Physiology & Behavior 119 (2013) 175–184

Contents lists available at SciVerse ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Maternal immune activation affects litter success, size and neuroendocrine responses related to behavior in adult offspring

Susannah S. French [a,1], Emily M. Chester [b,1], Gregory E. Demas [b,*]

[a] Department of Biology, Utah State University, 5305 Old Main Hill, Logan, UT 84322, United States
[b] Department of Biology, Center for the Integrative Study of Animal Behavior, Indiana University, 1001 E. 3rd St., Bloomington, IN 47405-3700, United States

HIGHLIGHTS

- We quantified sickness during pregnancy in hamsters given different doses of LPS.
- We assessed the resulting effects on litter success, size, and offspring development.
- Pregnancy success decreased and litter size was reduced with increasing doses of LPS.
- Offspring from LPS treated dams showed greater cortisol responses to stress.
- Cortisol levels in both sexes of offspring were related to defensive behaviors.

ARTICLE INFO

Article history:
Received 30 October 2012
Received in revised form 2 May 2013
Accepted 20 June 2013

Keywords:
Lipopolysaccharide (LPS)
Cortisol
Testosterone
Epigenetic effects

ABSTRACT

It is increasingly evident that influences other than genetics can contribute to offspring phenotype. In particular, maternal influences are an important contributing factor to offspring survival, development, physiology and behavior. Common environmental pathogens such as viral or bacterial microorganisms can induce maternal immune responses, which have the potential to alter the prenatal environment via multiple independent pathways. The effects of maternal immune activation on endocrine responses and behavior are less well studied and provide the basis for the current study. Our approach in the current study was two-pronged: 1) quantify sickness responses during pregnancy in adult female hamsters experiencing varying severity of immune responsiveness (i.e., differing doses of lipopolysaccharide [LPS]), and 2) assess the effects of maternal immune activation on offspring development, immunocompetence, hormone profiles, and social behavior during adulthood. Pregnancy success decreased with increasing doses of LPS, and litter size was reduced in LPS dams that managed to successfully reproduce. Unexpectedly, pregnant females treated with LPS showed a hypothermic response in addition to the more typical anorexic and body mass changes associated with sickness. Significant endocrine changes related to behavior were observed in the offspring of LPS-treated dams; these effects were apparent in adulthood. Specifically, offspring from LPS treated dams showed significantly greater cortisol responses to stressful resident–intruder encounters compared with offspring from control dams. Post-behavior cortisol was elevated in male LPS offspring relative to the offspring of control dams, and was positively correlated with the frequency of bites during agonistic interactions, and cortisol levels in both sexes were related to defensive behaviors, suggesting that changes in hypothalamo–pituitary–adrenal axis responsiveness may play a regulatory role in the observed behavioral differences. Overall, the results of this study provide evidence that maternal immune activation can exert marked effects on offspring physiology and behavior.

1. Introduction

Although genetic influences on a wide range of behaviors are well documented, it is becoming increasingly evident that non-genetic (e.g., environmental) influences also contribute to offspring phenotype, including behavior. While the role of environmental factors has been traditionally under-appreciated, recent studies have highlighted the importance of epigenetic (i.e., "above the genome") effects on an organisms' phenotype. In particular, maternal influences such as energy/nutrient availability, oxygen levels, and hormone concentrations are important contributing factors to offspring survival, development, and potentially behavior (reviewed in: [1,2]).

Pregnancy in eutherian mammals presents a life history stage characterized by prolonged physiological association between mother and offspring. Long gestational periods result in extended prenatal exposure to maternal influences. Further, placental buffering capabilities change throughout gestation varying embryo/fetal susceptibility temporally to maternal influences, such as maternal immune system activation [3]. Whereas some influences, such as morphological abnormalities, are apparent in utero, increasing evidence suggests that

* Corresponding author. Tel.: +1 812 856 0158; fax: +1 812 855 6705.
E-mail address: gdemas@indiana.edu (G.E. Demas).
[1] Both authors have contributed equally to this research and manuscript.

0031-9384/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.physbeh.2013.06.018

certain physiological and behavioral maternal effects may not manifest until later in development [4,5]. For example, exposure to prenatal stress can alter stress responsiveness in mammalian offspring [6]. Further, prenatal exposure to maternal stress, food restriction, or elevated glucocorticoid levels impairs male sexual behavior in rats [4]. Therefore environmental perturbations that influence the mother during pregnancy and lactation, can in turn affect offspring development.

In particular, activation of the maternal hypothalamo–pituitary–adrenal axis (HPA) during pregnancy can result in profound effects on offspring development, including altered gonadal steroid production, stress responsiveness, growth rate, and immunocompetence of offspring from both live-bearing and egg-laying species [7–12]. For example, prenatal stress in albino rats (Ola strain) results in decreased body mass and growth rates from birth [13]. There are many common environmentally relevant phenomena that have the capacity to activate the HPA axis and thus affect physiology and behavior. For example, predation attempts, resource limitation, social competition, and certain immunological responses to pathogens or parasites can all alter HPA axis activity (reviewed in: [14–16]). Therefore, immune-related maternal effects on offspring are common within an organism's natural environment and an important contributor to individual phenotype, including behavior.

While various environmental perturbations can alter the prenatal environment and thus contribute to the development of offspring behavior, the specific physiological mechanisms underlying these effects are unclear. Accumulating evidence suggests that neonatal or early-life exposure to elevated cytokines during an immune response can result in prolonged neural and behavioral abnormalities (reviewed in: [17,18]). Further, emerging evidence suggests that elevated cytokine concentrations during pregnancy cause marked changes to fetal brain development and therefore likely downstream changes in offspring behavior [5,19]. In fact, maternal immune activation may contribute to several psychiatric conditions including autism, and proinflammatory cytokines (i.e., interleukin [IL]-6, tumor necrosis factor [TNF]-α) play an important role in the regulation of social behavior such as aggression [20,21]. Activation of an innate immune response results in a cascade of molecular and biochemical events that involve activation of specific immune cells (e.g., neutrophils, macrophages), mobilization of complement proteins and production of cytokines, soluble molecules that aid in the targeting and destruction of the pathogen. Furthermore, this immune response can trigger the HPA, axis resulting in elevated glucocorticoids, which could in turn affect offspring [12,22]. Therefore, common environmental pathogens such as viral or bacterial organisms can induce maternal immune responses which have the potential to immensely alter the embryonic/fetal environment via multiple pathways [12,23,24]. It is also apparent that maternal immune activation can induce offspring brain pathology, having direct implications for behavior, and in extreme cases, miscarriage [5,25,26].

Although much progress has been made over the last decade in our understanding of the effects of maternal immune activation on neuroendocrine and behavioral responses, considerable gaps in our knowledge persist. For example, much of the work examining experimentally-induced immune activation has focused on biochemical and physiological changes occurring within the organism [27]. Substantially less is known regarding how specific behavioral phenotypes, particularly social behaviors, are affected by immune activation. In the current study, we examined the effects of maternal immune activation on reproductive success and offspring endocrine and behavioral development using the Siberian hamster (*Phodopus sungorus*) as a model system. To induce an immune response we utilized lipopolysaccharide (LPS), a molecule present on the outer coat of Gram-negative bacteria, which acts on Toll-like receptors (TLR) present on immune cells (specifically TLR4) [28]. LPS elicits an inflammatory and pyrogenic response without actually exposing the animal to an infectious agent [29,30].

Prenatal infection may enhance offspring immunity according to the transgenerational priming of immunity theory, which may be adaptive because the offspring are likely to encounter similar infectious agents [31,32]. Therefore, fetal exposure to a maternal innate immune response may increase the ability of the offspring to respond to an innate immune challenge through altered circulating immune components. We tested the immunocompetence of the offspring in adulthood with a bacterial killing assay as a functional measure of innate immunity. We also tested the ability of the animals to respond to an antigenic challenge. Testing both the innate and humoral arms of the immune system provides a broader understanding of the immune system and how it was altered during development.

The approach of the current study was two-pronged: 1) quantify sickness response during pregnancy in adult female hamsters in response to varying levels of immune responsiveness (i.e., varying doses of LPS), 2) measure the effects of maternal immune activation on pregnancy success and litter size, and 3) assess the effects of maternal immune activation during pregnancy on offspring development, endocrine responses, and behavior during adulthood. Specifically, we assessed the sickness response of pregnant females exposed to relatively low and high doses of LPS, measuring food intake, body mass and temperature, and pregnancy success. We subsequently assessed offspring initial birth mass, growth rate, and adult immunocompetence (i.e., bactericidal ability and KLH-antibody response), resident–intruder aggression test and associated steroid hormone concentrations (i.e., cortisol, testosterone), all factors known to be associated with maternal stress and potentially immune activation.

