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The Effect of Infection Risk on Female Blood Transcriptomics

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Running title: Effects of infection risk

Abstract

Defenses against pathogens can take on many forms. For instance, behavioral avoidance of diseased conspecifics is widely documented. Interactions with these infectious conspecifics can also, however, lead to physiological changes in uninfected animals, an effect that is much less well understood. These changes in behavior and physiology are particularly important to study in a reproductive context, where they can impact reproductive decisions and offspring quality. Here, we studied how an acute (3 h) exposure to an immune-challenged male affected female blood transcriptomics and behavior. We predicted that females paired with immune-challenged males would reduce eating and drinking behaviors (as avoidance behaviors) and that their blood would show activation of immune and stress responses. We used female Japanese quail as a study system because they have been shown to respond to male traits, in terms of their own physiology and egg investment. Only two genes showed significant differential expression due to treatment, including an increase in the threonine dehydrogenase (TDH) transcript, an enzyme important for threonine breakdown. However, hundreds of genes in pathways related to activation of immune responses showed coordinated up-regulation in females exposed to immune-challenged males. Suppressed pathways revealed potential changes to metabolism and reduced responsiveness to glucocorticoids. Contrary to our prediction, we found that females paired with immune-challenged males increased food consumption. Water consumption was not changed by treatment. These findings suggest that even short exposure to diseased conspecifics can trigger both behavioral and physiological responses in healthy animals.

1. Introduction

Animals employ an array of strategies to reduce infection burden. The most familiar and well-studied of those are the ones that take place upon infection, which can involve drastic changes in both physiology and behavior (Lopes et al., 2021). However, animals also respond to the perceived *risk* of infection. Behavioral avoidance of parasites, of parasite cues or of parasitized conspecifics, for instance, is documented in several species (Cremer et al., 2007; Lopes, 2020; Lopes et al., 2022; Meunier, 2015). A less well understood prophylactic strategy consists of changes in physiology in situations or environments with high risk of infection (reviewed in Lopes, 2022). One example of this comes from studies in fruit flies (*Drosophila melanogaster*) which show changed neuropeptide F signaling in the brain when exposed to parasitoid wasps (Kacsoh et al., 2013). This change in physiology results in modified oviposition behavior which protects larvae from parasitism. Another study showed that healthy canaries (*Serinus canaria domestica*) housed in visual and auditory contact with canaries infected with *Mycoplasma gallisepticum* (MG) developed immune responses (increased complement activity and higher heterophil counts) without ever becoming infected (Love et al., 2021). Increased circulating corticosterone (an endocrine response to stress) has been found in healthy female mice housed across from parasitized males (Curno et al., 2009). These types of studies illustrate the potential for anticipatory physiological responses to increase fitness of the individuals perceiving high risk of parasitism by enhancing their own survival upon infection and the survival of their offspring. It is of interest therefore to understand when these types of responses occur, what they look like and how they may impact individual fitness and disease transmission.

In this study, we used female Japanese quail (*Coturnix japonica*) to assess the effects of a short-term (acute) interaction with an immune-challenged male. We quantified these effects at the level of the blood transcriptome and behavior. We chose this study species because female Japanese quail are sensitive to male characteristics and characteristics of their social environment in ways that can influence their physiology, fertilization success, paternity, and offspring traits (Adkins-Regan, 1995; Adkins-Regan et al., 2013; Adkins–Regan and MacKillop, 2003; Correa et al., 2011; Langen et al., 2019, 2017; Persaud and Galef Jr., 2005). For our study, females were either paired with a healthy male or with a male underdoing a simulated infection (i.e., challenged with an antigen) and then, after 3 h of cohabitation, a blood sample was collected. We chose to study the blood transcriptome not only because it allows for assessment of immune activation and the contribution of immune cell subpopulations (Chaussabel, 2015; Li et al., 2016; Prokop et al., 2021; Tabone et al., 2021), but also because it can reflect additional (non-immune) phenotypic characteristics (Schmidt et al., 2020) and because it has been shown to predict tissue-specific expression for several other tissues (e.g., lungs, muscles, spleen, etc.) (Basu et al., 2021). In other words, blood transcriptomics can provide a global picture of an animal's physiological state.

