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Sex is associated with differences in oxidative stress and susceptibility to severe hepatic encephalopathy in bile-duct ligated rats

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List of abbreviation

BDL: bile-duct ligation

CHE: covert hepatic encephalopathy

CLD: chronic liver disease

GS: glutamine synthetase

HE: hepatic encephalopathy

MRI: magnetic resonance imaging

OHE: overt hepatic encephalopathy

ROS: reactive oxygen species

Abstract

Hepatic encephalopathy (HE) is a debilitating neurological complication of chronic liver disease (CLD). Hyperammonemia plays an important role in HE's pathogenesis, acting synergistically with systemic oxidative stress. During CLD, muscle plays a compensatory role in detoxifying ammonia, and therefore muscle loss leads to an increase in the risk of developing HE. With most animal studies involving males, sex's impact on the development of CLD and associated complications such as HE and muscle loss remains unknown. Therefore, we aimed to identify the impact of sex on CLD, HE, and muscle mass loss in a rodent model of CLD.

Liver injury markers, hyperammonemia, oxidative stress, muscle mass and ammonia clearance were measured in female and male bile-duct ligated (BDL) rats. In addition, covert HE was assessed in females while ammonia-precipitated severe HE was assessed in female and male BDL rats, and male BDL rats treated with allopurinol (100mg/kg), an antioxidant (xanthine oxidase inhibitor).

Female BDL developed CLD and HE (impaired motor-coordination and night activity) compared to respective SHAM. Hyperammonemia and muscle ammonia clearance were similar between female and male BDL. However, only female BDL rats did not develop muscle loss, brain edema, and short-term memory impairment (vs. female SHAM) and systemic oxidative stress and decreased albumin levels (vs. male BDL). Furthermore, both female BDL and allopurinol-treated male BDL rats were protected against ammonia-induced overt HE. In conclusion, female and male BDL rats develop distinct features of CLD and HE, with systemic oxidative stress playing a pivotal role in the susceptibility to ammonia precipitated overt HE.

Introduction

Hepatic encephalopathy (HE) is a frequent neurological complication that develops during chronic liver disease (CLD). This neuropsychiatric syndrome manifests with a wide range of symptoms: from subclinical (covert HE (CHE)) such as impaired memory, decreased reaction time and motor incoordination to clinically detectable (overt HE (OHE)) such as lethargy, ataxia, gross disorientation and coma. As much as 80% of patients with CLD suffer from CHE, and more importantly, this underestimated phenomenon leads to a 4-fold increased risk of developing OHE with a 30% risk within the first year (Montgomery and Bajaj 2011; Patidar *et al.* 2014). HE remains the primary cause of hospital readmissions which in addition to accounting for a substantial amount of costs, is also associated with poorer prognosis and higher mortality compared to other complications of cirrhosis (Jepsen *et al.* 2010). The burden of HE is multidimensional imposing a significant economic charge to the patient, patients' caregivers, healthcare systems, and society.

The pathogenesis of HE is complex and multifactorial. Amongst the various pathogenic factors involved in the development of HE, ammonia toxicity is central. Since the ailing liver during liver disease is no longer capable of detoxifying ammonia via the urea cycle, hyperammonemia arises (Vierling *et al.* 2016). Ammonia freely crosses and diffuses through biological membranes and rapidly enters the brain causing deleterious effects. Elevated ammonia is shown to cause changes in pH, membrane potential and cell metabolism and is associated with the presence and the worsening of HE (Bosoi and Rose 2009; Vierling *et al.* 2016; Ong *et al.* 2003). Ammonia neurotoxicity has been demonstrated to be associated with an increase in brain water in patients with HE

(Cudalbu and Taylor-Robinson 2019; Shah *et al.* 2008; Winterdahl *et al.* 2019). Similarly, in a rat model of CLD, our group has shown an increase in brain water in 6-week bile-duct ligated (BDL) male rats which is prevented following attenuation of elevated blood ammonia (Bosoi *et al.* 2011). Subsequently, ammonia lowering therapies are the mainstay strategy for the treatment of HE (Rose 2012).

In addition to ammonia, other factors such as oxidative stress (ROS) which arises from the imbalance of pro-oxidant and antioxidant capacity, play an essential role in the onset of HE (Giménez-Garzó *et al.* 2018; Görg *et al.* 2010). Systemic oxidative stress has been synergistically implicated with hyperammonemia in the presence of HE in patients as well as the development of brain edema in male animal models of CLD and HE (Montoliu *et al.* 2011; Bosoi *et al.* 2012).

The muscle plays an important compensatory role for ammonia detoxification during CLD since it houses glutamine synthetase (GS), an ammonia removing enzyme which amidates glutamate to glutamine. However, sarcopenia, defined by loss of muscle mass and function, is another common complication occurring during CLD (Nardelli *et al.* 2019). Subsequently, sarcopenia further decreases the capacity to clear ammonia in CLD and is therefore associated with a higher risk of HE, a worse prognosis and a higher mortality (Bhanji *et al.* 2018; Nardelli *et al.* 2019).

Although our understanding on the underlying pathophysiological mechanisms of HE has increased considerably over the last decades, the impact of sex on the natural course of CLD and HE remains undefined. This is partially due to the fact that the number of females inflicted with CLD (and HE) is lower compared to males and therefore the impact of sex becomes statistically difficult to evaluate in clinical studies

(Dickinson *et al.* 2012). As a result, clinical studies investigating CLD and HE involve primarily male patients (Xie *et al.* 2018; Ong *et al.* 2003; Poveda *et al.* 2010), and therefore the impact of sex is difficult to assess. Similarly, in pre-clinical studies in CLD and HE, mostly include male animals (Clément *et al.* 2021; Ochoa-Sanchez *et al.* 2021).

Therefore, we aimed to use the well characterized BDL rat (male) model of CLD and HE in female rats and to evaluate the impact of sex on the pathogenesis of CLD, HE, muscle mass loss as well as the susceptibility of severe HE.

Material and methods

The experimental design is summarized in Fig. 1.

Bile duct ligation (BDL)

Female and male Sprague-Dawley rats (200-225g) were purchased from Charles River and kept two per cage in a 12h light/dark cycle, with free access to water and rodent chow (TD.2819, 18% protein – Envigo, USA). Bile duct ligation or SHAM surgery was performed as described by Bosoi et. al (Bosoi *et al.* 2011). After 48 hours of acclimation, rats were anesthetized with isoflurane (4% induction and 2.5% maintenance, oxygen at 0.9L/min). The rats were shaved, the incision site was sterilized and infiltrated with the local anesthetic bupivacaine 2mg/kg. Then, a midline incision was done to open the skin and muscle, and the common bile duct was identified, isolated, and a ligature was placed distally. After the first ligature, 50µl of formalin was injected inside the bile duct to prevent the duct inflation, and a proximal ligature was rapidly done. A resection of the bile duct was made between the ligatures, and the rat abdominal wall was closed in layers with 4.0 (muscle) and 6.0 silk (skin). SHAM rats

underwent the same procedure, except for the formalin injection, placement of ligatures, and resection of the bile duct. After surgery, rats were allowed to recover in an incubator at 27 °C for at least 1 hour. Rats received analgesic treatment before and up to 72 hours after the surgery with buprenorphine (sc. 0.05 mg/kg, every 12 hours), carprofen (sc. 5mg/kg, every 24 hours), and gabapentin (orally, 30mg/kg every 12 hours). A total of 50 BDL (16 females and 34 males) and 16 SHAM surgeries (8 females and 8 males) were performed. No randomization was performed for the experiments, there was no pre-determined exclusion criteria and blinding was performed only for the novel-object recognition test. Number of animals per group was decided based on previous publications (Bosoi *et al.* 2011; Ochoa-Sanchez *et al.* 2021; Clément *et al.* 2021). The analgesia and anesthesia were decided according to the Institutional Animal Care and Use Committee at the CRCHUM. All studies were approved by the Institutional Animal Care and Use Committee at the CRCHUM (ethics approval no. 4l015049CR).

