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

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

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

It is known that GF mice have delayed intestinal transit, with documented two times less cholecystokinin (CCK)-like immunoreactivity in the small intestine from rapid degradation of CCK, compared to normally colonized mice, and that CCK acts to promote propulsive activity of the intestine (30, 34, 35, 50, 57, 61). The slower intestinal transit observed in GF mice is reminiscent of the altered peristaltic function in humans and experimental animals with cholesterol gallstone disease (36, 37, 58, 61). Although dysfunction in gallbladder and small intestinal motility has been linked to cholesterol gallstone disease, little is known about how hypomotility of the gallbladder influences “black” pigment gallstone formation (36, 37, 58). Gallbladder dysfunction has been reported in conditions associated with the formation of “black” pigment gallstones, including liver cirrhosis, truncal vagotomy and administration of total parenteral nutrition, and in conditions more often associated with cholesterol gallstones such as obesity and/or type II diabetes (4, 36, 37, 49, 54, 58). With recognized delayed intestinal 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 mΩcm⁻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),
The washed gallstones remaining in the reservoir were re-suspended in the Chelex-treated water (180 µL), transferred to acid-washed (2 M HCl) quartz EPR tubes and frozen in liquid nitrogen prior to analysis and stored at -80°C. A sample of commercial bilirubin [98% (EmM/453 = 60); Sigma-Aldrich] was prepared in Chelex-treated Milli-Q water and frozen in liquid nitrogen prior to analysis. EPR spectra (X-band, 9 GHz) were recorded on a Bruker EMX spectrometer with an ER 4199HS cavity. An ESR900 cryostat outfitted with a Cernox sensor was employed for all measurements. Unless noted otherwise, the modulation amplitude and frequency was 1 mT at 100 kHz. Samples of twice washed gallstones (4 samples pooled into 1 sample) and undiluted gallbladder bile (1 individual sample) from 14-month-old female GF SW mice, as well as gallstones washed five times (4 samples pooled into 1 sample) from 15-month-old GF SW mice were also analyzed.

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

**Screening for Hematopoietic or Metabolic Abnormalities**

Following an overnight fast and carbon dioxide euthanasia, post-mortem cardiac blood was collected for complete blood count (CBC) from 11 female and 12 male GF SW mice (10.8 ± 0.6 months old), and 3 female and 3 male SPF SW mice (12 months old), and for serum chemistry analysis from 11 female and 15 male GF SW mice (12.7 ± 1.1 months old), and 4 female and 5 male SPF SW mice (10 months old). CBCs were measured using a Hemavet 950FS analyzer (Drew Scientific, Waterbury, CT) and serum was sent
to IDEXX Laboratories (Memphis, TN) for a chemistry panel of 21 analytes [Table 5; 3 analytes (bicarbonate, creatine kinase, gamma-glutamyl transferase) excluded due to insufficient quantity for comparison].

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

Glucose tolerance testing (GTT) was performed on 9-month-old GF (n = 6 females, 5 males) and SPF (n = 6 females, 6 males) SW mice. Mice were fasted overnight, weighed, and baseline glucose was measured in blood obtained by tail nick, followed by intraperitoneal injection of 1 gram of 10% dextrose per kg body weight. Blood glucose levels were measured using a glucometer (AlphaTRAK, Abbott Laboratories, Abbott Park, IL) at time 0, 15, 30, 60, 90, and 120 min post glucose dosing. Additional serum samples were collected 2 days later from these same mice after an 8 hr fast for measurement of serum glucose and insulin levels by the Mouse Metabolism Core (MMC; Baylor College of Medicine, Diabetes and Endocrinology Research Center, Houston, TX). Cardiac blood was collected following carbon dioxide 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
determine significance using a chi-squared test to compare the favored logistic models, with or without the set of interaction variables. Confounders were defined as covariates that, when added to the favored model, resulted in ≥10% change in the slope of the major exposure, microbial status. Further, a stratified logistic regression analysis was performed as described above and was segregated by microbial status, with age as the major exposure and sex and body weight as covariates.

