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Does Sex Matter in Liver Surgery? Comparison of Severity Assessments between Female and Male Rats after Partial Hepatectomy: A Pilot Study

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Sex difference · Severity assessment · Open field · Fecal corticosterone metabolites · Partial hepatectomy

Abstract
Introduction: Current animal-based biomedical research, including studies on liver function and disease, is conducted almost exclusively on male animals to mitigate confounding effects of the estrous cycle. However, liver diseases afflict both men and women, so translational research findings should also be applicable to female patients. This pilot study investigated sex differences in objective and subjective severity assessment parameters in rats following 50% partial hepatectomy. ***Materials and Methods:*** This study was performed using Wistar Han rats, in which measurements of body weight, spontaneous motor activity in the open field (OF) (movement distance, movement velocity, rearing frequency), and fecal corticosterone metabolites were conducted at baseline and at multiple times after partial hepatectomy. Subjective postsurgical severity assessments were conducted using modified score sheets. Blood parameters such as leukocyte count and serum aspartate aminotransferase, as well as estrogens and testosterone were measured from samples obtained during partial hepatectomy and at sacrifice. In addition, the amount of resected liver tissue was measured at partial hepatectomy, and the proliferated liver was weighed at sacrifice. ***Results:*** Fecal corticosterone metabolite concentrations differed significantly between males and females at baseline and following hepatectomy. Also, leukocyte counts and estrogen concentrations were significantly different between sexes before partial hepatectomy. Alternatively, there were no sex differences in severity assessments, body weight changes, and behavior in the OF at any measurement time point. Liver weight was significantly different in males and females at the time point of partial hepatectomy and sacrifice. ***Conclusion:*** The results of this pilot study suggest that males and females respond similarly following partial hepatectomy. Examination of both sexes is very important for translation to humans, where both men and women suffer from liver disease. Furthermore, the use of both sexes in animal-based research would improve the utilization of the animal breeding in terms of the 3 Rs. However, due to some limitations, larger scale investigations including a broader spectrum of pathophysiolological, behavioral, and pharmacokinetic measures are planned.

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Introduction

Laboratory animals are widely used as model organism of human disease [1–3]. This includes, for example, testing of tissue compatibility of medical devices in rabbits, studying protective effects in rat livers after ischemia reperfusion injury, as well as conducting research on immunological topics in pigs [4–6]. The number and sex of animals used in these experiments must be justified according to current ethical guidelines and thorough literature reviews, as well as robust biometric planning and fundamental scientific desire. For this purpose, researchers can use the PREPARE Guidelines for assistance [7]. Unless a study focuses on a physiological or pathological process restricted to one sex (e.g., prostatitis, endometriosis, pregnancy), the justification for sex selection is often weak or absent [8–11]. A frequent rationale for the choice of experimental animal sex is the potential confounding influence of hormonal changes that occur during the estrus cycle, so many studies are restricted to males only [12–14]. Alternatively, females are generally less aggressive [15–17] and so are easier to handle and can be group-housed, which is economically beneficial and, most importantly, a form of natural environmental enrichment to fulfill the animals' ethological needs. Nevertheless, both circadian and estrus-related hormonal cycles have distinct influences on female and male animals from embryogenesis through adulthood, but these sex-dependent cycles are not sufficient enough in the experimental design if the process or disease under study is not of hormonal origin or treated by hormone therapy [18, 19]. Sex-specific hormonal conditions can also affect recovery and outcome after surgery [20, 21]. For instance, it has been reported that estrogen is hepatoprotective and improves survival following liver ischemia reperfusion (IR) possibly by suppressing cellular oxidative stress [22–24]. Furthermore, sexual dimorphisms influence the tolerance to various hepatic pathologies and recovery following major liver surgery [20]. In addition, the liver regulates sex hormone levels through metabolism, so liver-related diseases may differentially influence the reparative effects of sex hormones and thus the progression of disease or surgical recovery [25, 26].

In Germany, more than 80,000 cases of liver disease are treated every year [27]. According to the Center for Cancer Registry Data, Germany, around 9,000 new cases of liver cancer are diagnosed annually, and there are roughly 8,000 disease-related deaths [28]. Approximately 1/3 of these patients are female (i.e., more than 2,900 cases in 2017) [28], so translational research must be equally applicable to this group [21].

End-stage liver disease requires liver transplantation, and according to the European Liver Transplant Registry, 29.5% of transplantation patients are female [29]. Partial liver resection is an alternative to complete organ transplantation in some cases, and the pathophysiological responses to this treatment have been studied extensively in animal models, especially in rodents [30]. A comprehensive systematic review of existing literature from the mid-1930s to 2020 on partial liver resection in experimental rats and mice [30] performed in advance of the current study revealed that male rats are used almost exclusively, while only 4 of 115 studies examined females and only 2 included both sexes [12] with no evidence-based reasons for sex selection provided by the authors.

While the choice of male rats may be justified to ensure comparability with prior studies, this raises the question of whether the existing literature is applicable to female patients [31–33]. As a project of the German Research Council funded research group for 2591 "Severity Assessment in Animal-Based Research," this pilot study examined differences in objective outcome metrics and subjective severity assessments between male and female Wistar Han rats following partial hepatectomy to determine if estrus-related changes are a significant physiological source of bias. Our previous study using the open field (OF) test indicated that postsurgical severity depends on the extent of liver resection [34], so in the current study, 50% partial hepatectomy was used in both groups. The main research question of this study was if there are sex-specific differences after performing a 50% liver resection by using postoperative severity assessment methods in rats. This leads to the question of whether it is justified to use only one sex in this research approach in the future.

