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Early genistein exposure of California mice and effects on the gut microbiota–brain axis

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Abstract

Human offspring encounter high amounts of phytoestrogens, such as genistein (GEN), through maternal diet and soy-based formulas. Such chemicals can exert estrogenic activity and thereby disrupt neurobehavioral programming. Besides inducing direct host effects, GEN might cause gut dysbiosis and alter gut metabolites. To determine whether exposure to GEN affects these parameters, California mice (*Peromyscus californicus*) dams were placed 2 weeks prior to breeding and throughout gestation and lactation on a diet supplemented with GEN (250 mg/kg feed weight) or AIN93G phytoestrogen-free control diet (AIN). At weaning, offspring socio-communicative behaviors, gut microbiota and metabolite profiles were assayed. Exposure of offspring to GEN-induced sex-dependent changes in gut microbiota and metabolites. GEN exposed females were less likely to investigate a novel female mouse when tested in a three-chamber social test. When isolated, GEN males and females exhibited increased latency to elicit their first call, suggestive of reduced motivation to communicate with other individuals. Correlation analyses revealed interactions between GEN-induced microbiome, metabolome and socio-communicative behaviors. Comparison of GEN males with AIN males revealed the fraction of calls above 20 kHz was associated with daidzein, α -tocopherol, *Flexispira* spp. and *Odoribacter* spp. Results suggest early GEN exposure disrupts normal socio-communicative behaviors in California mice, which are otherwise evident in these social rodents. Such effects may be due to GEN disruptions on neural programming but might also be attributed to GEN-induced microbiota shifts and resultant changes in gut metabolites. Findings indicate cause for concern that perinatal exposure to GEN may detrimentally affect the offspring microbiome–gut–brain axis.

Key Words

- ▶ phytoestrogens
- ▶ brain
- ▶ intestinal bacteria
- ▶ diet
- ▶ xenoestrogens
- ▶ rodent models
- ▶ bioinformatics
- ▶ autism
- ▶ ASD

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Introduction

Many infants that are fed soy-based formulas are also exposed to plant phytoestrogens/isoflavones, including genistein (GEN). Additionally, fetuses can be exposed to GEN and other phytoestrogens through the maternal diet. Such chemicals can act similarly to estradiol (E2) and presumably disrupt normal developmental processes, including gonad and brain sexual differentiation. Thus, animal model and human epidemiological studies have been initiated to examine how this chemical affects neonatal development, especially for the reproductive and neurobehavioral systems (Dinsdale & Ward 2010). Early exposure to isoflavones in soy formula is associated with adverse female reproductive consequences later in life (Jefferson & Williams 2011, D'Aloisio *et al.* 2013, Upson *et al.* 2016). Girls exposed during infancy to soy formula show reduced female-typical play behavior, but comparable disruptions are absent in boys (Adgent *et al.* 2011). A potential linkage between feeding infant soy formula and subsequent risk for autistic behaviors has been reported (Westmark 2013). Similarly, animal model studies have reported evidence that early exposure to GEN can lead to reproductive (Jefferson *et al.* 2007, Cimafranca *et al.* 2010) and behavioral abnormalities such as increased defensive behaviors and demasculinization in male mice (Wisniewski *et al.* 2005). Developmental exposure of rodents to GEN is associated with increased anxiety and aggressive behaviors, along with altered brain development (Santti *et al.* 1998, Rodriguez-Gomez *et al.* 2014).

In pigs, a soy formula diet affects the intestinal epithelial lining, microbial populations and intestinal epithelial barrier as well as anti-inflammatory markers (Yeruva *et al.* 2016). Rodent and human studies suggest that GEN or soy-based foods can alter the gut microbiome (Piacentini *et al.* 2010, Fernandez-Raudales *et al.* 2012, Nakatsu *et al.* 2014, Bai *et al.* 2016, Smith-Brown *et al.* 2016, Wu *et al.* 2016, Cross *et al.* 2017, Paul *et al.* 2017). Soy-fed neonatal White Dutch Landrace pigs show linkages between diet-responsive intestinal metabolites and gut microbes (Piccolo *et al.* 2017). In a human study, the plasma metabolome of vegans living in a Western society and consuming a soy-rich diet differed from that of omnivores, whereas the gut microbial profile between the two groups of individuals was relatively similar (Wu *et al.* 2016). Other studies in mice have shown that direct consumption of GEN and exposure during pre- or postnatal life is associated with gut microbiota changes, and these alterations are linked to metabolic and cognitive changes

(Huang *et al.* 2018, Lopez *et al.* 2018, Zhou *et al.* 2018). Metabolites produced by gut microbes may influence host function (Rosenfeld 2015, Dodd *et al.* 2017, Schugar *et al.* 2017, van de Wouw *et al.* 2017, Zheng *et al.* 2017, Zhou & Fang 2018). However, these above studies examining effects of GEN on the gut microbiome did not examine changes in host or microbial metabolites.

