Impaired Cholecystokinin-Induced Gallbladder Emptying Incriminated in Spontaneous "Black" Pigment Gallstone Formation in Germfree Swiss Webster Mice

Stephanie E. Woods[1], Monika R. Leonard[2], Joshua A. Hayden[3], Megan B. Brophy[3], Kara R. Bernert[2], Brigitte Lavoie[4], Sureshkumar Muthupalani[1], *Mark T. Whary[1], Gary M. Mawe[4], Elizabeth M. Nolan[3], Martin C. Carey[2], and James G. Fox[1]

Author Contributions: Conceived and designed the experiments: SEW MRL JAH BL MTW GMM EMN MCC JGF. Performed the experiments: SEW MRL JAH MBB KRB BL SM. Analyzed the data: SEW. Wrote the paper: SEW.

[1] Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA 02139
[2] Division of Gastroenterology, Brigham and Women's Hospital, Department of Medicine, Harvard Medical School, Boston, MA 02115
[3] Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139
[4] Department of Neurological Sciences, University of Vermont, Burlington, VT 05405

Running Head: Gallbladder Impairment in GF SW Mice with Pigment Gallstones

* Corresponding Author:

Mark T Whary, DVM, PhD, DACLAM
Division of Comparative Medicine
Massachusetts Institute of Technology
77 Massachusetts Avenue, 16-825A
Cambridge, MA 02139
mwhary@mit.edu
Phone 617-253-9435
Fax 617-258-5708

ABSTRACT

"Black" pigment gallstones form in sterile gallbladder bile in the presence of excess bilirubin conjugates ("hyperbilirubinbilia") from ineffective erythropoiesis, hemolysis or induced enterohepatic cycling (EHC) of unconjugated bilirubin. Impaired gallbladder motility is a less well-studied risk factor. We evaluated the spontaneous occurrence of gallstones in adult germfree (GF) and conventionally housed specific pathogen-free (SPF) Swiss Webster (SW) mice. GF SW mice were more likely to have gallstones than SPF SW mice, with 75% and 23% prevalence, respectively. In GF SW mice, gallstones were observed predominately in heavier, older females. Gallbladders of GF SW mice were markedly enlarged, contained sterile "black" gallstones comprised of calcium bilirubinate and <1% cholesterol, and had low-grade inflammation, edema and epithelial hyperplasia. Hemograms were normal, but serum cholesterol was elevated in GF compared to SPF SW mice, and serum glucose levels were positively related to increasing age. Aged GF and SPF SW mice had deficits in gallbladder smooth muscle activity. In response to cholecystokinin (CCK), gallbladders of fasted GF SW mice showed impaired emptying (females: 29%; males: 1% emptying), whereas SPF SW females and males emptied 89% and 53% of volume, respectively. Bilirubin secretion rates of GF SW mice were not greater than SPF SW mice, repudiating an induced EHC. Gallstones likely developed in GF SW mice due to gallbladder hypomotility, enabled by features of GF physiology, including decreased intestinal CCK concentration and delayed intestinal transit, as well as an apparent genetic predisposition of the SW stock. GF SW mice may provide a valuable model to study gallbladder stasis as a cause of "black" pigment gallstones.

Keywords: “Black” Pigment Gallstones, Germfree Mice, Impaired Gallbladder Motility, Cholecystokinin

INTRODUCTION

Gallstone disease affects more than 20 million people in the United States and results in more than 700,000 cholecystectomies annually (32, 45, 46). Although not widely studied, pigment gallstones are observed in a variety of clinical conditions, and may account for up to 20-25% of gallstones among patients that undergo cholecystectomy in the Western world (19, 37, 55). While “brown” pigment gallstones form in septic bile, “black” pigment gallstones develop classically in sterile bile with the critical risk factor of hyperbilirubinbilia, defined as biliary hypersecretion of bilirubin conjugates, due principally to chronic hemolysis secondary to multiple syndromes, or ineffective erythropoiesis as seen with vitamin B12 and folate deficiencies (38, 48, 54, 55). Hyperbilirubinemia may also occur with prolonged intestinal transit, antibiotic therapy and ileal dysfunction from induced enterohepatic cycling (EHC) of unconjugated bilirubin (UCB), wherein UCB enters the enterohepatic circulation to be reconjugated, and resecreted into bile (18, 53-56).

A pathophysiological role for intestinal bacteria, or the lack thereof, in “black” pigment gallstone formation has not been well-documented, but may involve altered intestinal mucosal barrier function, and changes in intestinal bilirubin deconjugation and formation of urobilinoids, facilitating EHC of UCB (9, 47, 54, 55, 59). The Division of Comparative Medicine at M.I.T. maintains a germfree (GF) Swiss Webster (SW)

84 breeding colony to facilitate embryo transfer rederivation of other lines of mice into a GF
85 status, and periodically purchases conventionally housed specific pathogen-free (SPF)
86 SW mice for controls in various research studies. SW mice are customarily used as an
87 inexpensive outbred stock for biomedical research, transgenic technology, and as sentinel
88 mice for monitoring infectious diseases in research colonies. Interestingly, necropsies of
89 adult female and male GF SW mice from our colony revealed 100% prevalence of
90 markedly enlarged gallbladders, with 75% containing gallstones morphologically
91 consistent with "black" pigment gallstones of humans, whereas SPF SW mice
92 demonstrated 23% gallstone prevalence and normal sized gallbladders.

93 It is known that GF mice have delayed intestinal transit, with documented two
94 times less cholecystokinin (CCK)-like immunoreactivity in the small intestine from rapid
95 degradation of CCK, compared to normally colonized mice, and that CCK acts to
96 promote propulsive activity of the intestine (30, 34, 35, 50, 57, 61). The slower intestinal
97 transit observed in GF mice is reminiscent of the altered peristaltic function in humans
98 and experimental animals with cholesterol gallstone disease (36, 37, 58, 61). Although
99 dysfunction in gallbladder and small intestinal motility has been linked to cholesterol
100 gallstone disease, little is known about how hypomotility of the gallbladder influences
101 "black" pigment gallstone formation (36, 37, 58). Gallbladder dysfunction has been
102 reported in conditions associated with the formation of "black" pigment gallstones,
103 including liver cirrhosis, truncal vagotomy and administration of total parenteral
104 nutrition, and in conditions more often associated with cholesterol gallstones such as
105 obesity and/or type II diabetes (4, 36, 37, 49, 54, 58). With recognized delayed intestinal
106 transit in GF mice and the indefinite association of "black" pigment gallstones with

gallbladder dysfunction in humans, we postulated that GF SW mice may provide a unique, spontaneous animal model to investigate the role of the gut microbiota and impaired gallbladder motility in "black" pigment gallstone formation in humans.

In turn, we characterized gallstone disease in GF and SPF SW mice by demographic profiling, logistic regression analysis, various gallbladder bile and gallstone analyses, and gallbladder and liver histology. Mice were screened for hematopoietic abnormalities, and conjugated and unconjugated bilirubin levels in hepatic bile determined to rule out ineffective erythropoiesis or hemolysis, and induced EHC of UCB, respectively. The proposed mechanism of impaired gallbladder motility was probed by determination of fasting gallbladder volumes and bile pH, screening for metabolic abnormalities such as diabetes, and evaluation of calcium ion (Ca^{2+}) activity of gallbladder smooth muscle and gallbladder responsiveness to exogenous CCK.

METHODS

Mice

GF outbred Tac:SW mice were obtained from Taconic Farms (Germantown, NY) and maintained as a breeding colony in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. One hundred and twenty-five female and 99 male GF SW mice were bred periodically and aged further for purposes of this study (age range: 5 - 22 months; 10.7 ± 0.2 months old) (Table 1). For comparison to GF SW mice, SPF mice representing the same outbred genetic stock but colonized with intestinal microbiota were evaluated. Seventy-five female and 53 male SPF SW mice were purchased from Taconic as retired breeders (age range: 8 - 15

months; 10.1 ± 0.2 months old) (Table 1). SPF SW mice were free of exogenous murine viruses, bacterial pathogens and parasites, and animal use was approved by the Institutional Animal Care and Use Committees of the collaborating institutions.

Husbandry

GF SW mice were housed in sterile isolators in open-top polycarbonate cages on autoclaved hardwood bedding and fed autoclaved water and diet (Purina 5021, Purina Mills, St. Louis, MO) *ad libitum*. The diet had a guaranteed analysis of not less than 20% crude protein and 9% crude fat, and not more than 5% crude fiber and 6.5% ash. Macroenvironmental conditions included a 14:10 light / dark cycle and temperature maintenance at 68 ± 2ºF. Weekly microbiologic monitoring of interior isolator surfaces, feed, water, and feces confirmed absence of all aerobic and anaerobic bacteria and fungi. SPF SW mice were housed in a barrier facility in standard, non-autoclaved microisolator cages under similar environmental conditions. To standardize nutrition, these mice were fed the same autoclaved diet for the duration of their lives. SPF status was monitored by a sentinel program.

Determination of Gallbladder Volume and Bile pH

Mice were euthanized using carbon dioxide and at necropsy, relative gallbladder size and gross evidence of gallstones (relative size, approximate amount and color) were recorded.

Gallbladder volume (μL) and pH of gallbladder bile were determined for fasted GF (n = 6 females, 6 males; 12.0 ± 0.9 months old) and SPF (n = 14 females, 15 males; 11.2 ± 0.6 months old) SW mice. Mice were anesthetized by intraperitoneal injection of a cocktail of anesthetics in 9% NaCl, containing ketamine (80 mg/kg), xylazine (8 mg/kg), acepromazine (2 mg/kg) and atropine (0.012 mg/kg), and terminal

cholecystectomies were performed as previously described (18). Following gallbladder removal, mice were euthanized by anesthetic overdose, followed by bilateral thoracotomy. Gallbladder bile was drained into tared 200 μL microcentrifuge tubes, and gallbladder volumes were quantified gravimetrically by equating weight and volume (i.e., 1 mg = 1 μL). Immediately afterwards, gallbladder bile pH was measured by a micro pH electrode (Microelectrodes Inc., Bedford, NH).

Gallbladder Bile and Gallstone Analyses

To characterize gallbladder bile sediment and gallstone morphology, fresh and previously frozen (-70°C) gallbladder bile samples from GF SW mice with (n = 5 females, 2 males; 16.9 ± 2.0 months old) or without (n = 3 males; 10.3 ± 2.3 months old) gross evidence of gallstones were evaluated microscopically under direct light. These samples from GF SW mice were compared to bile of 7 SPF SW mice (n = 3 females, 4 males; 10 months old) lacking gross evidence of gallstones and one 15-month-old SPF SW female mouse with gallstones, though the latter sample was kept at room temperature for an extended period of time prior to analysis. Additionally, fresh gallbladder tissue, bile and gallstones from seven 11-month-old GF SW female mice (n = 5 with gallstones) were examined by direct light and polarized light microscopy.

Gallstones from two 15-month-old and two 22-month-old GF SW females and one 15-month-old GF SW male were sent to the Laboratory for Stone Research (Newton, MA) for compositional analysis by polarized light microscopy and infrared spectroscopy.

To determine cholesterol content of gallstones, microcentrifuge tubes containing gallstones in bile from 9-month-old GF (n = 5 females, 4 males) and SPF (n = 3 females) SW mice were centrifuged for 15 min in a tabletop microcentrifuge (ISC BioExpress,

Kaysville, UT). After bile supernatant was removed, gallstones were washed by vortexing thoroughly with 200 µL of 1% (w/v) Na tauroursodeoxycholate (NaTUDC). Then, samples from GF SW males and from SPF SW females were pooled into 1 sample per group, whereas gallstones from female GF SW mice were combined into 2 samples. Samples were washed 3 more times with 200 µL NaTUDC and then layered carefully onto a Nuclepore polycarbonate membrane filter (47 mm, 0.2 µm), washed with 5 mL double distilled water, and filter dried under house vacuum. Filter residue was carefully scraped with the flat edge of a metal spatula and transferred to a tared aluminum weighing dish that had been dried under house vacuum at 60˚C for 24 hours (hr). Dried gallstone samples were then resuspended in 150 µL isopropanol. Clumps were broken gently with a glass stirring rod, samples vortexed for 4 min and incubated at 37˚C for 2 hr in a shaking water bath. Immediately prior to analysis, 450 µL acetonitrile was added to each sample. Gallstones were analyzed for cholesterol content by a modified HPLC method using a Kinetex C18 column (2.6 µm particle size; Phenomenex, Torrance, CA) and eluting with acetonitrile:isopropanol (3:1, v/v) (52).

Gallstones from 12-month-old GF (n = 3 females, 3 males; pooled into 1 sample) and SPF (n = 1 female) SW mice were also analyzed by electron paramagnetic resonance (EPR) spectroscopy. Gallbladder bile supernatant was removed and gallstones were washed five times with Chelex-treated water. The water was obtained from a Milli-Q purification system (18.2 $m\Omega cm^{-1}$) and treated with Chelex resin (Biorad, 10 g/L, stirred for >1 hr and filtered) to remove contaminating metal ions prior to use. For washing, the gallstones were suspended in 180 µL of Milli-Q water in the sample reservoir of a centrifugal filter device, gently vortexed, and centrifuged [10,000 rpm x 5 minutes (min),

199 20°C]. The washed gallstones remaining in the reservoir were re-suspended in the
200 Chelex-treated water (180 μL), transferred to acid-washed (2 M HCl) quartz EPR tubes
201 and frozen in liquid nitrogen prior to analysis and stored at -80°C. A sample of
202 commercial bilirubin [98% (EmM/453 = 60); Sigma-Aldrich] was prepared in Chelex-
203 treated Milli-Q water and frozen in liquid nitrogen prior to analysis. EPR spectra (X-
204 band, 9 GHz) were recorded on a Bruker EMX spectrometer with an ER 4199HS cavity.
205 An ESR900 cryostat outfitted with a Cernox sensor was employed for all measurements.
206 Unless noted otherwise, the modulation amplitude and frequency was 1 mT at 100 kHz.
207 Samples of twice washed gallstones (4 samples pooled into 1 sample) and undiluted
208 gallbladder bile (1 individual sample) from 14-month-old female GF SW mice, as well as
209 gallstones washed five times (4 samples pooled into 1 sample) from 15-month-old GF
210 SW mice were also analyzed.

211 Additional gallstones and gallbladder bile from GF (n = 2 females, 4 males; 12
212 months old) and SPF (n = 3 females; 11 months old) SW mice were aseptically collected
213 for culture under aerobic and anaerobic (gas mix) conditions to confirm absence of
214 gallbladder infection.

215 **Screening for Hematopoietic or Metabolic Abnormalities**

216 Following an overnight fast and carbon dioxide euthanasia, post-mortem cardiac blood
217 was collected for complete blood count (CBC) from 11 female and 12 male GF SW mice
218 (10.8 ± 0.6 months old), and 3 female and 3 male SPF SW mice (12 months old), and for
219 serum chemistry analysis from 11 female and 15 male GF SW mice (12.7 ± 1.1 months
220 old), and 4 female and 5 male SPF SW mice (10 months old). CBCs were measured
221 using a Hemavet 950FS analyzer (Drew Scientific, Waterbury, CT) and serum was sent

222 to IDEXX Laboratories (Memphis, TN) for a chemistry panel of 21 analytes [Table 5; 3
223 analytes (bicarbonate, creatine kinase, gamma-glutamyl transferase) excluded due to
224 insufficient quantity for comparison].

