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Impact of environmental conditions on the levels of stress and breeding performance in Wistar rats: conventional environment versus environmentally controlled housing

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Abstract: It has been established that providing comparable and standard environmental conditions to house experimental rats is of high importance. However, there is growing evidence that rats prefer higher temperatures owing to their thermoneutral zone that lies between 28-34°C. This experiment validates the stress levels of Wistar rats of both sexes housed in conventional conditions about 24-31°C and a relative humidity of 70-92% in a tropical animal facility when compared to rats housed in controlled temperatures of 20-24°C with humidity maintained between 30-70%. Adequate air-changes to maintain ammonia-free environment was provided with a power-exhaust system in a conventional setup and with the individually ventilated caging system in the environmentally controlled group. To assess stress, faecal corticosteroid metabolite assay was done in naïve animals and in a set of animals that underwent retro-orbital bleeding under general anaesthesia. Mothering ability, fecundity and preweaning mortality were also compared between animals housed in two different conditions. Results showed no differences in stress hormone levels between the groups. However, faecal weights differed between males and females in both naïve and orbital bled groups. Animals housed in controlled environment clearly had better breeding data with lesser preweaning mortality in comparison to the conventionally housed rats. Further studies are required to elucidate whether the results are comparable when conducted during all seasons of the year at different locations across the globe.

Keywords: controlled environment; conventional housing; faecal corticosteroid metabolites; laboratory rats; stress.

Introduction

The quality of animal housing plays a key role in ensuring reproducibility, bringing in animal welfare and providing results with authenticity in biomedical research. There is an evolving scientific basis that the rats prefer higher temperatures of the order of 28-34°C [1] to suit their thermoneutral zone. However, to balance animal welfare and efficient management of facilities demanding intense housing and comply with the accepted animal care standards, rats in laboratories are housed between 20-24°C. Owing to financial constraints, operative ease, and limited resources, conventional housing is practiced widely in developing countries. However, differences had been reported in specific pathogen-free animals making animals prone to infections [2] on exposure to an uncontrolled environment. Further, it is also found that the thermoneutral zone will not be the same under different experimental conditions [1]. Apart from these findings, as a general cliché and to filter out confounding infective agents from entering the facility and maintain reproducibility within the laboratories globally, housing animals under environmentally controlled conditions had been a widely accepted practice. Owing to the scarce data in this topic and to generate more information on the effect of variable environment on stress levels of laboratory animals, a study was planned using Wistar rats of both sexes housed in standard-controlled and conventional conditions. Stress level assay was done using a non-invasive technique of faecal corticosteroid assay. In experimental animals, fecal corticosteroids are elevated in stress and can be used as a marker for diagnosing stress [3]. To compare and rule out the differences between the quantity of feces shed by males and females, faecal weights were also compared. The study was conducted in a set of animals without any treatment and also in a set of animals that were bled using retro-orbital venous puncture under Isoflurane gas anesthesia. It was hypothesized that the conventionally housed animals would be more stressed than the animals housed under standard controlled conditions.

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Materials and Methods

Animal care

The study was sanctioned by the Institutional Animal Ethics Committee in adherence with the "Breeding of and Experiments on Animals (Control and Supervision) Rules, 1998" under guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The animals were housed individually in standard dimension polysulfone cages of 800 cm^2 and height of 18.5 cm in both conventional (Conv) and environmentally controlled (Env-Cont) set-ups. In both the set-ups, ad libitum commercial pelleted food (Safe rodent diet-D131, Augy, France) and drinking water were provided throughout the study period. The cage changes were done once every five days, and for corticosteroid assay, bedding with the faecal matter was collected in plastic bags and was stored in -20°C. Each cage was provided with polysulfone red-colored enrichment rat tubes (Citizen Industries, Gujarat) and nesting material (Enviro Dri, Shepherd Specialty Papers, US) and replaced once every five days. The light intensity was calibrated not to cross 325 Lux above one-meter height in animal rooms, and a light-dark ratio of 10:14 was maintained with automated controls. Health monitoring in the colony was done by following the Federation of European Laboratory Animal Science Association (FELASA) Guidelines [4], and the animals were free from pathogens.

