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# Long-term effects of cage-cleaning frequency and bedding type on laboratory rat health, welfare, and handleability: a cross-laboratory study

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Short title: Effects of hygiene and bedding on rat welfare

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## Summary

Cage-cleaning is necessary for a hygienic environment, but since rats communicate using scent, they could suffer if their cages are cleaned too frequently. Male rats (Sprague–Dawley and Wistar) were kept for 5 months across 4 animal units. Their cages were cleaned twice-weekly, weekly, or every two weeks, and contained either aspen woodchips or absorbent paper bedding. Aggression, injuries and general health, weight gain, chromodacryorrhoea (a stress-related harderian gland secretion), handleability, and lung pathology were monitored, as was in-cage ammonia. Cleaning frequency had no clear impact on rat welfare, although frequent cleaning decreased ammonia concentrations and handleability, and non-aggressive skirmishing was highest in weekly cleaned rats. Surprisingly, bedding type did not affect ammonia, but all ammonia readings were unexpectedly low. However, rats kept on aspen had greater sneezing rates and lung pathology than those on paper bedding, but also had higher body weights. The results raise concerns about aspen bedding, which is relatively inert compared with other wood beddings, but nevertheless more harmful than paper. Animal unit significantly affected 8 of the 11 variables tested, having interactive effects on 5 of them. The study also demonstrates the interactive effects of different animal units, casting doubt on the feasibility of standardisation. We explored multiple variables of interest, so all findings require confirmation through further work. Nevertheless, cage-cleaning rates seem to affect socially housed male rats little, while bedding type has important effects on rat health.



**Keywords**: beddings; husbandry; respiratory pathology; rodents; welfare

Maintaining hygiene in rodent cages is necessary to keep animals and the humans working with them healthy. The most common concern arising from unclean cages is ammonia. The acceptable environmental limit for ammonia exposure in humans in the UK is 25 ppm per 8 h working day (Health and Safety Executive 1994). In contrast, there are no official guidelines for laboratory animals, but sensitivity thresholds may vary between species. Unlike human workers, laboratory animals remain in the animal unit, potentially exposed to ammonia for 24 h per day, every day. Moreover, in-cage ammonia levels tend to be much higher than those in the room. Nevertheless, in rodents, as in humans, high or prolonged ammonia exposure can cause respiratory and ocular damage (Broderson *et al.* 1976, Gamble & Clough 1976, Serrano 1971; Van Winkle & Balk 1986), and potentially damage skin in prolonged contact with soiled bedding (Berg *et al.* 1986, St. Claire *et al.* 1997). At concentrations of 100 and 300 ppm, ammonia has also been shown to cause lethargy in mice and rats (Tepper *et al.* 1985). Other potential concerns arising from soiled rodent cages include high levels of carbon dioxide (Hoglund & Renstrom 2001, Perkins & Lipman 1995, Reeb *et al.* 1998), the build-up of harmful micro-organisms (Borrello *et al.* 1998, Tuffery 1957), and accumulation of allergens from rodent urinary proteins that could harm human workers (Kacergis *et al.* 1996, Thulin *et al.* 2002).

As well as the potential health benefits, frequent cage-cleaning provides handling experience for the animals, improving handleability (Chantry 1964, Holson *et al.* 1991), and potentially reducing animal stress during experimental, veterinary and husbandry manipulations. Cage-cleaning also allows close inspection of the animals, enabling illnesses and injuries to be discovered and dealt with promptly.

However, since rodents rely heavily on scent for recognising and communicating with conspecifics (Doty 1986, Hurst *et al.* 2001, Singh *et al.* 1987),

they might benefit from a more stable in-cage olfactory environment. For example, rats use urine to signal dominance and mark out territories (Garcia-Brull *et al.* 1993, Krames *et al.* 1969), so when these signals are removed, aggression might result. Indeed, in male mice, aggression peaks after cage-cleaning (Gray & Hurst 1995, Van Loo *et al.* 2000), and this can be serious. In contrast, familiar rats tend not to wound each other, and their apparently aggressive 'skirmishing' behaviour can often be a form of play (Pellis & Pellis 1987, Burn *et al*. in press), and therefore presumably neither very stressful nor injurious. Nevertheless, in rats, cleaning does cause acute increases in cardiovascular parameters (Doerning 1998, Schnecko *et al.* 1998), and general activity (Burn *et al*. in press, Duke *et al.* 2001, Saibaba *et al.* 1996). These acute 'stress' responses could be due to the physical disturbance and handling associated with cleaning, as well as olfactory disruption.

In the long-term, frequent cleaning causes chronic stress in mice; more frequent cleaning reduces weight gain (Beynen & Vantintelen 1990) and increases pup-mortality (Peters *et al.* 2002, Reeb-Whitaker *et al.* 2001). If lower cleaning rates benefit rodents, their application would also be more economical for animal units, leading to less waste and reduced workloads for technicians.

Only one, small-scale, study has examined long-term effects of different cleaning frequencies in rats (Cisar & Jayson 1967). Here, twice-weekly cleaned breeder-rats produced a greater number of usable offspring, and pup weight-gain was higher than for weekly cleaned rats, although cannibalism was also higher. This suggests that the pups benefited from the increased hygiene conferred by the more frequent cleaning, while the mothers might have perceived a higher level of threat and instability. We know of no studies that have examined the effect of cage-cleaning rates on the welfare of rats in experimental facilities (usually all males).

Current practice regarding cage-cleaning frequency varies from 3-times weekly to every 2 weeks (fortnightly), with twice-weekly and weekly cleaning being most common in behavioural research (CB pers. obs.) and 2–3 times weekly being most common in toxicological studies (Wilson *et al.* 1995). Some guidelines recommend 'frequent' cage-cleaning to maintain hygiene (Home Office 1989, Wolfensohn & Lloyd 2002). In contrast, others suggest that scent marks should be maintained, either through less frequent cleaning (Hansen *et al.* 2000, Jennings *et al.* 1998) or by retaining parts of the substrate (Home Office 1995, Van Loo *et al.* 2003, Waiblinger 2002).

This study therefore aimed to investigate the long-term impact of cage-cleaning frequency on rat health and welfare. An absorbent paper bedding was used (Burn & Mason 2005), as well standard aspen woodchips, in an attempt to suppress ammonia and other pollutants, allowing infrequent cleaning. For practical relevance, male rats of the two most commonly used stocks of rat in the UK were chosen, and the experiment was repeated in four university animal units. Although within-cage parameters were standardised as much as practically possible, the units themselves were not expressly standardised, since they should have approximated the degree of standardisation between university units generally. Our aim was thus to verify that any long-term effects of cleaning frequency on rat health and welfare were generally applicable. Also, cross-laboratory studies are extremely rare, so we were interested in the effects of 'Unit'.