It is increasingly being recognized that the gut microbiome through production of metabolites and other potential mechanisms can affect the host's neurobehavioral state, which has been coined the microbiome gut–brain axis (Clarke *et al.* 2013, Borre *et al.* 2014, Stilling *et al.* 2014, Rosenfeld 2015). The first evidence associating gut microbiota disturbances and neurobehavioral disorders originated from germ-free (GF) mice that lack a resident gut microbiome. Such mice demonstrate disruptions in the hypothalamic–pituitary–adrenal gland (HPA) axis that is attenuated with bacterial reconstitution or fecal transplantation (Sudo *et al.* 2004). GF mice are more anxious, less exploratory and show cognitive and social deficits (Diaz Heijtz *et al.* 2011, Gareau *et al.* 2011, Neufeld *et al.* 2011, Clarke *et al.* 2013, Desbonnet *et al.* 2014).

Based on past studies, we hypothesized that developmental exposure to GEN might affect the gut microbiome, metabolome and socio-communicative behaviors either directly or indirectly via the microbiome–gut–brain axis. To test this hypothesis, California mice (*Peromyscus californicus*), who are generally quite social and monogamous, were developmentally exposed to GEN or a phytoestrogen-free control diet and the gut microbiota, metabolome and socio-communicative behaviors were examined at weaning. We chose this species over conventional inbred mouse strains to replicate the genetic diversity present in most human populations.

Materials and methods

Animals and treatments

Adult (60–90 days of age) California mouse females and males, free of common rodent pathogens, were purchased from the *Peromyscus* Genetic Stock Center (PGSC) at the University of South Carolina (Columbia, SC, USA). After shipment to the University of Missouri, they were placed in quarantine, along with sentinel mice, for a minimum of 8 weeks to ensure that they did not carry any transmissible and zoonotic diseases. No diseases have been identified to date in any sentinel or colony animals. Once the animals were deemed disease-free, they were then moved to the

Animal Sciences Research Center (ASRC). At this facility, we currently have our own breeding colony established. Additional animals have been purchased from the PGSC to maintain the outbred status of the line and similar procedures were followed as listed above. All experiments were approved by the University of Missouri Animal Care and Use Committee (Protocol #8693) and performed in accordance with the recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

To reduce any background bisphenol A (BPA) exposure, weaned animals were housed either in polystyrene (for breeder pairs) or polypropylene cages (for F₁ offspring) (Allentown Inc, Allentown, NJ, USA), provided glass water bottles and BPA-free water. Two weeks prior to breeding, virgin females, 8–12 weeks of age were randomly assigned to receive one of two diets: (1) a low phytoestrogen AIN 93G diet supplemented with 7% by weight corn oil (as a main fatty acid source) rather than soy oil to minimize potential phytoestrogenic contamination (control) or (2) the same AIN93G diet supplemented with 250mg/kg GEN, which has previously been shown to affect various parameters in mice (Dolinoy 2006, Dolinoy *et al.* 2007, Rosenfeld *et al.* 2013). This amount of GEN results in similar circulating concentrations of genistein as in humans consuming soy-enriched diets (Fritz *et al.* 2002). Upon weaning (30 days of age) all animals were placed on the AIN diet. This approach was used to replicate the maternal diet exposure of fetuses and neonates to GEN, which can then be transmitted across the placenta and in the milk, respectively. Maternal GEN supplementation was continued until pups were weaned as brain development extends throughout the postnatal period in rodents (Rice & Barone 2000, Howdeshell 2002).

One male and one female offspring from each litter were selected for subsequent analyses. For all analyses, samples from the same individuals were examined to allow for correlation analyses to be performed. Number of replicates included five AIN females, five GEN females, three AIN males and three GEN males. To determine how many replicates were needed to detect the significant differences, especially for the metabolome and microbiome portions of the study, we consulted previous studies in this area beforehand (Javurek *et al.* 2016, Paul *et al.* 2017, Okamoto *et al.* 2018, Rock *et al.* 2018). Moreover, we also did our own power analysis with SAS Power and Sample Size application (PSS, SAS Analysis, Cary, NC, USA) that verified this number of replicates should be sufficient to detect differences. Based on our previous microbiome and metabolome studies (Javurek *et al.* 2016,

Rosenfeld *et al.* 2018, Vieira-Potter *et al.* 2018), we also sought to test all samples at the same time to avoid any potential confounding batch effects.

Social behavior testing

Upon weaning (PND30), the pups underwent a sociability and preference for social novelty three-chambered test, designed to identify social deficits (Moy *et al.* 2004). We chose this time period to elucidate the potential immediate effects perinatal exposure to GEN might exert on socio-communicative behaviors with deficits in these traits considered one type of autistic-like behaviors in rodent models of autism (Crawley 2007, Silverman *et al.* 2010). With the interest as to whether xenoestrogens and endocrine disrupting chemicals as a whole are associated with increased risk of autism in children (Braun *et al.* 2014, Schug *et al.* 2015), we also sought to test the same life period as to when such disorders are commonly diagnosed in children. The three-chambered test is routinely used to examine for social behavioral deficits in weanling and older mice (Yang *et al.* 2007, Ryan *et al.* 2010, Ey *et al.* 2012, Brielmaier *et al.* 2014).

