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Characterization of Different Colony Housing Concepts in Rat Repeated-Dose Toxicity Studies

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Abstract:

Background: In non-clinical safety assessment, repeated-dose toxicity studies in rats remain a regulatory requirement. Housing conditions should support species-specific behaviors such as exploration, locomotion, and social interaction while maintaining study robustness and operational feasibility. To identify an enriched housing environment that preserves data integrity and practical feasibility while improving animal welfare, we compared stress indicators and data quality head-to-head between conventional Type IV cages (2–3 rats per cage), a Rat Colony Cage (RCC) comprising four vertically connected platforms (15 rats), and a Toxicology Rat Colony Cage (TRCC) comprising four horizontally connected Type IV cages (15 rats) in a full toxicity study with the immunosuppressant drug Methotrexate (MTX) used as a reference compound.

Results: Animals housed in the RCC and, to a lesser extent the TRCC gained more body weight, exhibited lower adrenal gland weight, showed lower neutrophil – lymphocyte ratios and habituated faster to an unfamiliar environment than rats in conventional cages, indicating improved welfare. Overall, the variability of outcome measures did not increase on average in either colony housing system. The only notable finding that was revealed by histopathology was the occurrence of mild-to-moderate atrophy of prostate and seminal vesicles in some RCC-housed animals. Finally, the well-established reversible effects of MTX on hematological and immunological parameters were consistently detected irrespective of housing system.

Conclusion: The results suggest that refinement can be achieved without compromising data integrity in repeated-dose toxicity studies using either colony housing system. While most welfare indicators showed greater improvement in the RCC, the TRCC offers enhanced operational flexibility for the conduct of toxicity studies.

Keywords

Animal welfare, drug development, environmental enrichment, methotrexate, non-clinical safety, pathology, rat housing, refinement, toxicology

Background

The refinement of animal use remains an important objective in non-clinical safety assessment of new drug candidates, particularly in the context of general toxicity studies. These studies are still required by regulatory authorities prior to investigations in humans and later in support of clinical development (1, 2). As a default rodent species, rats are most frequently used. It is generally assumed that healthy animals that are not subjected to chronic stress and whose species-specific needs are adequately met provide results that are more relevant and predictive for the human situation (3). Beyond procedural refinements, housing conditions are increasingly recognized as a critical determinant of animal welfare but also a potential source of biological variation that can affect study interpretation (4, 5). Rats are highly social animals whose welfare and physiological homeostasis depend on stable group dynamics and environmental complexity (6, 7). Conventional small-group housing can restrict natural behaviors and compromise rodent well-being, potentially affecting the validity and generalizability of study data. Insufficient housing and enrichment may also exacerbate disease severity, morbidity, and mortality in pharmacological models (8). Environmental enrichment (EE) has been shown to reduce corticosterone levels and adverse cardiovascular effects, while promoting exploratory and anti-anxiety behaviors. However, resource limitation, high stocking density, or introduction of unfamiliar animals may increase aggression (6). Providing a home cage with more space, greater structural complexity, distinct functional areas, and sustained interactions within larger social groups is expected to increase overall activity, including manageable physical and social challenges. When moderate, such challenges may constitute chronic mild eustress, promoting adaptation, resilience, and well-being rather than causing harm (9). In the context of an experimental study with all necessary interventions, e.g. blood sampling, ophthalmology examination and frequent dosing, such socially driven eustress may foster stress coping (10). Otherwise, large colony housing can also facilitate the establishment of social hierarchies, associated with behavioral and physiological differences between animals, potentially increasing biological variability (11). Dominance hierarchies can create biologically distinct subpopulations, with

72 subordinate males showing lower testosterone and higher corticosterone levels, potentially increasing
73 variability in endocrine and reproductive endpoints (12).
74 Socializing 48 rats of mixed experimental groups has previously been implemented in a multi-level "Rat
75 Colony Cage" (RCC) for use in models of osteoarthritis. Besides refinement in animal welfare, it
76 fostered the interaction between joint pain and osteoarthritis progression by stimulated spontaneous
77 activities and finally improved the translational value of the model (13, 14). More recently, colony
78 housing of 10 rats across two levels showed no meaningful effects on routine behavioral, clinical, or
79 histopathological parameters over 28 days (15). As different study types impose distinct requirements
80 on housing systems, toxicity studies demand flexibility for accommodating treatment groups of varying
81 sizes, separating cohorts with different treatment initiation times, facilitating frequent handling, and
82 isolating compromised animals. To address these needs, we developed the Toxicology Rat Colony Cage
83 (TRCC), a modular housing system comprising horizontally interconnected standard cage units,
84 multiple access doors, and movable dividers that allow cohorts to be separated or reunited as required.
85 Before finally implementing such fundamental changes in housing conditions in regulated repeated-
86 dose toxicity studies, three key questions were addressed in this head-to-head comparison:
87 (i) Is colony housing operationally feasible for conducting repeated-dose toxicity studies with the
88 typically required group sizes and complete collection of all necessary endpoints (ii) Is data quality,
89 including parameter variability, treatment effect size and background finding frequency equivalent to
90 that obtained under conventional housing (Type IV)? (iii) Does the transition to colony housing result
91 in welfare outcomes that provide a robust scientific and ethical rationale for its implementation? To
92 address these questions, a four-week repeated-dose toxicity study followed by a four-week recovery
93 period was conducted according to OECD TG 407 and ICH M3(R2) (16, 17) to compare TRCC and RCC
94 with standard Type IV housing fully randomized and head-to-head. Methotrexate was selected as the
95 reference substance due to its well-characterized pharmacology, toxicology and established clinical
96 use. Hematological toxicity represents one of its primary and clinically most relevant effects, with
97 myelosuppression constituting its most serious complication (18). The capacity to reliably detect the

backtranslation of these well-established effects across different housing conditions serves as an important internal validity benchmark. Beyond demonstrating the comparability of colony housing and conventional cages, with respect to toxicological data quality, the study was designed to support a decision on which of the two colony systems, TRCC or RCC, offers the most favorable balance between animal welfare, operational feasibility, and data quality.

Results

Study feasibility and indicators of animal welfare across different housing types

A full 4-week repeated-dose toxicity study, with daily oral gavage administration, was successfully carried out in both the Toxicology Rat Colony Cage (TRCC) and Rat Colony Cage (RCC) housing environments. Animals in all three housing systems were well socialized throughout the study, with no cage-related territorial aggression observed. Cooperativity during handling was assessed throughout the study using a predefined scoring scheme and revealed no housing- or treatment-related differences in movement/defensive behavior, body tension, or vocalization. All outcome measures required for pivotal regulatory toxicity studies and listed in Table 4 were successfully collected from all animals at all scheduled time points across all three housing conditions. No treatment-related mortality occurred in any housing group; however, one vehicle-treated rat housed in a Type IV cage was prematurely sacrificed on study day 15 due to significant skin wounds of unknown origin. Toxicokinetic evaluation confirmed that, following oral administration on study days 1 and 28, methotrexate (MTX) was not detectable in vehicle-treated animals, whereas all MTX-treated animals showed systemic exposure for at least 1–2 hours post-dose (see Additional File 2).

During the vehicle treatment period (study day 1 -28), rats showed an average cumulative body weight gain of 68.0 g ± 16.9 g, 74.5 g ± 19.7 g, and 76.2 g ± 19.6 g in the Type IV, TRCC, and RCC, respectively, suggesting a slightly higher weight gain in colony- versus conventional housed (Type IV) control animals (Figure 1 A). Until the end of the application free period at study day 56, the mean cumulative body weight gain was 100.2 g ± 25.7 g, 122.6 g ± 25.5 g, and 116.0 g ± 15.4 g in Type IV, TRCC, and RCC, respectively, and therefore still higher in colony housed rats.

Increased adrenal gland weight can indicate prolonged elevation of corticosterone production (19, 20). In vehicle-treated animals, relative adrenal gland weights were consistently lower in both colony-cage groups than in the Type IV group, although the differences were not statistically significant (Figure 1B). The differences were more pronounced on study day 29, when mean weights were 0.0226 ± 0.0025 % in Type IV, 0.0202 ± 0.002 % in TRCC and 0.0203 ± 0.0024 % in RCC. On study day 57, the corresponding values were 0.0184 ± 0.0025 %, 0.017 ± 0.0019 % and 0.0176 ± 0.0029 %, respectively. As elevated neutrophil-to-lymphocyte ratios (NLRs) have been reported in chronically stressed rats, it was assessed as an additional physiological parameter related to animal welfare (21). On study day 29, vehicle-treated animals showed in the RCC highly significant lower NLRs ($p = 0.0025$) while animals in the TRCC showed similar NLRs compared to conventionally housed animals (Figure 1C). On study day 57, NLRs in the remaining five animals tended to be increased relative to study day 29 across all three housing conditions. However, variability was higher, and no statistically significant differences were detected.

