Physiological profile of juvenile rats: effects of cage size and cage density

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Although there is a general consensus that housing conditions affect the well-being of laboratory animals, the ideal cage size and density for housing laboratory rodents has not been established. The authors investigated the effects of cage size and cage density on growth, organ development, metabolic profile, and hemogram in juvenile Sprague-Dawley rats. Larger cages and increased cage density were associated with depressions in body weight and in the weights of several organs. In general, increasing group size and density correlated more strongly with detrimental effects on the growth of females than males, although hemogram values indicated that males are more prone to emotional stress and immune suppression than females in response to increasing group size and crowding.

Housing should be designed to provide an environment that allows animals to maintain their health and achieve optimal growth. Housing conditions, such as population size and density, present potential welfare issues. These variables can have a major impact on the animals and, consequently, interfere with the outcome of experiments. As pointed out by Woolverton *et al.*[1], well-being is often referred to as 'psychological comfort', a term too vague to be demonstrably attained. Instead, welfare regulations should be based on observable and attainable changes in behavior or physiological variables.

Group size is crucial for learning and social development in young animals, as demonstrated by the sometimes devastating behavioral consequences for animals housed individually. On the other hand, larger-group housing may lead to aggression, trauma, and disease transmission[1]. Studies involving mice[2] and chickens[3] demonstrate that stress-related parameters based on hierarchical social structure and productivity change in a quadratic manner as population size increases. Gas accumulation[4], increases in heat and humidity[5], and decreased feed intake and feed utilization[6], all consequences of increasing cage size and cage density, also interfere with growth. In addition to these factors, physical activity may be limited in a crowded cage environment because of immobilization[7]. This is particularly important for health status because physical activity promotes function of the immune system by altering corticosterone production, which may mediate the phagocytic function of macrophages[8]. Moreover, increasing population size may trigger competitive behavior, such as attempts to escape from cages (in females) and chewing the cage bars and aggressive grooming (in males)[9].

Crowding, defined as increasing the animal density per cage, is also an important factor influencing the animals because it may limit feed intake and physical activity, and suppress growth[10]. Crowding can also lead to decreased excitatory responses and increased defecation rates, as well as adrenocorticotropin surge[11]. In one study, rats housed 10 per cage (as compared to those housed 5 per cage) showed dramatic growth depression accompanied by decreased thymus weight and increased adrenal gland and testis weights (though there was no change in feed intake)[12], suggesting that the growth-depressing effect of crowding could be related to alterations in hormonal profile and basal metabolism. To evaluate the mechanism by which crowding depresses growth, Restrepo and Armario[13] assigned rats into either crowded cages with *ad libitum* feeding or uncrowded cages with restricted feeding. Despite the growth-depressing effect, neither crowding nor food

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TABLE 1 | The effects of cage size, cage density, and gender on body and organ weights in juvenile rats

Groups[a]			Body weight and organ weights[b]											
CS	CD	S	BW (g)	Heart (g)	Lung (g)	Stomach (g)	SI (cm)	LI (cm)	Liver (g)	Kidney (g)	Spleen (g)	Adrenal (g)	Testis (g)	Ovary (g)
SC (*n*=36)			182.1±4.2	0.72±0.02	1.00±0.03	1.10±0.03	103.7±1.6	18.7±0.4	7.98±0.21	0.68±0.02	0.41±0.01	0.020±0.001	1.26±0.5	0.051±0.006
LC (*n*=108)			165.0±5.4	0.64±0.02	1.00±0.05	1.06±0.02	105.1±1.3	19.4±0.5	6.60±0.18	0.66±0.02	0.44±0.02	0.020±0.001	1.29±0.06	0.041±0.004
	ND (*n*=48)		181.0±5.5	0.72±0.03	1.07±0.05	1.13±0.02	104.6±1.2	19.0±0.4	7.95±0.25	0.71±0.02	0.46±0.02	0.020±0.001	1.29±0.05	0.049±0.007
	HD (*n*=96)		166.1±4.5	0.64±0.02	0.93±0.02	1.03±.02	104.2±1.6	19.1±0.5	6.63±0.14	0.63±0.02	0.38±0.01	0.020±0.001	1.26±0.06	0.043±0.004
		M (*n*=72)	192.5±3.4	0.74±0.02	1.06±0.05	1.14±0.02	107.1±1.7	18.8±0.4	7.73±0.19	0.74±0.01	0.44±0.02	0.018±0.001	1.28±0.06	-
		F (*n*=72)	154.6±3.6	0.62±0.02	0.94±0.02	1.02±0.02	101.7±0.9	19.3±0.5	6.85±0.24	0.60±0.01	0.40±0.01	0.022±0.001	-	0.046±0.006
ANOVA			*P*<F											
CS			0.0001	0.0006	0.92	0.22	0.48	0.27	0.0001	0.06	0.14	0.57	0.48	0.009
CD			0.0002	0.004	0.005	0.0006	0.80	0.83	0.0001	0.0001	0.0003	0.62	0.35	0.09
S			0.0001	0.0001	0.02	0.0002	0.008	0.50	0.0001	0.0001	0.06	0.0003	-	-
CS × CD			0.11	0.59	0.28	0.29	0.40	0.88	0.002	0.54	0.0002	0.15	0.15	0.06
CS × S			0.003	0.42	0.04	0.78	0.74	0.47	0.002	0.005	0.57	0.20	-	-
CD × S			0.82	0.04	0.03	0.95	0.41	0.20	0.26	0.07	0.006	0.99	-	-
CS × CD × S			0.12	0.66	0.14	0.03	0.09	0.46	0.01	0.03	0.10	0.42	-	-

[a]CS, cage size (SC, small cage: 24 × 40 × 20 cm in width × depth × height; LC, large cage: 50 × 58 × 30 cm in width × depth × height); CD, cage density (ND, normal density: 160 cm^2/rat; HD, high density: 80 cm^2/rat); S, gender (M, male, F, female). Rats were separated by gender and assigned randomly to cages so that 6 and 18 rats were raised at ND in SC and 12 and 36 rats at HD in LC.
[b]Data were obtained at 10 weeks of age. BW, body weight; SI, small intestine; LI, large intestine. Values were presented as mean±SE by cage type, cage density, and gender. Values for kidney, adrenal gland, testis, and ovary were means of the right and left organs.

restriction altered the pituitary-adrenal axis. However, both treatments decreased insulin, growth hormone, somatomedin C, and thyroid-stimulating hormone.

In many studies, both group-size and cage-density effects are confounded in terms of feeder space, floor area per animal, or both. Moreover, gender-specific responses to altering cage size and cage density have not been determined. The objective of our investigation is to elaborate some of these unknowns by evaluating the effects of cage size, cage density, and gender on growth, organ development, metabolic profile, and hemogram in juvenile rats.

METHODS

Animals and housing

The protocol in this experiment was reviewed and approved by the Research Animal Ethics Committee of Atatürk University. We obtained 72 male and 72 female post-weanling Sprague-Dawley rats weighing an average of 45.3±4.5 g (37.5–57.6 g and 3 weeks of age) from Atatürk University's ATADEM Breeding Facility after confirmation that they were free of major pathogens (*Salmonella* spp., *Shigellae* spp., *Leptospira* spp., *Streptobacillus moniliformis, Spirillum minus, Mycobacterium tuberculosis, Pastorella pseudotuberculosis,* and *Sarcoptes scapiei*). We randomly assigned the rats to one of four housing scenarios: small cage at normal density (24 × 40 × 20 cm at 160 cm^2/rat (6 rats per cage)), small cage at high density (24 × 40 × 20 cm at 80 cm^2/rat (18 rats per cage)), large cage at normal density (50 × 58 × 30 cm at 160 cm^2/rat (12 rats per cage)), and large cage at high density (50 × 58 × 30 cm at 80 cm^2/rat (36 rats per cage)). All cages were aluminum with wire-mesh floors. The rats were housed under these conditions until 10 weeks of age. Males and females were housed separately.