Chronic liver disease assessment

Plasmatic liver markers

Euthanasia was performed in the afternoon at week six after surgery. Blood from the left heart ventricle from male and female BDL and SHAM rats anesthetized with isoflurane (4% induction and 3% maintenance, oxygen at 0.9L/min) was collected with a heparinized syringe. Blood samples were centrifuged at 4800 rpm for 5 minutes, and plasma was snap-frozen and kept at -80°C until analysis. Alanine aminotransferase (ALT); RRID (ref 04718569-190), aspartate aminotransferase (AST); RRID (ref 04657543-190), alkaline phosphatase (ALKP); RRID (ref 04657373-190), bilirubin;

RRID (ref 05795648-190), and albumin; RRID (ref 04657357-190) were measured using the COBAS system (c111, Roche, USA). Plasma was snap frozen and ammonia was measured with a kit (Randox Laboratories, USA); RRID (ref AM-1015) within one month.

Liver histology

During euthanasia, the liver was dissected and weighed. The right lobe was dissected and a small piece was immersed in 10% formalin for 24h. Fixed liver tissue was blocked in paraffin, sliced (4µM) and stained with hematoxylin-eosin (H&E) and evaluated for bile duct proliferation, hepatocytes coverage, and activated Kupffer cells.

CHE assessment

Behavioral tests

All behavioral tests were conducted during the light phase (with exception of the night activity test), at 5 weeks following either BDL or SHAM surgery in female rats. First, rats were submitted to Novel Object recognition test (morning), followed by elevated plus maze and open field tests (afternoon). On the next two days, rats were submitted to the rota-rod test (afternoon). On the next day, rats were submitted to the night activity test.

Anxiety

The anxiety tests are based on the rat's expected conflict between exploring a new environment and fear of an unprotected open space. Thus, an anxious animal will avoid the open areas and stay close to the walls or in hidden areas. The elevated plus

maze (EPM) and the open-field (OF) test were used to assess anxiety. Both anxiety tests were conducted with female BDL and SHAM rats, with dim light conditions, and rats were allowed to acclimate for 1 hour before the tests.

The elevated plus maze (EPM) test

The elevated plus maze arena consists of a cross-shaped maze with four 45cm^2 arms, two open and two closed. The maze was divided into three areas: open arms, closed arms, and center. For the test, rats were placed in the center, and their ambulatory movements were recorded for 5 minutes and analyzed with the SMART video tracking system (Panlab). Total time (seconds) and %distance in the open arms were used to assess anxiety.

The open-field (OF) test

One hour after the EPM test, rats were placed in the OF arena, consisting of a black square box (90 cm^2). The box was divided into three areas: walls, a center, and an intermediate area. For the test, the rats were placed in the corner, facing the wall, and their ambulatory movements were recorded for 5 minutes and analyzed with the SMART video tracking system (Panlab). Total time (seconds) and % distance in the center of the OF were used to assess anxiety.

Novel object recognition (NOR) test

The novel object recognition test was used to evaluate short term memory (STM). After 1 hour of acclimation in a dim-lit room, rats were placed in an empty open field arena (60x45x33cm) for 5 minutes for habituation. One hour later rats were placed in the same arena containing two identical objects (A + A) for 5 minutes for familiarization. One hour later, one of the objects was changed (A + B) and the rats

were returned to the arena for STM assessment. The STM assessment was recorded by a video camera is expressed as % time exploring (sniffing, touching) the novel object (B) divided by the total exploration time (A + B). The analysis of the NOR test was blinded since the experimenter was unaware of the rats groups during the analysis.

Rota-rod test

Motor coordination was measured by the rota-rod test. The rota-rod device consists of a cylinder (7 cm of diameter) that turns with increasing speed and has a sensor that records rats' falls (ROTOR-ROD™ System, San Diego Instruments). On the first day of rota-rod, rats were first habituated and trained to the equipment. Rats were placed on the cylinder without rotation for 5 minutes, and then the rota-rod was started, with a linear increase of speed reaching 40 rpm over 5 minutes. The trial was finished when the rat fell, and each rat was allowed four trials with 3 minutes of rest between trials. On the following day (test day), the rats were submitted to the same protocol, and the latency to fall (the highest from the four trials) was recorded.

Activity test

Rats were placed in plexiglass boxes (42x42x22 cm) surrounded by an infrared bean system to record ambulatory activity (Omnitech-electronic Inc). Rats were provided with a thin layer of bedding material and free access to food and water and were allowed to acclimate for 4 hours before the start of the measurements. The activity was recorded for 12 hours (from 18h30 to 6h30) during the dark (active) phase, and total activity was measured as total distance (centimeters) over 12 hours.

Brain edema

Brain water content from the frontal cortex of female BDL and SHAM rats was measured 6 weeks after surgery by densitometry as described by Marmarou et al. (Marmarou *et al.* 1978). Briefly, kerosene and bromobenzene gradient density columns were prepared and calibrated with different concentrations of potassium sulfate (known densities). After blood collection, isoflurane anesthetized rats (4% induction and 3% maintenance, oxygen at 0.9L/min) were decapitated and the frontal cortex from fresh brains was dissected on ice, and 2mm^3 pieces (4 pieces for each rat) were placed and allowed to stabilize for 1 minute in the columns. Water content was measured as the tissue density, using the average of the four pieces.

Body parameters

Bodyweight and food intake

Bodyweight and food consumption of female BDL and SHAM rats were measured weekly until the 6th week after surgery. Since rats were housed two per cage, food intake was given as the mean of the cage per week for each rat.

Body composition

Fat and lean mass of 6 weeks-female BDL and SHAM rats were measured before euthanasia in non-sedated rats by EchoMRi 700® Body Composition Analyzer (R & D EchoMRi LLC).

Muscle strength

Muscle strength was measured in the afternoon, using a digital force gauge (Chatillon® DFE-010; AMETEK TCI Division). Rats were supported at the thorax and the base of the tail, placed with the limbs gripping the mesh pull bars, and slowly pulled

backward until releasing the bar. The mesh pull bars for forelimb measurements were placed horizontally, while for hindlimb strength, the mesh pull bars were placed at a 45-degree angle. Muscle strength was recorded as the maximum strength after three trials.

Muscle circumference and weight

During euthanasia, the right gastrocnemius muscle from isoflurane anesthetized (4% induction and 5% maintenance, oxygen at 0.9L/min) male and female rats was localized the circumference was measured using a measuring tread. After dissecting the gastrocnemius, wet weight was measured using a precision scale. Gastrocnemius muscle was then collected, snap-frozen and kept at -80°C. When comparing muscle weight from male and female rats, BDL muscle weight was normalized with respective SHAMs to account for normal body size differences present in male vs. female rats.

GS activity

The glutamine synthetase (GS) activity assay is based on the detection of inorganic phosphate from adenosine 5-triphosphate (ATP) by GS according to Gawronski et. al. (Gawronski and Benson 2004). First, gastrocnemius muscle tissue was lysed with zirconia beads in RIPA buffer (trisaminomethane (Tris) 50mM, sodium chloride 150 mM, ethylenediaminetetraacetic acid (EDTA) 1 mM, sodium dodecyl sulfate (SDS) 0.1% and proteinase inhibitor cocktail 1/500), at 3000 RPM during 6 minutes at 4°C and protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, 500-0116). Following protein dosage, 40 µg of muscular proteins or potassium phosphate dibasic were incubated with Tris 10 mM pH 7.5, magnesium chloride 5 mM, monosodium glutamate 25 mM and ammonium chloride

5 mM. The reaction was started by adding ATP 10 mM, and after 5 minutes, 12 % ascorbic acid and 2 % ammonium molybdate tetrahydrate were added. After 5 minutes, the reaction was stopped with 2 % sodium citrate tribasic dihydrate and 2% acetic acid, and absorbance was read at 655 nm on a microplate reader (Bio-Tek Synergy HT). Since GS activity is measured via the production of inorganic phosphate from the utilisation of ATP, GS activity results are expressed by nmol of phosphate.

