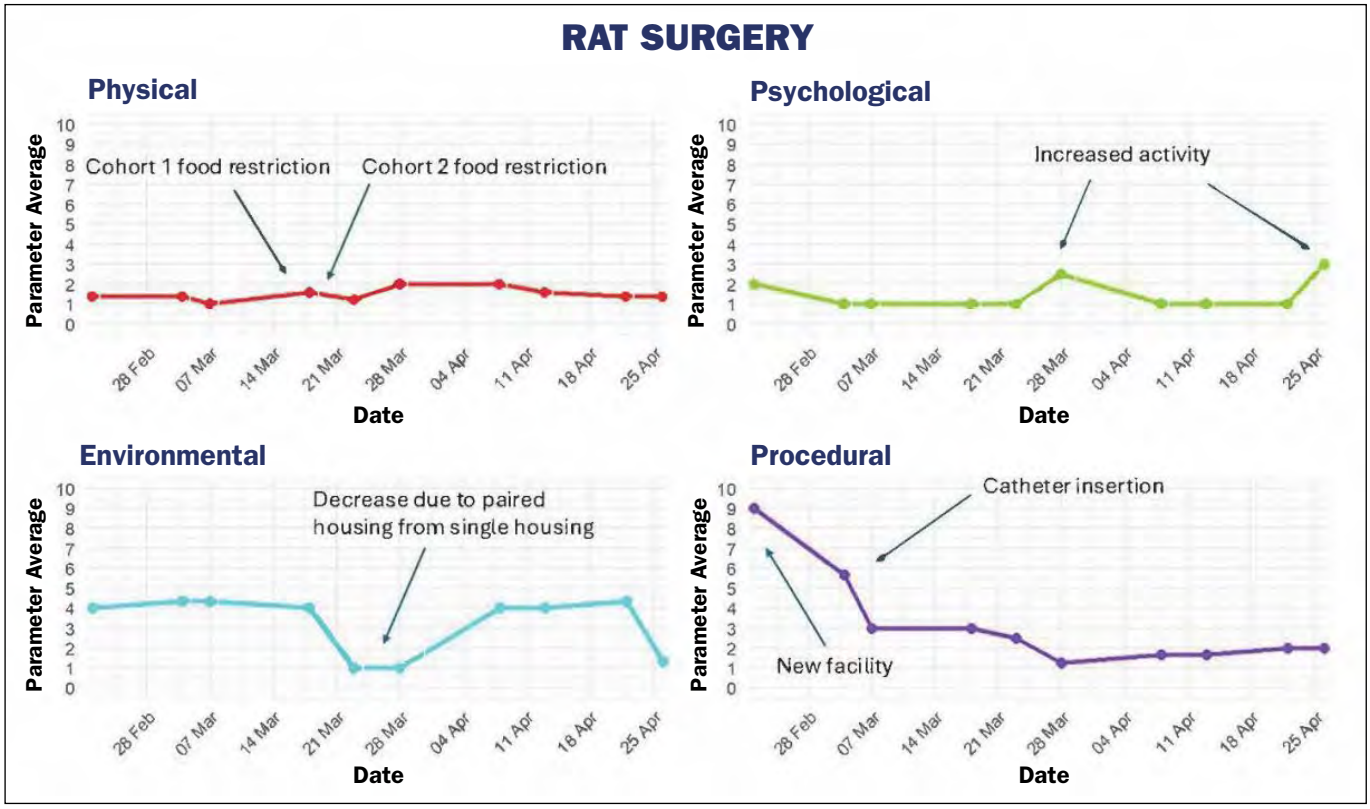

Figure 4. Study 3: AWAG scores plotted for each parameter in individual rats undergoing surgery (catheterisation) and drug self-administration.

Mouse 62's AWAG score dropped after going on water restriction in late May 2022 but returned to a more constant score in the middle of June.

The AWAG score overall increased hours after surgery due to inactivity however the week after recovery scores were lowered again. This suggests that similarly to the rats in study 3, the surgery did not have a long-term effect on the mice due to effective use of analgesics, post operative care and good aseptic techniques. It is difficult to determine the cause of mouse 61's decline and any other conclusions as the sample size is so small (n=3).

