The University of Hong Kong

Laboratory Animal Unit

Final Report on the Study of the Effects of Stocking Density on the Performance and Well-being of Mice

Summary:

The Guide for the Care and Use of Laboratory Animals, NRC, 2011 (Guide) has recommendations on the amount of floor space required for each mouse in a cage but also states that the space recommendations presented are based on professional judgment and experience only and thus adjustment to the floor space should be assessed, reviewed and modified as necessary based on performance indices. A study was therefore carried out to evaluate the effect of increased cage stocking density on performance and well-being of mice housed at the LAU. Multiple studies conducted by others have concluded that mice were less stressed when housed more densely. In our study, mice were housed in cages with "floor area / animal" ratios of 0.68, 0.85, 1.07, 1.14 and 1.42 times that recommended by the Guide. Three strains of outbred and inbred mice (ICR, C57BL/6N, and BALB/c) were evaluated throughout 11-23 weeks for health and well-being including mortality, body weight, fecal corticosterone level, organ weights, behavioral observations, cage ammonia level and bedding condition. Five transgenic mouse strains (CKS1, CD55, Boy/J, IRF4 and SRA) were also evaluated for health and well-being including mortality, fecal corticosterone level at body weights reaching 20-25g, 25-30g and >30g, organ weights, behavioral observations and cage sanitation. Significantly reduced adrenal weight in BALB/c mice and CD55 and SRA transgenic mice and kidney weight in Boy/J transgenic mice suggest reduced stress in these animals. Significantly increased testicular weight of CD55 transgenic mice housed in a higher stocking density may indicate increased reproductive capacity in those mice. Allogrooming was noted at nighttime videotaping of mice housed in stocking density of 4 and 5 mice/cage. For the other traits studied, we have tested that increased housing density had no significant effect. These results concluded that the performance and well-being of mice are not compromised when they are housed at a floor area/animal ratio lower than that recommended by the Guide and in some strains, a smaller floor area/animal is even beneficial to the animals. Therefore our results indicate that mice can be housed at a higher stocking density (i.e. 5 adult mice per cage with a floor area of size 330cm^2) than that recommended by the Guide.

Objective:

The aim of this project was to evaluate the effects of an increase in stocking density in mouse cages when compared to that recommended by the Guide for the Care and Use of Laboratory Animals, NRC, 2011 (Guide). This serves to assess and review the performance indices of space allocations, and to provide scientific justification for alternatives to the recommendations.

Background

The Guide for the Care and Use of Laboratory Animals, NRC, 2011 (Guide) has recommendations on the amount of floor space that should be allocated to each mouse in a cage: an adult mouse of 15-25g required 77.4cm^2 and that of over 25g required 96.7cm^2 (Table1). In the section under "Space" in the Chapter on "Environment, Housing and Management", the Guide states that "space allocations should be assessed, reviewed , and modified as necessary by the IACUC (animal ethic committee) considering the performance indices include health, reproduction, growth, behavior, activity and use of space[1]". The Guide also states that "space recommendations presented are based on professional judgment and experience. Adjustments to the amount and arrangement of space recommended should be reviewed and approved by the IACUC and should be based on performance indices related to animal wellbeing and research quality." These guidelines suggested by the Guide are adopted by the AAALAC International.

As a comparison to the Guide, the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (ETS No. 123) Appendix A, 2006 has similar recommendations but a higher density allocation to each animal (Table 1).

The number of animals which can be housed in a standard mouse cage used by the Laboratory Animal Unit (LAU) is also listed in Table 1. Based on the recommendations of the Guide and ETS, a LAU cage (NKP M2 cage of size 330cm^2 or Tecniplast 1144B cage of 335cm^2) can hold 4.2 or 4.7 mice per cage respectively for mouse of body weight up to 25g, and 3.4 or 4.1 mice per cage respectively for mouse of body weight ≥25g.

Multiple publications, including studies performed by the Jackson Laboratory and the Harlan Laboratories (both are reputable global commercial suppliers of laboratory mice), have investigated the effects of housing density [3-9]. These studies have shown that mice were less stressed when housed more densely, as assessed by mortality, behavior, immune function, adrenal weight and heart weight. Van Loo and colleagues [7,10] have demonstrated that mice, especially males, are less aggressive when they are offered with a reduced floor space per mouse. Overall, the studies that examined the effects of housing animals at different densities concur that the performance and well-being of mice are not compromised when they are housed at densities higher than that recommended by the Guide. Taken together, a number of strains have already been tested including C57BL/6, BALB/c, DBA/2J, 129S1, and C57BL/6J x 129S1/SvImJ mice. A summary of recent publications on the effects of stocking density on wellbeing of laboratory mice is attached at Appendix 1.

The Guide has recommended a bigger space allocation per mouse as the body weight increased (Table 1). For instance, a mouse of 20g would require 77.4cm^2 per animal while one at 25g would require 96.7cm^2 of space per animal. In such circumstance, mice will have to be re-mixed in different cages with other mice when body weight increases. However, a lot of experimental design requires a consistent grouping of animals during the study period and any negative impact of remixing of mice might confound the experimental data. A study investigated the common practice of mixing mouse litters to obtain equal group sizes has revealed that both male and female mice in undisturbed litters thrived better than those in disturbed litters and a higher variance of body weight was observed in disturbed litters than in undisturbed ones[11]. In addition, fighting is more likely to be observed in re-mixing groups of mice, especially in males. This illustrates mixing of mice in order to achieve a strict stocking density has negative effects on the animal wellbeing and the results in consequence, confound research data.

In the current study, we have evaluated the effect of stocking density on the performance and wellbeing of outbred, inbred and transgenic mice held in the LAU. Mice with body weight up to 30g were housed in cages with "floor area / animal" ratios of 0.68, 0.85, 1.07, 1.14 and 1.42 times that recommended in the Guide. We measured the mortality, body weight, behavior, fecal corticosterone and organ weights (adrenal glands, heart, kidney and testes). The cage microenvironment has also be evaluated by measuring of the ammonia level and cage sanitation.

Table 1. Recommended cage space for mice housed in groups by the Guide and ETS No. 123 and the corresponding number of mice which can be housed in "LAU Cage" (i.e. "NKP M2" and "Tecniplast 1144B" cages used by the Laboratory Animal Unit)

	The Guide[1] (USA)		ETS No. 123[2] (Europe)	
Weight	Floor area/animal (cm^2)	No. of animals in a LAU Cage (330-335cm^2)	Floor area/animal (cm^2)	No. of animals in a LAU Cage (330-335 cm^2)
<10	38.7	8.5		
Up to 15	51.6	6.4		
Up to 20	77.4	4.2	60	5.5
20-25			70	4.7
Up to 25				
25-30	96.7	3.4	80	4.1
>30			100	3.3

Materials and Methods

Mice

Both outbred (ICR) and inbred (BALB/c and C57BL/6N) mice in the Specific Pathogen Free Breeding Area and transgenic mice (CKS1, CD55, Boy/J, IR4 and SRA knockout) in Minimal Disease Area of the Laboratory Animal Unit were studied. Mice were fed ad libitum with irradiated standard laboratory chow (PicoLab Rodent Diet, 5053, St. Louis MO) and UV treated/ 1µ filtered water. The animal room is maintained at 20-26^{o}C, 30-70% humidity and a 12:12 hour light: dark cycle beginning at 07:00. The animal room is supplied with HEPA-filtered air of at least 15 air changes per hour. Autoclaved Aspen chip (NorthEastern, USA) is used for bedding and changed biweekly. Enrichment (EnviroDri®, NJ USA) is given to each cage and the animals are housed in standard polypropylene mouse cage (NKP M2, Coalville, UK) of size 330cm^2.

Study protocol

Altogether 60 male and female mice for each strain of ICR, C57Bl/6N and BALB/c were randomly assigned to one of the 3 density groups at weaning (21 days): 3, 4 or 5 mice per cage. These density groups provide floor space per mouse of 110, 83 and 66 cm^2, corresponding to 1.42, 1.07 and 0.85 times respectively for mice up to 25 g, and 1.14, 0.85 and 0.7 times respectively for mice >25g when compared to that recommended by the Guide. Each density group has 5 replicate cages per sex for a total of 30 cages per strain. Animals are monitored daily for mortality, morbidity, aggressive behavior (barbering, fighting and tail biting), compulsive behavior (whisker-picking and barbering) and stress (body condition and facial expression[12]). In addition to the daily monitoring, each cage will be filmed overnight using an infra-red camera to assess the behavior of the animals at night time. Similarly, transgenic mice includes the CKS1, CD55, Boy/J, IR4 and SRA knock out strains of weight 20-25g, 25-30g and >30g will be studied. The study will be conducted until the mice reach >25g for strains which have reached a maximum body weight and >30g for others; and all mice will be euthanized at the end of the experiment.

In addition to daily monitoring, mice will be weighed weekly to determine the body weight gain in all groups. One fecal pellet will be collected from each mouse during cage change at day 21 (weaning as baseline for inbred and outbred mice), and when the mean body weight of the group reaches 20-25g, 25-30g and >30 (for outbred, inbred and transgenic mice). The fecal samples collected will be processed for quantification of corticosterone concentration.

Corticosterone is a major indicator of stress. It is secreted by the cortex of the adrenal gland and produced in response to the stimulation of the adrenal cortex by the adrenocorticotropic hormone (ACTH). By measuring the fecal corticosterone level in mice, the stress level of the mouse can be evaluated. At the end of the experiment, mice were

randomly chosen from each density group to obtain the organ (kidney, heart, adrenal gland) weight. Adrenal weight is often used as a measure of stress [13,14]. A lower organ weight was previously noted as housing density increased[9] and a lower organ weight indicates less stress of the animals.