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

Where ANCOVAs were not performed, GF and SPF SW mice were compared and further analyzed within both microbial statuses by presence of gallstones and sex. Age and body weight values were also compared between GF and SPF SW mice analyzed using a two-sample test of group means assuming equal variance (two-tailed), and reported as mean ± standard error. Glucose tolerance testing data was analyzed using a two-sample test of group means (two-tailed), for comparison between groups at baseline and to determine the level of statistical significance when the difference between the mean area under the curves (AUC), determined by the trapezoidal rule with baselines 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: \( F(1, 35) = \)}
10.31, p<0.01] (Figure 1D-E). When analysis was stratified by microbial status, differences in bile pH according to sex were found to be non-significant, whereas significant effects were maintained on gallbladder volume in both GF (p<0.01) and SPF (p<0.001) SW mice, with females (GF SW mice: 229.4 ± 20.2 µL; SPF SW mice: 124.0 ± 15.2 µL) containing greater gallbladder volumes than males (GF SW mice: 130.9 ± 21.6 µL; SPF SW mice: 25.6 ± 14.0 µL) (Figure 1D-E).

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

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

Stratified logistic regression revealed the odds of developing gallstones for female GF SW mice was 3 times those of males, controlled for age and body weight (p<0.01) (Table 3). Further, a one month increase in age and a one gram increase in body weight of GF SW mice increased the odds of developing gallstones by 23% (p<0.01) and 8% (p<0.01), respectively (Table 3). Of the 169 GF SW mice with gallstones, 105 (62%) were females and 64 (38%) were males of similar age. Of the 55 mice without gallstones, 20 (36%) were females, and 35 (64%) were males. Stratified logistic regression analysis found no significant predictability for presence of gallstones in SPF 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
from GF SW mice with Chelex-treated Milli-Q water resulted in a loss of the signals attributed to the transition metal ions observed in twice washed gallstones and in gallbladder bile. The loss of the signals was gradual (i.e., decreased signal intensities with more washing); after five washes, the transition metal ions were either undetectable or significantly reduced (<10% of intensity), compared to gallstones washed twice. In contrast to prior studies, our results show that the transition metal ion signals likely arise from the gallbladder bile rather than the gallstones (7, 13).

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

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

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

No statistically significant differences in serum chemistry analytes analyzed by 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 \( \text{Ca}^{2+} \) transients under resting conditions and in response to agonist application. \( \text{Ca}^{2+} \) flashes correspond to synchronous smooth muscle action potentials, which are initiated by interstitial cells of Cajal in the gallbladder, and \( \text{Ca}^{2+} \) waves are transient increases in \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) waves observed; however, carbachol induced rhythmic, synchronized \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) 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
This study documented gallstones morphologically and compositionally consistent with “black” pigment gallstones of humans in 84% of females and 65% of males, for an overall prevalence of 75% (169/224) in GF SW mice (23, 33). The classic etiologic associations between “black” pigment gallstones in humans and chronic hemolysis and ineffective erythropoiesis were not detected in GF SW mice, as hemograms reflected normal erythroid values and morphology. Likewise, GF SW mice did not have increased concentration, secretion rate or % of UCB in hepatic bile, showing a lack of EHC of UCB. Markedly enlarged gallbladders were observed in GF SW mice with impaired CCK-induced gallbladder emptying and inactive Ca\(^{2+}\) responses, consistent with an inherent abnormal gastrointestinal physiology in GF mice characterized by slower intestinal transit (9, 34, 35, 50, 59). The combination of impaired responsiveness to CCK, weak basal smooth muscle activity and excess sediment may have contributed to biliary stasis, though a strictly mechanical effect on gallbladder motility due to presence of gallstones is highly unlikely, as GF SW mice with and without gallstones had enlarged fasting gallbladders and impaired gallbladder emptying in response to CCK. Exposure to gut microbiota also appeared to protect against the formation of “black” pigment gallstones, as only 30 of 128 SPF SW mice developed gallstones (23%). Our findings suggest genetic, age, sex and body weight predispositions, and impaired gallbladder motility, along with a microbiota-associated protective component to the pathogenesis of “black” pigment gallstone formation in SW mice.

