

Effect of Overcrowding on Hair Corticosterone Concentrations in Juvenile Male Wistar Rats

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In many species, chronic stress due to overcrowding during the juvenile period triggers several metabolic and behavioral pathologies in adulthood. The aim of this study was to determine whether a chronic stress condition (overcrowding) induces changes in plasma and hair corticosterone concentrations, overall growth, and organ weights in young Wistar rats. The experimental subjects were divided into 2 groups (control and overcrowded); the overcrowded subjects were exposed to overcrowding during days 38 through 65 after birth. Plasma and hair corticosterone concentrations were higher in overcrowded rats compared with control subjects. In addition, overcrowding reduced body and organ weight gains. These results demonstrate that measuring the concentration of corticosterone in hair samples is an effective, noninvasive method for monitoring chronic stress in rats.

Overcrowding is defined as the coexistence of individuals in an overly confined space. In rats, overcrowded living conditions leads to changes in physical and psychologic health,⁵⁵ increased HPA activity,²⁶ and variations in the body and organ weights of juvenile⁵⁸ and adult rats.^{20,37} Some studies have reported that overcrowding-related increases in blood corticosterone levels affect body weight and the weights of immunologic but not visceral organs;^{7,54} these changes can be related to somatic responses to the imposed stressor.^{8,26,58}

The juvenile period is important as a transitional time from childhood to adulthood¹² because this period involves physiologic maturation.⁴⁵ Despite difficulty in defining the juvenile stage for male rats,^{29,52} most researchers agree that adult stage begins on the 60th day after birth, when animals reach physical maturity, implying increased gonadotropin releasing hormone and activation of the HPA.²⁹

Researchers quantify glucocorticoids in various types of samples, including plasma, saliva, feces, urine and hair.^{1,21} Glucocorticoid deposition in hair has been proposed as an accurate indicator of chronic stress in several mammalian species.³¹ Quantifying glucocorticoid levels in hair is a noninvasive sampling method, and samples can be stored at room temperature, but the most relevant aspect is that hair samples indicate chronic (rather than acute) production of glucocorticoids.³⁹

Hair follicles contain glucocorticoids, a peripheral component of the HPA.^{4,43} Corticosteroids potentially can be deposited in the hair shaft in 2 ways. One hypothesis is that steroids found in hair are dependent on bloodstream corticoid levels; another hypothesis suggests that steroid levels are directly related to the local production of glucocorticoids in follicles.⁴² However, the contribution of local glucocorticoid synthesis to the amount of hormone measured in hair is not yet clear.

A recent study⁴¹ showed that the exposure of adult male rats to chronic immobilization stress or chronic unpredictable stress resulted in increased corticosterone levels in the animals' hair. Although that study was important for validating the use of hair to assess long-term HPA activity in rodents, further research is needed to confirm and extend those findings to other stressors and age groups. Therefore, the purposes of the present study were to determine corticosterone levels in the plasma and hair of juvenile male Wistar rats under overcrowded conditions and to determine how the stress of overcrowding influenced the body and organ weights of the animals.

Materials and Methods

The present research was conducted in the Neuroscience and Behavior Laboratory at the University of Los Andes (Bogotá, Colombia). The protocol was approved by the bioethics committee of the National University of Colombia and the IACUC of the Universidad de Los Andes.

Animals and housing conditions. Male Wistar rats (age, 38 d; $n = 18$) bred inhouse at the university's laboratory animal facility were used in the study. Experimental subjects were maintained under a 12:12-h light:dark cycle (lights on, 0700), 21 ± 2 °C room temperature, and humidity control (40–60 %). Food (Laboratory Autoclavable Rodent Diet 5010, LabDiet, St Louis, MO) and water were freely available. Rats were allocated randomly into 2 groups ($n = 9$ each). The control group was housed in acrylic boxes (15 cm × 31cm × 18 cm; 3 rats per box; 155 cm² per rat), whereas overcrowded rats were housed in similar but smaller boxes (12 cm × 12 cm × 18 cm; 3 rats per box; 48 cm² per rat). Boxes contained sterilized poplar shavings (Anestcol, Bogotá, Colombia), which were replaced daily. All animals were handled briefly during each of the 3 days prior to blood and hair sampling to reduce the stress on the collection day. The experiment ended on postnatal day (PND) 65.