A behavioral indicator that has been associated with enrichment-reared rats, is a shorter duration of novelty exploration activity, e.g. in an empty cage, that can indicate faster habituation to an uncommon situation (22). On study day 22 we placed single rats from their respective home cage conditions into the ActiMot2 test device, consisting of an empty novel cage with activity tracking. Within the first 30 minutes, 9880 counts ± 1864, 7786 counts ± 1962 and 5598 counts ± 1426 were detected from Type IV cage, TRCC or RCC rats respectively (Figure 1D), indicating significantly lower initial exploration activity in colony cages. From 30 to 60 minutes, exploration activity declined in all rats, while RCC rats still showed lowest activity (319 counts ± 155) compared to 2656 counts ± 2245 in TRCC and 1958 counts ± 1804 in Type IV housed rats. Of note, the distress-associated readouts exploration activity, adrenal weights and NLRs showed a similar pattern with the same housing related effect direction in the methotrexate-treated groups (see Additional file 2). Overall, welfare-related measures indicated lower levels of distress in colony-housed rats compared with conventionally housed rats, with differences being more pronounced in the RCC than in the TRCC.

Contribution of housing on the integrity of standard outcome measures

To evaluate whether colony housing compromised data integrity in the present toxicity study, we compared housing systems with respect to differences in variability and mean values of clinical pathology and organ weight outcome measures. For illustration of housing effects on outcome variability, the ratio of standard deviation (SD) values between respective colony and Type IV cages from vehicle treated rats on study day 29 was calculated and displayed in a forest plot (Figure 2).

In the comparison of TRCC and Type IV cages (Figure 2A), 10 parameters had 95% confidence intervals (CIs) excluding 1, indicating notable differences in variability. Of these, 5 parameters showed higher variability in TRCC and 5 showed higher variability in Type IV cages. In the RCC versus Type IV comparison (Figure 2B), 14 parameters differed substantially in variability, 11 were more variable in RCC and 3 were more variable in Type IV cages. Among the parameters for which differences in variability were observed, only the monocyte counts consistently showed higher variability in both colony cage systems than in conventional housing. Eosinophil counts, on the other hand, were the only parameter that was more stable in both colony cages and thus more variable in conventional housing in both comparisons. Additional differences were restricted to individual comparisons. As a descriptive summary across parameters, the mean SD ratio was 1.05 for TRCC and 1.14 for RCC. Taken together, while particularly in RCC cages a trend towards increased variability was observed, no consistent elevation in overall variability attributable to colony housing could be demonstrated across all parameters and housing types, indicating that colony housing does not systematically increase the variability of outcome measures in toxicity studies.

Next, we investigated whether there are parameters whose mean values are influenced by housing conditions. To this end, we compared absolute values on study day 29 from vehicle-treated groups between conventional cage housing and colony cage systems using Welch's t-tests, with Benjamini-Hochberg (BH) correction for multiple testing applied separately across all 48 parameters within each housing comparison (Type IV vs. TRCC, Type IV vs. RCC). Adjusted p-values revealed significant differences only for inorganic phosphate in serum in the RCC comparison. Therefore, we also present

unadjusted p-values for exploratory purposes. Parameters with unadjusted p values below 0.05 were distributed across hematology, clinical chemistry, and organ weights. Across all three domains, 5 parameters showed nominal differences in the comparison between Type IV- and TRCC-housed rats (Table 1), whereas 10 parameters showed nominal differences in the comparison between Type IV- and RCC-housed rats (Table 2).

Table 1. Housing effects of TRCC versus Conventional Cage.

Domain	Parameter	n	Conventional Cage Vehicle	TRCC Vehicle	Difference [95% CI]	p (unadj.)	p (BH-adj.)
Hematology	Eosinophils [10^3/µL]	15	0.13 ± 0.04	0.1 ± 0.03	0.03 [0.00, 0.06]	0.038	0.427
Hematology	Basophils [10^3/µL]	15	0.05 ± 0.02	0.04 ± 0.01	0.01 [0.00, 0.02]	0.046	0.427
Hematology	Hemoglobin [g/dL]	15	15.92 ± 0.50	15.55 ± 0.48	0.37 [0.01, 0.74]	0.046	0.427
Clinical Chemistry	Inorganic Phosphate [mmol/L]	15	2.24 ± 0.17	2.39 ± 0.14	-0.15 [-0.26, -0.04]	0.012	0.427
Organ Weights	Adrenals [%]	10	0.0226 ± 0.0025	0.0202 ± 0.002	0.0024 [0.0002, 0.0045]	0.031	0.427

Parameters with a nominal (unadjusted) p-value below 0.05 in comparison between conventional Type IV and TRCC-housed vehicle-treated animals on study day 29 (two-sided Welch's t-test). p-values were adjusted for multiple testing across all 48 parameters using the Benjamini-Hochberg (BH) procedure. None reached significance after adjustment. Data is presented as mean ± SD.

185 ***Table 2. Housing effects of RCC versus Conventional Cage***

Domain	Parameter	n	Conventional Cage Vehicle	RCC Vehicle	Difference [95% CI]	p (nominal)	p (BH-adj.)
Hematology	Neutrophils [10^3/µL]	15	1.20 ± 0.32	0.85 ± 0.24	0.35 [0.14, 0.57]	0.002	0.051
Hematology	Basophils [10^3/µL]	15	0.05 ± 0.02	0.08 ± 0.04	-0.03 [-0.05, -0.01]	0.011	0.104
Clinical Chemistry	Inorganic Phosphate [mmol/L]	15	2.24 ± 0.17	2.59 ± 0.21	-0.35 [-0.49, -0.21]	<0.001	<0.001
Clinical Chemistry	Potassium [mmol/L]	15	4.38 ± 0.27	4.69 ± 0.31	-0.3 [-0.52, -0.09]	0.008	0.104
Clinical Chemistry	Calcium [mmol/L]	15	2.50 ± 0.08	2.58 ± 0.09	-0.08 [-0.15, -0.02]	0.009	0.104
Clinical Chemistry	Albumin [g/L]	15	39.77 ± 1.17	38.73 ± 1.52	1.05 [0.03, 2.07]	0.045	0.214
Organ Weights	Seminal Vesicles [%]	10	0.334 ± 0.1025	0.207 ± 0.1151	0.127 [0.025, 0.230]	0.018	0.137
Organ Weights	Thyroids with Parathyroids [%]	10	0.007 ± 0.0011	0.006 ± 0.0009	0.001 [0.000, 0.002]	0.029	0.188
Organ Weights	Prostate [%]	10	0.262 ± 0.0402	0.207 ± 0.0691	0.055 [0.001, 0.109]	0.046	0.214
Organ Weights	Adrenals [%]	10	0.0226 ± 0.0025	0.0203 ± 0.0024	0.0023 [0.000, 0.005]	0.046	0.214

186 *Parameters with a nominal (unadjusted) p-value below 0.05 in the comparison between conventional Type IV and RCC-housed*
187 *vehicle-treated animals on study day 29 (two-sided Welch's t-test). p-values were adjusted for multiple testing across all 48*
188 *parameters using the Benjamini-Hochberg (BH) procedure. Only inorganic phosphate remained statistically significant after*
189 *adjustment (BH-adjusted p < 0.05). Data is presented as mean ± SD.*

190 Overall, fewer nominal differences were observed in the TRCC comparison than in the RCC comparison,
191 where clinical chemistry parameters accounted for most of the findings. Based on unadjusted p-values,
192 inorganic phosphate and adrenal gland weight showed consistent nominal differences across both

comparisons. After BH adjustment, only inorganic phosphate in the RCC comparison remained statistically significant, and no parameter remained significant in the TRCC comparison. The complete statistical results for all parameters, including those with unadjusted p values > 0.05, are included in the supplementary data (Additional file 3). Ophthalmology investigation revealed no spontaneous or treatment-related findings across all housing conditions (Additional file 4). Also, Functional Observational Battery (FOB) testing was feasible with no treatment-related findings and no increase in incidental observations in colony versus standard housing (Additional file 5). Given the small sample size (n=10-15), these findings were interpreted as exploratory rather than confirmatory evidence of statistical significance.

Next, we compared the occurrence of histopathology findings between housing types. Focal to multifocal perivascular granulocytic infiltrates with a minimal to moderate severity were observed in the lung, with a slightly higher incidence in the RCC caging system (Additional file 6), still considered to be incidental in nature. Single rats (3/20) housed in the RCC housing system showed an atrophy (mild to moderate) of the accessory sexual glands (prostate and seminal vesicle). Two other rats within the same housing system exhibited a mild to moderate reduced secretion of either the prostate or seminal vesicles (Table 3, Figure 3). A lower mean prostate weight and greater variability in prostate weight in the RCC group were already evident in the comparisons presented in Table 2 and Figure 2B, which is related to the lower weights in those single rats showing atrophic changes or decreased secretion.