Materials and Methods

Ethics Statement

The study was performed in accordance with the German animal welfare law (Tierschutzgesetz) and European Union (EU)-Directive 2010/63/EU [35], and the protocol was approved by the Governmental Animal Care and Use Committee (Reference No.: 84-02.04.2017.A304; Landesamt für Natur, Umwelt und Verbraucherschutz Recklinghausen, Northrhine Westphalia, Germany). According to Federation of European Laboratory Animal Science Associations guidelines, all study participants were prior trained in rat care and the monitoring of rat-specific behaviors to reduce stress [36].

Animals

Seven female and seven male Wistar Han rats (Janvier S.A.S., Saint-Berthevin Cedex, France; delivery body weight of both sexes: 150–175 g; age, males: 5–6 weeks and females: 5–7 weeks; BW at

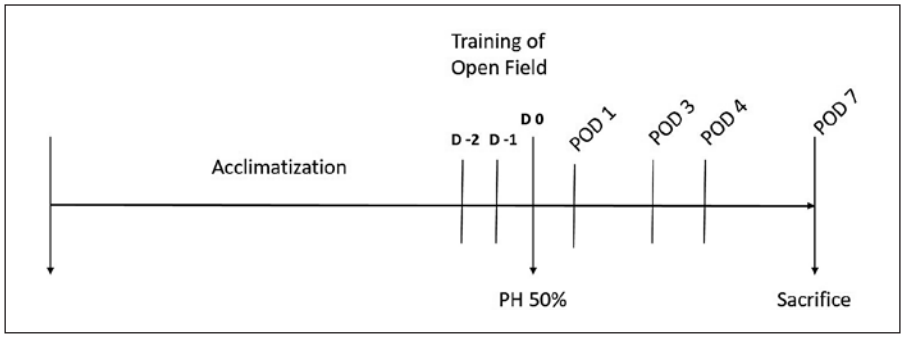

Fig. 1. Schematic timeline of experiments. The day of surgery is indicated as D0. All animals were received at least 1 week before D-2. POD, postoperative day; D, study day; PH 50%, 50% partial hepatectomy.

study start, males: 241 g ± 14 g and females: 196 g ± 7 g) were included according to a priori calculated group size in the corresponding project via power calculation using the software. G*Power (version 3.1; freeware, Heinrich Heine University of Düsseldorf, Düsseldorf, Germany; www.gpower.hhu.de) [34, 37]. The age of the animals in all our experiments was determined by a prior performed systematic review by Zieglowski et al. [34]. So, the age was corresponding to the literature as well as to our previous studies. Animals were group-housed by sex in filter-top cages (type 2000, Tecniplast, Buguggiate, Italy) in a controlled environment (12-h/12-h light-dark cycle, 22°C ± 2°C, 50% ± 20% relative humidity) according to Federation of European Laboratory Animal Science Associations guidelines [36, 38]. Experiments on males and females were time shifted so that groups were not housed in the same room simultaneously, thereby mitigating potential influences of pheromones. Animals were housed with red tunnels (tunnel Ø 155 × 75 mm, #3084014, Zoonlab GmbH, Castrop-Rauxel, Germany) for handling and cage enrichment and with low-dust wood granulate as bedding (Rettenmeier Holding AG, Wilburgstetten, Germany). Food and sweetened drinking water (Ja! Süßstoff flüssig, Rio Mints & Sweeteners B.V., Utrecht, The Netherlands; 3.8 mL sweetener in 735 mL drinking water) were provided ad libitum. The sweetener was added to mask the bitter taste of postsurgical analgesics administered via the drinking water. For habituation, sweetened water was also used throughout the presurgical housing period.

Experimental Design

As shown in Figure 1, rats were acclimatized to the new housing conditions for at least 1 week after delivery. OF tests were conducted for two consecutive days prior to surgery and again on postoperative days (PODs) 1, 3, 4, and 7. Severity assessments were performed for 7 consecutive days post-surgery and included scoring described by Morton et al. [39].

All surgeries were performed under general inhalation anesthesia with subcutaneous antibiotics and analgesia. Analgesia was additionally administered via drinking water immediately after surgery for the first 3 PODs. Animals were sacrificed by exsanguination under general anesthesia on POD 7. During surgeries, the blood and feces were collected to investigate changes in blood parameters and fecal corticosterone metabolites (FCMs). Feces were also collected during OF tests for FCM analysis.

Measurement of Sex Hormones and Estrus Stage Determination

Estrogen and testosterone concentrations were determined in the serum from blood samples collected during partial hepatectomy and at sacrifice with ELISA Kits (Rat Estrogen ELISA Kit, Catalog No.: MBS7606809, Mybiosource, San Diego, CA, USA, and Testosterone rat/mouse ELSIA, REF:DEV9911, Demeditec, Kiel, Germany).

The estrous cycle was examined on the day of partial hepatectomy and on the day of sacrifice. Briefly, a sterile cotton swab was prewettened with sterile saline, inserted in the vagina, and then smeared on a microscope glass slide. The sloughed cells were stained using the Papanicolaou protocol, and stages of the estrous cycle were determined as previously described [40].

Surgical Intervention

All surgeries (liver resection and sacrifice) were performed under sterile conditions. Animals were administered inhalational anesthesia (induction by 5 vol% Isoflurane [Abbott GmbH, Wiesbaden, Germany] in 5 L/min O_2 and maintenance by 2 vol% Isoflurane in 2 L/min O_2). Analgesics (metamizole; Novaminsulfon-ratiopharm® 1 g/2 mL; Ratiopharm GmbH, Ulm, Germany; 100 mg/kg, s.c., single dose) and antibiotics (Cefuroxime; Fresenius Kabi, Bad Homburg, Germany 16 mg/kg, s.c.) were also administered before surgery according to national recommendations in Germany [41].