Blood samples and ELISA. On PND 38 and 65, the rats were removed individually from their cages for approximately 5 min and transported to the next room to obtain blood samples. The rats were gently restrained while the veterinarian used ice-cold heparinized capillary tubes (Brand, Wertheim, Germany) and needles (21-gauge, 32 mm) to collect blood from

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the right saphenous vein. The samples were maintained at 0 °C, centrifuged for 10 min at 600 × g (MicroCL 17 Microcentrifuge, Thermo Scientific, Waltham, MA), and plasma extracted and stored at -20 °C. The plasma was evaluated by ELISA (catalog no. ADI-901-097, Enzo Life Sciences, Exeter, United Kingdom), plates were read at 490 nm, and corticosterone concentrations were determined by using the kit standards and Gen5 software (BioTek, Winooski, VT).

Hair samples and hormone analysis. To obtain hair samples without hair follicles, the right flank area of each animal was shaved on the first and last days of the experiment (PND 38 and 65, respectively). Hair samples were processed as previously described.¹⁶ Briefly, the samples were rinsed 3 times (5 min each) in isopropanol (5 mL) at room temperature and dried for 4 d; 150- to 200-mg samples then were pulverized in a grinding ball mill (Retsch model MM200, Verder Scientific, Newtown, PA). Samples of powdered hair (49.5 to 50.5 mg each) were incubated overnight in methanol (1 mL) to extract corticosterone. Methanol was evaporated (SpeedVac, Savant, Thermo Fisher); samples were reconstituted in 5% methanol in deionized water (1 mL) and purified over C18 columns (Supelco Supel Select 30 mg × 1 mL, Sigma-Aldrich, St Louis, MO). The columns were activated by using methanol (1 mL) and deionized water (1 mL), samples were added to the columns, and columns were rinsed with 5% methanol (1 mL). Finally, samples were eluted into methanol (1 mL) and dried under vacuum for 3 h. This protocol was validated by demonstrating parallelism of authentic corticosterone standards with serially diluted purified rat hair extracts (data not shown). Corticosterone in the purified hair samples were quantified by using enzyme immunoassay kits (catalog no. K014-H1, DetectX, Arbor Assays, Ann Arbor, MI). Absorbancies were read at a wavelength of 450 nm, and corticosterone concentrations were determined by using the standard curve and Gen5 software (BioTek).

Euthanasia and organ collection. To assess the effects of overcrowding stress on organ weight and to determine the relationship between organ weight changes and plasma and hair corticosterone levels, rats were anesthetized with ketamine (90 mg/kg IP) and xylazine (10 mg/kg IP) at the end of the study and then euthanized by means of cardiac perfusion (200 mL saline solution 0.9% followed by 200 mL 4% paraformaldehyde). Heart, lungs, kidneys, and adrenal glands were dissected and carefully weighed.

Statistical analysis. For statistical analysis, corticosterone concentrations in hair (pg/mg) and plasma (ng/mL) were log-transformed to normalize the data. For hair corticosterone, plasma corticosterone, and body weight data, 2-way nested mixed-design ANOVA was used, with treatment as a between-subjects variable, day as a within-subjects variable, and cage as a nested variable within treatment group. Significant effects in the mixed ANOVA were followed by posthoc univariate ANOVA to determine specific differences related to treatment and day. Univariate ANOVA with treatment as a between-subjects variable and cage as a nested variable within treatment group were performed on the organ weight values expressed either as absolute weights or relative weights (organ weight divided by overall body weight). Right and left adrenal gland weights were combined to yield a single value for each animal for the purpose of statistical analysis. Due to technical problems, no organ weight data were available from one of the control rats, which can be seen in the adjusted error df values for the organ weight ANOVA. Hair or plasma corticosterone concentrations as predictors of organ weights were assessed by using a mixed linear-regression model with organ weight, corticosterone

concentration (hair or plasma), and treatment as fixed effects variables and cage as a random effects variable. A similar analysis was performed to assess plasma corticosterone as a predictor of hair corticosterone. All statistical analyses were performed by using SPSS (IBM, Armonk, NY), and a *P* value less than 0.05 was considered significant. In the present experiment, all error bars indicate 1 SD.