211 ***Table 3. Summary table of histopathology findings***

	Conventional Cage		TRCC		RCC	
Treatment	Vehicle	MTX	Vehicle	MTX	Vehicle	MTX
Number of animals	10	10	10	10	10	10
Prostate						
Atrophy, mild					2	1
Secretion, reduced, mild					1	
Seminal vesicle						
Atrophy, mild					1	
Atrophy, moderate					1	1
Secretion, reduced, moderate					1	

212

213 **Comparison of treatment-related outcome measures across housing systems**

214 Multiple nominal differences ($p < 0.05$; uncorrected) between vehicle- and MTX-treated groups were
215 identified across housing systems (Additional File 7). None were considered toxicologically relevant,
216 consistent with a dose below an adverse effect level. Most reflected the expected MTX pharmacology,
217 predominantly affecting hematological parameters in line with its antifolate activity and effects on
218 rapidly dividing cells. Accordingly, myelosuppression is recognized as the most sensitive and clinically
219 relevant manifestation of MTX toxicity (18, 23). Based on this mechanistic rationale, the statistical
220 analysis of treatment effects across housing systems was focused and therefore restricted to body
221 weight gain, hematology and immunology parameters including only 18 parameters (Additional File
222 8). For graphical presentation (Figure 4), nine parameters were selected with a focus to avoid

redundancy and still represent clinically and mechanistically most relevant hematological and immunological responses. The treatment comparison revealed reduced body weight gain with MTX treatment in all three housing systems (Figure 4A). The reduction was greatest in RCC (approximately 28%, compared with 22% in TRCC and 19% in Type IV) and remained significant after BH correction only in RCC (adjusted p = 0.009). Reticulocyte counts were also lower in the MTX groups in all housing systems (Figure 4B), reaching statistical significance in TRCC (p = 0.027) and RCC (p = 0.038), but not in Type IV (p = 0.069). Mean hemoglobin concentrations were lower in all MTX groups (Figure 4C); this difference remained significant only in RCC (p = 0.034). Conversely, platelet counts were higher in all MTX groups (Figure 4D), reaching statistical significance after BH correction only in RCC (p = 0.007). Neutrophil counts were lower with MTX treatment in all three housing systems (Figure 4E), by approximately 18% in Type IV, 8% in TRCC, and 18% in RCC. However, none of these differences remained significant after BH correction (adjusted p = 0.130, 0.546, and 0.168, respectively).

Among immunological endpoints, serum immunoglobulin A (IgA) was lower under MTX in each housing system (Figure 4F): by approximately 30% in Type IV, 42% in TRCC and 54% in RCC. These differences remained significant after BH adjustment in TRCC (p = 0.013) and RCC (p = 0.001), but not in Type IV (p = 0.060). Natural killer (NK)-cell counts were higher in MTX-treated animals in all three systems (Figure 4G), with only the difference in TRCC reaching adjusted significance (p = 0.027). T-helper and cytotoxic T-cell counts were lower under MTX across all housing systems (Figures 4H and 4I). Both reductions remained significant only in the RCC group (adjusted p = 0.009 and p = 0.005, respectively).

Among the nine endpoints selected for Figure 4, none showed a statistically significant MTX–vehicle difference after BH adjustment in Type IV at the end of treatment. Three endpoints were significant in TRCC and seven in RCC (all except neutrophils and NK cells, see Additional File 8). At the end of recovery (study day 56 for body weight gain and day 57 for blood-derived endpoints), none of these endpoints showed a significant MTX–vehicle difference after BH adjustment in any housing system. Nevertheless, mean body weight gain remained numerically lower in the RCC MTX group.

Discussion

Effects of Colony-Cage Housing on Animal Welfare and the Conduct of Toxicological Studies

A key prerequisite for implementing colony housing in regulated toxicity studies is its practical feasibility without compromising data integrity, no substantially prolonged timelines, and a beneficial impact on animal welfare. In the present study, both colony housing systems were successfully integrated into a standard 28-day repeated-dose toxicity study, with all predefined endpoints collected as planned. Since animals need time for orientation in the more complex colony cages, a longer habituation period with a minimum of two weeks from animal delivery to study start was seen as necessary. Further, targeted staff training is seen as an important factor to ensure procedural efficiency and compliance. No increased inter-animal aggressions or injuries were observed in any cage throughout the study period and structured assessment using a predefined scoring scheme likewise revealed no indication of negatively altered behavior when handling colony-housed animals. Aggressive behavior of rats found by others was predominantly under conditions of resource limitation, excessive density, or when unfamiliar animals are introduced into an established group (24, 25). Moreover, chronic stress arising from unmet basic needs may itself be a trigger for aggression (7). Colony cages implement the concept of environmental enrichment (EE) through increased space, social contact, and sensory stimulation (6), thereby addressing the gap between the natural behavioral needs of the animals and the limited opportunities to express these under conventional laboratory conditions.

One critical requirement in toxicity studies is to ensure comparable systemic exposure to the test item across treated animals, with no exposure in vehicle-treated control animals (26). To avoid cross contamination, we did not socialize animals of different treatment groups in the same colonies. No exposure was detected in vehicle-treated animals, whereas all MTX-treated animals showed exposure for at least 1–2 hours. Beyond feasibility, colony housing was associated with a consistently more favorable welfare status across clinical, endocrine, immunological, and behavioral measures. Body weight gain, a known indicator of general health condition and negatively influenced by chronic stress

(17, 19), was previously shown to be increased by housing improvements with EE (27). In this study, colony-housed control rats gained more weight than contemporary controls housed in Type IV cages, suggesting that the increased social competition and structural obstacles (e.g., vertical level changes) present in TRCC and RCC cages do not prevent sufficient food intake to support healthy growth in individual rats. It remains unclear whether the trend toward greater body weight gain in the colony-cage environment is driven by social pressure that promotes competitive physical development and consequently higher body weights, cooperative behaviors, such as food sharing previously described in rats, that can contribute to this effect (28, 29) or a stress resilience during the study in general. The introduction of EE has been shown to attenuate a decrease in body weight gain that was caused by a chronic variable stress (CVS) paradigm (27). Since routine study procedures inevitably expose animals to a certain degree of stress, EE implemented by colony housing may help to achieve resilience to stress-induced negative effects on weight gain. Mean IgA levels showed different trajectories between study days 29 and 56 within colony- versus conventional housing conditions in vehicle treated animals. In Type IV-housed animals, mean levels were lower on day 56 than on day 29, whereas the opposite pattern was observed in colony-housed rats (Additional file 3). These differences are presented descriptively, as no within-group statistical comparison between the two time-points was performed but support the hypothesis that long term colony housing reduces chronic stress and its suppressive effects on immunoglobulin production (30, 31). The absence of housing related differences on study day 29 and their emergence by study day 57 suggests that these effects accumulate gradually and become detectable after a prolonged housing period. Although most evidence for stress-induced IgA suppression derives from secretory IgA measurements (32–35), the concordant serum IgA findings strengthen the biological plausibility of the observed housing effect. Additional support is provided by the neutrophil-to-lymphocyte ratio (NLR), a validated indicator of physiological distress (21). On study day 29, RCC-housed animals showed significantly lower NLRs than Type IV controls, indicating reduced stress burden.

Furthermore, a non-significant but for all dose groups and time points consistent decrease of adrenal gland weight within colony housed animals was seen. Rats exposed to chronic stress often exhibit adrenal enlargement and increased basal plasma corticosterone despite normal plasma adrenocorticotropic hormone (ACTH) levels. Moreover, patients with depression were shown to exhibit increased basal plasma cortisol and enlarged adrenals (20, 36–38). Lower adrenal weights under colony housing may indicate reduced basal stress, but this was not supported by statistical significance, morphology, or changes in other stress-sensitive organs such as the thymus.

Although some enrichment studies reported increased adrenal weight and corticosterone, these changes were accompanied by reduced responses to mild stressors and more adaptive coping (39–41), supporting the stress inoculation hypothesis that enrichment may promote resilience (42). Differences in enrichment protocols may explain divergent endocrine responses: previous studies frequently changed enrichment items to maintain novelty (39–41), whereas in our study enrichment was changed weekly in both housing conditions. Thus, the additional space, movement, social interaction, and retreat options of colony housing may have contributed to favorable behavioral and welfare outcomes. colony-housed rats showed lower initial exploration and faster habituation in the ActiMot test, consistent with reports that socially or enriched-reared rats exhibit reduced exploration due to more rapid habituation in novel environments than isolation-reared animals (22). Similarly, RCC housed versus Type IV housed rats were previously shown to display shorter exploration times in a light–dark box test (43).

Overall, the results regarding body weight, serum IgA, NLR, adrenal gland weight and physical activity consistently point into a direction of less distress of animals kept in colonies, particularly in the RCC. The favorable effect direction across those various dimensions examined for at least one cage type and time point supports the general conclusion, despite the individual limitations of each parameter taken on its own. The RCC generally conferred greater benefits than the TRCC, possibly due to a combination of greater space allowance and enhanced structural complexity (6, 44).