Mice were singly housed. Physical, psychological, environmental and procedural AWAG scores were recorded for each mouse weekly for a period of 14 weeks. Each data point represents the cumulative AWAG scores plotted as an aggregate for each of the 4 AWAG parameter in each individual mice undergoing surgery. AWAG scores were based on scoring definition illustrated in table 2, ranging from 1 (nothing observed) to 10 (very severe clinical signs). The factors profile and reasoning of choice of factor profiles based on which AWAG parameters are based are seen in table 1. Important events (e.g. single, cleaning, surgery, water restriction) are annotated.

Discussion

The study has demonstrated that the use of AWAG in a laboratory animal facility is beneficial in providing data that assists in assessing the impact of laboratory and experimental procedures in rodents. Overall, the scores were relatively low indicating the Animal Welfare was generally satisfactory. The physical and psychological AWAG parameters were the least variable even following highly invasive procedure such as surgery, where scores of the rats and mice did not significantly change most of the time. This observation suggests that with correct analgesics, post operative care and good aseptic techniques, positive welfare can be observed following surgery which was the most invasive procedure that AWAG was used for in this study.

Of interest some discrepancy was noted between researchers' observation of the rodents and the AWAG scores recorded. From the researcher's notes for the rat study (study 3), more negative behaviour was noted when restraining prior to recording sessions took place, with high frequency vocalisations, which is indicative of anxiety.[51] Although noticeable in certain rats, some physical problems with the VAB caps were not seen when recording the AWAG, likely due to lack of expertise. Better collaboration with researchers would allow for more accurate scoring. Handling during scoring could also increase accuracy but at the cost of the welfare to the animal.