The liver was exposed via midline incision and 750 µL of blood was subsequently collected from the abdominal vena cava. The left lateral lobe, left part of the medial lobe, and the caudal lobe were ligated with a 4/0 silk thread (Resorba Medical GmbH, Nürnberg, Germany) and resected from the remaining liver tissue with scissors and weighed. After ensuring that there was no bleeding, the abdominal cavity was rinsed with prewarmed sterile saline, and the muscle and skin were closed in layers using continuous suture with 5/0 Prolene and single knots of 4/0 Vicryl (ETHICON, Johnson & Johnson Medical GmbH, Neuss, Germany), respectively. According to data from the commercial animal breeder (Janvier S.A.S.), initial liver weight accounts for 2.7% of the total body weight in females and 2.8% in males. These values were used to calculate liver weight and cell proliferation at multiple times prior to sacrifice and to assess the association with FCM values.

After surgery, animals received 100% oxygen for 15 min in an intensive care unit (Vetario; Brinsea Products Ltd., North Somerset, UK). As postsurgical analgesia, rats received metamizole (Novaminsulfon-ratiopharm® 1 g/2 mL; 400 mg/kg/day) in sweetened drinking water immediately after surgery for 3 days (POD 1–3). Antibiotic prophylaxis (cefuroxime, 16 mg/kg, s.c.) was administered once daily up to POD 3 [37, 42]. On the day of sacrifice, the abdomen was reopened under deep general anesthesia via midline incision, and final blood samples were collected from the abdominal vena cava. The remaining liver was dissected, weighted, and

fixed in formalin for histology. Animals were then sacrificed under deep isoflurane anesthesia (induction by 5 vol% isoflurane [Abbott GmbH, Wiesbaden, Germany] in 5 L/min O_2 and maintenance by 2 vol% isoflurane in 2 L/min O_2) by exsanguination.

Hematological Parameters

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase, glucose, and urea were measured from blood samples collected during partial hepatectomy and at sacrifice using a VITROS 350® Integrated System Analyzer (Ortho Clinical Diagnostics, NJ, USA). Leukocyte counts were determined in EDTA-treated blood samples using the α MEK-6450 K analyzer (Nihon Kohden Europe GmbH, Rosbach vor der Höhe, Germany).

Liver Weight

Since this experiment is based on the 50% liver resection model, the initial weight of the liver was recalculated based on the resected liver mass and determined approximately by multiplying the factor 2. Afterward, the ratio of liver weight to body weight (gram liver weight per gram body weight) was calculated.

Severity Scoring

The modified severity scoring system included subscores for body weight change, general health status, spontaneous locomotor behavior, readiness to walk, consequences of the surgical procedure, and wound healing. Scores for these individual factors were determined 3 times per day on PODs 1–3 and once daily on PODs 4–7. Animals were classified based on the total score for each measurement session as in "mild distress" (≥5 points), "moderate distress" (≥10), or "severe distress" (≥20 points). The animals were scored at least once every morning, and the body weight was determined before the OF test. Animals scoring ≥15 were exempted from the OF test on that day. A score of 20 was considered the humane end point, necessitating immediate euthanasia [43]. A body weight decrease ≥20% from postsurgical body weight, cramps, paralysis, breathing difficulties, icterus, diarrhea lasting >48 h, loss of thermoregulation, permanent crouching with closed eyes, repetitive suture dehiscence, severe wound infection, hemostasis, and severe tongue swelling with opened mouth were also considered humane end points.

Analyzing FCMs

Fecal samples were collected during each OF test and prior to surgery during anesthesia induction. Samples were stored at −80°C until extraction [44]. Thawed samples were analyzed using the 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay [45], which has been successfully validated for rats. The species-specific time delay between increased blood corticosterone and elevated FCMs (approximately 12 h in rats) means that measured FCM levels represent adrenocortical activity on the preceding day.

OF Test

The OF was a 72 cm × 72 cm box opened at the top to allow filming by a video camera (Media Recorder 4, NOLDUS, Wageningen, The Netherlands; Camera GigE monochrome, 1/1"; lens: Lens Std CS mount, 4.5–12.5 mm 1/2," Basler AG, Ahrensburg, Germany) as described by Zieglowski et al. [34]. The animals were tested in 10-min sessions during the first 2 h of the light phase. Videos were analyzed using Ethovision XT 14, version 15.0.1416 (NOLDUS, Wageningen, The Netherlands) to measure total distance moved, movement velocity, and supported rearing behavior (defined as leaning against the OF wall to raise the head). Between test runs, the surfaces of the OF were cleaned of urine and fecal deposits and disinfected with Antifect (0297; Schülke & Mayr GmbH, Germany).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism for Windows version 7.04 (GraphPad Software, San Diego, CA, USA). Group means were compared by two-way analysis of variance with post hoc Sidak multiple comparisons tests. Liver weights were analyzed by a paired parametric *t* test. A p value < 0.05 was considered significant for all pair-wise comparisons. Estrogen concentrations are expressed in pg/mL and testosterone concentrations in ng/mL. Both are presented as individual values with mean lines. Prior to analysis, OF distance and rearing were normalized to the first presurgical training, FCMs to values from fecal samples collected during the second presurgical OF training, and body weight to immediate postsurgical baseline. FCM concentrations were measured in μg per g feces, per g liver weight as calculated assuming a body weight: liver weight ratio of 2.7% for females and 2.8% and μg/g feces. OF parameters, FCMs, and body weight are presented as line graphs of mean % change from baseline ± standard deviation. AST, leukocytes, FCM (%), severity score, as well as liver weight are presented as boxplots with mean and upper and lower limits.

Results

Hematological Parameters

As shown in Figure 2a, serum AST and ALT (for ALT see Data Availability Statement) were comparable between sexes prior to 50% hepatectomy ($p > 0.05$). On POD 7 (the day of sacrifice), serum AST concentrations were higher in both male and female groups relative to corresponding baselines but still within physiological ranges and did not differ significantly between groups [46]. In contrast, leukocyte counts were significantly different between groups at the time of partial hepatectomy ($p < 0.05$) but were within the physiological range [46] (shown in Fig. 2b). At sacrifice, counts were still elevated without being significant between groups.