Muscle ammonia clearance and glutamine production

Two days after recovery from the ammonia challenge (afternoon), female and male BDL rats were anesthetized with isoflurane (4 % induction and 2.5 % maintenance) with an oxygen flow rate of 0.9L/min, and the left femoral vein and iliac artery were catheterized for blood collection. Blood was taken in heparinized tubes, centrifuged at 4800 rpm for 5 minutes, and plasma was snap-frozen and kept at -80°C until analysis.

Plasma ammonia was measured by a kit (Randox Laboratories), and glutamine was measured by the EnzyChrom Glutamine Assay kit (BioAssay Systems, EGLN-100).

Plasmatic oxidative stress

Reactive oxygen species in plasma were measured in female and male rats using the 2',7'-dichlorofluorescein diacetate (DCFDA) test (Invitrogen, D399). Briefly, DCFDA (10 µM) was incubated with hydroxylamine (1 M, pH 8.5) for 30 minutes and then added to 10 µl of plasma in triplicates in a 96 well plate. Fluorescence (λ_{exc}485 ηm and λ_{emi} 520 ηm) was measured every 2 minutes, up to 10 minutes, and the slope of

time/readings was calculated. Results are expressed as relative fluorescent units (RFU).

Brain oxidative stress – total antioxidant capacity

During euthanasia, brains were carefully dissected and kept at -80 °C until experiments. Total antioxidant capacity was measured by the total antioxidant capacity kit (Sigma-Aldrich, CS0790) according to manufacture's instructions. Briefly, brains' cortex and hippocampus were lysed with assay buffer and, after protein dosage using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories), 150 µg of protein was incubated with ABTS solution and hydrogen peroxide at the advised concentrations for 3 minutes. Stop solution was added and absorbance was read at 405 ηm. Results are given as total antioxidant capacity in mM.

Ammonia-induced OHE

An ammonia challenge was performed to induce OHE in female and male BDL rats (N=6) as well as male BDL rats treated for 10 days (from day 25 to day 35 after BDL surgery) with either vehicle (saline) (N=9) or allopurinol (100mg/kg intraperitoneally) (N=8) (Bosoi *et al.* 2012). In the afternoon, after baseline blood sampling, rats were injected with a sublethal dose of 6 mmoles/kg of ammonium acetate subcutaneously. Mental status was evaluated every 5 minutes after the injection for mild lethargy (lack of spontaneous ambulatory movement, but capable of moving when manipulated), severe lethargy (inability to perform ambulatory movement even if manipulated, righting reflex delayed but present), and loss of righting reflex. Severe

lethargy and loss of righting reflex were defined as an episode of OHE. Since multiple blood draws were required, alternate locations were used and blood was taken from both the saphenous vein and tail vein in heparinized tubes at baseline and during episode or at the peak of mental status impairment for the rats that did not had an episode. For female rats, blood was drawn time-matched with male rats' episodes. All samples were collected up to 60 minutes after injection and average time of blood sampling was not different between the groups. Blood samples were centrifuged at 4800 rpm for 5 minutes, and plasma was snap-frozen and kept at -80°C until analysis.

Statistic

Data are expressed as mean ± standard deviation (SD) or percentages, and *p*-values < 0.05 were considered statistically significant. Normality was assessed graphically. Statistical significance was tested using parametric t-test for all variables except body weight, food intake and incidence of OHE episode. The chi-square test was used to compare the incidence of OHE episodes. Repeated measures two-way ANOVA was used to compare body weight and food intake. If the interaction between time and group was found significant, groups (BDL vs. SHAM) were compared at each time point using Sidak's multiple comparisons test to control for multiplicity. No tests for outliers were conducted. All statistical tests were two-tailed. Sample size was determined using an online sample size calculator based on published studies ((Bosoi et al. 2011), with alpha = 0.05, power of 95% and enrollment rate of 1. NS= non-significant and df= degrees of freedom. Statistical analysis was done using GraphPad Prism 8 (La Jolla, CA, USA).

Results

BDL-induced CLD

Six-weeks following surgery, female BDL rats developed elevated plasma AST ($p=0.0001$; $t=5.2$; $df=14$), ALKP ($p=0.001$; $t=4.03$; $df=14$), bilirubin ($p<0.0001$; $t=17.76$; $df=14$) and lower albumin ($p<0.0001$; $t=5.4$; $df=14$) vs. female SHAM controls. ALT was also elevated but was not significant different ($p=0.009$; $t=1.8$; $df=14$). Blood ammonia ($p=0.005$; $t=3.3$; $df=14$) and ROS ($p=0.002$; $t=1.8$; $df=14$) levels were also significantly higher in female BDL vs SHAM controls (Table 1). Accordingly, liver histology revealed tissue disorganization, with hepatocytes necrosis and reduced number of hepatocytes, proliferation of bile ducts and inflammation (activated Kupfer cells) in female BDL vs. female SHAMs (Fig. 2A-B and Table 2).

CHE assessment

Female BDL rats showed impaired motor coordination evaluated using the rota-rod test, with lower average latency to fall compared to female SHAM controls (135 ± 25 seconds vs 200 ± 68 seconds; $p=0.02$; $t=2.6$; $df=14$) (Fig. 3A). Night activity, total distance travelled, was also lower in female BDL rats compared to female SHAMs (17033 ± 6454 cm vs 31180 ± 8310 cm; $p=0.003$; $t=3.6$; $df=13$) (Fig. 3B). Level of anxiety (OF test: $p=1$; $t=1.8$; $df=14$, EPM test: $p=0.07$; $t=2$; $df=14$) as well as short-term memory ($p=0.6$; $t=0.6$; $df=14$) were similar in female BDL rats vs female SHAMs (Fig. 3C-3E). Additionally, female BDL rats did not acquire brain edema with similar degrees of brain water found in both female BDL and SHAMs (77.93 ± 0.30 % and 77.95 ± 0.30 % respectively; $p=0.8$; $t=0.2$; $df=14$) (Fig. 3F).

Food consumption and muscle mass

There was a significant interaction between time and surgery (BDL and SHAM) for body weight (p=0.0005; F=4.5; df=6) and food intake (p=0.04; F=2.4; df=5), so we compared the groups at each time point. When we fixed the time, there was no difference observed between BDL and SHAM for either body weight (p=0.3; F=1.2; df=1) or food intake (p=1; F=0.0003; df=1) (Fig. 4A). Female BDL rats had higher lean mass compared to SHAM (241.7 ± 16.2 g vs 221.7 ± 16.5 g respectively; p=0.03; t=2.5; df=14), while fat mass was lower in BDL vs SHAMs (33.3 ± 7.4 g vs 56.4 ± 13.6 g respectively; p=0.0008; t=4.2; df=14) (Fig. 4B). Female BDL and SHAM rats showed no difference in gastrocnemius muscle circumference (BDL 4.3 ± 0.2 cm vs. SHAM 4.7 ± 0.5 cm; p=0.09; t=1.8; df=14) and weight (BDL 1.8 ± 0.2 g vs. SHAM 1.9 ± 0.2 g; p=0.2; t=1.4; df=14) (Fig. 4C), as well as similar muscle strength in forelimbs (BDL 1680 ± 295 g vs. SHAM 1758 ± 167 g; p=0.5; t=0.7; df=14) and hindlimbs (BDL 1289 ± 337 g vs. SHAM 1400 ± 214 g; p=0.4; t=0.8; df=14) (Fig. 4D).