Results

Corticosterone deposition in hair. The assay results confirmed that the hair of male Wistar rats contains corticosterone. ANOVA revealed significant main effects of treatment ($F = 10.69$; $df = 1.4$; $P = 0.031$) and day ($F = 13.56$; $df = 1.16$; $P = 0.002$) and a significant treatment×day interaction ($F = 7.94$; $df = 1.16$; $P = 0.012$). Posthoc testing further revealed no significant difference between the control and overcrowded groups on PND 38 but significantly higher hair corticosterone concentrations in the overcrowded group (40.5 ± 9.8 pg/mg) compared with the control group (19.7 ± 9.8 pg/mg) on PND 65 ($F = 35.64$; $df = 1.4$; $P = 0.004$; Figure 1). The nested factor of cage within group had no significant effect on this or any other outcome measure.

Effect of overcrowding during the juvenile period on plasma corticosterone. Plasma corticosterone showed a significant main effect of treatment ($F = 9.80$; $df = 1.4$; $P = 0.035$) and a significant treatment×day interaction ($F = 9.26$; $df = 1.16$; $P = 0.008$). Posthoc testing showed that the groups did not differ on PND 38, whereas the overcrowded group had significantly higher plasma corticosterone concentrations (470.2 ± 113.2 ng/mL) than did the control group (232.6 ± 78.7 ng/mL) on PND 65 ($F = 28.70$; $df = 1.4$; $P = 0.006$ (Figure 2).

Relationships between plasma and hair corticosterone concentrations. Multiple linear regression analyses of data from control and overcrowded rats showed that plasma corticosterone concentrations did not significantly predict hair corticosterone at either PND 38 or PND 65.

Effect of overcrowded group on body weight. Analysis of body weight data showed significant main effects of treatment ($F = 42.48$; $df = 1.4$; $P = 0.003$) and day ($F = 955.29$; $df = 4, 64$; $P < 0.001$) and a significant treatment×day interaction ($F = 46.52$; $df = 4, 64$; $P < 0.001$). Posthoc analyses further revealed no statistically significant group differences in weight on PND 39 or PND 44, but weight in the overcrowded group was significantly lower than that of the control group on PND 52 ($F = 19.50$; $df = 1, 4$; $P = 0.012$), PND 58 ($F = 113.63$; $df = 1.4$; $P < 0.001$), and PND 65 ($F = 84.26$; $df = 1.4$; $P = 0.001$; Figure 3). Mean body weight at PND 65 (the end of the study) was 347.5 ± 26.371 g for the control group compared with 266.5 ± 9.634 g for the overcrowded group.

Effects of overcrowding on absolute and relative organ weights. Treatment effects on organ weights varied by organ and depended on whether absolute or relative (that is, organ weight divided by body weight) weight was analyzed (Figures 4 and 5). For heart and kidney, organ weight was significantly lower in the overcrowded compared with the control group when expressed as absolute values (heart: $F = 9.09$; $df = 1.3867$; $P = 0.041$; kidney: $F = 227.94$; $df = 1.3384$; $P < 0.001$) but not when expressed as relative weights. A different pattern occurred with the lungs, where absolute weight did not differ between groups but was significantly higher in the overcrowded group than the control group when relative values were analyzed ($F = 10.02$; $df = 1.3842$; $P = 0.036$). Finally, neither absolute nor relative adrenal gland weight differed between groups.