Here, we tested the hypothesis that interactions with animals experiencing infection-related symptoms can affect physiological and behavioral responses of healthy animals. We predicted that the blood of females paired with an immune-challenged male but not of those paired with healthy males, would show activation of immune and stress responses. We predicted activation of immune responses because this is a type

of anticipatory response found in other studies (reviewed in Lopes, 2022). More specifically, given that the innate arm of the immune system constitutes the first line of defense in a primary infection, we predicted that transcriptomic signatures related to innate immune defenses would be activated in the females exposed to immune-challenged males, and could include signatures of cell types involved in this response, of proinflammatory signals, and of elements of the acute phase response. As highlighted in the introduction, being co-housed with a diseased conspecific could constitute a stressor (Curno et al., 2009). We therefore predicted activation of stress responses. The secretion of glucocorticoids, induced upon activation of the hypothalamic-pituitary-adrenal axis, is one of the classic endocrine responses to stress (Sapolsky et al., 2000). Since glucocorticoids can affect immune regulation in complex ways (Cain and Cidlowski, 2017), we briefly outline those relationships here. It is proposed that at low concentrations and in the short-term, glucocorticoids can have an immunostimulatory effect (in particular, of the innate immunity), and that at high concentrations and in the longer-term, glucocorticoids can inhibit both the innate and adaptive arms of the immune response (Cain and Cidlowski, 2017). Glucocorticoids bind to more than one receptor, but the effects of glucocorticoids on immune regulation are exerted through the glucocorticoid receptor. For this reason, synthetic glucocorticoids, such as dexamethasone or prednisone, have been produced to have high specificity to the glucocorticoid receptor, and are used as potent immunoregulators. In terms of behavior, since avoidance of contaminated food and drinking sources has been found in mammals and a few bird species (Lopes et al., 2022), we predicted that

females exposed to immune-challenged males would show reduced feeding and drinking behaviors relative to females exposed to controls, in order to avoid parasitism.

2. Materials and Methods

2.1. Animals

Animals were maintained in a long day light cycle (14 L: 10 D) and the room temperature was consistently 21˚C. Lights were on at 7:00. A long day light cycle was selected to ensure the animals were in breeding condition, which was critical for ongoing parallel experiments studying effects on eggs. Animals used as part of this experiment were hatched in our facilities from fertilized Japanese quail eggs purchased from AA Lab Eggs, Inc. (Westminster, CA, USA). Egg incubation and animal rearing conditions were as described in de Bruijn et al. (2020). Adults were fed *at libitum* with Mazuri® Exotic Gamebird Breeder (St. Louis, MO, USA). Birds were 89.8 ± 10.2 days of age at the start of the experiment (range 71 – 108 days).

This experiment was approved by Chapman University Institutional Animal Care and Use Committee (Protocol # 2018-02) and was conducted according to the Association for the Assessment and Accreditation of Laboratory Animal Care Guidelines.

2.2. Experimental design

To test how females react behaviorally and physiologically to immune-challenged males, 31 female Japanese quails were used. Fifteen females were exposed to immune-challenged males (henceforth, male LPS treatment) and sixteen were exposed

to control saline-treated males (henceforth, male saline treatment). As an immune challenge for this experiment, we used lipopolysaccharide (LPS). LPS is a constituent of the outer membrane of the majority of Gram-negative bacteria and, once administered, it elicits an immune response, along with sickness behaviors (Lopes et al., 2021) but it cannot be transmitted to nearby animals. This immune response to LPS is characterized by activation of Toll-like receptor signaling pathways and culminates in the production of proinflammatory cytokines, which are involved in the production of sickness behaviors (Lopes et al., 2021). At 9:00, male quail were weighed and either injected intramuscularly with LPS (2 mg/kg; Sigma L4005 in endotoxin-free sterile PBS) or an equivalent amount of saline (endotoxin-free sterile PBS). They were then moved to experimental cages for 1 hour, which is a sufficient time for male Japanese quail to develop sickness behavior symptoms (Gormally et al., 2022). In male quail, these include increased time spent resting, and reduced eating and drinking bouts (Gormally et al., 2022). At this time, female quail were moved into the experimental cages where they remained for 3 hours. Behavior was recorded remotely via security cameras. At the end of this part of the experiment, blood samples from the brachial vein (~60 µL) were taken from all animals. Samples were collected within 3 min of entering the enclosures. Blood samples were centrifuged to separate plasma and red blood cells; aliquots of each were stored at -80˚C. The behaviors and physiology of the males used in this experiment were analyzed as part of a separate study (Gormally et al., 2022).