225 Because a predisposition to diabetes mellitus was previously reported for Tac:SW
226 mice, GF and SPF SW mice were screened for glucosuria, fasting hyperglycemia (>300
227 mg/dL) and glucose intolerance (29, 39, 40), and pancreata were examined histologically.
228 Naturally voided urine was collected in sterile polycarbonate caging, or via post-mortem
229 cystocentesis from 11 female and 3 male GF SW mice (11.0 ± 0.7 months old), and 5
230 female SPF SW mice (12 months old). Clinical urinalysis dipsticks (Multistix 10 SG,
231 Siemens Healthcare Diagnostics, Tarrytown, NY) were used to measure protein, glucose,
232 leukocytes, nitrites, ketones, bilirubin, blood and urobilinogen. Specific gravity was
233 measured when a sufficient urine volume was collected.

234 Glucose tolerance testing (GTT) was performed on 9-month-old GF (n = 6
235 females, 5 males) and SPF (n = 6 females, 6 males) SW mice. Mice were fasted
236 overnight, weighed, and baseline glucose was measured in blood obtained by tail nick,
237 followed by intraperitoneal injection of 1 gram of 10% dextrose per kg body weight.
238 Blood glucose levels were measured using a glucometer (AlphaTRAK, Abbott
239 Laboratories, Abbott Park, IL) at time 0, 15, 30, 60, 90, and 120 min post glucose dosing.

240 Additional serum samples were collected 2 days later from these same mice after
241 an 8 hr fast for measurement of serum glucose and insulin levels by the Mouse
242 Metabolism Core (MMC; Baylor College of Medicine, Diabetes and Endocrinology
243 Research Center, Houston, TX). Cardiac blood was collected following carbon dioxide
244 euthanasia. Sera from 12-month-old GF (n = 4 females, 4 males) and SPF (n = 3

females, 3 males) SW mice were collected for glucose and glycated hemoglobin (HbA1c) levels performed by the Comparative Pathology Laboratory (CPL; University of California, School of Veterinary Medicine, Davis, CA).

Histology

Abdominal organs were evaluated grossly at necropsy and gallbladder, liver, pancreas and kidneys were fixed in buffered 10% formalin and processed for histology. Formalin-fixed tissues were evaluated from GF SW mice with gallstones (n = 11 females, 7 males; 14.1 ± 1.3 months old), without gallstones (n = 1 female, 7 males; 10.5 ± 1.2 months old), and from SPF SW mice without gallstones (n = 6 females, 6 males; 9.5 ± 0.3 months old). Tissues were embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin (H&E), and evaluated by a board-certified veterinary pathologist blinded as to sample identity. Gallbladders were graded semi-quantitatively on a scale of 0 (normal) to 3 (severe) for histomorphological changes, including inflammation, edema, hyalinosis, metaplasia, hyperplasia and dysplasia. The liver, pancreas and kidneys were qualitatively assessed for any relevant pathology. Because mild liver lesions were observed in some mice, liver sections were further assessed on a scale of 0 to 4 for lobular and portal inflammation, and dysplasia/neoplasia. The number of lobes with >5 inflammatory foci was used to calculate a cumulative hepatitis index score, as previously described (41).

Gallbladder Muscle Activity

Calcium imaging studies were performed as previously described in greater detail (25). Age-matched GF and SPF SW female mice (10 months old; n = 4 per group) were anesthetized with isoflurane, exsanguinated and underwent cholecystectomy. Gallbladders were opened and mounted serosa side up between two pieces of Sylgard

(Dow Corning, Midland, MI) connected by metal pins. Mounted tissues were incubated in HEPES buffer containing 10 μM fluo-4 AM and 2.5 μg/ml pluronic acid for 45 min at room temperature, and then rinsed in HEPES buffer for at least 30 min to allow de-esterification. The fluo-4- loaded gallbladders were placed in an imaging chamber and superperfused with aerated physiological saline solution (PSS). Ca^{2+} transients were visualized using a Nikon TMD inverted microscope with a 60x water immersion lens attached to a Noran Oz laser confocal system. After a 20 min equilibration period, basal Ca^{2+} activity was recorded over periods of 30 to 60 seconds (15-30 frames per second), from three to seven fields per gallbladder. To measure agonist-induced Ca^{2+} activity, carbachol (3 μM in PSS) was superfused over the tissue and Ca^{2+} transients were recorded every few minutes over a 20 min period. Data were analyzed using SparkAN, a custom software program written at the University of Vermont, and also compared to baseline data obtained from 7-10-week-old SPF SW males.

Responsiveness to Exogenous Cholecystokinin

Fasted GF (n = 9 females, 10 males; 8 months old) and SPF (n = 8 females, 7 males; 8 months old) SW mice were administered cholecystokinin octapeptide (CCK) to evaluate gallbladder emptying. Under injectable anesthesia described above, mice were injected intravenously with 2 μL/g of CCK solution (10^{-5} mg/mL sulfated CCK; Tocris Bioscience, Bristol, UK) in sterile PBS, pH 7.4. After 20 min, cholecystectomies were performed and gallbladder volumes (μL) were determined as described above. Age-matched fasted controls (GF SW mice: n = 9 females, 9 males; SPF SW mice: n = 7 females, 9 males) received an injection of sterile PBS or no injection.

Analysis of Conjugated and Unconjugated Bilirubin in Hepatic Bile

Conjugated and unconjugated bilirubin concentrations (μM) and secretion rates (nmol/hr) in hepatic bile were determined for unfasted GF (n = 19 females, 8 males; 11.1 ± 0.1 months old) and SPF (n = 15 females, 7 males; 11.3 ± 0.5 months old) SW mice. Mice were induced with an anesthetic cocktail administered intraperitoneally as described above. Following cannulation of the hepatic bile duct, hepatic biliary outputs and secretion rates were assessed as previously described (17). To prevent actinic and oxidative degradation of bilirubin, hepatic bile was kept in the dark and/or under red lights. Hepatic biliary species were determined and quantified by HPLC using the method of Spivak and Yuey (44). Percent UCB (%) was calculated by dividing the concentration of UCB by the sum of the concentrations of all individual bilirubin species (i.e., all mono- and di-conjugates, plus UCB). Secretion rates were normalized to 1 hr of hepatic bile flow.

Statistics

Table 1 provides demographic data on SW mice with and without gallstones. Logistic regression was performed to determine the likelihood of SW mice having gallstones (binary variable), controlling for microbial status (GF or SPF; binary variable), age (continuous variable), sex (binary variable) and body weight (continuous variable), and was reported through adjusted (crude) odds ratios (OR), 95% confidence intervals (95% CI), and p-values of the overall test of the model and each parameter estimate. For each covariate, the likelihood-ratio chi-squared test for parameter estimates was used to compare the full logistic model to a model excluding the covariate of interest. The favored model included only covariates found to contribute to the predictability of the model. All possible interactions in the favored model were evaluated as a set to

314 determine significance using a chi-squared test to compare the favored logistic models,
315 with or without the set of interaction variables. Confounders were defined as covariates
316 that, when added to the favored model, resulted in ≥10% change in the slope of the major
317 exposure, microbial status. Further, a stratified logistic regression analysis was
318 performed as described above and was segregated by microbial status, with age as the
319 major exposure and sex and body weight as covariates.

320 Presence of gallstones, microbial status, age, sex and body weight were tested
321 against individual quantitative analytes to determine significant effect(s) by analysis of
322 covariance (ANCOVA), also with separate ANCOVAs performed for both microbial
323 statuses. Adjusted means were calculated for both microbial statuses, with the
324 continuous variables (age, body weight) fixed at their means; data was reported as
325 adjusted mean ± standard error. Percentage data (hematocrit, HbA1c, unconjugated
326 bilirubin) were arcsin transformed prior to analysis; reported adjusted mean ± standard
327 error reflects untransformed data.

328 Where ANCOVAs were not performed, GF and SPF SW mice were compared
329 and further analyzed within both microbial statuses by presence of gallstones and sex.
330 Age and body weight values were also compared between GF and SPF SW mice
331 analyzed using a two-sample test of group means assuming equal variance (two-tailed),
332 and reported as mean ± standard error. Glucose tolerance testing data was analyzed using
333 a two-sample test of group means (two-tailed), for comparison between groups at
334 baseline and to determine the level of statistical significance when the difference between
335 the mean area under the curves (AUC), determined by the trapezoidal rule with baselines
336 set at zero, of two groups was considered. Median pathology scores were compared

between groups using a Mann-Whitney two-sample rank-sum test. To analyze gallbladder muscle activity, a one-way analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons between groups was used.

Statistical analysis was performed using STATA/IC 13.0 for Mac (StataCorp; College Station, TX) and Prism Version 5.0 (GraphPad Software; La Jolla, CA), with $p<0.05$ considered statistically significant.

RESULTS

GF SW mice had markedly enlarged gallbladders, irrespective of gallstones

Necropsy of GF SW mice revealed that 169 of 224 mice (75%) showed gallbladders containing grossly visible, variably sized gallstones numbering from few to numerous (Table 1; Figure 1A-C). Fasted and non-fasted GF SW mice had markedly enlarged gallbladders that commonly measured 1.0 cm long by 0.5 cm wide (Figure 1A-B). Most SPF SW mice (77%; 98/128) displayed normal appearing gallbladders with no gross evidence of gallstones (Table 1). However, 15 female and 15 male (23%) gallbladders contained gallstones (Table 1).

GF SW mice (n = 12; 5 females, 4 males with gallstones) exhibited greater gallbladder volumes (179.0 ± 18.8 μL; SPF SW mice: 73.6 ± 11.3 μL) and lower pH of gallbladder bile (6.8 ± 0.1; SPF SW mice: 7.4 ± 0.1) compared to SPF SW mice (n = 29; 1 female, 5 males with gallstones). Statistically significant differences were unrelated to presence of gallstones, age or body weight, but related to microbial status [gallbladder volume: $F(1, 35) = 20.37$, $p<0.001$; gallbladder bile pH: $F(1, 35) = 11.56$, $p<0.01$] and sex [gallbladder volume: $F(1, 35) = 28.51$, $p<0.0001$; gallbladder bile pH: $F(1, 35) =$

360 10.31, $p<0.01$] (Figure 1D-E). When analysis was stratified by microbial status,
361 differences in bile pH according to sex were found to be non-significant, whereas
362 significant effects were maintained on gallbladder volume in both GF ($p<0.01$) and SPF
363 ($p<0.001$) SW mice, with females (GF SW mice: 229.4 ± 20.2 μL; SPF SW mice: 124.0
364 ± 15.2 μL) containing greater gallbladder volumes than males (GF SW mice: 130.9 ±
365 21.6 μL; SPF SW mice: 25.6 ± 14.0 μL) (Figure 1D-E).

366 **Gallstones developed predominantly in obese, older female GF SW mice**

367 Using logistic regression, the odds of developing gallstones for GF SW mice was 11
368 times those of SPF SW mice, controlled for age and body weight ($p<0.001$) (Table 2).
369 Additionally, a one month increase in age and a one gram increase in body weight of SW
370 mice increased the odds of developing gallstones by 15% ($p<0.01$) and 5% ($p<0.01$),
371 respectively (Table 2). Sex was found non-predictive in the full model, and no
372 interaction or confounding was demonstrated.

373 Stratified logistic regression revealed the odds of developing gallstones for female
374 GF SW mice was 3 times those of males, controlled for age and body weight ($p<0.01$)
375 (Table 3). Further, a one month increase in age and a one gram increase in body weight
376 of GF SW mice increased the odds of developing gallstones by 23% ($p<0.01$) and 8%
377 ($p<0.01$), respectively (Table 3). Of the 169 GF SW mice with gallstones, 105 (62%)
378 were females and 64 (38%) were males of similar age. Of the 55 mice without
379 gallstones, 20 (36%) were females, and 35 (64%) were males. Stratified logistic
380 regression analysis found no significant predictability for presence of gallstones in SPF
381 SW mice, controlling for age, sex and body weight.

Gallstone morphologic features and composition were consistent with "black" pigment gallstones

Gallstones were variable in size (all less than 1 mm), and their color ranged from yellow to dark brown to black. On average, gallstones from GF SW mice were grossly dark in color and durable (Figure 1A-C), whereas SPF SW gallstones were pale and friable.

Gallstones from GF SW mice viewed under direct light microscopy had well defined smooth edges and were yellow to light brown on the outside, with a more pigmented, darker brown core (Figure 1F). Using polarized light microscopy, the outermost aspect of the gallstones was almost translucent and revealed speckles of birefringent material, but not distinct crystals (Figure 1H-I). Direct light microscopy of 1 gallstone sample from an SPF SW mouse showed a few gallstones that were much lighter in color and lacked a dark core (Figure 1G). Direct light microscopy of gallbladder bile from GF and SPF SW mice lacking visible gallstones revealed pale to light brown, amorphous sediment, which was also present in the bile from GF SW mice with gallstones (Figure 1F).

Gallstones from a 15-month-old female GF SW mouse analyzed by the Laboratory for Stone Research by polarized light microscopy and infrared spectroscopy were composed of "100%" calcium bilirubinate; note that no crystalline substances were observed, and acid or neutral salts were not defined, but was likely $Ca(HUCB)_2$ based on gallbladder bile pH. The remainder of gallstones submitted for analysis contained non-crystalline, undefined proteinaceous material.

Cholesterol content was <1% cholesterol content in all gallstone samples analyzed (GF SW females: 0.7%; GF SW males: 0.6%; SPF SW females: 0.1%). Aerobic and anaerobic cultures of GF and SPF SW gallstones and gallbladder bile were negative.

EPR spectroscopic analysis supported the presence of bilirubin radicals in SW gallstones

Previous reports have indicated the presence of EPR-detectable transition metals ions, specifically Mn^{2+}, Cu^{2+} and Fe^{3+}, as well as bilirubin radicals in "black" pigment gallstones (7, 13). In our study, EPR-detectable species were identified in samples of gallstones that were washed two (GF SW) and five (GF and SPF SW) times, and in gallbladder bile (GF SW). EPR spectroscopic analysis of the gallstones washed five times from GF and SPF SW mice revealed features consistent with those observed for commercial bilirubin: the signal centered at g = 2.00 indicates a radical species and is attributed to the presence of bilirubin radicals (Figure 2, Top Panel).

Signals from EPR-detectable transition metal ions attributed to Mn^{2+} (g = 2.01, a = 8.9 mT), Cu^{2+} (g = 2.27, a = 16 mT), and Fe^{3+} (g = 4.31) were observed in twice washed gallstones and gallbladder bile obtained from GF SW mice (Figure 2, Middle Panel). Signals from Mn^{2+} and Cu^{2+} are visible in the g = 2 region of the spectra, and the expected hyperfine patterns (4-line, a = 16 mT from the I = 3/2 Cu nucleus; 6-line, a = 8.9 mT from the I = 5/2 ^{55}Mn nucleus) from these individual species overlap considerably. The observed pattern of lines around g = 2.01 for a gallbladder bile sample (*vide infra*) could be accurately reproduced by the summation of spectra obtained for aqueous solutions of Mn^{2+} and Cu^{2+} (obtained from commercial atomic absorption standard solutions) (Figure 2, Bottom Panel). Thorough washing (5 times) of gallstones

426 from GF SW mice with Chelex-treated Milli-Q water resulted in a loss of the signals
427 attributed to the transition metal ions observed in twice washed gallstones and in
428 gallbladder bile. The loss of the signals was gradual (i.e., decreased signal intensities
429 with more washing); after five washes, the transition metal ions were either undetectable
430 or significantly reduced (<10% of intensity), compared to gallstones washed twice. In
431 contrast to prior studies, our results show that the transition metal ion signals likely arise
432 from the gallbladder bile rather than the gallstones (7, 13).

433 Consistent with the presence of bilirubin radicals, a sharp signal at $g = 2.00$ was
434 also observed in the twice washed gallstone and gallbladder bile samples. In contrast to
435 the transition metal ion signals, this radical signal persisted in the gallstones washed five
436 times, indicating that the signal likely arises from a species in the gallstones themselves
437 (Figure 2, Middle Panel). The possibility of another radical species, or the presence of
438 other radicals that are not detectable under these conditions, cannot be ruled out from
439 these experiments.