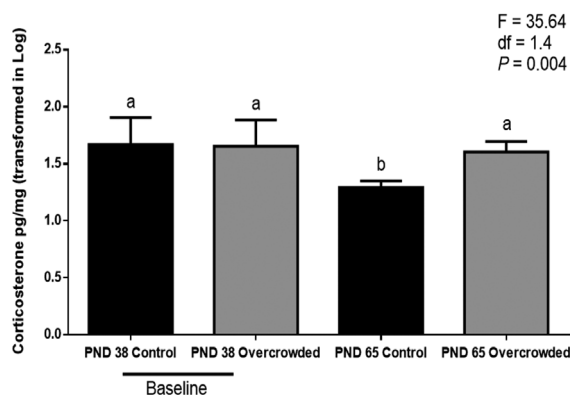


Figure 1. Hair corticosterone levels of control rats ($n = 9$) and overcrowded rats ($n = 9$). Baseline values are those before overcrowding (postnatal day [PND] 38); those for PND 65 represent 27 d of overcrowding. Concentration data were log-transformed. For hair corticosterone analyses, 2-way nested mixed design ANOVA was used; different letters indicate that the values differ significantly ($P < 0.01$). Error bars indicate 1 SD. Posthoc univariate ANOVA were used to determine specific group differences after overall ANOVA.

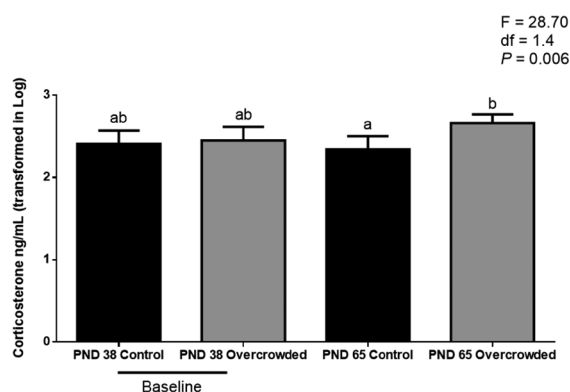


Figure 2. Plasma corticosterone levels of control rats ($n = 9$) and overcrowded rats ($n = 9$). Baseline values are those before overcrowding (postnatal day [PND] 38); those for PND 65 represent 27 d of overcrowding. Concentration data were log-transformed. For plasma corticosterone analyses, 2-way nested mixed design ANOVA was used; different letters indicate that the values differ significantly ($P < 0.01$). Error bars indicate 1 SD. Posthoc univariate ANOVA were used to determine specific group differences after overall ANOVA.

Relationships between organ weights and plasma and hair corticosterone concentrations. Multiple linear-regression analyses revealed that neither plasma nor hair corticosterone concentration predicted organ weight (absolute or relative) at PND 38 or 65.

Discussion

Social hierarchy as well as territory defense determines individual vulnerability when an animal is exposed to stressful conditions.⁵ Accordingly, we used overcrowded housing as a stress model in young rats. A previous study has demonstrated the utility of quantifying plasma glucocorticoids for assessing stress over short periods of time.⁴⁷ In addition, the glucocorticoid concentration in plasma displays a circadian rhythm and is susceptible to environmental perturbations.³¹ Measuring cortisol or corticosterone levels in plasma, saliva, urine, or feces reflects glucocorticoid concentrations over short time frames (minutes, hours, or days) after exposure to a stressor event. To assess the long-term effects of events, new tools that have been validated for monitoring include glucocorticoid levels in