Faecal corticosteroid assay of naive rats

A study on the effect of housing on naive rats was done with twenty-eight Wistar outbred animals (14 males and 14 Females) (Sctb: WI) of 3-4 months of age and weight range 230g-400g were divided into four groups with an equal number of males and females. One group of males and females (7 males and 7 females) were housed in conventional housing. With 7 males and 7 females, the other group was housed in Individually Ventilated Cages (IVC) in a controlled environment with temperature 20-24°C and relative humidity between 30-70% with 12-15 air changes hour. After an acclimatizing time of 10 days, 24 hours after a cage change with fresh bedding, faecal samples from each cage were separately collected, labeled, and stored at -20°C for corticosteroid assay done as previously mentioned [5].

Faecal corticosteroid assay of rats that underwent retro-orbital bleeding

A study on the effect of housing on retro-orbital bleeding was done with a separate set of twenty-eight Wistar outbred animals (14 males and 14 Females) (Sctb: WI-https://www.nap.edu/labcode/search_codes_full.php?labcode_id=8045&user_id=56073) of 3-4 months of age and of weight range 230g-400g were divided into four groups with an equal number of males and females and the housing conditions, and management was all the same as mentioned in the naive animal study. Under Isoflurane anesthesia (1.5–2%) (Forane, Abbott India Limited, Mumbai, India) in oxygen (0.5–1 l/min) administered through the face mask using a precision vaporizer (E-Z system corporation, Palmer, PA), the rats were scruffed with the left hand and retro-orbitally bled from the left eye using a capillary tube with the right hand. As soon as a few drops started to flow out of the capillary tube, the bleeding was stopped, and wet saline gauze was pressed against the eye, and immediately the animals were left back to cages with fresh bedding. Animals were examined closely until they fully recovered from anesthesia. After 24 hours, faecal samples were collected, labeled and stored as mentioned for the naive animal group for corticosteroid assay as previously described [5]. The animals in each group were either housed conventionally since birth or in a controlled environment, and the study was completed within one month after initiation.

Estimation of litter size and preweaning mortality

Female breeders were assigned randomly into two groups with thirty animals per group. One group of 30 female rats was housed in conventional rat cages housed under conventional conditions since their birth. The other group was housed under a controlled environment with 20-24°C and relative humidity between 30-70% with 12-15 air changes per hour. A number of young ones born to each female (litter-size) after breeding with a male rat housed under respective conditions (1:1 male: female ratio) were recorded. Further, the number of young ones dead before weaning (preweaning mortality) on day 21 after birth was also recorded. These data were statistically analysed.

Statistical analysis

Statistical analysis was done using GraphPad Prism 9.0.0 (121) for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. All the data were analyzed for normality with the Shapiro-Wilk test. The data that followed Gaussian distribution were analyzed using One-Way ANOVA followed by Tukey's multiple comparisons test. The data that was not following normal Gaussian distribution were analyzed using the non-parametric counterpart with the Kruskal Wallis test followed by Dunn's multiple comparisons test. Data are expressed as Means± SEM and graphs presented with individual data points where $P<0.05$ was considered statistically significant. In addition, Mann Whitney U test was carried out for non-Gaussian data to find differences between the number of pups born (litter-size) and preweaning mortality on pups of dams housed in conventional compared to those in the controlled environment.

Results

Faecal corticosteroid metabolite levels and faecal weights in animals housed without blood collection

None of the naive animal groups differed in faecal corticosteroid metabolites concentration measured in

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nmol/g (H (4) = 0.4195 P = 0.9362; Effect size = 0.211). Pairwise comparisons are shown in **figure 1**.