Estrogens and Testosterone

Estrogen concentrations on the day of liver resection were not different from those at sacrifice in the female group (shown in Fig. 3a). The males differed significantly between the days of liver resection and sacrifice ($p < 0.001$). There was a significant difference between female and male animals on the day of liver resection ($p < 0.0001$).

Testosterone concentrations (shown in Fig. 3b) in females were below the detection limit of the ELISA Kit

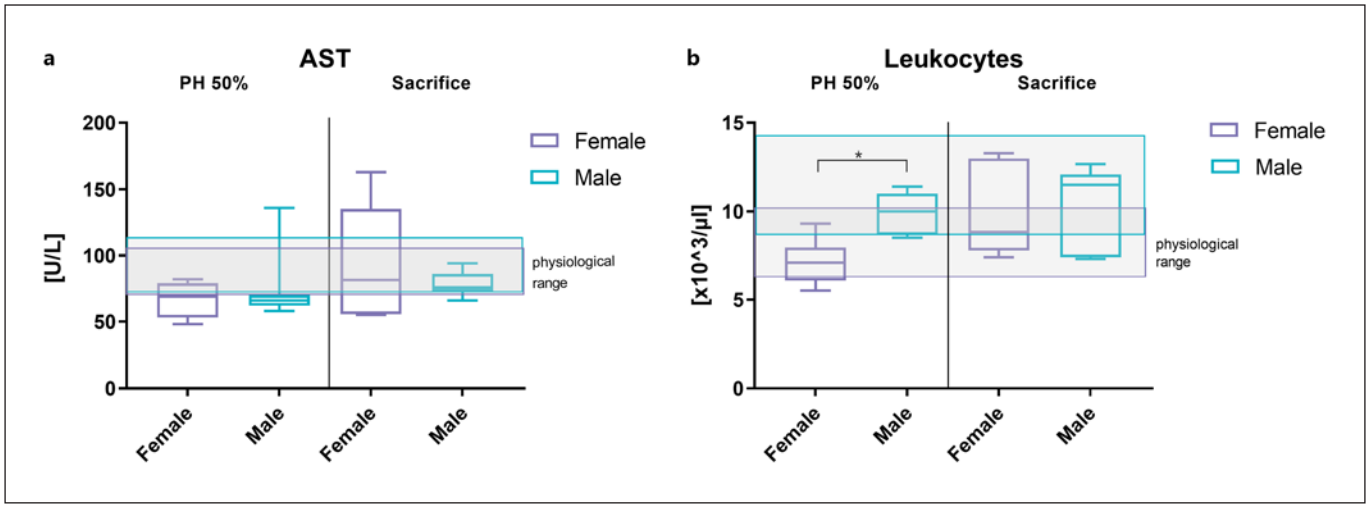

Fig. 2. Changes in hematological parameters following 50% partial hepatectomy and comparison between male and female rat groups. Gray bars represent physiological ranges. **a** Boxplots of AST (in U/L). There were no significant group differences in serum AST. **b** Boxplots of leukocyte count (cells $\times 10^3/\mu L$). Counts differed significantly in blood samples taken during PH 50% ($^*p_{adj} = 0.0120$).

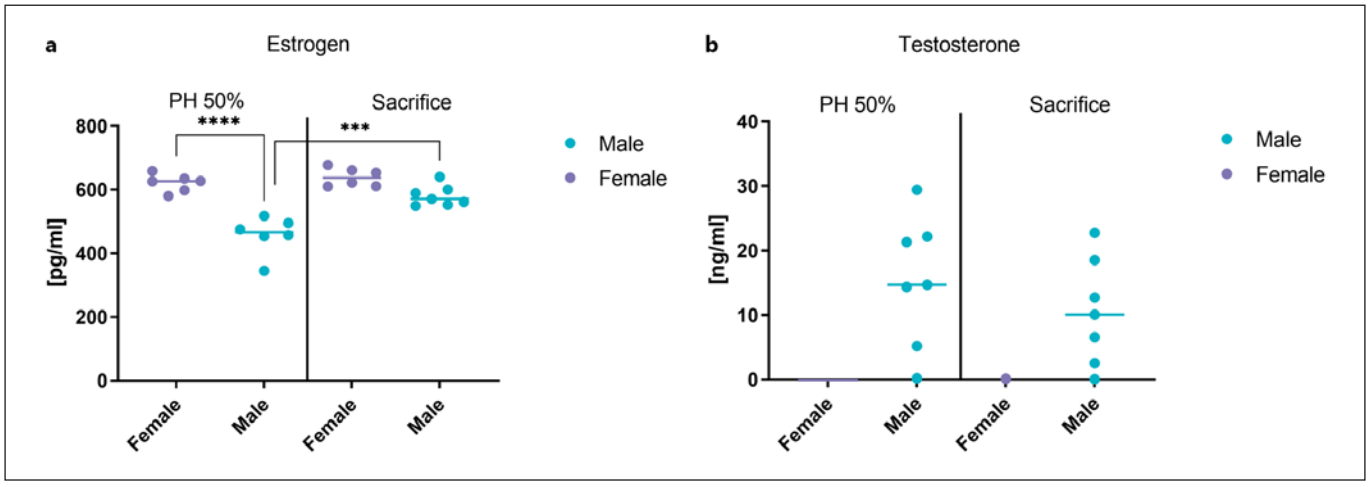

Fig. 3. Estrogen and testosterone concentrations in the serum. Lines represent mean and dots represent individual values. **a** Estrogens are expressed in pg/mL; significant differences in sex on PH 50% ($^{****}p_{adj} = <0.0001$) and on day of liver resection and sacrifice in male animals ($^{***}p_{adj} = 0.0002$). **b** Testosterone is expressed in ng/mL; no significant differences on study days in male animals.