Ammonia metabolism and ROS in female vs. male BDL rats

Compared to female BDL rats, male BDL rats presented lower gastrocnemius muscle mass (92.7 ± 12.2 % vs 69.8 ± 10.7 % respectively; p=0.001; t=4; df=14) (Fig. 5A). Nevertheless, levels of hyperammonemia were similar between male and female BDL (127.6 ± 81.7 µmol/L vs. 104.7 ± 37.9 µmol/L respectively, p=0.5; t=0.6; df=10) (Fig. 5B). Interestingly, muscle GS activity was upregulated in male BDL rats vs. BDL females (6.62 ± 1.33 nmol of phosphate vs 4.78 ± 0.79 nmol of phosphate; p=0.01; t=2.9; df=14) (Fig. 5C). Ammonia clearance (male BDL; 21.40 ± 10.80 µmol/L vs. female BDL; 10.07 ± 17.18 µmol/L; p=0.2; t=1.3; df=9) (Fig. 5D) and glutamine production across the muscle was not significantly different between male and female BDL rats (0.124 ± 0.306

mM for male BDL vs. -0.118 ± 0.231 mM female BDL; p=0.2; t=1.4; df=7) (Fig. 5E). Significantly lower levels of systemic ROS were found in female vs. male BDL rats (7.16 ± 2.67 RFU for female BDL vs 26.43 ± 15.09 RFU for male BDL; p=0.01; t=3.1; df=10) (Fig. 5F) and higher levels of albumin were found in female BDL rats vs male BDL rats (26.10 ± 2.05 g/L for female vs 16.96 ± 2.10 g/L for male BDL; p=0.006; t=3.4; df=12) (Fig. 5G). Brain ROS levels were not significantly different between female and male BDL rats (pre-frontal cortex female BDL; 0.21 ± 0.03 mM vs male BDL; 0.21 ± 0.07 mM; p=1; t=0.02; df=10, and hippocampus female BDL; 0.20 ± 0.07 mM vs. male BDL; 0.21 ± 0.06 mM; p=0.9; t=0.1; df=10) (Fig. 5H-I).

Ammonia-induced episode of OHE in female vs. male BDL rats

Upon the acute injection of ammonia, 67% of the male BDL rats developed severe HE (OHE episode) while 0% of the female BDL rats incurred an episode (p=0.0005; df=1) (Fig. 6A). During the episode, the rise in plasma ammonia was similar (non-significant) in male and female BDL rats (610.0 ± 200.2 µmol/L in females vs. male BDL; 918.6 ± 361.7 µmol/L; p=0.1; t=1.8; df=10) (Fig 6B). Plasma ROS levels during the ammonia challenge were significantly lower in female BDL rats compared to male BDL rats (16.79 ± 9.13 RFU vs 98.53 ± 31.07 RFU respectively; p=0.0001; t=6.2; df=10) (Fig. 6C). To further understand the role of ROS in the onset of ammonia-induced OHE, male BDL rats were treated with the antioxidant allopurinol, previously shown to be effective in reducing systemic ROS in BDL rats (Bosoi *et al.* 2012). Allopurinol treatment in male BDL rats lead to protection against ammonia-induced episodes compared to vehicle treated male BDL rats (25% developed episodes in allopurinol-treated vs. 67% in vehicle-treated male BDL rats; p=0.01; df=1) (Fig. 6D).

Discussion

Six weeks following BDL, female rats developed CLD, comparable to what has been previously reported in male BDL rats. Furthermore, female BDL rats developed similar parameters of neurological impairment as previously observed in males except for anxiety, loss of short-memory and brain edema for which females were safeguarded. Comparable levels of hyperammonemia were found in both female and male BDL even though females did not endure a loss of muscle mass as observed in males. Female BDL rats were naturally protected against ammonia-induced episode of OHE, whereas male BDL rats were not. Systemic oxidative stress was found to play a deciphering role in protecting female BDL rats since systemic ROS were significantly higher in male BDL rats and when treated with allopurinol (antioxidant; xanthine oxidase inhibitor), male BDL rats did not develop severe neurological impairment following ammonia-challenge. Increased antioxidant capacity in female BDL rats, possibly due to higher levels of albumin vs. male BDL (data not shown), reduces the sensibility to ammonia-induced OHE. This sex-specific increased antioxidant capacity found in females and resistance against HE suggests reducing oxidative stress with antioxidants could be an important treatment strategy for the prevention of HE in males, who are at higher risk.

The use of female animals in pre-clinical research is often underrepresented, resulting in significant lack of knowledge on the impact of sex in sickness and therefore creating a care gap. Historically, female animals were rarely chosen for studies since it was believed they would lead to increased variability compared to males. Subsequently, the lack of inclusion of female models in pre-clinical research became a growing

concern (Beery and Zucker 2011), and it was later proven that the variability found in females is in fact not higher than in males (Becker *et al.* 2016). However, what became clear was that there are fundamental differences between males and females in response to disease and that addressing these differences would result in sex-specific management and therapeutic interventions. In liver disease, differences between male and females have been found in patients with CLD, including etiology of disease, mortality predictors, liver transplantation access, and gut microbiota (Saboo *et al.* 2020; Sarkar *et al.* 2015). However, the impact of sex-specific differences in the onset of CLD and HE remains largely unknown.

In this study, we described for the first time, a female rat model of CLD and HE. Our group has ample experience with this well characterized animal model of CLD and HE in male rats. Even though female BDL rats established CLD and HE after 6 weeks, they developed distinct features compared to male BDL rats. Since the liver is the main metabolic organ, liver disease negatively impacts body composition by changing energy homeostasis and inducing a hypermetabolic state. Consequently, sarcopenia develops which leads to increased mortality and higher susceptibility to episodes of OHE (Bhanji *et al.* 2018). While sarcopenia is a well-established condition in male patients with CLD and HE, there is evidence that female patients are less affected (Bhanji *et al.* 2018; Tandon *et al.* 2012). In pre-clinical research, our group already demonstrated male animal models of CLD and HE develop loss of muscle mass (Bosoi *et al.* 2017). In the current study, supporting clinical observations, female BDL rats did not develop loss of muscle mass and function. It is known that muscle plays an important compensatory role in clearing ammonia during CLD and therefore, a reduction in muscle mass or

function leads to a further decline in the capacity to clear ammonia. However, despite no loss of muscle mass in female BDL, blood ammonia levels were comparable between female vs male BDL. Furthermore, muscle ammonia clearance and subsequent glutamine production via GS was not higher in female BDL compared to males. However, male BDLs do develop a compensatory overexpression of GS in the muscle (Jover-Cobos et al. 2014), which could explain the similar levels of glutamine as well as in ammonia clearance in muscle of both males and female BDL rats.Diverse factors could explain the increased expression of GS in muscle of male BDL. First, muscle mass loss in males could stimulate the upregulation of muscle GS in response to preventing ammonia levels from increasing (Jover-Cobos *et al.* 2014). Secondly, GS expression could be regulated by androgenic hormones, such as testosterone. Indeed, in a study assessing sex-specific hepatic GS, its protein expression and activity were found to be higher in male vs. female rats (Sirma *et al.* 1996). In agreement, removing male sex-hormones by gonadectomy in male rats decreased hepatic GS to levels similar to females (Sirma *et al.* 1996).

CHE is defined by a broad spectrum of subtle neurological changes arising due to liver dysfunction encompassing impairments in memory, motor coordination, activity and mood. However, little is known about the impact of sex on CHE. Levels of anxiety were similar in female BDL vs SHAMs, contrary to what has been demonstrated in male BDL (Clément *et al.* 2021) whereas female BDL had reduced overall activity, similar to what has been observed in male BDL (Bosoi *et al.* 2011). There is evidence that female patients with cirrhosis and CHE have worse quality of life, poor sleep quality and increased anxiety compared to male patients (Popović *et al.* 2015; Labenz *et al.* 2020;

Labenz *et al.* 2018). However, the methods for evaluating anxiety in rodents and patients are evidently different, which could also explain the discrepancy. In addition, it is possible that alternative tests or protocols for anxiety could be more specific for the assessment of anxiety in females. The tests and protocols used in the present study have been validated and characterized to assess anxiety in male rats. Therefore, future studies merit to be considered in regards to sex-specific anxiety tests. Furthermore, anxiogenic behaviour in rats can be influenced by hormonal changes caused by the estrous cycle (ter Horst *et al.* 2012). However, when female BDL and SHAM rats were grouped by estrous cycle, anxiety levels did not differ between phases (Suppl. Fig. 1A-B). In addition, female and male rats can respond differently to stressors and therefore the assessment of stress hormones such as corticosterone could provide additional insights on the different anxiety levels between male and female BDL rats. The breadth of CHE symptoms and behavioral variances can manifest differently in male vs. female BDL rats. Recognition memory evaluated by the NOR test encompasses the identification of a familiar stimulus, assessing episodic memory, thus the ability to recall specific episodes. Contrary to what has been previously demonstrated in males (Ochoa-Sanchez *et al.* 2021), female BDL rats did not develop impairment in short-term memory. It is plausible that the difference is due to the type of memory assessed, since females have better performance on episodic memory, while males perform better on spatial memory tests (Loprinzi and Frith 2018). Since the neurophenotype of female and male BDL rats differs (females; lack of anxiety, brain edema and short-term memory loss), it would be interesting to evaluate the assessment of sex-specific cognitive tasks which could increase sensitivity and specificity to CHE testing.