Data Quality

Overall, colony housing did not relevantly increase variability (mean SD ratios: TRCC, 1.05; RCC, 1.14), with observed differences being parameter-specific rather than driven by housing condition. This is notable because the greater physical, sensory, cognitive, and social stimulation provided by colony housing can influence behavioral, endocrine, physiological, neurobiological, and immunological parameters (6), potentially shifting the biological baseline from which treatment effects are measured. However, conventional housing is itself not biologically neutral and may adversely affect physiology, behavior, and stress responsiveness (45, 46). Thus, EE-induced changes may represent a shift toward a more physiologically representative state, consistent with evidence that environmental context affects reproducibility and translational validity (47). By promoting physical activity, social interaction, and cognitive stimulation, EE may better reflect a healthy human lifestyle and improve translation to the human situation (48–54). This is in line with systematic reviews and meta-analyses that do not support a general increase in variability under enriched conditions, provided that species-appropriate enrichment is implemented and documented precisely and transparently (55). One other relevant aspect of data consistency is that the widespread use of historical control data generated under conventional housing conditions may limit comparability when EE alters baseline values. The colony housing related differences of mean values detected in this study were very limited. Phosphate was significantly elevated in RCC, while calcium showed only a nominal increase. Both parameters trended in the same direction in TRCC, but without reaching significance. This similar pattern in both cages could reflect altered metabolism and calcium–phosphate homeostasis under enriched housing (56–58). As discussed for animal welfare related effects, a slight trend for reduced adrenal weights has been observed. The limited effects on stress-sensitive organs may reflect the comparator, that was not intended as a stress model (59). Nevertheless, several welfare-related measures indicated reduced distress under colony housing. Finally, the most notable histopathology findings were restricted to accessory sexual glands (prostate and seminal vesicles) in single RCC animals, consisting of atrophy and reduced secretion. These findings coincided with lower prostate and seminal vesicle weights and

weight variability. A plausible explanation is social hierarchy-related suppression of the Hypothalamic–Pituitary–Gonadal (HPG) axis in subordinate males within larger groups (60). The absence of comparable findings in TRCC suggests a cage design-specific rather than general colony-housing effect. Given the small group sizes, these findings should be considered exploratory and with caution. Recently, another variant of enriched colony housing has also been shown to preserve all key toxicological endpoints relevant for safety assessment, including clinical pathology, body weight, organ weights, and histopathology (15). Taken together the findings are consistent with the existing literature and support the view that EE and colony housing do not systematically compromise data quality, but it must be regarded as a husbandry-related experimental variable with potential design specific effects, requiring standardization and transparent reporting (61).

Impact of Housing on Treatment-Related Findings

To assess whether housing conditions modulate treatment-related effects, the immunosuppressant MTX was used as a reference compound due to its well-characterized pharmacology and established adverse effects on the hematopoietic and immune systems. In contrast to Patel et al. (62), no toxicologically relevant pathology findings in target organs liver, kidney, lungs and testes were observed at the dose level investigated. Consequently, the present study cannot evaluate the suitability of colony housing under conditions associated with overt toxicity, as encountered in dose-range finding or pivotal good laboratory practice (GLP) toxicity studies. Animals experiencing substantial toxicity may respond differently to the increased social and environmental complexity of colony cages, which require navigation of a multi-level habitat and interactions with a larger number of conspecifics. Therefore, future studies employing higher and toxicologically more relevant dose levels are warranted. Nevertheless, toxicity studies also aim to characterize non-adverse, pharmacologically mediated effects occurring at clinically relevant exposures. Such endpoints may be particularly sensitive to environmental factors and lifestyle-related changes associated with markedly different housing conditions (63). Across all housing systems, MTX treatment was associated with changes in hematological and immunological parameters consistent with its known pharmacology

(Figure 4). After adjustment for multiple testing, more of the selected endpoints showed treatment induced differences in colony-housed animals than in Type IV-housed animals. This pattern should not, however, be interpreted as evidence of a stronger treatment effect under colony housing, because treatment effects were not directly compared between housing systems. IgA levels and B-cell counts also tended to be higher at baseline in vehicle-treated colony-housed animals than in Type IV-housed controls (Additional File 3). Although this observation may be compatible with reduced stress-related immunosuppression (64), the present data do not establish the underlying mechanism or its contribution to the MTX response. Overall, Colony housing did not impair detection of MTX-related hematological and immunological effects, which were pharmacologically expected but not toxicologically relevant.

Conclusions

Both colony housing systems were successfully implemented in a 28-day toxicity study without compromising feasibility, data integrity, or sensitivity for detecting treatment-related effects. Welfare-related outcomes consistently improved, particularly in RCC-housed animals. Minor housing-related baseline shifts likely reflect physiological adaptation rather than impaired data quality. Based on the overall balance of welfare benefits, data quality, practicality and option for flexible animal separation, the TRCC appears most suitable for regulatory toxicity studies. As this study was limited to males and only a single non-toxic dose level, confirmation in independent studies including both sexes and higher dose levels is warranted.

Methods

Ethics and quality Statement

The study was designed and conducted in general compliance with current international study guidelines ICH M3, ICH S3A and OECD TG 407 (16, 17, 26) for pharmaceutical drug development. All procedures were approved by the animal protection authorities of the local district government (Regional Authorities of Hessen (Regierungspräsidium Darmstadt), Germany) under the reference

DA4/2011. The study was conducted under Good Research Practices (GRP) conditions, a Merck internal Quality Management System.

Animals and Study Design

Ninety Male Crl:WI (Han) rats were obtained from Charles River Laboratories (Sulzfeld, Germany) with a mean age of 8 weeks at delivery and at study start a mean body weight of 244.9 g (range: 213.2–279.7 g). Directly after arrival and prior to the acclimation period (13-16 days) animals were randomly allocated to six groups (n = 15/group) using the Laboratory Management System Provantis (Instem, Staffordshire, United Kingdom), ensuring comparable body weight distributions across six groups, defined by housing system (Type IV, Toxicology Rat Colony Cage [TRCC], or Rat Colony Cage [RCC]) and treatment (vehicle or Methotrexate [MTX]). Scheduled necropsies were performed on study day 29 (main cohort, n = 10/group) and study day 57 (recovery cohort, n = 5/group). Animals were monitored daily for clinical signs throughout the study. At scheduled and unscheduled necropsy, animals were euthanized by isoflurane overdose followed by CO_2 asphyxiation. Animals were identified by subcutaneously implanted microchip transponders and additionally by tail tattooing, both placed under brief isoflurane anesthesia. Upon arrival, the animals' health certificates were reviewed and archived, and the animals were examined to confirm their health status. Animals were housed under specific pathogen-free (SPF) conditions, with SPF status confirmed by routine health monitoring using the Rat 3R FELASA Quarterly Serology + Quarterly PCR panels (IDEXX, Kornwestheim, Germany). Full study design is described in Supplementary Methods S10 (Additional file 1).

Test Item, Formulation, and Administration

MTX (10 mg/mL injection solution, Ratiopharm, Ulm, Germany) was diluted in 0.9% NaCl solution (B. Braun, Melsungen, Germany) stored at Room Temperature protected from light and was administered within 3 hours once daily by oral gavage at a volume of 5 mL/kg. The initial MTX dose was 0.2 mg/kg (study days 1–14). As no relevant body weight loss or overt clinical signs were observed during this period, the dose was increased to 0.25 mg/kg for the rest of the treatment phase (study days 15–28).

Housing and Husbandry

Animals were housed in Tecniplast 1500U Eurostandard Type IV S polycarbonate cages (480 x 375 x 210 mm, floor area: 1,500 cm², Tecniplast S.p.A., Buguggiate, Italy) in gangs of 2–3 males and in the TRCC and RCC in colonies of 15 males. The TRCC consists of four standard Type IV cages interconnected by a horizontal transfer tube placed above, providing a total floor area of 9,651 cm² per unit. For more flexibility, pushers are introduced that allow separation of two or single cages if needed. The integration of several doors allows caretaker to reach the animals at any location within the tubes. The RCC is a multi-level colony housing system with a total floor area of 22,246 cm² per unit, as described in detail by Brenneis et al. (43). Schematic illustrations of both colony cage systems are provided in Figure 5. All animals were maintained under a 12-hour light/dark cycle at 22 ± 2°C and a targeted relative humidity of 45–65%. Softwood granulate bedding was used throughout. Standard rodent diet (Ssniff V1534, Ssniff Spezialdiäten, Soest, Germany) and tap water were provided ad libitum. Five enrichment items were provided on an alternating schedule: Metal shelters (Industry Montage Service IMS, Germany) were present in all cages throughout the study. In addition, each cage contained two further enrichment items, consisting of either a corner home (Datesand) or SAFE(R) Crinklets Natural (J. Rettenmaier & Söhne GmbH & Co. KG), combined with either a play tunnel or an aspen brick. For urinalysis, animals were transferred to individual metabolic cages for rats >300 g (3701M081, Tecniplast S.p.A., Buguggiate, Italy) and food-deprived for approximately 18–22 hours prior to clinical pathology blood sampling. Animals undergoing behavioral assessments were temporarily housed individually in Type III cages for a duration of approximately 2 hours.