(0.066 ng/mL) on the day of liver resection. On the day of sacrifice, 5 out of 6 values of the samples were below the detection limit. One sample showed a concentration of 0.12 ng/mL. Values of male animals showed no significant difference between both time points.

Liver Weight

As shown in Figure 4, the ratio of liver weight per gram body weight differed significantly between the days of partial hepatectomy and sacrifice in male ($p < 0.01$) rats.

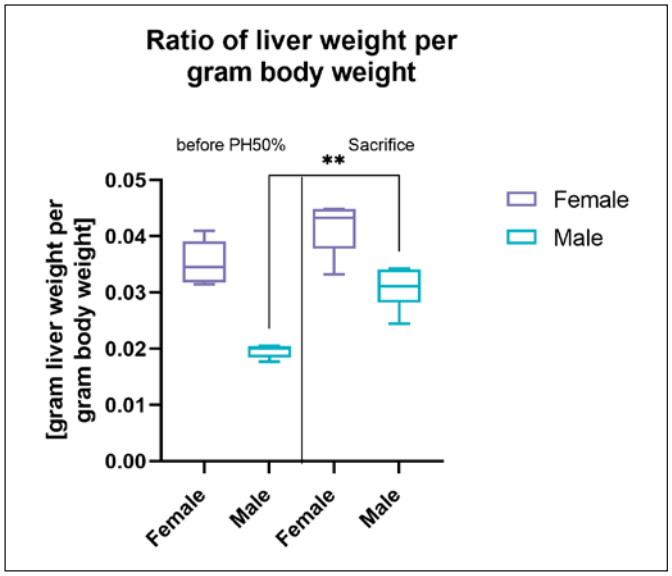

Fig. 4. Liver weight in relation to the body weight. Boxplots of reconstructed ratio of liver weights per gram body weight before partial hepatectomy of 50% and on sacrifice day in grams. There were significant differences in males (**$p < 0.0049$) from the time point before partial hepatectomy and on sacrifice day.

Body Weight and Severity Score

Body weight was lower in both groups on the first day post-surgery relative to baseline (shown in Fig. 5a). On POD 2, female body weight began to increase, whereas male body weight reached its lowest level. Female rats reached and exceeded baseline body weights on POD 3, while male body weights exceeded baseline on POD 4. The body weights of all animals in both groups exceeded individual baselines on POD 5, 6, and 7. However, there were no significant differences in body weight relative to baseline (%) between groups at any postsurgical time point.

Similarly, there were no significant group differences in postsurgical severity scores (shown in Fig. 5b). Mean severity scores were highest on POD 1 in both groups. For all animals, the severity score was highest on POD 1 and decreased progressively until POD 7. One male rat had a severity score of 12 on POD 1 (moderate distress), and one female had a severity score of 11 on POD 7. Necropsy revealed innate bilateral morphological kidney pathology in this female; consequently, results were excluded from analysis. Males showed the same morphological changes at euthanasia (POD 7) as the females. Frequently, adhesions of the liver lobes with the omentum majus and the gastric surface were visible. Otherwise, the mean and individual severity score mean did not exceed ≤5, indicating only mild distress after hepatectomy for both sexes.

Fecal Corticosterone Metabolites

At all time points, FCM concentrations expressed relative to liver weight were higher in males than in females ($p < 0.001$). As shown in Figure 6a, both groups, FCM concentrations peaked on POD 1 and decreased progressively until values were below baseline on POD 7. The FCM per fecal weight was also higher in males than females, differing significantly on the day of surgery ($p < 0.01$). In both groups, FCM per g feces peaked on POD 1. We observed FCM values in females were lower in samples collected from the second OF training than the values in males. As shown in Figure 6b, groups exhibited significant differences on PH 50% ($p < 0.01$), POD 1 ($p < 0.0001$), POD 3 ($p < 0.01$), and POD 4 ($p < 0.05$). In both groups, values were below baseline on POD 7. When expressed as % changes from baseline (shown in Fig. 6c), groups still exhibited significant differences ($p < 0.01$). Postsurgical values fell below baseline on POD 4 in females but not until POD 7 in males.

Open Field Activity

Total distance moved (shown in Fig. 7a), movement velocity [see Data Availability Statement], and supported rearing behavior (shown in Fig. 7b) in the OF were also compared between sexes before and after hepatectomy. Total distance and rearing behavior were normalized to individual baseline values from the first presurgical OFs. In both groups, the distance increased during the second training. In both groups as well, the distance moved was lowest on POD 1 but did not differ from baseline. On POD 3, mean moved distance exceeded mean baseline values in both groups and increased continuously in both groups until POD 7, reaching a similar peak ($p > 0.05$). Changes in movement velocity exhibited a similar pattern in both groups, and there were no significant group differences at any examination time point. The frequency of rearing increased from the first OF training to the second in both groups. Both groups also demonstrated lower rearing frequencies on POD 1. In males, this was the lowest value, while females exhibited the lowest rearing frequencies on POD 1 and POD 3. In both groups, rearing frequency increased progressively thereafter. In females, rearing frequency exceeded baseline on POD 4 while in males, rearing frequency did not exceed baseline until POD 7. There were no within-group or between-group differences, however.