Diet and management

We fed the rats a conventional pellet diet formulated to meet nutrient requirements for growing rats[14]. Each ration consisted of 38.5% corn, 10.7% rye, 4.0% wheat bran, 35.0% soybean meal, 4.3% sunflower meal, 2.5% fish meal, 2.8% sunflower oil, 1.0% limestone, 0.30% salt, 0.25% vitamin-mineral premix, 0.15% methionine, and 0.5% sodium bicarbonate and contained 23.6% crude protein, 3.4% crude fiber, 5.7% fat, and 7.5% ash. Feed was offered *ad libitum* and water was constantly available via glass bottles with rubber nipples. Room temperature and humidity were maintained at 20–24 °C and 58%, respectively, and all animals were exposed to 12:12-hour light:dark cycle during the experimental period[15]. Cages were cleaned twice weekly.

Sample collection and analytical procedure

We measured body weights (BW) and took blood samples from the heart under anesthesia using a non-terminal procedure following a 24-hour fast at the end of the experiment. We collected blood samples in additive-free vacutainers (BD vacutainer SST, BD Vacutainer Systems Preanalytical Solutions, Belliver Industrial Estate, London, UK) for blood chemistry analysis. We obtained serum following centrifugation at 3,000*g* for

TABLE 2 | Net effects of cage size and density on body and organ weights of juvenile rats

Net effect of large cage	Weight parameter	Net effect of high density
–9.4%	Body	–8.2%
–11.1%	Heart	–11.1%
–17.3%	Liver	–16.6%
–19.6%	Ovary (average of right and left)	–12.2%
–2.9%	Kidney (average of right and left)	–11.3%
N/A	Lung	–13.1%
N/A	Emptied stomach	–8.8%
N/A	Spleen	–17.4%
N/A	Adrenal gland (average of right and left)	N/A
N/A	Testis (average of right and left)	N/A

15 min at 20 °C, aliquots of which were stored at –20 °C until analyses could be done for glucose, total protein (TP), albumin, creatinine, triglyceride, cholesterol, very low-density lipoprotein (VLDL), alkaline phosphatase (ALP), calcium, and phosphorus using spectrophotometric methods with commercial kits (DDS, Diasis Diagnostic Systems Co., Istanbul, Turkey). We also put blood samples into vacutainers with K_3-EDTA for hemogram. Within one hour after sampling, whole-blood samples were subjected to flow cytochemistry (Coulter STKS Hematology Flow Cytometer, Beckman Coulter, Miami, FL) for neutrophil, lymphocyte, eosinophil, basophil, erythrocyte, and platelet counts, hemoglobin concentration, and hematocrit value.

Following sedation by intraperitoneal injection of xylazine hydrochloride (5 mg/kg) (Rompun, Bayer, Istanbul, Turkey), we anesthetized the rats with 2% sevoflurane (Sevorane, Abbott Laboratories, Istanbul, Turkey). Six rats chosen randomly from each group were then euthanized by exsanguinations under anesthesia. Internal organs were excised, blotted, and then weighed.

Statistics

We normalized blood cell data by log transformation before statistical analyses. All data were then subjected to three-way ANOVA using the MIXED procedure[16]. The linear model included the main effects of cage size, cage density, and gender, as well as their interactions. The random term for the statistical analyses was the assignment of the rats to the cages. The effects were considered to be significant at $P<0.05$.

RESULTS

Mortality, growth, and organ development

There were no deaths among the rats raised in small cages at normal density, but the mortality rate at high density was 16.7% for both males and females. Mortality rates for males and females raised in large cages, however, were 16.7% and 11.1% at normal density and 44.4% and 41.7% at high density, respectively. Deaths in small cages occurred during the last two weeks of the experiment, whereas those in large cages occurred sporadically throughout the experiment (data not shown). Deaths were caused by bruising and retarded growth, not infection.

At three weeks of age (post-weaning), the body weight of males (46.7±5.1 g) and females (43.8±3.5 g) did not differ significantly. **Table 1** contains the raw data for body and organ weights with respect to cage size, cage density, and gender for the rats in this study. **Table 2** presents the net effect of cage size and cage density on the body and organ weights of rats raised at normal versus high density and in small versus large cages. We found lower weights for every organ we weighed in rats raised at high density or in large cages as compared to their counterparts raised at low density or in small cages, respectively. We also found that the body or organ weights of the male rats, regardless of housing conditions, were greater than those of the female rats, with the exception of the weights of the adrenal glands, which were greater in the females (**Table 3**).

Figures 1 and **2** depict the effects of cage density and gender on the body and organ weights of the rats raised in either small or large cages. Increasing cage density generally correlated to comparatively lower liver, spleen, and organ weights, except in the cases of the spleen in rats raised in small cages and the ovaries in rats raised in large cages, which both showed weights less than two percent greater than those of their counterparts in the other size of cage (**Fig. 1**). High cage density was associated with a gender-neutral comparative drop

TABLE 3 | Female-to-male ratio of body and organ weights in 10-week-old Sprague-Dawley rats

	Female	Male
Body	1	1.25
Heart	1	1.19
Lung	1	1.13
Emptied stomach	1	1.12
Liver	1	1.13
Kidney (average of right and left)	1	1.23
Spleen	1	1.1
Adrenal gland (average of right and left)	1	0.82

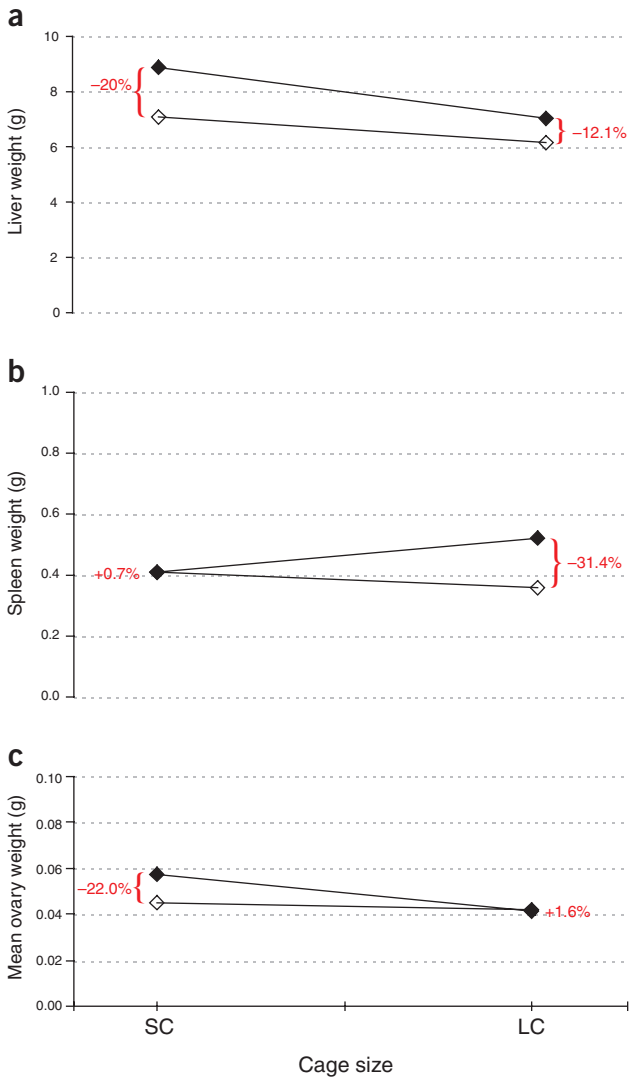

FIGURE 1 | The effects of doubling cage density on the liver, spleen, and ovary weights of rats raised in small or large cages. (**a**) Liver weights ($P<0.002$), (**b**) spleen weights ($P<0.0002$), and (**c**) ovary weights ($P<0.06$). SC = small cage (24 × 40 × 20 cm); LC = large cage (50 × 58 × 30 cm); ND, ♦ = normal density (160 cm^2/rat); HD, ◊ = high density (80 cm^2/rat). Red numbers represent the percentage differences in organ weight between rats raised at normal versus high density in either small or large cages.