In-Life Observations and Behavioral Assessments

A complete schedule of all measurements and planned animal numbers is provided in Table 4. Handling-related behavior was assessed on each working day (Monday–Friday) throughout the study using a predefined scoring scheme. Full scoring criteria are provided in Supplementary Methods S1 (Additional file 1).

Table 4. Schedule of Measurements performed within this study and planned animal numbers.

Domain	Observation/ Measurements	Timepoint (study day)	Animals
In-Life	Mortality & Clin. Signs	Daily	Day1-28: n=15 Day29-57: n=5
	Appearance and Behaviour	Daily	Day1-28: n=15 Day29-57: n=5
	Body Weight	Twice weekly	Day1-28: n=15 Day29-57: n=5
	Functional Observation Battery	Week 4 Week 8	n=9 n=3
	Motor Activity	Week 4 Week 8	n=9 n=3
	Ophthalmological Assessment	Day -1 & Day 26	n=5
Laboratory	Clinical Pathology (hematology, immune cell phenotyping*, clinical chemistry, urinalysis)	Day 29 Day 57	n =15 n= 5
	Toxicokinetics	Day 1: 1, 2, 4, 6 and 24 hours after administration Day 28: predose, 1, 2, 4, 6 and 24 hours after administration	n=10 n=10
Pathology	Gross Pathology	Day 29 Day 57	n=10 n=5
	Organ Weights	Day 29 Day 57	n=10 n=5
	Histopathology	Day 29 Day 57	n=10 n=5
Special	Corticosterone	Day 14 Day 29 Day 40 Day 57	n=10 n=10 n=5 n=5
	Immunoglobulin A	Day 29 Day 57	n=10 n=5

*n=10 for immune cell phenotyping on Day 29

Behavioral investigations consist of Functional Observational Battery (FOB) test, Ophthalmological- and Motor Activity assessment. For study day, groups and group sizes see Table 4. Detailed procedures for those assays are provided in Supplementary Methods S2-4 (Additional file 1).

Clinical Pathology and Toxicokinetics

Standard clinical pathology assessments (hematology, clinical chemistry, and urinalysis) were conducted at the timepoints specified in Table 4. The parameters analyzed, abbreviations, measurement methods and sample handling procedures are described in Supplementary Methods S5 (Additional file 1). For toxicokinetic assessment, blood samples (~20 µL) were collected by tail vein venipuncture at pre-specified timepoints (Table 4). Methods are described in Supplementary Methods S9 (Additional file 1).

Stress and Immune Biomarkers as indicators for animal welfare related effects

Serum corticosterone was quantified using a competitive enzyme-linked immunosorbent assay (ELISA) (Demeditec, DEV9922, Kiel, Germany) and IgA was quantified using a sandwich ELISA (Bethyl Laboratories, Montgomery, Texas, United States of America) at a 1:1000 dilution. Both assays were performed in duplicate with four-parameter logistic (4PL) curve fitting and according to the respective manufacturer's instructions. Sampling time points are specified in Table 4. Detailed assay procedures are provided in Supplementary Methods S6 (Additional File 1).

Necropsy, Organ Weights, and Histopathology

Scheduled necropsies were performed on study day 29 (main cohort, n = 10/group) and study day 57 (recovery cohort, n = 5/group). Organ weights were recorded for all animals. The list of organs examined, tissue processing, sectioning, staining, and histopathological evaluation procedures are described in Supplementary Methods S7 (Additional file 1).

Statistical Analyses

All available valid observations were analysed. One recovery animal in group 2 was euthanized on day 15 and one blood sample from a recovery animal in group 5 was excluded because of clotting. Endpoint specific sample sizes are reported in Table 4, the figure legends, and Additional file 1 (Supplementary Methods S8 and S10). No single primary outcome or formal a priori sample size calculation was prespecified, and housing comparisons were exploratory. Housing and treatment effects were assessed using Welch’s t tests with Benjamini Hochberg correction. Housing related

variability was assessed using SD ratios with bootstrap confidence intervals. Four prespecified welfare endpoints were analysed using one way ANOVA followed by Dunnett's test. Analyses were performed separately for each time point. Individual animals were treated as the units of analysis despite being group housed. Given the small group sizes, results should be interpreted with caution. Analyses were performed using GraphPad Prism 10.2.1, R 4.3.1 in RStudio (65), and Microsoft Excel. R was used for data preparation, Welch's tests, bootstrap analyses, and the variability forest plot, using dplyr, tidyr, purrr, and boot as the main packages. The remaining figures and Dunnett's tests were generated in GraphPad Prism. Further statistical details are provided in Additional file 1 (Supplementary Methods S8).

List of abbreviations

4PL — four parameter logistic

ACTH — adrenocorticotropic hormone

ANOVA — analysis of variance

ARRIVE — Animal Research: Reporting of In Vivo Experiments

BCa — bias-corrected and accelerated

BH— Benjamini-Hochberg

CI; CIs — confidence interval; confidence intervals

CRAMPED — cold, rotund, abnormal, male-biased, poorly-surviving, enclosed and distressed

CVS — chronic variable stress

EE — environmental enrichment

ELISA — enzyme-linked immunosorbent assay

EU — European Union

FOB — Functional Observational Battery

GLP — good laboratory practice

GRP — good research practice

HPA — hypothalamic-pituitary-adrenal

HPG — hypothalamic-pituitary-gonadal

ICH — International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

IgA — Immunoglobulin A

MTX — methotrexate

NK — natural killer

NLR — neutrophil-to-lymphocyte ratio

OECD — Organisation for Economic CO-operation and Development

RCC — Rat Colony Cage

SD — standard deviation

TG — Test Guideline

TRCC — Toxicology Rat Colony Cage

Figure Legends

Figure 1. Animal welfare indicators across housing systems. (A) Body weight gain relative to study day 1 at study day 28 (n = 15 per group) and study day 56 (n = 5 per group). (B) Relative adrenal gland weight on study day 29 (n = 10 per group) and 57 (n = 5 per group). (C) Ratio of absolute neutrophil and lymphocyte counts at study day 29 (n = 15 per group) and 57 (n = 5 per group, except RCC: n = 4) (D) Laser beam counts indicating exploratory locomotor activity during 0–30 min and 30–60 min intervals (n = 9 per group). Panels A, B, C and D show mean + SD with individual values. For each panel and time point, data were analyzed separately by one-way ANOVA with Dunnett's post-hoc test for comparison to Type IV control. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, **** $p < 0.0001$.

Figure 2. Variability of standard clinical pathology parameters and organ weights of control-main group animals. Forest plot showing the SD Ratio (Colony Cage / Type IV) with 95% confidence intervals derived via bias-corrected and accelerated (BCa) bootstrapping (10,000 iterations). Panel (A) shows comparisons for Type IV vs. TRCC and (B) Type IV vs. RCC. A ratio <1 indicates greater variability in Type IV cages. A ratio >1 indicates greater variability in the respective colony cage system (TRCC or RCC). Parameters are grouped by domain: hematology (11 parameters), clinical chemistry (17 parameters), and organ weights (13 parameters). For all hematology and clinical chemistry parameters, n = 15 animals per group were included, for organ weights, n = 10 animals per group were included. The x-axis is log-transformed to symmetrize the scale around equality (SD Ratio = 1, dashed line). 95% CIs excluding 1 indicated a notable difference in variability between the two housing systems for the corresponding parameter (indicated in red). The “mean SD-ratio” row shows the arithmetic mean. Blood parameter abbreviations are defined in Supplementary Methods S5 (Additional file 1).

Figure 3. Incidental histopathological findings in male reproductive organs of vehicle-treated RCC housed rats. (A) Prostate (ventral lobe, 20x). Left: Normal epithelium in a Type IV-housed animal showing tall columnar epithelial cells with basally located nuclei, abundant apical secretory droplets, and homogeneously stained eosinophilic luminal secretion. Right: RCC-housed animal with mild diffuse epithelial atrophy characterized by reduced cell size, isoprismatic cell morphology, decreased secretory droplets and markedly reduced luminal secretion. (B) Seminal vesicle (20x): Left: Type IV-housed animal with normal epithelium. Right: RCC-housed animal with mild diffuse atrophy characterized by reduced cytoplasm, loss of secretory droplets, and diminished luminal secretion.