One of the goals of the current project was to also to determine if associations existed between GEN-induced gut microbiota changes (experimental detailed below) and behavioral alterations. Past studies have shown that gut microbiota changes induced by other maternal environmental factors are strongly associated with social behavioral deficits (Buffington *et al.* 2016, Bharwani *et al.* 2017, Sgritta *et al.* 2019). Buffington *et al.* (2016) also established causation of maternal high-fat diet (MHFD)-induced gut microbiota changes and social deficits in offspring by supplementing this group of offspring with a single bacterium, *Lactobacillus reuteri*, that abolished the social deficits and neuropathological changes induced by MHFD. Further, this group confirmed that MHFD-induced gut microbiota changes led offspring social impairments by performing fecal microbial transfer from MHFD mice to GF mice not exposed to this *in utero* change, which resulted in social deficiencies in the recipient mice that recapitulated those seen in donor individuals exposed to the MHFD. With an interest as to whether developmental exposure to GEN-induced gut microbiota may be linked with neurobehavioral changes, we chose to examine socio-communication behaviors that have already proven vulnerable to shifts in gut microbiota resulting from other *in utero* manipulations.

The test uses a three-chambered apparatus in which openings between the chambers allow the animal to move

throughout the apparatus for the duration of the test. The outer two chambers contain wire mesh cups to hold the ‘stranger’ mice, either Stranger 1 or Stranger 2. The stranger mice originated from different litters than that of the test animal and were of the same sex, approximate age and treatment group as the test individual. For Trial 1 (habituation trial), the left and right chambers were empty. The pup was placed in the center chamber and video-recorded for a 5-min acclimation period using a Logitech Carl Zeiss Tessar HD 1080P (Newark, CA, USA) camera mounted onto a Joby-Gorilla Pod Original Tripod (Daymen US Inc., Petaluma, CA, USA). The pup was then removed, and the two stranger mice were placed under their respective cups for a 5-min acclimation period. For Trial 2, a stranger mouse (Stranger 1) was placed on the right side, and the left side contained an empty cup. The test mouse was then placed in the center chamber and recorded for 10 min. For Trial 3, the previous mouse (Stranger 1) was again placed on the right side, and a novel mouse (Stranger 2) was placed on the left side. The test mouse was again placed in the center chamber and recorded for 10 min.

For these tests, we opted to use conspecific stranger mice, that is, those developmentally exposed to the same maternal treatment, to avoid potential confounding effects that could originate due to stranger individuals from different treatment groups being more or less dominant than the experimental animal. In other words, by mixing experimental and stranger mice, any potential social deficits might also be attributed to aversion of the experimental mouse to interact with strangers of different perinatal exposure background, which is of particular concern in California mice where social dominance can be influenced by extrinsic factors (Rosenfeld & Trainor 2014). Between each trial, all animals were returned to their cages and the apparatus was cleaned with a 70% ethanol solution. Data collected from this test were analyzed using Observer software version 11.5 (Noldus, Leesburg, VA, USA), and the animal's location in the three-chambered test was coded along with behaviors such as rearing, grooming and nose-to-nose contact with Stranger 1 mouse (trials 2 and 3) and Stranger 2 mouse (trial 3).

Ultrasonic vocalization measurements

Immediately following the sociability test, the animal was placed in a clean, empty cage and transferred to a polypropylene box containing polypropylene panels lined with 2 inches of convoluted acoustic foam paneling (Soundproof Cow, Chambersburg, PA, USA).

Audio was recorded for 5 min using an Avisoft Bioacoustics CM16/CMPA40-5V microphone (Gliencke, Germany) plugged into a National Instruments USB 6351 data collection board, which was further connected to a Dell OptiPlex 7010 (Dell Incorporated, Roundrock, TX, USA). Data were collected using a custom code written by the laboratory of Dr Katrin Schenk at Randolph College using MATLAB software (MathWorks, Natick, MA, USA) as detailed previously (Johnson *et al.* 2017, 2018). These recordings capture audible calls as well as ultrasonic vocalizations (USVs) that are above 20 kHz and out of the hearing range for humans. Vocalizations were segmented (separated from the background noise) and the number of syllables, syllable durations, syllable's median frequencies, average syllable power and power percent below and above 20 kHz were determined using the analysis program (MATLAB software) designed by Dr Katrin Schenk.

Collection of fecal samples and isolation of fecal microbial DNA

After the behavioral tests were completed, each animal was placed in a cage alone without any bedding. Four to five fecal boli were collected from each animal and placed in a 7 mL polypropylene vial (Fisher Scientific). As these animals are part of a larger ongoing study to examine for potential longstanding social behavioral deficits, cognitive impairments and anxiogenic behaviors throughout the lifespan, they were not euthanized at the completion of these experiments. Thus, brain, intestine and terminal blood samples were not collected at this time.

The fecal microbial DNA was isolated using the PowerFecal DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. The quantity of DNA isolated was measured using Qubit 3.0 Fluorometer (Life Technologies). The number of replicates ($n=3-5$ per group) tested is comparable to that used in other maternal diet and offspring gut microbiome studies that showed that such sample sizes can result in statistical differences between offspring groups (Ma *et al.* 2014, Thorburn *et al.* 2015).