2.3. Gene expression

2.3.1. RNA extraction, library preparation and sequencing

RNA was extracted from a subset of randomly selected samples from the male LPS (n = 10) and male saline (n = 9) treatments. An aliquot of 20 µL of blood (the spun down layer after removing plasma, consisting of red blood cells, white blood cells and platelets; also known as packed cells) was placed in 1 mL of QIAzol lysis reagent (Qiagen, item #79306). Cell disruption was done by agitation of this solution in a beaded tube (ZR BashingBeads Lysis Tubes, Zymo Research, item # S6003-50) on a Benchmark Scientific Beadbug 6 homogenizer for 20 sec at a speed of 7 m s^{-1}. This process was repeated once, after a 5 min rest. After chloroform precipitation, the aqueous layer was used to isolate total RNA using the Zymo Clean and Concentrator Kit-5 (Zymo Research, item # R1013), following manufacturer's instructions, including the DNase I treatment step. Frozen RNA samples were sent to Novogene Corporation Inc. (Chula Vista, CA, USA), where RNA quantity, purity and integrity were assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), cDNA libraries were produced using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA) and cDNA fragments (150–200 bp in length) were then purified using the AMPure XP System (Beckman Coulter, Beverly, USA). Paired-end sequencing of libraries (PE150; Illumina Novaseq 6000) was performed following standard protocols. An average of 45 million paired-end raw reads were obtained for each sample. Raw reads were subjected to the following filtering procedures: reads with adapter contamination, reads where uncertain nucleotides (N) constituted more than 10 % of the read, and reads where more than 50 % of the read contained low-quality nucleotides (Qscore < 5), were removed. This resulted in an average of 41.7 million clean reads.

2.3.2. Mapping and differential gene expression analysis

An average of 88.04% of clean reads (i.e., post adapter removal and quality filtering) were mapped to the Japanese quail reference genome (Coturnix_japonica_2.0, INSDC Assembly Mar 2016, downloaded from Ensembl), representing an average of 36.73 million mapped reads per sample (Supplementary Table S1). HISAT2 was used for mapping and HTSeq to count the number of mapped reads to each gene. Prior to differential expression analysis, we filtered out low expression genes by removing from the dataset genes with 10 or less counts in 90 % or more of the samples, resulting in 8,284 genes. Principal component analysis using variance stabilized transformed counts revealed the presence of two outlier samples (both from male saline treatment; outlier samples were separated by a distance of 25 % on PC1 from any other sample, Fig. S1), which were removed from downstream analysis. The DESeq2 R package v. 1.32 (Love et al., 2014) was used for differential gene expression analysis to understand the effect of male treatment on female blood gene expression. Resulting p-values from Wald tests were adjusted using the Benjamini–Hochberg procedure to control for false discovery rate due to multiple testing. Genes were considered as statistically differentially expressed when adjusted p-values were < 0.05.The volcano plot of all genes in this analysis was produced using base R. All statistical analysis (in this and following sections) were carried out in R v. 4.2.1 (R Core Team, 2022).

2.3.3. Gene Set Enrichment Analysis (GSEA)

While single gene analysis (such as the one we performed using DESeq2) may reveal large individual differences in expression due to treatment, it can often miss smaller coordinated changes in expression in gene sets. These coordinated changes, however, can have stronger impacts on cellular and molecular pathways than larger changes in single genes (Subramanian et al., 2005). In order to determine whether gene sets sharing the same Gene Ontology (GO) categories were up or down regulated in females exposed to LPS males relative to females exposed to saline males, we used Gene Set Enrichment Analysis (GSEA) using the package clusterProfiler v.4.4.4 (Wu et al., 2021). As input for the analysis, first, all genes (without pre-filtering of low expression counts) were subjected to DESeq2 analysis, modeled as described in section 3.2.3. Of those genes, we then used all genes that had Ensembl IDs that matched to official gene symbols (a total of 11,054 genes), after filtering any genes with duplicate names (only 39). Scores were obtained by multiplying the sign of fold change (i.e., the direction of change) by the negative $\log_{10}$ p-value, following (Reimand et al., 2019). These scores were rank ordered by decreasing value and this ranked list used as the input for GSEA. GSEA was performed using the gseGO function, with the genome wide annotation for Human (v.3.15.0) (Carlson, 2022), using the Biological Process (BP) GO term collection, a minimum set size of 30 genes and maximum set size of 500, 1000 permutations, and using FDR as the adjustment method for multiple comparisons, and an adjusted p-value cutoff of < 0.05. Dotplots for enrichment results were also prepared using the clusterProfiler package.