To assess the microenvironment (i.e. cage bedding), the degree of soiling was evaluated by at least 2 staff (technician and area head); blinded scoring of the bedding for dirtiness on a scale of 1 to 5 (Table 2) was carried out, with a score of 5 being the most heavily soiled and 1 being the least soiled in cages which are changed on a biweekly basis. The ammonia level of each cage after scoring was measured from the center at the top of the cage.

Table 2 Criteria for scoring of soiled bedding at cage changing

Score	Criteria
1	Overall bedding is dry (including corners) and less than 10% of surface bedding is covered by feces
2	Bedding at 1 out of 4 corners of the cage is wet, and feces cover 11-25% of bedding surface
3	Bedding at 2 corners of the cage are wet, and feces cover 26-40% of bedding surface
4	Bedding over one side of the cage or over 3 corners of the cage are wet, and feces cover 41-50% bedding surface
5	Bedding is wet in general and feces cover over 50% of the bedding surface

Animal Ethics

This study protocol was approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) of The University of Hong Kong under Protocol No. 4037-16.

Extraction procedure for testing of fecal corticosterone.

Fecal pellet collected in 1.5ml centrifuge tube from each mouse in the cage was extracted according to the method described previously by Touma, 2004[15]. Briefly, all fecal pellets from the same cage were homogenized in a petri dish before extraction. An aliquot of

approximately 0.05g was then shaken with 1 mL of 80% methanol (methanol: distilled water – 8:2) 30 min on a multi-vortex. After centrifugation (10 min at 2500 g), the supernatant was stored at -80^{o}C freezer until analysis.

Enzyme immunoassay.

Amount of corticosterone present in the mouse fecal pellet was quantified using a commercially available corticosterone ELISA Kit (ENZO Life Sciences, NY, USA). The antibody of this ELISA Kit cross-reacted with various steroids as follows: corticosterone (100%); deoxycorticosterone (28.6%); progesterone (1.7%); testosterone (0.13%); tetrahydrocorticosterone (0.28%); aldosterone (0.18%); cortisol (0.046%); pregnenolone (<0.03%); β-estradiol (<0.03%); cortisone (<0.03%) and 11-dehydrocorticosterone acetate (<0.03%). All samples diluted in 1:10 were assayed in duplicate.

ELISA plates were read at 405 nm on a plate reader (Instrument). Concentration was determined as percentage bound by using a standard curve ranging from 32 to 20,000 pg/mL (kit sensitivity is 26.99 pg/mL). Values were expressed based on the total feces collected over a time period and as picogram corticosterone per 0.05 g of feces.

Study Analysis

Analysis of whether increased density has any effect will be performed for each strain separately due to the possibility that density might affect some strains more than the others. Likewise, males and females were analyzed separately. The parameters measured were reported as mean ± standard error (SEM) unless otherwise stated. Student's *t*-test and Bonferroni test was performed to determine the significant differences between stocking density groups using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, and IBM SPSS Statistics for Windows, Version 23.0, NY: IBM Corp.

Result and Discussion

Mortality and Morbidity

The study was performed on male and female outbred (ICR), inbred (C57BL/6N, BALB/c) and transgenic (CKS1, CD55, Boy/J, IR4 and SRA) mice. No mortality nor morbidity was observed during the study except in BALB/c male, IRF4 transgenic, and Boy/J transgenic mice. Two BALB/c males which were housed in stocking density consisting 3 mice/cage died at 11 and 15 weeks old, one BALB/c mouse housed in stocking density containing 5 mice/cage also died at 14 weeks old. On post mortem, no significant finding was noted. Mortality of mice differs from strain to strain and it was reported to be 0.1% to 6.3% in inbred strains during the first 8 months of their life span[9]. Our findings in mortality rate for BALB/c mice in this study was 5% which is consistent with the findings of others.

Three IRF4 transgenic mice from each cage containing a stocking density of 3 mice/ cage were found dead at 5 months (n=2) and 7 months (n=1) old, while two IRF4 transgenic mice in cage containing 4 mice/ cage died at 6 months old and one male IRF4 transgenic mouse died in cage containing 5 mice/cage at 9 months old. IRF4 transgenic mice display immune system abnormalities involving development of both T and B cells and the average life span is 4 months of age[16]. The age of the IRF4 knockout mice which died during this study was older than the average life span of 4 months. Thus we can infer that the mortality of these mice in our study was not related to the stocking density. One IRF4 mouse from stocking density group of 3 mice/ cage developed a subcutaneous mass at neck region at 8 months old and one from 4 mice/ cage was found to have rectal prolapse at 2 months of age. No mouse from the 5 mice/ cage stocking density group was found to have any symptoms of disease. , One Boy/J mouse from the stocking density group of 4 mice/cage developed rectal prolapse at 8 months old. Morbidity was not noted in any of the other stocking density groups. We can conclude that increasing stocking density does not seem to play a role in causing the mortality and morbidity of the animals.

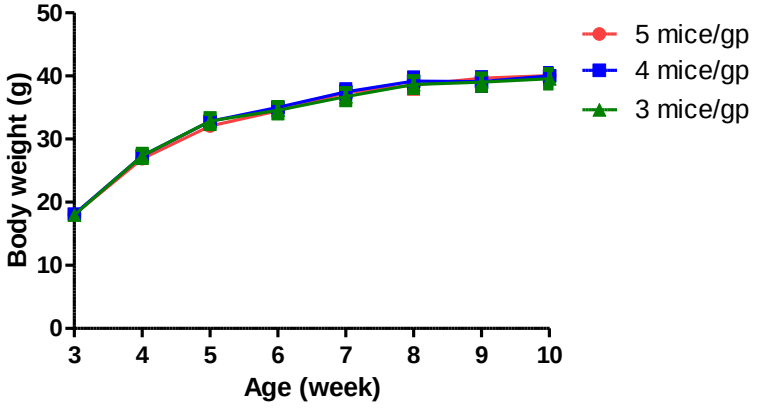
Growth

The body condition of all mice during the study period was good. There is no significant difference in the body weight (Fig 1a-f) between mice housed in stocking density containing 3, 4 and 5 mice/cage for both outbred and inbred mice at any time point from 3 weeks until they reach a body weight of 25 g (for females) and 30g or over (for males). Reduced weight gain for a given density group may reflect an effect of chronic stress and this was not observed among all density groups in this study.

Transgenic mice were not specifically bred for this study and therefore longitudinal growth of these animals was not recorded.

Fig 1. Mean body weight (± SEM) of a) ICR male, b) ICR female mice at 3 to 10 weeks of age, (c) C57Bl/6N male, d) C57Bl/6N female, e) BALB/c male and f) BALB/c female in stocking density of 4 and 5 mice/cage compared to that of 3 mice/ cage (recommended stocking density by the Guide for mice >25g) . A replicate of 5 cages per group.

a)



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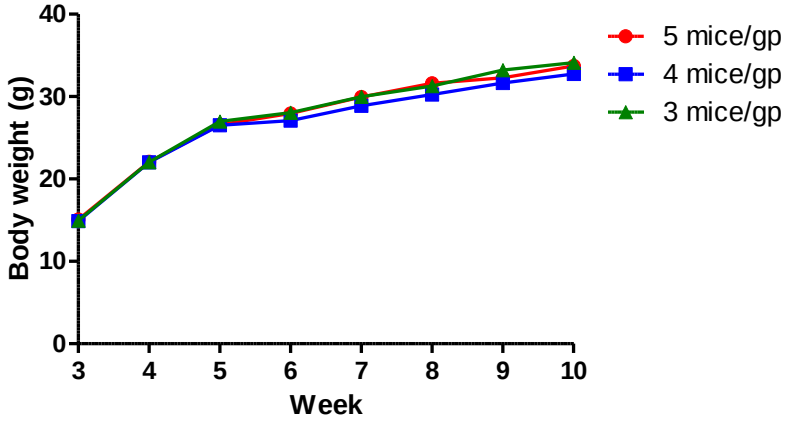

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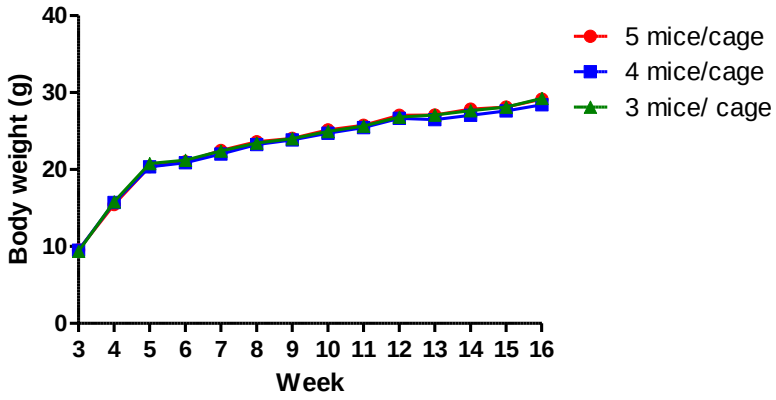
Body weight (g)
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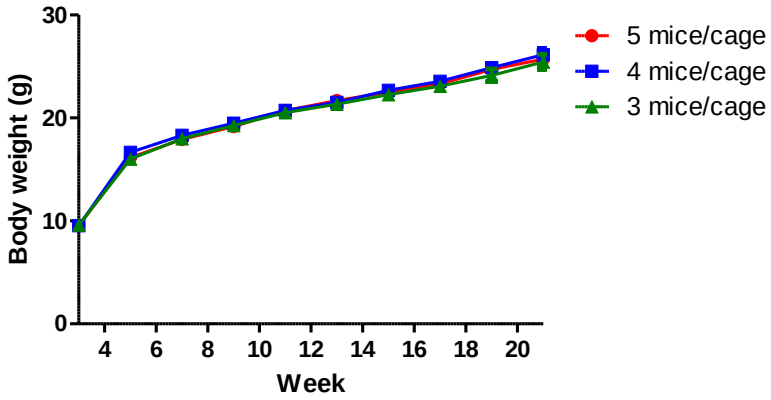
3 mice/ cage