in heart, lung, kidney, and spleen weights in all rats (**Fig. 2**). Increasing cage size, independent of density, also corresponded to lower body, lung, liver, and kidney weights in male rats, whereas female rats demonstrated a similar general trend toward lower weights in those areas, though to a lesser degree and with some exception (lung and kidney weights) (**Fig. 3**).

Metabolic profile

Table 4 summarizes the effects of cage size, cage density, and gender on serum chemistry. Cage size did not appreciably affect serum concentrations of glucose, TP, albumin, creatinine, and triglyceride. Rats raised in small cages had higher serum cholesterol, ALP, calcium, and phosphorus concentrations and lower VLDL concentrations than rats raised in large cages (**Table 4**, **Fig. 4**). Except for serum triglyceride (47.4% increase), other blood metabolites were not consistently responsive to doubling cage density across cage sizes (**Table 4**, **Fig. 4**). Females had a 1.24-fold greater creatinine concentration than males, whereas males had 1.57-fold greater ALP activity than females; there was no gender effect on other blood metabolites (**Table 4**, **Fig. 5**).

Hemogram

Differences in hemogram measurements in relation to cage size, cage density, and gender are shown in **Table 5**. The only differences in hemogram values for larger cages and higher density were a 2.2% lower hematocrit value and a 2.9% higher erythrocyte count, respectively. White blood cell counts for males and females were similar. However, males had a 7.5% greater erythrocyte count, 5.4% greater hemoglobin concentration, 6.4% greater hematocrit value, and 14.5% lower platelet count than females. The hemogram values of males and females did not differ with respect to cage density (**Table 5**).

Higher cage density correlated to significant differences in lymphocyte and total leukocyte counts, with significantly higher numbers of both in rats raised in small cages at high density and lower numbers of both in rats raised in large cages at high density (**Fig. 6**). Males raised in large cages had significantly fewer neutrophils than their counterparts in small cages, whereas females in large cages actually had higher neutrophil counts (**Fig. 7**).

DISCUSSION

Cage size

Housing conditions with respect to population size and density have long been welfare concerns because they may impose alterations in physical and social activities, especially in situations involving long-term confinement. Group formation is known to be important in social and cognitive development, physical activity, health, and growth, but the optimum number of rats per group still remains to be determined. The effects of group size on laboratory animals has already been studied by comparing individual versus small-group housing. Standard size cages (24 × 40 × 20 cm) have a recommended density of six juvenile rats per cage[15]. Mering *et al.*[17] compared groups of one to four rats per cage and showed that despite the lack of difference in final body weight, adrenal gland weight varied with group size. Klir *et al.*[18], however, kept male Wistar rats in various group sizes (1–4, 6, and 8 rats per cage) and reported that rats housed in groups of three and four had the greatest body weights, suggesting that there is a curvilinear relationship between group size and growth.

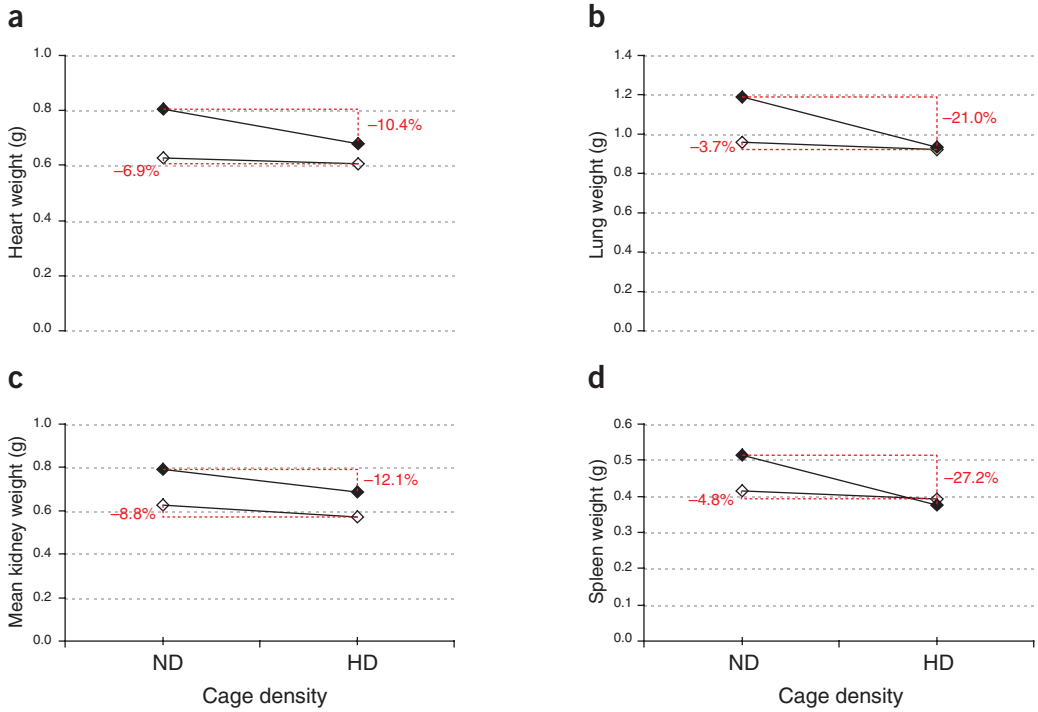

FIGURE 2 | Gender-specific effects of cage density on the heart, lung, kidney, and spleen weights of rats raised at either normal or high density. (**a**) Heart (P<0.04), (**b**) lung (P<0.03), (**c**) kidney (P<0.07), and (**d**) spleen (P<0.006). ND = normal density (160 cm^2/rat); HD = high density (80 cm^2/rat); M, ♦ = male; F, ◊ = female. Red numbers represent the gender-specific percentage differences in the organ weights of rats raised at normal versus high density.