16S rRNA sequencing

The University of Missouri DNA Core Facility prepared bacterial 16S ribosomal DNA amplicons from extracted fecal DNA by amplification of the V4 hypervariable region of the 16S rDNA with universal primers (U515F/806R) flanked by Illumina standard adapter sequences (Caporaso *et al.* 2011, Walters *et al.* 2011). Universal primer sequences

are available at proBase (<http://www.microbial-ecology.net/probebase/>) (Loy *et al.* 2007). A forward primer and reverse primer employing unique, dual indexes were used in each PCR reaction. PCR reactions (50 µL) contained 100 ng of genomic DNA, forward and reverse primers (0.2 µM each), dNTPs (200 µM each) and Phusion High-Fidelity DNA Polymerase (1 U). PCR amplification was performed as follows: 98°C (3:00)+(98°C (0:15)+50°C (0:30)+72°C (0:30))×25 cycles+72°C (7:00). Amplified product (5 µL) from each PCR reaction was combined and thoroughly mixed to prepare a single pool. Pooled amplicons were then purified by addition of Axygen AxyPrep MagPCR Clean-up beads (50 µL) to an equal volume of 50 µL of the amplicon library pool and incubated at room temperature for 15 min. Products were placed on a magnetic stand for 5 min and supernatant (95 µL) removed and discarded. Each well was washed by addition of 200 µL of freshly prepared 80% EtOH, incubated at room temperature for 30 s and supernatant was removed. Wash steps were repeated once and the plate was allowed to dry on the magnetic stand for 15 min. The dried pellet was resuspended in Qiagen EB Buffer (32.5 µL), incubated at room temperature for 2 min, and then placed on the magnetic stand for 5 min. The supernatant (30 µL) was transferred to a low binding microcentrifuge tube for storage. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system, quantified with a Qubit fluorometer using a quant-iT HS dsDNA reagent kit (Invitrogen), and diluted according to Illumina's standard protocol for sequencing on the MiSeq.

Bioinformatics and amplicon analyses

Paired-end Illumina MiSeq DNA reads were joined using `join_paired_ends.py` and combined using `add_qiime_labels.py` from QIIME 1.9.1 (Magoc & Salzberg 2011). Uclust (Edgar 2010) was used to clean contigs and remove those with $E > 0.5$ (http://drive5.com/usearch/manual/exp_errs.html). Contigs were clustered to 97% identity against DNA sequences in the Greengenes database (DeSantis *et al.* 2006), version 13_8, using the QIIME (Caporaso *et al.* 2010), version 1.9.1, script `pick_open_reference_otus.py`, which obviates chimera and PCR error detection with all reads clustered. After OTU picking, we filtered out OTUs with less than two observation counts using the script `filter_otus_from_otu_table.py`. For alpha-diversity in F_1 samples, Chao1 (species richness), Shannon (species diversity) values and rarefaction matrices were calculated and plotted using the `alpha_rarefaction.py` script in the

QIIME package (Caporaso *et al.* 2010). Measurements of beta-diversity were facilitated by the QIIME script `beta_diversity.py` and `jackknifed_beta_diversity.py` with PCoA plots generated. Visualization of taxonomy bar-charts were generated using `summarize_taxa_through_plots.py` from QIIME packages. Permutational multivariate analysis of variance (PERMANOVA) was used to examine for significant difference between groups.

For the subsequent differential abundant OTU and taxonomy analysis, we used the DESeq2 (Love *et al.* 2014) and negative binomial Wald test using scripts `differential_abundance.py` as implemented in QIIME. We selected significant OTUs based on an adjusted P value at the 0.05 level mapping to the Greengenes database. Regulation was determined by assigned $\log_2$ fold change calculated from differential analysis. With these methods, relevant pairwise comparisons were performed, such as F-GEN vs F-AIN and M-GEN vs M-AIN.

Gut metabolome analyses

Metabolomics analyses were performed with the fecal samples from all individuals, as detailed previously (Vieira-Potter *et al.* 2018) and were used to assess how developmental exposure to GEN affects gut bacterial metabolite profiles. An alkane mix was used for quality control (QC) samples for validation of instrument performance (Sigma). 10 µL of H_2O containing 1 µg/µL ribitol (internal standard that was previously validated) and 500 µL of 80% methanol were added to 10 ± 0.06 mg of each fecal sample. Samples were vortexed for 5 s, sonicated for 15 min shaken for 2 h on an orbital shaker at 140 rpm, and finally centrifuged at 13,000 g for 15 min. 400 µL of supernatant was collected into a glass vial, dried under a gaseous nitrogen stream, methoximated in pyridine with 40 µL of 15 mg/mL methoxyamine hydrochloride and then trimethylsilylated with 40 µL MSTFA (N-methyl-N-(trimethyl-silyl)trifluoroacetamide)+1% TMCS (chlorotrimethylsilane) reagent. The derivatized extracts were analyzed, as described previously (Deda *et al.* 2017). Non-targeted metabolic profiling was performed using an Agilent 6890 GC coupled to a 5973N MSD mass spectrometer with a scan range from m/z 50 to 650 (Agilent Technologies, Inc.). Separation was achieved with a temperature program of 80°C for 2 min, then ramped at 5°C/min to 315°C and held at 315°C for 12 min, a 60 m DB-5MS column (J&W Scientific, 0.25 mm ID, 0.25 µm film thickness) and a constant flow of 1.0 mL/min of helium gas. Data were deconvoluted with AMDIS and annotated through mass spectral and retention index matching to

our in-house constructed spectra library. The unidentified components were then searched and characterized using spectral matching to a commercial NIST17 mass spectral library. The combined identifications were saved as an .ELU file, and the abundance of the ions were extracted using custom MET-IDEA software (Lei *et al.* 2012). Abundances were then normalized to the ribitol internal standard and used for statistical comparisons.