440 **Hemograms and urinalysis were normal in GF SW mice, but serum cholesterol was**
441 **elevated, and serum glucose was positively related to increasing age**

442 Of the 23 GF and 6 SPF SW mice evaluated for CBC, 9 female and 7 male GF SW mice
443 had gallstones, while only 1 female SPF SW mouse showed gallstones. There were no
444 statistically significant differences in CBC analytes related to presence of gallstones,
445 microbial status, age, sex or body weight in SW mice, and all analytes were comparable
446 to reference values (Table 4) (14, 21).

447 No statistically significant differences in serum chemistry analytes analyzed by
448 IDEXX from 26 GF SW and 9 SPF SW mice were related to presence of gallstones (GF

SW mice: n = 10 females, 6 males with gallstones; SPF SW: n = 0 with gallstones), but microbial status, age, sex and body weight had significant effect(s) (Table 5). Serum chemistries were unremarkable except for elevated serum cholesterol in GF SW, and elevated serum glucose in GF and SPF SW mice compared to the reference values (Table 5) (21, 39, 40). Differences in serum cholesterol were related to microbial status [$F(1, 23) = 4.96$, $p<0.05$], with GF SW mice (245 ± 12 mg/dL) having higher values than SPF SW mice (174 ± 28 mg/dL), controlled for presence of gallstones, age, sex and body weight. Differences in serum glucose were related to increasing age [$F(1, 29) = 15.29$, $p<0.001$], with the effect pronounced in GF SW mice (238 ± 14 mg/dL, $p<0.01$; SPF SW mice: 219 ± 27 mg/dL). The remaining differences (indirect bilirubin, alanine aminotransferase, blood urea nitrogen, phosphorus) were evaluated but not clinically meaningful, as noted in Table 5.

Urine samples from GF SW mice (n = 14; 8 females, 1 male with gallstones) appeared grossly normal and were negative for bilirubin and glucose. Urobilinogen levels were ≤ 0.2 mg/dL, which was the lowest detectable limit of the urinalysis strip. Ketonuria (5.0 to 80 mg/dL) was observed in 5 female mice, 4 of which had gallstones, and protein levels varied from none to 100 mg/dL. Urine pH was 6.0 in all samples, and the specific gravity of 5 urine samples ranged from 1.010 to 1.025. Urine samples from 5 female SPF SW retired breeders, 2 of which had gallstones, were also negative for glucose, and were otherwise within normal clinical limits.

Glucose tolerance testing in GF and SPF SW mice was normal

Glucose tolerance testing of 9-month-old GF (n = 11; 5 females, 4 males with gallstones) and SPF (n = 12; 3 females, 1 male with gallstones) SW mice was normal (Figure 3).

There were no significant differences in baseline blood glucose between groups, except that GF SW males (174 ± 8 mg/dL) had slightly higher levels compared to GF SW female mice (141 ± 11 mg/dL) ($p<0.05$) (Figure 3). The mean AUCs of all groups were statistically the same (Figure 3). There was no significant difference between the body weights of the GF (48.8 ± 1.0 grams) and SPF (48.5 ± 2.0 grams) SW mice evaluated for diabetes, including by sex, though mice were obese.

Additionally, there were no significant differences in serum glucose (GF SW mice: 167 ± 24 mg/dL; SPF SW mice: 210 ± 22 mg/dL) or insulin (GF SW mice: 2.6 ± 0.9 ng/mL; SPF SW mice: 3.5 ± 0.8 ng/mL) levels of 9-month-old SW mice, related to presence of gallstones, microbial status, age, sex or body weight. There was also no significant difference in HbA1c levels (GF SW mice: 4.3 ± 0.2 %; SPF SW mice: 4.1 ± 0.2 %) of 12-month old SW mice (GF SW mice: n = 8; 4 females, 4 males with gallstones; SPF SW mice: n = 6; 1 female with gallstones), but increasing body weight positively related to serum glucose [$F(1, 9) = 8.08$, $p<0.05$] in SPF SW mice (162 ± 28 mg/dL, $p<0.05$; GF SW mice: 251 ± 22 mg/dL). Note that two HbA1c levels were below the detectable limit, so the lowest registered levels were used for statistical analysis (GF SW mouse: <3.83 %; SPF SW mouse: <3.59 %).

GF SW mice developed low-grade gallbladder and portal inflammation, compared to SPF SW mice

Of the 26 GF SW mice evaluated histologically, 18 mice had gallstones, though presence of gallstones had no effect on gallbladder lesion scores. Tissue samples from SPF SW mice with gallstones were not evaluated histologically, but 12 SPF SW mice without gallstones were examined. Compared to SPF SW mice that had none to minimal

gallbladder pathology (Figure 4E-F), GF SW mice had mild to moderate (i.e. low-grade) inflammation of the gallbladder (median: 1.0; range: 0.3-2.5; $p<0.001$), with mononuclear infiltrates consisting predominantly of lymphocytes, plasma cells and macrophages, with variable numbers of neutrophils and mast cells (Figure 4A-D). Mild to moderate edema (median: 1.0; range: 0.0-2.0; $p<0.05$) and epithelial hyperplasia (median: 1.0; range: 0.0-2.0; $p<0.01$) had also developed, while hyalinosis, metaplasia (GF SW males > females; $p<0.05$) and dysplasia were absent or minimal (Figure 4A-D).

SPF SW mice showed no or only minimal inflammation in the liver, while GF SW mice displayed significantly higher hepatitis index scores (median: 0.5; range: 0.0-4.0; $p<0.05$) than SPF SW mice consisting of minimal to mild mononuclear portal inflammation (median: 0.5; range: 0.0-2.0; $p<0.001$), minimal to mild biliary hyperplasia (associated with gallstones, $p<0.05$), and variable hepatocellular fatty change in a few mice. Three GF SW mice had unrelated liver pathology, including vascular lesions and lymphoma, and hence were not used for quantitative liver lesion analysis.

The pancreas of most mice was normal with adequate size and distribution of islets. However, in a few mice, there was some segmental lobular reduction in islet size/number, and small perivascular and periductal mononuclear cellular aggregates in one or two foci, with or without intra-islet infiltration. The kidneys of a majority of GF and SPF SW mice contained variable degrees of background pathological changes consistent with lymphoma and glomerulonephritis/nephropathy. Of those mice evaluated histologically, GF SW mice (13.0 ± 1.0 months old) were older than SPF SW mice (9.5 ± 0.3 months old) ($p<0.05$), but body weights were the same.

Aged GF and SPF SW mice had decreased basal activity and altered agonist-induced activation of the gallbladder smooth muscle

Gallbladder smooth muscle activity can be assessed by evaluating Ca^{2+} transients under resting conditions and in response to agonist application. Ca^{2+} flashes correspond to synchronous smooth muscle action potentials, which are initiated by interstitial cells of Cajal in the gallbladder, and Ca^{2+} waves are transient increases in Ca^{2+} release from intracellular stores (2, 3, 26). Gallbladder smooth muscle activity was evaluated in 4 10-month-old female GF SW mice with gallstones and 4 age-matched female SPF SW mice, 1 with gallstones. Basal activity of both aged GF and SPF SW mouse gallbladder smooth muscle was quiescent, with only occasional Ca^{2+} waves observed; however, carbachol induced rhythmic, synchronized Ca^{2+} flashes were present in 3 of 4 preparations from both groups (Figure 5). The frequencies of the agonist-induced flashes in aged GF (0.32 ± 0.06 Hz) and SPF (0.42 ± 0.01 Hz) SW mice were comparable, but were slower than the Ca^{2+} flash frequencies observed in 7-10-week-old SPF SW mice (0.63 ± 0.02 Hz; $p<0.05$) 2-10 min after the application of the agonist. In young SPF SW mice, the peak flash frequency in response to carbachol occurred within 2-10 min, and this was also observed in aged SPF SW mice. However, in 2 of the 3 responsive aged GF SW mice, the peak in flash frequency was not reached until 15-18 min.

GF SW mice demonstrated impaired gallbladder emptying in response to CCK

GF (n = 19; 7 females, 8 males with gallstones) and SPF (n = 15; 1 female, 3 males with gallstones) SW mice were evaluated for responsiveness to exogenous CCK by determination of % gallbladder emptying through comparison of gallbladder volumes to mice receiving no CCK (GF SW mice: n = 18; 7 females, 9 males with gallstones; SPF

SW mice: n = 16; 0 females, 5 males with gallstones). Data from control mice injected with sterile PBS or no injection were combined into one group after it was determined that gallbladder volumes were identical between control groups.

Significant differences in gallbladder volume determined by ANCOVA were unrelated to presence of gallstones, age or body weight, but related to microbial status [control mice: $F(1, 29) = 35.82$, $p<0.0001$; experimental mice: $F(1, 29) = 31.60$, $p<0.0001$] and sex [control mice: $F(1, 29) = 8.82$, $p<0.01$] (Figure 6). GF SW mice showed greater gallbladder volumes in both CCK dose groups (control mice: 170.1 ± 10.5 μL; experimental mice: 142.4 ± 12.4 μL), compared to SPF SW mice (control mice: 64.3 ± 11.3 μL; experimental mice: 15.0 ± 14.7 μL) (Figure 6). When analysis was stratified by microbial status, a difference in gallbladder volume in GF SW controls due to sex was found to be non-significant, whereas a significant effect was maintained in SPF SW controls ($p<0.0001$), with females (87.3 ± 15.4 μL) possessing greater gallbladder volumes than males (43.8 ± 11.5 μL) (Figure 6).

No significant difference was found in gallbladder volume related to CCK dose group in GF SW mice, but there was a difference in SPF SW mice ($p<0.0001$), where SPF SW mice receiving CCK (15.0 ± 14.7 μL) showed lower gallbladder volumes than mice in the control group (64.3 ± 11.3 μL) (Figure 6). Compared to SPF SW mice, GF SW mice exhibited substantially reduced gallbladder emptying in response to CCK; GF SW female mice demonstrated 29.0% emptying compared to 89.0% emptying in SPF SW female mice, and only 1.2% emptying occurred in GF SW males, with 53.4% emptying in SPF SW males (Figure 6).

SW mice showed no evidence of induced enterohepatic cycling of unconjugated bilirubin

GF (n = 27; 18 females, 8 males with gallstones) and SPF (n = 22; 3 females, 1 male with gallstones) SW mice were evaluated for EHC of UCB by determination of bilirubin concentrations (μM), bilirubin secretion rates (nmol/hr) and % UCB in the hepatic bile. Significant differences were unrelated to presence of gallstones or body weight, but related to microbial status [conjugated bilirubin concentration: $F(1, 43) = 11.66$, $p<0.01$], age [UCB concentration: $F(1, 43) = 6.12$, $p<0.05$; UCB secretion rate: $F(1, 43) = 4.39$, $p<0.05$; inverse relationships], and sex [conjugated bilirubin concentration: $F(1, 43) = 14.38$, $p<0.001$; % UCB: $F(1, 43) = 13.28$, $p<0.001$] (Figure 7). GF SW mice had lower conjugated bilirubin concentrations (87.6 ± 16.3 μM) compared to SPF SW mice (193.0 ± 19.0 μL) (Figure 7A).

When analysis was stratified by microbial status, differences in % UCB due to sex in GF SW mice, and differences in UCB concentration and secretion rate due to age in GF and SPF SW mice were found non-significant. Significant effects were maintained on conjugated bilirubin concentrations in both GF ($p<0.01$) and SPF ($p<0.05$) SW mice, with females (GF SW mice: 111.4 ± 16.8 μM; SPF SW mice: 216.8 ± 20.7 μM) having greater conjugated bilirubin concentrations than males (GF SW mice: 33.7 ± 22.8 μM; SPF SW mice: 139.1 ± 22.4 μM). Likewise, % UCB in SPF SW mice was greater in males (females: 0.34 ± 0.09 %; males: 0.66 ± 0.10 %; $p<0.05$) (Figure 7A,C).

DISCUSSION

584 This study documented gallstones morphologically and compositionally consistent with
585 "black" pigment gallstones of humans in 84% of females and 65% of males, for an
586 overall prevalence of 75% (169/224) in GF SW mice (23, 33). The classic etiologic
587 associations between "black" pigment gallstones in humans and chronic hemolysis and
588 ineffective erythropoiesis were not detected in GF SW mice, as hemograms reflected
589 normal erythroid values and morphology. Likewise, GF SW mice did not have increased
590 concentration, secretion rate or % of UCB in hepatic bile, showing a lack of EHC of
591 UCB. Markedly enlarged gallbladders were observed in GF SW mice with impaired
592 CCK-induced gallbladder emptying and inactive Ca^{2+} responses, consistent with an
593 inherent abnormal gastrointestinal physiology in GF mice characterized by slower
594 intestinal transit (9, 34, 35, 50, 59). The combination of impaired responsiveness to
595 CCK, weak basal smooth muscle activity and excess sediment may have contributed to
596 biliary stasis, though a strictly mechanical effect on gallbladder motility due to presence
597 of gallstones is highly unlikely, as GF SW mice with and without gallstones had enlarged
598 fasting gallbladders and impaired gallbladder emptying in response to CCK. Exposure to
599 gut microbiota also appeared to protect against the formation of "black" pigment
600 gallstones, as only 30 of 128 SPF SW mice developed gallstones (23%). Our findings
601 suggest genetic, age, sex and body weight predispositions, and impaired gallbladder
602 motility, along with a microbiota-associated protective component to the pathogenesis of
603 "black" pigment gallstone formation in SW mice.

604 The apparent genetic predisposition and age related increases in prevalence of
605 "black" pigment gallstones in GF SW mice are similar to the epidemiology of pigment
606 gallstone disease in humans (5, 32). In humans, genetic factors may be responsible for at

607 least 25% of symptomatic gallstone disease, although the true role of heredity is likely
608 underestimated due to undetected asymptomatic prevalence (22, 32, 46). SW mice are an
609 outbred stock with a long history of experimental study since 1932; however, a known
610 genetic predisposition to "black" pigment gallstones in either the GF or SPF status has
611 not been noted (6). Given that pigment gallstones have only been observed in our colony
612 of GF SW mice, and not in 3 other strains of GF mice on distinct genetic backgrounds,
613 the mechanism(s) underlying formation in SW mice may involve one or more
614 spontaneous mutations affecting gastrointestinal physiology, glucoregulatory function or
615 lipopigment metabolism.

616 Specifically, physiologically important mutations or altered regulation may have
617 occurred in genes of the gut-liver axis, such as fibroblast growth factor 15 (FGF15) and
618 CCK, which regulate gallbladder filling and emptying, respectively (8, 11, 37). A recent
619 study established a mechanism in GF SW mice whereby increased tauro-beta-muricolic
620 acid acts as a naturally occurring farnesoid X receptor (FXR) antagonist, with subsequent
621 downregulation of FGF15 (42). In a non-sterile gut, bile acids are known to induce
622 FGF15 synthesis and suppress CCK secretion, with FGF15 opposing actions of CCK on
623 the gallbladder (8, 42). It has been previously shown that GF mice have a lower
624 concentration of CCK in the intestinal tract and delayed intestinal transit (34, 35, 50).
625 One of the roles of the commensal gut microbiota may be to increase CCK concentration,
626 in order to maintain intestinal transit to promote colonization resistance to pathogenic
627 bacteria (34, 35, 50). The interaction between FGF15 and CCK in GF mice has not been
628 studied directly, but it is likely that the above described downregulation of FGF15 and

629 the lower concentration of CCK in the intestinal tract in GF mice both play a role in
630 gallbladder dysfunction (34, 35, 42).