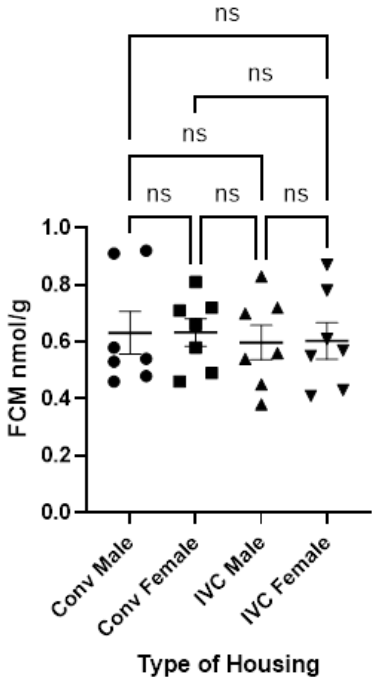

Figure 1. Faecal corticosteroid metabolites (FCM) measured in nmol/g from naive animal groups housed in conventional and environmentally controlled set-up. Data presented with individual data points with Mean with SEM (n per group = 7).

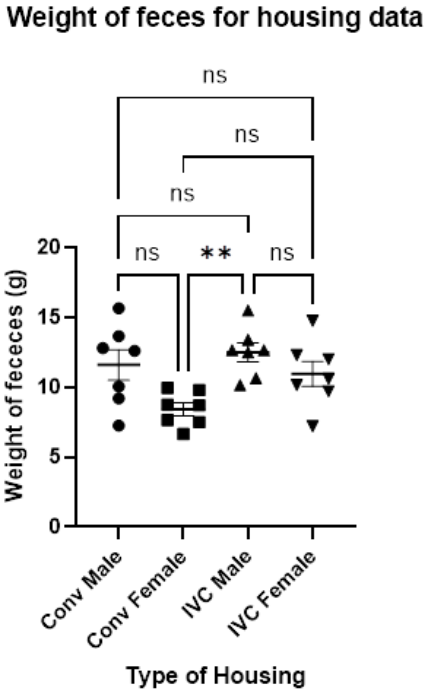

Figure 2. Faecal weights from naive rats housed in conventional and environmentally controlled set-up. P<0.05 was considered as statistically significant. Data presented with individual data points with Mean with SEM (n per group = 7).

Faecal weights from naive rats differed significantly between the groups (F (3,4) = 4.592, P = 0.0112). Post-hoc comparisons with Tukey's multiple comparisons test suggested that the conventionally housed females had lesser faecal weights than the males housed in IVC under controlled conditions (**figure 2**). Effect size observed was 0.19, and Tukey's multiple comparisons tests showed differences between females housed in conventional systems and males housed in a controlled environment (P = 0.0086).

Faecal corticosteroid metabolites and faecal weights in animals housed subjected to blood collection

Among the groups that underwent retro-orbital bleeding under isofluorane anesthesia, no differences were observed (F (3, 24) = 1.073, P = 0.3792) in the levels of faecal corticosteroid metabolites. Pairwise comparisons are shown in **figure 3**.

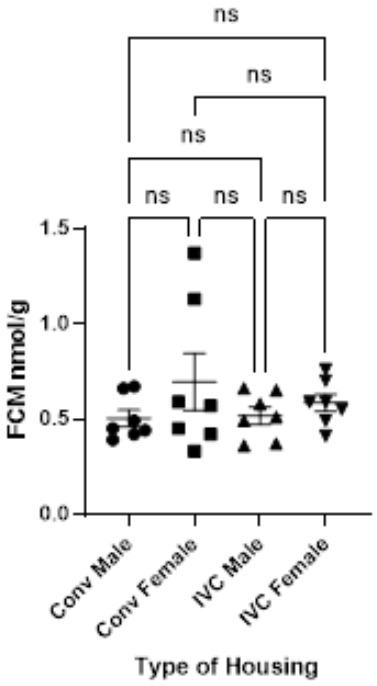

Figure 3. Faecal corticosteroid metabolites (FCM) measured from animals bled retro-orbitally under isofluorane anesthesia housed in conventional and environmentally controlled set-up in nmol/g. Data presented with individual data points with Mean with SEM (n per group = 7).

Faecal weights from groups that underwent bleeding differed significantly (F (3, 24) = 4.216, P = 0.0157) where the faecal weights of conventionally housed males that underwent bleeding were more than the conventionally housed females that were bled, as per posthoc Tukey's multiple comparison test (**figure 4**).