2.4. Behavior

Female drinking and eating behaviors were quantified for the first 20 min of each hour (total of 60 min). Drinking was quantified as the total number of pecks at the water bottle spout during the 60 min of observation. Eating was quantified as the total number of pecks at the food dish or at the floor (because sometimes the food scatters on the cage floor when animals feed) during the 60 min of observation. Both behaviors were scored by one observer blind to the treatments, using the BORIS software (Friard and Gamba, 2016). For drinking, the behavior of one animal could not be collected because the drinking spout was obstructed from view, so drinking behavior for that animal was not included in the analysis. Each behavior was first modeled as a function of treatment, using the Poisson distribution. Because overdispersion was detected in both models, both were ultimately modeled using a quasi-Poisson regression. The drop1 function was then applied to each model to obtain the main effect of treatment. Plots for behavioral data represent estimated marginal means and 95 % confidence intervals and were produced using the ggeffects package (Lüdecke, 2018).

3. Results

Only two genes were differentially expressed at p_{adj}-value < 0.05 (Supplementary Table S2) (Fig. 1). A novel gene (ENSCJPG00005001560; a long non-coding RNA) was downregulated in females exposed to LPS-treated males. The other gene was L-threonine 3-dehydrogenase, mitochondrial (TDH). This gene showed increased expression in females exposed to LPS-treated males.