Brain edema is considered to be implicated in the pathogenesis of HE. However, its precise role is unknown since the incidence of brain edema in HE patients is not uniform (Winterdahl *et al.* 2019; Joshi *et al.* 2014). Therefore not all manifestations of CHE are related to brain edema. For example, not all animal models of CHE develop brain edema (Cauli *et al.* 2013). The portal-cava anastomosis (PCA) rat (type B model of HE) shows neurological impairments characteristic of HE without the presence of brain edema (Bémeur *et al.* 2016). In male BDL rats, brain edema is present (Bosoi *et al.* 2014b), an entity which we did not observe in female BDL rats. Interestingly, in animal models of traumatic brain injury, females are partly protected due to their hormonal profile, developing delayed brain edema compared to males (O'Connor *et al.* 2006). However, in our female BDL model, the estrous cycle, which is governed by the hormonal profile, is impaired (Suppl. Fig.1E). This suggests a hormonal imbalance, which is a known complication of CLD resulting in lower estrogen levels in females (Mahmoud 2018; Välimäki *et al.* 1984). The impact of hormonal dysregulation on brain physiology in cirrhosis remains to be thoroughly investigated. Our group has demonstrated that the onset of brain edema in male BDL rats is a result of the synergistic interaction between hyperammonemia and systemic oxidative stress (Bosoi *et al.* 2012; Bosoi *et al.* 2014a; Bosoi and Rose 2013). We demonstrated that either reducing blood ammonia levels with an ammonia lowering agent or reducing ROS with an antioxidant, lead to a prevention of brain edema in male BDL rats. Since the hyperammonemia levels were similar in male vs female BDL rats, our data suggest the lower systemic ROS levels in females is the plausible explanation for the protection against brain edema.

Many of the precipitating factors of OHE in clinic, such as constipation and gastrointestinal bleeding, lead to an increase in blood ammonia (Vilstrup *et al.* 2014). Therefore, we induced an episode of OHE with an acute injection (s.c.) of ammonium acetate. The majority of male BDL rats developed severe HE (severe lethargy and loss of righting reflex) following the ammonia challenge. Whereas none of the female BDL rats developed OHE. This protection was independent of ammonia levels (baseline; pre-injection and during episode) since in both cases, ammonia levels were similar in male and female BDL. However, systemic ROS levels were significantly higher at baseline (pre-injection) and significantly higher during the episodes in male BDL rats. To strengthen the causal role of ROS in precipitating OHE, we treated male BDL rats with allopurinol, an antioxidant acting as a xanthine oxidase inhibitor, which our group has previously shown to reduce systemic oxidative stress (Bosoi *et al.* 2012). Treatment of allopurinol to male BDL rats lead to a significant decrease in episodes of OHE compared vehicle-treated male BDL rats. This strongly suggests systemic oxidative plays an important role in the development of OHE.

The further induction of ROS following ammonia-injection in male BDL rats is evidently ammonia-dependent. Our group has demonstrated that the onset of CHE involves systemic ROS and hyperammonemia as independent factors (Bosoi and Rose 2013). However, it has been shown that higher levels of blood ammonia, as seen during acute liver failure (ALF) due to hepatic devascularization in rats, or exposure to high concentrations of ammonia *in vitro* (higher than 500uM) can cause ROS levels to rise (Bosoi and Rose 2013). Nevertheless, the ammonia-injection only lead to an increase in systemic ROS and the onset of OHE in male BDL rats. This suggests, together with

lower systemic ROS levels at baseline (pre-ammonia injection), that females contain a higher antioxidant capacity in order to promptly neutralize the generated ROS induced from the acute injection of ammonia. Antioxidant capacity refers to the cumulative antioxidant action in a biological system and is impacted by the quantity of antioxidants produced and available in regards to generation of ROS in the system. The increased quantity of albumin observed in female BDL rats reflects a higher antioxidant capacity. In circulation, albumin is an effective antioxidant which has the capacity to control ROS in two distinct ways. First, albumin contains methionine and cysteine residues that actively bind to reactive oxygen and nitrogen species. The reduced cysteine groups in albumin are considered the largest pool of thiols in circulation, stressing albumin's importance as a potent antioxidant (Roche *et al.* 2008). Secondly, albumin binds to ligands such as iron and copper and prevent the formation of ROS (Roche *et al.* 2008). Increased albumin in female vs. male BDL rats is likely due to innate higher albumin in female rats, with higher albumin being found in female SHAMs compared to male SHAMs (data not shown). In humans, it has been shown that females have a higher antioxidant capacity compared to males (Brunelli et al. 2014). However, in humans, albumin is not found to be higher in females compared to males (Weaving et al. 2016) and therefore the mechanisms of a potential protection against ROS in females may not solely be dependent on albumin quantity and therefore alternate ROS protection mechanisms may be involved, including albumin quality. Other antioxidant enzymes, molecules and radicals as part of the antioxidant system might also play a role in the sex-specific protection against brain edema and OHE but this remains to be investigated.

In addition to attenuating a rise in ROS levels leading to protection against OHE episodes in female BDL, the lack of brain edema must not be neglected as key factor involved in the reduced susceptibility to severe HE. Male BDL rats with brain edema(Bosoi *et al.* 2011) are susceptible to ammonia-toxicity as they developed OHE episodes. However, male BDL rats treated with allopurinol which leads to a decrease in brain water (Bosoi *et al.* 2012), were also protected against OHE episodes. Understand the role of brain in the susceptibility or risk of developing OHE remains to be thoroughly investigated.

Without a doubt, ammonia remains a key pathogenic factor in the pathogenesis of HE and therefore reducing ammonia remains a primary therapeutic strategy. However, we have demonstrated in our study that ROS can increase the sensitivity of ammonia toxicity and therefore reducing systemic oxidative stress should also be a treatment target. Interestingly, therapies such as vitamin C and zinc supplementation, considered to have antioxidant effects, have been shown to be superior compared to lactulose to treat HE in patients (Mousa *et al.* 2016). However, the effect of the treatments by sex was not evaluated.

In conclusion, the female rats following BDL develop similar hepatic damage/injury compared to the well-characterized male BDL rat. However, even though both female and male BDL rats develop HE, females have distinct features such as lack of anxiety and brain edema and intact short-term memory. In addition, sex-dependent differences were found at the muscle level since even though similar levels of hyperammonemia were found in female and male BDL rats, females were safeguarded against muscle mass loss while males developed a loss of muscle together with an

upregulation in GS activity. A higher systemic antioxidant capacity found in females naturally protected female BDL rats from enduring an ammonia-induced episode of OHE. Our results suggest the occurrence and development of CLD-induced complications differs between male and females and that sex-specific management of patients merits further attention.

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Author contributions

MMO, MT and CFR contributed for the study's concept and design. MMO, AMA and CRB carried out the experiments. MMO, AMA, CRB, MT and CFR analysed the data. MMO, MT and CFR wrote the paper. CFR funded and supervised the study. All authors participated on the paper's revision and approval.