Effect size observed was 0.19, and Tukey's multiple comparisons tests showed differences between females housed in a conventional system and males housed in a controlled environment (P = 0.035).

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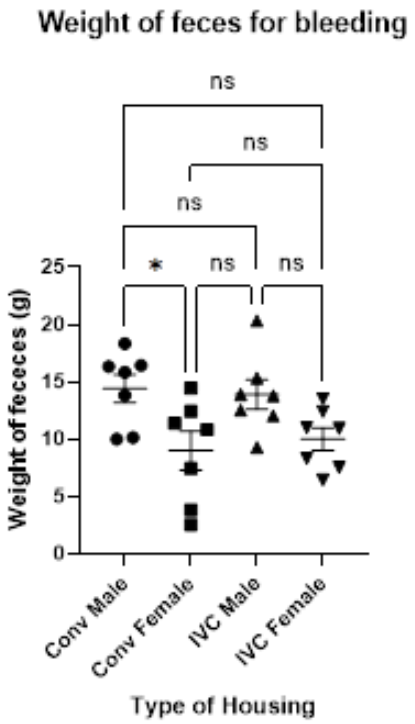

Figure 4. Faecal weights from rats bled retro-orbitally under isofluorane anesthesia housed in conventional and environmentally controlled set-up. P<0.05 was considered as statistically significant. Data presented with individual data points with Mean with SEM (n per group = 7).

Litter size and preweaning mortality rate in animals housed in different conditions

A significant difference was observed in preweaning mortality rates between animals housed in conventional housing compared to the controlled environment. Mothers housed in a controlled environment presented litters with considerably lesser preweaning mortality (P = 0.0041) (**figure 5**).

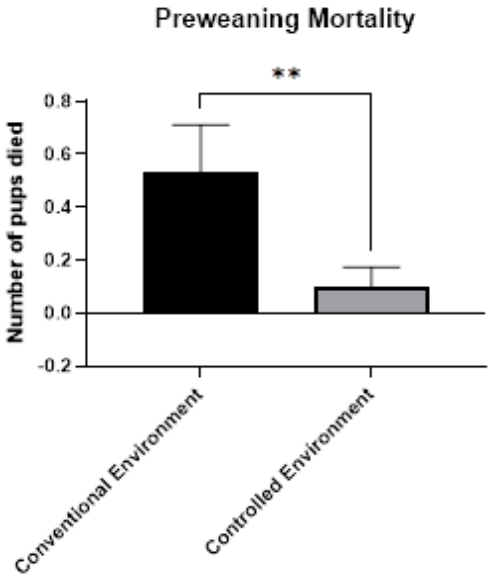

Figure 5. Number of pups died before weaning when mother rats are housed in conventional and environmentally controlled set-up. P<0.05 was considered as statistically significant. Data presented with individual data points with Mean with SEM (n per group = 30).

However, litter size did not differ between groups (n=30 per group) of mother rats housed in conventional in comparison to the animals housed in a controlled environment (P = 0.9) (**figure 6**).

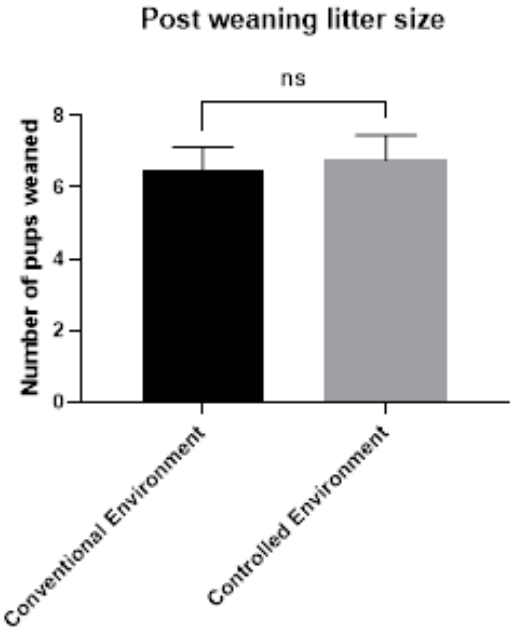

Figure 6. Number of healthy pups weaned out when mother rats are housed in conventional and environmentally controlled set-up. Data presented with individual data points with Mean with SEM (n per group = 30).