2. Materials and methods

2.1. Experiment 1A and B: effects of immune activation on maternal physiology and behavior

2.1.1. Animals and housing

Sixty-nine adult nulliparous female Siberian hamsters were obtained from our breeding colony at Indiana University. All animals were initially group-housed (2–4 per cage with same sex siblings upon weaning at 21 days of age). Ten days before the start of the experiment, animals were housed individually in polypropylene cages (28 × 17 × 12 cm). Conditions were maintained at 16:8 (light:dark) hour photoperiod, temperature (20 ± 2 °C), and humidity (50 ± 10%). All animals were given ad libitum access to food (Purina rat chow, St. Louis, MO) and water throughout the study. All animals were treated in accordance with the Bloomington Institutional Animal Care and Use Committee (BIACUC).

2.1.2. Maternal immune activation

To examine the effects of mounting a costly immune response on offspring investment, adult female hamsters were randomly assigned to one of two treatment groups in each of two separate studies. A) In the first experiment animals were injected on gestational day (GD) 11 (±2 days; i.e., GD 10–13) with either high dose of LPS 0.7 mg/kg (high dose, n = 12) or saline vehicle 0 mg/kg (control, n = 15). Because of the low pregnancy success and the desire to measure offspring effects a second iteration of the experiment was run using a lower dose of LPS. B) In the second experiment animals were injected on GD 11 (±2 days) with either low dose of LPS 0.07 mg/kg (low dose, n = 12) or saline vehicle 0 mg/kg (control, n = 15). Gestational day was assigned with the second day of pairing the male and female as GD 0. Males and females were paired for 4 days in the cage of the female, after which the male was removed. All pairs were given a cotton nestlet to create a nest. Variation arose due to the fact that hamsters do not have regular estrous cycles, which introduces some uncertainty in the date of conception. Injections occurred on GD 11 (±2 days) because this time point is late enough to reduce the risk of spontaneous abortion and early enough to affect brain development during a period

of peak neurogenesis [33] in the offspring. Food intake was monitored daily by weighing the remaining food pellets and female body mass every three days throughout the study except for the four days while the female was paired with the male. The dams' rectal body temperatures were monitored during 24 and 48 h following the LPS injection. Colonic temperature was measured with a pre-lubricated thermoprobe (Physitemp Thermalert TH-5) inserted ~12 mm into the rectum. Time from removal from cage to return was 20 s or less for each animal, minimizing handling stress on the animal.

2.1.3. Blood sampling

Blood samples were collected 2 h following the injection in order to assess circulating cortisol concentrations in control and LPS-treated animals. Animals were anesthetized with isoflurane, and blood samples were collected via the retro-orbital sinus within 3 min of leaving their cage. Blood samples were allowed to clot at room temperature for 1 h, and then clots were removed and the samples were centrifuged (at 4 °C) for 30 min at 2500 rpm. Serum aliquots were extracted and stored at − 80 °C until subsequent assays.

2.1.4. Statistical analyses

The responses to maternal immune activation in females (i.e., body temperature, food intake, body mass) were subjected to a one-way analysis of variance (ANOVA) with treatment (LPS or saline vehicle) as a factor.

2.2. Experiment 2: effects of maternal immune activation on offspring physiology and behavior

2.2.1. Offspring rearing and housing

In order to examine the effects of maternal immune activation on development, immune function and behavior of the offspring, young from the low dose and control treatments (i.e., Experiment 2B only; experiment in which the offspring survived to parturition) were reared to adulthood. Environmental conditions were maintained as described above. Weekly litter mass measurements were taken until weaning at 21 days of age. Upon weaning, animals were housed with no more than 2 same sex siblings, and the total mass of all animals in a cage was recorded weekly. Hamsters were tested for their responses to a social interaction and immune challenge in adulthood.

2.2.2. Hormone assays

Total serum cortisol concentrations were measured to assess stress reactivity of the offspring. Serum cortisol was measured at two time points: one week prior to resident–intruder interaction and 30 min post resident–intruder interaction. Although the resident–intruder model introduces some variation to the stress response, it also presents a more biologically relevant stressor than other alternatives (i.e., confinement). Cortisol is the predominant glucocorticoid produced in Siberian hamsters at ~100× that of corticosterone [34]. Cortisol concentrations were determined with a commercially available enzyme immunoassay (EIA) kit (Correlate-EIA™, Assay Designs, Ann Arbor, MI). Samples were diluted 1:20 with assay buffer and were run in duplicate for each sample. This assay was previously validated for use with Siberian hamsters [70] and is highly specific for cortisol. The cross-reactivity of corticosterone is 27.68% and other steroid hormones are <0.1%. The sensitivity of the assay is 56.72 pg/ml. Intra-assay variability was 9.9%.

Serum testosterone was measured in male offspring samples at 3 time points: 1 week prior to resident–intruder interaction, 30 min post resident–intruder interaction and recovery (10 days following resident–intruder interaction). Testosterone concentrations were determined with the EIA kit (Correlate-EIA™, Assay Designs, Ann Arbor, MI). This assay was previously validated for use with Siberian hamsters [35] and is highly specific for testosterone. The cross-reactivity of the assay for 19-hydroxytestosterone is 14.64%, androstendione is 7.20%, dehydroepiandrosterone is 0.72%, and estradiol is 0.40%, all other steroid hormones are <0.001%. The inter-assay variability was 3.96%.

2.2.3. Resident–intruder aggression test

At 15 weeks of age (i.e., young adulthood), the male and female offspring from low LPS dose and control dams were observed in a resident–intruder interaction with a single same-sex hamster. Individuals were placed in the cages of individually housed aggressive resident (i.e., territorial) hamsters for 5 min. Sessions occurred within the first 2 h of the dark phase of the light:dark cycle under red light (see below). All interactions were observed in order to determine whether resident aggressors attacked the experimental animals and if the experimental animals displayed any submissive behaviors. All experimental trials were performed under low illumination (25 W), red light conditions, which allowed for sufficient light during video recording and observations without disturbing the behavior of the hamsters. To identify the experimental offspring from the residents, small patches of fur were shaved on the dorsal surface. Behavioral interactions were scored using ODlog™ software (Macropod) by a trained observer blind to maternal treatment. The numbers of attacks (a lunge with body contact) and bites (mouth contact) by the resident and defensive behaviors (i.e., fleeing and on-back submissive posture) by the experimental animal were characterized.

2.2.4. Immunizations and enzyme-linked immunosorbent assays

Immune responses of the offspring were assessed by measuring specific antibody production and the ability of blood serum components (e.g., complement proteins) to kill *Escherichia coli*. A blood sample was collected (as described in Experiment 1) one week prior to the start of all social interaction trials in order to assess baseline circulating cortisol and bacterial killing ability. Thirty minutes following the social interaction, a post-stress blood sample was taken. Next, all hamsters received a single subcutaneous injection of keyhole limpet hemocyanin (KLH; Calbiochem, San Diego, CA), 100 μg KLH in 0.1 ml saline. KLH is an innocuous respiratory protein derived from the giant keyhole limpet (*Megathura crenulata*). KLH was used because it generates a robust antigenic response in rodents, but does not make the animals sick (e.g., inflammation or fever) [36]. Blood samples were drawn on Day 10 post-immunization as described above for anti-KLH IgG levels, the most abundant antibody produced. Serum aliquots were stored at −80 °C until assayed for anti-KLH IgG levels.