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Tables

	BDL	**SHAM**	**P value**
Liver weight (g)	29.1 ± 3.9	9.1 ± 0.8	***p<0.001
Blood markers of liver injury			
ALT (U/L)	40.9 ± 20.9	26.3 ± 8.5	NS
AST (U/L)	331.2 ± 140.9	68.7 ± 18.7	***p<0.001
ALKP (U/L)	361.3 ± 193.5	81.7 ± 30.8	**p<0.01
Blood markers of liver function			
Albumin (g/L)	29.9 ± 5.8	49.3 ± 8.4	***p<0.001
Bilirubin (µmol/L)	192.0 ± 30.4	1.1 ± 0.8	**p<0.001
Ammonia (µmol/L)	111.8 ± 51.8	50.7 ± 8.7	**p<0.01
ROS (fluorescence)	41.36 ± 20.9	5.24 ± 4.8	*p<0.05

Table 1: Markers of liver disease in female rats. Liver weight and liver enzymes at week 6 after BDL or SHAM surgery in female BDL rats. N=8 rats per group. Parametric

t-test, numbers expressed as means ± SD, significance reached when $p<0.05$. NS = non-significant. ALT, alanine aminotransferase; AST, aspartate transaminase; ALKP, alkaline phosphatase.

	BDL	**SHAM**	**p value**
Necrosis (0-3)	1.8 ± 1	0 ± 0	$p<0.05$
Bile-duct proliferation (%)	66.7 ± 16.3	0 ± 0	$p<0.001$
Inflammation (0-4)	2 ± 0.9	0 ± 0	$p<0.01$

Table 2: Evaluation of liver histology in female rats. Presence of necrosis, bile-duct proliferation and inflammation at week 6 after BDL or SHAM surgery in female BDL rats. N=3 SHAM and N=6 BDL rats per group. Parametric t-test, numbers expressed as means ± SD, significance reached when $p<0.05$.

Figure legends

Fig. 1. Experimental design.

Three groups were used for the experiments. Six-week female BDL and SHAM rats for the characterization of the model, five-week female and male BDL rats for the muscle ammonia challenge and ammonia clearance (OHE assessment), and five-week male BDL vehicle and male BDL allopurinol rats for the ammonia clearance (OHE assessment).

Fig. 2. Liver histology of female rats.

Hematoxylin and eosin staining in livers of female rats after 6 weeks of bile duct ligation

(A) or SHAM (B) surgery. ★ = hepatocytes ↖ = bile ducts and ▾ = Kupfer cells.

Fig. 3. Hepatic encephalopathy assessment after BDL or SHAM surgery in female rats.

In the rota-rod test, female BDL rats showed altered motor coordination (A) and impaired night activity (B), compared to female SHAM rats. Female BDL rats had no anxiety measured by the open filed (OF) and the elevated plus maze (EPM) tests vs. SHAM rats (C and D). Short term memory was not impaired in female BDL vs. SHAM, (E). Brain edema (F) was not present in female BDL rats compared to female SHAM rats, N=8 per group. Parametric t-test, numbers expressed as means ± SD, significance reached when $p<0.05$. NS = non-significant.

Fig. 4. Body parameters in female rats after BDL or SHAM surgery. Female BDL rats showed no changes of body weight and food intake (A) vs. female SHAM through the duration of the model. Body composition analysis (B) showed higher lean mass and lower fat mass in female BDL vs. SHAM. No muscle loss (C) assessed by weight or circumference was observed in female BDL rats vs SHAM controls as well as no loss of muscle strength (D) from either forelimbs or hindlimbs. N=8 rats per group. Two-Way ANOVA and parametric t-test, numbers expressed as means ± SD, significance reached when $p<0.05$. NS = non-significant.

Fig. 5. Higher muscle mass does not lower ammonia levels in female vs. male BDL rats. Male BDL rats had lower percent muscle mass vs. female BDL rats, normalized to respective SHAM groups (A) N=6, but no changes in baseline ammonia

levels (B). Muscle GS activity (C) was higher in BDL male vs. female, and muscle ammonia clearance (D) and glutamine production (E) were not different. Female BDL rats have lower baseline oxidative stress with lower baseline ROS (F) and higher baseline albumin levels (G) compared to male BDL rats. Brain antioxidant capacity was similar between male and female BDL in cortex (H) and hippocampus (I). N=5-6 rats per group. Parametric t-test, numbers expressed as means ± SD, significance reached when $p<0.05$. NS = non-significant.

Fig. 6. Lower ROS protects against ammonia induced OHE. When challenged with ammonia, female BDL rats were naturally protected against neurological impairment compared to male BDL ($p<0.001$) (A). Female rats have similar plasma ammonia (B) and lower plasma ROS levels during the ammonia challenge (C) compared to male BDL. In addition, Male BDL rats treated with the antioxidant allopurinol were protected against ammonia induced neurological impairment compared to male BDL vehicle rats ($p<0.05$) (D). N=6-9 rats per group. Parametric t-test and Chi-square test. Numbers expressed as means ± SD or percentages, significance reached when $p<0.05$. NS = non-significant.