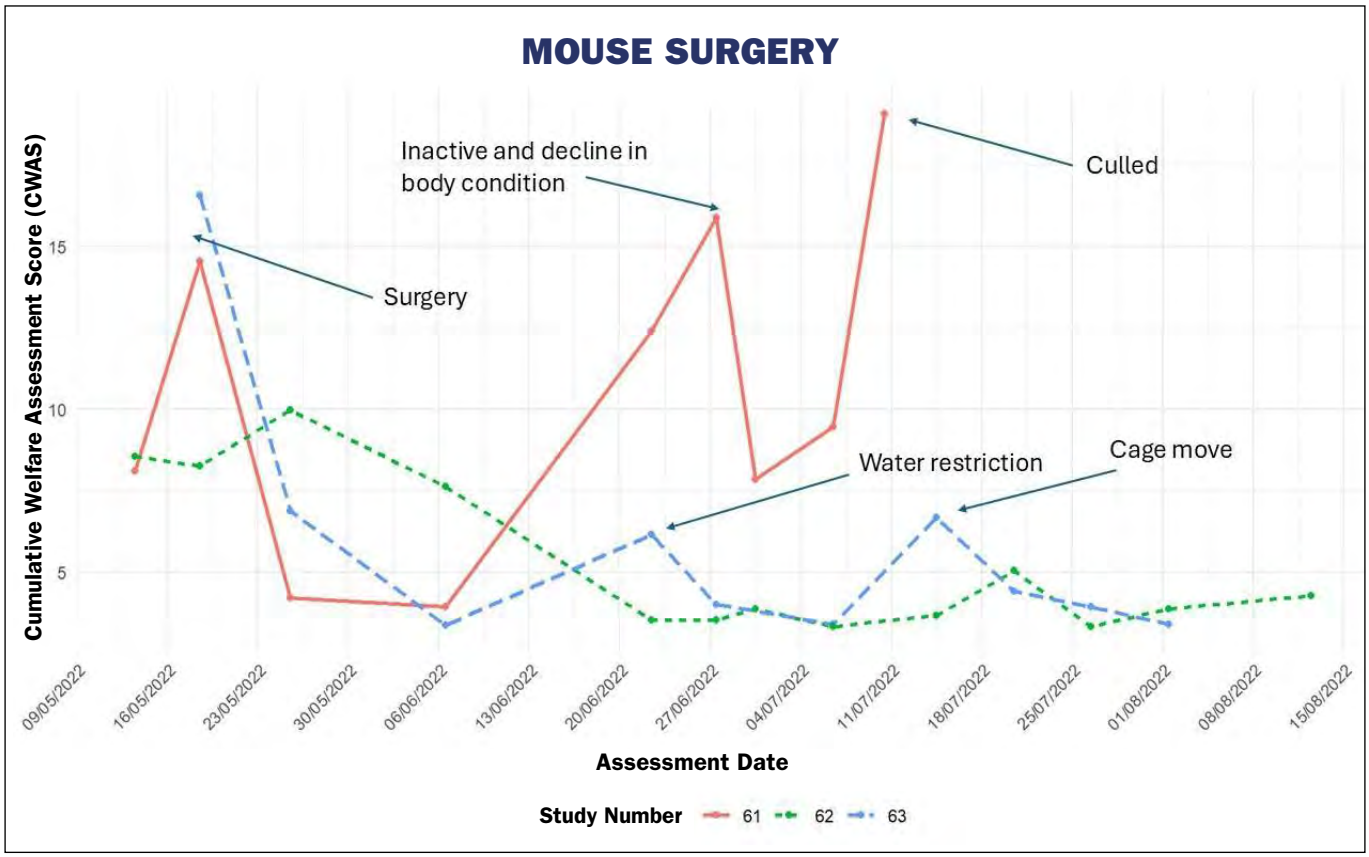

Figure 5. Study 4: Cumulative AWAG scores plotted as an aggregate for each of the 4 AWAG parameter in individual mice undergoing surgery (headpost surgery) and drug self-administration.

Husbandry activities also appeared to have a short-term impact on the animal's wellbeing as there were increased psychological scores observed during cleaning out and cage changing. It is plausible that this is due to noises and smells from cleaning products or technicians disturbing the mice which lead to an increase in movement and bar climbing as an exploratory behaviour. Males were the most active as females were left caring for their litters, in agreement with Newson et al observation.[52]

In terms of data recording and observations it was felt that measuring one point in time during the day at approximately weekly intervals led to difficulties in determining how the behaviour of animals may change throughout the day. For example, some behaviour and physical signs that were seen and recorded in the original study researcher's laboratory notes on the rats were not seen when recording findings on AWAG. This could be due to the animals not being handled when recording the scores as the animals were just observed in the cage under reverse light conditions. Indeed, behaviour may be different when handling as it has been shown promotes fear responses even if handled regularly. This is likely to be due to humans not being seen as a positive interaction compared with rodents owned domestically, who experience proper socialisation. Certainly, positive social interaction such as tickling in rats has been shown to decrease anxiety.[53,54] In the future, better collaboration with researchers could help to increase accuracy of results and see how the behaviour changes over the day. More work should also be conducted scoring animals using the AWAG whilst also handling them as part of study related procedures and this could be achieved by training researchers in the use of AWAG.

As discussed, the relatively high environmental score detected in C57BL/6J mice on some days, was thought to reflect their behaviour leading to disorganised and scattered nests. This is consistent with previous studies on nesting mice which have suggested that how mice shape their nests could be strain based as well as based on temperature and the amount of bedding given. In one study, Gaskill *et al*[55] found that C57BL/6J mice tended to change the shape of the nest relating to temperature and required more bedding to build a better nest than CRL:CD1(ICR). In the same study C57BL/6J also preferred nesting at 26°C to 30°C which is higher than most strains.

With reference to mouse 61's decline in study 4 more research would need to be performed to observe these mice to see if there is any way to intervene with future decline, such as rehousing a timid mouse with a previous littermate. The impact of water restriction should also be better researched as the results were inconclusive due to only one of the surgery mice undergoing it.