Figure 4. MTX-related changes across housing systems. Mean percent change relative to the respective vehicle group is shown for animals housed in Type IV or RCC cages. Panel (A) shows cumulative body weight gain assessed on study days 28 and 56. Panels (B–I) show blood-derived endpoints assessed on days 29 and 57: reticulocytes (B), hemoglobin (C), platelets (D), neutrophils (E), serum IgA (F), NK cells (G), T-helper cells (H), and cytotoxic T cells (I). At the end of treatment, n = 15/group for body weight gain and hematology (Type IV MTX: n = 14) and n = 10/group for IgA and immune-cell phenotyping. At the end of recovery, n = 5/group except for Type IV MTX (n = 4 for all endpoints) and RCC vehicle (n = 4 for hematology and immune-cell phenotyping and n = 5 for body weight gain and IgA). Asterisks indicate differences versus vehicle (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; two-sided Welch's t-tests, with Benjamini–Hochberg adjustment across all 18 evaluated parameters separately for each housing system and assessment period). Nine of the 18 evaluated parameters are displayed.

Figure 5. Schematic overview of investigated colony housing systems. (A) Scheme and photograph of the prototype Toxicology Rat Colony Cage (TRCC), consisting of four standard type IV cages (Cage 1–4) interconnected horizontally by an overhead large tube (gray) that can be accessed through short vertical tubes (blue). Green triangles in the grey tube indicate doors providing access for animal care staff. Black squares represent closable sliding doors that can grant or restrict the animals' access between compartments. (B) Scheme and photograph of the Rat Colony Cage (RCC), consisting of 4 levels that are interconnected vertically via jump holes (blue) between levels 1 to 2 or 3 to 4 or via a staircase (green) between levels 2 to 3.

Additional Files Legends

Additional file 1. (.docx). *Supplementary Methods*. Detailed descriptions of the methods and study procedures reported in the manuscript.

Additional file 2. (.pdf). *Supplementary Plots* showing methotrexate plasma concentrations and animal welfare indicators in methotrexate treated animals, each accompanied by a title and figure legend.

Additional file 3. (.pdf). *Housing Effects*. Tables comparing vehicle-treated animals between housing groups for hematological, clinical chemistry, organ-weight, and immunological parameters using Welch's t-tests. P values were adjusted for multiple testing using the Benjamini–Hochberg procedure across the parameters included in the housing-effects analysis.

Additional file 4. (.pdf). *Ophthalmological Results*. Tables presenting the results of the ophthalmological assessments.

Additional file 5. (.xlsx). *Functional Observational Battery Results*. Description: Tables presenting the results of the functional observational battery (FOB) assessments.

Additional file 6. (.docx). *Detailed Histopathological Findings.* Tables presenting detailed histopathological findings in the affected reproductive organs and lungs.

Additional file 7. (.pdf). *Methotrexate Effects in all domains.* Tables comparing methotrexate-treated and vehicle-treated animals within each housing group using Welch's t-tests. The analysis includes hematological, clinical chemistry, organ-weight, and immunological parameters. P values were adjusted for multiple testing using the Benjamini–Hochberg procedure across this broader parameter panel.

Additional file 8. (.pdf). *Pharmacologically known Methotrexate Effects on Hematological and Immunological Parameter Panel.* Tables comparing methotrexate-treated and vehicle-treated animals within each housing group using Welch's t-tests. This focused analysis includes hematological and immunological parameters only. P values were adjusted for multiple testing using the Benjamini–Hochberg procedure across this smaller parameter panel. Adjusted p values may therefore differ from those for the same endpoints in Additional file 4.

Additional file 9. (.Rmd; viewable in RStudio). *R Scripts*. Scripts for data preparation, variability and treatment-effect analyses, and visualization of results as forest plots.

Additional file 10. (.pdf). *Animal Research: Reporting of In Vivo Experiments* (*ARRIVE) 2.0 Essential 10 Author Checklist*. Completed reporting checklist indicating where each ARRIVE Essential 10 item is addressed in the manuscript.

Declarations

Ethics approval and consent to participate

All animal procedures were approved by the competent animal protection authority of the local district government, the Regierungspräsidium Darmstadt, Germany, under reference number DA4/2011. The study was conducted in accordance with applicable institutional and national regulations for the care and use of laboratory animals.

Consent for publication

Not applicable

Availability of data and materials

Data generated or analyzed during this study are included in this published article and its additional files. Except for pharmacokinetic and bioanalytic analysis, all samples and documents to be archived are stored in the archives of the Test Facility Merck Darmstadt in accordance with the valid regulations for archival storage. The documentation of the bioanalytic evaluation is stored in the archive of the Contributing Laboratory (Swiss BioQuant, Reinach Switzerland). The raw datasets, which are not included in additional files, are available from the corresponding author on reasonable request.

Competing interests

All authors are employees of Merck Healthcare KGaA.

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Authors' contributions

JPW planned and performed the special investigations, analyzed the data, and wrote the manuscript. AK was responsible for gross and histopathological investigations and the analysis and interpretation of the results. SK was responsible for clinical pathology investigations and the analysis and interpretation of the results. SJ contributed to the study concept and data interpretation. CB conceived

and designed the study, supervised the investigative laboratory, and wrote the manuscript. All authors reviewed and approved the final manuscript for publication.