Figure 1

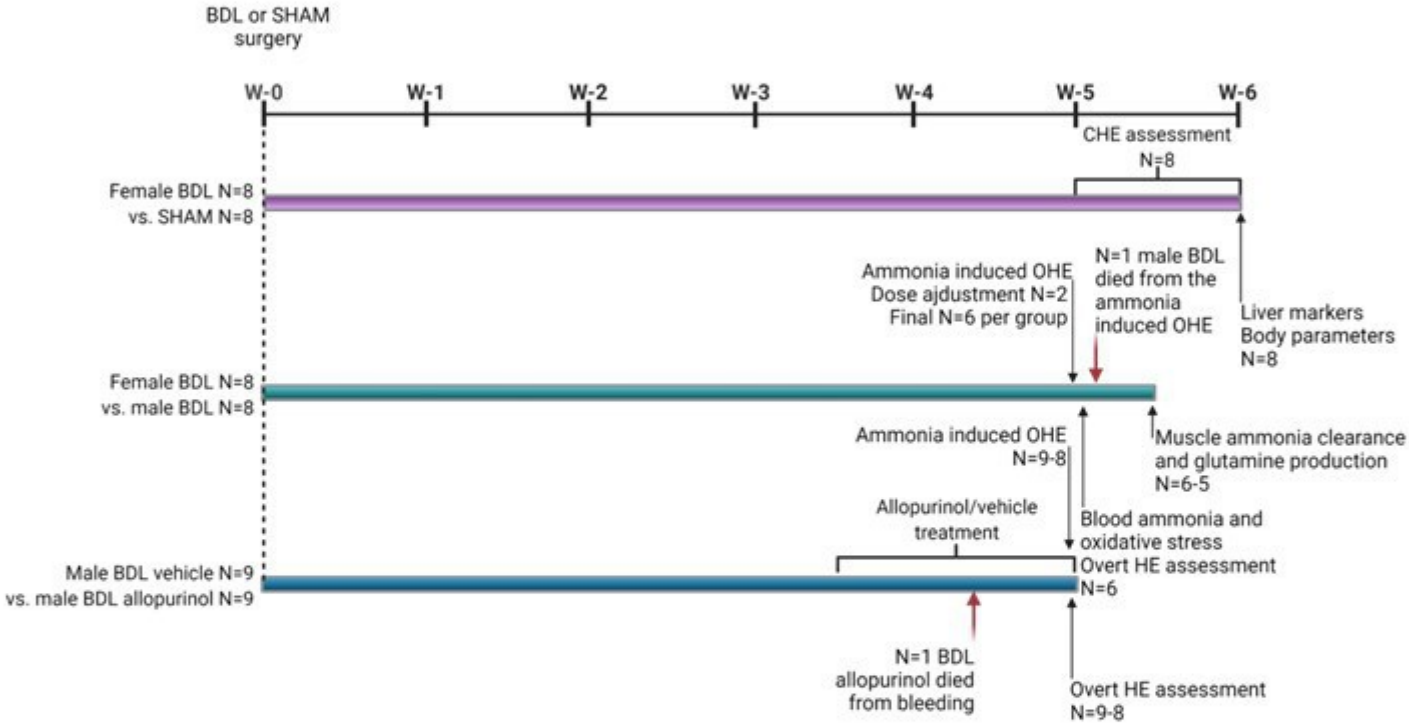

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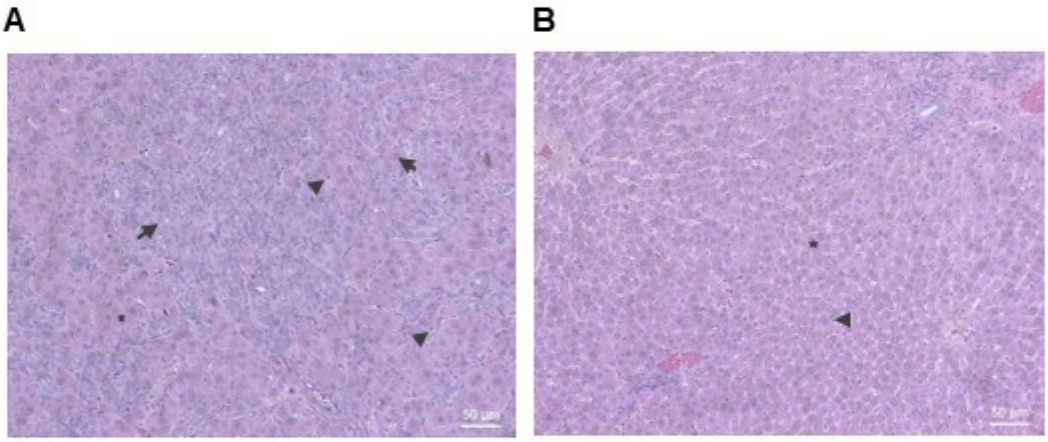
Figure 2