Activity and stereotypic behaviour were high in almost all cages that underwent food restriction, with some cases of food aggression in study 3 also being noticed. Although not all behaviour was seen, it is possible that this may have been a Pavlovian response to the humans observing the rodents, mistaking them for the researchers that usually feed them. All the rodents were fed at the same time daily when on food restriction but circadian rhythms could also be linked as these have been seen to increase stereotypic behaviour[56] in mice. In humans, circadian rhythms have been linked to microbiota-gut-brain axis, increasing pathology of disease and mental health disorders when the rhythm is in disarray.[57,58,59] Other studies have suggested a link between the biota changes and an increase in activity in mice[60,61] and gut metabolites have been found to increase anxiety in mice.[62] Most studies have been done using germ-free animals, however there is evidence to suggest that biota also changes with food restriction.[63] It cannot be ruled out that circadian rhythms, behaviour and the gut microbiome are all linked but more work would need to establish how food and water restrictions impact mice and rats particularly as there is little research understanding the additional behavioural impact of water restriction in rodents.

The AWAG shows good observational data of rats and mice in studies and could be used beneficially to demonstrate the impact of refinements on improving the level of Animal Welfare. For example, the data suggests that surgical procedures which one would probably expect to have a significant impact on welfare scores, do not. This is due to the pre and post operative care routines. From the results presented, the AWAG would be a useful model to help monitor scientific procedures which can be used to help researchers understand how research conditions impact and help interventions to improve Animal Welfare. There is also possible use in study or transit environments to refine methods and help reduce stress when transporting animals.

Although it would be unlikely for the AWAG to be used daily due to time constraints, integration of the tool into other commonly used databases and telemetry devices that are being developed to monitor rodent activity and facial expressions[64,65] would be beneficial. The visual aspect is useful at showing how the behaviour and health of animals change over time. Though it can be difficult to compare the scores of multiple graphs using the system, with further development this could be refined further.

Recently, artificial intelligence and computer monitoring have been used to help measure the environmental variables and score behaviour in livestock species.[66] In the future this could be applied to rodents allowing for time efficient and remote scoring. However it could be expensive given the number of cameras needed for all the cages to be seen. As a solution, cameras could be used on a small number of cages that make up the representative sample size of the population.

We have demonstrated that the AWAG is a useful model not just for assessing the impact of experimental procedures on rats and mice but also for monitoring the impacts of routine husbandry procedures, environmental changes, transportation and changes in social groupings in animals prior to entering studies. There are potential benefits and impacts of this system in relation to the implementation of the 3Rs and the ethical evaluation of the harm/benefit analysis for any research proposals. AWAG can be used to help researchers understand how their study protocols may negatively impact on animals, by providing data to assist with decisions relating to interventions designed to improve Animal Welfare, study design and reproducibility of data. Whilst the role of AWAG is not to monitor daily husbandry practices we have demonstrated that it is effective in showing how changes in routines (e.g. cleaning, different technicians, environmental changes, study protocols) can impact on the animals' welfare and this knowledge could be used to educate Animal Technicians and researchers in areas for consideration to help improve Animal Welfare and have a positive impact on data reproducibility. Further research should be performed to investigate changes in activity levels and behaviour in rats and mice that appear to take place during periods of food and water restriction.

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References

1 **Wolfensohn, S., Sharpe, S., Hall, I. *et al.*** (2015). Refinement of welfare through development of a quantitative system for assessment of lifetime experience. Animal Welfare 24: 139-149.

2 **Dennis, M., Charlton, M., Elam, S. *et al.*** (2024). Facility design and Management: Strategies for High Level Biocontainment Laboratory Animals Special Edition 0(0) 1–9 http://doi.org/10.1177/00236772241247104

3 **Justice, W.S.M., O'Brien, M.F., Szyszka, O. *et al.*** (2017). Adaptation of the Animal Welfare Assessment Grid (AWAG) for Monitoring Animal Welfare in Zoological Collections. The Veterinary Record, [online] 181(6), p.143. doi:10.1136/vr.104309.

4 **Wolfensohn, S., Shotton, J., Bowley, H. *et al.*** (2018). Assessment of Welfare in Zoo Animals: Towards Optimum Quality of Life. Animals, [online] 8(7), p.110. doi:10.3390/ani8070110

5 **Brouwers Stijn P. and Duchateau Marie José H.M.** (2021). Feasibility and validity of the Animal Welfare Assessment Grid to monitor the welfare of zoo-housed gorillas Gorilla gorilla gorilla Journal of Zoo and Aquarium Research 9(4) https://doi.org/10.19227/jzar.v9i4.607

6 **Free, D., Justice, W.S., Smith, S.J. *et al.*** (2022). An Approach to Assessing Zoo Animal Welfare in a Rarely Studied Species, the Common Cusimanse Crossarchus obscurus. Journal of Zoological and Botanical Gardens, 3(3), pp.420-441.