The apparent genetic predisposition and age related increases in prevalence of “black” pigment gallstones in GF SW mice are similar to the epidemiology of pigment gallstone disease in humans (5, 32). In humans, genetic factors may be responsible for at
least 25% of symptomatic gallstone disease, although the true role of heredity is likely underestimated due to undetected asymptomatic prevalence (22, 32, 46). SW mice are an outbred stock with a long history of experimental study since 1932; however, a known genetic predisposition to “black” pigment gallstones in either the GF or SPF status has not been noted (6). Given that pigment gallstones have only been observed in our colony of GF SW mice, and not in 3 other strains of GF mice on distinct genetic backgrounds, the mechanism(s) underlying formation in SW mice may involve one or more spontaneous mutations affecting gastrointestinal physiology, glucoregulatory function or lipopigment metabolism.

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

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

Evaluation of spontaneous and agonist-activated Ca\(^{2+}\) transients (increases in
intracellular [Ca\(^{2+}\)]) in gallbladder smooth muscle has previously been validated as a
useful approach for evaluating muscle activity (2, 3). Normal gallbladder smooth muscle
activity is typically associated with rhythmic, spontaneous Ca\(^{2+}\) flashes that correspond to
action potentials occurring simultaneously in all cells of a muscle bundle, and are used as
an index of basal smooth muscle tone of the gallbladder (2, 3). Additionally, transient,
spontaneous Ca\(^{2+}\) waves represent Ca\(^{2+}\) release from inositol triphosphate channels (2).
Aged GF and SPF SW mice both had deficits in basal and agonist-induced gallbladder
smooth muscle activity, compared to young SPF SW mice. Defects in gallbladder
muscle function may reflect oxidative stress damage observed with older age, among
other factors, and promote formation of a small nucleus of precipitated calcium
bilirubinate, the principal component of “black” pigment gallstones, with subsequent
growth by accretion (2, 11, 20). Furthermore, free radical attack of singlet oxygen may
have contributed to polymerization and oxidation of calcium bilirubinate, wherein free
radical signal amplitude likely generated from UCB was linearly correlated with pigment content of gallstones (4, 13, 54).

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

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

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References


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, the spectra may not accurately reflect the true parameters of the metal ions.
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 ($n = 11; 79.8 \pm 6.9$) compared to SPF SW mice ($n = 12; 93.8 \pm 6.9$). **B:** GF SW mice with gallstones ($n = 9; 80.5 \pm 7.9$) compared to GF SW mice without gallstones ($n = 2; 77.0 \pm 15.0$). **C:** GF SW females ($n = 6; 84.2 \pm 9.8$) compared to GF SW males ($n = 5; 74.6 \pm 9.9$). 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

<table>
<thead>
<tr>
<th>Microbial Status</th>
<th>n</th>
<th>Gallstone Prevalence</th>
<th>Age (months)</th>
<th>a Body Weight (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± SE</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td></td>
<td>Mean ± SE</td>
<td>Range</td>
</tr>
<tr>
<td>GF SW Mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>224</td>
<td>75%</td>
<td>10.7 ± 0.2</td>
<td>5 - 22</td>
</tr>
<tr>
<td>Gallstones</td>
<td>169</td>
<td></td>
<td>11.1 ± 0.2</td>
<td>5 - 22</td>
</tr>
<tr>
<td>No Gallstones</td>
<td>55</td>
<td></td>
<td>9.7 ± 0.4</td>
<td>5 - 17</td>
</tr>
<tr>
<td>Females</td>
<td>125</td>
<td>84%</td>
<td>11.0 ± 0.3</td>
<td>5 - 22</td>
</tr>
<tr>
<td>Males</td>
<td>99</td>
<td>65%</td>
<td>10.4 ± 0.3</td>
<td>5 - 17</td>
</tr>
<tr>
<td>SPF SW Mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>128</td>
<td>23%</td>
<td>10.1 ± 0.2</td>
<td>8 - 15</td>
</tr>
<tr>
<td>Gallstones</td>
<td>30</td>
<td></td>
<td>10.2 ± 0.4</td>
<td>8 - 15</td>
</tr>
<tr>
<td>No Gallstones</td>
<td>98</td>
<td></td>
<td>10.1 ± 0.2</td>
<td>8 - 15</td>
</tr>
<tr>
<td>Females</td>
<td>75</td>
<td>20%</td>
<td>10.5 ± 0.3</td>
<td>8 - 15</td>
</tr>
<tr>
<td>Males</td>
<td>53</td>
<td>28%</td>
<td>9.6 ± 0.3</td>
<td>8 - 14</td>
</tr>
</tbody>
</table>