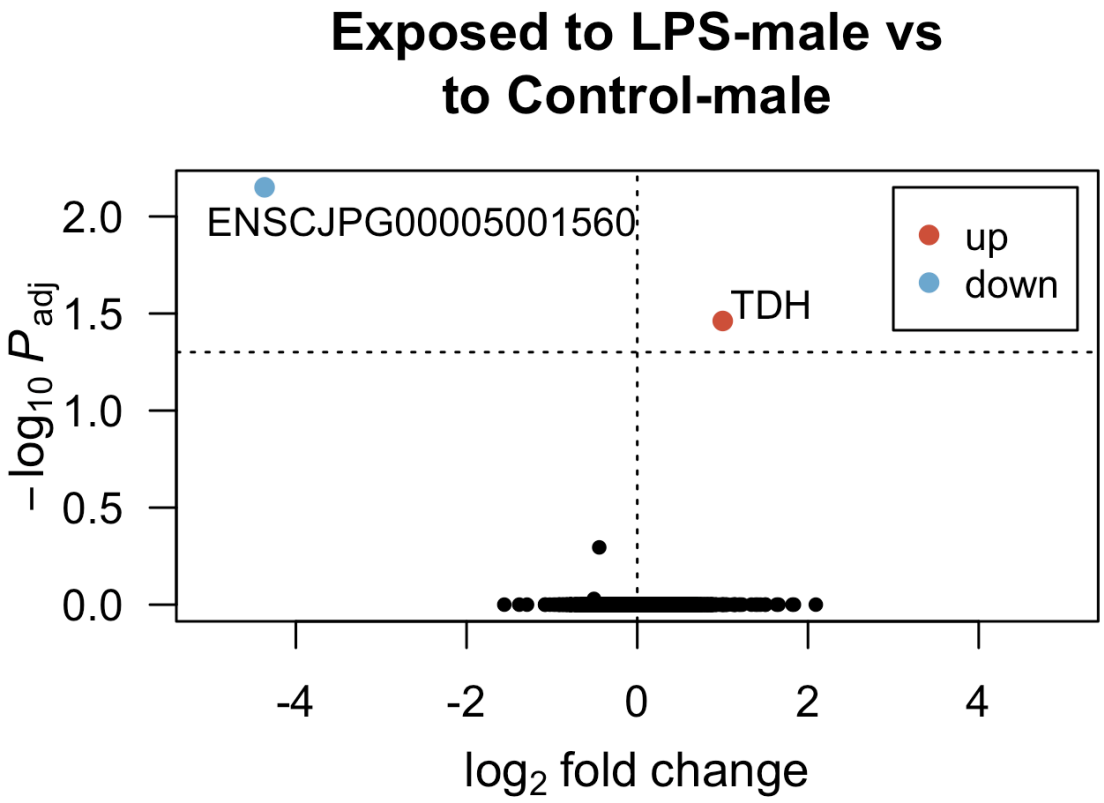

Fig. 1 – Volcano plot representing $\log_2$ fold change against the corresponding negative $\log_{10}$ padj-value of all genes analyzed. Red circles represent genes that were up-regulated in blood of females exposed to LPS-treated males (male LPS treatment) relative to saline-treated males (male saline treatment), while blue circles represent down-regulated genes and black circles genes that did not meet the p_{adj}-value < 0.05 cutoff (dashed horizontal line).

GSEA of the biological processes (BP) from Gene Ontology revealed activation and suppression of selected pathways in females exposed to LPS-males relative to females exposed to saline-males (Supplementaty Table S3). Within the up-regulated pathways (Fig, 2), most were related to the inflammatory response (such as cytokine production, innate immune response, toll-like receptor signaling pathway, and various

leukocyte-related pahtways, including leukocyte migration, leukocyte chemotaxis, leukocyte activation, and leukocyte differentiation). Activated pathways related to specific leukocyte cell types, included: lymphocyte activation, T cell activation, mononuclear cell migration, myeloid leukocyte activation, and granulocyte migration. Supressed pathways were fewer in number and involved long-chain fatty acid transport, cellular response to ketone and response to dexamethasone (Fig. 2).