7 **Malkani, R., Paramasivam, S. and Wolfensohn, S.** (2022). Preliminary Validation of a Novel Tool to Assess Dog welfare: the Animal Welfare Assessment Grid. Frontiers in Veterinary Science, 9, https://www.frontiersin.org/articles/10.3389/fvets.2022.940017 doi:10.3389/fvets.2022.940017.

8 **Malkani, R., Paramasivam, S., Wolfensohn S.** (2024a). How does chronic pain impact the lives of dogs: an investigation of factors that are associated with pain using the Animal Welfare Assessment Grid (AWAG) Front. Vet. Sci. Sec. Animal Behavior and Welfare Volume 11 https://www.frontiersin.org/articles/10.3389/fvets.2024.1374858

9 **Malkani, R., Paramasivam, S., Wolfensohn S.** (2024b). A Multidimensional Evaluation of the Factors in the Animal Welfare Assessment Grid (AWAG) That Are Associated with, and Predictive of, Behaviour Disorders in Dogs. Animals 2024, 14, 528. https://doi.org/10.3390/ani14040528

10 **Ryan, M., Waters, R. and Wolfensohn, S.** (2021). Assessment of the Welfare of Experimental Cattle and Pigs Using the Animal Welfare Assessment Grid. Animals, 11(4),p.999 doi:10.3390/ani11040999

11 GOV.UK (2023). Annual Statistics of Scientific Procedures on Living Animals, Great Britain, 2023. [online] GOV.UK. Available at: https://www.gov.uk/government/statistics/statistics-of-scientific-procedures-on-living-animals-great-britain-2003

12 **Bryda, E.C.** (2013). The Mighty Mouse: the Impact of Rodents on Advances in Biomedical Research. Missouri Medicine, [online] 110(3), pp.207–11. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3987984/

13 **Poole, T.** (1997). Happy Animals Make Good Science. Laboratory Animals, 31(2), pp.116–124. Doi:10.1258/002367797780600198

14 **Jirkof, P.** (2017). Focus on Reproducibility. [online] Available at: https://www.nature.com/articles/laban.1216.pdf

15 **Peterson, N., Nunamaker, E. and Turner, P.** (2017). To Treat or Not to Treat: the Effects of Pain on Experimental Parameters. Comparative Medicine, (online) 67(6). Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5713161/pdf/cm2017000469

16 **Wolfensohn, S.** (2020). Too Cute to Kill? the Need for Objective Measurements of Quality of Life. Animals, 10(6), p.1054. doi:10.3390/ani10061054

17 **Wolfensohn, S.** (2023). Humane Endpoints and End of Life in Primates Used in Laboratories. In: Robinson, L.M., Weiss, A. (eds) Nonhuman Primate Welfare. Springer, Cham. https://doi.org/10.1007/978-3-030-82708-3_16

18 Legislation.gov.uk. (2020). Animals (Scientific Procedures) Act 1986. [online] Available at: https://www.legislation.gov.uk/ukpga/1986/14/introduction

[19] **Russell, W.M.S. and Burch, R.L.** (1959). The principles of humane experimental technique. [online Wheathampstead: Universities Federation For Animal Welfare. Available at: https://caat.jhsph.edu/principles/the-principles-of-humane-experimental-technique

[20] **Clemence, M.** (2019). 'Public attitudes to animal research in 2018' IPOS, Available at: https://www.ipsos.com/en-uk/public-attitudes-animal-research-2018

[21] **Graham, M.L. and Prescott, M.J.** (2015). The Multifactorial Role of the 3Rs in Shifting the harm benefit Analysis in Animal Models of Disease. European Journal of Pharmacology, [online] 759, pp.19–29. doi:10.1016/j.ejphar.2015.03.040.