Serum anti-KLH antibody concentrations were assayed using an enzyme-linked immunosorbent assay (ELISA). For measurement of IgG concentrations microtiter plates were coated with antigen by incubating overnight at 4 °C with 0.5 mg/ml KLH in sodium bicarbonate buffer (pH 9.6) [37]. Plates were washed with phosphate buffered saline (PBS) (pH 7.4) containing 0.05% Tween 20 (PBS-T) at pH 7.4, then blocked with 5% non-fat dry milk in PBS overnight at 4 °C to reduce non-specific binding, and washed again with PBS-T. Thawed serum samples were diluted 1:20 with PBS-T, and 150 μl of each serum dilution was added in duplicate to the wells of the antigen-coated plates. Positive control samples (pooled sera from hamsters previously determined to have high levels of anti-KLH antibody, similarly diluted with PBS-T) were added in duplicate. Plates were sealed, incubated at 37 °C for 3 h, and then washed with PBS-T. Secondary antibody (alkaline phosphatase-conjugated-anti hamster IgG diluted 1:500 with PBS-T (Rockland, Gilbertsville, PA)) was added to the wells, and the plates were sealed and incubated for 1 h at 37 °C. Plates were then washed again with PBS-T and 150 μl of the enzyme substrate *p*-nitro-phenyl phosphate (Sigma, St Louis, MO; 0.1 mg/ml in diethanolamine substrate buffer) was added to each well. Plates were protected from light during the enzyme–substrate reaction. The optical density (OD) of each well was determined using a plate reader (Bio-Rad, Benchmark Richmond, CA) equipped with a 405 nm wavelength filter, and the

mean OD for each sample was expressed as a ratio of its plate positive control OD for statistical analysis.

2.2.5. Bacterial killing assay

As a functional assessment of an animal's ability to clear a bacterial infection, we utilized an ex vivo bacterial killing assay, based on a modification [38] of a previously published protocol [39]. This assay quantifies the relative number of *E. coli* colony forming units (CFU) that grow after incubation with serum. Differences in CFU presumably represent differences in serum proteins. Briefly, *E. coli* (ATCC #8739, Microbiologics, St. Cloud, MN) (1 pellet = 10^7 CFU) was added to 40 ml 1 M sterile PBS warmed to 35–37 °C and vortexed to create a bacterial stock solution, which was activated by incubation for 30 min at 37 °C. Serum samples were diluted 1:40 in glutamine enriched CO_2-independent media (Invitrogen Corp., Carlsbad, CA). This dilution was validated for serum with a dose response curve prior to the experiment. The stock bacteria solution (500,000 CFU/ml) was diluted with sterile 1 M PBS to create a 50,000 CFU/ml working solution. To obtain estimates of bacterial numbers (i.e., positive control), the working solution was diluted 1:10 with glutamine enriched CO_2-independent media. For each sample, the working solution was added at a 1:10 ratio to the diluted serum sample. The bacteria/serum cocktails were incubated for 30 min at 37 °C. All samples were vortexed and 50 μl was added to petri plates in duplicate and spread with a flame-sterilized spreader. All plates were stored upside down overnight at 37 °C. Following incubation, bacteria colonies were counted on each plate, and duplicates were averaged. The mean value for each sample was expressed as a percent of bacteria killed relative to the control plates in which no killing occurred.

2.2.6. Statistical analyses

Offspring measures were analyzed using a nested design (i.e., litters nested within treatment = litters [treatment]), and were subjected to one-way ANOVAs for treatment [40]. An initial ANOVA revealed sex differences and so for all analyses the sexes were analyzed separately. If significant differences were found, a Tukey's Honestly Significant Differences (HSD) test was performed for pair-wise comparisons. Repeated measures were used to analyze data collected over time (i.e., body temperature, body mass, food intake, cortisol). If a significant main effect was found, a univariate Greenhouse–Geisser test was conducted. Bacterial killing and IgG data did not meet assumptions of normality and were arcsine transformed (appropriate for proportional data). Behavioral and hormonal data were log transformed if they did not meet the assumptions of normality. A difference at the level of $p < 0.05$ was considered statistically significant. All analyses were performed using JMP version 7.0.1 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Experiment 1A and B: maternal immune activation

Prior to LPS treatment in both Experiment A high dose and Experiment B low dose groups, body temperature (high: $t = -0.61$, $p > 0.05$; low: $t = 1.16$, $p > 0.05$), food intake (high: $F_{1,\ 22} = 0.215$, $p > 0.05$; low: $F_{1,\ 43} < 0.001$, $p > 0.05$) and body mass (high: $F_{1,\ 22} = 0.194$, $p > 0.05$; low: $F_{1,\ 43} = 0.008$, $p > 0.05$) did not differ compared with their respective controls. In response to treatment, however, the LPS-treated pregnant females displayed a significant hypothermic response (Fig. 1A). The mean body temperature of the high LPS dose group dropped 5 °C at 24 h post-injection (Time × Treatment, $F_{1,22} = 4.87$, $p < 0.05$). The low dose and control groups did not significantly vary following the injections over time (within subjects, $F_{2.4,\ 102.5} = 1.22$, $p = 0.30$), and the low dose group was significantly lower overall than the control group (between subjects, $F_{1,42} = 6.66$, $p = 0.01$; Fig. 1B). Activation of the immune response by LPS induced a host of sickness behaviors, including anorexia. Specifically, food intake was reduced significantly in the high dose group ($F_{1,\ 19} = 104.8$, $p < 0.0001$) and low dose group ($F_{1,\ 42} = 24.89$ $p < 0.001$) for two days (Fig. 2A, B), before returning to normal control levels. Additionally, body mass was significantly reduced in high dose females following treatment (Time × Treatment: $F_{1.428,\ 27.13} = 42.02$ $p < 0.0001$) and low dose females ($F_{1,42} = 3.98$, $p = 0.05$; Fig. 2C, D). Pregnancy success was noted as the birth of at least one live pup. The number of successful births was reduced by treatment with LPS (Table 1, Fig. 3), and analysis of the low dose group reveals that the initial pup number (LPS = 5.58 ± 0.54; control = 6.8 ± 0.39; $t = 1.82$, $p = 0.04$; Table 1) and mass (LPS = 9.23 ± 0.88; control = 11.21 ± 0.69; $t = 1.78$, $p = 0.04$; Table 1) of the successful litters were reduced in pups from LPS treated dams.

3.2. Experiment 2: effects on offspring development and behavior

3.2.1. Endocrine responses

With respect to endocrine disturbances, sexes were analyzed separately because hormone levels are known to vary by sex. Pre-behavioral interaction cortisol concentrations, although not significantly affected by maternal treatment, trended towards being different in both sexes, although in opposite directions (female $F_{Treatment\ 1,46} = 2.83$, $p = 0.10$, $F_{Litter[Treatment]} = 1.13$, $p = 0.38$; male $F_{Treatment\ 1,50} = 2.42$, $p = 0.13$, $F_{Litter[Treatment]} = 1.53$, $p = 0.14$; Table 2). Post-behavioral cortisol concentrations, however, were significantly elevated in the male offspring of LPS-treated dams relative to offspring of vehicle-treated dams ($F_{Treatment\ 1,50} = 17.22$, $p < 0.01$, $F_{Litter[Treatment]} = 1.69$, $p = 0.09$; Table 2), but not in female offspring ($F_{Treatment\ 1,46} = 0.26$, $p = 0.61$, $F_{Litter[Treatment]} = 1.41$, $p = 0.21$; Table 2). Additionally, the cortisol response (i.e., change in cortisol from pre to post-behavior levels) was significantly greater in female offspring from LPS-treated dams relative to female offspring from control dams ($F_{Treatment\ 1,46} = 6.18$, $p = 0.02$, $F_{Litter[Treatment]} = 1.02$, $p = 0.48$; Fig. 4). The same effect was seen in male offspring ($F_{Treatment\ 1,50} = 4.54$, $p = 0.04$; Fig. 4), but males also varied among litters within treatment ($F_{Litter[Treatment]} = 2.02$, $p = 0.04$), suggesting that both treatment and litter are important for development of the stress response. Male testosterone levels were not affected by maternal LPS treatment ($F_{Treatment\ 1,50} = 0.29$, $p = 0.59$, $F_{Litter[Treatment]} = 1.89$, $p = 0.06$; Table 2), but a greater change in testosterone from pre to post encounter (i.e., stress levels) correlated positively with fleeing ($F_{1,50} = 7.99$, $p < 0.01$) and frequency of submissive behaviors ($F_{1,50} = 4.44$, $p = 0.04$), and no other behaviors (p is above 0.42 in all cases).