JNC_15661_Figure2_reviewed.JPG

Figure 3

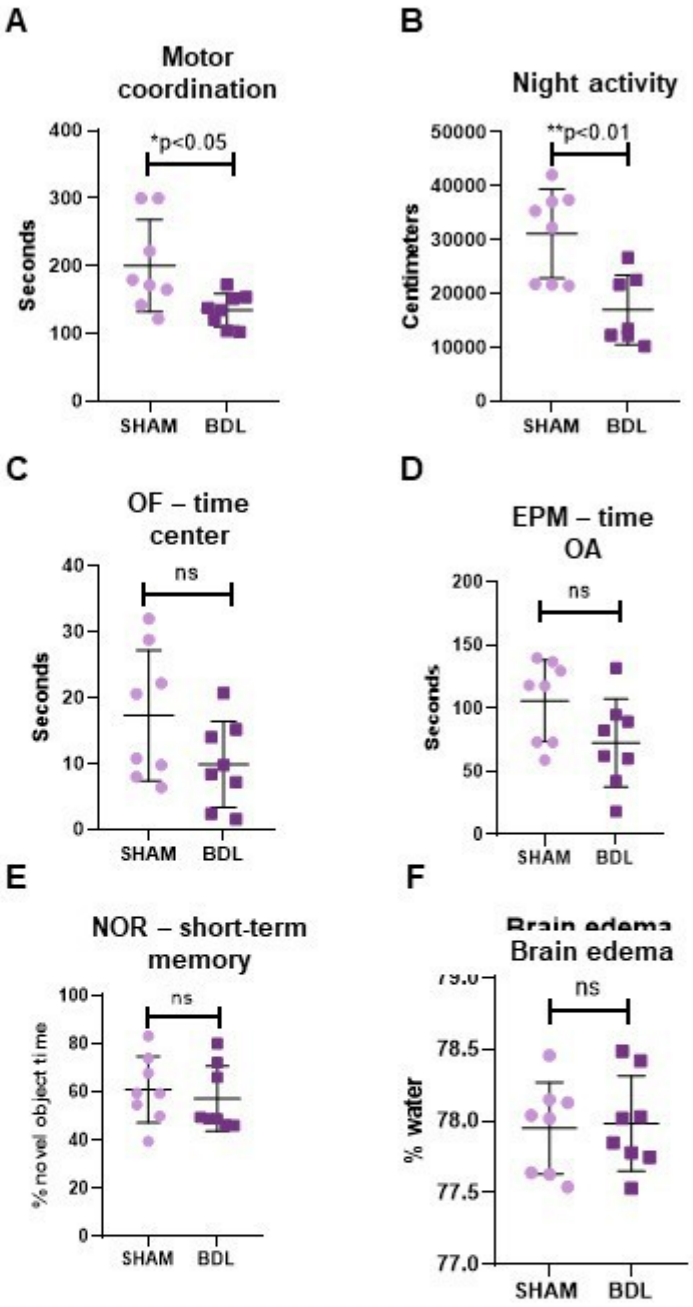


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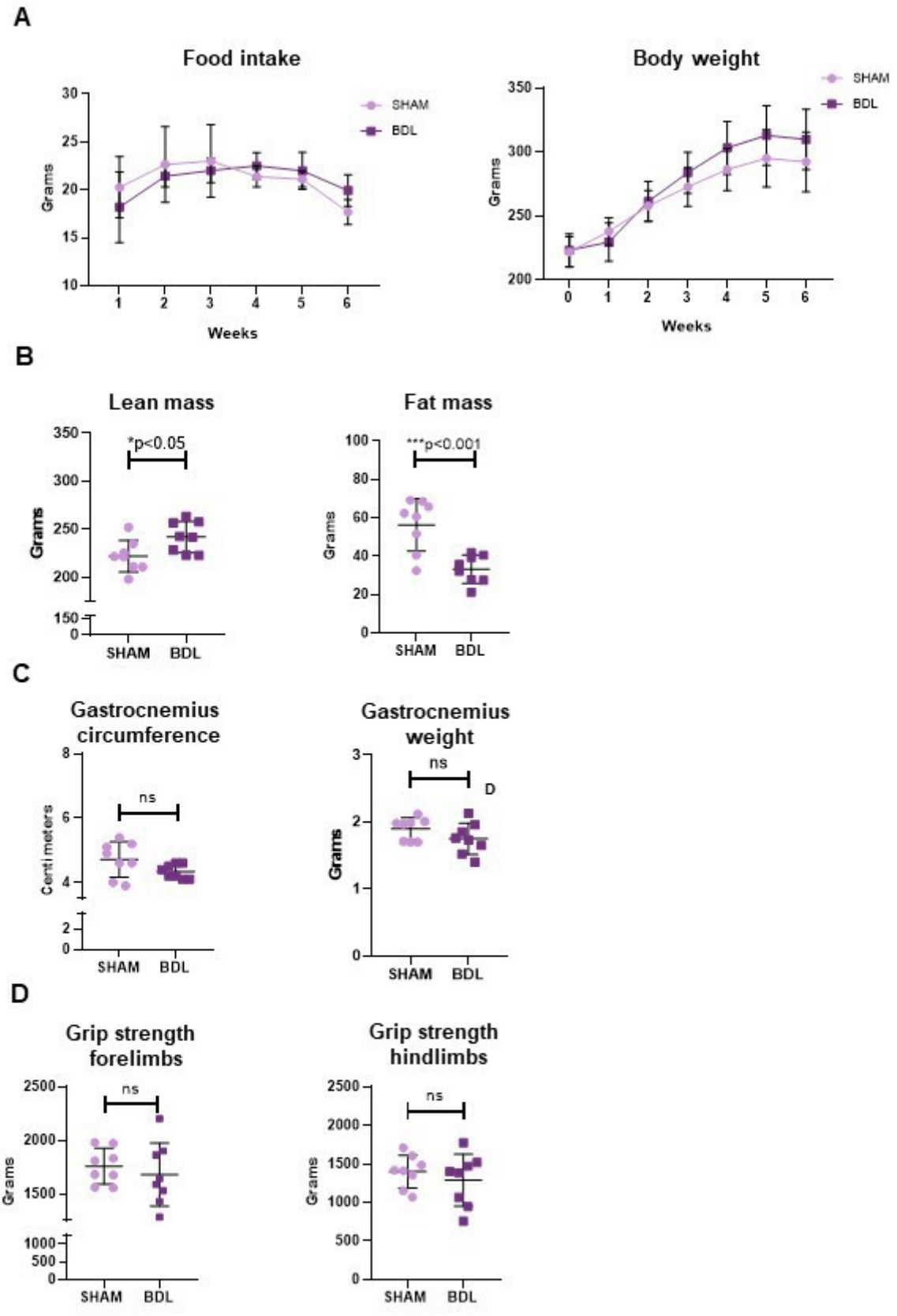
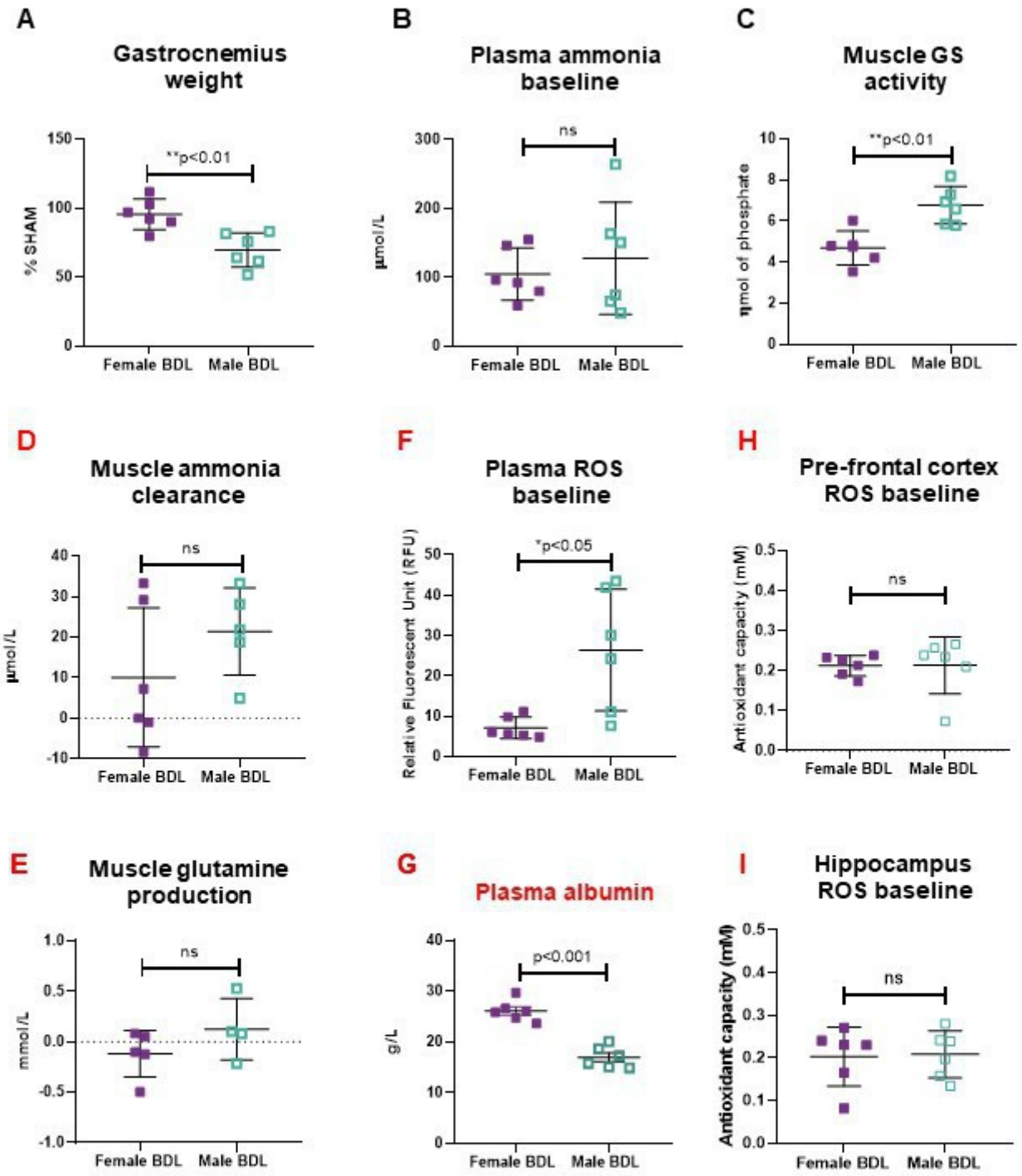
Figure 4



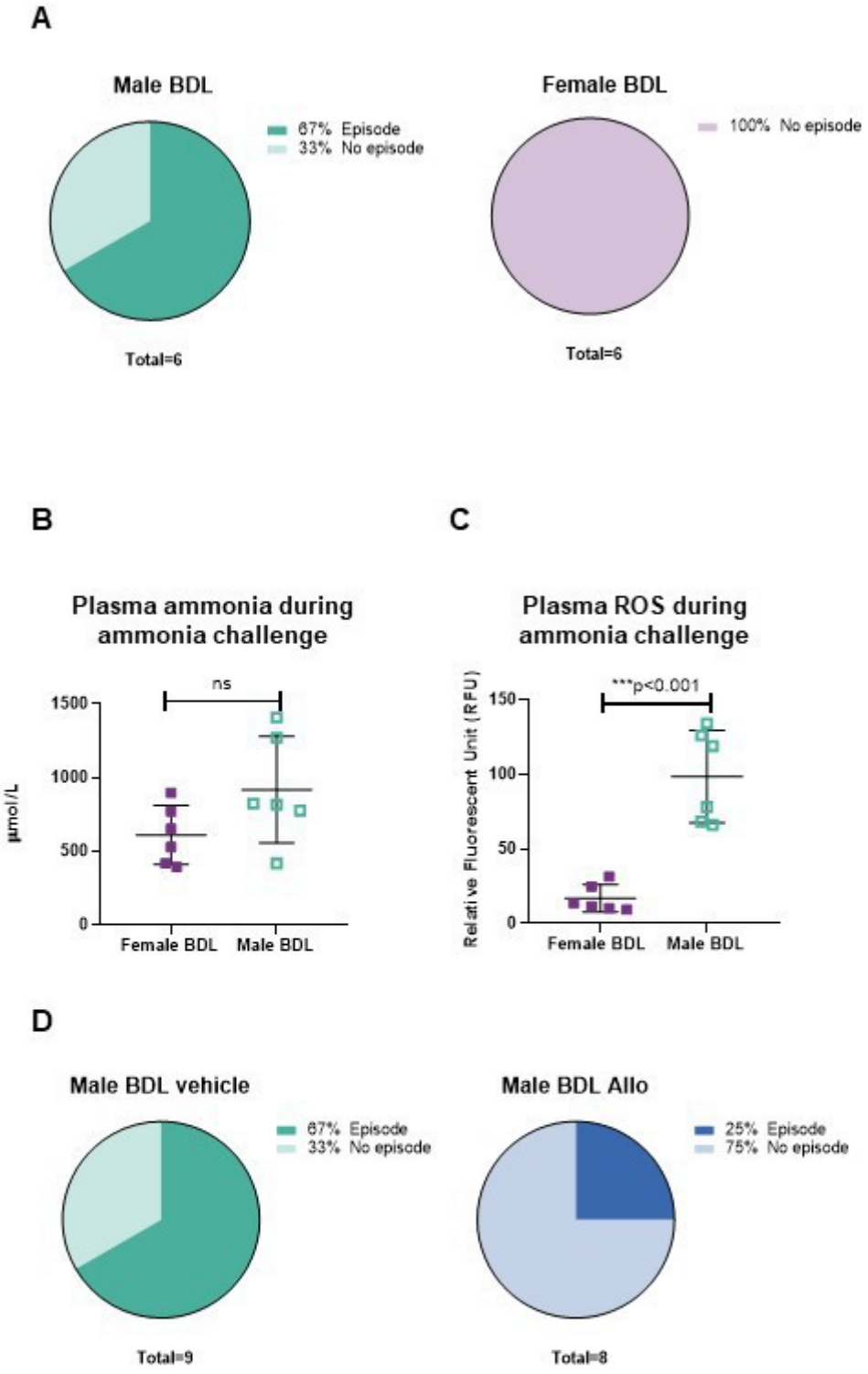
JNC_15661_Figure4_reviewed.JPG

Figure 5



JNC_15661_Figure5_reviewed.JPG

Figure 6



JNC_15661_Figure6_reviewed.JPG