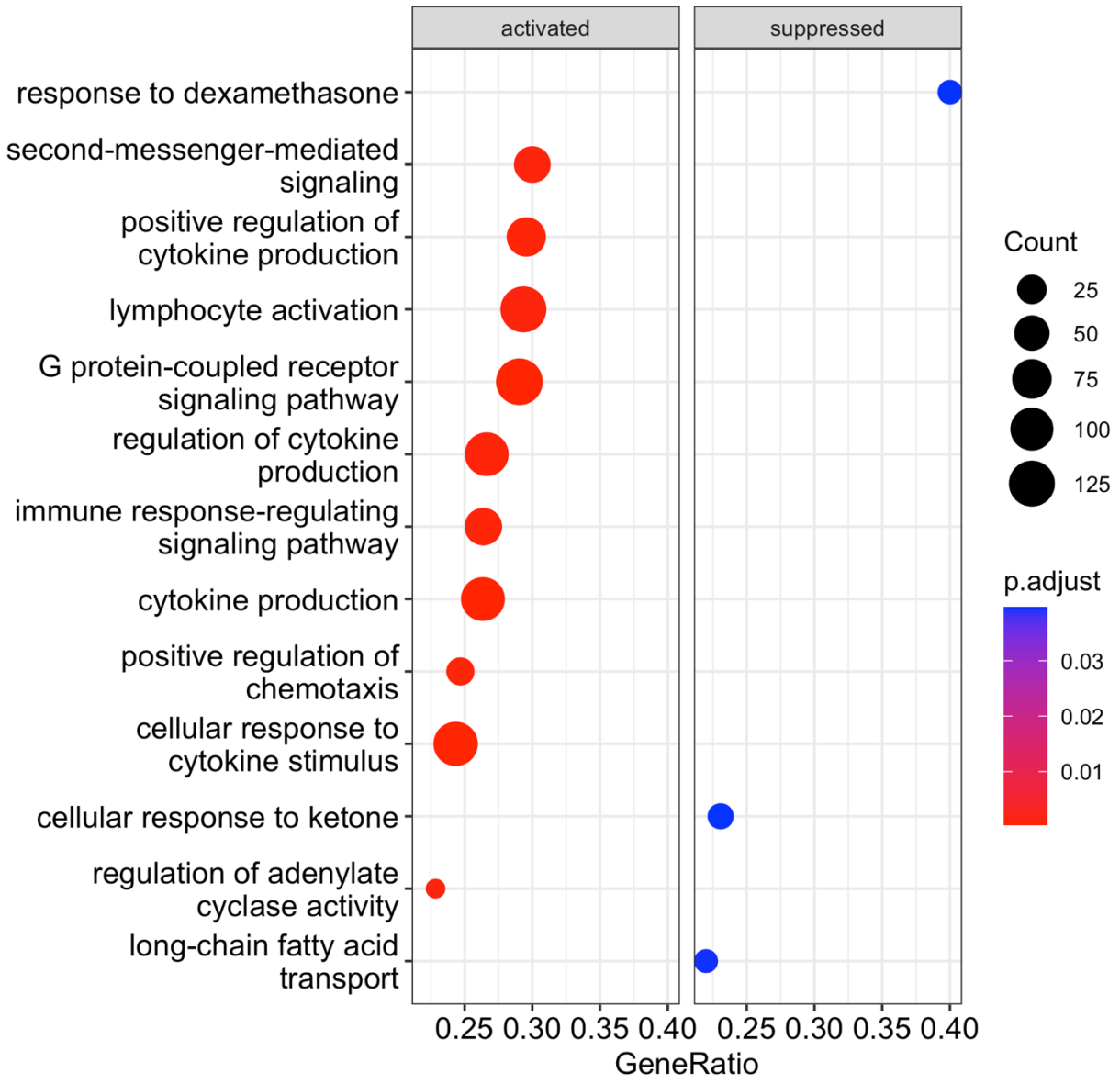

Fig. 2 – Dotplot of 10 most significant activated and supressed GO:BP pathways. Only three terms are shown for suppressed pathways because these were the only terms found. The GeneRatio axis represents the number of input genes that are annotated in a given term over the size of that term (gene set).

We quantified eating and drinking behaviors in response to the male treatments, as a way to assess whether females behaviorally avoided infection. While the number of pecks at food was significantly increased in females exposed to males treated with LPS relative to females exposed to controls ($\chi^2 = 7.87$, $p = 0.0050$, d.f. = 1; Fig. 3A), the number of pecks at the water source was not different between the treatments ($\chi^2 = 0.65$, $p = 0.42$, d.f. = 1; Fig. 3B).