d)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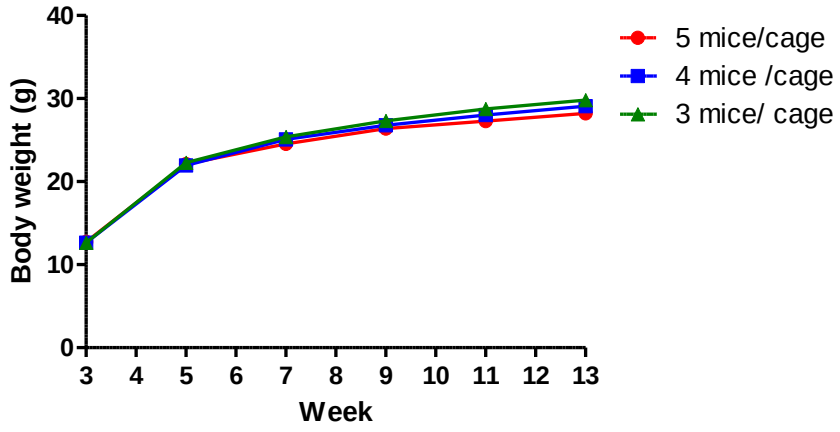
Body weight (g)
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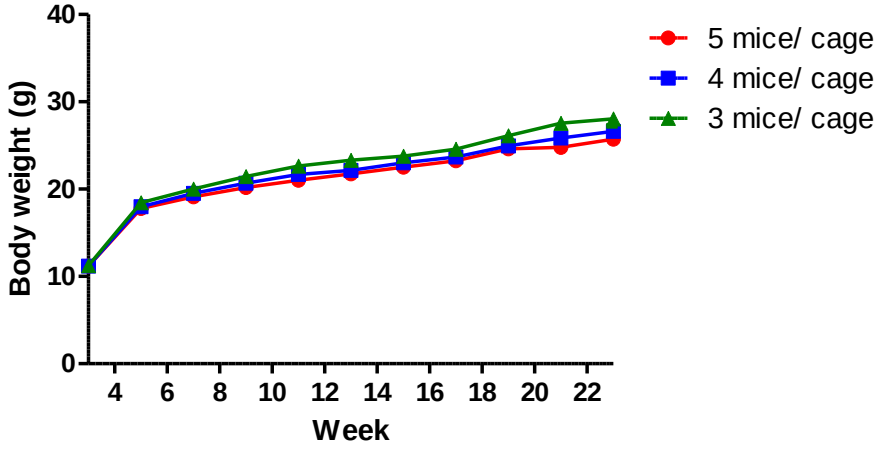
f)



Fecal corticosterone and organ weight

The stress level of mice in each density group was evaluated by measurement of fecal corticosterone level (Fig 2a (i) to (vi) and 2b).

The fecal corticosterone level differs from strains to strains and males to females. When the effect of housing density was evaluated, we found that except for BALB/c female mice, there was no significant difference in the fecal corticosterone level among all three density groups at each time point of mice reaching 15-20g (inbred and outbred strains), 20-25g (both inbred, outbred and transgenic strains), 25-30g (both inbred, outbred, and transgenic strain) and >30g (outbred and transgenic CKS1 and CD55 strains). BALB/c female mice housed in the 4 mice/ cage group had a higher ($p=0.02$) fecal corticosterone level when compared to 3 mice/cage and 5 mice/cage group (Fig 2a). However, there is no significant difference between those mice housed in 3 mice/ group and 5 mice/group and thus there is no evidence to suggest that increased housing density would lead to an increase of fecal corticosterone level in this strain. In addition, at body weight >30g, the levels of fecal corticosterone were comparable among all groups indicating the increase of fecal corticosterone level in mice housed in 4 mice /cage group at 25-30g of body weight was only transient.

Inbred and outbred strains tend of have a higher fecal corticosterone level at weaning (at 3 weeks old and <15g of body weight) than that taken at older stage. The fecal corticosterone level in all strains of mice decreased as mice settled down in each of the density groups after separation from their dam. The higher fecal corticosterone level in these mice could be due to activities related to weaning (for example the introduction to a new cage and new diet, and mixing of litters). The corticosterone level in C57BL/6N male mice at weaning was significantly higher ($p= 0.028$) in stocking density of 5 mice/cage.

However this effect of higher corticosterone level did not sustain, and the levels were all comparable when samples were taken at later time points. The higher level of corticosterone level could be attributed to the mixing of different litters during the random allocation of mice to different groups. It was reported by Doolittle et. al. (1976) that mice thrived worse when litters were being mixed to obtain a certain group size[11]. Similarly, when the fecal corticosterone level in transgenic mice was studied (Fig. 2b), we found there was no significant difference in the level among different density groups.

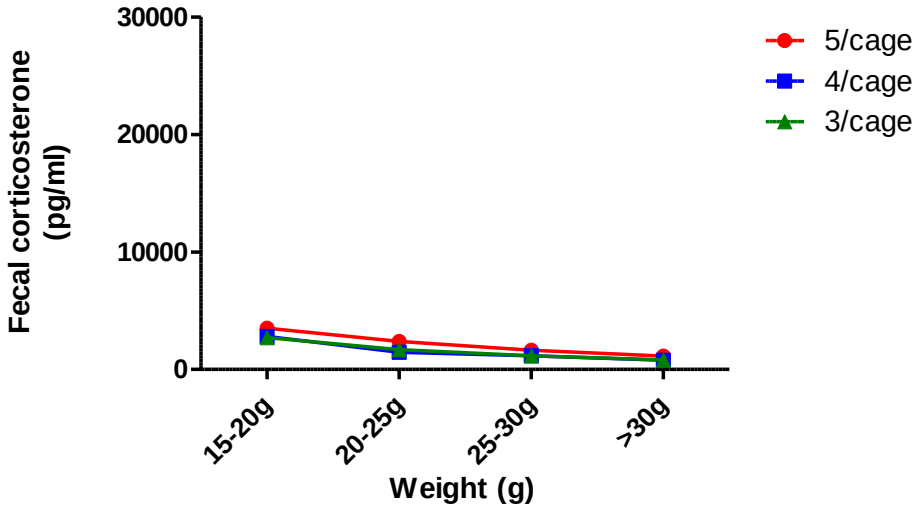
We analyzed the organ weights of the animals at the end of the experiment. Heart, kidney, adrenal glands and testes were dissected and weighed in each group. All organs' weights were within the normal range [19] (Fig 3a). The adrenal weight of BALB/c female mice housed in 5 mice per group was significantly lower when compared to those from mice housed in 3 mice /group ($p=0.0002$) and 4 mice/ group ($p=0.0107$). The results were consistent when considering the fecal corticosterone level (Fig 2a (vi)). Despite the result not reaching statistically significance, the level of fecal corticosterone in BALB/c mice housed in 5 mice /cage was lower compared to other density groups. Adrenal weight is often used as a measure of stress, and it reflects the amount of chronic stress hormone production[9,13, 17, 18]. The decreased adrenal weight that was observed in BALB/c mice at over 20 weeks of age suggests that mice housed in higher stocking density were less stressed. Similar findings were reported by Morgan et. al that adrenal weight decreased as female inbred mice (including BALB/c, B6 and DBA strains), were housed more densely in a cage [9].

The organ weights for transgenic mice were also studied (Fig 3b). The weight of adrenal glands for CD55 and SRA male knockout mice was significantly lower in animals housed in 5 mice/cage when compared to animals housed in 3 mice/cage ($p=0.02$ and $p=0.049$, respectively) (Fig 3b(i)). The weight of the adrenal glands of SRA knockout mice housed in 5 mice/cage was also significantly decreased when compared to mice housed in 4 mice/ cage ($p=0.03$). This result is consistent with our other finding that decreased adrenal weight was noted in the higher density group of BALB/c female mice. Apart from the adrenal glands, CD55 knockout mice housed in 5 mice/ cage was also found to have a higher testicular weight when compared to mice housed in 4 mice/cage and 3 mice/ cage ($p<0.0001$). Testicular weight is highly correlated with sperm production [20]. Therefore CD55 mice housed in a higher density suggests an increased reproductive capacity. This transgenic CD55 mice were also found to have a heavier heart weight in higher density group (5 mice/ cage). SRA knockout mice housed in higher density group, on the other hand, was found to have a lighter heart. Boy/J transgenic mice housed in 5 mice /cage had a smaller kidney weight when compared to the lower stocking density group. Decreased kidney weight was also found by Morgan et. al. (2014) in inbred mice housed in higher stocking density[9].

Taken together, increased stocking density from 3 mice/cage to 5 mice/cage does not lead to increase of stress in the animals. In contrary, our findings suggested some mouse strains housed in a higher density (5 mice/ cage) do exhibit less stress.