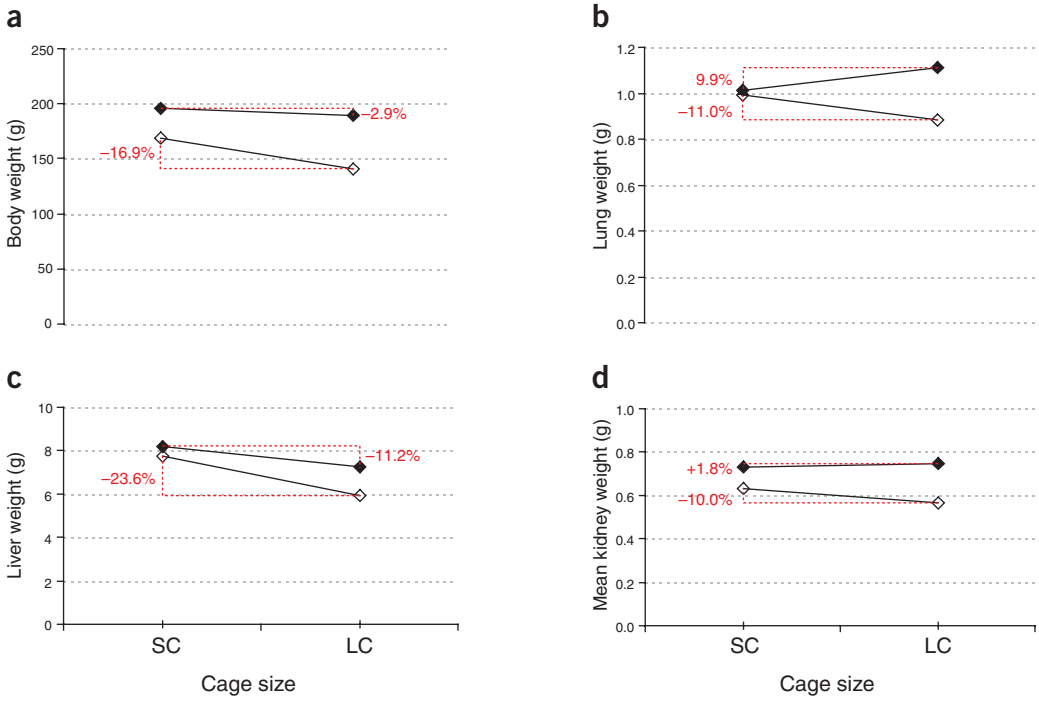

FIGURE 3 | Gender-specific effects of cage size on body, lung, liver, and kidney weights of rats raised in either small or large cages. (**a**) Body weight (P<0.003), and weights of (**b**) lung (P<0.04), (**c**) liver (P<0.002), and (**d**) kidney (P<0.005). SC = small cage (24 × 40 × 20 cm); LC = large cage (50 × 58 × 30 cm); M, ♦ = male; F, ◊ = female. Red numbers represent the gender-specific percentage differences in the organ and body weights of rats raised in small versus large cages.

As group size increases, housing conditions may also change, thereby depressing growth and development[19]. Ventilation has a positive effect on air quality and animal comfort (through odor and humidity reduction) for caged mice[20,21]. These issues involving the internal cage environment may be more pertinent to growth depression when animals are housed in large cages. Moreover, compared with hamsters housed in small cages, those housed in large cages were shown to have a greater fever index following injection of fever-inducing lipopolysaccharide[22]. Greater population size is also more detrimental to the behavior-related physiological and biochemical parameters of males than females[23]. Reducing group size for juvenile rats causes decreased locomotion and lower propensity for exploration[24]. Spangenberg *et al.*[25] examined welfare-related parameters in rats housed in groups of eight (150 × 210 cm) and individually (42 × 26 cm). 'Larger space', as described by Spangenberg, correlated with stimulated physical activity and various social behaviors; improved muscle strength, citrate synthase (oxidative capacity), and muscle glycogen content; and did not affect the ratio of corticosterone and creatinine. These results, however, are ambiguous because the groups of mice differed in both cage size and cage density, thereby confounding the results.

Group size may also influence the potency of the immune system. Jessop *et al.*[26] reported no difference in splenic lymphocyte proliferative responses to phytohemagglutinin in mice housed as a group (five/cage) and individually. In a similar experiment, however, Salvin *et al.*[27] reported that mice housed as a group (five/cage) had a greater capacity to phagocytose dead *Candida albicans*, spleens that produced more macrophage colony stimulating factor, peritoneal macrophages that released greater quantities of interleukin-1 *in vitro* into the surrounding medium, a greater capacity to migrate toward a chemotactic stimulus, and higher titers of IgM hemagglutination antibody to sheep erythrocytes than mice housed individually.

Several variables have been proposed as stress indices (including blood cells, hormones, growth, and mortality) in poultry[28]. Gross and Siegel[29] reported that the number of lymphocytes decreased and the number of heterophils increased in response to stress in chickens. Post *et al.*[30] postulated that the heterophil-to-lymphocyte ratio was a more reliable stress indicator than corticosterone. These indicators are also valid for laboratory animals[31,32] because the stress-induced level of glucocorticoid hormone modulates leukocyte function[33]. However, Rabin *et al.*[34] reported that individually housed mice produced more antibody-forming spleen lymphocytes to sheep erythrocytes than group-housed mice, and that corticosterone levels were not related to the intensity of immune response.

In this experiment, large cages contained more rats than the 'group sizes' tested in other studies. We found that larger cage size was related to body weight depression, with a greater impact on females than males (**Table 1**). Despite no change in stomach, lung, kidney,

TABLE 4 | The effects of cage size, cage density, and gender on serum chemistry parameters in juvenile rats

Groups[a]			Blood metabolites[b]									
CS	CD	S	Glucose	TP	Albumin	Creatinine	TG	Chol	VLDL	ALP	Ca	P
SC (*n*=36)			202.4±4.9	5.70±0.10	3.28±0.04	0.45±0.04	69.9±9.3	56.3±1.6	16.90±1.00	394.2±23.1	11.51±0.05	7.58±0.18
LC (*n*=108)			196.0±5.4	5.72±0.07	3.26±0.02	0.38±0.02	61.9±4.8	49.8±1.4	20.65±1.00	347.2±21.1	11.32±0.06	7.02±0.25
	ND (*n*=48)		205.0±5.1	5.72±0.08	3.27±0.03	0.42±0.03	53.2±4.3	54.4±1.5	17.70±1.28	369.8±21.1	11.42±0.07	7.37±0.21
	HD (*n*=96)		193.4±5.0	5.70±0.09	3.28±0.03	0.41±0.03	78.4±8.5	51.7±1.7	19.85±0.79	371.6±23.8	11.42±0.05	7.23±0.24
		M (*n*=72)	201.5±6.0	5.62±0.08	3.24±0.03	0.37±0.02	63.8±4.3	52.8±1.7	18.65±0.70	453.3±17.4	11.46±0.06	7.45±0.26
		F (*n*=72)	196.9±4.1	5.80±0.08	3.31±0.03	0.46±0.04	67.8±9.4	53.3±1.5	18.91±1.32	288.1±13.5	11.38±0.06	7.15±0.18
ANOVA			*P*<F									
CS			0.36	0.85	0.73	0.06	0.41	0.004	0.007	0.03	0.01	0.04
CD			0.10	0.87	0.89	0.79	0.01	0.23	0.11	0.93	0.96	0.59
S			0.51	0.14	0.13	0.02	0.68	0.81	0.85	0.0001	0.29	0.25
CS × CD			0.06	0.54	0.64	0.008	0.29	0.57	0.02	0.14	0.65	0.002
CS × S			0.06	0.91	0.39	0.02	0.18	0.89	0.09	0.95	0.03	0.56
CD × S			0.92	0.34	0.96	0.39	0.18	0.44	0.93	0.17	0.65	0.88
CS × CD × S			0.26	0.37	0.18	0.30	0.14	0.10	0.13	0.19	0.0002	0.0004

[a]CS, cage size (SC, small cage: 24 × 40 × 20 cm in width × depth × height; LC, large cage:-50 × 58 × 30 cm in width × depth × height); CD, cage density (ND, normal density: 160 cm^2/rat; HD, high density: 80 cm^2/rat); S, gender (M, male, F, female). Rats were separated by gender and assigned randomly to cages so that 6 and 18 rats were raised at ND in SC and 12 and 36 rats at HD in LC.
[b]Data were obtained at 10 weeks of age. TP, total protein; TG, triglyceride; Chol, cholesterol; VLDL, very low-density lipoprotein; ALP, alkaline phosphatase, Ca, calcium; P, phosphorus. Unit is mg/dl for all metabolites, expect for ALP (U/l). Values were presented as mean±SE by cage type, cage density, and gender.