To assess the rats' health, we monitored weight gain, injuries, and signs of illness or loss of condition. In addition we assessed sneezing rates and lung pathology, to assess damage from ammonia and other pollutants, and measured in-cage ammonia directly. Activity levels were recorded to add information about the other variables

(e.g. were rats that had higher sneezing rates or lung pathology more lethargic?). To assess welfare, we measured aggression (a common outcome of cage-cleaning in mice as mentioned above) *versus* 'play', checked for stomach ulceration, and measured chromodacryorrhoea. Chromodacryorrhoea ('red/bloody tears') is a dark red stress-related secretion from the Harderian gland, which appears around the eyes and nose in response to restraint (Harkness & Ridgway 1980), joint pain (Kerins *et al.* 2003), morphine withdrawal (Hepburn *et al.* 1997, Rohde & Basbaum 1998), bright light (Hugo *et al.* 1987), and even mild disturbances (Mason *et al.* 2004). Finally, we examined how the different cleaning rates affected handleability, primarily as a practical consideration.

## Materials and methods

### Animals and housing

The subjects were 160 Wistar and 160 Sprague–Dawley male rats (Harlan, Bicester, UK). They weighed 50-70 g at the start of the experiment, and were 3-4 weeks of age. They were housed in single-stock groups of four in polycarbonate cages (L x W x H: 45-50 x 32 x 20-25 cm), containing a paper 'Des Res' shelter and a wooden chewing block (Lillico, Surrey, UK). The resulting 80 cages were allocated to four university conventional mixed-species animal units (A, B, C and D) in a balanced design, so each animal unit contained 20 cages. Cages of each treatment were randomly placed within the racks, and their positions were rotated every 2 weeks, after observations and weighing were completed. The experiment ran for 20 weeks.

Environmental parameters varied between animal units but were within UK Home Office limits (Home Office 1995). Specifically, across the four animal units, the ranges were 19-23 $^{o}$C for temperature, 40-65% for humidity, and 15-25 air

changes per hour for ventilation. However, Home Office limits may occasionally have been exceeded in Unit D due to building work. Light:Dark ratios were 12:12 in two units (Unit A: 7am to 7pm; Unit C: 8am to 8pm) and 14:10 in the other two (Lights on in Unit B: 6am to 8pm; Unit D: 5am to 7pm). All rats were provided with each unit's normal pelleted rat chow (Unit A: RM1 (E) pelleted diet (Special Diet Services, Witham, UK); Units B and C: RM3 pelleted diet, (Special Diet Services); and Unit D: FFG (Harlan Teklad, Bicester, UK)) and water *ad libitum*. Two of the animal units also provided seed mixtures (Units A and C: forage mix and mixed corn (Lillico); and Unit A: peanuts) in the bedding on a weekly basis. Cage-cleaning involved replacement of the cage body and all the bedding, although two of the animal units retained the cage lid and two did not. The shelters and chewing blocks were changed fortnightly, when all the cages were cleaned, but shelters were also changed at other times because most rats destroyed them within a few days.

Individually marking the rats was not a priority here because cagemates were statistically non-independent, and cage was our unit of replication. Also, non-invasively marking the rats would confer extra handling experience whenever the marks required renewing, making the rats unrealistic models of typical laboratory animals.

**Treatments**

Cages were cleaned twice-weekly, weekly, or fortnightly. They were either supplied with the aspen woodchips normally used by their respective animal units (Units A, B and D: grade 8 (Lillico); Unit C: QC bedding (B&K Universal ltd, Hull, UK)), or with absorbent paper bedding (Alpha-dri$^{TM}$ (Lillico), or occasionally a very similar product, Omega-Dri$^{TM}$ (Harlan Teklad)). The treatments were therefore as follows.

1. Twice-weekly cleaning, with aspen woodchip bedding
2. Twice-weekly cleaning, with Alpha-Dri bedding
3. Weekly cleaning, with aspen woodchip bedding
4. Weekly cleaning, with Alpha-Dri bedding
5. Fortnightly cleaning, with Alpha-Dri bedding

Aspen bedding was not used with the fortnightly cleaning frequency because previous studies had indicated that ammonia might reach levels unsafe for the humans, and perhaps the animals (Broderson *et al.* 1976, Carissimi *et al.* 2000, Ishii *et al.* 1998, Perkins & Lipman 1995). Technicians were instructed to fill all cages to a 2 cm depth of bedding.

**Body weight and condition**

The rats were weighed fortnightly until puberty (at 9-10 weeks), after which time they were weighed every 4 weeks. They were weighed at least 1 h after cleaning. Body weights, and all other measurements unless stated, were expressed as 'per cage' values because individual rats were not distinguishable.

During weighing, the rats were physically inspected for wounds. Wounds were counted, their locations on the body were noted, and the severity of each scored on a scale of 1-3 (see Table 1). Any rough or dirty pelages and areas of hair loss were noted, along with any rats that appeared lethargic or unusually resistant to handling. Cages with signs of diarrhoea were also noted every fortnight.

**Behavioural observations**

Starting from the third fortnight of the experiment, when the rats were 9-10 weeks old, behaviour was recorded for 45 min both on the day before cages of all

treatments were cleaned (i.e. when the cages were dirtiest), and immediately after cleaning had taken place. These observations were made between 8.30 and 10.30 am on both days (specific timings were pre-arranged with the animal unit technicians). The behaviours of interest, skirmishing and sneezing, were recorded whenever they were noticed during the 45 min observation period. This was possible because they could be heard, although sneezing bouts of short duration could sometimes not be located. On each of these days (18 in total), three scan samples were also taken for each cage, at 0, 15 and 30 min respectively, to record how many rats were active and how many were resting in each cage. Since resting behaviour is characteristically of a longer duration than sneezing and skirmishing, it should not have required such intensive observation.

Recording the data live meant that the observer's presence might have influenced the rats. However, observations were usually made while technicians carried out routine husbandry procedures in close proximity to the cages, so the rats would have been subjected to the presence of humans at these times, even without the observer. All behavioural and subjective observations were carried out by the same observer (CB) to ensure consistency. The observer was formally blind to the treatments for each cage, although the degree of soiling often made the treatments obvious.

**Chromodacryorrhoea scoring**

Chromodacryorrhoea was scored after each behavioural observation period. The rats were brought to the cage-front by the observer calling to them and gently tapping the bars. Their noses and eyes could then be easily seen, and were subjectively scored on a scale of 0 to 5, as shown in Table 1. The nose and each eye were scored separately. In addition, when the rats were physically inspected for

wounds, the area in $cm^3$ of chromodacryorrhoea that was visible as pale pink smudges on the fur was estimated.

**Ammonia measurements**

Ammonia concentrations were measured using a gas detection pump with glass tubes that detected ammonia at either 2-30 or 5-100 ppm (Shawcity Ltd, Oxfordshire, UK). Measurements were taken on the day before cleaning at 12, 16 and 18 weeks into the experiment. To obtain readings that reflected the concentrations within the undisturbed cages, the cages were left *in situ*, and the tubes were inserted between the bars at the front of the cage, where the spare water bottle would fit. The tube was held about 5 cm above the bedding to sample air at the level of an adult rat's head.