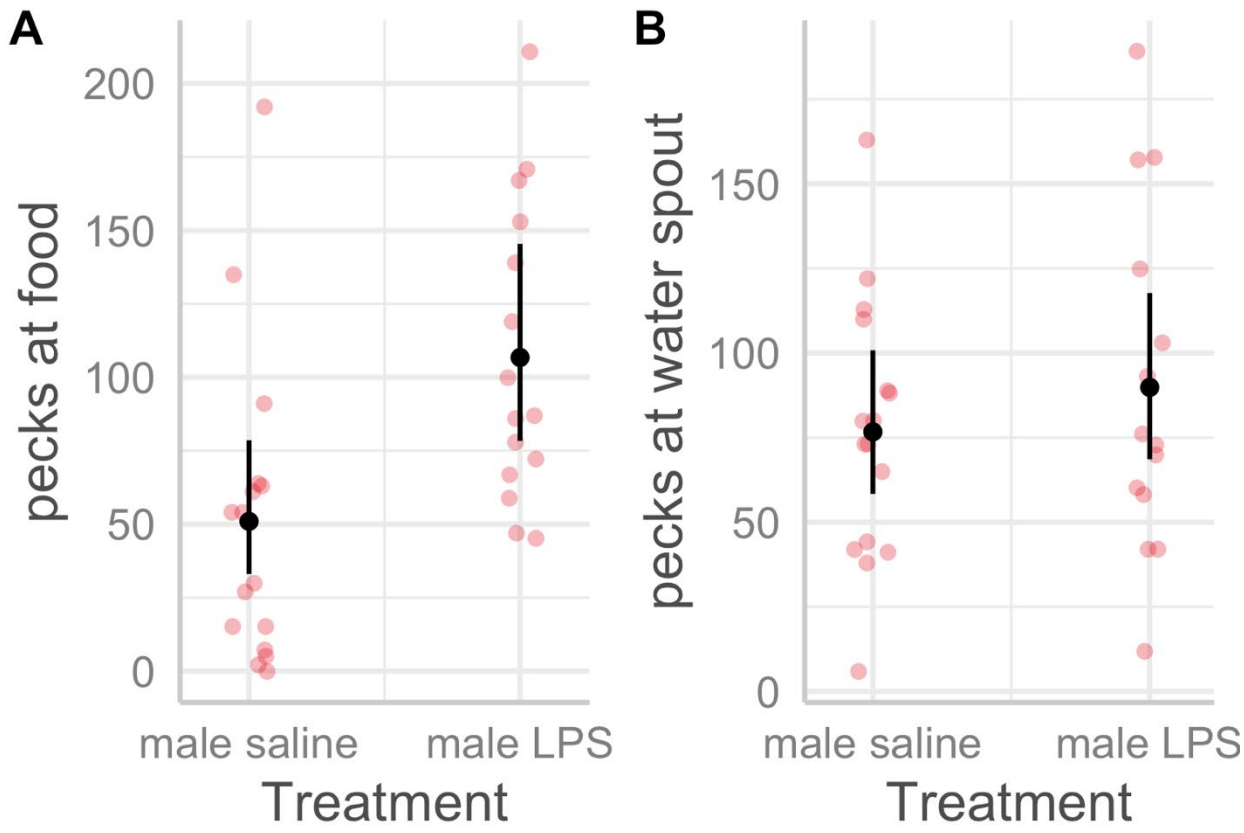

Fig. 3 – Estimated marginal means of eating (A) and drinking (B) behaviors of females exposed to the different male treatments. Red circles represent raw data.

4. Discussion

This study was aimed at assessing whether females exposed to immune-challenged males would show rapid changes in their physiological responses, relative to females exposed to control males. When we tested the blood transcriptome for individual gene differences in expression due to treatment, we found only two genes that met our adjusted p-value cutoff. However, smaller but coordinated changes in sets of genes can affect cellular and molecular pathways in much more dramatic ways than large changes in single genes (Subramanian et al., 2005). When we studied

coordinated changes in expression in gene sets, the results revealed up-regulation of hundreds of genes involved in activation of immune responses, as well as a few repressed pathways involved in pathways related to metabolic changes and to response to glucocorticoids. Female behaviors were also changed, but, in opposition to our predictions, females exposed to LPS-treated males increased eating behaviors. Combined, these results suggest that social information from males undergoing an inflammatory challenge can affect both the physiology and behavior of female Japanese quail.

Females paired with LPS-treated males had higher expression of TDH. TDH codes for threonine dehydrogenase, an enzyme that may be important in L-threonine catabolism. TDH is a functional enzyme in chicken and quail and it's activity levels vary depending on diet, increasing as the protein content of the diet increases (Akagi et al., 2004; Davis and Austic, 1982; Yuan et al., 2000; Yuan and Austic, 2001; Yuan and E. Austic, 2001). In quail, the liver levels of this enzyme increase in fasted animals and in animals fed a threonine-rich diet (Akagi et al., 2004). In case TDH expression levels in the blood are predictive of expression patterns of this gene in the liver, the increased expression of TDH in females exposed to LPS-treated males could therefore reflect changes in food consumption. We had initially hypothesized that females may have reduced ingestive behaviors, such as eating and drinking, in order to avoid becoming parasitized. Contrary to this, rather than showing reduced ingestive behaviors, we found that eating was increased in females exposed to LPS-treated males relative to those exposed to controls. The increase in TDH expression may therefore be explained by relative differences in food consumption between the