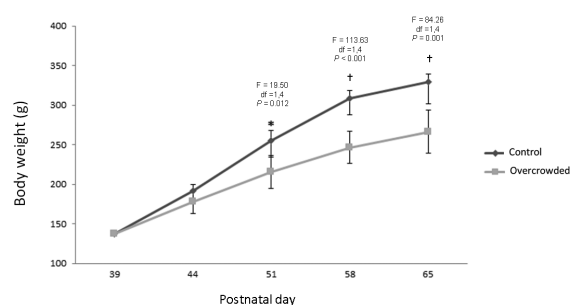


Figure 3. Body weight of male juvenile Wistar rats on postnatal day (PND) 38 (before overcrowding); PND 44, 51, and 58 (during overcrowding); and PND 65 (after overcrowding). Gray line corresponds to the control group ($n = 9$); the green line corresponds to the overcrowded group ($n = 9$). For analysis of body weight, 2-way nested mixed-design ANOVA was used; *, $P < 0.05$; †, $P < 0.01$. Error bars indicate 1 SD. Posthoc univariate ANOVA were used to determine specific group differences at each time point.

hair, which provide a noninvasive and effective method for diagnosing chronic stress.^{16,39,41,44,59} Our current results clearly show that overcrowded housing from PND 38 to 65 significantly increased the hair corticosterone concentration of juvenile male Wistar rats.

Our data indicate that hair corticosterone concentrations in rats follow a similar dynamic as cortisol in hair from other mammals.^{13,15,17, 28,31,33,44,48,49,60} A recent study confirmed the presence of corticosterone in the hair of adult Sprague–Dawley rats.^{41,59} Hair corticosterone levels were higher in rats exposed to restraint, chronic unpredictable stress, and chronic immobilization than in control animals.⁵⁹ In addition, hair corticosterone concentrations were elevated in rats that underwent electrode implantation, and variations in serum and hair corticosterone levels occurred in mice exposed for 30 d to repeated social defeat.⁵⁹ To our knowledge, our current results are the first evidence of corticosterone deposition in the hair of juvenile male Wistar rats, and are important for extending previous findings to a different type of social stressor (that is, overcrowding) in a rodent species.

We found no relationship between plasma and hair corticosterone levels at either of 2 time points (PND 38 and 65). Recent experiments in rats and mice reported correlations between blood and hair corticosterone levels,⁵⁹ but studies in other species found no correlation between blood and hair or feather corticosteroid concentrations in humans,⁴⁰ dogs with chronic exposure to glucocorticoids,³⁶ and birds.¹⁹ Perhaps this lack of correlation is simply because plasma levels represent a ‘snapshot’ of acute HPA activity, whereas hair and feathers provide measures of chronic activity. Hair glucocorticoid concentrations vary more gradually than does the plasma concentration and therefore might represent a better method of monitoring glucocorticoid concentrations over prolonged time periods.³⁶

Corticosterone measured in growing hair after it has been shaved might come from the surrounding plasma or might be synthesized in response to a local-type HPA axis.^{24,42} Although adrenalectomy of Sprague–Dawley rats significantly reduced hair corticosterone levels, the hormone was still detected⁴¹. This finding first prompts consideration of the length of time necessary for complete clearance of all corticosterone from the skin and hair follicles. Second, low levels of corticosterone in the plasma might come from organs other than adrenal glands⁵¹ to potentially accumulate in hair. Third, the residual hair corticosterone in the adrenalectomized rats might reflect local synthesis, as has been suggested regarding cortisol levels in human hair.²⁴ Additional research is needed to test these hypotheses, and the