a Eight body weight values not provided.
<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Multivariate Full Model (n = 344)</strong></td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Microbial Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GF</td>
<td>11.44 (10.04)</td>
<td>6.57 - 19.93 (6.03 - 16.71)</td>
<td>&lt; 0.001 (&lt; 0.001)</td>
</tr>
<tr>
<td>SPF</td>
<td>Reference Group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (months)</td>
<td>1.14 (1.16)</td>
<td>1.04 - 1.26 (1.07 - 1.27)</td>
<td>&lt; 0.01 (&lt; 0.01)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.55 (1.39)</td>
<td>0.92 - 2.60 (0.91 - 2.12)</td>
<td>0.10 (0.13)</td>
</tr>
<tr>
<td>Male</td>
<td>Reference Group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b Body Weight (grams)</td>
<td>1.05 (1.03)</td>
<td>1.02 - 1.09 (1.01 - 1.06)</td>
<td>&lt; 0.01 (&lt; 0.05)</td>
</tr>
<tr>
<td><strong>Multivariate Reduced Model (n = 344)</strong></td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Microbial Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GF</td>
<td>10.98</td>
<td>6.36 - 18.98</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Age (months)</td>
<td>1.15</td>
<td>1.05 - 1.27</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>b Body Weight (grams)</td>
<td>1.05</td>
<td>1.02 - 1.08</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

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

* Eight body weight values not provided.

* 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

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Multivariate Full Model (n = 220)</strong></td>
<td>&lt; 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (months)</td>
<td>1.23 (1.22)</td>
<td>1.08 - 1.40 (1.08 - 1.39)</td>
<td>&lt; 0.01 (&lt; 0.01)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>3.16 (2.87)</td>
<td>1.58 - 6.29 (1.53 - 5.40)</td>
<td>&lt; 0.01 (&lt; 0.01)</td>
</tr>
<tr>
<td>Male</td>
<td>Reference Group</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Body Weight (grams)</strong></td>
<td>1.08 (1.07)</td>
<td>1.04 - 1.13 (1.03 - 1.12)</td>
<td>&lt; 0.001 (&lt; 0.01)</td>
</tr>
</tbody>
</table>

*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

<table>
<thead>
<tr>
<th>Complete Blood Count</th>
<th>GF SW Mice</th>
<th>SPF SW Mice</th>
<th>Reference Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female (n=11)</td>
<td>Male (n=12)</td>
<td>Female (n=3)</td>
</tr>
<tr>
<td>White Blood Cell Count (10^3/ul)</td>
<td>4.9 ± 0.8</td>
<td>4.3 ± 0.7</td>
<td>4.0 ± 1.4</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.2 ± 0.3</td>
<td>1.7 ± 0.2</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>Bands</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2.6 ± 0.5</td>
<td>2.5 ± 0.5</td>
<td>2.6 ± 0.9</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Red Blood Cell Count (10^6/uL)</td>
<td>10.3 ± 0.3</td>
<td>10.7 ± 0.3</td>
<td>9.7 ± 0.5</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>48.8 ± 1.5</td>
<td>49.7 ± 1.4</td>
<td>51.9 ± 2.6</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.6 ± 0.3</td>
<td>14.2 ± 0.3</td>
<td>13.9 ± 0.6</td>
</tr>
<tr>
<td>Platelet Count (10^3/uL)</td>
<td>1239 ± 147</td>
<td>1426 ± 131</td>
<td>1417 ± 246</td>
</tr>
<tr>
<td>Mean Corpuscular Volume (fL)</td>
<td>47.7 ± 1.0</td>
<td>46.4 ± 0.9</td>
<td>53.3 ± 1.8</td>
</tr>
<tr>
<td>Mean Corpuscular Hemoglobin (pg/cell)</td>
<td>13.3 ± 0.3</td>
<td>13.2 ± 0.2</td>
<td>14.3 ± 0.4</td>
</tr>
<tr>
<td>MCH Concentration (g/dL)</td>
<td>27.9 ± 0.6</td>
<td>28.5 ± 0.6</td>
<td>26.8 ± 1.1</td>
</tr>
</tbody>
</table>