3.2.2. Growth and behavioral responses

We measured growth rate (change in body mass over time) and tested male and female social behavior of the offspring of the low dose group and respective controls at 15 weeks of age, using the resident–intruder paradigm (see below). Initial growth rate of offspring did not differ by maternal treatment ($F_{1,25} = 0.39$, $p > 0.05$). For behavioral trials no more than two randomly chosen offspring of each sex were used from a single litter. There was no significant direct effect of maternal LPS treatment or litter [treatment], however, on any of the pup behaviors of either sex (p is above 0.11 in all cases). Specifically, in male offspring there was a positive relationship between baseline cortisol (which tended to be higher in males from LPS treated dams, see below) and defensive behavior ($F_{1,50} = 7.78$, $p < 0.01$). The opposite relationship was present in females where pre-behavior cortisol (which tended to be lower in offspring from LPS treated dams) correlated negatively with defensive behavior ($F_{1,46} = 3.51$, $p = 0.06$; Fig. 5A), suggesting that LPS-treated offspring of both sexes engaged in more defensive behaviors. In males, stress cortisol levels positively correlated with frequency of bites ($F_{1,50} = 3.59$, $p = 0.06$; Fig. 5B), and inversely to grooming behaviors ($F_{1,50} = 3.89$, $p = 0.05$). Similar effects were not seen in female offspring (p is above 0.28 in all cases). There was no relationship with hormones affected by maternal treatment on

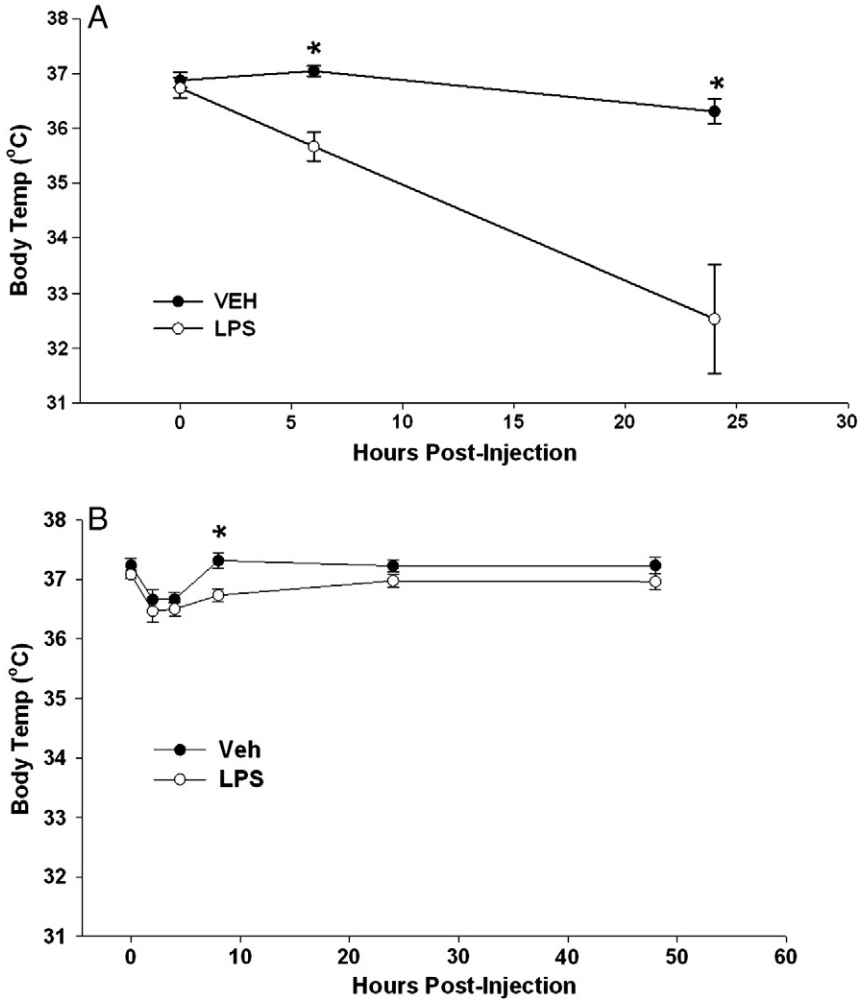

Fig. 1. Maternal body temperature during LPS response. Body temperature (°C) in pregnant females over the time (h) following LPS injection from 2 separate studies in: A) high dose (upper panel) and B) low dose (lower panel) dams. Open circles represent LPS-treated females and closed circles represent vehicle-treated females. Asterisks denote statistically significant time points ($\alpha = 0.05$).

other social behaviors (i.e., escape and attack) during the offspring interaction (p is above 0.09 in all cases).

3.2.3. Immune function

We tested the response to a novel antigen by measuring anti-KLH IgG antibodies ten days following inoculation. We found no difference in the antibodies produced in the offspring of control and low dose dams (female $F_{Treatment\ 1,46} = 0.09$, $p = 0.77$, $F_{Litter[Treatment]} = 1.11$, $p = 0.40$; male $F_{Treatment\ 1,50} = 0.33$, $p = 0.57$, $F_{Litter[Treatment]} = 1.37$, $p = 0.22$; Table 2). In addition, we examined bacterial killing ability in the offspring, a functional measure of immunity. In female offspring, bacterial killing varied significantly by treatment ($F_{Treatment\ 1,46} = 19.78$, $p < 0.01$; Fig. 6) and among litters within treatment ($F_{Litter[Treatment]} = 21.75$, $p < 0.01$). In male offspring there was no difference in bacterial killing ability according to treatment ($F_{Treatment\ 1,49} = 2.64$, $p = 0.12$; Fig. 6) but it did vary among litters within treatment ($F_{Litter[Treatment]} = 25.72$, $p < 0.01$) and was inversely related to baseline male testosterone levels ($F_{1,49} = 5.34$, $p = 0.03$).

4. Discussion

Overall, the current study demonstrates that maternal immune activation with LPS results in significant gestational effects including 1) reduced pregnancy success, 2) reduced litter size and mass and 3) endocrine abnormalities in adult offspring that are correlated with certain behaviors including defensive behavior and dominant/subordinate relationships. Specifically, treatment with LPS reduced pregnancy success in a dose-dependent manner, and those females that were successful had smaller litters both in number of pups and overall litter mass. Furthermore, offspring of LPS treated dams that reproduced successfully showed altered cortisol levels which were related to defensive behaviors in both sexes. Both cortisol levels and cortisol responsiveness were affected by maternal immune activation in both sexes; testosterone levels, however, remained unaffected. Bacterial killing ability, a functional measure of innate immunity, was also affected by maternal immune activation. Surprisingly, there was no effect of maternal treatment on offspring growth or offspring humoral immunity. Furthermore, the LPS-induced immune activation in pregnant females in this study is markedly different to that of non-pregnant females [41] or males of the same species from previous studies [42,43], where pregnant females in the current study experienced a hypothermic response to LPS as compared to the more typically reported hyperthermia (i.e., fever).

4.1. Maternal effects

Activation of the immune response by LPS induced a suite of sickness responses, including anorexia. Specifically, food intake was significantly reduced in the high dose LPS group for two days and low LPS group for one day, before returning to normal baseline levels. Additionally, body mass was significantly reduced as a result of the anorexic behavior in high dose LPS females following treatment.