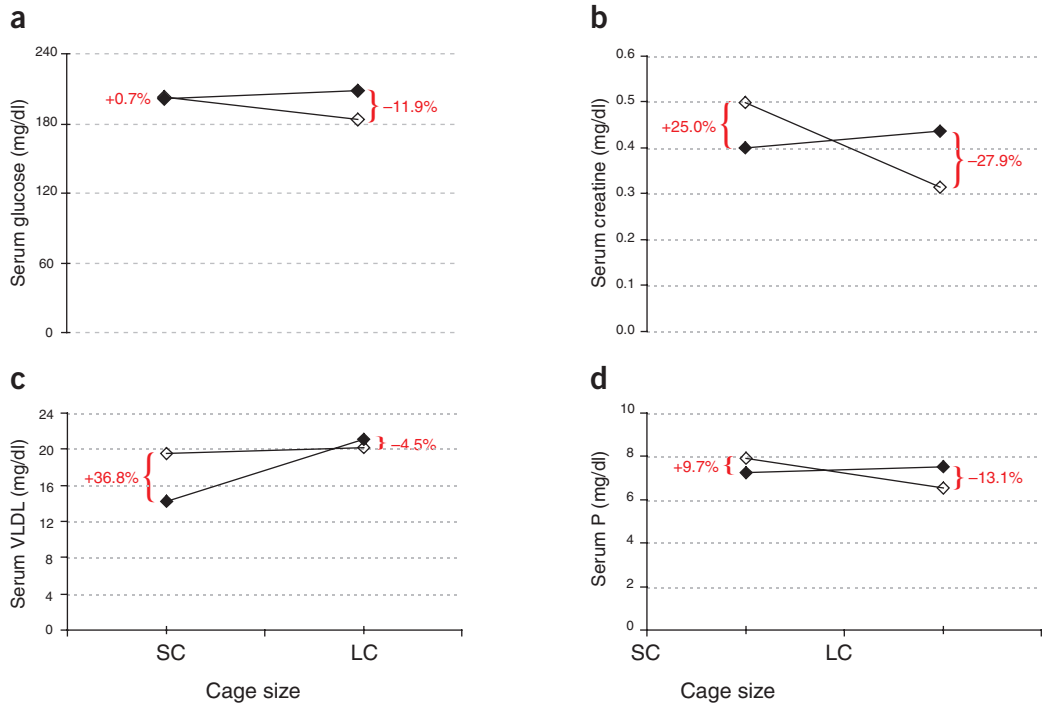

FIGURE 4 | The effects of doubling cage density on the serum concentrations of glucose, creatinine, VLDL, and phosphorus in rats raised in small or large cages. (**a**) Serum glucose concentration ($P<0.06$), (**b**) serum creatinine concentration ($P<0.008$), (**c**) serum VLDL concentration ($P<0.007$), and (**d**) serum phosphorus concentration ($P<0.002$). SC = small cage (24 × 40 × 20 cm); LC = large cage (50 × 58 × 30 cm); ND, ♦ = normal density (160 cm^2/rat); HD, ◊ = high density (80 cm^2/rat). Red numbers represent the percentage differences in the concentrations of various serum substances in rats raised at normal versus high density in large or small cages.

spleen, and adrenal gland weights, there were significant depressions in the heart, liver, and ovary weights. These depressions were pronounced, especially for rats raised at high density (**Fig. 1**) and for females (**Fig. 3**). The only blood metabolites altered by cage size were creatinine, ALP, calcium, and phosphorus concentrations (**Table 4**). Moreover, in response to increasing cage size, reductions in these variables were greater for rats raised at high density than for rats raised at normal density (**Fig. 4**) and for females than for males (**Fig. 5**). Except for hematocrit value, cage size did not affect any hemogram values (**Table 5**). However, in response to increasing cage size, there was an increase in lymphocyte count for rats raised at high density as opposed to a decrease for rats raised at normal density (**Fig. 6**). Also, the neutrophil-to-lymphocyte ratio increased for males but decreased for females with respect to increasing cage size (**Fig. 7**). Briefly, our data showed that increasing

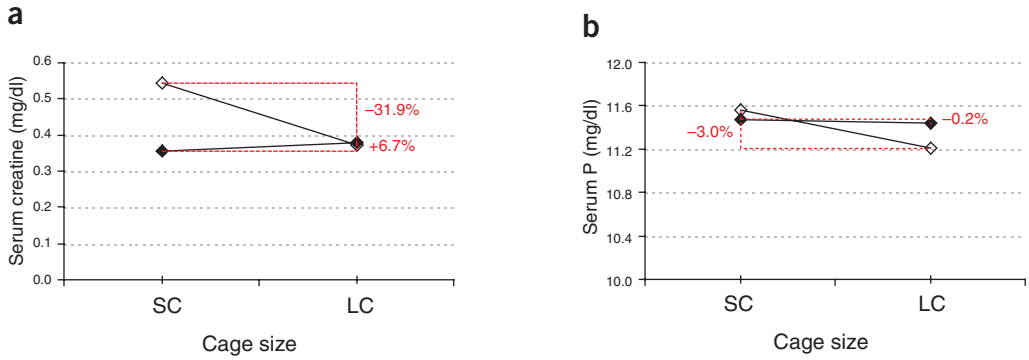

FIGURE 5 | Gender-specific effects of cage size on the serum concentrations of creatinine and calcium in rats. (**a**) Serum creatinine concentration ($P<0.02$) and (**b**) serum calcium concentration ($P<0.03$). SC = small cage (24 × 40 × 20 cm); LC = large cage (50 × 58 × 30 cm); M, ♦ = male; F, ◊ = female. Red numbers represent the gender-specific percentage differences in serum concentrations of creatinine or calcium in rats raised in small versus large cages.

TABLE 5 | The effects of cage size, cage density, and gender on hemogram in juvenile rats

Groups[a]			Hemogram values[b]										
CS	CD	S	Neutrophil (10^3/μl)	Lymphocyte (10^3/μl)	N:L	Monocyte (10^3/μl)	Eosinophil (10^3/μl)	Basophil (10^3/μl)	Leukocyte (10^3/μl)	RBC (10^6/μl)	PLT (10^3/μl)	Hb (g/dl)	HCT (%)
SC (*n*=36)			0.51±0.10	6.12±0.42	0.087±0.016	0.017±0.004	0.011±0.003	0.58±0.29	7.25±0.49	7.73±0.08	834±28	14.14±0.13	40.5±0.5
LC (*n*=108)			0.35±0.08	5.31±0.41	0.070±0.017	0.009±0.004	0.014±0.003	0.42±0.14	6.10±0.44	7.67±0.10	844±20	14.04±0.15	39.6±0.5
	ND (*n*=48)		0.47±0.11	5.48±0.40	0.089±0.020	0.018±0.005	0.012±0.003	0.44±0.26	6.42±0.44	7.59±0.10	818±19	14.00±0.16	39.7±0.5
	HD (*n*=96)		0.39±0.07	5.95±0.44	0.068±0.013	0.009±0.002	0.014±0.003	0.57±0.21	6.93±0.50	7.81±0.08	860±27	14.19±0.12	40.4±0.4
		M (*n*=72)	0.38±0.09	5.97±0.40	0.063±0.014	0.014±0.004	0.011±0.003	0.60±0.27	6.97±0.42	8.00±0.07	782±19	14.48±0.11	41.9±0.3
		F (*n*=72)	0.48±0.09	5.46±0.44	0.094±0.018	0.012±0.004	0.017±0.004	0.41±0.16	6.38±0.52	7.40±0.07	895±23	13.70±0.12	39.2±0.4
ANOVA							***P*<F**						
CS			0.20	0.14	0.46	0.17	0.39	0.61	0.07	0.52	0.74	0.56	0.05
CD			0.54	0.39	0.37	0.11	0.71	0.68	0.42	0.02	0.18	0.26	0.15
S			0.44	0.34	0.18	0.81	0.09	0.55	0.35	0.0001	0.0005	0.0001	0.0001
CS × CD			0.69	0.0007	0.08	0.65	0.24	0.15	0.02	0.92	0.82	0.30	0.90
CS × S			0.09	0.55	0.05	0.12	0.95	0.51	0.59	0.26	0.11	0.06	0.33
CD × S			0.20	0.64	0.38	0.62	0.92	0.79	0.42	0.30	0.62	0.72	0.36
CS × CD × S			0.41	0.58	0.52	0.12	0.56	0.04	0.10	0.30	0.70	0.89	0.63