631 Furthermore, our study showed that female GF SW mice are 3 times more likely
632 to develop pigment gallstones than males. Both GF and SPF SW female mice had greater
633 fasting gallbladder volumes compared to males, which may be due to the inhibitory effect
634 of progesterone on the contractility of gastrointestinal smooth muscle, including the
635 gallbladder, acting through multiple signaling pathways (24). Gallbladder stasis can
636 occur in pregnant women and is due to high progesterone increasing fasting residual
637 gallbladder volume and decreased emptying capacity (24).

638 Evaluation of spontaneous and agonist-activated Ca^{2+} transients (increases in
639 intracellular [Ca^{2+}]) in gallbladder smooth muscle has previously been validated as a
640 useful approach for evaluating muscle activity (2, 3). Normal gallbladder smooth muscle
641 activity is typically associated with rhythmic, spontaneous Ca^{2+} flashes that correspond to
642 action potentials occurring simultaneously in all cells of a muscle bundle, and are used as
643 an index of basal smooth muscle tone of the gallbladder (2, 3). Additionally, transient,
644 spontaneous Ca^{2+} waves represent Ca^{2+} release from inositol triphosphate channels (2).
645 Aged GF and SPF SW mice both had deficits in basal and agonist-induced gallbladder
646 smooth muscle activity, compared to young SPF SW mice. Defects in gallbladder
647 muscle function may reflect oxidative stress damage observed with older age, among
648 other factors, and promote formation of a small nucleus of precipitated calcium
649 bilirubinate, the principal component of “black” pigment gallstones, with subsequent
650 growth by accretion (2, 11, 20). Furthermore, free radical attack of singlet oxygen may
651 have contributed to polymerization and oxidation of calcium bilirubinate, wherein free

652 radical signal amplitude likely generated from UCB was linearly correlated with pigment
653 content of gallstones (4, 13, 54).

654 Irrespective of grossly observable gallstones, GF SW mice developed mild to
655 moderate gallbladder inflammation, edema, and epithelial hyperplasia, and mild portal
656 inflammation, compared to SPF SW mice. Gallbladder inflammation may have resulted
657 from the toxic or immune response-modulating properties of UCB, and/or from free
658 radical-mediated oxidative stress (43, 54). Cholecystitis in cholesterol gallstone disease
659 has been associated with impaired gallbladder motility, including altered CCK-induced
660 smooth muscle contraction, but has not been found to contribute to gallbladder stasis in
661 "black" pigment gallstone formation (16, 31, 37, 51). We reason that the observed mild
662 pathology in the gallbladders of GF SW mice both contributed to and resulted from
663 impaired gallbladder motility.

664 The increased prevalence of "black" pigment gallstones, particularly in older and
665 heavier female GF SW mice, is consistent with a previous report by our group.
666 Gallstones lacking cholesterol content were found as an incidental finding in aged, obese
667 female SPF SW mice that were part of a breeding colony used to characterize a male-
668 predominant SPF SW mouse model of non-insulin dependent diabetes mellitus (29).
669 Type II diabetes mellitus was not substantiated in GF or SPF SW mice by normal glucose
670 tolerance testing, mean fasting serum glucose levels below 300 mg/dL, an absence of
671 glucosuria, and normal insulin and HbA1c levels (29, 39, 40). Although, there was a
672 positive relationship between serum glucose and age in GF SW mice. Hyperglycemia
673 inhibits bile secretion from the liver and impairs gallbladder contraction, leading to bile
674 stasis and gallstone formation, and is augmented by diabetic autonomic neuropathy (5,

12, 37, 54). One study found that diabetic Taiwanese were twice as likely to develop presumed pigment gallstones, compared to non-diabetic patients (5, 15). Increased risk for both pigment and cholesterol gallstones in humans with diabetes mellitus is most likely due to metabolic syndrome, and is confounded by age, obesity and a family history of gallstones (5, 45, 46). Epigenetic factors, specifically variations in gut microbiota, have been causally linked to the development of diabetes (1, 16, 28). A potential genetic predisposition to diabetes and/or a tendency for development of metabolic syndrome in SW mice, combined with differences in exposure to microbes, all likely play a role in the observed variations in glucoregulatory function and lipid metabolism in SW mice.

As in cholesterol gallstone disease, cholesterol may also play a role in the observed increased fasting gallbladder volumes and impaired CCK-induced gallbladder emptying in GF SW mice documented in this study. Biliary hypersecretion of cholesterol can cause gallbladder immotility, and prolonged intestinal transit may allow for hyperabsorption of cholesterol from the gut (27, 36, 37, 58, 60, 61). Cholesterol incorporation into the sarcolemmal membranes of gallbladder and intestinal smooth muscle cells decreases turnover of CCK-1R, the cognate receptor of CCK-8, with subsequent interrupted ligand-receptor interaction, thus impairing muscle contraction through blocked CCK signaling (10, 36, 37, 58, 61). This relationship has been elucidated with a targeted deletion of CCK-1R in mice showing increased gallstone susceptibility, delayed small intestinal transit and increased biliary cholesterol secretion, and more recently in CCK knockout mice with enlarged fasting gallbladder volumes and impaired postprandial response of the gallbladder (57, 58, 61). Also, increased

susceptibility to cholesterol gallstones in GF mice compared to mice with indigenous microbiota was related to larger gallbladders and gallbladder inflammation (16).

One study using human subjects found that patients with "black" pigment gallstones had moderately impaired gallbladder motility characterized by delayed and incomplete postprandial emptying, but these patients had normal fasting gallbladder volumes and biliary cholesterol saturation indices (36). Irrespective that Portincasa et al. reported human patients with "black" pigment gallstones do not have excess biliary cholesterol, this mechanism is still worthwhile to explore in GF SW mice with known delayed intestinal transit and increased serum cholesterol levels (36, 37). The hypomotile gallbladder of GF SW mice may not only be prolonging the residence time of UCB, but also of cholesterol (36, 58). Defective interaction of CCK with CCK-1R may also explain why GF SW mice did not respond to exogenously administered CCK as robustly as SPF SW mice. Another possibility is that, because of the lower CCK concentration in the small intestine of GF mice, receptors may be present in lower numbers. A combination of decreased intestinal concentration of CCK and density of CCK-1 receptors, from cholesterol incorporation in the gallbladder and/or receptor downregulation, may both contribute to the major defects observed in gallbladder motility and subsequent "black" pigment gallstone formation in GF SW mice.

This study documents a systematic and detailed description of a new animal model of "black" pigment gallstone formation, and suggests additional experiments to elucidate the molecular mechanism(s) that are responsible for cholelithogenesis. Further studies could probe the possibility of biliary cholesterol supersaturation as a factor in the observed impaired gallbladder motility in GF SW mice. This work could involve

complete hepatic and gallbladder bile chemistry profiles of GF and SPF SW mice, tests to determine cholesterol content in the gallbladder smooth muscle, and gene expression analyses, most importantly of CCK-1R. Future experiments should also explore the altered gut-liver axis in a sterile gut, specifically the interplay of FGF15 and CCK on gallbladder function, and the gallstone protective components of the commensal microbiota.

We theorize that features of GF physiology, including decreased intestinal CCK concentration and delayed intestinal transit, as well as an apparent genetic predisposition of the SW stock, contributed to the spontaneous formation of "black" pigment gallstones in GF SW mice. It is likely that histomorphological alterations in the gallbladder, progesterone in females, increasing serum glucose with age, obesity and a predisposition to diabetes and/or metabolic syndrome, and elevated serum cholesterol all played a role in the increased fasting residual gallbladder volume, weak basal gallbladder smooth muscle activity and impaired CCK-induced gallbladder emptying. GF SW mice should continue to be a valuable animal model to study impaired gallbladder motility as one contributing cause of "black" pigment gallstones in humans in the absence of hyperbilirubinbilia.

ACKNOWLEDGMENTS

We thank Shikshya Shrestha and Jon David P. Andrade for assistance with cholecystectomies, gallbladder bile collection and analysis, as well as their helpful suggestions and critical insights. We also thank Carlos Umana for his precise and dedicated care of the GF SW mouse colony and for greatly assisting with GTT performed

743 under aseptic conditions, and Oscar Acevedo for GF SW husbandry. Lastly, we
744 acknowledge and thank the necropsy technicians (Lenzie Cheaney, Michelle Graffam,
745 Christian Kaufman, Melissa Mobley, Amanda Potter and Jesse Schacht) who have
746 assisted with sample and data collection.

747

748 **GRANTS**

749 This work was largely supported by NIH training grant T32-OD10978-26 and P30-
750 ES002109 to JGF. We also acknowledge BCM Diabetes and Endocrinology Research
751 Center grant (P30 DK079338), DK080480 and DK62267 to GMM, the services of the
752 Mouse Metabolism Core, DK036588 to MCC, and the Kinship Foundation Searle
753 Scholar Award to EMN.

754

755 **References**

756

757 1. **Alam C, Bittoun E, Bhagwat D, Valkonen S, Saari A, Jaakkola U, Eerola E,**
758 **Huovinen P, and Hanninen A**. Effects of a germ-free environment on gut immune
759 regulation and diabetes progression in non-obese diabetic (NOD) mice. *Diabetologia* 54:
760 1398-1406, 2011.
761 2. **Balemba OB, Heppner TJ, Bonev AD, Nelson MT, and Mawe GM**. Calcium
762 waves in intact guinea pig gallbladder smooth muscle cells. *Am J Physiol Gastrointest*
763 *Liver Physiol* 291: G717-727, 2006.
764 3. **Balemba OB, Salter MJ, Heppner TJ, Bonev AD, Nelson MT, and Mawe**
765 **GM**. Spontaneous electrical rhythmicity and the role of the sarcoplasmic reticulum in the
766 excitability of guinea pig gallbladder smooth muscle cells. *Am J Physiol Gastrointest*
767 *Liver Physiol* 290: G655-664, 2006.
768 4. **Cahalane MJ, Neubrand MW, and Carey MC**. Physical-chemical pathogenesis
769 of pigment gallstones. *Semin Liver Dis* 8: 317-328, 1988.
770 5. **Chen CY, Lu CL, Huang YS, Tam TN, Chao Y, Chang FY, and Lee SD**. Age
771 is one of the risk factors in developing gallstone disease in Taiwan. *Age Ageing* 27: 437-
772 441, 1998.
773 6. **Chia R, Achilli F, Festing MF, and Fisher EM**. The origins and uses of mouse
774 outbred stocks. *Nat Genet* 37: 1181-1186, 2005.

7. **Chikvaidze E, Tabutsadze T, Gogoladze T, Datuashvili G, and Iremashvili B**. Ternary complexes of albumin-Mn (II)-bilirubin and Electron Spin Resonance studies of gallstones. *Georgian Med News* 11-15, 2009.
8. **Choi M, Moschetta A, Bookout AL, Peng L, Umetani M, Holmstrom SR, Suino-Powell K, Xu HE, Richardson JA, Gerard RD, Mangelsdorf DJ, and Kliewer SA**. Identification of a hormonal basis for gallbladder filling. *Nat Med* 12: 1253-1255, 2006.
9. **Coates ME, and Gustafsson BE** editors. *The Germ-free Animal in Biomedical Research*. London: Laboratory Animals LTD, 1984, p. 141-213, 291-332.
10. **Cong P, Pricolo V, Biancani P, and Behar J**. Effects of cholesterol on CCK-1 receptors and caveolin-3 proteins recycling in human gallbladder muscle. *Am J Physiol Gastrointest Liver Physiol* 299: G742-750, 2010.
11. **Debray D, Rainteau D, Barbu V, Rouahi M, El Mourabit H, Lerondel S, Rey C, Humbert L, Wendum D, Cottart CH, Dawson P, Chignard N, and Housset C**. Defects in gallbladder emptying and bile acid homeostasis in mice with cystic fibrosis transmembrane conductance regulator deficiencies. *Gastroenterology* 142: 1581-1591 e1586, 2012.
12. **Ding X, Lu CY, Mei Y, Liu CA, and Shi YJ**. Correlation between gene expression of CCK-A receptor and emptying dysfunction of the gallbladder in patients with gallstones and diabetes mellitus. *Hepatobiliary Pancreat Dis Int* 4: 295-298, 2005.
13. **Elek G, and Rockenbauer A**. The free radical signal of pigment gallstones. *Klin Wochenschr* 60: 33-35, 1982.
14. **Everds NE**. Hematology of the Laboratory Mouse. In: *The Mouse in Biomedical Research: Normative Biology, Husbandry, and Models*, edited by Fox JG, Barthold SW, Davisson MT, Newcomer CE, Quimby FW, and Smith AL. San Diego, CA: Elsevier, Inc., 2007, p. 133-170.
15. **Feldman M, and Feldman M, Jr.** The incidence of cholelithiasis, cholesterosis, and liver disease in diabetes mellitus: an autopsy study. *Diabetes* 3: 305-307, 1954.
16. **Fremont-Rahl JJ, Ge Z, Umana C, Whary MT, Taylor NS, Muthupalani S, Carey MC, Fox JG, and Maurer KJ**. An analysis of the role of the indigenous microbiota in cholesterol gallstone pathogenesis. *PLoS One* 8: e70657, 2013.
17. **Freudenberg F, Broderick AL, Yu BB, Leonard MR, Glickman JN, and Carey MC**. Pathophysiological basis of liver disease in cystic fibrosis employing a DeltaF508 mouse model. *Am J Physiol Gastrointest Liver Physiol* 294: G1411-1420, 2008.
18. **Freudenberg F, Leonard MR, Liu SA, Glickman JN, and Carey MC**. Pathophysiological preconditions promoting mixed "black" pigment plus cholesterol gallstones in a DeltaF508 mouse model of cystic fibrosis. *Am J Physiol Gastrointest Liver Physiol* 299: G205-214, 2010.
19. **Ghumman CA, Moutinho AM, Santos A, Tolstogouzov A, and Teodoro OM**. TOF-SIMS study of cystine and cholesterol stones. *J Mass Spectrom* 47: 547-551, 2012.
20. **Gomez-Pinilla PJ, Pozo MJ, and Camello PJ**. Aging impairs neurogenic contraction in guinea pig urinary bladder: role of oxidative stress and melatonin. *Am J Physiol Regul Integr Comp Physiol* 293: R793-803, 2007.