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>
<thead>
<tr>
<th>Serum Chemistry</th>
<th>GF SW Mice</th>
<th>SPF SW Mice</th>
<th>Reference Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female (n=11)</td>
<td>Male (n=15)</td>
<td>Female (n=4)</td>
</tr>
<tr>
<td>Lipid &amp; Carbohydrate Metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2° Cholesterol (mg/dL)</td>
<td>221.5 ± 18.2</td>
<td>264.7 ± 14.9</td>
<td>150.3 ± 28.8</td>
</tr>
<tr>
<td>3a*** Glucose (mg/dL)</td>
<td>226.0 ± 22.3</td>
<td>246.4 ± 16.4</td>
<td>207.7 ± 27.1</td>
</tr>
<tr>
<td>Hepatic Function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Bilirubin (mg/dL)</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Direct Bilirubin (mg/dL)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>3a**, 5a** Indirect Bilirubin (mg/dL)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>3.1 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>3.2 ± 0.2</td>
<td>3.3 ± 0.1</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>Total Protein (g/dL)</td>
<td>6.3 ± 0.2</td>
<td>6.5 ± 0.2</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>2** Alanine Aminotransferase (IU/L)</td>
<td>45.7 ± 9.0</td>
<td>35.4 ± 7.1</td>
<td>91.8 ± 14.0</td>
</tr>
<tr>
<td>Alkaline Phosphatase (IU/L)</td>
<td>85.3 ± 7.5</td>
<td>71.4 ± 5.9</td>
<td>59.2 ± 11.7</td>
</tr>
<tr>
<td>Aspartate Aminotransferase (IU/L)</td>
<td>143.8 ± 33.2</td>
<td>75.9 ± 27.0</td>
<td>133.1 ± 52.4</td>
</tr>
<tr>
<td>Renal Function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dL)</td>
<td>17.4 ± 1.1</td>
<td>21.1 ± 0.9</td>
<td>21.5 ± 1.7</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Electrolytes, Acid-Base Balance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>10.1 ± 0.2</td>
<td>10.1 ± 0.2</td>
<td>10.4 ± 0.3</td>
</tr>
<tr>
<td>Chloride (mEq/L)</td>
<td>108.9 ± 1.3</td>
<td>111.3 ± 1.0</td>
<td>104.9 ± 1.8</td>
</tr>
<tr>
<td>4b Phosphorus (mg/dL)</td>
<td>8.6 ± 0.5</td>
<td>10.1 ± 0.4</td>
<td>8.5 ± 0.6</td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td>9.6 ± 0.7</td>
<td>10.2 ± 0.5</td>
<td>8.5 ± 1.0</td>
</tr>
<tr>
<td>Sodium (mEq/L)</td>
<td>154.1 ± 2.2</td>
<td>157.2 ± 1.6</td>
<td>152.4 ± 3.0</td>
</tr>
</tbody>
</table>

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 6 GF and/or 7 SPF found responsible for significant effect(s) by ANCOVA stratified by microbial status; 1 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.
**Basal Activity**

Young SPF SW Mouse

Aged GF SW Mouse

Aged SPF SW Mouse

**Agonist-Induced Activity**

2 min after carbachol

16 min after carbachol

9 min after carbachol
"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.