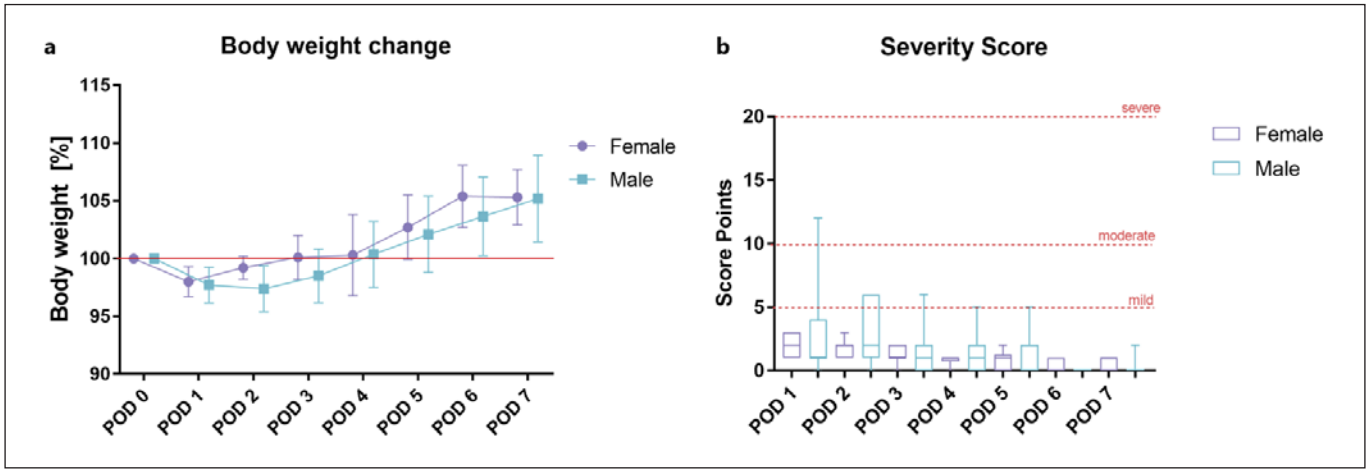

Fig. 5. Changes in body weight and severity scores. **a** Body weight change (% relative to baseline). There were no significant group differences relative to baseline after surgery. **b** Severity scores also did not differ between groups.

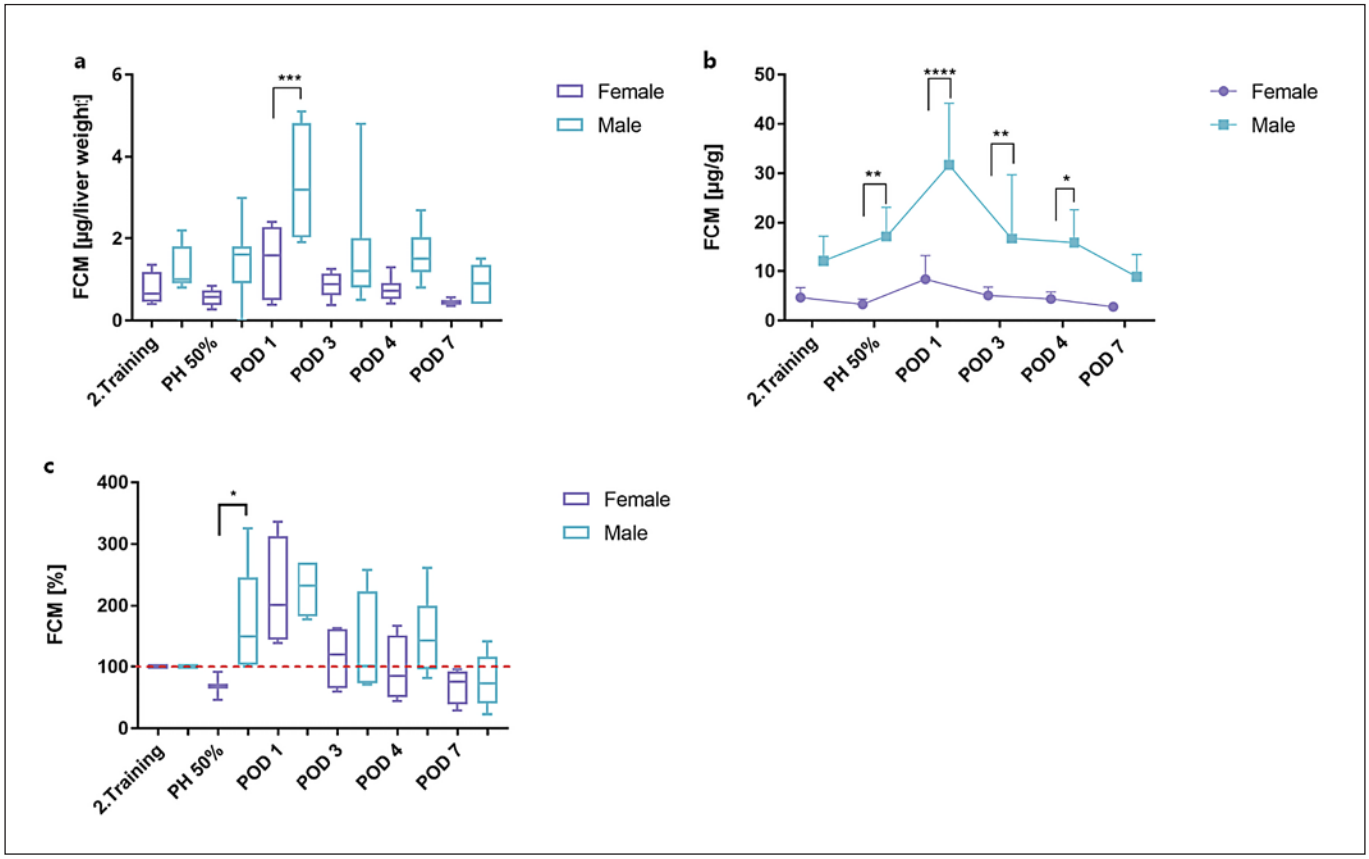

Fig. 6. Comparison of FCM levels. **a** FCMs as expressed in µg/g liver weight. There was a significant group difference on POD 1 (***p_{adj} = 0.0008). **b** FCMs as expressed in µg/g fecal weight. There were significant differences between sexes during PH 50% (**p_{adj} = 0.0047), on POD 1 (****p_{adj} < 0.0001), on POD 3 (*p_{adj} = 0.0117), and POD 4 (*p_{adj} = 0.0192). **c** FCM levels expressed as % change from baseline (red line). There was a significant difference on POD 1 (*p_{adj} = 0.0292).