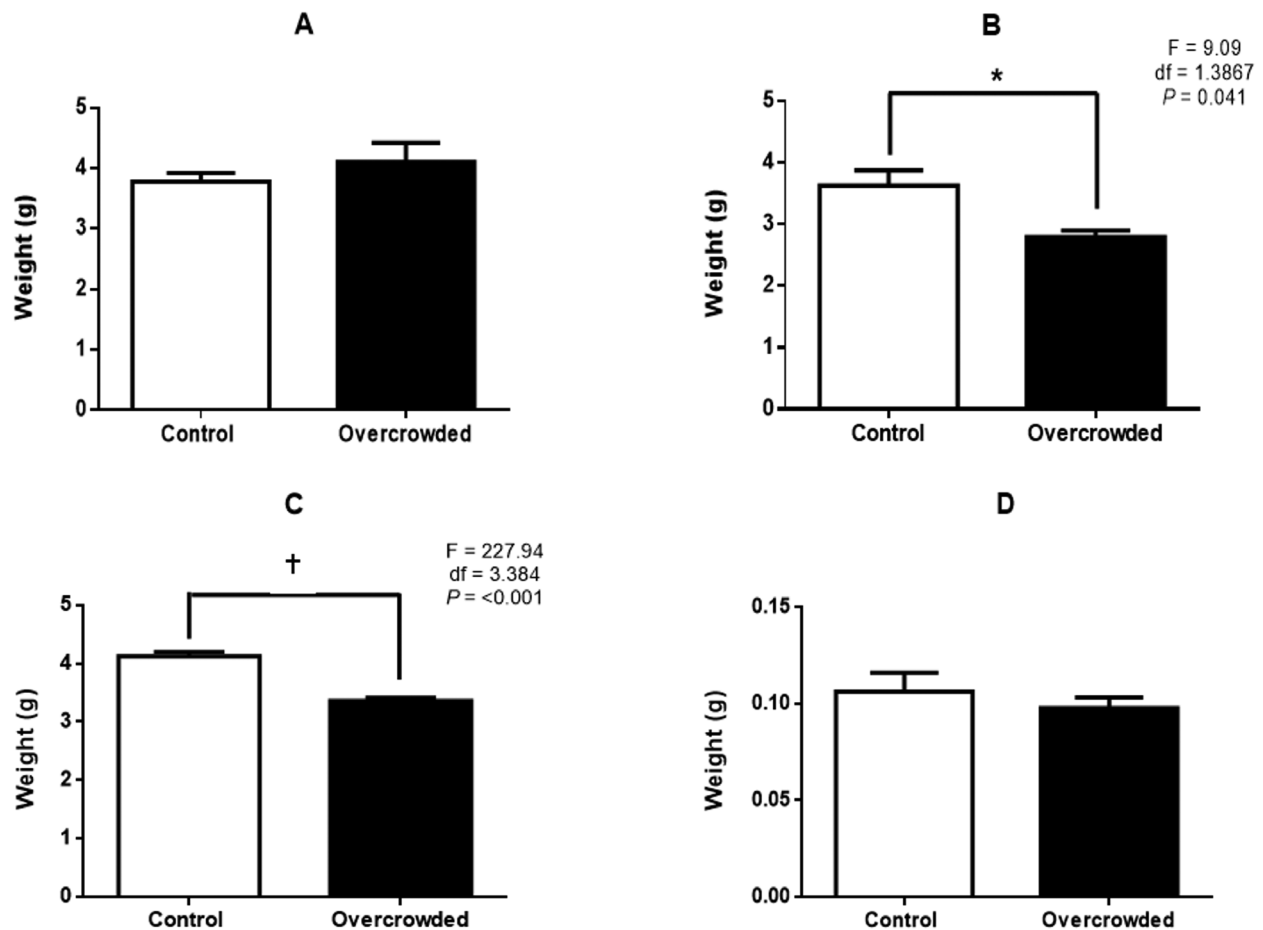


Figure 4. Absolute weight of (A) lung, (B) heart, (C) kidneys, and (D) adrenal glands of control rats ($n = 8$) and overcrowded rats ($n = 9$) on postnatal day 65 (after overcrowding). *, $P < 0.05$; †, $P < 0.01$; error bars, 1 SD.

mechanism underlying corticosterone synthesis in the rat hair shaft is unknown currently.

In primates, hair cortisol levels are higher during infancy and decrease over time until adulthood.¹⁸ These findings support our results showing higher corticosterone levels on PND 38 than PND 65 in control rats. However hair corticosterone levels should be monitored throughout life to confirm a similar pattern in rats as in primates.