[a]CS, cage size (SC, small cage: 24 × 40 × 20 cm in width × depth × height; LC, large cage: 50 × 58 × 30 cm in width × depth × height); CD, cage density (ND, normal density: 160 cm^2/rat; HD, high density: 80 cm^2/rat); S, gender (M, male, F, female). Rats were separated by gender and assigned randomly to cages so that 6 and 18 rats were raised at ND in SC and 12 and 36 rats at HD in LC.
[b]Data were obtained at 10 weeks of age. N:L, neutrophil:lymphocyte ratio; RBC, erythrocytes; PLT, platelets; Hb, hemoglobin; HCT, hematocrit. Values were presented as mean±SE by cage type, cage density, and gender.

group size had an adverse effect on growth and organ development, which were more notable for rats raised in normal stocking density than for rats raised in high stocking density and for females than for males.

Cage density

The adverse effects of crowding on laboratory animal welfare are related to limited physical activity (a cause of aggressiveness), alteration of the microenvironment (such as humidity, temperature, and air quality in the cage), and suppression of immune potency. An earlier study by Muraoka *et al.*[35] showed that increasing cage density from two to five rats was associated with growth suppression without affecting liver, kidney, heart, and femur weights. Armario *et al.*[11], however, showed that crowded rats (ten/cage) had lower body weight than control rats (three/cage). Moreover, crowding decreased food intake and increased water intake but did not alter the weights of the thymus, liver, or endocrine glands (though the testes were affected). In another experiment[10], no changes in adrenal gland weight and corticosterone concentration were observed among male rats subjected to crowding (from three to nine rats per cage), but the rats did show depression in growth and increased testis weight.

Crowding may cause aggressive behaviors by affecting neuroendocrine mechanisms, as reflected in elevated dopamine (but not norepinephrine or serotonin) concentration in the diencephalon[36], and aggravated anxiety[24]. Similar to the effects of increasing group size, increasing cage density from four to eight mice per cage was shown to suppress immune potency through decreasing lymphocyte count, increasing neutrophil count, decreasing superoxide production activity and phagocytic activity of neutrophils, and increasing IgG levels[37]. However, Peters and Festing[38] reported no adverse effects on body or adrenal gland weight in mice after increasing cage density from 60 cm^2 per mouse (optimal) to 27 cm^2 per mouse. The response of stress-related variables (such as corticosterone) differs between males and females as cage density[39] and cage size[40] increases, with a greater response in males than in females in terms of motor activity.

In the present experiment, higher cage density was associated with greater mortality, especially among rats raised in larger group size. Doubling cage density resulted in depressions in body weight and the weights of heart, lung, stomach, liver, kidney, and spleen (**Table 1**). We observed this result at a greater magnitude for males than for females (**Fig. 2**). However, the adrenal gland and genital organ weights were independent of cage density. Three parameters, triglyceride versus cage density (**Table 4**), serum creatinine versus cage size, and calcium concentrations versus cage size, showed greater magnitudes of difference in female as compared with male rats. These gender-specific variations may reflect a differential response to limited physical activity in female versus male rats[41]. Most stress-related hemogram measurements (**Table 5**) deteriorated when cage density was doubled, an indication of the adverse effects of crowding

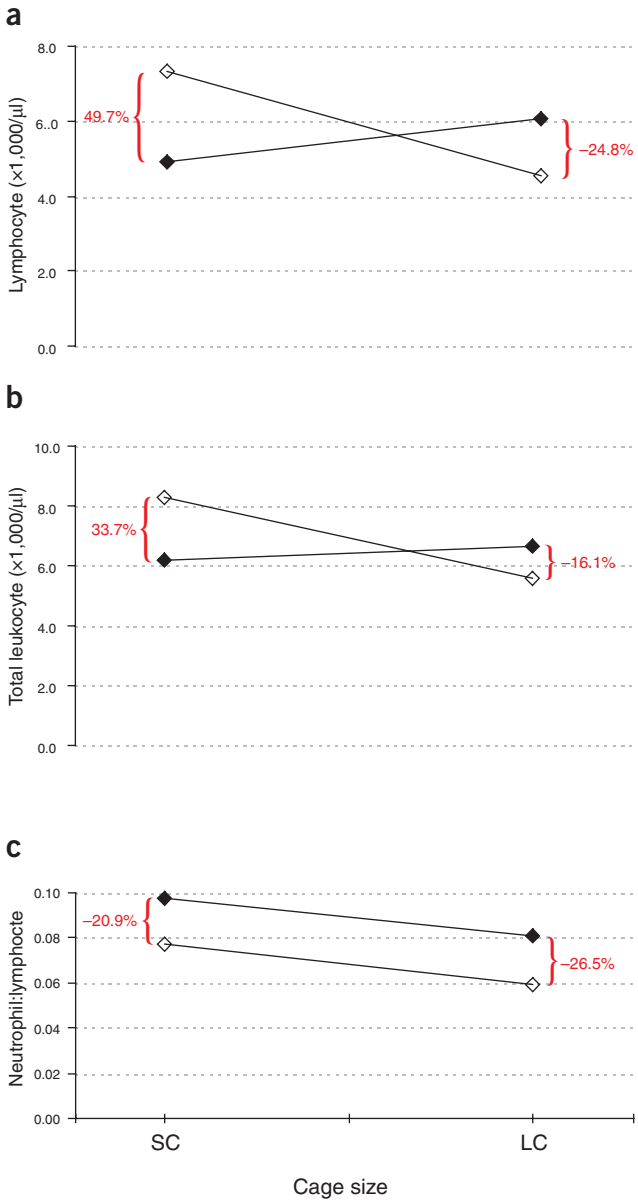

FIGURE 6 | The effects of doubling cage density on lymphocyte, leukocyte, and neutrophil cell populations in rats raised in either small or large cages. (**a**) Lymphocyte count ($P<0.0007$), (**b**) total leukocyte count ($P<0.02$), and (**c**) neutrophil:lymphocyte ratio ($P<0.08$). SC = small cage (24 × 40 × 20 cm); LC = large cage (50 × 58 × 30 cm); ND, ♦ = normal density (160 cm^2/rat); HD, ◊ = high density (80 cm^2/rat). Red numbers represent the percentage differences in cell population parameter in rats raised at normal versus high density in small or large cages.

on immune system-related parameters of the rats raised in large cages (**Fig. 6**) and females (**Fig. 7**).