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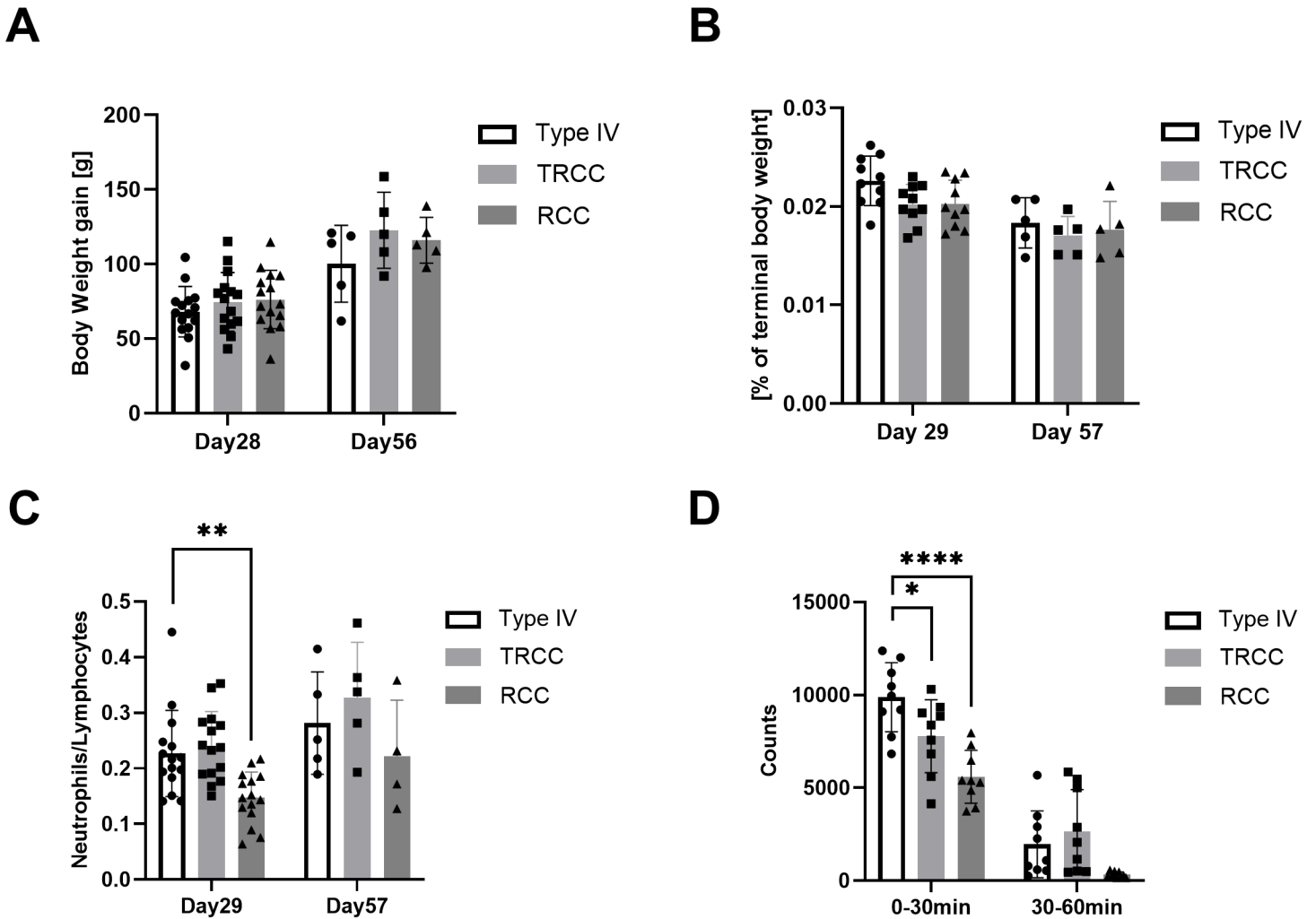
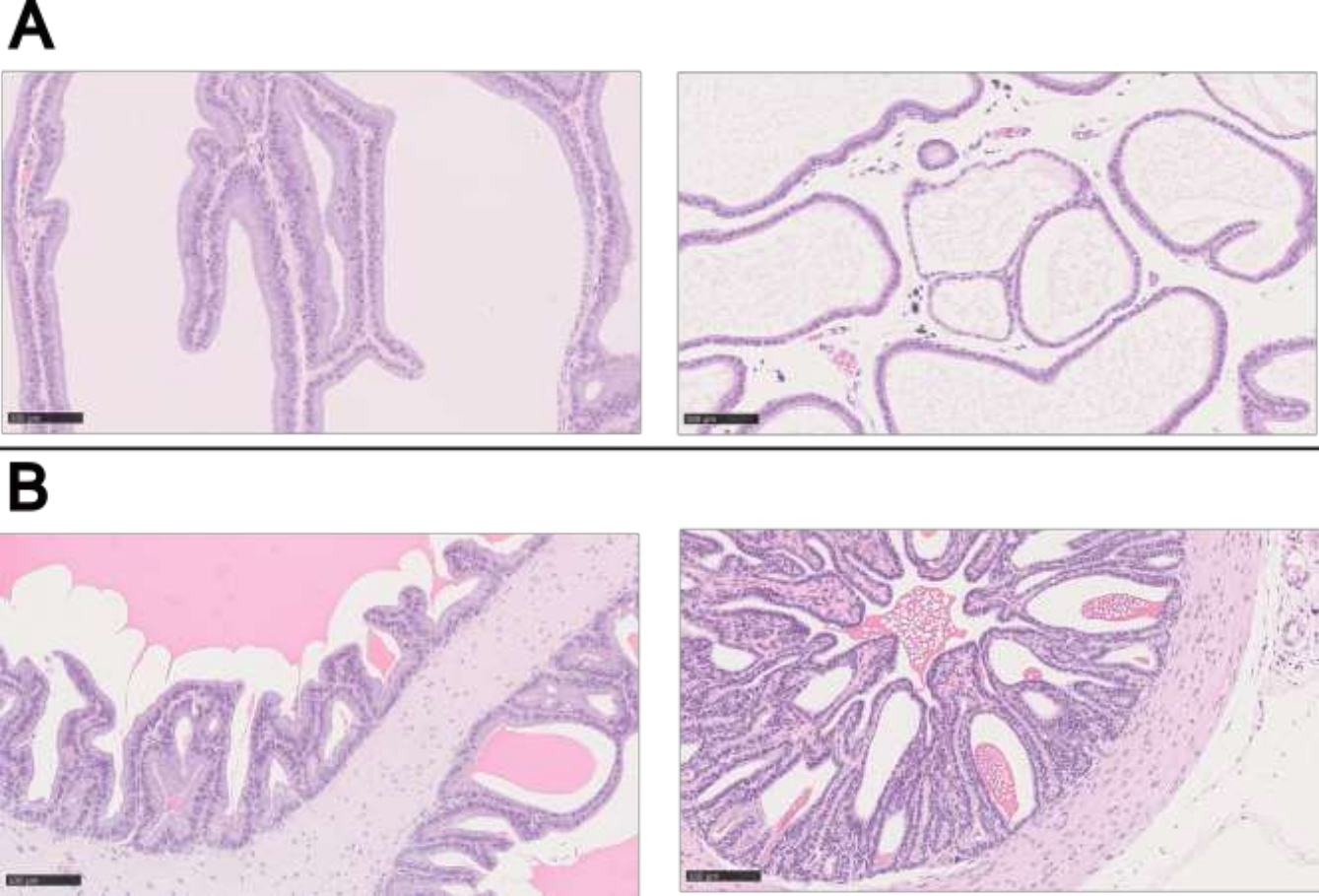
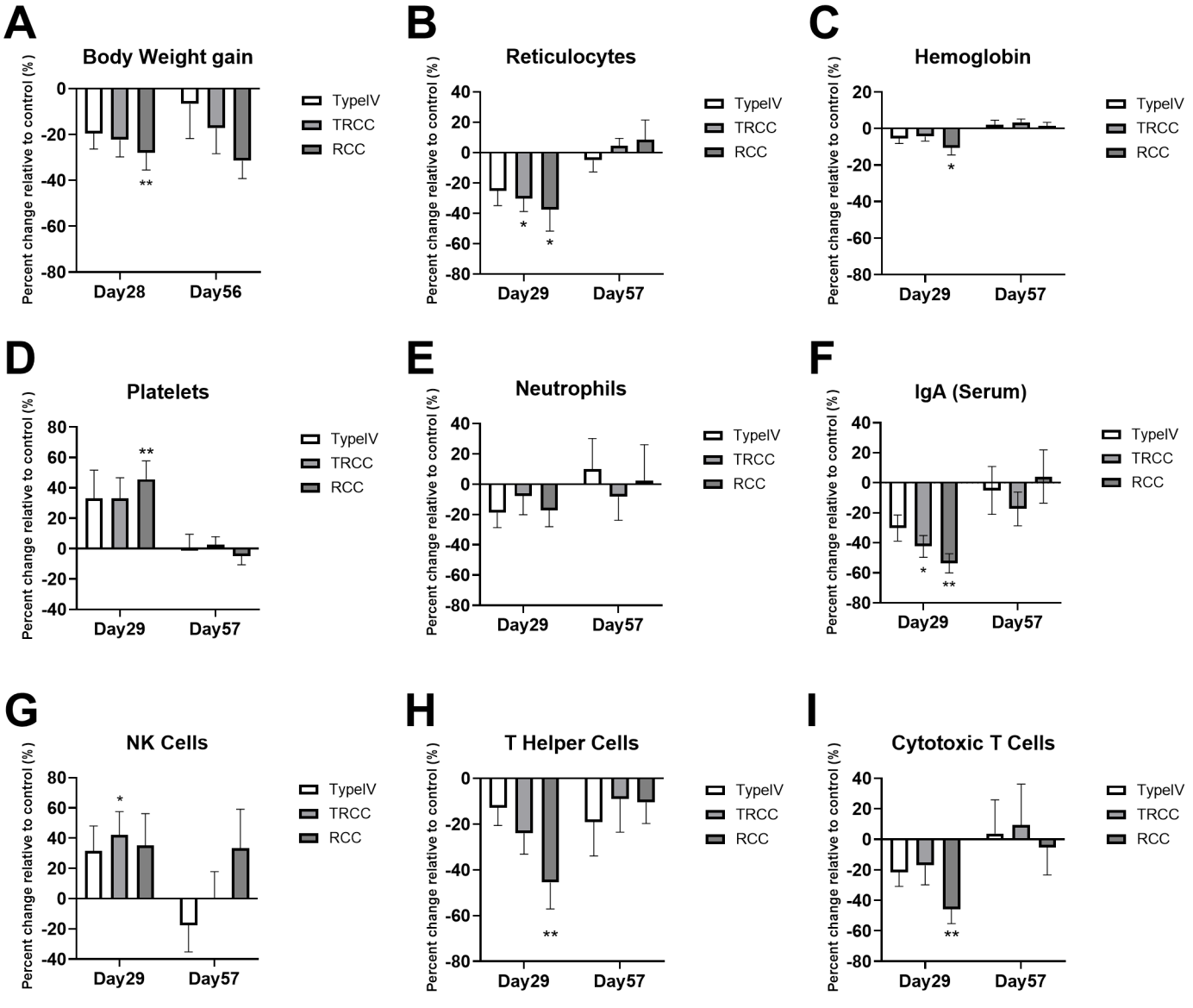
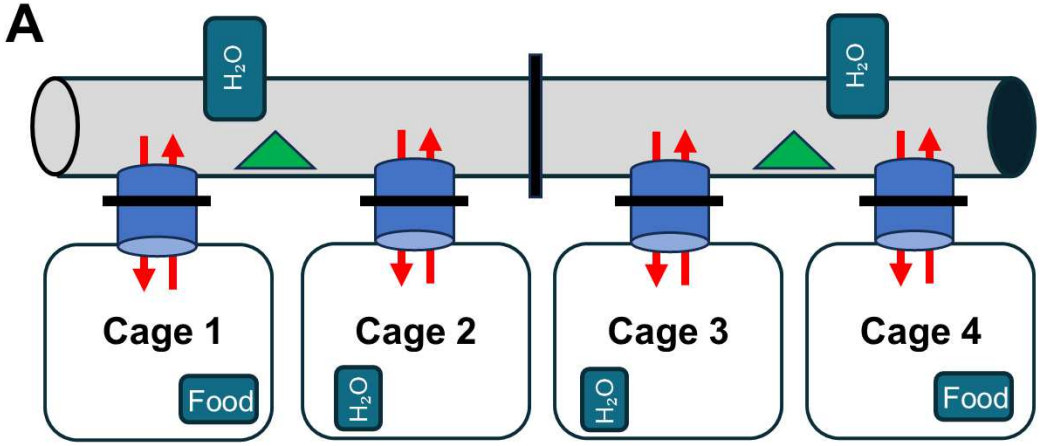