**Handling**

After 20 weeks, rats were selected for handling by the technician in their animal unit. Due to constraints on technicians' time, one rat per cage was handled. For each cage, the rat selected was either the first, second, third or fourth rat initially picked up, balanced across treatments, to avoid testing only the rats that were easiest to catch. The technician was blind to each rat's treatment group. The technician scored each rat as to how tense or relaxed it seemed (Table 1), and then put it into the restraint position. The observer (CB) touched the rat's belly in the area of an intraperitoneal injection to confirm that the rat was restrained securely. Ease of restraint, amount of squeaking, and attempts to bite were scored by the technician according to the systems detailed in Table 1.

## Histopathology

After 5 months, one rat per cage was anaesthetised with inhaled isoflurane (IsoCare, AnimalCare Ltd), given at 4% in oxygen at 6 l/min. The rat was then given an overdose of 1 ml pentobarbital (Euthatal, Merial Animal Health Ltd, Harlow) by intraperitoneal injection.

From each rat, the entire head, trachea, and lungs were placed into 10% neutral-buffered formalin. The stomach of each rat was opened, and those with visible irregularities or redness were also fixed for microscopic assessment of gastric ulceration. Following fixation, soft tissue and the mandibles were removed from the head and a 0.5 cm cross-section of the nasal cavities was taken immediately cranial to the eye. Single cross-sections of the lungs were taken across the entire width of one caudal lobe, the contralateral anterior lobe, and the mid-level trachea (and oesophagus). Stomachs were sectioned through the centre of each sample, which included the entire length of the specimen and both anatomical zones of the mucosa.

Tissues were processed, paraffin wax-embedded and sectioned at 4 µm. All sections were stained with haematoxylin and eosin. Sections were encoded and examined by an accredited veterinary histopathologist (MJD) without knowledge of the experimental groups. The main pathological changes in each section were recorded, including the distribution and nature of any inflammatory change. The lesions of the trachea, and anterior and caudal lung lobes were subjectively graded on a scale of 0–3 according to their severity (Figure 1).

## Statistical analyses

The software used was Minitab$^{TM}$ version 13.20 (Minitab Ltd, Pennsylvania, USA). Variables included in statistical models were cleaning frequency, bedding type,

rat stock, and animal unit. Unit was analysed as a fixed effect because we were interested in the relative influences of each one. Repeated measures were summarised by a mean value per cage over the entire study period, unless time effects were relevant. Parametric data were square-root transformed where necessary, and were analysed in two ways, as follows.

Firstly, to examine the effects of bedding, Unit, rat stock, and their interactions, the fortnightly (Alpha-Dri only) treatment was excluded, and a general linear model (GLM) was used, comparing the twice-weekly and weekly treatments. For non-normal data, the non-parametric equivalents used were the Mann-Whitney test or logistic regressions. Secondly, to investigate the effects of cleaning frequency, the three Alpha-Dri treatments were compared, again using a GLM. The Kruskall–Wallis test or logistic regression were used as the equivalent non-parametric tests. In both parametric models and logistic regressions, animal unit and stock and all their interactions were also included. Effects of time were examined using a repeated-measures analysis of variance, with cage and time as additional factors. In one GLM that showed non-orthogonality (chromodacryorrhoea, which required body weight to be included in the model), sequential sums of squares were used to calculate the F-ratios, and the sequence of variables in the model was rearranged to test the robustness of results.

Regressions were performed to test for correlations between parametric data, and for non-normal data Spearman rank correlations were used.

A split-half analysis was also carried on the resting/activity data to verify whether or not the instantaneous observations taken had been sufficient to build up consistent information about the cages. The regression between the odd and even

observations was significant (T = 4.68; $n = 80$; $P < 0.001$) allowing this variable to be used with confidence.

Multiple variables of interest were tested, but no adjustment was made for this multiple testing because the study was of an exploratory nature, and the risk of making a Type II error was therefore to be avoided (Bender & Lange 2001).

## Results

In total, 11 variables were tested (stomach ulceration, nasal pathology, loss of condition, and obvious symptoms of illness were rarely or never seen). Only the statistically significant effects of cage-cleaning rate, bedding type, and animal unit will be reported below.

Unfortunately, in Unit B, 12 Sprague–Dawley and 2 Wistar rats died during the experiment. The mortalities spanned all treatment groups, with no obvious pattern. Symptoms preceding death included noticeably increased sneezing rates and rasping breath, lethargy, pilo-erection, and weight loss. The first four rats to develop these symptoms were given antibiotics, but when they failed to recover, any further rats developing the symptoms were euthanased for humane reasons. A *post-mortem* screening of one of these rats (4 months of age) showed infection with *Mycoplasma pulmonis*, and intercurrent infections of Kilham rat virus and a rat parvovirus.

Data from the eight affected cages were only included for the period when four apparently healthy rats were still present. For measurements taken at the end of the study (final body weight, handleability and histopathology), data from this animal unit were excluded completely because there were too many missing values for statistical evaluation.

Details of the known pathogens in each animal unit are included in Table 2, for comparative purposes.

**Cage-cleaning frequency**

Pre-cleaning ammonia concentrations were, unsurprisingly, highest in the fortnightly treatment and lowest in the twice-weekly one ($F_{2, 24} = 11.09$; $P = <0.001$) (Figure 2). The concentrations were all relatively low, rarely exceeding 25 ppm even in the fortnightly cleaned cages.

Skirmishing was more frequent in the weekly cleaned cages than in those cleaned twice-weekly or fortnightly ($F_{2, 24} = 4.50$; $P = 0.022$) (Figure 3). All skirmishing bouts observed were more like the play-fighting described by Pellis and Pellis (1987) than serious aggression. That is, the target of attack was the nape of the neck rather than the rump, (non-injurious) biting was only observed on one occasion, and pilo-erection was never observed. Furthermore, skirmishing frequency did not correlate with incidence of wounds or with chromodacryorrhoea. The frequencies of skirmishing bouts did not decrease significantly during the course of the experiment, although they were consistent over time within cages ($F_{79, 424} = 2.04$; $P = <0.001$).

During the handleability tests, rats cleaned fortnightly struggled, squeaked and bit less than the rats cleaned more frequently ($F_{2, 18} = 4.17$; $P = 0.032$) (Figure 4). There was no obvious relationship with ‘tension’ scores, however.

There were no other significant effects of cage-cleaning frequency.

**Bedding material**

Sneezing rates were significantly higher on aspen bedding than on Alpha-Dri ($F_{1, 32} = 13.53$; $P = 0.001$) (Figure 5). This was not due to the respiratory symptoms of the *Mycoplasma pulmonis* infection in Unit B, because the effect was still significant

when it was excluded from analysis ($F_{1, 24} = 5.87$; $P = 0.023$). The difference between sneezing rates on the two beddings was already apparent at the start of formal data collection, when the rats had spent 6 weeks housed on their respective beddings ($F_{1, 32} = 10.00$; $P = 0.003$). Sneezing rates did not correlate with ammonia concentrations, chromodacryorrhoea, activity levels or body weight.