21. **Jacoby RO, Fox JG, and Davisson M**. Biology and Diseases of Mice. In: *Laboratory Animal Medicine*, edited by Fox JG, Anderson LC, Loew FM, and Quimby FW. San Diego, CA: Elsevier, Inc., 2002, p. 43-44.
22. **Katsika D, Grjibovski A, Einarsson C, Lammert F, Lichtenstein P, and Marschall HU**. Genetic and environmental influences on symptomatic gallstone disease: a Swedish study of 43,141 twin pairs. *Hepatology* 41: 1138-1143, 2005.
23. **Kim IS, Myung SJ, Lee SS, Lee SK, and Kim MH**. Classification and nomenclature of gallstones revisited. *Yonsei Med J* 44: 561-570, 2003.
24. **Kline LW, and Karpinski E**. Progesterone inhibits gallbladder motility through multiple signaling pathways. *Steroids* 70: 673-679, 2005.
25. **Lavoie B, Balemba OB, Godfrey C, Watson CA, Vassileva G, Corvera CU, Nelson MT, and Mawe GM**. Hydrophobic bile salts inhibit gallbladder smooth muscle function via stimulation of GPBAR1 receptors and activation of KATP channels. *J Physiol* 588: 3295-3305, 2010.
26. **Lavoie B, Balemba OB, Nelson MT, Ward SM, and Mawe GM**. Morphological and physiological evidence for interstitial cell of Cajal-like cells in the guinea pig gallbladder. *J Physiol* 579: 487-501, 2007.
27. **Lavoie B, Nausch B, Zane EA, Leonard MR, Balemba OB, Bartoo AC, Wilcox R, Nelson MT, Carey MC, and Mawe GM**. Disruption of gallbladder smooth muscle function is an early feature in the development of cholesterol gallstone disease. *Neurogastroenterol Motil* 24: e313-324, 2012.
28. **Le Roy T, Liopis M, Bruneau A, Rabot S, Bevilacqua C, Martin P, Walker F, Bado A, Perlemuter G, Cassard-Doulcier AM, and Gerard P**. Gut microbiota transplantation demonstrates its causal role in the development of type 2 diabetes and fatty liver. *Abstracts of The International Liver Congress 2012 - 47th Annual Meeting of the European Association for the Study of the Liver* S23, 2012.
29. **Lemke LB, Rogers AB, Nambiar PR, and Fox JG**. Obesity and non-insulin-dependent diabetes mellitus in Swiss-Webster mice associated with late-onset hepatocellular carcinoma. *J Endocrinol* 199: 21-32, 2008.
30. **Levant JA, Kun TL, Jachna J, Sturdevant RA, and Isenberg JI**. The effects of graded doses of C-terminal octapeptide of cholecystokinin on small intestinal transit time in man. *Am J Dig Dis* 19: 207-209, 1974.
31. **Martinez-Cuesta MA, Moreno L, Morillas J, Ponce J, and Esplugues JV**. Influence of cholecystitis state on pharmacological response to cholecystokinin of isolated human gallbladder with gallstones. *Dig Dis Sci* 48: 898-905, 2003.
32. **Nakeeb A, Comuzzie AG, Martin L, Sonnenberg GE, Swartz-Basile D, Kissebah AH, and Pitt HA**. Gallstones: genetics versus environment. *Ann Surg* 235: 842-849, 2002.
33. **Ostrow JD**. The etiology of pigment gallstones. *Hepatology* 4: 215S-222S, 1984.
34. **Pen J, and Welling GW**. The concentration of cholecystokinin in the intestinal tract of germ-free and control mice. *Antonie Van Leeuwenhoek* 47: 84-85, 1981.
35. **Pen J, and Welling GW**. Influence of the microbial flora on the amount of CCK8- and secretin21-27-like immunoreactivity in the intestinal tract of mice. *Comp Biochem Physiol B* 76: 585-589, 1983.
36. **Portincasa P, Di Ciaula A, Vendemiale G, Palmieri V, Moschetta A, Vanberge-Henegouwen GP, and Palasciano G**. Gallbladder motility and cholesterol

865 crystallization in bile from patients with pigment and cholesterol gallstones. *Eur J Clin*
866 *Invest* 30: 317-324, 2000.
867 37. **Portincasa P, Di Ciaula A, Wang HH, Palasciano G, van Erpecum KJ,**
868 **Moschetta A, and Wang DQ**. Coordinate regulation of gallbladder motor function in the
869 gut-liver axis. *Hepatology* 47: 2112-2126, 2008.
870 38. **Portincasa P, Moschetta A, Berardino M, Di-Ciaula A, Vacca M, Baldassarre**
871 **G, Pietrapertosa A, Cammarota R, Tannoia N, and Palasciano G**. Impaired
872 gallbladder motility and delayed orocecal transit contribute to pigment gallstone and
873 biliary sludge formation in beta-thalassemia major adults. *World J Gastroenterol* 10:
874 2383-2390, 2004.
875 39. **Quimby FW**. The Mouse. In: *The Clinical Chemistry of Laboratory Animals*,
876 edited by Loeb WF, and Quimby FW. Philadelphia, PA: Taylor & Francis, 1999, p. 3-32.
877 40. **Quimby FW, and Luong RH**. Clinical Chemistry of the Laboratory Mouse. In:
878 *The Mouse in Biomedical Research: Normative Biology, Husbandry, and Models*, edited
879 by Fox JG, Barthold SW, Davisson MT, Newcomer CE, Quimby FW, and Smith AL. San
880 Diego, CA: Elsevier, Inc., 2007, p. 171-216.
881 41. **Rogers AB, Boutin SR, Whary MT, Sundina N, Ge Z, Cormier K, and Fox**
882 **JG**. Progression of chronic hepatitis and preneoplasia in Helicobacter hepaticus-infected
883 A/JCr mice. *Toxicol Pathol* 32: 668-677, 2004.
884 42. **Sayin SI, Wahlstrom A, Felin J, Jantti S, Marschall HU, Bamberg K,**
885 **Angelin B, Hyotylainen T, Oresic M, and Backhed F**. Gut microbiota regulates bile
886 acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally
887 occurring FXR antagonist. *Cell Metab* 17: 225-235, 2013.
888 43. **Shiesh SC, Chen CY, Lin XZ, Liu ZA, and Tsao HC**. Melatonin prevents
889 pigment gallstone formation induced by bile duct ligation in guinea pigs. *Hepatology* 32:
890 455-460, 2000.
891 44. **Spivak W, and Yuey W**. Application of a rapid and efficient h.p.l.c. method to
892 measure bilirubin and its conjugates from native bile and in model bile systems. Potential
893 use as a tool for kinetic reactions and as an aid in diagnosis of hepatobiliary disease.
894 *Biochem J* 234: 101-109, 1986.
895 45. **Stinton LM, Myers RP, and Shaffer EA**. Epidemiology of gallstones.
896 *Gastroenterol Clin North Am* 39: 157-169, vii, 2010.
897 46. **Stokes CS, Krawczyk M, and Lammert F**. Gallstones: environment, lifestyle
898 and genes. *Dig Dis* 29: 191-201, 2011.
899 47. **Su Y, Wu S, Fan Y, Jin J, and Zhang Z**. The preliminary experimental and
900 clinical study of the relationship between the pigment gallstone and intestinal mucosal
901 barrier. *J Gastroenterol Hepatol* 24: 1451-1456, 2009.
902 48. **Trotman BW, Bernstein SE, Bove KE, and Wirt GD**. Studies on the
903 pathogenesis of pigment gallstones in hemolytic anemia: description and characteristics
904 of a mouse model. *J Clin Invest* 65: 1301-1308, 1980.
905 49. **Tsunoda K, Shirai Y, Wakai T, Yokoyama N, Akazawa K, and Hatakeyama**
906 **K**. Increased risk of cholelithiasis after esophagectomy. *J Hepatobiliary Pancreat Surg*
907 11: 319-323, 2004.
908 50. **van der Zee R, and Welling GW**. The effect of exogenous CCK-8 on the transit
909 time and colonization resistance of decontaminated mice. *Antonie Van Leeuwenhoek* 47:
910 82-84, 1981.

51. **van Erpecum KJ, Wang DQ, Moschetta A, Ferri D, Svelto M, Portincasa P, Hendrickx JJ, Schipper M, and Calamita G**. Gallbladder histopathology during murine gallstone formation: relation to motility and concentrating function. *J Lipid Res* 47: 32-41, 2006.
52. **Vercaemst R, Union A, Rosseneu M, De Craene I, De Backer G, and Kornitzer M**. Quantitation of plasma free cholesterol and cholesteryl esters by high performance liquid chromatography. Study of a normal population. *Atherosclerosis* 78: 245-250, 1989.
53. **Vítek L, and Carey MC**. Enterohepatic cycling of bilirubin as a cause of 'black' pigment gallstones in adult life. *Eur J Clin Invest* 33: 799-810, 2003.
54. **Vítek L, and Carey MC**. New pathophysiological concepts underlying pathogenesis of pigment gallstones. *Clin Res Hepatol Gastroenterol* 36: 122-129, 2012.
55. **Vítek L, and Ostrow JD**. Bilirubin chemistry and metabolism; harmful and protective aspects. *Curr Pharm Des* 15: 2869-2883, 2009.
56. **Vítek L, Zelenka J, Zadinova M, and Malina J**. The impact of intestinal microflora on serum bilirubin levels. *J Hepatol* 42: 238-243, 2005.
57. **Wang DQ, Schmitz F, Kopin AS, and Carey MC**. Targeted disruption of the murine cholecystokinin-1 receptor promotes intestinal cholesterol absorption and susceptibility to cholesterol cholelithiasis. *J Clin Invest* 114: 521-528, 2004.
58. **Wang HH, Portincasa P, Liu M, Tso P, Samuelson LC, and Wang DQ**. Effect of gallbladder hypomotility on cholesterol crystallization and growth in CCK-deficient mice. *Biochim Biophys Acta* 1801: 138-146, 2010.
59. **Wostmann BS**. Germfree and Gnotobiotic Animal Models: Background and Applications. Boca Raton, FL: CRC Press, Inc., 1996, p. 19-41, 54-57.
60. **Xiao ZL, Chen Q, Biancani P, and Behar J**. Abnormalities of gallbladder muscle associated with acute inflammation in guinea pigs. *Am J Physiol Gastrointest Liver Physiol* 281: G490-497, 2001.
61. **Xie M, Kotecha VR, Andrade JD, Fox JG, and Carey MC**. Augmented cholesterol absorption and sarcolemmal sterol enrichment slow small intestinal transit in mice, contributing to cholesterol cholelithogenesis. *J Physiol* 590: 1811-1824, 2012.

Figure 1. Gallbladders of germfree (GF) Swiss Webster (SW) mice were markedly enlarged and 75% contained gallstones grossly and microscopically consistent with "black" pigment gallstones. **Panel A**: Twelve-month-old female GF SW mouse with dilated gallbladder containing gallstones (arrow). **B**: Excised gallbladder with gallstones from a 12-month-old female GF SW mouse. Gallstones were present in varying number, size and color, but were often dark brown to black, as pictured here. Black bar indicates 1 cm. **C**: Eppendorf tube containing gallstones and gallbladder bile from a 12-month-old female GF SW mouse. **D**: Gallbladder volumes (μL) and **E**: gallbladder bile pHs of SW mice were reported as adjusted mean ± standard error, with age and body weight fixed at their means (n = 41; mean age: 11.4 months; mean body weight: 44.9 grams). Asterisks indicate level of significance of differences in gallbladder volume and bile pH, related to microbial status, with *** $p<0.001$, ** $p<0.01$; note that the gallbladder bile samples of GF SW mice were acidic. Statistically significant differences in analytes related to sex in the overall model were noted (#), and if also found significant when stratified by microbial status, were marked by a difference in letters (a-b; c-d) (gallbladder volume: GF SW mice: $p<0.01$, SPF SW mice: $p<0.001$). **F**: Gallstones and sediment in gallbladder bile from a 15-month-old female GF SW mouse at 100x magnification, under direct light. **G**: Direct light microscopy of a gallstone from a 15-month-old female specific pathogen-free (SPF) SW mouse viewed at 200x magnification. **H**: Polarized light microscopy at 40x magnification of gallstones present on the mucosal surface of the gallbladder of an 11-month-old GF SW mouse; one gallstone appears to be broken. **I**: High magnification view (100x) of a gallstone in gallbladder bile from an 11-month-old GF SW mouse viewed under polarized light.

Figure 2. Electron paramagnetic resonance (EPR) spectra of gallstones and gallbladder bile. Top Panel: Bilirubin (**A**) and gallstone samples from germfree (GF) (**B**) and specific pathogen-free (SPF) (**C**) Swiss Webster (SW) mice, highlighting the organic radical observed at $g = 2$. **A**: A sample of commercial bilirubin (10 μM in Chelex-treated Milli-Q water) contained a derivative EPR signal centered at $g = 2.00$. Additional features were observed at $g = 2.04$ and $g = 1.98$. **B**: EPR spectrum of gallstones obtained from GF SW mice. Spectrum B contained a derivative feature centered at $g = 2.0$ attributed to bilirubin radicals. An additional feature was observed at $g = 1.98$. Multiple additional features that display weak signal intensities were observed at lower field and may indicate the presence of additional EPR-detectable species in the sample. **C**: EPR spectrum of gallstones from one SPF SW mouse. The spectrum is scaled by 5x to facilitate comparison with spectra A and B. A derivative feature centered at $g = 2.00$ was also observed, and multiple weak features were present in the baseline. Instrument conditions: temperature, 5 K; microwaves, 20.1 μW at 9.4 GHz; modulation amplitude, 1 mT. **Middle Panel**: EPR spectra of gallstones and gallbladder bile from GF SW mice. **D**: Spectrum of twice washed gallstones. **E**: Spectrum of undiluted gallbladder bile. **F:** Spectrum of gallstones washed five times. Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz. **Bottom Panel**: Expanded view of the EPR signals in the $g = 2$ region from spectrum E. The Mn^{2+} and Cu^{2+} signals were obtained from standards of each metal ion (prepared in Milli-Q water). The $Mn^{2+} + Cu^{2+}$ spectrum was generated through a linear combination of the Mn^{2+} and Cu^{2+} standard spectra. Because it was necessary to record the spectra under non-ideal spectroscopic

conditions (higher power) to observe and maximize signals for the transition metal ions, the radical signal at $g \sim 2$ is saturated, resulting in loss of the characteristic derivative signal that is apparent under ideal spectroscopic conditions in the Top and Middle Panels. Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz.

Figure 3. Normal glucose tolerance testing results from 9-month-old germfree (GF) SW (Swiss Webster) and specific pathogen-free (SPF) SW mice. The mean area under the curves (AUC) of all groups compared were statistically the same, including not pictured SPF SW mice with or without gallstones, and SPF SW females or males. **Panel A**: GF SW mice (n = 11; 79.8 ± 6.9) compared to SPF SW mice (n = 12; 93.8 ± 6.9). **B**: GF SW mice with gallstones (n = 9; 80.5 ± 7.9) compared to GF SW mice without gallstones (n = 2; 77.0 ± 15.0). **C**: GF SW females (n = 6; 84.2 ± 9.8) compared to GF SW males (n = 5; 74.6 ± 9.9). Mean baseline blood glucose values were significantly higher in GF SW male mice, compared to GF SW female mice, * $p<0.05$.

Figure 4. H&E images of the range of gallbladder lesions in germfree (GF) Swiss Webster (SW) (A - D) compared to specific pathogen-free (SPF) SW (E & F) mice. **Panel A**: Gallbladder of an 8-month-old male GF SW mouse with gallstones, showing mild sub-epithelial inflammation, edema and epithelial hyalinosis (intensely eosinophilic granular hyaline-like cytoplasmic alteration). **B**: Gallbladder of an 8-month-old female GF SW mouse without gallstones showing moderate mixed (lymphocytic and granulocytic) inflammation of the epithelium and stroma with minimal papillary epithelial projections. **C**: Low magnification image of a gallbladder of an 8-month-old

male GF SW mouse without gallstones, showing prominent papillomatous epithelial hyperplasia, scattered inflammatory cells and edema in the sub-epithelial space/stroma. **D**: Higher magnification of C, showing hyperplastic long columnar epithelium with mostly basal oval nuclei, abundant eosinophilic to vacuolated (mucous) cytoplasm, and an intra-glandular protein cast (arrow). **E and F**: Low and high magnification images of a gallbladder of a 10-month-old male SPF SW mouse with sparse inflammatory cells and mild papillary epithelial hyperplasia. **Bars**: A, B and F = 80 μM; C and E = 160 μM; D = 40 μM.

Figure 5. Gallbladder smooth muscle activity is disrupted in aged germfree (GF) and specific pathogen-free (SPF) Swiss Webster (SW) mice. Ca^{2+} transient recordings from pairs of gallbladder smooth muscle cells (gray and black) showing an age-related disruption in spontaneous activity. Gallbladder smooth muscle cells in young SPF SW mice exhibit synchronized rhythmic Ca^{2+} flashes (upper left panel). Ca^{2+} flash activity is absent in 10-month-old GF and SPF SW mice (center and bottom left panels), where only Ca^{2+} waves were detected. Carbachol (3 uM) induced Ca^{2+} flashes in all 3 groups of mice once peak frequency was reached (right panels; time point indicated above each trace).