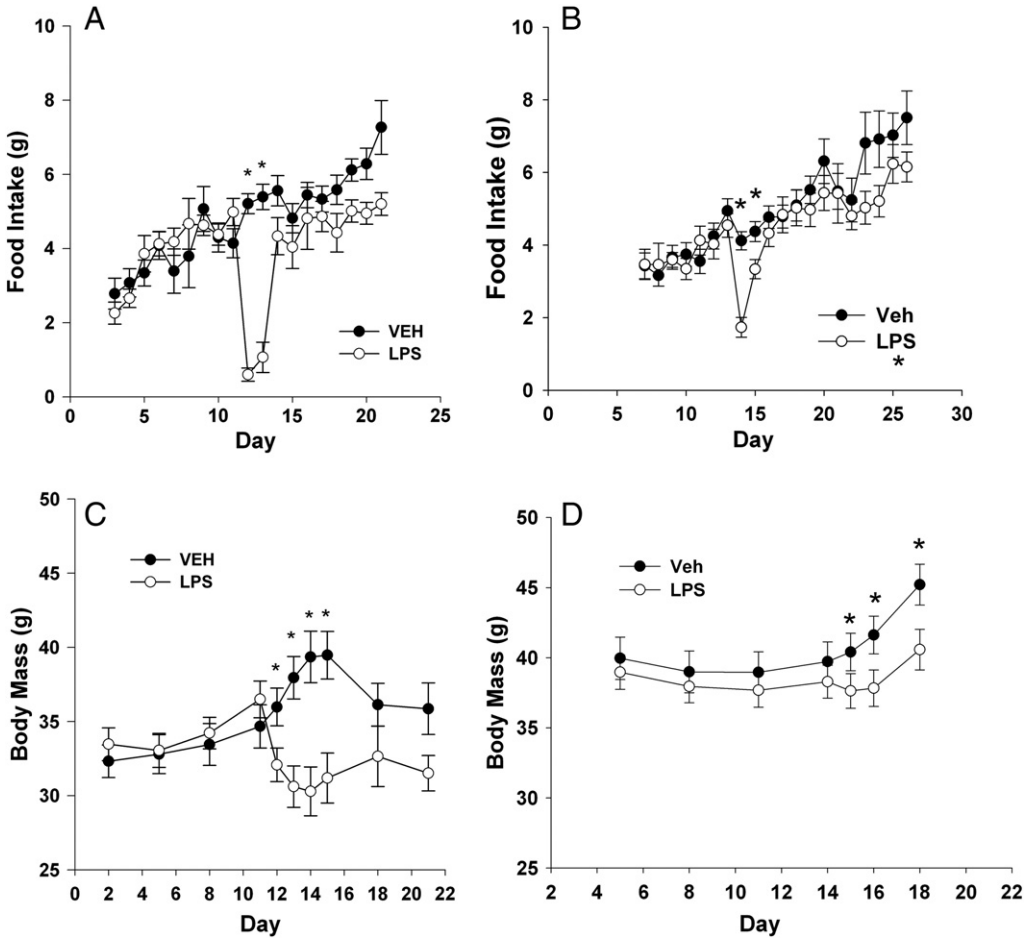

Fig. 2. Food intake and body mass. Food intake in A) high dose (left upper panel) and B) low dose (right upper panel), and body mass in C) high dose (left lower panel) and D) low dose (right lower panel) dams during pregnancy. Injections of LPS or vehicle occurred on Day 11. Asterisks denote statistically significant time points ($\alpha = 0.05$).

This response has been well documented in previous studies where LPS induced a decrease in food intake, which resulted in a subsequent decrease in body mass [44]. Further, in the current study, the effect was dose-dependent, where increasing the LPS dose exacerbated the anorexic response and resulted in weight loss of females.

While LPS typically causes a fever response in non-pregnant animals, the LPS-treated pregnant hamsters displayed a hypothermic response following treatment. The mean body temperature for the high LPS groups dropped 5 °C at 24 h post-injection, whereas the low group displayed an attenuated yet significant reduction in body temperature of a half of a degree over the same time period. This may be a function of LPS dose, as higher doses in Wistar rats are known to induce hypothermic responses [45,46]. Alternatively, this could also be a function of reproductive or energetic state. For example, similar hypothermic responses were found in pregnant rats and guinea pigs [47–49]. However, this response is markedly different from the typical fever response found in males and non-pregnant females of this species using a comparable LPS dose, as well as other mammalian species, including rats and mice [48]. The proximate cause of this hypothermic response remains unclear, however several studies in humans and rats reported elevated anti-inflammatory cytokines (i.e., IL-1ra) during late pregnancy which could account for suppressed fever responsiveness [48,50,51]. Fevers are induced by prostaglandin E_2, and therefore reduction of the production of this prostaglandin during immune activation in late pregnancy may also induce fever suppression [52]. At an ultimate level of analysis, reduced fever is hypothesized to be a protective mechanism to shield the fetus from adverse pyrogenic conditions (reviewed in: [24,53]), or potentially preserve energy resources from costly febrile response for the developing fetuses.

Table 1
Number of dams, proportion of successful pregnancies, average litter mass (g), and average litter number per treatment group.

Treatment	Number of female	Proportion of successful pregnancies	Litter mass (g)	Number of offspring
High LPS	12	8.3	9.8	5
Vehicle	12	66.7	10.06 ± 0.63	6.5 ± 0.38
Low LPS	26	46.1	9.23 ± 0.88	5.58 ± 0.54
Vehicle	19	78.9	11.21 ± 0.69	6.8 ± 0.39

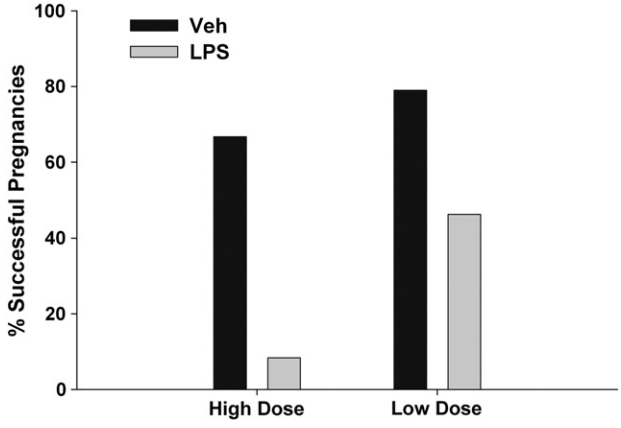

Fig. 3. Pregnancy success. LPS treatment decreased pregnancy success in both high and low dose animals relative to vehicle-treated control animals.

Table 2
Pre and post-behavioral interaction serum testosterone ng/ml (±SEM), serum cortisol ng/ml (±SEM), and immunoglobulin G antibody response in male and female offspring of either vehicle (VEH) or lipopolysaccharide (LPS)-treated dams.

Sex	Maternal treatment	Number	Pre-behavior testosterone (ng/ml)	Post-behavior testosterone (ng/ml)	Pre-behavior cortisol (ng/ml)	Post-behavior cortisol (ng/ml)	IgG
Male	Vehicle	33	19.28 ± 2.37	22.48 ± 2.89	73.76 ± 4.48	69.73 ± 2.52	57.89 ± 4.13
	LPS	21	17.31 ± 2.67	24.76 ± 4.76	80.01 ± 6.05	87.30 ± 5.37	65.77 ± 4.58
Female	Vehicle	28	–	–	99.95 ± 7.17	89.92 ± 6.21	59.17 ± 5.65
	LPS	20	–	–	83.03 ± 5.65	90.31 ± 6.85	58.00 ± 5.56

Finally, the extreme drop in body temperature in the high dose animals may be indicative of sepsis as shown by Romanovsky et al. [45,46]. High levels of endotoxins such as LPS can induce septic shock through inflammatory agents and lead to hypothermia, sickness behaviors, miscarriage and even death. In rats, the LD_{50} for LPS in rats was 5 mg/kg [54]. Our high dose of 0.7 mg/kg LPS was lower than that in non-pregnant rats which induced sepsis but did not cause significant mortality (1 mg/kg; [54]). Hamsters may be more sensitive to LPS in general or specifically during pregnancy, when energy costs are high.

The more pronounced effects of LPS-induced immune activation during pregnancy in the current study were the effects on pregnancy success and litter size. First, the number of successful births was significantly reduced by treatment with the low dose of LPS (i.e., 78.9% vehicle, 46.1% low dose successful pregnancies), and reduced even further with increasing sickness severity (i.e., 66.7% vehicle, only 8.33% high dose successful pregnancies). Other studies show similar effects where maternal immune activation, and not fetal exposure to LPS, induced miscarriage in BALB/c mice [27] and LPS-hyporesponsive strain C3H/HeJ mice [26]. Furthermore, the above studies linked LPS-induced pro-inflammatory cytokines (e.g., TNFα) and nitric oxide production to fetal death [26,27]. These factors may play a role in the dose-dependent effect on pregnancy success in the current study. Finally, low dose dams that reproduced showed reduced litter size in both the number of pups and total litter mass relative to litters born of control dams. Therefore even those females that had successful pregnancies (i.e., at least one live pup born) had impaired reproductive output relative to control females.

