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Abstract

When animals are sick, their physiology and behavior change in ways that can impact their offspring. Research is emerging showing that infection risk alone can also modify the physiology and behavior of healthy animals. If physiological responses to environments with high infection risk take place during reproduction, it is possible that they lead to maternal effects. Understanding whether and how high infection risk triggers maternal effects is important to elucidate how the impacts of infectious agents extend beyond infected individuals and how, in this way, they are even stronger evolutionary forces than already considered. Here, to evaluate the effects of infection risk on maternal responses, we exposed healthy female Japanese quail to either an immune-challenged (lipopolysaccharide, LPS, treated) mate or to a healthy (control) mate. We first assessed how females responded behaviorally to these treatments. Exposure to an immune-challenged or control male was immediately followed by exposure to a healthy male, to determine whether treatment affected paternity allocation. We predicted that females paired with immune-challenged males would avoid and show aggression towards those males, and that paternity would be skewed towards the healthy male. After mating, we collected eggs over a 5-day period. As an additional control, we collected eggs from immune-challenged females mated to healthy males. We tested eggs for fertilization status, embryo sex ratio, as well as albumen corticosterone, lysozyme activity, and ovotransferrin, and yolk antioxidant capacity. We predicted that immune-challenged females would show the strongest changes in the egg and embryo metrics, and that females exposed to immune-challenged males would show intermediate responses. Contrary to our predictions, we found no avoidance of

immune-challenged males and no differences in terms of paternity allocation. Immune-challenged females laid fewer eggs, with an almost bimodal distribution of sex ratio for embryos. In this group, albumen ovotransferrin was the lowest, and yolk antioxidant capacity decreased over time, while it increased in the other treatments. No differences in albumen lysozyme were found. Both females that were immune-challenged and those exposed to immune-challenged males deposited progressively more corticosterone in their eggs over time, a pattern opposed to that shown by females exposed to control males. Our results suggest that egg-laying Japanese quail may be able to respond to infection risk, but that additional or prolonged sickness symptoms may be needed for more extensive maternal responses.

Introduction

The effects of infectious agents extend beyond the effects caused to the infected individuals. The best example of this is behavioral avoidance of parasitism. When in the presence of parasites, parasitized individuals or their cues, or contaminated environments, both vertebrates and invertebrates are able to change their behaviors in order to reduce parasitism (Cremer et al. 2007; Meunier 2015; Lopes 2020; Lopes et al. 2022). Interestingly, a smaller body of research suggests that, when in situations where the risk of infection is high, the physiology of animals can also change. For example, in humans, the mere visualization of disease cues (e.g., photograph of a person sneezing) can lead to stronger cellular immune responses to a bacterial challenge (Schaller et al. 2010). More recently, a study in canaries (*Serinus canaria domestica*) showed that

uninfected canaries provided with visual (eye lesions), behavioral, and auditory cues from canaries infected with *Mycoplasma gallisepticum* (MG) developed immune responses without ever becoming infected (Love et al. 2021). The current experience of a worldwide pandemic (COVID-19) underscores the urgent need to understand how widespread these types of effects are.

Physiological responses to high disease risk situations may be more prominent at specific life history stages, such as when animals are reproducing and the physiological changes they experience can impact their offspring through maternal effects (Agrawal et al. 1999). Indeed, social exposure to an immune-challenged conspecific led to production of larger eggs by females of a non-social insect species (*Tenebrio molitor*) (Gallagher et al. 2018). Also, after cohabitation with parasitoid wasps (which infect fruit fly larvae), fruit flies (*Drosophila melanogaster*) primed the immune responses of their offspring (Bozler et al. 2020). In addition, female mice that were exposed to disease risk during pregnancy (exposure to *Babesia microti* infected males through a mesh partition) had offspring that were faster at resolving an infection (Curno et al. 2009). Responses produced by reproducing animals exposed to high infection risk may therefore be critically important for offspring development and survival, as well as in terms of priming offspring for high disease environments. Despite this likely importance, these responses remain underexplored and poorly understood.