The pathology scores of the caudal lung sections were also significantly greater in rats kept on the aspen chip bedding than on the Alpha-Dri ($F_{1, 42} = 4.61$; $P = 0.038$), with 48% of the rats on aspen having moderate–severe pathology compared with 26% of those on Alpha-Dri (Figure 6 a and b). The caudal lung pathology correlated positively with that of the anterior lung ($R^2 = 27.6\%$; $T_{2, 57} = 2.77$; $P = 0.008$), but the tracheal pathology showed no relationship with either lung section. None of the pathology scores correlated with sneezing, chromodacryorrhoea, body weight, ammonia concentration or activity levels (regardless of whether Unit B was included in analyses or not).

The nature of the lung pathology was an interstitial pneumonia characterised by macrophage infiltration with fewer neutrophils and eosinophils. This was generally a mild and diffuse change affecting the caudal more than the anterior lung lobes. Some samples had foci of more severe change involving consolidation of lung tissue, and these lesions invariably involved the caudal lobes. Two sections showed large aggregates of highly vacuolated macrophages within the severely affected foci. Where the lung was not collapsed, there were occasional sections with prominent alveolar macrophages. The bronchi were not involved in this inflammatory change. In most sections there were prominent accumulations of bronchial associated lymphoid tissue.

Most of the tracheal sections were histologically normal, although some samples showed mild to moderate focal tracheitis. The dominant inflammatory cell in

these lesions was the macrophage. The tracheal epithelium was always intact and there was no evidence of squamous metaplasia or hyperplasia. In some samples there was a mild mixed mononuclear infiltration of the lamina propria. Overall, there was no evidence of inflammatory change to the nasal mucosa in these rats.

Rats kept on aspen bedding were heavier than those on Alpha-Dri ($F_{1, 24}$ = 8.92; $P$ = 0.006) (Figure 7). This bedding effect was significant from the first weighing session, only 2-5 days after the rats arrived in their respective animal units ($F_{1, 24}$ = 6.02; $P$ = 0.022). However, these early body weights did not predict adult weight when included in the GLM, while bedding type remained significant ($F_{1, 21}$ = 6.59; $P$ = 0.018).

Surprisingly, bedding had no significant effects on ammonia production ($F_{3, 32}$ = 0.03; $P$ = 0.855) (see Figure 2).

**Animal unit**

As shown in Table 3, animal unit had a significant interactive or main effects on 8 of the 11 testable variables. This contrasts with the effects of rat stock, which had no main effects on any of the variables, and only 4 interactive effects all of which were with Unit.

There were significant animal unit by stock interactions on body weight ($F_{2, 21}$ = 12.63; $P$ = <0.001), skirmishing ($F_{3, 32}$ = 7.80; $P$ = <0.001), resting levels ($F_{3, 32}$ = 3.31; $P$ = 0.032) and chromodacryorrhoea ($F_{3, 28}$ = 8.75; $P$ =<0.001) (Figure 8 a-d). For ammonia, there was a significant interaction between animal unit and cleaning frequency in the GLM that tested for bedding effects: it was only in Units A and B that cages tested 6 days after cleaning had more ammonia than cages tested after 2-3

395 days ($F_{3, 32}$ = 3.54; $P$ = 0.026) (Figure 8e). There was also a tertiary interaction
396 between stock, animal unit and bedding for body weight ($F_{2, 21}$ = 3.80; $P$ = 0.039).

397 ## Discussion

398 ### Cleaning frequency

399 Our main interests were the effects of cage-cleaning frequency and the two
400 bedding types on the net health and welfare of the rats, and the effects of the different
401 animal units on these parameters.

402 Cleaning frequency only affected 3 of the 11 measurable variables: ammonia
403 concentration, skirmishing and handleability. As expected, more ammonia was
404 produced in the less frequently cleaned cages, although levels remained low relative
405 to previous studies (cf. Carissimi *et al.* 2000, Perkins & Lipman 1995). Skirmishing
406 was highest in the weekly cleaned treatment compared with either extreme, but as it
407 was play-like, non-injurious and seemingly unstressful, it was ambiguous as a welfare
408 indicator.

409 With respect to handleability, the rats in the fortnightly treatment were easier
410 to restrain, and squeaked and bit significantly less than those cleaned more frequently.
411 This has obvious practical implications, but the welfare indications are not clear cut.
412 At first sight it might seem that the fortnightly rats were ‘calmer’ (e.g. due to
413 potentially weaker negative-associations with humans). However, in a previous study,
414 rats that had never been handled showed higher anxiety-related scores in open field
415 tests than those handled twice-weekly, indicating that at least in those contrasting
416 handling schedules, experience of handling reduces rats’ fear (Holson *et al.* 1991).
417 Also, rats cage-cleaned more frequently settled-down more quickly after cage-
418 cleaning (Burn *et al*. in press). Therefore, it is possible that the fortnightly cleaned rats

were showing a passive stress response to being handled, perhaps just as they might 'freeze' in novel situations (e.g. Toates 1995).

Our methodology did not allow us to discriminate between individual rats, so there remains the possibility that different cleaning frequencies might have affected some rats differently from others. This could be particularly likely in the context of dominant and subordinate rats being affected differently by the removal of dominance scent marks. However, it would not be practicable to clean the cages of dominants at a different frequency from the cages of subordinates, since dominance ranks are obviously hierarchies that exist within the same cages. Therefore our findings are of practical relevance: of the commonly used cage-cleaning frequencies examined here, there appears to be no *overall* welfare benefit or harm associated with any of the frequencies. In future work it might be interesting to assess the effects of different cleaning frequencies on individual rats to build up a more detailed understanding of their welfare.

**Bedding**

Surprisingly, bedding type had no effects on ammonia generation, despite differing in absorbency (Burn & Mason 2005). However, bedding had some other unexpected but potentially important effects. Sneezing and respiratory pathology were significantly higher in rats housed on aspen than on Alpha-Dri. These results are surprising because aspen is generally regarded as relatively non-toxic, but to our knowledge, all previous relevant studies have been brief, and did not use a paper bedding comparable to Alpha-Dri or Omega-Dri (Holland & Griffin 2000, Odynets *et al.* 1991, Törrönen *et al.* 1989).

442 The histological analysis did not allow us to identify the agent responsible for
443 the pathology, but candidates could include (a) micro-organisms inherent in the
444 material (Ewaldsson *et al.* 2002, Kaliste *et al.* 2004), (b) toxic volatile substances
445 associated with the source material or processing products (Odynets *et al.* 1991,
446 Törrönen *et al.* 1989), and (c) cellulose dust (Milton *et al.* 1990, Tatrai *et al.* 1995,
447 Kaliste *et al.* 2004). Ammonia was not the cause because, not only did it not differ
448 with bedding type, but it did not correlate with sneezing or respiratory pathology.