Fig 2a. Mean fecal corticosterone level ± SEM in i) ICR male ii) ICR female, iii) C57BL/6N male, iv) C57BL/6N female, v) BALB/c male and vi) BALB/c female at <15g (weaning), 20-25g, 25-30g and >30g. A replicate of 5 cages per group. No significant difference was observed in any of the density groups at each body weight range except in C57BL/6N male mice at 15g and BALB/c female mice at 20-25g. *indicates a significant difference of p≤0.05

i)



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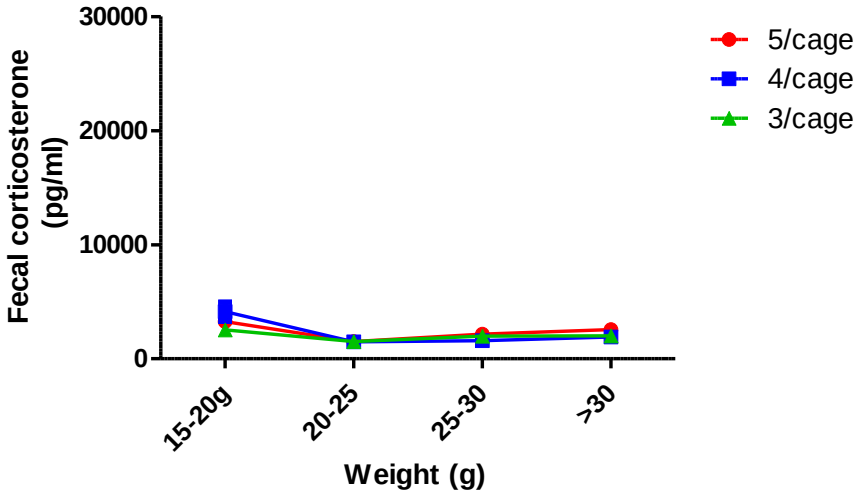

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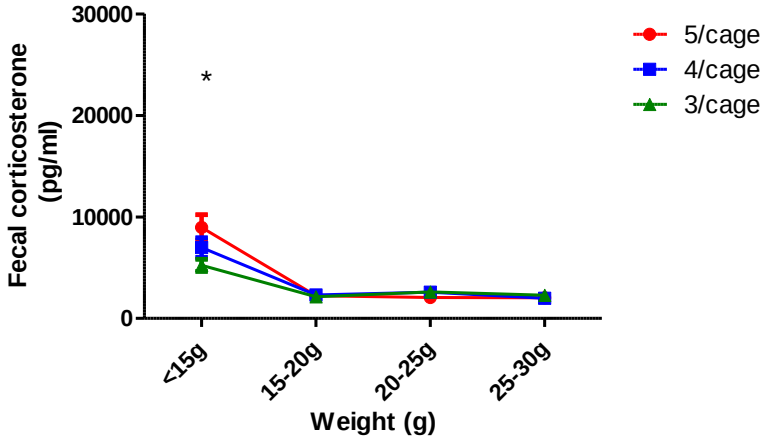
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Fecal corticosterone (pg/ml)
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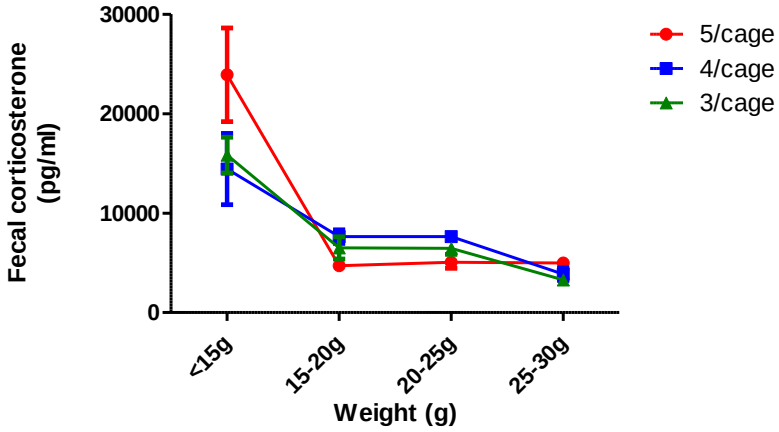
iv)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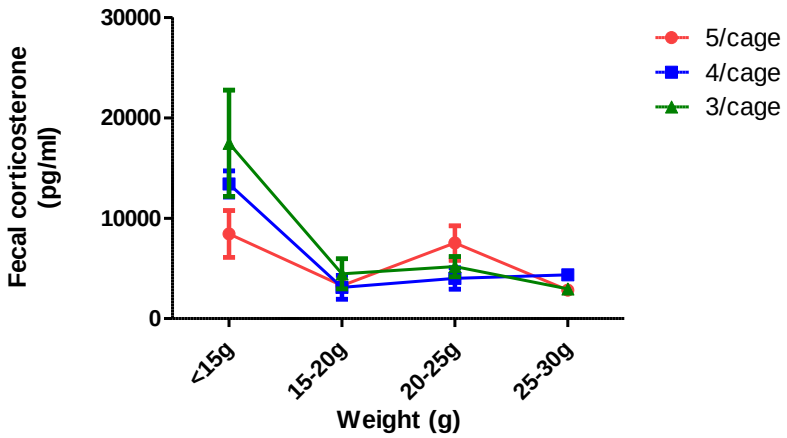
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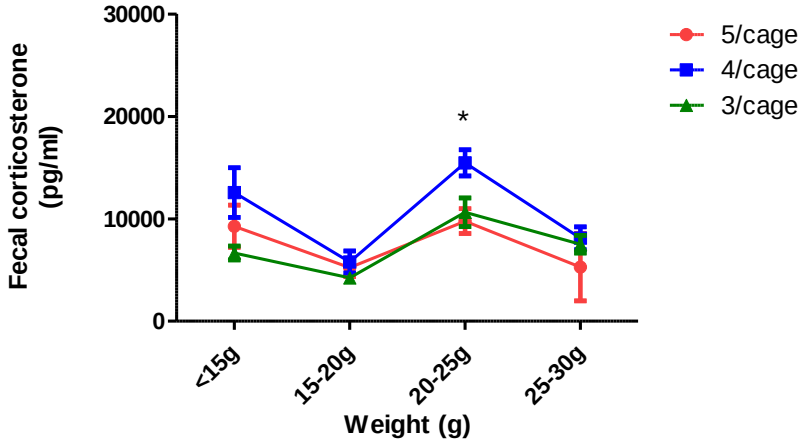
vi)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4/cage
3/cage

Fig 2b. Mean fecal corticosterone level ± SEM in transgenic mice (CKS1, CD55, Boy/J, IRF4 and SRA) at 20-25g, 25-30g and >30g. A replicate of 3-7 cages per group. No significant difference ($p > 0.05$) was observed in any of the density groups of all the strains studied.

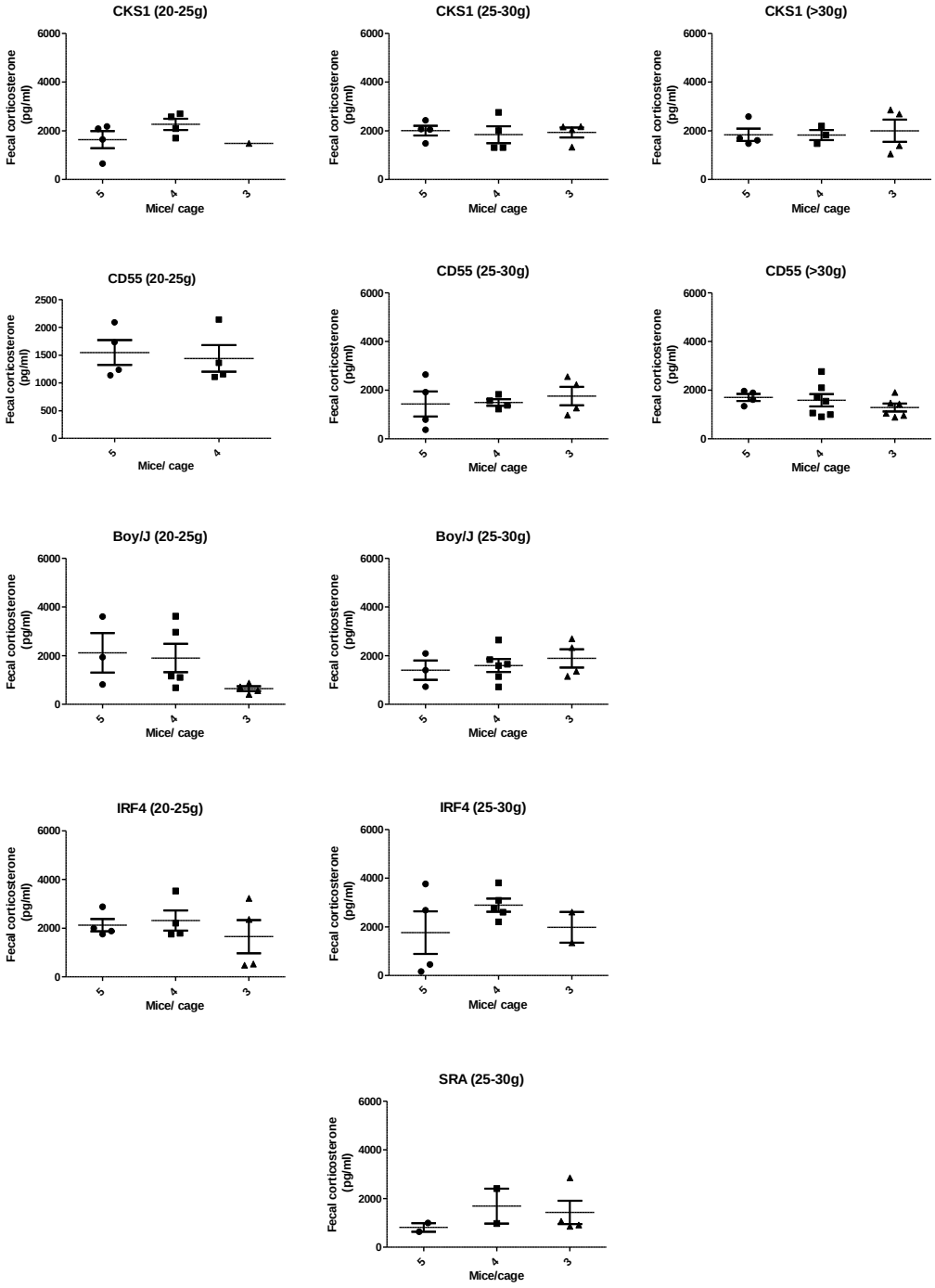
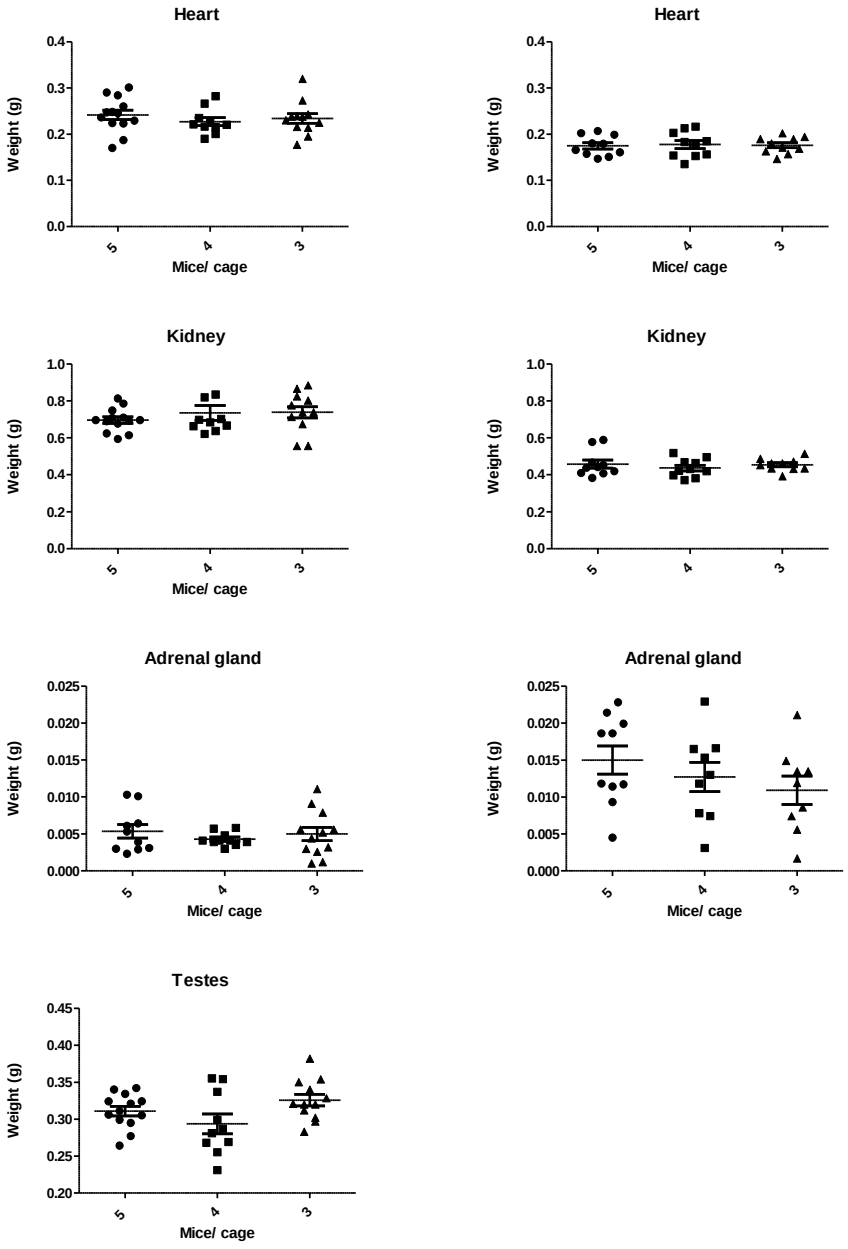
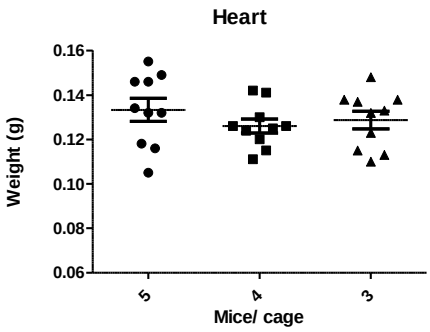