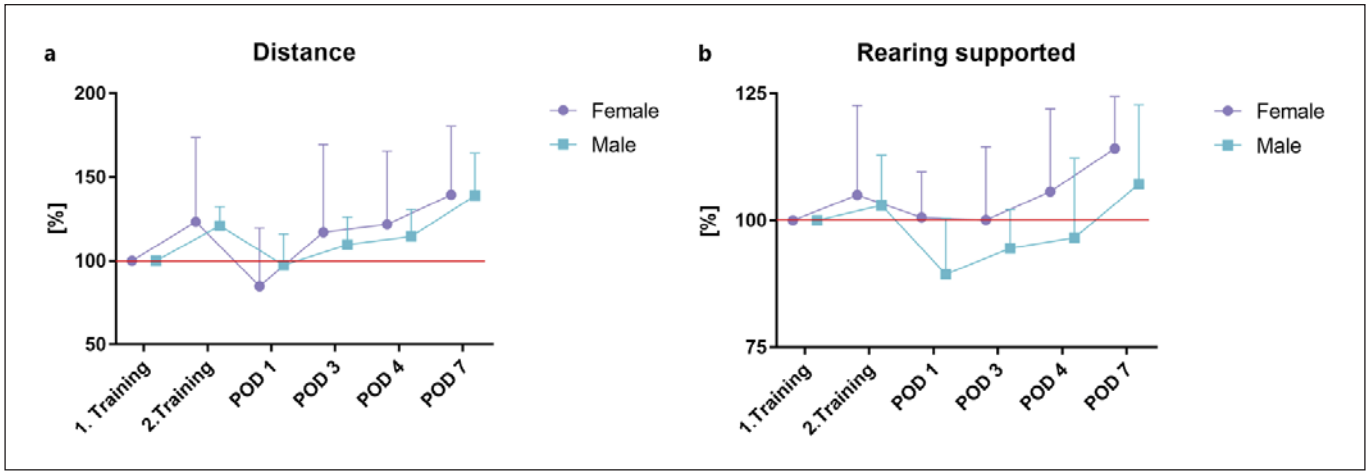

Fig. 7. Comparison of behavior between females and males. **a** Distance traveled (% of first baseline session). There were no significant differences. **b** Rearing (% of baseline). Again, there were no significant differences between sexes.

Discussion

Roughly 30% of liver disease patients are female, so translational research on animal models must be applicable to both sexes. However, only about 5% of prior studies have included female animals, and most of them were published after 2010 [12, 30]. This slow increase may be due to implementation of the ARRIVE guidelines in 2010, suggesting that these guidelines may have fulfilled the stated aim of improving the quality of research reports involving animals [47]. Furthermore, increased public pressure is being exerted to implement the 3 Rs in research as well as in animal husbandry. In addition, the sensitization of politicians by animal rights activists is attracting attention and demanding changes. A systematic review by Nakatake et al. [12] (unpublished data) also indicated that the vast majority of rodent studies on liver IR injury (97.4%) were conducted on males only, despite recommendations for the use of both sexes in animal-based liver disease research.

Therefore, the purpose of this pilot study was to investigate if there are sex-specific differences after performing a 50% liver resection by using postoperative severity assessment methods in rats. In this context, it is not expected that the selected age of the animals has an influence on the expression and thus the presented results of severity assessment, which is the main focus of the study. A severity assessment system takes numerous parameters into account. Among these, body weight change is considered critical and so is routinely included [37, 43, 48]. However, the experimental procedure alone may alter body weight, at least temporarily, independently of effects on health status. To avoid possible surgery-related body weight changes in the present study, weights were normalized to that measured immediately after surgery. Both groups showed a similar trend in postsurgical body weight change, but females recovered lost weight faster than males. However, there were no significant sex differences in normalized body weight at any measurement point. Flecknell et al. [49] reported that the reduced body weight after laparotomy was due to lower water and food consumption, suggesting that delayed food intake may have contributed to the reductions in body weight following hepatectomy. Stress, per se, may also contribute; as in accord with our findings, Faraday et al. [48] reported greater body weight changes from stressful interventions in male than female rats.

In contrast to body weight and other easily measurable parameters, general health status and behavioral changes are generally scored subjectively [50, 51] and so are heavily influenced by rater experience and a variety of more transient physiological and emotional factors [43, 52]. To reduce rater-specific variation of score point, only two raters (experience <5 years) scored the severity score. In the current study, severity scores were generally higher in males than in females. In the majority of animals (12 of 14), postoperative distress was rated as "mild" according to existing recommendations for evaluating the outcomes of abdominal surgery [35]. None of the female rats included in the statistical analysis exhibited moderate or

severe distress, while two males were deemed in moderate distress, mainly due to self-manipulation of sutures. In fact, most of the males (5 of 7) disturbed their surgical wounds, while none of the females did so. Thus, "wound healing" subscores were higher in males than females (see Data Availability Statement). In this study, no animal reached a humane end point. Only one female had to be subsequently excluded from the analysis due to morphological kidney abnormalities in the dissection, after reaching the end of the study. However, no cases of moderate/severe distress were observed at POD 6 and POD 7, and there were no significant differences in total scores between sexes at any time.