4.2. Offspring effects

Significant endocrine changes related to behavior were observed in the offspring of LPS-treated dams; and these effects were observed even into adulthood. The present finding is particularly interesting given that the LPS dose was relatively modest in terms of effects to the mother, resulting in only a small change in food intake and maternal body mass. Thus, even minor perturbations can alter litter size, pregnancy success, and offspring phenotype. Because only one litter from the high dose group of LPS treated dams survived to parturition, all offspring studies were performed in litters from the low dose LPS group, where there were sufficient litters for comparison. However, changes induced by the low-dose of LPS in the gestational environment of pregnant females were sufficient to alter litter size and offspring physiology and likely behavior. The potential mediators of the observed effects include, but are not limited to, altered resource availability, cytokine profiles, glucocorticoid levels, or maternal care behavior (reviewed in: [5,55]). Therefore, we measured offspring growth rate, humoral and innate immune parameters, cortisol and testosterone levels, and tested male and female behavior at 15 weeks of age (adulthood), using the resident–intruder paradigm. Initial litter size (i.e., number of pups and total litter mass) did differ by maternal treatment, however, initial body mass and growth rate of offspring only differed according to sex and not according to maternal treatment. While some previous studies

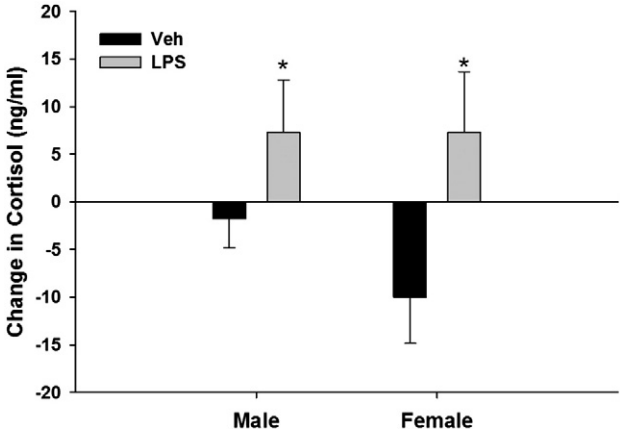

Fig. 4. Offspring cortisol. Magnitude of the change in cortisol concentrations in male and female offspring of LPS and vehicle-treated dams before and after resident-intruder aggression. These data represent surviving offspring from low dose LPS-treated dams. Asterisks denote statistically significant differences ($\alpha = 0.05$).

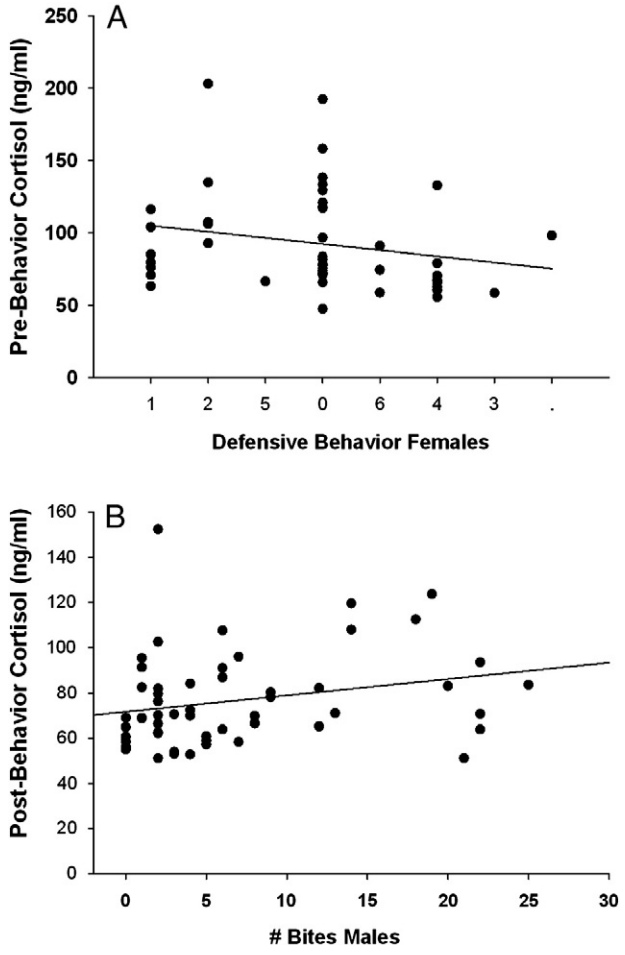

Fig. 5. Relationship between defensive and bite behavior and cortisol is sex dependent. A) Correlation between frequency of defensive behavior and pre-behavior serum cortisol in females, and B) correlation between frequency of bites and post-behavior serum cortisol in males ($\alpha = 0.05$).

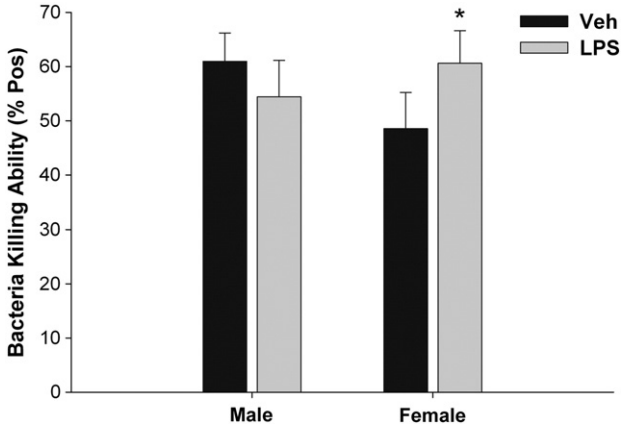

Fig. 6. Bacterial killing ability. Percent bacteria killed in male and female offspring of LPS and vehicle-treated dams. These data represent surviving offspring from low dose LPS-treated dams. Asterisks denote statistically significant differences ($\alpha = 0.05$).

found an effect of prenatal LPS exposure on offspring body size and growth [56], others did not [57].

While we found no effect on offspring body size or growth rate in the present study, we did find that maternal LPS treatment affected endocrine characteristics which are related to behavior during adulthood. No significant effect of maternal immune activation on behavior was observed (although several trends were noted), but behaviors were significantly correlated with LPS-induced cortisol changes. The fact that effects were not observed early in development, but instead manifested later in life in endocrine abnormalities is highly significant. One possible mediator of the behavioral effects of LPS is potential differences in maternal care that are due to differences in initial pup number. The litter size from the LPS-treated dams was smaller, and these pups may therefore have received more grooming, which is known to affect HPA activity and adult behavior. Increasing evidence across taxa suggests that prenatal exposure to stressors, such as LPS, has the potential to result in behavioral abnormalities later in life (reviewed in: [6]).

We also found significant endocrine variations related to prenatal LPS exposure. Post-behavior cortisol concentrations were altered in a sex-dependent manner where cortisol was elevated in male offspring of LPS-treated dams relative to male offspring of vehicle-treated dams, but no difference was seen in female offspring. In both sexes, pre-behavior cortisol was related to defensive behavior, suggesting that it may play a regulatory role in the observed behavioral differences. However, the direction of the relationship between defensive behavior and basal cortisol varied by sex so that it was positive in males and negative in females. Post-encounter cortisol was also positively correlated with bites during encounters in male offspring, but a similar relationship was not present in females. Interestingly, a similar paradigm exists in Syrian hamsters (*Mesocricetus auratus*), whereby vasopressin, an important hormone for the expression of many social behaviors, stimulates aggression in males but inhibits aggression in females [58]. This pattern has also been found in other small mammalian species [59]. Alternatively, the change in cortisol from baseline to stress-induced levels was greater in LPS treated relative to control offspring of both sexes; however this effect in males was also partially driven by litter suggesting that both LPS treatment and litter are important for development of stress response. Similar effects of neonatal LPS treatment on corticosterone were observed in Wistar rats, and were shown to affect sex steroid hormones and reproductive development into adulthood [60]. Although male offspring testosterone levels were not affected by maternal treatment, they did correlate positively with both fleeing and frequency of submissive behaviors.

Prenatal exposure to LPS resulted in similar elevations in basal levels of an alternative glucocorticoid, corticosterone, and upstream adrenal corticotropin-releasing hormone (CRH) in male Wistar rats [57]. Neonatal rats exposed to LPS also showed elevated corticosterone levels relative to control animals, but only in response to an acute stressor [61]. One potential mechanism for this effect is that LPS exposure could alter maternal care behaviors which are known to influence offspring stress responses and behavior in other mammals [55,62]. Regardless, these results suggest that deregulation of the HPA axis may be at least partially responsible for the altered endocrine responses in offspring prenatally exposed to LPS, although the specific mechanism is not known. Further, the opposite direction of this association in males and females suggests that the deregulation may be sex-dependent.