307 treatments, given that the food the animals were being fed during the experiment had
308 20 % protein content.
309 As predicted, we found evidence of activation of immune response pathways.
310 Even though in their study, Love and colleagues (2021) did not find differences in
311 expression of the proinflammatory cytokines IL-6 and IL-1 between animals exposed to
312 healthy relative to sick conspecifics, terms related to production of both of these
313 cytokines were among the activated pathways in our study. This difference could be
314 related to the timeline chosen to evaluate cytokine production. The first time point
315 evaluated by Love and colleagues occurred 2 days after exposure to the infected
316 animals. It is possible that changes in proinflammatory cytokines are therefore early
317 responses to exposed to sick conspecifics. Also, while the majority of the activated
318 pathways were indicative of activation of innate immunity and included terms for cellular
319 types of the innate immune system (e.g., myeloid leukocyte activation, granulocyte
320 migration), which fits our predictions, pathways related to T-cell activation and
321 differentiation (T-cells are lymphocytes and part of the adaptive immune system) were
322 also detected. The Love et al. (2021) study found changes in leukocyte cell counts, with
323 increased heterophils (a type of granulocyte) and fewer lymphocytes in birds exposed to
324 diseased animals relative to those exposed to controls. While we cannot speak to
325 changes in cell counts, it has been shown that many cell types can activate T cells
326 under inflammatory conditions (Kambayashi and Laufer, 2014; Lin and Loré, 2017).
327 One of the suppressed pathways (long-chain fatty acid transport) in our study
328 suggests that at least some alterations of metabolic pathways took place in the females
329 exposed to LPS-treated males. Given the energetic and nutritional costs associated with

mounting an immune response (Zuk and Stoehr, 2002), metabolic changes are not unexpected. Indeed, while immune responses were not quantified, at least two studies (one in *Drosophila nigrospiracula* flies and the other one in the California killifish *Fundulus parvipinnis*) have shown an increase in metabolic rates of animals exposed to but uninfected by parasites (Horn et al., 2020; Nadler et al., 2021). To be used as an energy source, long-chain fatty acids need to be transported into the mitochondria (Vockley, 2020) and, therefore, a suppression of this pathway points to reduced use of long-chain fatty acids as energy source in females exposed to LPS-treated males relative to females exposed to controls. Changes in metabolism of these females could be a consequence or a cause of the increased feeding behavior.

Two additional suppressed pathways (cellular response to ketone and response to dexamethasone) hint at the possibility that the response to stress was altered in females exposed to LPS-treated males. Dexamethasone is a ketone and a synthetic glucocorticoid, that acts as a potent and selective glucocorticoid receptor agonist, thereby activating the downstream pathways triggered by binding to this receptor (Timmermans et al., 2019). Whereas this result may seem contradictory to our prediction that these females would show activation of stress responses, it does not necessarily imply changes to the activation of the stress response (in terms of glucocorticoid release). Instead, it implies that females exposed to LPS-treated males are potentially less responsive to glucocorticoids or to activation of the glucocorticoid receptor. In other words, even if corticosterone (the major avian glucocorticoid) were to be released, these females would be less responsive to its downstream effects. Because prolonged exposure or exposure to high concentrations of glucocorticoids is

353 immunosuppressive (Cain and Cidlowski, 2017), one could speculate that the decrease
354 in responsiveness to glucocorticoids could help sustain the activation of immune
355 responses in the longer term. In addition, reduced responsiveness to glucocorticoids
356 could also have contributed to the changes in fatty acid metabolism discussed above
357 (Macfarlane et al., 2008).
358
359 **5. Conclusion**
360 In conclusion, our study suggests that brief exposure to infection risk affects
361 female physiological and behavioral responses. While we considered the blood to
362 contain a global picture of the physiological state of our study animals, future studies
363 that directly analyze other tissues, such as the liver or spleen (as organs important for
364 the production of acute phase responses and immune cells, respectively), will provide
365 more specific insights into which organs are responsive to acute interactions with
366 diseased conspecifics. For example, physiological changes due to infection risk
367 observed in other studies took place at the level of the brain, kidneys, adrenal glands,
368 liver, and likely the spleen (Curno et al., 2009; Kacsoh et al., 2013; Love et al., 2021).
369 Finally, given some of the differences highlighted between ours and the other avian
370 study (Love et al., 2021), it will be interesting for future studies to examine in more detail
371 the temporal aspects of these anticipatory responses to infection risk.
372
373 **Funding**

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Data availability statement

RNA-seq mapping statistics, lists of DEGs, GSEA results, and behavioral data have been uploaded as part of the supplementary material. Sequencing datasets generated and analyzed during the current study are deposited in the NCBI Gene Expression Omnibus (GEO) repository, with record GSE174094 [dataset will be made publicly available once manuscript is accepted for publication].

Authorship

PCL devised the experiments, helped collect data, analyzed data, and wrote the initial draft of the manuscript. BMGG ran the experiment, collected data, wrote the methods section, and edited the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

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