Figure 6. Germfree (GF) Swiss Webster (SW) mice showed impaired cholecystokinin (CCK)-induced gallbladder emptying, compared to specific pathogen-free (SPF) SW mice. Gallbladder volumes (μL) of SW mice were reported as adjusted mean ± standard error, with age and body weight fixed at their means (control

mice: n = 34; mean age: 8.0 months; mean body weight: 55.0 grams; experimental mice: n = 34; mean age: 8.0 months; mean body weight: 55.7 grams). Asterisks indicate level of significance of differences in gallbladder volumes of control and experimental mice, related to microbial status, with **** $p<0.0001$. Statistically significant differences in gallbladder volume related to sex in the overall model were noted (#), and if also found significant when stratified by microbial status, were marked by a difference in letters (a-b) (SPF SW mice: $p<0.0001$). A difference in numbers (1-2) denotes a statistically significant difference in gallbladder volume between SPF SW control and experimental mice ($p<0.0001$).

Figure 7. Germfree (GF) Swiss Webster (SW) and specific pathogen-free (SPF) SW mice were comparable in concentration, secretion rate and % of unconjugated bilirubin (UCB) in hepatic bile. Panel A: Bilirubin concentrations (μM), **B**: secretion rates (nmol/hr) and **C**: % UCB of hepatic bile of SW mice were reported as adjusted mean ± standard error, with age and body weight fixed at their means (n = 49; mean age: 11.2 months; mean body weight: 54.8 grams). Asterisks indicate level of significance of difference in conjugated bilirubin concentration, related to microbial status, with ** $p<0.01$. Statistically significant differences in analytes related to sex in the overall model were noted (#), and if also found significant when stratified by microbial status, were marked by a difference in letters (a-b; c-d) (conjugated bilirubin concentration: GF SW mice: $p<0.01$, SPF SW mice: $p<0.05$; % UCB: SPF SW mice: $p<0.05$).

Table 1. Demographic profile of germfree (GF) and specific pathogen-free (SPF) Swiss Webster (SW) mice

Microbial Status	*n*	Gallstone Prevalence	Age (months)		[a] Body Weight (grams)	
			Mean ± SE	Range	Mean ± SE	Range
GF SW Mice						
All	224	75%	10.7 ± 0.2	5 - 22	48.6 ± 0.6	28.6 - 75.6
Gallstones	169		11.1 ± 0.2	5 - 22	49.8 ± 0.7	31.3 - 75.6
No Gallstones	55		9.7 ± 0.4	5 - 17	45.1 ± 1.1	28.6 - 63.8
Females	125	84%	11.0 ± 0.3	5 - 22	48.0 ± 0.8	28.6 - 75.6
Males	99	65%	10.4 ± 0.3	5 - 17	49.3 ± 0.9	29.9 - 66.7
SPF SW Mice						
All	128	23%	10.1 ± 0.2	8 - 15	49.6 ± 0.8	28.6 - 86.8
Gallstones	30		10.2 ± 0.4	8 - 15	50.9 ± 1.3	40.0 - 68.6
No Gallstones	98		10.1 ± 0.2	8 - 15	49.1 ± 0.9	28.6 - 86.8
Females	75	20%	10.5 ± 0.3	8 - 15	50.3 ± 1.1	28.6 - 86.8
Males	53	28%	9.6 ± 0.3	8 - 14	48.6 ± 1.0	33.3 - 63.9

[a] Eight body weight values not provided.

Table 2. Logistic regression models of the relationship between microbial status [germfree (GF), specific pathogen-free (SPF)] of Swiss Webster (SW) mice and presence of gallstones

	OR	95% CI	*p*-value
[a] **Multivariate Full Model** (*n* = 344)			**< 0.0001**
Microbial Status			
GF	11.44 (10.04)	6.57 - 19.93 (6.03 - 16.71)	< 0.001 (< 0.001)
SPF		Reference Group	
Age (months)	1.14 (1.16)	1.04 - 1.26 (1.07 - 1.27)	< 0.01 (< 0.01)
Sex			
Female	1.55 (1.39)	0.92 - 2.60 (0.91 - 2.12)	0.10 (0.13)
Male		Reference Group	
[b] Body Weight (grams)	1.05 (1.03)	1.02 - 1.09 (1.01 - 1.06)	< 0.01 (< 0.05)
[c] **Multivariate Reduced Model** (*n* = 344)			**< 0.0001**
Microbial Status	10.98	6.36 - 18.98	< 0.001
Age (months)	1.15	1.05 - 1.27	< 0.01
[b] Body Weight (grams)	1.05	1.02 - 1.08	< 0.01

[a] In multivariate full model, odds ratios (ORs), 95% confidence intervals (CIs) and p-values are reported as adjusted (crude).

[b] Eight body weight values not provided.

[c] Favored model; excludes sex found non-significant by likelihood-ratio chi-squared test.

Table 3. Logistic regression model of the relationship between independent variables and presence of gallstones in germfree (GF) Swiss Webster (SW) mice

	OR	95% CI	*p*-value
[a] **Multivariate Full Model** (*n* = 220)			**< 0.0001**
Age (months)	1.23 (1.22)	1.08 - 1.40 (1.08 - 1.39)	< 0.01 (< 0.01)
Sex			
Female	3.16 (2.87)	1.58 - 6.29 (1.53 - 5.40)	< 0.01 (< 0.01)
Male		Reference Group	
[b] Body Weight (grams)	1.08 (1.07)	1.04 - 1.13 (1.03 - 1.12)	< 0.001 (< 0.01)

[a] In multivariate full model, odds ratios (ORs), 95% confidence intervals (CIs) and p-values are reported as adjusted (crude). Multivariate full model is favored model; no covariates found non-significant by likelihood-ratio chi-squared test.

[b] Four body weight values not provided.

Table 4. Complete blood count analytes from germfree (GF) and specific pathogen-free (SPF) Swiss Webster (SW) mice

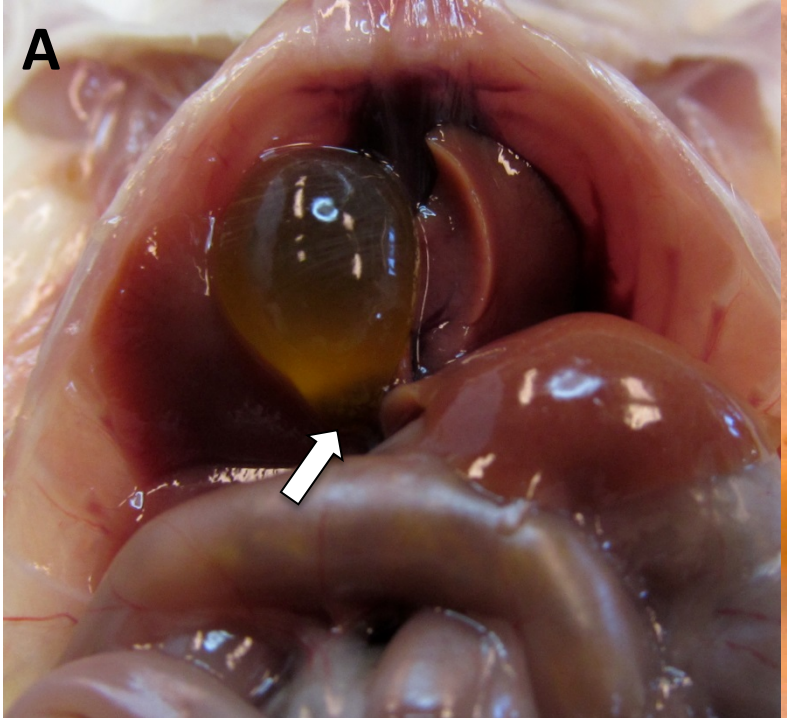
Complete Blood Count	GF SW Mice		SPF SW Mice		Reference Values
	Female (*n*=11)	Male (*n*=12)	Female (*n*=3)	Male (*n*=3)	
White Blood Cell Count (10^3/ul)	4.9 ± 0.8	4.3 ± 0.7	4.0 ± 1.4	4.3 ± 1.4	5.1 - 11.6
Neutrophils	1.2 ± 0.3	1.7 ± 0.2	1.2 ± 0.5	1.7 ± 0.5	0.3 - 4.3
Bands	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	none to few
Lymphocytes	2.6 ± 0.5	2.5 ± 0.5	2.6 ± 0.9	2.5 ± 1.0	3.2 - 8.7
Monocytes	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 - 0.3
Eosinophils	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 - 0.4
Basophils	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 - 0.2
Red Blood Cell Count (10^6/uL)	10.3 ± 0.3	10.7 ± 0.3	9.7 ± 0.5	10.2 ± 0.6	7 - 11
Hematocrit (%)	48.8 ± 1.5	49.7 ± 1.4	51.9 ± 2.6	52.7 ± 2.7	35 - 52
Hemoglobin (g/dL)	13.6 ± 0.3	14.2 ± 0.3	13.9 ± 0.6	14.5 ± 0.6	10 - 17
Platelet Count (10^3/uL)	1239 ± 147	1426 ± 131	1417 ± 246	1604 ± 255	900 - 1600
Mean Corpuscular Volume (fL)	47.7 ± 1.0	46.4 ± 0.9	53.3 ± 1.8	52.0 ± 1.9	45 - 55
Mean Corpuscular Hemoglobin (pg/cell)	13.3 ± 0.3	13.2 ± 0.2	14.3 ± 0.4	14.2 ± 0.5	15 - 18
MCH Concentration (g/dL)	27.9 ± 0.6	28.5 ± 0.6	26.8 ± 1.1	27.4 ± 1.1	30 - 38

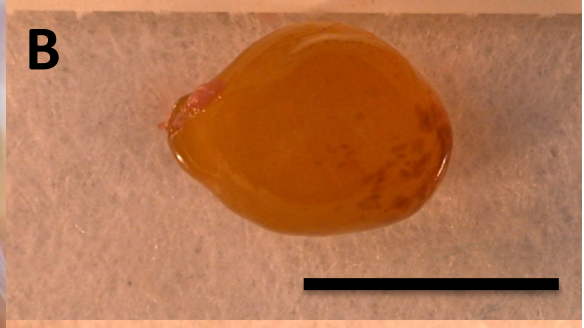
There were no statistically significant differences in analytes determined by ANCOVA related to presence of gallstones, microbial status, age, sex or body weight. GF SW and SPF SW data represent adjusted mean ± standard error, where age and body weight are fixed at their means (n = 29; mean age: 11.1 months; mean body weight: 44.7 grams). Of those analyzed, 16 GF SW mice and one SPF SW mouse had gallstones. Reference data for SW mice are not published, and reference intervals provided represent normative data for the mouse, and are not specific for strain, sex, or age (14, 21).

Table 5. Serum chemistry analytes from germfree (GF) and specific pathogen-free (SPF) Swiss Webster (SW) mice

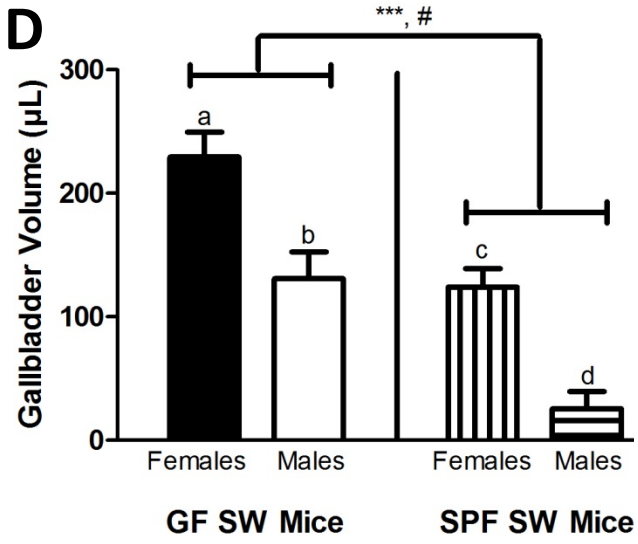
Serum Chemistry	GF SW Mice		SPF SW Mice		Reference Values
	Female (*n*=11)	Male (*n*=15)	Female (*n*=4)	Male (*n*=5)	
Lipid & Carbohydrate Metabolism					
[2*] Cholesterol (mg/dL)	221.5 ± 18.2	264.7 ± 14.9	150.3 ± 28.8	193.5 ± 32.1	114 ± 56
[3a***] Glucose (mg/dL)	226.0 ± 22.3	246.4 ± 16.4	207.7 ± 27.1	228.1 ± 32.0	112 ± 38
Hepatic Function					
Total Bilirubin (mg/dL)	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.4 ± 0.2
Direct Bilirubin (mg/dL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
[3a*; 5a**] Indirect Bilirubin (mg/dL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	N/A
Albumin (g/dL)	3.1 ± 0.1	3.1 ± 0.1	2.8 ± 0.2	2.8 ± 0.2	N/A
Globulin (g/dL)	3.2 ± 0.2	3.3 ± 0.1	3.1 ± 0.3	3.3 ± 0.3	N/A
Total Protein (g/dL)	6.3 ± 0.2	6.5 ± 0.2	5.8 ± 0.3	6.0 ± 0.4	4.4 ± 1.1
[2**] Alanine Aminotransferase (IU/L)	45.7 ± 9.0	35.4 ± 7.1	91.8 ± 14.0	81.5 ± 15.7	99 ± 86
Alkaline Phosphatase (IU/L)	85.3 ± 7.5	71.4 ± 5.9	59.2 ± 11.7	45.3 ± 13.1	39 ± 26
Aspartate Aminotransferase (IU/L)	143.8 ± 33.2	75.9 ± 27.0	133.1 ± 52.4	65.2 ± 58.5	196 ± 133
Renal Function					
[2*; 4a*] Blood Urea Nitrogen (mg/dL)	17.4 ± 1.1	21.1 ± 0.9	21.5 ± 1.7	25.2 ± 1.9	38 ± 20
Creatinine (mg/dL)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	1.1 ± 0.5
Electrolytes, Acid-Base Balance					
Calcium (mg/dL)	10.1 ± 0.2	10.1 ± 0.2	10.4 ± 0.3	10.4 ± 0.3	8.9 ± 2.1
Chloride (mEq/L)	108.9 ± 1.3	111.3 ± 1.0	104.9 ± 1.8	107.4 ± 2.0	125 ± 7.2
[4b*] Phosphorus (mg/dL)	8.6 ± 0.5	10.1 ± 0.4	8.5 ± 0.6	10.0 ± 0.7	8.3 ± 1.5
Potassium (mEq/L)	9.6 ± 0.7	10.2 ± 0.5	8.5 ± 1.0	9.1 ± 1.1	8.0 ± 0.9
Sodium (mEq/L)	154.1 ± 2.2	157.2 ± 1.6	152.4 ± 3.0	155.6 ± 3.3	166 ± 8.6

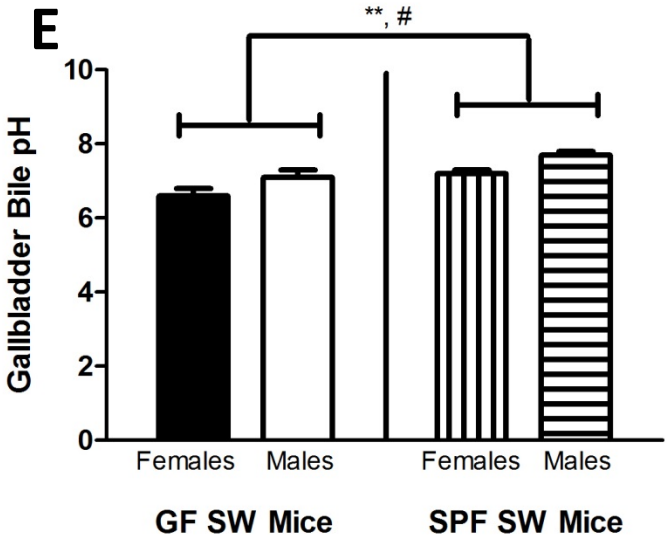
Statistically significant differences in analytes determined by ANCOVA are noted and relate to [1] presence of gallstones, [2] microbial status, [3] age (direct relationship), [4] sex or [5] body weight (inverse relationship), with [a] GF and/or [b] SPF found responsible for significant effect(s) by ANCOVA stratified by microbial status; * $p<0.05$, ** $p<0.01$, *** $p<0.001$. GF SW and SPF SW data represent adjusted mean ± standard error, where age and body weight are fixed at their means (n = 35; mean age: 12.0 months; mean body weight: 43.6 grams). Sixteen GF SW mice had gallstones, while no SPF SW mice analyzed had gallstones. Reference data for SW mice are not published, and reference values provided represent mean ± standard deviation obtained from adult male CD-1 mice (21, 39, 40); N/A indicates no data available.