The high variability in hair corticosterone concentrations on PND 38 might reflect interindividual variation in the expression of the glucocorticoid-metabolizing enzyme 11 β -hydroxysteroid dehydrogenase 2 within the skin.¹⁴ Another potential source of variability is prenatal stress,^{3,32} which might enhance fetal exposure to corticosterone through increased concentrations in amniotic fluid⁵⁰ and placental transport.⁵⁷ Social roles and group restructuring are important factors that influence variability in plasma corticosterone levels.^{11,13} The plasma level variability on PND 65 corroborates that physiologic dynamics are constantly altered by physical and social factors,⁵ such as the overcrowded conditions in our study.

The scientific literature contains numerous examples of significant changes in the body weights of subjects that underwent chronic social stress^{2,5,30} and of overcrowding stress in Wistar rats.^{9,27,34} The present study found differences in body weight between the control and overcrowded groups that might reflect social (social defeat, overcrowding), physical (minimum living space, handling), or environmental (temperature, humidity, ammonia) factors involved in overcrowded housing. In addition, we found differences in the weights of several organs, although

the nature of the difference depended on whether absolute or relative weight was compared. The appropriateness of analyzing organ weight results as absolute data or indexed by body weight has been an issue in several studies,^{35,56} and different approaches may be appropriate for different organs.⁶ Here we observed that overcrowding caused significant decreases in absolute (but not relative) heart and kidney weights, no change in absolute weight but a significant decrease in the relative weight of lung tissue, and no change in either absolute or relative adrenal weights. Therefore, the growth of the heart and kidneys was affected to the same degree as was overall body growth, whereas the lungs seem to have been somewhat protected from the effects of overcrowding because they had a different pattern of weight reduction from heart and kidneys. A previous study similarly reported a decrease in kidney weight that closely paralleled the reduction in body weight in young chronically stressed Wistar rats.⁸ Those authors further associated the weight decrease with less glomerular volume and fewer nephrons per kidney;⁸ however, the responses of kidneys exposed to overcrowding have not been studied extensively. Differences in heart weight between the overcrowded and control groups might reflect differences in physical activity, housing area, and opportunity to express natural behavior.⁴⁶ The failure of lung weight to respond to overcrowding was surprising, especially considering that a previous study found reduced lung weight in rats exposed to high-density caging.⁵⁸ One possible reason for this difference is that the group size was much greater in the previous study (18 subjects per cage) than in the current investigation (3 rats per cage). Although we did not monitor ammonia concentrations in