Statistical analyses

Behavioral results

Behaviors in the three-chamber social testing and vocalization parameters were analyzed as detailed previously (Johnson *et al.* 2017, 2018). Data were analyzed using SAS version 9.4 Software (SAS Institute). Mean differences were determined using Fisher's Protected Least Significant Difference (LSD), as described by Steel (Steel 1997). The LSD was only calculated if the overall F test was significant (Chew & United 1977, Saville 1990). All data are presented as actual means ($\bar{x}$). The error bars for all figures and reported data represent the standard error of the mean (S.E.M.).

Metabolome

Multivariate statistical analyses such as 2D PCA, ANOVA, box plots and Volcano plots were performed with the MetaboAnalyst 3.0 program after data pre-treatments, i.e., normalization to the sum, log transformation and Pareto scaling (<http://www.metaboanalyst.ca/>). Changes in metabolite abundances were considered statistically significant when their *P* values were ≤ 0.05 . This program was also used to determine the overall metabolite changes in the GEN vs CON (AIN) groups and pathways predicted to be affected in the GEN male and female offspring. A Venn diagram was generated using <http://www.interactivenn.net/> (Heberle *et al.* 2015).

Integrative correlation analyses

We used the mixOmics R package (Rohart *et al.* 2017) to correlate the bacterial genera changes simultaneously with fecal metabolome and behavioral results, which enabled the integration of the microbiome, metabolome and mouse behavioral, social and vocalization data. We conducted sparse discriminant analysis with partial least square regression with function 'block.splsda'. The circos plot was generated using the 'circosPlot' function with correlations calculated using the method from González *et al.* (2012) and 0.9 correlation was used as the cutoff.

It shows together the correlation between each individual features pairs from all four datasets.

Results

Socio-communicative behaviors

As detailed above, F1 male and female California mice were tested in three trials in the three-chamber social test with T1 trial being considered the habituation period. In T2 trial, one stranger mouse placed on one side of the chamber and an empty cup on the other, no treatment differences in location within the apparatus (left, middle or right) or nose-to-nose contact with Stranger 1 mouse were detected. However, in T3 trial, when a second stranger mouse was introduced, differences in nose-to-nose contact for females emerged with AIN females predictably spending more time investigating the novel Stranger 2 mouse than Stranger 1 mouse ($P=0.001$, Fig. 1). In contrast, GEN-exposed females (F-GEN) spent equal time in this trial with Stranger 1 and Stranger 2 mice, suggesting reduced social behavior or interest in seeking out novel individuals.

For vocalization responses, a significant main effect based on maternal treatment was detected for latency to exhibit first call ($P\leq 0.05$), but no significant interactive effects for maternal treatment $\times$ sex were observed. Thus, for this category, male and female data within each treatment group were combined together. When vocalization responses were measured after the three-chambered social test, F1 GEN-exposed individuals took twice as long to elicit their first syllable (call) as AIN control mice ($P=0.03$, Fig. 2),

Figure 1

Number of times F1 females engaged in nose-to-nose contact in T3 trial with Stranger 1 and 2 mice. AIN females spent more time investigating a novel, Stranger 2, mouse than previously acquainted mouse, Stranger 1. However, such differences were not observed in GEN-exposed females who spent equal time in this trial in contact with Stranger 1 and Stranger 2 mice, suggestive of reduced social behaviors. * $P=0.001$ based on time spent with Stranger 2 mouse for AIN females vs GEN females.

supporting the idea that developmental exposure to GEN reduces socio-communicative behaviors. None of the other vocalization parameters differed between the two groups or based on offspring sex.

Gut microbiota

Based on *t*-test analyses, no overall differences in α -diversity (chao-1 and Shannon analyses) were observed based on developmental exposure of males or females to GEN (Fig. 3). Bar plots of operational taxonomic units (OTU) in F-AIN, F-GEN, M-AIN and M-GEN revealed some potential but not overt bacterial differences (Fig. 3, as several bacteria are represented by Fig. 3C, a legend for the various bacteria are provided in Supplementary Figs 1, 2, 3 and 4 (see section on [supplementary data](#) given at the end of this article to allow for each to be legible). Weighted PERMANOVA analyses of the PCA plot revealed significant differences based on GEN exposure and offspring sex ($P=0.003$) (Fig. 3D). All four groups are shown in this figure to compare the α - and β -diversity across all groups.

Further pairwise examination with DESeq2 (Love *et al.* 2014) and negative binomial Wald test, revealed that select bacteria were altered based on GEN exposure and offspring sex. Relative amounts of *Lactobacillus* spp., *Ruminococcus flavefaciens*, Bacteroidales and Clostridiales were reduced in GEN-exposed females, whereas Rikenellaceae, Ruminococcaceae, Lachnospiraceae and *Lactococcus* spp. were elevated in this group (Supplementary file 1). In males, GEN decreased the relative abundance of *Flexispira* spp., Clostridiales, Bacteroidales, *Odoribacter* spp. and Desulfovibrionaceae but increased the relative abundance of Lachnospiraceae and *Allobaculum* spp. (Supplementary file 2).

Figure 2

Latency to first syllable (call). GEN-exposed males and females took twice as long to elicit their first syllable (call) as AIN individuals. * $P=0.03$.