A
Body Weight gain [g]
200
150
100
50
0
Day28
Day56
Type IV
TRCC
RCC
B
[% of terminal body weight]
0.03
0.02
0.01
0.00
Day 29
Day 57
Type IV
TRCC
RCC
C
**
Neutrophils/Lymphocytes
0.5
0.4
0.3
0.2
0.1
0.0
Day29
Day57
Type IV
TRCC
RCC
D
****
*
Counts
15000
10000
5000
0
0-30min
30-60min
Type IV
TRCC
RCC


A
B
Hematology
Clinical Chemistry
Organ Weight
Parameter
Estimate [95% CI]
MONO 1.34 [1.01, 1.75]
WBC 1.23 [0.88, 1.86]
LYM 1.12 [0.82, 1.55]
PLT 0.99 [0.81, 1.23]
HGB 0.94 [0.75, 1.14]
HCT 0.93 [0.74, 1.16]
NEU 0.88 [0.57, 1.39]
RET 0.83 [0.63, 1.08]
RBC 0.81 [0.62, 1.03]
BASO 0.67 [0.49, 0.99]
EOS 0.57 [0.46, 0.71]
K 2.06 [1.57, 3.14]
ASAT 1.93 [1.34, 2.96]
GLDH 1.72 [1.37, 2.13]
AP 1.62 [1.12, 2.19]
UREA 1.37 [0.89, 2.09]
TBIL 1.33 [0.93, 1.95]
GLUC 1.05 [0.73, 1.44]
CREA 1.03 [0.72, 1.31]
CL 1.01 [0.77, 1.29]
ALAT 0.96 [0.76, 1.23]
NA 0.90 [0.61, 1.26]
CA 0.85 [0.61, 1.16]
TRIG 0.85 [0.65, 1.14]
IP 0.82 [0.62, 1.08]
PROT 0.77 [0.52, 1.08]
ALB 0.71 [0.57, 0.88]
CHOL 0.63 [0.45, 0.95]
Epididymides 1.54 [0.94, 2.45]
Heart 1.37 [0.92, 1.96]
Kidneys 1.24 [0.80, 2.39]
Liver 1.19 [0.79, 1.93]
Spleen 1.17 [0.81, 1.72]
Thymus 1.08 [0.71, 1.76]
Prostate 1.02 [0.75, 1.47]
Term Bw 0.93 [0.65, 1.38]
Testes 0.83 [0.61, 1.08]
Adrenals 0.80 [0.56, 1.10]
Brain 0.78 [0.58, 1.07]
Thyroids Parath... 0.75 [0.52, 1.08]
Seminal Vesicles 0.59 [0.45, 0.79]
Mean SD-ratio 1.05 [0.95, 1.16]
0.25 0.50 1.0 2.0 4.0
SD Ratio (TRCC / TypeIV)
Parameter
Estimate [95% CI]
BASO 2.27 [1.66, 3.34]
MONO 1.59 [1.20, 2.07]
HCT 1.37 [1.10, 1.71]
HGB 1.29 [1.02, 1.57]
RBC 1.25 [0.96, 1.59]
RET 0.96 [0.73, 1.25]
LYM 0.91 [0.66, 1.25]
WBC 0.88 [0.63, 1.33]
NEU 0.74 [0.47, 1.17]
EOS 0.73 [0.58, 0.90]
PLT 0.61 [0.50, 0.76]
UREA 1.97 [1.28, 3.00]
GLUC 1.83 [1.28, 2.51]
TBIL 1.53 [1.07, 2.25]
AP 1.38 [0.95, 1.85]
PROT 1.37 [0.92, 1.91]
ALB 1.30 [1.05, 1.62]
GLDH 1.28 [1.01, 1.58]
IP 1.23 [0.92, 1.62]
CA 1.13 [0.81, 1.54]
K 1.11 [0.85, 1.70]
ASAT 1.03 [0.72, 1.58]
CL 0.96 [0.73, 1.23]
CHOL 0.95 [0.67, 1.43]
CREA 0.94 [0.66, 1.20]
ALAT 0.87 [0.69, 1.11]
TRIG 0.80 [0.62, 1.08]
NA 0.76 [0.51, 1.06]
Prostate 1.72 [1.26, 2.48]
Thymus 1.66 [1.10, 2.71]
Epididymides 1.25 [0.76, 1.99]
Brain 1.14 [0.84, 1.56]
Seminal Vesicles 1.12 [0.86, 1.52]
Adrenals 0.95 [0.67, 1.32]
Spleen 0.95 [0.66, 1.40]
Term Bw 0.95 [0.67, 1.40]
Liver 0.92 [0.61, 1.49]
Testes 0.81 [0.60, 1.06]
Thyroids Parath... 0.76 [0.52, 1.09]
Kidneys 0.69 [0.45, 1.33]
Heart 0.68 [0.45, 0.97]
Mean SD-ratio 1.14 [1.02, 1.25]
0.25 0.50 1.0 2.0 4.0
SD Ratio (RCC / TypeIV)


A
B

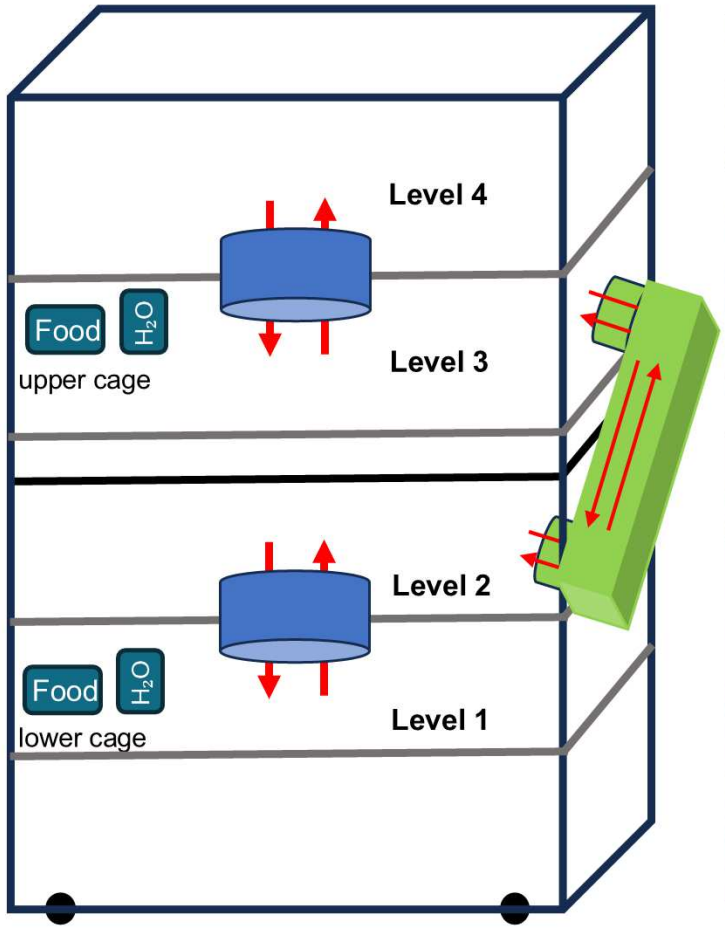

A
Body Weight gain
Percent change relative to control (%)
0
-20
-40
-60
-80
**
Day28
Day56
TypeIV
TRCC
RCC
B
Reticulocytes
Percent change relative to control (%)
40
20
0
-20
-40
-60
-80
*
*
Day29
Day57
TypeIV
TRCC
RCC
C
Hemoglobin
Percent change relative to control (%)
20
0
-20
-40
-60
-80
*
Day29
Day57
TypeIV
TRCC
RCC
D
Platelets
Percent change relative to control (%)
80
60
40
20
0
-20
-40
**
Day29
Day57
TypeIV
TRCC
RCC
E
Neutrophils
Percent change relative to control (%)
40
20
0
-20
-40
-60
-80
Day29
Day57
TypeIV
TRCC
RCC
F
IgA (Serum)
Percent change relative to control (%)
40
20
0
-20
-40
-60
-80
*
**
Day29
Day57
TypeIV
TRCC
RCC
G
NK Cells
Percent change relative to control (%)
80
60
40
20
0
-20
-40
*
Day29
Day57
TypeIV
TRCC
RCC
H
T Helper Cells
Percent change relative to control (%)
0
-20
-40
-60
-80
**
Day29
Day57
TypeIV
TRCC
RCC
I
Cytotoxic T Cells
Percent change relative to control (%)
40
20
0
-20
-40
-60
-80
**
Day29
Day57
TypeIV
TRCC
RCC

A


H2O
H2O
Cage 1
Cage 2
Cage 3
Cage 4
Food
H2O
H2O
Food



B


Level 4
Food
H2O
upper cage
Level 3
Level 2
Food
H2O
lower cage
Level 1



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