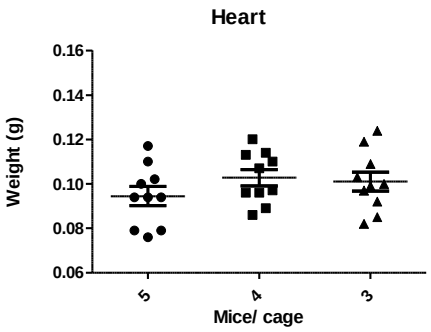
Fig 3a. Mean organ weight (+/- SEM) of i) ICR, ii) C57BL/6N, iii) BALB/c housed in cage with stocking density of 3,4 and 5 mice per cage. A replicate of 10 mice per group. There was no significant difference (p>0.05) in the organ weights among the different density groups except for BALB/c female mice in which the adrenal glands significantly weighed less in cages with 5 mice/ cage when compared to those with 3 mice/ cage (p=0.0002)

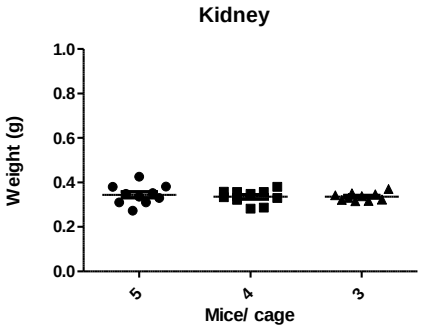
i) **Male** **Female**

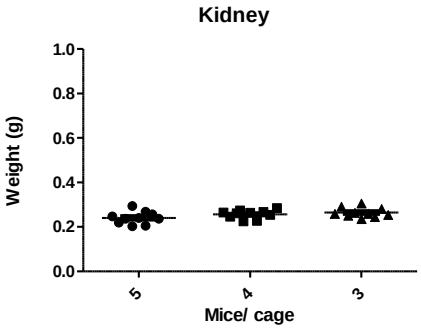


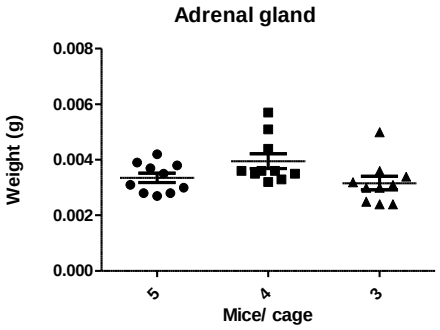
ii) Male Female

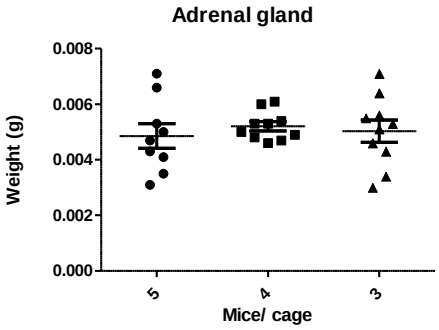


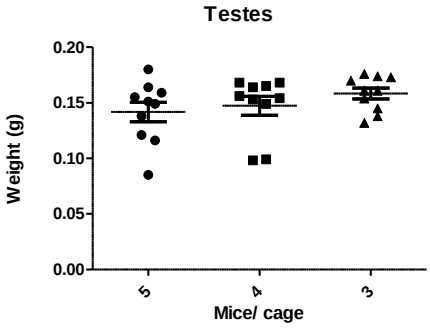




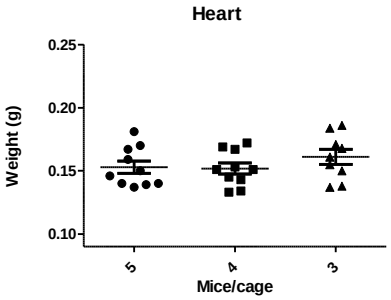


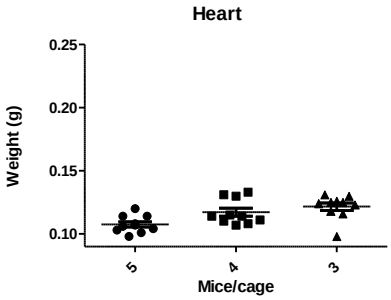


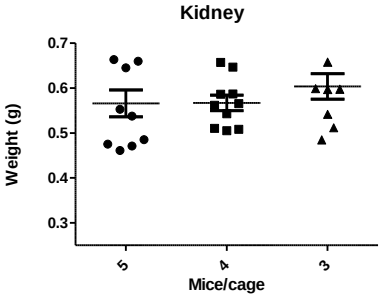


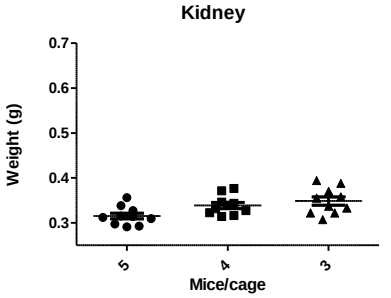


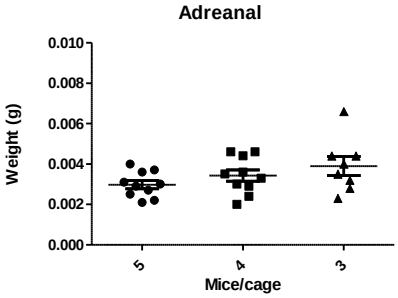
iii) Male **Female**

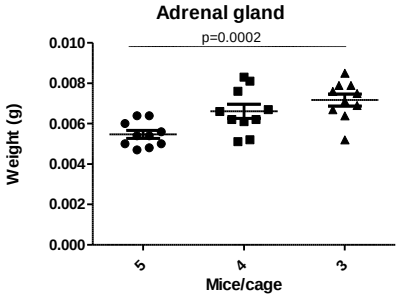


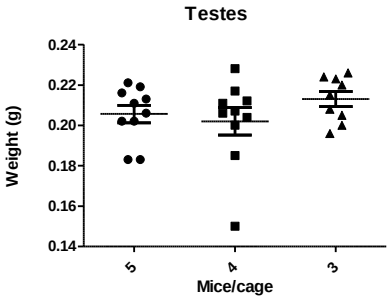




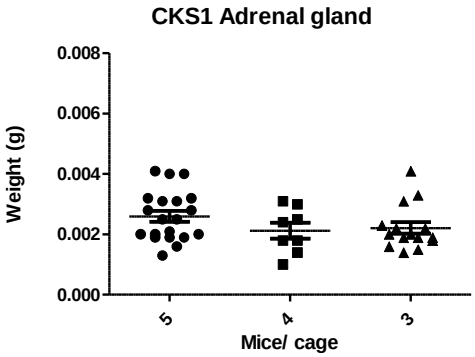


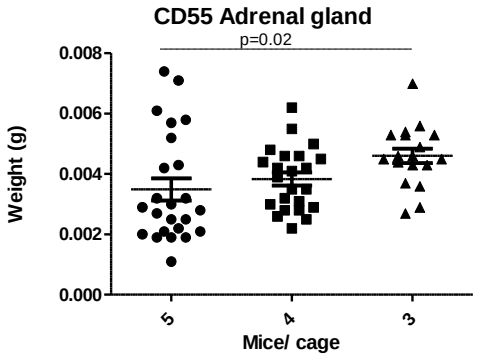


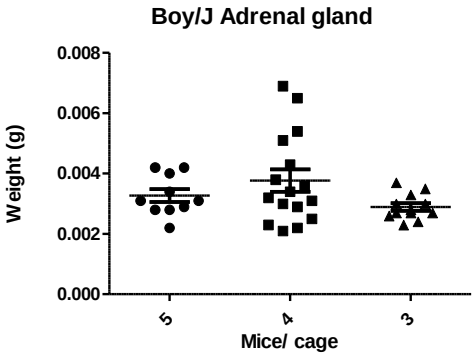


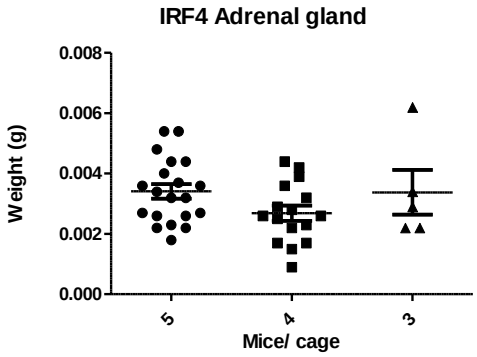


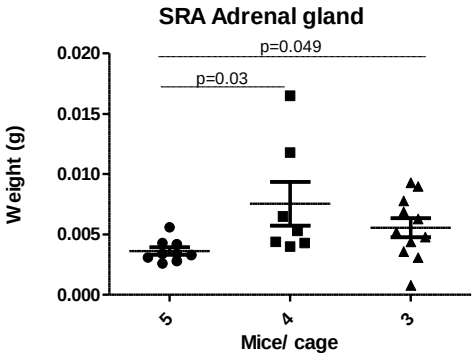
i) Fig 3b. Mean weight (+/- SEM) of i) Adrenal gland, ii) Testes, iii) Kidney, and iv) Heart of transgenic (CKS1, CD55, Boy/J, IRF4 and SRA) mice housed in cage with stocking density of 3,4 and 5 mice per cage. A replicate of 5 -24 mice in each group. Significant difference ($p<0.05$) between organ weights was observed in adrenal glands of CD55 and SRA; testes of CD55; kidney of Boy/J and heart of CD55 and SRA knockout mice.