The observed cortisol-related behavioral response was not surprising given that one of the primary physiological pathways modified by TLR4 activation via LPS is the HPA axis. Treatment with LPS results in significant increases in the release of CRH, and in turn, results in elevated adrenal glucocorticoid release, a well-characterized response [63]. Subsequently, glucocorticoids can exert a wide range of behavioral and physiological effects. For example, elevations in glucocorticoids may alter stress-related behaviors including anxiety, aggression and depression [64]. Prolonged elevation of glucocorticoids also suppresses various immune components, including inflammatory cytokines [65]. In the present study, bacterial killing ability (a functional innate immune response) was elevated in female but not male offspring from dams that underwent immune activation during pregnancy. This provides some support for the transgenerational priming of immunity hypothesis, in that a prenatal innate immune challenge resulted in enhanced innate immunity, at least in female offspring. The sex difference indicates altered signaling or reception of maternal factors depending on offspring sex following the immune challenge, which needs to be addressed in future studies. Surprisingly, we found no effect of maternal immune activation on KLH antibody response (humoral immunity). This suggests that an inflammatory challenge in mid-pregnancy does not affect the developing humoral immune system to the extent that effects are seen in adulthood. In previous studies prenatal stressors have had mixed results on adult humoral immunity. In rats, prenatal restraint stress resulted in an enhancement in offspring humoral immunity [66], but a conditioned stress response in mice during pregnancy produced offspring with reduced humoral immune responses [67]. This supports that the type and timing of a stressor affect the physiological response. Further, there is increasing experimental support that prenatal exposure to elevated maternal glucocorticoid levels can exert long-term developmental and behavioral alterations in offspring, including alterations in gonadal steroids, immune function and stress responsiveness [6,68].

4.3. Conclusions

Collectively, the results of these experiments provide evidence that stimulation of maternal immunity during pregnancy can disrupt pregnancy to the point of termination or manifest later in the adult social life of the offspring. Thus, experimental sickness stimulated by LPS is relatively short-term, but still capable of long-term phenotypic consequences for offspring, possibly through deregulation of the HPA axis. However it is important to note that whether these effects are mediated during gestation (i.e., exposure to LPS or maternal physiological changes) or post-gestation (i.e., maternal behavior) is not yet known. Consequently, infections occurring during pregnancy may result in immunological responses that could, if left unchecked or exacerbated, result in long term developmental and behavioral modifications to offspring. For example, exposure to common periodontal bacteria (*Fusobacterium nucleatum*) can cause premature delivery, still births, and reduced offspring survival in mice [69]. Therefore, by characterizing the long-term developmental and behavioral implications of maternal immune activation, we can identify potential early indicators

of behavioral syndromes as well as preventative maternal treatments concordant with immune activation.

It is important to note that the temporal design of many of the published studies varies greatly, which may account for disparate results. A study in C57BL6/J demonstrated that the timing of viral infection during pregnancy was an important determinant for the severity of offspring effects [3]. Therefore future studies should address how temporal differences in immune activation across pregnancy affect offspring behavior. Moreover, it will be important to identify causative agents mediating these behavioral changes (i.e., specific cytokines, cortisol) to understand the proximate mechanisms underlying the resultant behavioral abnormalities and subsequently employing pharmacological agents that target specific physiological mechanisms. This may allow for the eventual development of effective treatment strategies to attenuate behavioral abnormalities caused by maternal immune activation.

Acknowledgments

We thank Timothy Greives for help with testosterone assays. This work was in part supported by Common Themes in Reproductive Diversity Training Grant, NIH # HD 049336-04 and NSF grant IOB 0543798. This work was reviewed and approved by the Institutional Animal Care and Use Committee at Indiana University under protocol # 07-097.