Gut metabolome

GS-MS data were processed using AMDIS software. Metabolite identifications reported are either level 1 or 2 according to the Metabolomics Standards Initiative and specifically those related to metabolite identification (Sumner *et al.* 2007). A total of 528 components were detected and 278 were annotated by searching our in-house and NIST17 MS spectral libraries (Supplementary files 3 and 4). Two-dimensional (2D) principal component analyses (PCA) revealed male mice developmentally exposed to genistein were separated from the rest of the groups and some separation based on offspring sex (F vs M) and maternal treatment (GEN vs CON) was evident (Fig. 4). Differential clustering was confirmed by analyzing for significant differences with a Volcano plot for GEN-exposed females vs AIN control females and GEN-exposed males vs AIN control males (Fig. 5). The Volcano plot is a combination of fold change and *t*-test *P* values. Components with a fold change greater than 2 and a *t*-test *P* value less than 0.05 are considered significantly different. We report here *P* values rather than adjusted *P* values because while adjusted *P* value reduces the number of false positives, it also reduces the number of true discoveries. Since this is an exploratory experiment, we believe it is appropriate and necessary to use *P* values in order to avoid missing some significant metabolite differences.

In females, the relative accumulation of several metabolites was altered by developmental exposure to GEN (Supplementary file 5). Malonic acid and methyl heptadecanoate were increased in F-GEN individuals, whereas 4-aminobutyric acid, cysteine, homoserine and ornithine were decreased in this group (Fig. 6). 3-Amino isobutanoic acid was elevated in GEN-exposed males (M-GEN), but methyl linoleate, N2-acetyl-ornithine, daidzein, α -tocopherol and 2 β ,4 β -choestan-3-ol were reduced in this group (Fig. 7). The full list of metabolites altered in M-GEN is detailed in Supplementary file 5.

The overview of the significantly changed metabolites can be visualized in Supplementary Fig. 5 showing a number of overlap and unique metabolites that were significantly regulated in each comparison. The data show that very few common metabolites were found to be significantly change based on treatment $\times$ sex interactions.

Two pathways significantly affected in F-GEN individuals relative to F-AIN group include methionine and glutamate metabolism and carnitine synthesis (Fig. 8, FDR ≤ 0.05). Supplementary Figure 6 shows these two pathways and denotes those metabolites within them

Figure 3

Measures of α - and β -diversity in AIN and GEN-exposed F1 males and females. (A and B) Developmental exposure of males and females to GEN did not affect overall differences in species richness (as shown by Chao1 results) and diversity (as shown by Shannon results). The data were plotted using phyloSeq R package plot_richness function (McMurdie and Holmes 2013). (C) Bar plot analysis of the most abundant bacterial species in all three treatment groups for F1 males and females. (D) 3D PCoA analysis F1 male and female offspring results. Weighted PERMANOVA ($P=0.003$). All four treatment groups are included in these figures to allow for comparison of α - and β -diversity across all groups. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0214>.

that were affected by GEN exposure. After FDR correction, no overall pathways were significantly altered in M-GEN vs M-AIN.

Integrative correlation analyses between gut microbiota, gut metabolome and socio-communicative behaviors

To correlate the gut microbiota changes with gut metabolome and behavioral results, we used the mixOmics R package (Rohart *et al.* 2017). Four multi-analyses comparisons were tested: F-GEN vs F-AIN, M-GEN vs M-AIN, F-GEN vs M-GEN and F-AIN vs M-AIN. Comparison of F-GEN to F-AIN revealed that the only correlations that were significant at 0.9 were average vocalization power positively correlated with *Rikenellaceae*

and *Ruminococcaceae*, both of these bacteria were elevated in F-GEN (Fig. 9). Comparison of M-GEN with M-AIN revealed several significant correlations among the gut metabolome, gut microbiome and behaviors (social and vocalization) at 0.9 (Fig. 10). Some example correlations in GEN-exposed males vs control males include ornithine, 1-heptadecanol, 3 β ,5 β , cholestan-3-ol was associated with *Odoribacter spp.* and *Bacteroidales*. 1-heptadecanol was also associated with *Flexispira spp.*, *Odoribacter spp.*, and *Flexispira spp.* were associated with 3 β ,5 α , cholestan-3-ol and daidzein. All these bacteria and metabolites were positively associated with fraction of calls above 20 kHz, along with α -tocopherol.

When comparing within GEN and AIN groups, sex differences were evident. Comparison of M-GEN to F-GEN revealed such correlations as valine was associated with

Figure 4

2D PCA analysis of F1 gut metabolome results. The 2D PCA analysis shows some clustering based on developmental exposure to GEN and offspring sex. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0214>.

Clostridiales and *Ruminococcus gnavus* (Fig. 11). Cadaverine was associated with *Odoribacter spp.*, *Oscillospira spp.* and *Ruminococcus gnavus*. Cholic acid was associated with *Rikenellaceae*. Total number of calls was associated with cadaverine, *Odoribacter spp.*, *Oscillospira spp.* and *Ruminococcus gnavus*. Latency to produce the first call was associated with cholic acid and *Rikenellaceae*. Comparison of M-AIN vs F-AIN revealed fewer significant correlations (Fig. 12). One example was average power above and

below 20 kHz was positively associated with alanine. The individual pairwise comparisons for these four analyses are included in Supplementary Figs 7, 8, 9 and 10. Supplementary file 6 provides all of the comparisons, and those with correlations ≥ 0.9 are highlighted.