Discussion

Faecal corticosteroid assay is considered a non-invasive and sensitive tool to analyze stress levels in laboratory animals [6]. This study reveals that rats bred and reared in conventional cages have no added stress compared to rats housed in an environmentally controlled set-up. The higher ambient room temperatures or varying humidity have no impact on faecal corticosteroid metabolite levels. Contradictory reports exist regarding the temperature that shall be provided for the comfortable housing of mice [7]. Preference tests suggested that lower temperatures can distress them [8], whereas most standards recommend temperatures from 20-24°C. However, studies are rare in rats regarding the impact of the environment on animal stress levels. Further, much data is generated from animals reared in conventional facilities in developing nations across the globe. It is worthwhile to study the impact of varying temperatures on stress and animal health. Data generated throughout the year may be required to explore this fully. However, in areas with fewer variations in ambient temperature range, the data from this study can be a torch-bearer in elucidating that conventional housing cannot influence stress levels of acclimatized animals. It is

a well-established fact in rats that isofluorane anesthesia itself and retro-orbital blood collection can be stressful [9]. However, in the study, we found that the corticosteroid metabolite levels were unaffected post bleeding in conventional and controlled environmental set-ups; this agrees with a previous report in rats orbital bleeding under isofluorane anesthesia reported no elevation in faecal corticosteroid metabolites [9]. Faecal weights showed differences between males and females on two occasions. The body weights of males and females at the same age differ where males will have more bodyweight [10] and more fecal output. The differences are attributable to this fact rather than to the type of housing. Effects of temperature and humidity to act as a zeitgeber in rodents are ruled out by an in-depth review done on studies focusing on how circannual seasonality affects rodent behavior [11]. Rather than photo periodicity, temperature or humidity, it could be the presence of a Type-2 circannual clock or endogenous oscillator that brings in seasonal variations in observations made from these animals [11]. This could also be responsible for the lack of reproducibility of biomedical research using rodents in different seasons.

Birthrate comparisons suggest a clear advantage that a controlled environment has in rat breeding. On the other hand, the preweaning mortality was more due to the dwindling climatic conditions by stressing rodents and adversely affecting mothering ability in conventionally housed dams.

More studies are to be conducted to ascertain the findings since this study was done in a particular geographic location with a limited sample size of a single strain and species. The limitation faced by the present study is that we could not produce data year-round, and in future studies, we will fill this gap. Further, a wide array of strains and species are used in research, and so evidence lacks in this study on strains and species other than Wistar rats. This is a gap area to be worked on in the future. However, the data from this study suggests that a controlled environment is better for the breeders even though housing animals in a natural environment might impact experimental outcomes due to stress.

Conclusion

This study concludes that in Wistar rats, conventional housing and controlled-environment housing did not produce stress-level variations. However, a difference was noticed in preweaning mortality with a clear advantage of the housing in controlled-environment over conventional housing in breeding female Wistar rats. It is also noteworthy that the litter size did not show any differences between groups housed in conventional and controlled environments. Since the work was done at a single time point, more data is required to prove that this finding holds good throughout the year. This study is done in a tropical region with an almost 12:12 light-dark ratio with an environmental temperature ranging from 24-31°C and relative humidity from 70-92% during the study period.

Similar studies have to be conducted in regions with different environmental conditions to validate the consistency of this data. It is impossible to house animals under controlled conditions in parts of the world, where ambient temperature measures sub-zero. At the same time, the fact that rodents can live in a wide range of environmental conditions and can adapt shall be considered, which can negotiate with varying climatic conditions. This study provides data to establish that conventional housing doesn't produce any added stress to laboratory rats under the described geographical location.

Declarations

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Author Contribution: VSH planned and performed the experiments, did data analysis and wrote the manuscript; SKRS and SKR performed the experiments.

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Conflict of Interest: No potential conflict of interest is being reported by the authors.

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