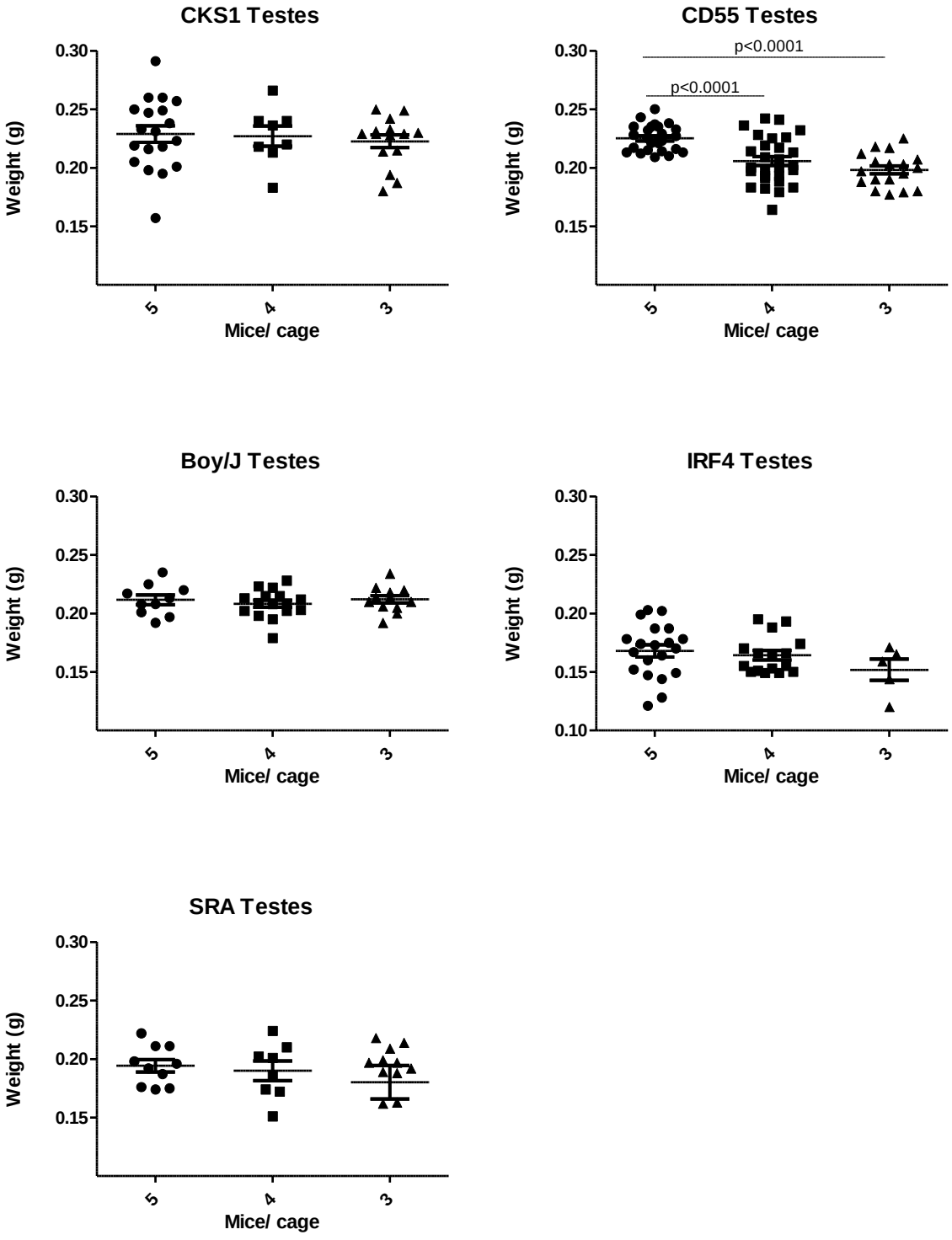




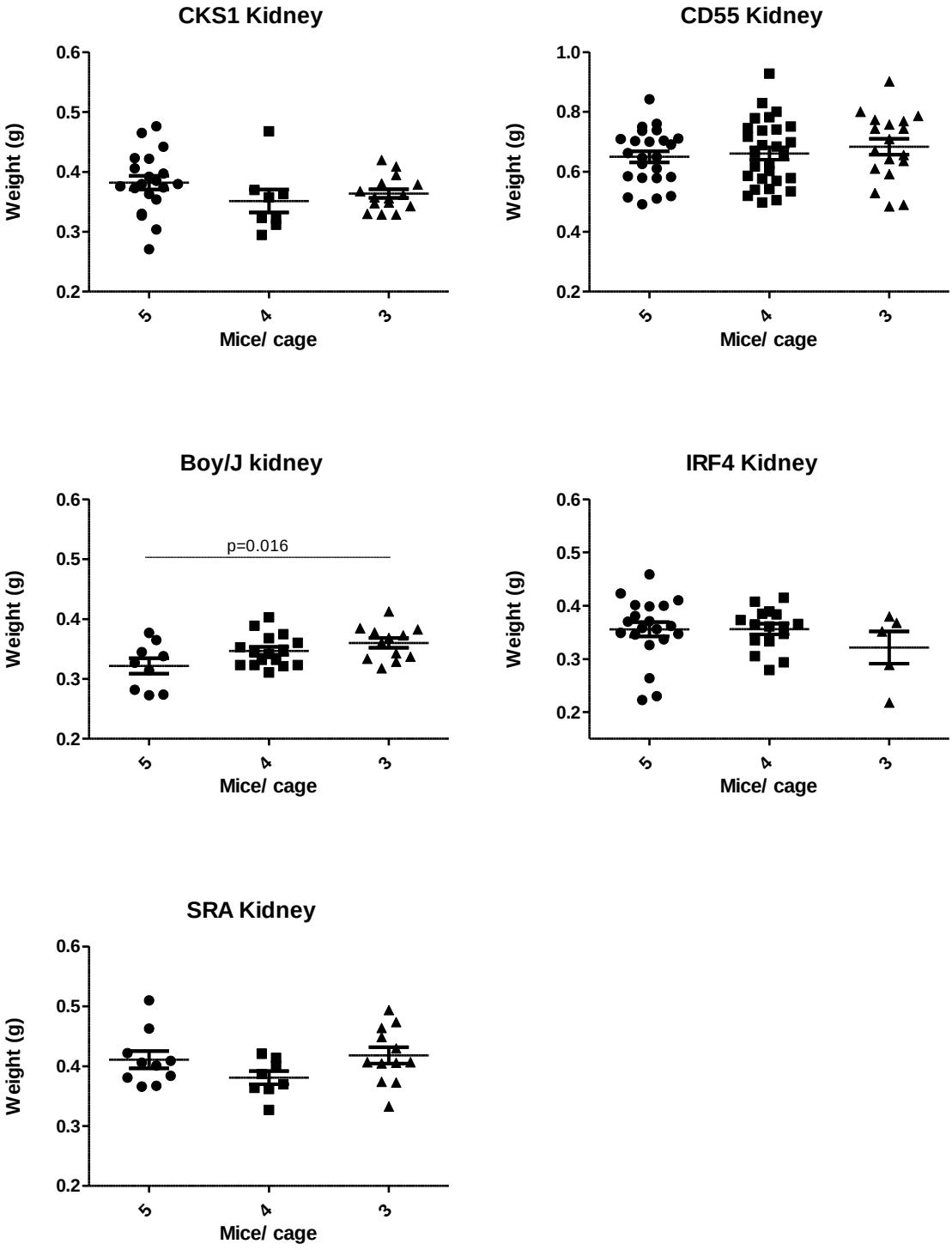




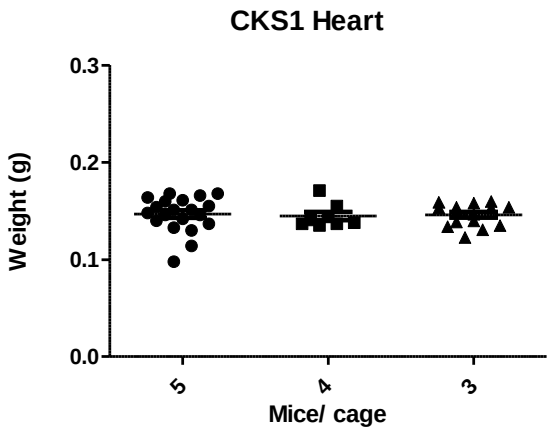
ii)

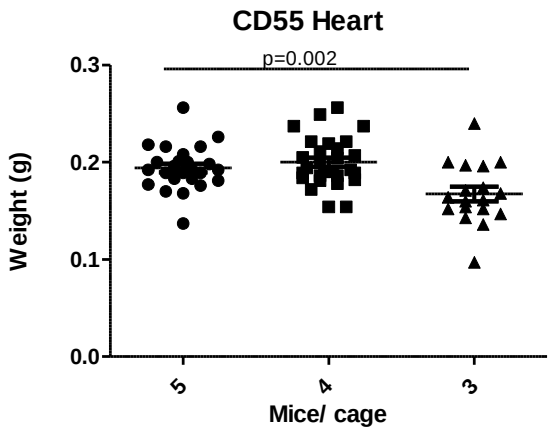

CKS1 Testes
Weight (g)
0.30
0.25
0.20
0.15
5
4
3
Mice/ cage
CD55 Testes
p<0.0001
p<0.0001
Weight (g)
0.30
0.25
0.20
0.15
5
4
3
Mice/ cage
Boy/J Testes
Weight (g)
0.30
0.25
0.20
0.15
5
4
3
Mice/ cage
IRF4 Testes
Weight (g)
0.30
0.25
0.20
0.15
0.10
5
4
3
Mice/ cage
SRA Testes
Weight (g)
0.30
0.25
0.20
0.15
5
4
3
Mice/ cage

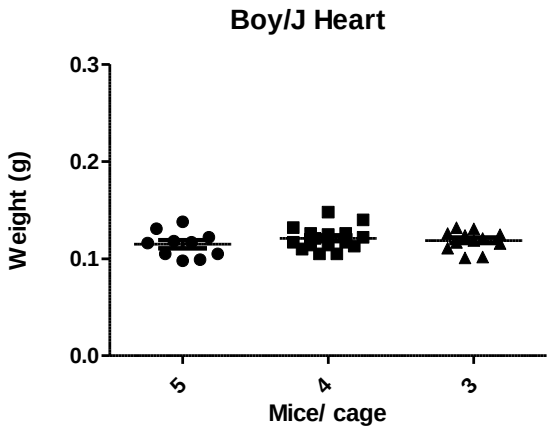
iii)

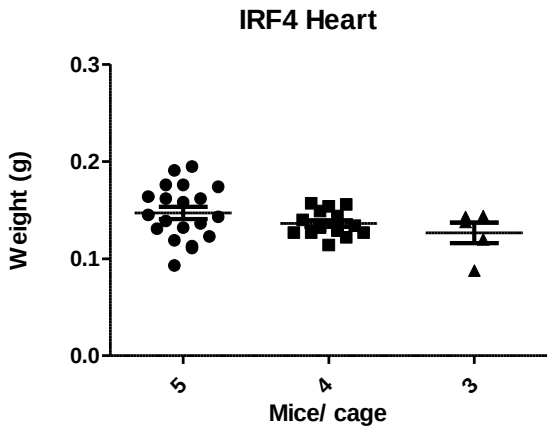

CKS1 Kidney
Weight (g)
0.6
0.5
0.4
0.3
0.2
5
4
3
Mice/ cage
CD55 Kidney
Weight (g)
1.0
0.8
0.6
0.4
0.2
5
4
3
Mice/ cage
Boy/J kidney
p=0.016
Weight (g)
0.6
0.5
0.4
0.3
0.2
5
4
3
Mice/ cage
IRF4 Kidney
Weight (g)
0.6
0.5
0.4
0.3
0.2
5
4
3
Mice/ cage
SRA Kidney
Weight (g)
0.6
0.5
0.4
0.3
0.2
5
4
3
Mice/ cage

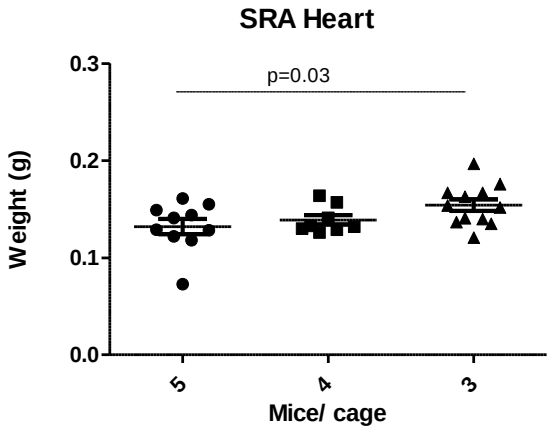
iv)


CKS1 Heart
Weight (g)
0.3
0.2
0.1
0.0
5
4
3
Mice/ cage


CD55 Heart
p=0.002
Weight (g)
0.3
0.2
0.1
0.0
5
4
3
Mice/ cage


Boy/J Heart
Weight (g)
0.3
0.2
0.1
0.0
5
4
3
Mice/ cage


IRF4 Heart
Weight (g)
0.3
0.2
0.1
0.0
5
4
3
Mice/ cage


SRA Heart
p=0.03
Weight (g)
0.3
0.2
0.1
0.0
5
4
3
Mice/ cage

Behavior

During daytime monitoring of the animals, no behavioral traits of barbering and whiskering nor aggressive behavior and stereotypic behavior of the animals were recorded. No fighting of the animals during daytime was reported except minor intermittent fighting behavior was observed in BALB/c male and female mice. For males, fighting was observed in three out of the five replicate cages in stocking density containing 5 and 3 mice/cage; four out of the five replicate cages in stocking density containing 4 mice/ cage. For females, fighting was reported in two out of the five replicate cages in stocking density containing 5 and 3 mice/cage; and four out of the five replicate cages in stocking density containing 4 mice /cage group. No injury was observed in these mice during the experimental period. Interestingly, when viewing the mice during nighttime using an infrared camera, minor intermittent fighting was only noted in one of the BALB/c male cages containing 3 mice/ group and it was not noted in the female mice. In addition, intermittent fighting was also noted during nighttime in C57BL/6N male mice housed in one of the 5 replicate cages with 5 mice/ cage, and in ICR male mice housed in one of the 5 replicate cages with 3 mice/ cage. Other publications have shown that mice given a smaller floor area per mouse are less aggressive than those given with bigger space [7,10] though this was not observed in our study.