Discussion

The first goal of the current study was to determine whether developmental exposure to GEN induces direct effects on subsequent socio-communicative responses. Secondly, we sought to determine whether early contact with this xenoestrogen might affect gut microbiota, fecal metabolites, and thereby result in indirect CNS effects via the microbiome–gut–brain axis. The current findings support previous human and rodent studies suggesting that phytoestrogens in soy formula and GEN can result in subsequent neurobehavioral disruptions, as we have seen with ethinyl estradiol (estrogen present in birth control pills) and BPA (Jasarevic *et al.* 2011, 2013, Williams *et al.* 2013, Johnson *et al.* 2015). Reduced social behaviors, as evidenced by willingness to interact with a novel mouse, were observed in female California mice developmentally exposed to GEN. GEN-exposed males and females took longer to vocalize when placed in isolation. Current rodent data are similar to human epidemiological reports suggesting that soy infant formula consumption is associated with reduced female-typical play behavior in girls and increases the risk of autistic behaviors (Adgent *et al.* 2011, Westmark 2013). In male rodents, developmental exposure to GEN increases anxiogenic and aggressive behaviors, in spite of

Figure 5

Volcano plots of metabolites that differ between GEN and AIN females and males. (A) This analysis revealed several metabolites that differed between GEN-exposed females and AIN females. (B) This analysis revealed several metabolites that differed between GEN-exposed males and AIN males. In each figure, the blue dots represent metabolites that were not significantly different between the two groups, whereas, the red dots represent those that are significantly different between the two groups ($P \leq 0.05$). Characterized metabolites are accordingly labeled. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0214>.

Figure 6

Traditional box plots for metabolites elevated in F-GEN vs. F-AIN groups ($P < 0.05$). The y-axes are log 10 values of the normalized instrument response for the labeled metabolites in F-GEN and F-AIN groups (x-axes). The program arbitrarily assigns color codes for the various groups. The box plot upper and lower brackets represents $\pm (1.58 * \text{interquartile range-IQR/squared root of sample size})$. A complete list of metabolites that differed between these two groups, as well as directionality, is included in Supplementary file 5. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0214>.

Figure 7

Traditional box plots for metabolites elevated in M-GEN vs. M-AIN groups ($P < 0.05$). The y-axes are log 10 values of the normalized instrument response for the labeled metabolites M-GEN vs M-AIN groups (x-axes). The program arbitrarily assigns color codes for the various groups. The box plot upper and lower brackets represents $\pm (1.58 * \text{interquartile range-IQR/squared root of sample size})$. A complete list of metabolites that differed between these two groups, as well as directionality, is included in Supplementary file 5. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0214>.

Such behavioral changes might be due to direct effects on the brain, especially the hypothalamus. Early exposure to GEN has been reported to cause vacuolization of oxytocin neurons in the neonatal mouse hypothalamus (Yoshimura *et al.* 2011). GEN may alter brain nitrenergic and vasopressinergic circuitry systems in rodents and Japanese quail (Scallet *et al.* 2003, Viglietti-Panzica *et al.* 2007, Ponti *et al.* 2017). The gonadotropin releasing hormone network and hypothalamic–pituitary–adrenal axis might also be affected by GEN exposure (Levy *et al.* 1995, Wojcik-Gladysz *et al.* 2005, Medigovic *et al.* 2012, Trifunovic *et al.* 2012, Arispe *et al.* 2013, Mueller & Heger 2014). While socio-communicative deficits observed in GEN-exposed California mice might be attributed to changes in these

Figure 10

Circos correlations among taxa altered in fecal microbial community of M-GEN vs M-AIN individuals and fecal metabolomic changes due to GEN exposure. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0214>.

hypothalamic pathways, such effects could be due to gut dysbiosis and shifts in gut metabolites.

Developmental exposure to GEN led to sex-dependent gut microbiota changes even though xenoestrogen did not affect the overall species richness and diversity. Our expectation was that GEN exposure would lead to similar gut microbiota changes as we previously identified in ethinyl estradiol (EE)-exposed female California mice (Javurek *et al.* 2016). However, the bacterial changes identified in GEN-exposed individuals did not resemble the profile identified in EE-exposed individuals. Instead, select bacteria showed the opposite pattern with the relative amounts of Rikenellaceae and *Prevotella* spp. increased in GEN-exposed females but reduced in EE-exposed females. Similarly, we previously found that exposure to bisphenol A led to a unique signature pattern of bacteria in male and female California mice (Javurek *et al.* 2016). A previous study in mice developmentally exposed to GEN showed that members of the Rikenellaceae family were increased by this phytoestrogen (Zhou *et al.* 2018), although this study did not separate offspring based on sex. Another study reported that perinatal exposure to GEN increased Enterobacteriales in PND 90 female offspring (Huang *et al.* 2018). This bacterial order

was not affected in the current studies with PND 30 offspring.