Gender

Differences in the growth rate and organ weights between males and females are well established. Responsiveness to stress may also vary by gender, which might be linked to behavioral reflexes[24,42]. Using broiler chicks, Marin *et al.*[43] showed males were more stress-susceptible than females, as reflected by increased corticosterone concentration and stressor-induced benzodiazepine receptor density in the brain. Under normal housing conditions, the blood chemistry[44] and hemogram[45] of males and females are not different[44]. Uribe *et al.*[46], however, reported that male Sprague-Dawley rats had greater ALP activity and glucose and P concentrations, whereas females had greater albumin. The same researchers[46] also reported that male and female rats had different leukocyte counts and hemoglobin concentrations.

In this experiment, mortality rate was independent of gender. In general, males had greater body and organ weights, except in the case of the adrenal gland (**Table 1**). In response to greater cage size, depression in organ weight for females was greater than for males (**Fig. 3**). However, these depressions were greater for males than for females in response to higher cage density (**Fig. 2**). These data suggest that males and females may have different social behavior and adapt differently to housing conditions. The incidence of greater adrenal weight, higher creatinine concentration in females as compared to males (**Tables 1**, **4**), as well as the greater increase in the neutrophil-to-lymphocyte ratios for males in response to altering housing conditions (**Table 5**, **Fig. 7**), may indicate that males and females have varying degrees of predisposition to stress and immune potency. The higher erythrocyte count, hemoglobin concentration, and hematocrit values for males as compared to females could be linked to greater heart and lung weights. Blood chemistry value, organ weights[46–48], and hemogram parameters[48–50] agreed with those in the literature, despite being affected by cage size, cage density, and gender.

CONCLUSIONS

In this experiment, the effects of group size and cage density on growth, organ development, metabolic profile, and hemogram of post-weanling to puberty-period male and female rats were evaluated without confounding floor area and feeder space per animal. Increasing population size and cage density were associated with greater mortality and caused depression in body and organ weights. In general, increasing group size and crowding had more detrimental effects on the growth of females than males. The immune system-related hemogram values, however, might indicate that male rats had a greater predisposition to emotional stress and infections than female rats. Group size and cage density should not exceed six growing rats per cage at a density of 160 cm^2 per rat. The effects of cage size and cage density may also have been worsened by grid floor in this experiment, the usage of which is no longer suggested. Future studies should deal with the carry-over effects

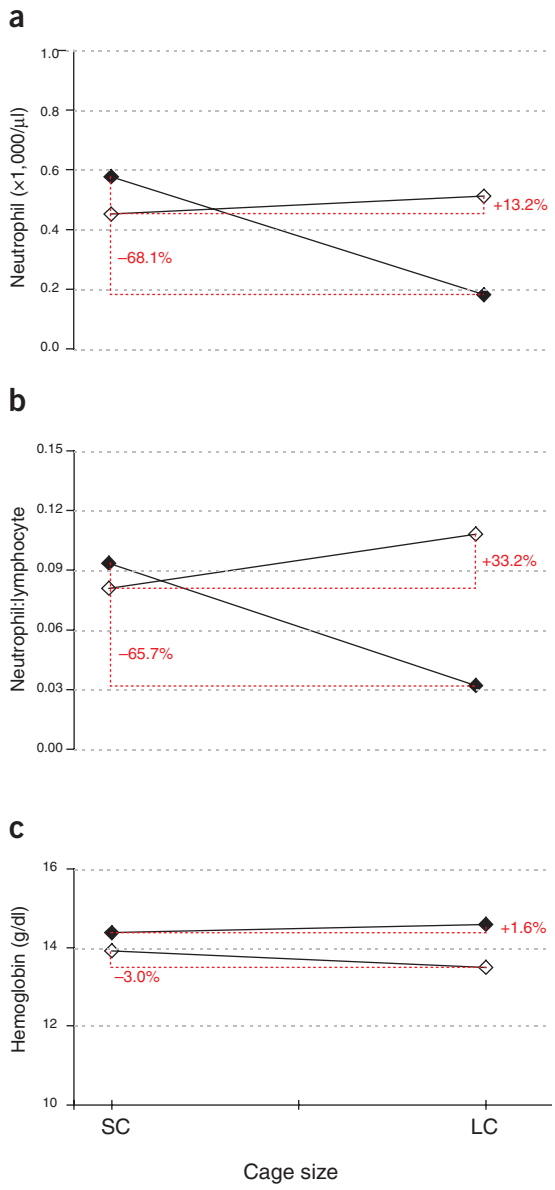

FIGURE 7 | Gender-specific effects of cage size on lymphocyte and neutrophil cell populations and hemoglobin concentration in rats raised in either small or large cages. (**a**) Neutrophil count ($P<0.09$), (**b**) neutrophil:lymphocyte ratio ($P<0.05$), and (**c**) hemoglobin concentration ($P<0.06$). SC = small cage (24 × 40 × 20 cm); LC = large cage (50 × 58 × 30 cm); M, ♦ = male; F, ◊ = female. Red numbers represent the gender-specific percentage differences in cell population parameter or hemoglobin concentration in rats raised in small versus large cages.

of group size and cage density on the productivity of juvenile animals.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Woolverton, W.L., Ator, N.A., Beardsley, P.M. & Carroll, M.E. Effects of environmental conditions on the psychological well-being of primates: a review of the literature. *Life Sci.* **44(14)**, 901–917 (1989).
2. Peng, X., Lang, C.M., Drozdowicz, C.K. & Ohlsson-Wilhelm, B.M. Effect of cage population density on plasma corticosterone and peripheral lymphocyte populations of laboratory mice. *Lab Anim.* **23(4)**, 302–306 (1989).
3. Keeling, L.J., Estevez, I., Newberry, R.C. & Correia, M.G. Production-related traits of layers reared in different sized flocks: the concept of problematic intermediate group sizes. *Poult. Sci.* **82(9)**, 1393–1396 (2003).
4. Serrano, L.J. Carbon dioxide and ammonia in mouse cages: effect of cage covers, population, and activity. *Lab. Anim. Sci.* **21(1)**, 75–85 (1971).
5. Anderson, A., Werboff, J. & Les, E.P. Effects of environmental temperature-humidity and cage density on body weight and behavior in mice. *Experientia* **24(10)**, 1022–1023 (1968).
6. Les, E.P. Cage population density and efficiency of feed utilization in inbred mice. *Lab. Anim. Care* **18(3)**, 305–313 (1968).
7. Monteiro, F., Abraham, M.E., Sahakari, S.D. & Mascarenhas, J.F. Effect of immobilization stress on food intake, body weight and weights of various organs in rat. *Indian J. Physiol. Pharmacol.* **33(3)**, 186–190 (1989).
8. Forner, M.A., Barriga, C., Rodriguez, A.B. & Ortega, E. A study of the role of corticosterone as a mediator in exercise-induced stimulation of murine macrophage phagocytosis. *J. Physiol.* **488(Pt3)**, 789–794 (1995).
9. Hurst, J.L., Barnard, C.J., Tolladay, U., Nevision, C.M. & West, C.D. Housing and welfare in laboratory rats: effects of cage stocking density and behavioural predictors of welfare. *Anim. Behav.* **58(3)**, 563–586 (1999).
10. Armario, A., Castellanos, J.M. & Balasch, J. Effect of crowding on emotional reactivity in male rats. *Neuroendocrinology* **39(4)**, 330–333 (1984).
11. Armario, A., Ortiz, R. & Balasch, J. Effect of crowding on some physiological and behavioral variables in adult male rats. *Physiol. Behav.* **32(1)**, 35–37 (1984).
12. Gamallo, A., Villanua, A. & Beato, M.J. Body weight gain and food intake alterations in crowd-reared rats. *Physiol. Behav.* **36(5)**, 835–837 (1986).
13. Restrepo, C. & Armario, A. Comparison of crowding and food restriction effects on growth, body weight gain and endocrine status in the rat. *Reprod. Nutr. Dev.* **29(3)**, 339–345 (1989).
14. National Research Council. *Nutrient Requirements of Laboratory Animals* 4th Edn. (National Academy Press, Washington, DC, 1995).
15. Canadian Council on Animal Care. *Guide to the Care and Use of Experimental Animals* Vol. I, 2nd Edn. (Bradda Printing Services Inc., Ottawa, ON, Canada, 1993).
16. SAS. *SAS® User's Guide: Statistics*, Version 7th. Statistical Analysis System Institute Inc., Cary, NC, USA, 1998).
17. Mering, S., Kaliste-Korhonen, E. & Nevalainen, T. Estimates of appropriate number of rats: interaction with housing environment. *Lab Anim.* **35(1)**, 80–90 (2001).
18. Klir, P., Bondy, R., Lachout, J. & Hanis, T. Physiological changes in laboratory rats caused by different housing. *Physiol. Bohemoslov* **33(2)**, 111–121 (1984).
19. Dawkins, M S., Donnelly, C.A. & Jones, T.A. Chicken welfare is influenced more by housing conditions than by stocking density. *Nature* **427(6972)**, 342–344 (2004).
20. Keller, L.S., White, W.J., Snider, M.T. & Lang, C.M. An evaluation of intra-cage ventilation in three animal caging systems. *Lab. Anim. Sci.* **39(3)**, 237–242 (1989).
21. Memarzadeh, F., Harrison, P.C., Riskowski, G.L. & Henze, T. Comparison of environment and mice in static and mechanically ventilated isolator cages with different air velocities and ventilation designs. *Contemp. Top. Lab. Anim. Sci.* **43(1)**, 14–20 (2004).
22. Kuhnen, G. The effect of cage size and enrichment on core temperature and febrile response of the golden hamster. *Lab Anim.* **33(3)**, 221–227 (1999).