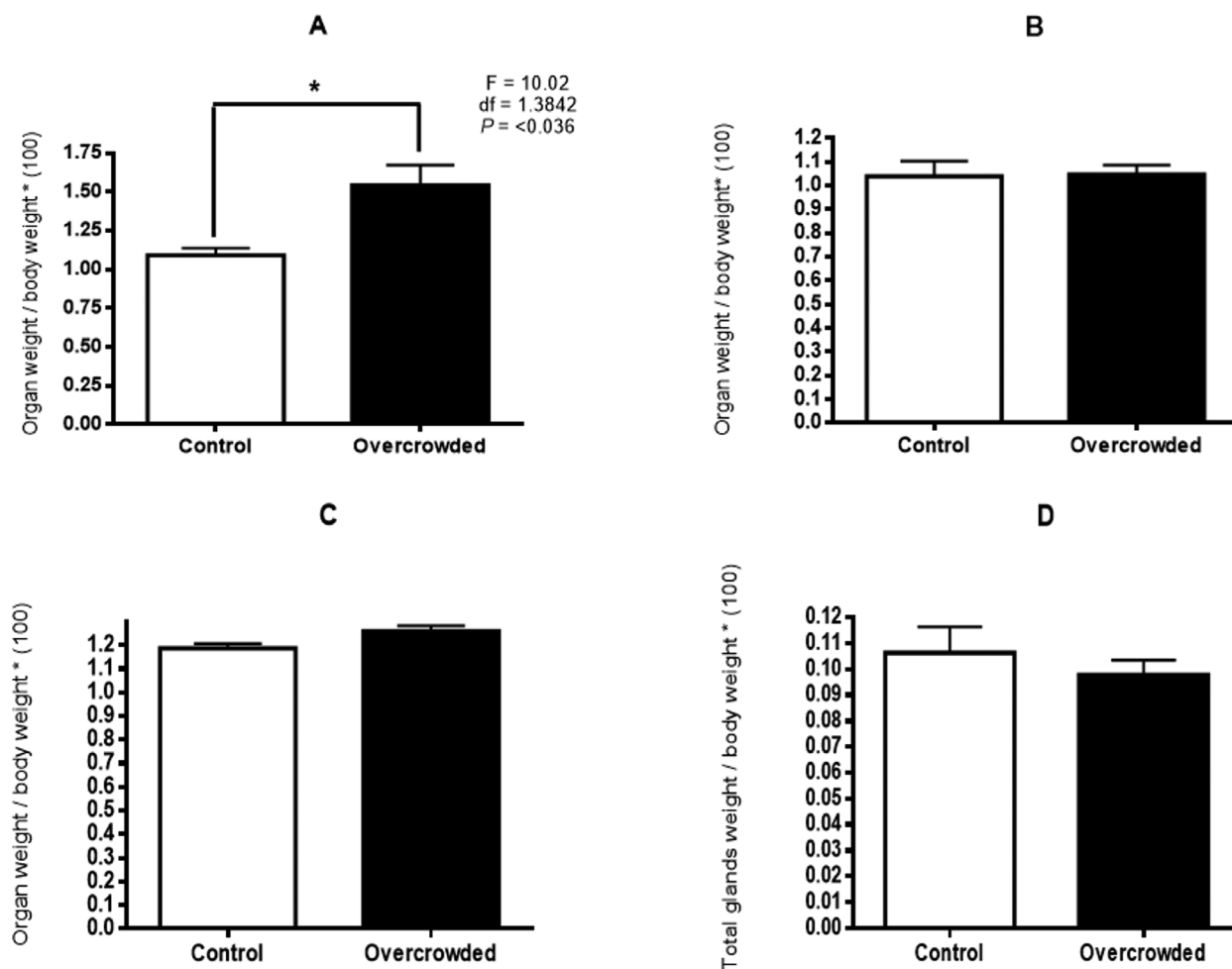


Figure 5. Relative weight of (A) lung, (B) heart, (C) kidneys, and (D) adrenal glands of control rats ($n = 8$) and overcrowded rats ($n = 9$) on post-natal day 65 (after overcrowding). *, $P < 0.05$; †, $P < 0.01$; error bars, 1 SD.

air, they should be considered given that previous studies have documented deleterious effects of ammonia on health in rats.^{10,23} The effect of overcrowding on adrenal gland weight likely depends on the particular experimental conditions. Whereas studies of chronic stress have often documented increases in adrenal weight,^{38,53} other authors found no such increase due to proportional changes (increase compared with decrease) in different zones of the adrenal cortex.²⁶ The same phenomenon may have occurred in the present study.

An important point is that measurement of glucocorticoid concentrations in hair cannot detect acute stress-associated effects;²² therefore concentrations of glucocorticoids in hair should be measured as a complementary method to salivary or plasma quantification.²⁵ Nevertheless, noninvasive methods to study chronic stress are important tools for monitoring physiologic conditions that might influence health and wellbeing.³⁹ Further studies should address the effects of larger cages and the relation of overcrowding on the hair corticosterone concentrations, body weight, and organ weights of both juvenile and adult animals.

In conclusion, corticosterone is present in the hair of Wistar rats during the juvenile period. The current data indicate that our technique for quantifying the levels of corticosterone in hair can be used to monitor corticosterone in Wistar rats over long periods of time. Overcrowding induced significant differences in the hair and plasma concentrations of corticosterone and lung, kidney, and heart weights of male juvenile Wistar rats. Future investigations are necessary to understand the relations

between cage size, housing conditions, and hair glucocorticoids in other laboratory species. Nevertheless, measurement of hair corticosterone can be a useful approach for researchers, veterinarians, and animal colony managers to assess chronic stress in laboratory rodents.

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