Positive behaviors including allogrooming, on the other hand, was also noted in mice during nighttime. Allogroming was recorded in one of the five replicate cages with the following stocking density group: 4 and 5 BALB/c female mice /cage, 4 BALB/c male mice/cage, 5 C57BL/6N female mice/ cage, 4 C57BL/6N male/ cage, 5 ICR male mice /cage and 5 ICR female mice/ cage. ICR male mice in two of the five replicate cages with 4 mice/ group were also being observed to have the allogrooming behavior. Interestingly, no allogrooming was recorded in cages containing 3 mice /cage during the observed period.

Circling was observed in one animal of one of the replicates consisting 4 ICR male mice/ cage but no mice in other groups have such behavior. Circling is observed to be a common stereotypic behavior in some ICR mice as held in the Laboratory Animal Unit and this behavior was not related to housing density.

Micro-environment (bedding condition and ammonia level)

To assess the microenvironment and the condition of soiled cages of different density groups, ammonia level was measured at the center of the cage and blinded scoring of the cages was performed by 2-3 staff of the Unit. Representative data of the ammonia level recorded in each density group was shown in Fig 4, the result showed no significant difference among all density groups in each strain. The soiled bedding scores were compared among groups and the results are shown in Table 3. When comparing the soiled bedding score for groups with 5 mice/cage and 3 mice/cage, there were significant difference in the scores between the different stocking density groups in ICR male and female mice. Cages with 5 mice/cage tend to have a higher bedding condition score. For

C57BL/6N mice, the bedding condition scores of both male and females were significantly higher in cages with 5 mice/cage when compared to 3 mice/cage at 25-30g (males) and 30g (females); but not when mice were younger at 25-30g (males) and 20-25g (females). For BALB/c male mice, the bedding condition score was significantly higher in cages with 5 mice/cage at 20-25g but was comparable in all stocking density groups with mice over 30g. The scores for BALB/c female mice were not significantly different among groups at 7,9,15 and 25 weeks old. (Table 3).