[22] **Fenwick, N., Griffin, G. and Gauthier, C.** (2009). The Welfare of Animals Used in science: How the 'Three Rs' Ethic Guides Improvements. The Canadian Veterinary Journal = La Revue Veterinaire Canadienne, [online] 50(5), pp.523–30. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2671878/

[23] **Honess, P. & Wolfensohn, S.** (2010). A matrix for the assessment of welfare and cumulative suffering in experimental animals. Alternatives to Laboratory Animals. 38(3): 205-212.

[24] **Wolfensohn, S. and Honess, P.** (2007). Laboratory animal, pet animal, farm animal, wild animal: Who gets the best deal? UFAW Symposium: Quality of Life: The Heart of the Matter. Animal Welfare 16 supplement 1, 117-123.

[25] **Mellor, D.J., Beausoleil, N.J., Littlewood, K.E. *et al.*** The 2020 Five Domains Model: Including Human-Animal Interactions in Assessments of Animal Welfare. Animals (Basel). 2020 Oct 14;10(10):1870. doi: 10.3390/ani10101870. PMID: 33066335; PMCID: PMC7602120.

[26] **Gray, S.J., Jensen, S.P. and Hurst, J.L.** (2002). Effects of Resource Distribution on Activity and Territory Defence in House mice, Mus Domesticus. Animal Behaviour, 63(3), pp.531–539. Doi:10.1006/anbe.2001.1932.

[27] **Weber, E.M., Dallaire, J.A., Gaskill, B.N., *et al.*** (2017). Aggression in group-housed Laboratory mice: Why can't We Solve the problem? Lab Animal, 46(4), pp.157-161. Doi:10.1038/laBAN.1219.

[28] **Medendorp, W.E., Petersen, E.D., Pal, A. *et al.*** (2018). Altered Behavior in Mice Socially Isolated During Adolescence Corresponds With Immature Dendritic Spine Morphology and Impaired Plasticity in the Prefrontal Cortex. Frontiers in Behavioral Neuroscience, 12(87). doi:10.3389/fnbeh.2018.00087.

[29] **Kappel, S., Hawkins, P. and Mendl, M.** (2017). To Group or Not to Group? Good Practice for Housing Male Laboratory Mice. Animals, (online) 7 (12), p.88. doi:10.3390/ani7120088.

[30] **Balcombe, J.P.** (2006). Laboratory Environments and rodents' Behavioural needs: a Review. Laboratory Animals, 40(3), pp.217–235 doi:10.1258/002367706777611488.

[31] **Makowska, I.J. and Weary, D.M.** (2016). The Importance of burrowing, climbing and standing upright for Laboratory Rats. Royal Society Open Science [online] 3(6). doi:10.1098/rsos.160136.

[32] **Turner, C.A., Yang, M.C. and Lewis, M.H.** (2002). Environmental enrichment: Effects on Stereotyped Behaviour and Regional Neuronal Metabolic Activity. Brain Research, 938(1-2),pp.15-21. doi:10.1016/s0006-8993(02)02472-1.

[33] **Phillips, D., Choleris, E., Ervin, K.S.J., *et al.*** (2016). Cage-induced Stereotypic Behaviour in Laboratory Mice Covaries with Nucleus Accumbens FosB/FosB Expression. Behavioural Brain Research, 301, pp.238–242. doi:10.1016/j.bbr.2015.12.035.

[34] **Becker, H.C., Anton, R.F. and Randall, C.L.** (1987). Stereotypic Wall Climbing in Mice during Ethanol withdrawal: a New Measure of Physical Dependence. Alcohol, 4(6), pp.443-447. doi: 10.1016/0741-8329(87)90083-8.

[35] **Boleij, H., Salomons, A.R., van Sprundel, M., *et al.*** (2012). Not All Mice Are Equal: Welfare Implications of Behavioural Habituation Profiles in Four 129 Mouse Substrains. PLoS ONE, 7(8), p.e42544. doi:10.1371/journal.pone.0042544.