23. Perez, C. *et al*. Individual housing influences certain biochemical parameters in the rat. *Lab. Anim.* **31(4)**, 357–361 (1997).
24. Arakawa, H. Age dependent effects of space limitation and social tension on open-field behavior in male rats. *Physiol. Behav.* **84(3)**, 429–436 (2005).
25. Spangenberg, E.M., Augustsson, H., Dahlborn, K., Essen-Gustavsson, B. & Cvek, K. Housing related activity in rats: effects on body weight, urinary corticosterone levels, muscle properties and performance. *Lab Anim.* **39(1)**, 45–57 (2005).
26. Jessop, J.J., Gale, K. & Bayer, B.M. Enhancement of rat lymphocyte proliferation after prolonged exposure to stress. *J. Neuroimmunol*. **16(2)**, 261–271 (1987).
27. Salvin, S.B., Rabin, B.S. & Neta, R. Evaluation of immunologic assays to determine the effects of differential housing on immune reactivity. *Brain Behav. Immun.* **4(3)**, 180–188 (1990).
28. Craig, J.V., Craig, J.A. & Vargas, J. Corticosteroids and other indicators of hens' well-being in four laying-house environments. *Poult. Sci.* **65(5)**, 856–863 (1986).
29. Gross, W.B. & Siegel, H.S. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. *Avian Dis.* **27(4)**, 972–979 (1983).
30. Post, J., Rebel, J.M. & ter Huurne, A.A. Automated blood cell count: a sensitive and reliable method to study corticosterone-related stress in broilers. *Poult. Sci.* **82(4)**, 591–595 (2003).
31. Del Pup, J. & Palmes, E.D. Effect of housing conditions on corticosterone levels in mice. *Arch. Environ. Health* **22(4)**, 493–495 (1971).
32. Squires, E.J. in *Applied Animal Endocrinology*, 192–229 (CABI Publishing, Cambridge, MA, 2003).
33. Stark, J.L. *et al*. Social stress induces glucocorticoid resistance in macrophages. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **280(6)**, R1799–R1805 (2001).
34. Rabin, B.S., Lyte, M. & Hamill, E. The influence of mouse strain and housing on the immune response. *J. Neuroimmunol.* **17(1)**, 11–16 (1987).
35. Muraoka, Y., Itoh, M. & Hayashi, Y. Effects of the population density on growth of SD-JCL rats. [Japanese]. *Jikken Dobutsu* **25(4)**, 283–289 (1976).
36. Holladay, S.D. & Edens, F.W. Effect of cage density and rank in peck order on brain regional monoamines in adult male *Coturnix coturnix japonica*. *Comp. Biochem. Physiol. A.* **87(2)**, 261–265 (1987).
37. Tsukamoto, K. *et al*. Effects of crowding on immune functions in mice. [Japanese]. *Nippon Eiseigaku Zasshi* **49(4)**, 827–836 (1994).
38. Peters, A. & Festing, M. Population density and growth rate in laboratory mice. *Lab Anim.* **24(3)**, 273–279 (1990).
39. Brown, K.J. & Grunberg, N.E. Effects of housing on male and female rats: crowding stresses male but calm females. *Physiol. Behav.* **58(6)**, 1085–1089 (1995).
40. Rock, F.M., Landi, M.S., Hughes, H.C. & Gagnon, R.C. Effects of caging type and group size on selected physiologic variables in rats. *Contemp. Top. Lab. Anim. Sci.* **36(2)**, 69–72 (1997).
41. Kannus, P. *et al*. Effects of immobilization, three forms of remobilization and subsequent deconditioning on bone mineral content and density in rat femora. *J. Bone Miner. Res.* **11(9)**, 1339–1346 (1996).
42. Eskola, S. & Kaliste-Korhonen, E. Nesting material and number of females per cage: effects on mouse productivity in BALB/c, C57BL/6J, DBA/2 and NIH/S mice. *Lab. Anim.* **33(2)**, 122–128 (1999).
43. Marin, R.H., Benavidez, E., Garcia, D.A. & Satterlee, D.G. Sex differences in central benzodiazepine receptor densities and circulating corticosterone release after acute stress in broiler chicks. *Poult. Sci.* **81(2)**, 261–264 (2002).
44. Tsuchiya, N. *et al*. Age-related changes and sex differences on the serum chemistry values in Sprague-Dawley rats. I. 6-30 weeks of age. [Japanese]. *Exp. Anim.* **43(5)**, 671–678 (1995).
45. Robel, G.L., Lochmiller, R.L., McMurry, S.T. & Qualls, Jr., C. W. Environmental, age, and sex effects on cotton rat (*Sigmodon hispidus*) hematology. *J. Wildl. Dis.* **32(2)**, 390–394 (1996).
46. Uribe, M. *et al*. Hematological serological valves, and organ weight in adult Sprague-Dawley rats. *Rev. Med. Chil.* **123(10)**, 1235–1242 (1995).
47. Wolford, S.T. *et al*. Age-related changes in serum chemistry and hematology values in normal Sprague-Dawley rats. *Fundam. Appl. Toxicol.* **8(1)**, 80–88 (1987).
48. Kahn, C.M. & Line, S. *The Merck Veterinary Manual* 9th edn. (Merial Ltd., Whitehouse Station, NJ, 2005).
49. Hebold, G. & Bleutel, H. Hematologic standards of female and male rats (Sprague Dawley). [German]. *Z. Versuchstierkd* **13(6)**, 316–320 (1971).
50. Leonard, R. & Ruben, Z. Hematology reference values for peripheral blood of laboratory rats. *Lab. Anim. Sci.* **36(3)**, 277–281 (1986).