We observed that bedding condition varied from strains to strains and age of the mice. For the current husbandry practice at the LAU, cage changing was performed every 2 weeks but when any cages are found to be soiled (i.e. reaching a bedding condition score of above 3), immediate cage changing will be performed. Therefore, bedding condition will not be affected by an increase of stocking density from 3 mice/cage to 5 mice/cage.

Fig 4. Ammonia level (+/- SEM) taken at the center of cage of a) ICR male; b) ICR female at 10 week, c) C57BL/6N male at 16 weeks, d) C57BL/6N female at 21 weeks, e) BALB/c male at 12 weeks and f) BALB/c female at 21 weeks old in cage with a stocking density of 3, 4 and 5 mice /cage. There was no significant difference in the cage ammonia level ($p>0.05$) among all density groups.

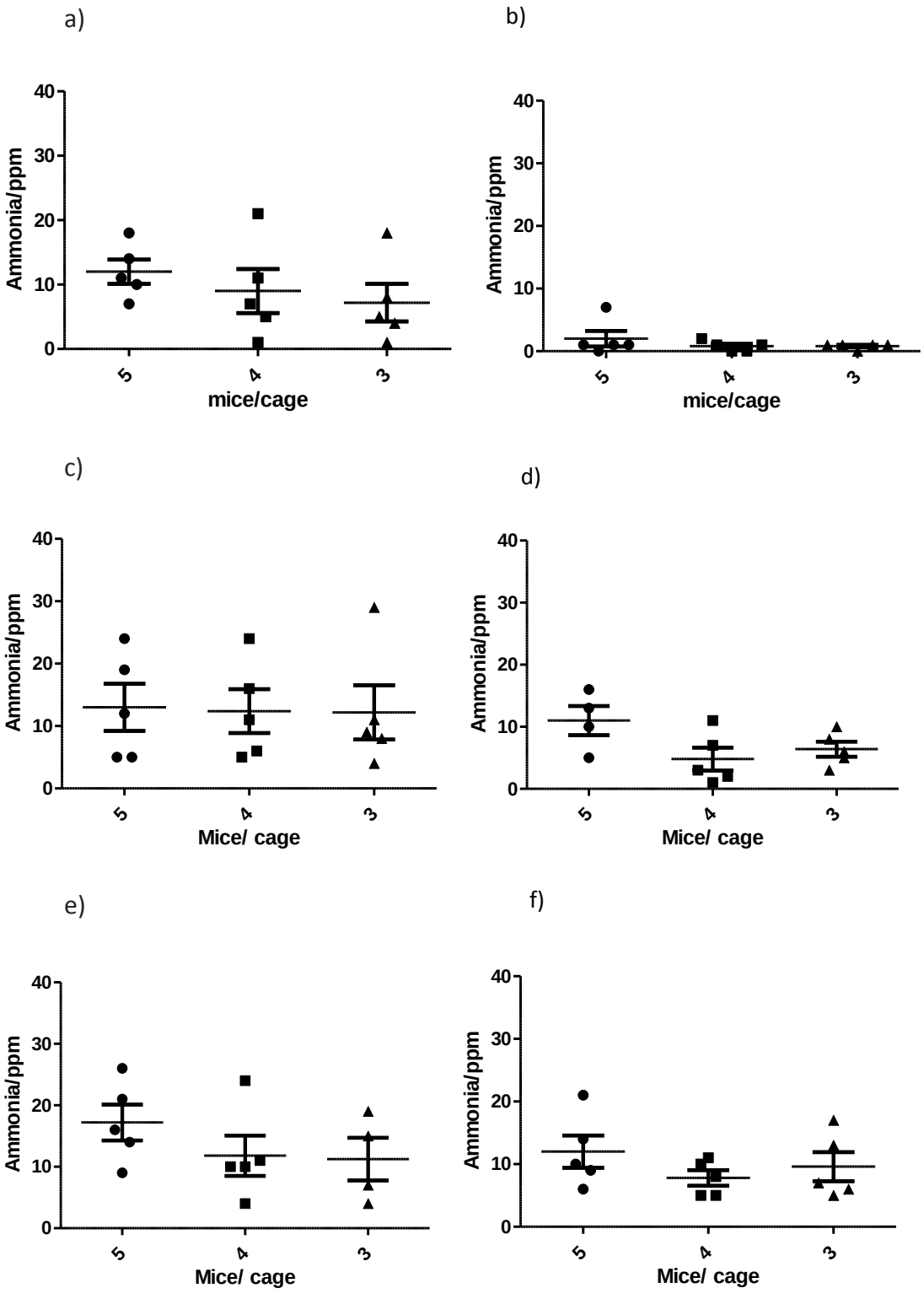


Table 3. Bedding condition score of ICR, C57BL/6N and BALB/c mice housed in different stocking densities. A replicate of 5 cages per group. *** p<0.0005; **p<0.005, **p<0.05

Strain	Age (weeks) / Weight (g)	Cage sanitation score	P values compared to 3 mice /cage	P values compared to 4 mice/ cage
ICR Male	5 / 30g	5/cage = 4.30±0.21 4/cage =4.1±0.10 3/cage = 2.4 ± 0.16	<0.0001***	0.0766
	11 / >30g	5/cage = 3.36±0.16 4/cage =3.2±0.13 3/cage = 1.9 ± 0.20	0.0005***	0.4751
ICR Female	5 / 25-30g	5/cage = 3.93±0.15 4/cage =2.93±0.19 3/cage = 2.57±0.22	<0.0001***	0.0004***
	10 / >30g	5/cage = 3.12±0.17 4/cage =2.76±0.24 3/cage = 2.14±0.14	0.0026**	0.0790
C57BL/5N Male	12/ 25-30g	5/cage = 2.3±0.15 4/cage =2.70±0.15 3/cage = 2.5±0.18	0.4118	0.0806
	16/ 30g	5/cage = 2.78±0.08 4/cage =2.60±0.11 3/cage = 2.10±0.10	0.0007***	0.2323
C57BL/6N Female	9 / 20-25g	5/cage = 2.83±0.19 4/cage =2.73±0.12 3/cage = 2.47±0.11	0.0941	0.6436
	22 / 25-30g	5/cage = 2.88±0.13 4/cage =2.40±0.24 3/cage = 2.30±0.12	0.0141*	0.1558
BALB/c Male	10/ 20-25g	5/cage = 2.27±0.11 4/cage =2.33±0.12 3/cage = 1.70±0.11	0.0009***	0.6770
	18/ >30g	5/cage = 2.56±0.21 4/cage =2.50±0.08 3/cage = 2.17±0.17	0.2284	0.7724
	20/ >30g	5/cage = 3.44±0.16 4/cage =3.3±0.09 3/cage = 3.08±0.22	0.2344	0.4557
BALB/c Female	7 / 15-20g	5/cage = 2.4±0.24 4/cage =2.4±0.22 3/cage = 2.13±0.20	0.3987	1.000
	9 / 20-25g	5/cage = 1.95±0.25 4/cage =2.25±0.30 3/cage = 2.20±0.30	0.5316	0.4543
	15/ 20-25g	5/cage = 2.70±0.23 4/cage =2.45±0.15 3/cage = 2.45±0.09	0.3420	0.3842
	25 / 25-30g	5/cage = 2.53±0.10 4/cage =2.47±0.15 3/cage = 2.47±0.15	0.7171	0.7171

Conclusion

When compared to the stocking density recommended by the Guide, our data indicated that BALB/c female, CD55 and SRA knockout mice had less stress when housed in groups of 5 compared to 3 in a standard LAU cage with floor area of 330cm^2. Another trait to study the testicular size also suggested that CD55 mice housed in groups of 5 had an increased reproductive capacity. For other strains studied, including outbred (ICR), inbred (BALB/c male, C57BL/6N) and transgenic (CKS1, Boy/J, and IRF4) mice, there was no significant effect on the performance and wellbeing when they were housed at higher densities of up to 5 adult mice in a standard LAU Cage These results concluded that the performance and well-being of mice are not compromised when they are housed at a floor area/animal ratio lower than that recommended by the Guide and in some strains, a smaller floor area/animal is even beneficial to the animals. A conclusion can thus be made to support that up to 5 mice of body weight ≥ 25g can be housed in a standard LAU Cage (i.e. NKP M2 and Tecniplast 1144B cage with floor area of 330cm^2 and 335cm^2 respectively).

The Guide has specifically stated that group-housed and social animals can share space such that the amount of space required per animal may decrease with increasing group size and thus larger groups may be housed at slightly higher stocking densities. At the same time, the Guide also recommends that performance indices should be taken into consideration when utilizing the recommended species-specific guidelines and the space allocations should be modified as necessary by the IACUC considering the performance indices. Therefore, the LAU would like to seek approval from the CULATR to hold mice at a floor area / animal lowerr than that recommended by the Guide as shown in Table 4 below.

Table 4. Stocking density recommended by the Guide and ETS No 123 and the proposed LAU stocking density

Body Weight (gm)	The Guide[1] (USA)		ETS No 123[2] (Europe)	Proposed LAU stocking density	
	Floor area (cm^2)/Animal	No. of animals in a LAU Cage (330-335cm^2)	No. of animals in a LAU Cage (330-335cm^2)	No. of animals/Cage (330-335cm^2)	Floor area/ animal of LAU: The Guide[1]
Up to 20	77.4	4.2	5.5	5	0.85
20-25			4.7		
25-30	96.7	3.4	4.1	5	0.68
>30			3.3		

Appendix 1: Summary of recent publications to study the effects of stocking density on wellbeing of laboratory mice

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