[36] GOV.UK (2015). Use, Keeping Alive and Re-use. [online] GOV.UK. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_dat a/file/660236/Use__Keeping_Alive_and_Re-use_Advice_Note.pdf

[37] GOV.UK (2006). Animal Welfare Act 2006. [online] Legislation.gov.uk. Available at: https://www.legislation.gov.uk/ukpga/2006/45/contents

[38] **Leung, V., Zhang, E. and Pang, D.S.** (2016). Real-time Application of the Rat Grimace Scale as a Welfare Refinement in Laboratory Rats. Scientific Reports, 6(1). doi:10.1038/srep31667.

[39] **Langford, D.J., Bailey, A.L., Chanda, M.L., *et al.*** (2010). Coding of Facial Expressions of Pain in the Laboratory Mouse. Nature Methods, [online] 7(6), pp.447–449. doi:10.1038/nmeth.1455.

[40] **Lee, J.D., Kamaruzaman, N.A., Fung, *et al.*** (2013). Dysregulation of the Complement Cascade in the hSOD1G93A Transgenic Mouse Model of Amyotrophic Lateral Sclerosis. Journal of Neuroinflammation, [online] 10(10), p.119. doi:10.1186/1742-2094-10-119.

[41] **Dupuis, L., Oudart, H., Rene, F., *et al.*** (2004). Evidence for Defective Energy Homeostasis in Amyotrophic Lateral sclerosis: Benefit of a high-energy Diet in a Transgenic Mouse Model. Proceedings of the National Academy of Sciences, [online] 101(30), pp.11159–11164. doi:10.1073/pnas.0402026101.

[42] **Engber, D.** (2011). The Trouble With Black-6. [online] Slate. Available at: http://www.slate.com/articles/health_and_science/the_mouse_trap/2011/11/black_6_lab_mice_and_the_history_of_biomedical_research.html

[43] Mouse Genome Sequencing Consortium (2002). Initial Sequencing and Comparative Analysis of the Mouse Genome. Nature, 420(6915), pp.520–562. doi:10.1038/nature01262.

[44] Envigo (n.d.). ICR (CRL:CD1(ICR)®) Outbred mice: Hsd:ICR (CRL:CD1(ICR)). [online] www.envigo.com. Available at: https://www.envigo.com/model/hsd-icr-Crl:CD1(ICR)

[45] Charles River (n.d.). CRL:CD1(ICR)® IGS Mouse | Charles River. [online] www.criver.com. Available at: https://www.criver.com/products-services/find-model/Crl:CD1(ICR)-igs-mouse?region=3671

[46] **Avvisati, A., Contu, L., Stendardo, E. *et al.*** (2016). Ultrasonic vocalization in rats self-administering heroin and cocaine in different settings:evidence of substance-specific interactions between drug and setting. Psychopharmacolgy, Volume 23, pp1501-1511.

[47] **Sarna, J.** (2000). The Dalila effect: C57BL6 mice barber whiskers by plucking. Behavioural Brain Research, 108(1), pp.39–45. doi:10.1016/s0166-4328(99)00137-0.

[48] NC3Rs (n.d.). Blood sampling: Vascular catheters | NC3Rs. [online] www.nc3rs.org.uk. Available at: https://www.nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-vascularcatheters#anchor_1

[49] **Krsiak, M., Valouchová, Z. and Zdárková, A.** (1974). Timidity Occurring on Social Interaction in singly-housed mice: the Role of the partner's Sex. Activitas Nervosa Superior, [online] 16(2), p.94. Available at: https://pubmed.ncbi.nlm.nih.gov/4472404/

[50] **Hurst, J.L. and West, R.S.** (2010). Taming Anxiety in Laboratory Mice. Nature Methods, 7(10), pp.825–826. doi:10.1038/nmeth.1500.

[51] **Gärtner, K., Büttner, D., Döhler, K., *et al.*** (1980). Stress Response of Rats to Handling and Experimental Procedures. Laboratory Animals, 14(3), pp.267–274. doi:10.1258/002367780780937454

[52] **Nevison, C.M., Hurst, J.L. and Barnard, C.J.** (1999). Why Do Male ICR(CRL:CD1(ICR)) Mice Perform bar-related (stereotypic) behaviour? Behavioural Processes, 47(2), pp.95–111. doi:10.1016/s0376-6357(99)00053-4.