An alternative to gauge physiological stress more objectively is measurement of serum corticosterone. However, blood sampling itself may be stressful and physiologically dangerous following invasive treatment involving blood loss. In addition, the half-life of corticosterone in the blood is very short, so stress responses may be missed. Therefore, we measured corticosterone metabolites in feces as a convenient, noninvasive alternative [44, 53]. Concentrations of FCMs were several-fold higher in males than females, in accord with the findings of Lepschy and colleagues [54, 55]. However, this method is indirect, and it is critical to distinguish between sex differences in stress hormone production and metabolism per se. Sex differences in FCM formation rather than differences in adrenocortical activity are thought to underlie differences in measured FCMs [55]. According to Cavigello et al. [56], sex differences in FCMs values may be due to different plasma corticosterone binding capacities and a slower rate of fractional clearance. Our results suggest that relative changes (%) in FCM are suitable to assess postoperative condition severity independent of differences in corticosterone metabolism and plasma binding capacity. Percentage changes in FCMs after surgery were similar in males and females, consistent with severity score results. Nevertheless, it is still unclear if liver resection has a differential effect on the rate of corticosterone metabolism, an issue that must be addressed in future studies.

Up to now, it is neither clarified in which way liver resection effects the metabolization of corticosterone nor sexual hormones, and therefore, further investigations are needed. Sex hormones from the ovaries and testes are transported via the bloodstream to receptors on their respective target cells. There, they trigger signal transduction pathways. Binding to liver cells, in particular, sex hormones are metabolized for possible regenerating processes [57]. To what extent, a 50% reduction of functional liver tissue affects estrogen and testosterone concentrations were analyzed in serum samples. When comparing estrogen concentrations on the day of liver resection with those of sacrifice, no significant change in estrogen could be seen in female rats. The remaining liver seems to be able to metabolize steroids to the same extent. However, there was a significant increase from liver resection to sacrifice in estrogen levels in the male animals, which may be related to the protective effect of estrogens [22–24]. These findings are in line with Francavilla et al. [58] whose studies investigated regenerating functions of sex hormones in male rat livers. Here, higher estrogen levels in the blood after 70% partial hepatectomy were detected. Besides estrogens, testosterone was analyzed.

Male testosterone values ranged between 0.19 ng/mL and 29.4 ng/mL. In cages with male animals, dominant males produce more testosterone than their social lower-ranking mates [16]. This could be one possible explanation of the larger standard deviation of the values within this group. However, there was no significant statistical difference between testosterone concentrations on the day of liver resection and the sacrifice in male animals. Our results are in line with the study of Francavilla et al. [58] who reported no significantly different testosterone concentrations in male rats 6 days after 70% liver resection. We could not measure testosterone in female serum samples (values below detection level except in one animal). Therefore, the role of testosterone in female rats within this model needs to be elucidated in further studies.

A possible minor hepatoprotective effect of estrogens can be noticed when considering liver weight [20]. Males and females showed a different ratio of liver weights per gram body weight before liver resection due to the different body weights. Males showed significantly different values on the day of sacrifice to the initial time point. Nevertheless, female rats were closer to their initial values than males. Concerning the estrogen concentrations, we found that on the day of sacrifice, the males had a significantly increased estrogen level compared to the liver resection, but they did not exceed the concentration of the females. The increase in male estrogen values might be due to the tissue proliferating and hepatoprotective effect of estrogen after liver resection. The females showed higher estrogen levels at each time point. This may be due to the hepatoprotective effect of estrogens, which allowed females to proliferate more liver tissue than males in our experiments [57, 59, 60]. Due to outstanding physiological control data, this important point should be investigated in further studies to confirm our findings. Further approaches for severity assessment independent of liver

function changes or other experimental factors include behavioral methods like the rat grimace scale and OF activity monitoring [34, 61, 62]. For example, assessment of changes in orbital tightening, nose and cheek bulge, ear position, and whisker movement (grimacing) can be performed in the rat grimace scale using automated video analysis software without experimenter interference or interaction [63–65]. Similarly, OF monitoring can reveal voluntary willingness to move (an indirect measure of pain) and anxiety-like behavior (as indicated by less frequent rearing and excessive grooming) [66–68].

Indeed, to augment the semi-objective scoring system, we monitored OF activity. According to Zieglowski et al. [34], the total distance moved, movement velocity, and rearing behavior are suitable metrics for evaluating general condition and best represent the voluntary willingness to move and physical condition after laparotomy. In the present pilot study, both sexes demonstrated reductions in OF activity after liver resection, consistent with severity scoring [34, 37]. Again, however, there were no significant differences between groups. These findings are in line with Scholl et al. [69], who found no difference in the distance moved between males and females in the OF, indicating similar levels of state anxiety. In contrast, however, Sturmann et al. [66] reported sex-specific differences in OF behavior after stressful episodes such as restraint and forced swim test. These differences may reflect the unique effects of behavioral/emotional stress (e.g., restraint) versus the physiological stress of surgery.

Conclusions

In summary, these findings revealed no major differences in the early response to liver resection between male and female rats. Thus, there is no justification for using male-only or male-predominant cohorts in studies on liver function and disease. Accordingly, for improved utilization of bred animals, males should be used as well as females in the sense of the 3 Rs principle.

Limitations

Limitations of the presented pilot study include the small sample size and the age of the rats. Furthermore, the relationship between FCM, estrogen and testosterone concentrations, and liver function is not completely clear. Larger scale studies with a systematic approach are thus required for confirmation.

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Statement of Ethics

The study was performed in accordance with the German animal welfare law (Tierschutzgesetz (TSchG)) and EU-Directive 2010/63/EU [35], and the protocol was approved by the governmental Animal Care and Use Committee (Reference No.: 84-02.04.2017.A304; Landesamt für Natur, Umwelt und Verbraucherschutz Recklinghausen, Northrhine Westphalia, Germany).

Conflict of Interest Statement

The authors declare no conflict of interest.

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Author Contributions

Leonie Zieglowski, Lisa Ernst, and René H Tolba designed the study. Anna Maria Kümmecke and Leonie Zieglowski performed the experiments and collected data. Rupert Palme analyzed FCMs. Anna Kümmecke wrote the manuscript, designed the graphics, and calculated the statistics. All authors reviewed and revised the paper.

Data Availability Statement

The data of this study are openly assessable in "Zendo" at https://doi.org/10.5281/zenodo.5118654.

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