449 In fact, the large differences between the animal units suggest that the harmful
450 agent(s) in the bedding might have interacted with the pathogens present in each unit
451 (Table 2), exacerbating or facilitating the development of respiratory symptoms. This
452 could also explain the high prevalence of caudal lung damage observed on both
453 beddings (only about 14% of the rats on aspen and 17% on Alpha-Dri had normal
454 caudal lung sections). Interestingly, sneezing and respiratory pathology did not
455 correlate, which could be partly due to the fact that sneezing was measured per cage
456 while lung pathology was scored per rat. The euthanasia of the clinically ill
457 individuals in Unit B could also have obscured any relationship by creating an
458 artificially low cut-off point. Nevertheless, it is possible that the sneezing and
459 respiratory pathology might have had different causal agents, both associated more
460 strongly with the aspen bedding.

461 The sneezing and respiratory pathology observed may have caused welfare
462 problems, e.g. discomfort or pain, although we saw no evidence of them causing
463 lethargy, weight loss, or increased chromodacryorrhoea. It is possible that the lack of
464 correlation between lung pathology and any of these other variables could be due to
465 the necessary exclusion of the clinically ill rats in Unit B, which may have been those
466 with the worst respiratory pathology.

Considering the impact of aspen on respiratory health, it was surprising to find that rats were heavier when kept on aspen than on Alpha-Dri. Reasons for this weight difference could include the fact that the rats nibbled and manipulated aspen bedding with their snouts more than Alpha-Dri (Burn *et al.* in press), which almost certainly included ingestion of the bedding. Pica, the ingestion of 'non-nutritive' substances, can occur for several reasons, including nutritional deficiency (Wallis de Vries 1996, Dunham *et al.* 1994). There is no *a priori* reason to suspect any deficiency in the rats, but even so aspen may have contained some nutritional incentive. Alternatively, the bedding could have been ingested for non-nutritive, 'hedonistic' reasons (the flavour or texture of the bedding, for example), and the weight gain could then have been due to the increased volume of fibre consumed, perhaps by increasing gut-fill or affecting gut development, as observed in pigs (Ramonet *et al.* 1999, Stanogias & Pearce 1985). No differences in the activity levels of the rats were found between the two beddings, but the weight differences could also relate to the beddings' thermal properties; if aspen prevented heat loss more effectively than Alpha-Dri, rats housed on aspen might have gained weight more rapidly for the same mass of food than those on Alpha-Dri. Further work could measure body length and condition, body fat, and gut contents, to assess the mechanisms behind these weight differences.

**Animal unit**

Animal unit affected 8 of the 11 variables tested, with significant interactive effects on 5 variables. In fact, only wound scores and two of the respiratory tissue scores showed no main or interactive unit effects. The differences between units could be striking. For example, the mean ammonia concentrations observed a fortnight after cleaning varied more than 30-fold between the most extreme units (Figure 8e).

The interactive effects of animal units are of particular concern because they demonstrate that the very direction and/or existence of significant differences can depend on the unit animals were kept in. For example, even a stock characteristic as stable as body weight can depend on the unit, as shown here by the typically lighter Wistar rats actually being heavier than the Sprague–Dawleys in Unit A (Figures 8 a). Reasons for this are unknown, but could include effects of that unit's diet or any of the other environmental conditions. Of course one possibility is that all the labels in animal Unit A were switched, particularly since 4 of the 5 interactions depend on this unit, but the unit has repeatedly assured us that this really was not possible. Furthermore, studies of mouse behaviour have shown that, in that species too, some strain differences can vary in magnitude and direction between different units, even when care is taken to standardise experimental and husbandry procedures (Crabbe *et al.* 1999, Salome *et al.* 2002, Wahlsten *et al.* 2003b). Assuming this is therefore a real interaction, the implications are serious.

We deliberately did not control the general environmental variables within the four animal units in this study, despite being careful to standardise in-cage variables, to test whether our results would generalise across similar systems. In university set-ups at least, units often house animals for several different researchers, and researchers rarely have complete control over environmental parameters, or even the diet that their animals are fed; in any case each researcher's requirements would often conflict with those of the other researchers using the unit. Moreover, some variables, such as ammonia concentrations, are not yet well-enough understood for accurate control to be possible. Unfortunately, here two of the animal units, Units B and D, experienced problems (disease and building works, respectively) that probably increased variation further. However, Tukey tests showed that the animal unit effects

were not solely due to these two animal units. The variation between the units was larger than we expected and raises questions about whether standardisation across units within the same university, let alone in different institutions and countries, is really possible.

Interestingly, of the 11 variables tested here, stock had no main effects. Strain differences are commonly acknowledged to affect many biological variables; for example, searching ISI Web of Science for the terms ‘strain differences’ and ‘rats’ revealed 725 records. In contrast, extensive searching for animal unit differences yielded just 2 meta-analyses (Haseman & Rao 1992, Kafkafi *et al.* 2003), and 6 cross-laboratory experiments (Crabbe *et al.* 1999, Lee & McClintock 1986, Salome *et al.* 2002, Wahlsten *et al.* 2003a, Wahlsten *et al.* 2003b, Wolfer *et al.* 2004). All of these studies, except one (Wolfer *et al.* 2004), found important inter-laboratory effects. In fact, inter-laboratory variation could be fairly widespread, given the frequency with which different research groups report conflicting findings. The prevalent implicit assumption among researchers that standardisation is generally achieved could therefore be very inaccurate.

## Conclusions

It seems that cage-cleaning frequency has little effect on the health and welfare of socially housed male rats overall, although it does affect ammonia levels, the frequency of non-aggressive skirmishing, and handleability. Since cleaning on a fortnightly regime seems not to benefit the rats significantly, we would hesitate to recommend its use on the basis that ammonia levels in other animal units are likely to be higher than those observed here (Broderson *et al.* 1976, Carissimi *et al.* 2000, Ishii *et al.* 1998, Perkins & Lipman 1995), and the technicians reported the odour of the

fortnightly cleaned cages as unpleasant. We have no welfare evidence to distinguish between the weekly and twice-weekly treatments, but weekly cage-cleaning might be preferable for cost and resource reasons.

Our findings suggest that paper bedding should be used instead of aspen chips, particularly in respiratory and dietary studies, to avoid the effects of aspen on the respiratory system and body weight. It should also be borne in mind that aspen might affect other bodily systems not examined here. Because of the health benefits of the paper bedding, its use might increase rat welfare, although we found no clear evidence that it increased welfare *per se* (as opposed to health) in this study. Also, we know of no preference studies that have used anything similar to Alpha-Dri, and in fact aspen beddings have to date been preferred by rats and mice when compared against a variety of other beddings (Mulder 1975, Odynets *et al.* 1991, Ras *et al.* 2002). Currently many medical and toxicity studies use wire cages to avoid interference from bedding materials, but it would be useful to discover if Alpha-Dri and similar products are as inert as wire; if so, perhaps their use could enrich the animals' environments without compromising the quality of research.