[53] **Costa, R., Tamascia, M.L., Nogueira, *et al.*** (2012). Handling of Adolescent Rats Improves Learning and Memory and Decreases Anxiety. Journal of the American Association for Laboratory Animal Science, [online] 51(5), pp.548–553. Available at: https://www.ingentaconnect.com/content/aalas/jaalas/2012/000000 51/00000005/art00003

[54] **LaFollette, M.R., Cloutier, S., Brady, C.M., *et al.*** (2020). Changing Human Behavior to Improve Animal Welfare: a Longitudinal Investigation of Training Laboratory Animal Personnel about Heterospecific Play or 'Rat Tickling'. Animals, 10(8), p.1435. doi:10.3390/ani10081435.

[55] **Gaskill, B.N., Gordon, C.J., Pajor, *et al.*** (2012). Heat or Insulation: Behavioral Titration of Mouse Preference for Warmth or Access to a Nest. PLoS ONE, [online] 7(3), p.e32799. doi:10.1371/journal.pone.0032799.

[56] **Hawkins, P. and Golledge, H.D.R.** (2018). The 9 to 5 Rodent – Time for Change? Scientific and Animal Welfare Implications of Circadian and Light Effects on Laboratory Mice and Rats. Journal of Neuroscience Methods, [online] 300, pp.20–25. doi:10.1016/j.jneumeth.2017.05.014.

[57] **Matenchuk, B.A., Mandhane, P.J. and Kozyrskyj, A.L.** (2020). Sleep, Circadian Rhythm, and Gut Microbiota. Sleep Medicine Reviews, p.101340. doi:10.1016/j.smrv.2020.101340.

[58] **Clapp, M., Aurora, N., Herrera, L., *et al.*** (2017). Gut Microbiota's Effect on Mental health: the gut-brain Axis. Clinics and Practice, [online] 7(4). doi:10.4081/cp.2017.987.

[59] **Guo, J., Song, C., Liu, Y., *et al.*** (2022). Characteristics of Gut Microbiota in Representative Mice strains: Implications for Biological Research. Animal Models and Experimental Medicine, [online] 5(4), pp.337–349. doi:10.1002/ame2.12257.

[60] **Belkaid, Y. and Hand, Timothy W.** (2014). Role of the Microbiota in Immunity and Inflammation. Cell, 157(1), pp.121–141. doi:10.1016/j.cell.2014.03.011.

[61] **Heijtz, R.D., Wang, S., Anuar, F., *et al.*** (2011). Normal Gut Microbiota Modulates Brain Development and Behavior. Proceedings of the National Academy of Sciences, 108(7), pp.3047–3052. doi:10.1073/pnas.1010529108.

[62] **Needham, B.D., Funabashi, M., Adame, M.D., *et al.*** (2022). A gut-derived Metabolite Alters Brain Activity and Anxiety Behaviour in Mice. Nature, [online] pp.1–7. doi:10.1038/s41586-022-04396-8.

[63] **Genton, L., Cani, P.D. and Schrenzel, J.** (2015). Alterations of Gut Barrier and Gut Microbiota in Food restriction, Food Deprivation and protein-energy Wasting. Clinical Nutrition, 34(3), pp.341–349. doi:10.1016/j.clnu.2014.10.003.

[64] **Skinner, M., Ceuppens, P., White, P. *et al.*** (2019). Social-housing and Use of double-decker Cages in Rat Telemetry Studies. Journal of Pharmacological and Toxicological Methods, 96, pp.87–94. doi:10.1016/j.vascn.2019.02.005.

[65] **Hansen, M.F., Baxter, E.M., Rutherford, K.M.D., *et al.*** (2021). Towards Facial Expression Recognition for On-Farm Welfare Assessment in Pigs. Agriculture, 11(9), p.847. doi:10.3390/agriculture11090847.

[66] **Bao, J. and Xie, Q.** (2022). Artificial Intelligence in Animal farming: a Systematic Literature Review. Journal of Cleaner Production, 331, p.129956. doi:10.1016/j.jclepro.2021.129956.