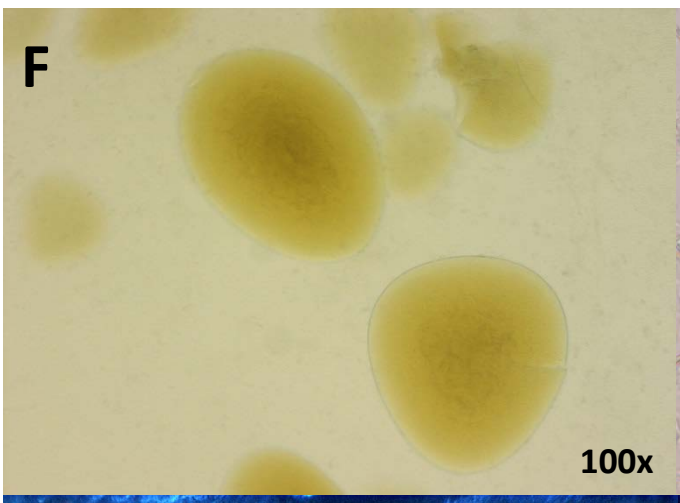

A

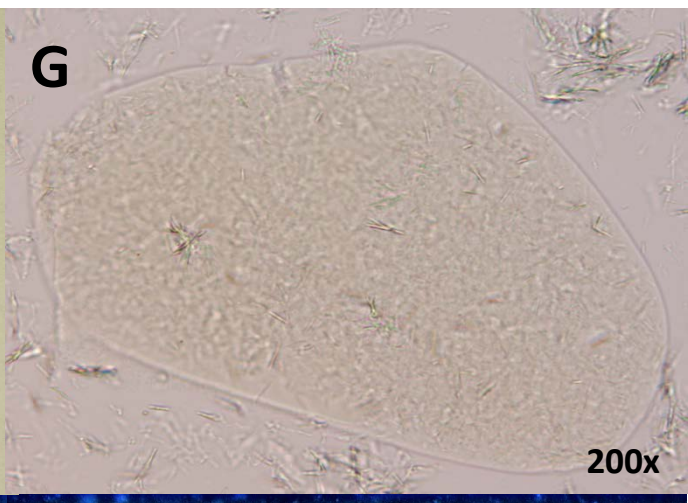

B

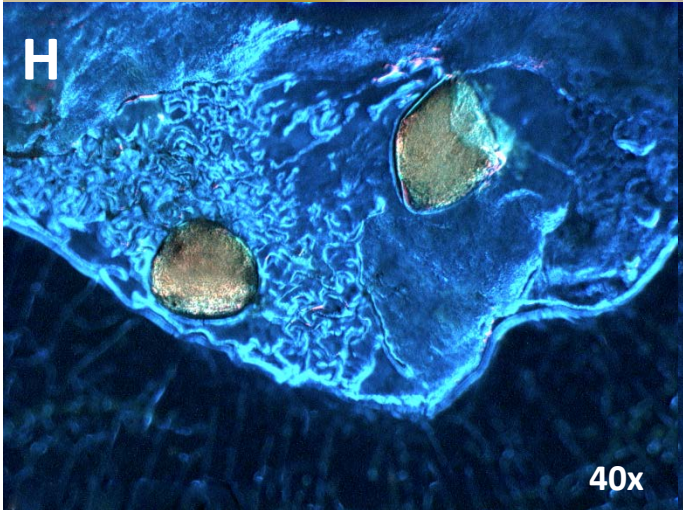

C

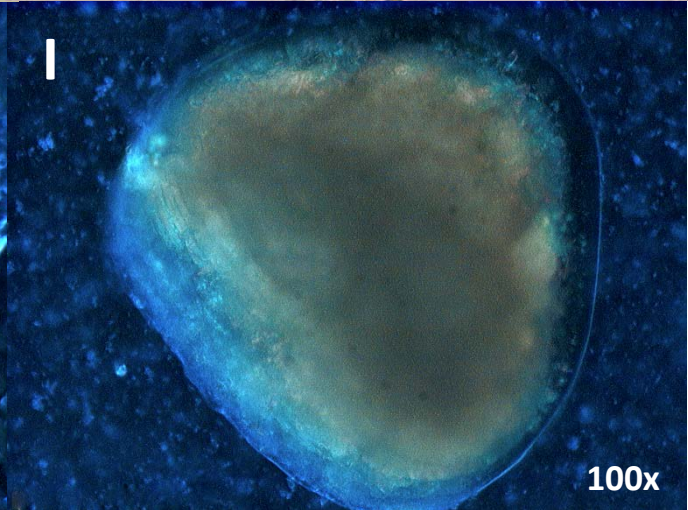

D
***, #
Gallbladder Volume (µL)
300
200
100
0
a
b
c
d
Females
Males
Females
Males
GF SW Mice
SPF SW Mice

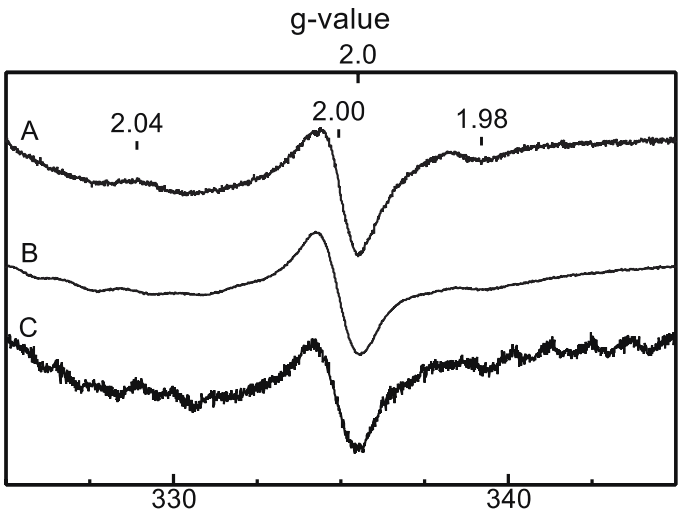

E
**, #
Gallbladder Bile pH
10
8
6
4
2
0
Females
Males
Females
Males
GF SW Mice
SPF SW Mice