The striking differences we found between the four animal units in this study raise serious concerns regarding the practicability of standardisation. Further work on the causes of inter-unit differences would help with standardisation and the interpretation of unit effects. Until these differences are understood, carrying out studies in more than one animal unit would often be beneficial to assess the generalisability and robustness of results (Würbel 2000). However, such studies would usually increase the number of animals used in experiments, due to the increased variation inherent within their design (Festing *et al*. 2002). Therefore, considerable refinement of animal housing, procedures and experimental design

would be of crucial importance for ethical reasons. Animal experiments are only justifiable if they provide valuable new information with real external validity.

Because the exploratory nature of this study required the use of multiple testing, there is a probability that at least one of these 'statistically significant' effects results from a Type I error. Further work will therefore be necessary to confirm these findings. Such studies could also build upon this work, for example, by identifying the mechanisms behind the effects of aspen on respiratory health and body weight, and by assessing whether any other aspects of health and welfare are affected by bedding type.

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## Figure and Table Captions.

Table 1. Subjective scoring systems used during the study. Wound severity was scored as described in the table, and the number of wounds was also counted. The amount of chromodacryrrhoea present on the nose was scored separately from the amount present around each eye. The rats' tension, ease of restraint, squeaking and biting were all assessed during a handleability test. Wound and chromodacryorrhoea scores were subjectively made by CB, while the scores taken during the handleability test were made by the technician carrying out the handleability test in each animal unit.

Table 2. The known pathogens present in each of the conventional mixed-species animal units. A, B, C and D. Rats in Unit B had to be euthanased before the end of the study due to an outbreak of *Mycoplasma pulmonis*, combined with a background of Kilham rat virus and a rat parvovirus.

Table 3. The effects of animal units on the 11 testable variables. Animal unit had significant main effects on 6 variables and interactive effects on 5. Only 3 variables were unaffected. NS = 'not statistically significant'; NA = 'not applicable'. When interactions are significant, main effects are not reported because they are redundant. The variables that were measured but are not mentioned in this table were too rarely observed for statistical analysis to be possible.

Figure 1. Representative sections of the caudal lungs of rats (x20) showing the scoring system for the severity of interstitial pneumonia. 0 = normal; 1 = mild; 2 = moderate; and 3 = marked inflammation. The tissues were paraffin wax-embedded, sectioned at 4 µm, and stained with haematoxylin and eosin. Scoring was carried out in a blinded fashion by MJD.

Figure 2. The mean (+/- S.E.) ammonia concentrations within rat cages on the days before cage-cleaning, with cages being cleaned twice-weekly, weekly or two-weekly. Day 0 is the day that cages were cleaned. Ammonia increased with days since cleaning ($F_{2, 24} = 11.09$; $P = <0.001$), but bedding type had no significant effect.

Figure 3. The effect of cage-cleaning frequency on mean (+/- S.E.) numbers of skirmishing bouts per cage per observation before cage-cleaning. Rats in weekly cleaned cages skirmished more than those in twice-weekly or fortnightly cleaned cages ($F_{2, 24} = 4.50$; $P = 0.022$).

Figure 4. Mean (+S.E.) struggling scores during a handleability test for rats cleaned twice-weekly, weekly or two-weekly. The struggling score was a weighted combination of the following subjective scores: ease of restraint, amount of squeaking, and whether or not the rat attempted to bite. The rats cleaned every two weeks were easier to handle than those cleaned more frequently ($F_{2, 18} = 4.17$; $P = 0.032$).

Figure 5. The mean (+S.E.) number of sneezing bouts per cage per observation, separated by bedding type and animal unit. There were significantly more sneezing bouts on aspen bedding than on Alpha-Dri ($F_{1, 32} = 13.53$; $P = 0.001$) in every animal unit, and the animal units were significantly different to each other ($F_{3, 32} = 7.66$; $P = 0.001$).

Figure 6. (a) The mean (+ S.E.) histopathology scores of the caudal lung sections of rats housed on aspen or Alpha-Dri bedding, and kept in three different animal units. The scores are as follows: 0 = normal; 1 = mild; 2 = moderate; and 3 = marked inflammation. Rats in Unit B had to be excluded from analyses because of high numbers of mortalities, which resulted in too many missing values. Rats on aspen had higher caudal lung pathology scores than those on Alpha-Dri in every animal unit ($F_{1, 42} = 4.61$; $P = 0.038$). (b) The percentage of rats attaining each histopathology score for each bedding type. All rats are included in this graph, and because there were more rats housed on Alpha-Dri ($n = 42$) than on aspen ($n = 29$), the absolute numbers of rats attaining each score are included within the columns. About 46% of the rats on aspen had moderate–marked interstitial pneumonia, compared with 26% of those on Alpha-Dri.

Figure 7. The mean (+S.E.) final body masses of the 4 rats per cage for each stock and bedding type. Rats kept on aspen bedding were heavier than those on Alpha-Dri ($F_{1, 24} = 8.92$; $P = 0.006$), and Sprague-Dawleys were heavier than Wistars ($F_{1, 24} = 13.81$; $P = 0.001$).

Figure 8. The interactive effects of animal unit. The mean (+S.E.) (a) final body mass, (b) skirmishing frequency, (c) resting frequency (number of rats resting per observation per cage; maximum of 4 rats), and (d) chromodacryorrhoea score of the 4 rats per cage for each stock in each animal unit. Sprague-Dawleys were significantly heavier ($F_{2, 21} = 12.63$; $P = <0.001$), skirmished less ($F_{3, 32} = 7.80$; $P = <0.001$), rested less ($F_{3, 32} = 3.31$; $P = 0.032$), and had more chromodacryorrhoea ($F_{3, 28} = 8.75$; $P = <0.001$) than Wistars in every unit except for Unit A, where the situation was reversed. (e) The mean (S.E.) ammonia concentration in each unit depending on cage-cleaning frequency. It was only in Units A and B that ammonia was higher at 6 days after cage-cleaning than at 2–3 days ($F_{3, 32} = 3.54$; $P = 0.026$).

961 **Table 1**

| Parameter | Score | Description |
|---|---|---|
| Wounds | 1 | A small superficial graze or scratch |
| | 2 | A small, shallow wound |
| | 3 | A deeper wound in need of medical treatment |
| Chromodacryorrhoea | 0 | No visible chromodacryorrhoea |
| | 1 | One small (<1 mm in diameter) droplet of chromodacryorrhoea |
| | 2 | One larger droplet or a few small droplets of chromodacryorrhoea |
| | 3 | A few large droplets or many small droplets of chromodacryorrhoea |
| | 4 | About 25-50% of the nose covered or the eye surrounded by chromodacryorrhoea |
| | 5 | Over 50% of nose covered in chromodacryorrhoea or eye surrounded by it. |
| Tension during handling | 0 | The rat's muscles are relaxed, and it does not resist manipulation |
| | 1 | Some resistance to manipulation |
| | 2 | The rat's muscle tone is tense and stiff, and it is resistant to manipulation |
| Ease of restraint during handling | 0 | No struggling |
| | 1 | Some struggling but still easy to restrain |
| | 2 | Much struggling and difficult to restrain |
| | 3 | Impossible to restrain within the 2 min period |
| Squeaking during handling | 0 | No squeaking |

| | | |
|---|---|---|
| | 1 | One loud squeak or up to three quiet squeaks |
| | 2 | More than one loud squeak or more than three quiet squeaks |
| Biting during handling | 0 | Rat does not appear to attempt to bring its mouth close to the technician's hand |
| | 1 | Rat appears to bring its mouth into contact with the technician's hand but does not bite successfully |
| | 2 | Rat makes contact between its teeth and the technician's hand, and bites |