Previous studies in animals and humans consuming soy products or exposed to GEN indicate that the gut bacterial changes might depend upon type of phytoestrogen exposure, host species examined and time and duration of exposure to phytoestrogens (Piacentini *et al.* 2010, Fernandez-Raudales *et al.* 2012, Nakatsu *et al.* 2014, Bai *et al.* 2016, Smith-Brown *et al.* 2016, Wu *et al.* 2016, Yeruva *et al.* 2016, Cross *et al.* 2017, Paul *et al.* 2017, Piccolo *et al.* 2017). GEN-induced changes in gut microbes might alter host responses, as shown previously (Cross *et al.* 2017). Rats provisioned with a phytoestrogen-rich diet demonstrated changes in fecal microbiota that were positively associated with improved cardiometabolic outcomes (Cross *et al.* 2017). In the current studies, GEN-induced gut microbe changes showed sex-dependent associations with socio-communicative behaviors. However, such correlations only indicate that these changes track together; they do not indicate causation. To establish causation that bacterial changes due to GEN exposure result in socio-communicative alterations, fecal microbial transfer from GEN-exposed individuals into GF mice that lack resident gut microbiota and who have not

Figure 11

Circos correlations among taxa altered in fecal microbial community of M-GEN vs F-GEN individuals and fecal metabolomic changes. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0214>.

been exposed to GEN would be needed, as was done for fecal samples from offspring exposed to MHFD to GF mice with GF recipients then developing similar social deficits as the donor mice (Buffington *et al.* 2016). Unfortunately, our current animal facility does not have the ability to maintain GF mice. Thus, we are currently exploring such studies through potential collaborations with those at other institutes who have a GF mouse facility.

By altering the gut microbiota, GEN likely affects gut metabolome profiles. Soy-fed neonatal White Dutch Landrace pigs show linkages between diet-responsive intestinal metabolites and gut microbes (Piccolo *et al.* 2017). However, the plasma metabolome of vegans living in a Western society and consuming a soy-rich diet differed from that of omnivores, whereas the gut microbial profile between the two groups of individuals was surprisingly similar (Wu *et al.* 2016). In female California mice, GEN exposure predominantly decreased several metabolites, including 4 (gamma)-aminobutyric acid (GABA), cysteine, homoserine, ornithine and glycine. Deficiencies in GABAergic synaptic pathways are linked with mouse models of autism and other human neurobehavioral disorders (Han *et al.* 2012, Politte *et al.* 2014, Tso *et al.* 2015, Chau *et al.* 2017). Methionine and glutamate metabolism

pathways might be disrupted in GEN-exposed females. Disturbances in glutamate metabolism are associated with autism and autistic-like behaviors in animal models of this disorder (Bristot Silvestrin *et al.* 2013, Fung & Hardan 2015, Wei *et al.* 2015). Several discrete metabolites were upregulated (such as 3-amino-isobutanoic acid) and downregulated (e.g. methyl linoleate, N2-acetyl-ornithine, daidzein, α -tocopherol and 3 β ,5 β -choesetan-3-ol) in males.

Importantly, the current studies are the first to show clear sex-dependent linkages between gut metabolites altered in response to developmental exposure to GEN and subsequent socio-communicative disorders. In our recent work, we similarly determined that SOY-induced gut metabolite shifts correlated with host cardiometabolic outcomes (Vieira-Potter *et al.* 2018). As with the correlations between gut microbes and neurobehavioral responses, the conclusion that changes in select metabolites cause subsequent neurobehavioral alterations cannot be definitively drawn. To establish causation, metabolites identified to be enriched with GEN exposure would need to be administered to control dams and offspring neurobehavioral responses examined throughout the lifespan. Such a post-metabolite approach was done with

As the animals were not collected at the end of these current studies, sufficient blood for plasma metabolome studies could not be collected. However, bacterial metabolites might be transmitted to the brain both through the circulatory system and vagal nerve conduction (Bercik *et al.* 2011, Petra *et al.* 2015, Rosenfeld 2015, Liu 2017, Bonaz *et al.* 2018, Jaglin *et al.* 2018). Gut microbiota changes are often associated with intestinal system pathologies, including increased gut permeability and influx of inflammatory cells that could also impact the brain (Rosenfeld 2015). Because animals were not killed at the completion of these current studies, no assessments could also be made on how developmental exposure to GEN affects the host gastrointestinal system. Future work will explore for such GEN-induced alterations.

Conclusions

Our study is the first to show that developmental exposure to GEN leads to socio-communicative deficits in California mice, especially in females. While such effects might be due to direct disruptions on the neural circuitry, especially in the hypothalamus, the observed changes might also be due to GEN stimulated alterations in the microbiome–gut–brain axis. Multi-integrative correlation analyses revealed several interactions between GEN-induced microbiome, metabolome and resulting changes in socio-communicative behaviors. Further work is needed to establish how much of the observed neurobehavioral changes are due to direct effects of GEN compared to those attributed to gut dysbiosis/shifts in gut metabolite profiles. Usage of GF mice and post-biotic supplementation of GEN-induced metabolites will be useful in teasing apart these diverging mechanisms. Probiotic and post-biotic supplementation approaches might be used to prevent or remediate behavioral disturbances due to early GEN exposure.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0214>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

B A M, L W S, T J and C S R designed the study. B A M, Y L, M J F, J M, W G H, A K S, N J B, S J S and Z L participated in data acquisition and analyses. B A M, Y L, J M, W G H, A K S, N J B, S J S, Z L, L W S, T J and C S R participated in interpretation of data. B A M, Y L, J M, N J B, S J S, Z L, L W S, T J and C S R drafted the manuscript. All authors revised the manuscript for intellectual content and approved the final version. C S R is the guarantor of this work.

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