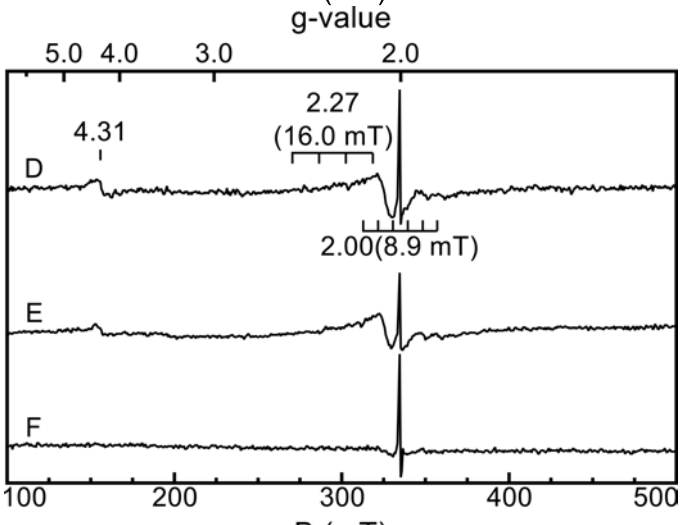

F
100x

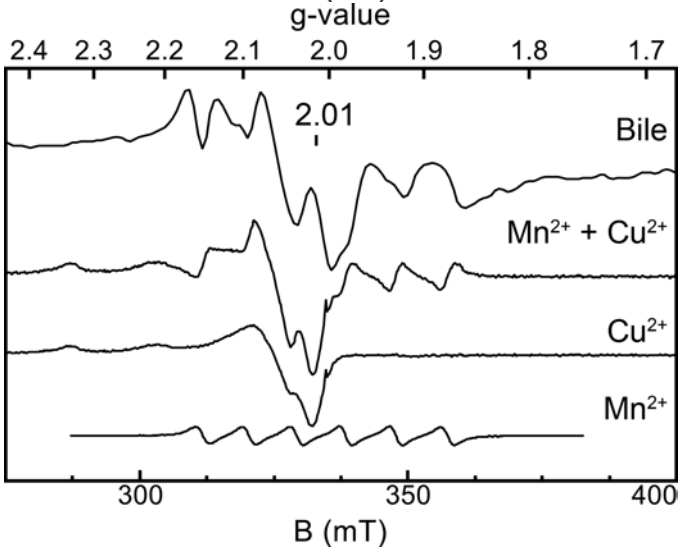

G
200x

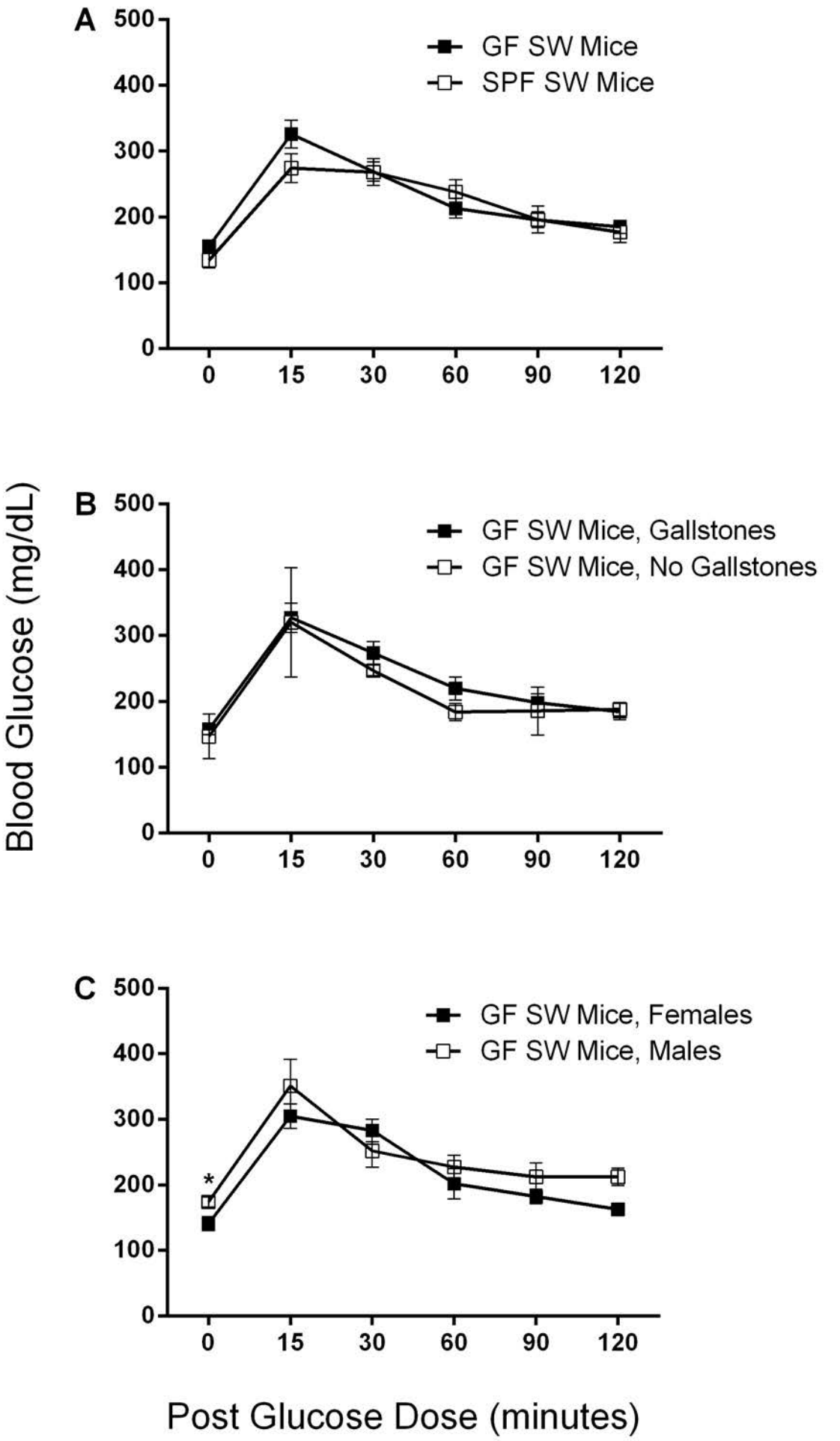

H
40x

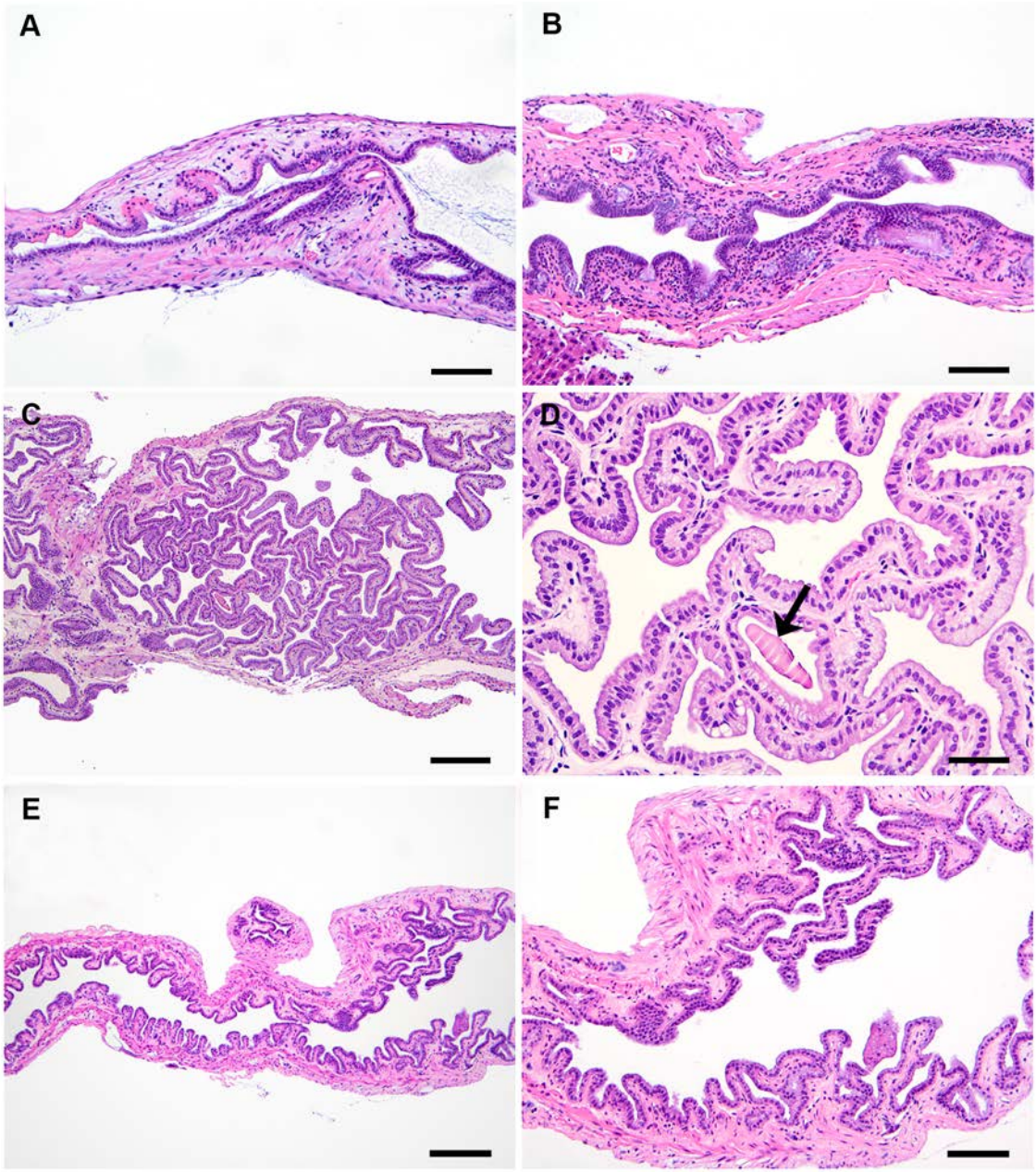

I
100x

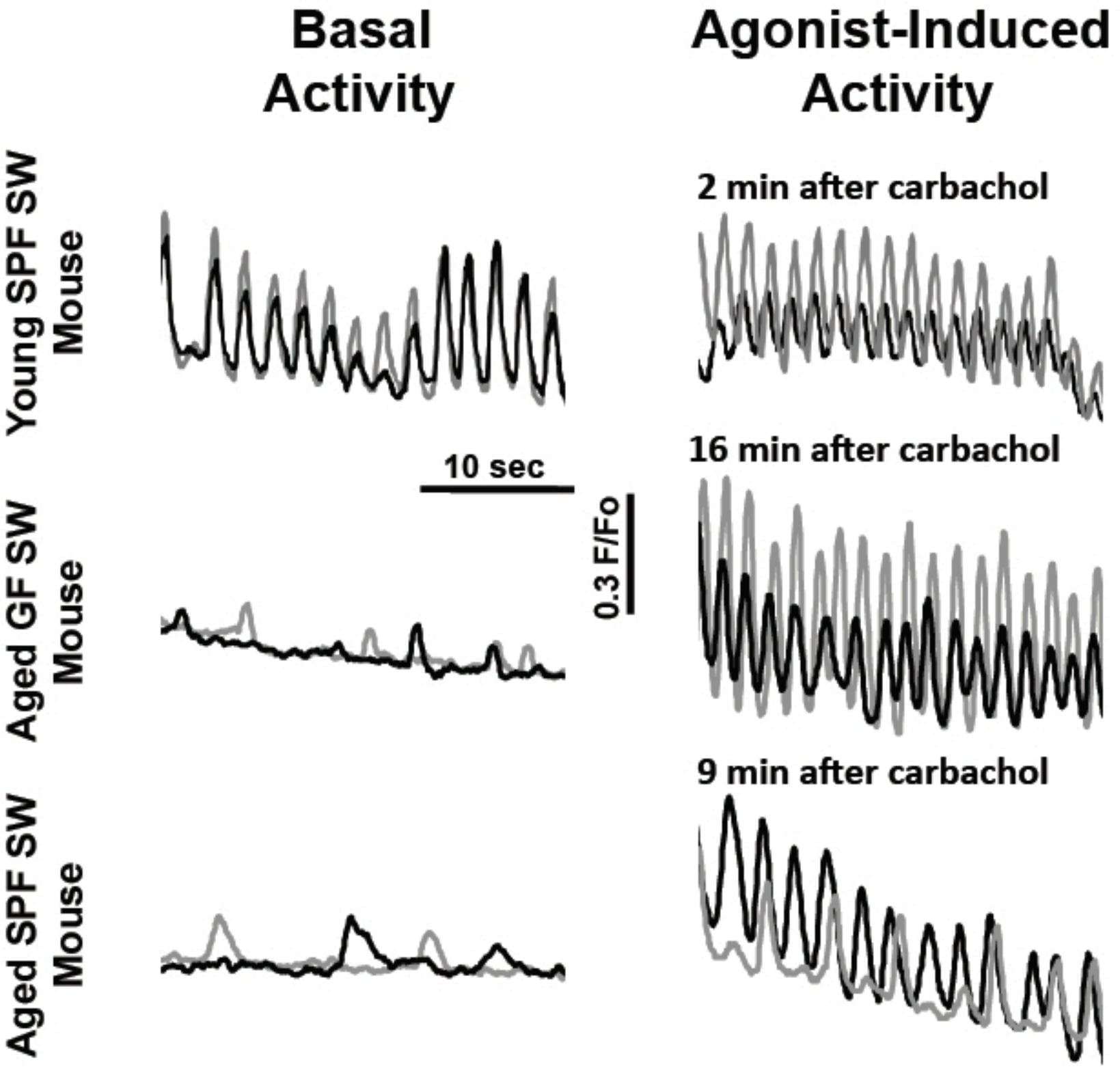

g-value
2.0
2.04
2.00
1.98
A
B
C
330
340
B (mT)

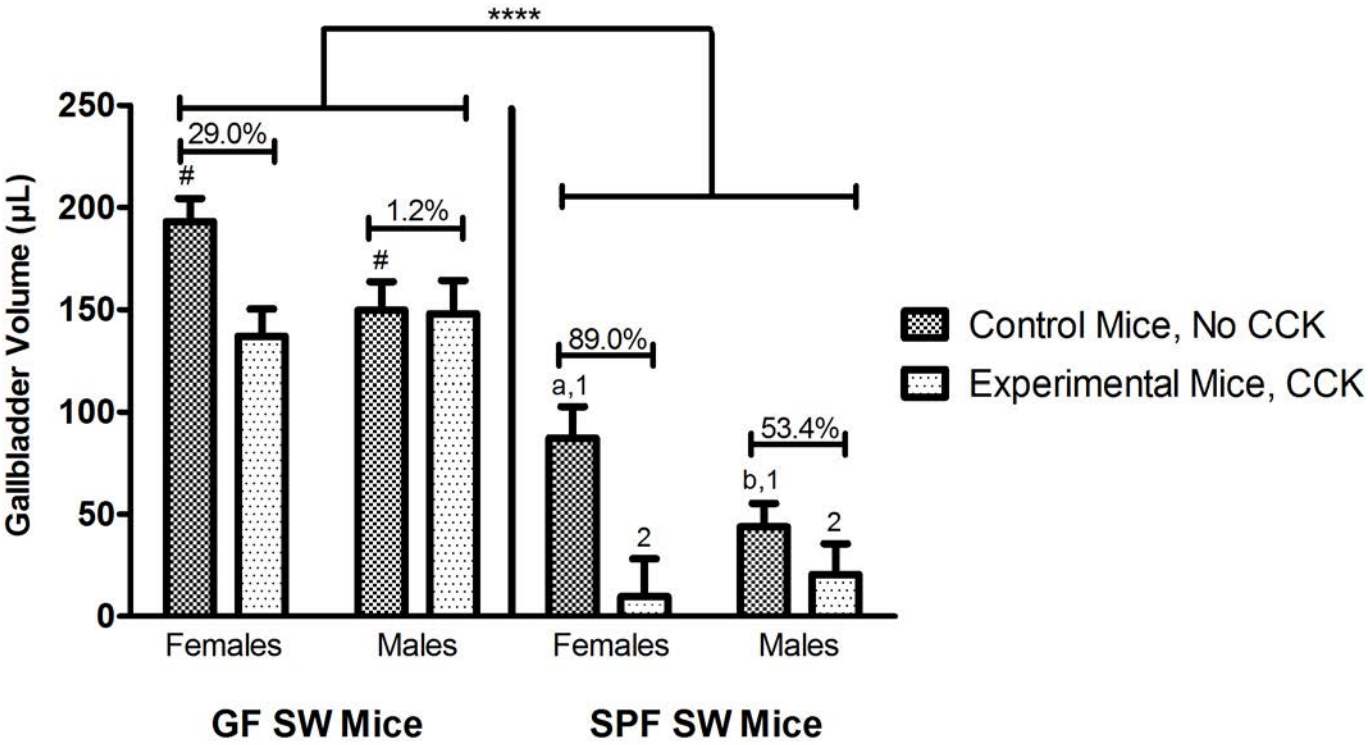

g-value
5.0
4.0
3.0
2.0
4.31
2.27
(16.0 mT)
D
2.00(8.9 mT)
E
F
100
200
300
400
500
B (mT)

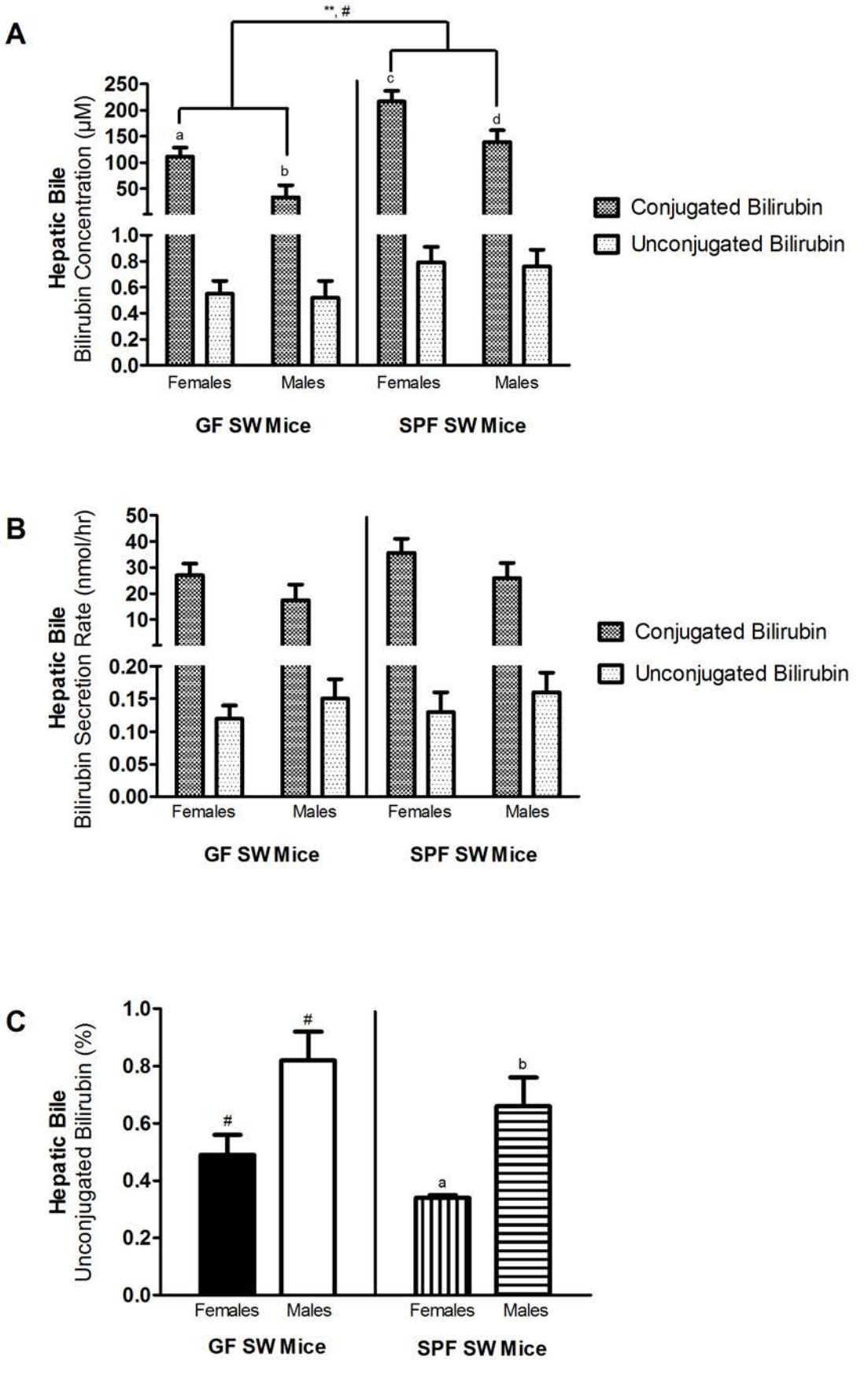

g-value
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
2.01
Bile
Mn2+ + Cu2+
Cu2+
Mn2+
300
350
400
B (mT)


A
500
400
300
200
100
0
GF SW Mice
SPF SW Mice
0
15
30
60
90
120
B
500
400
300
200
100
0
GF SW Mice, Gallstones
GF SW Mice, No Gallstones
0
15
30
60
90
120
Blood Glucose (mg/dL)
C
500
400
300
200
100
0
GF SW Mice, Females
GF SW Mice, Males
*
0
15
30
60
90
120
Post Glucose Dose (minutes)


A
B
C
D
E
F


Basal Activity
Agonist-Induced Activity
Young SPF SW Mouse
Aged GF SW Mouse
Aged SPF SW Mouse
10 sec
0.3 F/Fo
2 min after carbachol
16 min after carbachol
9 min after carbachol


****
250
200
150
100
50
0
Gallbladder Volume (µL)
29.0%
#
1.2%
#
89.0%
a,1
2
53.4%
b,1
2
Females
Males
Females
Males
GF SW Mice
SPF SW Mice
Control Mice, No CCK
Experimental Mice, CCK


A
**, #
Hepatic Bile
Bilirubin Concentration (μM)
250
200
150
100
50
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
d
Females
Males
Females
Males
GF SW Mice
SPF SW Mice
Conjugated Bilirubin
Unconjugated Bilirubin
B
Hepatic Bile
Bilirubin Secretion Rate (nmol/hr)
50
40
30
20
10
0.20
0.15
0.10
0.05
0.00
Females
Males
Females
Males
GF SW Mice
SPF SW Mice
Conjugated Bilirubin
Unconjugated Bilirubin
C
Hepatic Bile
Unconjugated Bilirubin (%)
1.0
0.8
0.6
0.4
0.2
0.0
#
#
a
b
Females
Males
Females
Males
GF SW Mice
SPF SW Mice

"Black" pigment gallstones form in sterile gallbladder bile in the presence of excess bilirubin conjugates from ineffective erythropoiesis, hemolysis or induced enterohepatic cycling (EHC) of unconjugated bilirubin. Impaired gallbladder motility is a less well-studied risk factor. We evaluated the spontaneous occurrence of gallstones in adult germfree (GF) and specific pathogen-free (SPF) Swiss Webster (SW) mice. GF SW mice were more likely to have gallstones than SPF SW mice, with 75% and 23% prevalence, respectively, and were observed predominately in heavier, older females. Gallbladders of GF SW mice were markedly enlarged, contained sterile "black" gallstones comprised of calcium bilirubinate and <1% cholesterol, and had low-grade inflammation, edema and hyperplasia. Hemograms were normal, but serum cholesterol was elevated in GF SW mice, and serum glucose levels were positively related to increasing age. Aged GF and SPF SW mice had deficits in gallbladder smooth muscle activity. In response to cholecystokinin (CCK), gallbladders of fasted GF SW mice showed impaired emptying (females: 29%; males: 1% emptying), whereas SPF SW females and males emptied 89% and 53% of volume, respectively. Bilirubin secretion rates of GF SW mice were not greater than SPF SW mice, repudiating an induced EHC. Gallstones likely developed in GF SW mice due to gallbladder hypomotility, enabled by features of GF physiology, including decreased intestinal CCK concentration and delayed intestinal transit, as well as an apparent genetic predisposition of the SW stock. GF SW mice may provide a valuable model to study gallbladder stasis as a cause of "black" pigment gallstones.