962

963 **Table 2**

964

| **Animal unit** | **Viruses** | **Bacteria** | **Protozoans and mycoplasmas** | **Parasites** |
|---|---|---|---|---|
| A | Minute virus of mice<br>Mouse hepatitis virus<br>Mouse parvovirus | *Pasteurella pneumotropica*<br>*Staphylococcus aureus* | *Spironucleus* sp.<br>*Trichomonas* sp. | 'Arthropods'<br>*Syphacia* spp. |
| B | Kilham rat virus<br>Rat parvovirus | *Haemophilus* sp.<br>*Pasteurella pneumotropica*<br>*Staphylococcus aureus* | *Mycoplasma pulmonis*<br>*Trichomonas* sp. | *Syphacia* spp. |
| C | Kilham rat virus<br>Rat parvovirus | *Haemophilus* sp.<br>*Pasteurella pneumotropica* | - | - |
| D | Minute virus of mice<br>Mouse hepatitis virus<br>Mouse parvovirus | *Pasteurella pneumotropica* | - | - |

965

966 **Table 3**

| Variable | Main effects of Unit (Statistic) | Direction of main effects (Highest to lowest) | Interactive effects (Statistic) | Interacting variable | Figure |
|---|---|---|---|---|---|
| Ammonia | - | - | $F_{3, 32} = 3.54$; $P = 0.026$ | Cage-cleaning rate | 8(e) |
| Body weight | - | - | $F_{2, 21} = 12.63$; $P < 0.001$ | Stock | 8(a) |
| Chromodacryorrhoea | - | - | $F_{3, 28} = 8.75$; $P < 0.001$ | Stock | 8(d) |
| Handleability | $F_{2, 24} = 4.72$, $P = 0.019$ | C>D>A | NS | - | - |
| Caudal histopathology | NS | NA | NS | - | - |
| Anterior histopathology | $F_{1, 42} = 5.23$, $P = 0.009$ | D>A>C | NS | - | - |
| Tracheal histopathology | NS | NA | NS | - | - |
| Resting/activity | - | - | $F_{3, 32} = 3.31$; $P = 0.032$ | Stock | 8(c) |
| Skirmishing | - | - | $F_{3, 32} = 7.80$; $P < 0.001$ | Stock | 8(b) |
| Sneezing | $F_{3, 32} = 7.66$, $P = 0.001$ | B>D>C>A | NS | - | - |
| Wounds | NS | NA | NS | - | - |

967

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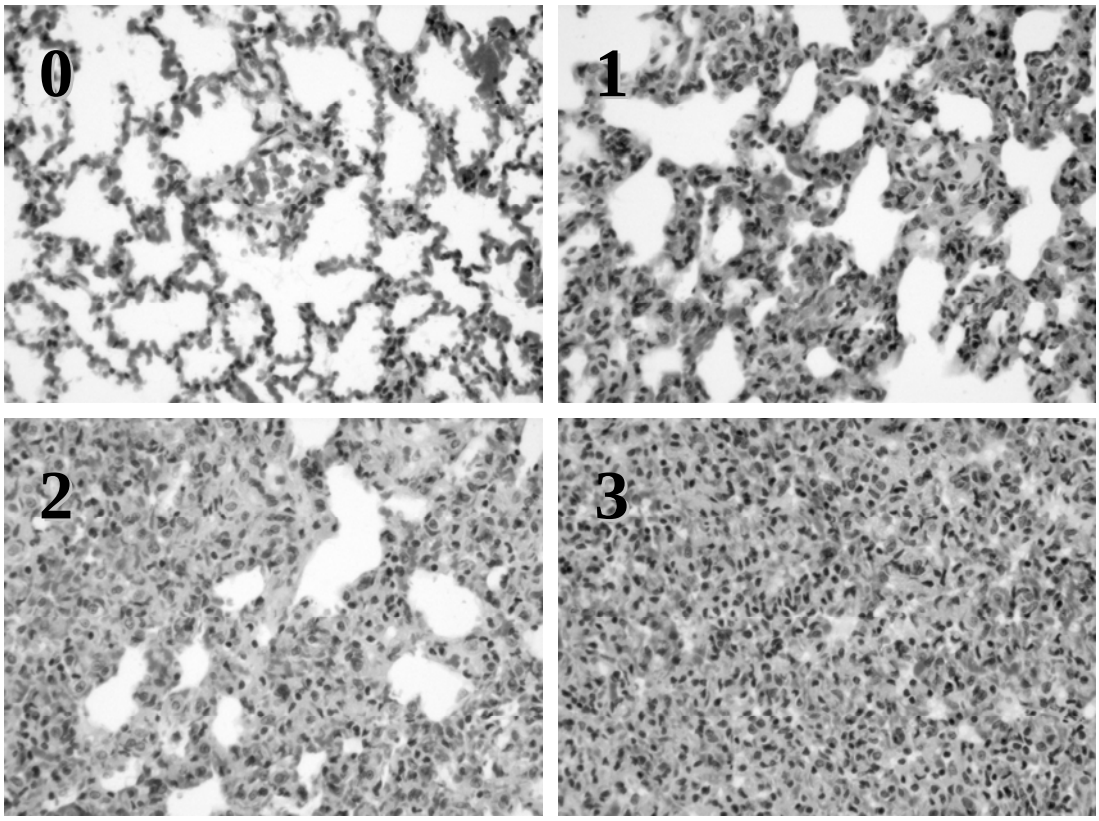
**Figure 1**



## Figure 2

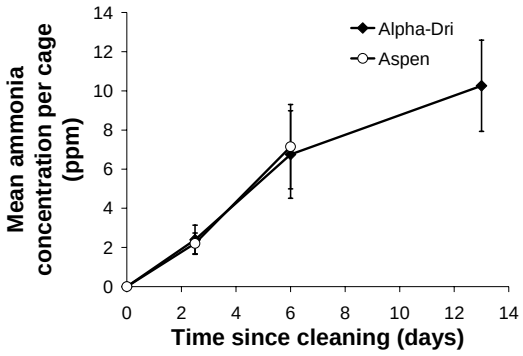
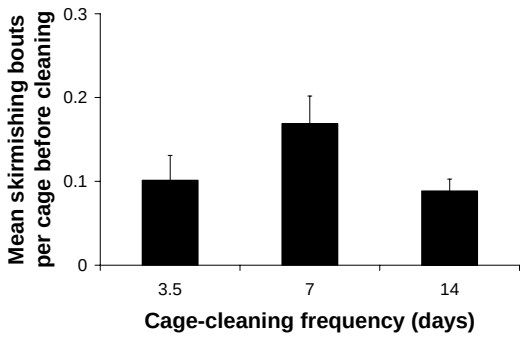
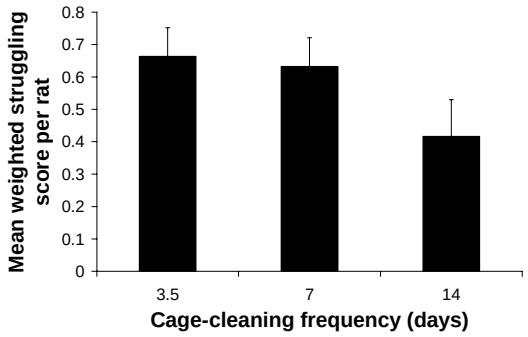