References

[1] Mousseau TA, Fox CW. The adaptive significance of maternal effects. Trends Ecol Evol 1998;13:403–7.
[2] Holekamp KE, Dloniak SM. Maternal effects in mammals. Chicago: University of Chicago Press; 2009 .
[3] Meyer U, Nyffeler M, Engler A, Urwyler A, Schedlowski M, Knuesel I, et al. The time of prenatal immune challenge determines the specificity of inflammation-mediated brain and behavioral pathology. J Neurosci 2006;26:4752–62.
[4] Rhees RW, Fleming DE. Effects of malnutrition, maternal stress, or ACTH injections during pregnancy on sexual behavior of male offspring. Physiol Behav 1981;27:879–82.
[5] Patterson PH, Xu W, Smith SEP, Devarman BE. Maternal immune activation, cytokines and autism. Autism 2009:289–307.
[6] Weinstock M. Does prenatal stress impair coping and regulation of hypothalamic–pituitary–adrenal axis? Neurosci Biobehav Rev 1997;21:1–10.
[7] Entringer S, Kumsta R, Hellhammer DH, Wadhwa PD, Wüst S. Prenatal exposure to maternal psychosocial stress and HPA axis regulation in young adults. Horm Behav 2009;55:292–8.
[8] Love OAP, Chin EAH, Wynne-Edwards KE, Williams TAD. Stress hormones: a link between maternal condition and sex-biased reproductive investment. Am Nat 2005;166:751–66.
[9] Hayward LS, Wingfield JC. Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. Gen Comp Endocrinol 2004;135:365–71.
[10] Cree A, Tyrrell CL, Preest MR, Thorburn D, Guillette LJ. Protecting embryos from stress: corticosterone effects and the corticosterone response to capture and confinement during pregnancy in a live-bearing lizard (*Hoplodactylus maculatus*). Gen Comp Endocrinol 2003;134:316–29.
[11] Meaney MJ, Szyf M, Seckl JR. Epigenetic mechanisms of perinatal programming of hypothalamic–pituitary–adrenal function and health. Trends Mol Med 2007;13:269–77.
[12] Welberg LAM, Seckl JR. Prenatal stress, glucocorticoids and the programming of the brain. J Neuroendocrinol 2001;13:113–28.
[13] Barlow SM, Knight AF, Sullivan FM. Delay in postnatal growth and development of offspring produced by maternal restraint stress during pregnancy in the rat. Teratology 1978;18:211–8.
[14] Moore IT, Jessop TS. Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. Horm Behav 2003;43:39–47.
[15] Reeder DM, Kramer KM. Stress in free-ranging mammals: integrating physiology, ecology, and natural history. J Mammal 2005;86:225–35.
[16] Romero LM, Reed JM, Wingfield JC. Effects of weather on corticosterone responses in wild free-living passerine birds. Gen Comp Endocrinol 2000;118:113–22.
[17] Bilbo SD, Schwarz JM. Early-life programming of later-life brain and behavior: a critical role for the immune system. Front Behav Neurosci 2009;3.
[18] Kentner AC, Pittman QJ. Minireview: early-life programming by inflammation of the neuroendocrine system. Endocrinology 2010;151:4602–6.
[19] Patterson PH. Maternal infection: window on neuroimmune interactions in fetal brain development and mental illness. Curr Opin Neurobiol 2002;12:115–8.
[20] Zalcman SS, Siegel A. The neurobiology of aggression and rage: role of cytokines. Brain Behav Immun 2006;20:507–14.
[21] Siegel A, Bhatt S, Bhatt R, Zalcman SS. Cytokines and aggressive behavior. Neuroimmunological Basis Behav Ment Disord 2009:235–59.
[22] Ebisui O, Fukata J, Murakami N, Kobayashi H, Segawa H, Muro S, et al. Effect of IL-1 receptor antagonist and antiserum to TNF-alpha on LPS-induced plasma ACTH and corticosterone rise in rats. Am J Physiol Endocrinol Metab 1994;266:E986–92.
[23] Smith SEP, Li J, Garbett K, Mirnics K, Patterson PH. Maternal immune activation alters fetal brain development through interleukin-6. J Neurosci 2007;27:10695–702.
[24] Mouihate A, Harré EM, Martin S, Pittman QJ. Suppression of the febrile response in late gestation: evidence, mechanisms and outcomes. J Neuroendocrinol 2008;20:508–14.
[25] Clark DA, Manuel J, Lee L, Chaouat G, Gorczynski RM, Levy GA. Ecology of danger-dependent cytokine-boosted spontaneous abortion in the CBA x DBA/2 mouse model. I. Synergistic effect of LPS and (TNF-α + IFN-γ) on pregnancy loss. Am J Reprod Immunol 2004;52:370–8.
[26] Silver R, Lohner W, Daynes R, Mitchell M, Branch D. Lipopolysaccharide-induced fetal death: the role of tumor-necrosis factor alpha. Biol Reprod 1994;50:1108–12.
[27] Athanassakis I, Aifantis I, Ranella A, Giouremou K, Vassiliadis S. Inhibition of nitric oxide production rescues LPS-induced fetal abortion in mice. Nitric Oxide 1999;3:216–24.
[28] Spencer SJ, Galic MA, Pittman QJ. Neonatal programming of innate immune function. Am J Physiol Endocrinol Metab 2011;300:E11–8.
[29] Dantzer R, Kelley KW. Twenty years of research on cytokine-induced sickness behavior. Brain Behav Immun 2007;21:153–60.
[30] Mouihate A, Pittman QJ. Neuroimmune response to endogenous and exogenous pyrogens is differently modulated by sex steroids. Endocrinology 2003;144:2454–60.
[31] Grindstaff JL, Hasselquist D, Nilsson J-Å, Sandell M, Smith HG, Stjernman M. Transgenerational priming of immunity: maternal exposure to a bacterial antigen enhances offspring humoral immunity. Proc R Soc Lond B Biol Sci 2006;273:2551–7.
[32] Moret Y. 'Trans-generational immune priming': specific enhancement of the antimicrobial immune response in the mealworm beetle, *Tenebrio molitor*. Proc R Soc Lond B Biol Sci 2006;273:1399–405.
[33] Finlay B, Darlington R. Linked regularities in the development and evolution of mammalian brains. Science 1995;268:1578–84.
[34] Reburn CJ, Wynne-Edwards KE. Hormonal changes in males of a naturally biparental and a uniparental mammal. Horm Behav 1999;35:163–76.
[35] Scotti M-AL, Belén J, Jackson JE, Demas GE. The role of androgens in the mediation of seasonal territorial aggression in male Siberian hamsters (*Phodopus sungorus*). Physiol Behav 2008;95:633–40.
[36] Dixon FJ, Jacot-Guillarmod H, McConahey PJ. The antibody responses of rabbits and rats to hemocyanin. J Immunol 1966;97:350–5.
[37] Demas GE, Drazen DL, Nelson RJ. Reductions in total body fat decrease humoral immunity. Proc R Soc Lond B Biol Sci 2003;270:905–11.
[38] French SS, Greives TJ, Zysling DA, Chester EM, Demas GE. Leptin increases maternal investment. Proc R Soc Lond B Biol Sci 2009;276:4003–11.
[39] Matson KD, Cohen AA, Klasing KC, Ricklefs RE, Scheuerlein A. No simple answers for ecological immunology: relationships among immune indices at the individual level break down at the species level in waterfowl. Proc R Soc Lond B Biol Sci 2006;273:815–22.
[40] Haseman JK, Bailer AJ, Kodell RL, Morris R, Portier K. Statistical issues in the analysis of low-dose endocrine disruptor data. Toxicol Sci 2001;61:201–10.
[41] Pyter LM, Samuelsson AR, Quan N, Nelson RJ. Photoperiod alters hypothalamic cytokine gene expression and sickness responses following immune challenge in female Siberian hamsters (*Phodopus sungorus*). Neuroscience 2005;131:779–84.
[42] Prendergast BJ, Hotchkiss AK, Bilbo SD, Nelson RJ. Peripubertal immune challenges attenuate reproductive development in male Siberian hamsters (*Phodopus sungorus*). Biol Reprod 2004;70:813–20.
[43] Bilbo SD, Drazen DL, Quan N, He L, Nelson RJ. Short day lengths attenuate the symptoms of infection in Siberian hamsters. Proc R Soc Lond B Biol Sci 2002;269:447–54.
[44] Kelley KW, Bluthé RM, Dantzer R, Zhou JH, Shen WH, Johnson RW, et al. Cytokine-induced sickness behavior. Brain Behav Immun 2003;17:112–8.
[45] Romanovsky AA, Székely M. Fever and hypothermia: two adaptive thermoregulatory responses to systemic inflammation. Med Hypotheses 1998;50:219–26.
[46] Romanovsky AA, Shido O, Sakurada S, Sugimoto N, Nagasaka T. Endotoxin shock: thermoregulatory mechanisms. Am J Physiol Regul Integr Comp Physiol 1996;270:R693–703.
[47] Zeisberger E, Merker G, Blähser S. Fever response in the guinea pig before and after parturition. Brain Res 1981;212:379–92.
[48] Ashdown H, Poole S, Boksa P, Luheshi GN. Interleukin-1 receptor antagonist as a modulator of gender differences in the febrile response to lipopolysaccharide in rats. Am J Physiol Regul Integr Comp Physiol 2007;292:R1667–74.
[49] Martin SM, Malkinson TJ, Veale WL, Pittman QJ. Fever in pregnant, parturient, and lactating rats. Am J Physiol Regul Integr Comp Physiol 1995;268:R919–23.
[50] Fofie AE, Fewell JE. Influence of pregnancy on plasma cytokines and the febrile response to intraperitoneal administration of bacterial endotoxin in rats. Exp Physiol 2003;88:747–54.
[51] Pillay V, Savage N, Laburn H. Interleukin-1 receptor antagonist in newborn babies and pregnant women. Pflugers Arch 1993;424:549–51.
[52] Spencer SJ, Mouihate A, Galic MA, Pittman QJ. Central and peripheral neuroimmune responses: hyporesponsiveness during pregnancy. J Physiol 2008;586:399–406.
[53] Chambers CD, Johnson KA, Dick LM, Felix RJ, Jones KL. Maternal fever and birth outcome: a prospective study. Teratology 1998;58:251–7.
[54] Spencer SJ, Field E, Pittman QJ. Neonatal programming by neuroimmune challenge: effects on responses and tolerance to septic doses of lipopolysaccharide in adult male and female rats. J Neuroendocrinol 2010;22:272–81.
[55] Meaney MJ. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Neuroscience 2001;24.

[56] Bakos J, Duncko R, Makatsori A, Pirnik Z, Kiss A, Jezova D. Prenatal immune challenge affects growth, behavior, and brain dopamine in offspring. Ann N Y Acad Sci 2004;1018:281–7.
[57] Reul JM, Stec I, Wiegers GJ, Labeur MS, Linthorst AC, Arzt E, et al. Prenatal immune challenge alters the hypothalamic–pituitary–adrenocortical axis in adult rats. J Clin Invest 1994;93:2600–7.
[58] Gutzler SJ, Karom M, Erwin WD, Albers HE. Arginine–vasopressin and the regulation of aggression in female Syrian hamsters (*Mesocricetus auratus*). Eur J Neurosci 2010;31:1655–63.
[59] de Vries GJ. Sex differences in vasopressin and oxytocin innervation of the brain. In: Inga DN, Rainer L, editors. Prog. Brain Res. Elsevier; 2008. p. 17–27.
[60] Walker AK, Hiles SA, Sominsky L, McLaughlin EA, Hodgson DM. Neonatal lipopolysaccharide exposure impairs sexual development and reproductive success in the Wistar rat. Brain Behav Immun 2011;25:674–84.
[61] Walker FR, Knott B, Hodgson DM. Neonatal endotoxin exposure modifies the acoustic startle response and circulating levels of corticosterone in the adult rat but only following acute stress. J Psychiatr Res 2008;42:1094–103.
[62] Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, et al. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic–pituitary–adrenal responses to stress. Science 1997;277:1659–62.
[63] Shanks N, Lightman SL. The maternal–neonatal neuro-immune interface: are there long-term implications for inflammatory or stress-related disease? J Clin Invest 2001;108:1567–73.
[64] Dantzer R, Aubert A, Bluthé RM, Gheusi G, Cremona S, Layé S, et al. Mechanisms of the behavioural effects of cytokines. Cytokines Stress Depress 1999:83–105.
[65] Dhabhar FS. Stress-induced augmentation of immune function—the role of stress hormones, leukocyte trafficking, and cytokines. Brain Behav Immun 2002;16:785–98.
[66] Klein SL, Rager DR. Prenatal stress alters immune function in the offspring of rats. Dev Psychobiol 1995;28:321–36.
[67] Gorczynski RM. Conditioned stress responses by pregnant and/or lactating mice reduce immune responses of their offspring after weaning. Brain Behav Immun 1992;6:87–95.
[68] Barbazanges A, Piazza PV, Le Moal M, Maccari S. maternal glucocorticoid secretion mediates long-term effects of prenatal stress. J Neurosci 1996;16:3943–9.
[69] Han YW, Redline RW, Li M, Yin L, Hill GB, McCormick TS. *Fusobacterium nucleatum* induces premature and term stillbirths in pregnant mice: implication of oral bacteria in preterm birth. Infect Immun 2004;72:2272–9.
[70] Demas GE, Johnson C, Polacek KM. Social interactions affect reproductive and immune responses in Siberian hamsters (*Phodopus sungorus*). Physiol Behav 2004;83:73–9.