Mean ammonia concentration per cage (ppm)
Time since cleaning (days)
Alpha-Dri
Aspen
0
2
4
6
8
10
12
14

Figure 3


Mean skirmishing bouts per cage before cleaning
0.3
0.2
0.1
0
3.5
7
14
Cage-cleaning frequency (days)

1026 Figure 4

1027




1028

Figure 5

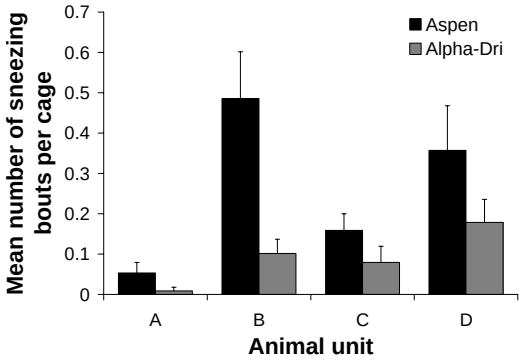

Mean number of sneezing bouts per cage
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A
B
C
D
Animal unit
Aspen
Alpha-Dri

Figure 6

(a) (b)

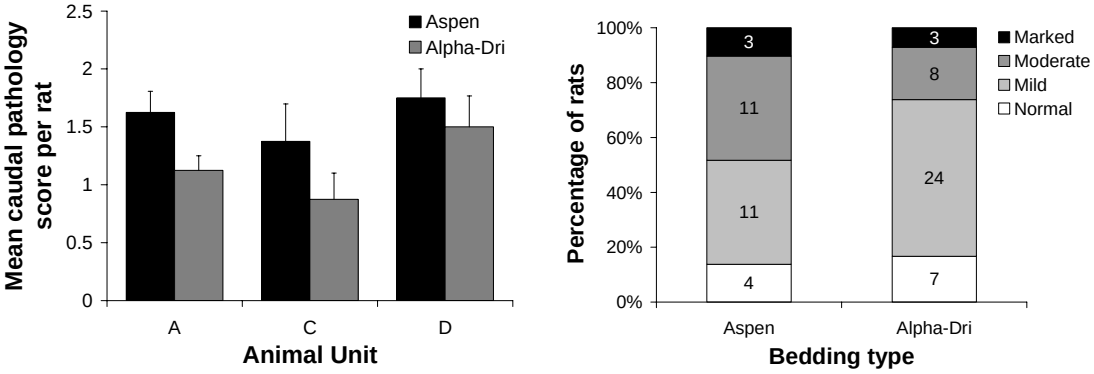

Mean caudal pathology score per rat
2.5
2
1.5
1
0.5
0
Aspen
Alpha-Dri
A
C
D
Animal Unit
Percentage of rats
100%
80%
60%
40%
20%
0%
3
11
11
4
3
8
24
7
Marked
Moderate
Mild
Normal
Aspen
Alpha-Dri
Bedding type

Figure 7

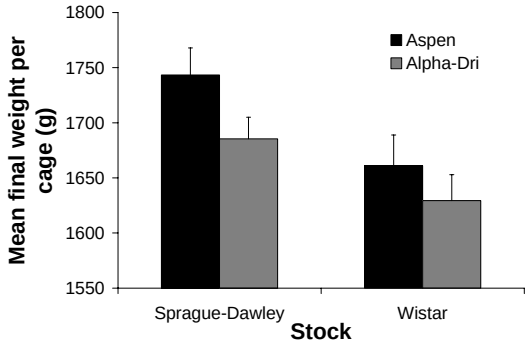
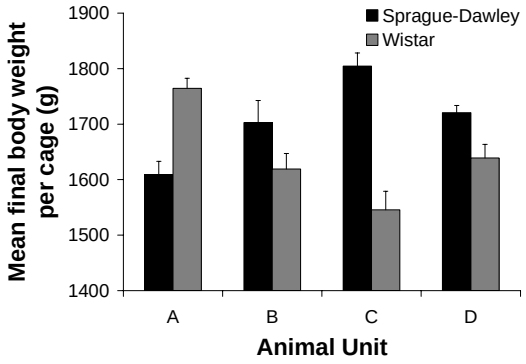
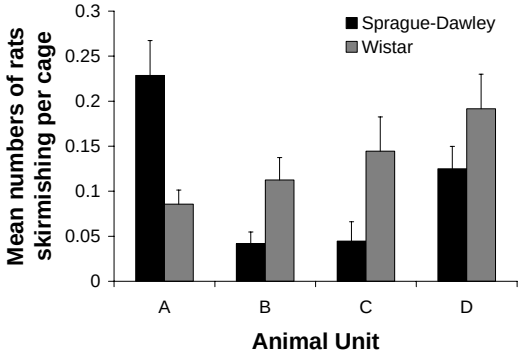

1800
1750
1700
1650
1600
1550
Mean final weight per cage (g)
Aspen
Alpha-Dri
Sprague-Dawley
Wistar
Stock

Figure 8

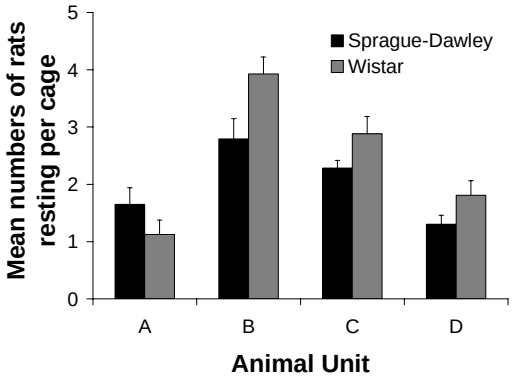
(a)



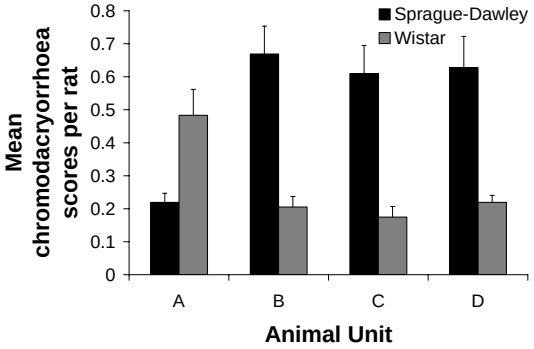

(b)




(c)




(d)




(e)