In this study, we set out to determine whether female exposure to an immune-challenged male would affect reproductive responses (such as number and mass of eggs laid, and fertilization rate), offspring sex ratio, and immune defenses deposited to the eggs. In addition, we tested whether these responses were comparable to

responses produced by immune-challenged females. As a study species, we used Japanese quail (*Coturnix japonica*). We chose this system for three main reasons: in this species, maternal immune activation (i.e., when the mother is experiencing an immune challenge) is known to affect immune defenses (antibodies) deposited to eggs and offspring phenotype (Grindstaff 2008); female Japanese quail are sensitive to their social environment (such as mate attractiveness, social instability and context of mating), which has been shown to affect the deposition of steroids into eggs, egg mass, paternity and fertilization success (Adkins-Regan 1995; Adkins–Regan and MacKillop 2003; Persaud and Galef Jr. 2005; Rutkowska and Adkins-Regan 2009; Guibert et al. 2010; Correa et al. 2011; Adkins-Regan et al. 2013); and, finally, Japanese quail are a precocial egg-laying species, which potentially places even more emphasis on the quality of the prenatal environment (the egg) for offspring fitness as compared to altricial species. As an immune challenge (for both males and females), we used lipopolysaccharide (LPS) treatments. LPS is a component of Gram-negative bacteria, known to rapidly trigger immune responses and behavioral symptoms of infection in Japanese quail and a wide range of species (Lopes et al. 2021). We predicted that females initially paired with an immune-challenged male but not those initially paired with healthy males, would show avoidance and aggressive behaviors towards the immune-challenged males, and that paternity of their eggs would be skewed towards the healthy male. Given the cost of mounting a strong immune response, we predicted that LPS-treated females would lay fewer eggs than females in other treatments, that their eggs would contain the highest levels of corticosterone and that egg immune defenses (lysozyme, ovotransferrin and antioxidant capacity) would be elevated in this

treatment. We further predicted that females exposed to LPS-treated males (i.e., high infection risk experience) would show changes in at least some of these egg allocations, but that they would be able to continue producing a larger number of eggs, since they would have information on disease risk but would not carry the burden of actually being sick. We also predicted that egg allocations could change over time after male exposure and that the way these changes occurred could depend on treatment.

Materials and Methods

Animals

Fertilized Japanese quail eggs were purchased from AA Lab Eggs, Inc. (Westminster, CA) and incubated at 37.5-38˚C and 50-60% humidity for 15 days (Ovation 56 EX Egg Incubator, Brinsea, Titusville, FL). On day 16, eggs were moved to a hatching incubator (1502 Sportsman, GOF Manufacturing, Savannah, GA). After hatching, chicks were housed together and provided food (Purina Medicated Start and Grow, Purina Animal Nutrition, Gray Summit, MO) and water *ad libitum*. To assist with thermoregulation and maintain a temperature of ~38˚C, heating lamps were attached to the sides and tops of the cages. At about 3 weeks of age, chicks were able to be sexed and therefore males and females were separated into group cages (~4-8 quail per cage, 100 x 40 x 50 cm) with bedding and food and water ad libitum. As male quail grew larger (~6-8 weeks) they were further separated to prevent aggression; same sex pairs of quail were housed in chicken wire-divided cages (50 x 40 x 50 cm).

Animals were maintained on a long day light cycle (14 L: 10 D) and the room temperature was consistently 21˚C. Quail were shifted to adult feed (Exotic Animal Nutrition, Mazuri, St. Louis, MO) at about 3 months of age. Females were 101 ± 23.6 days of age at the start of the experiment (range 79 – 144 days).

Experimental design

To test female responses to infection risk, we established three experimental treatments. Fifteen untreated females were exposed to LPS-treated males (male LPS), sixteen untreated females were exposed to control saline-treated males (male saline), and fifteen females were treated with LPS and exposed to untreated males (female LPS). At 9:00, male quail were weighed and either injected intramuscularly (i.m.) 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 isolation experimental cages for 1 hour, which is sufficient time to develop sickness behavior symptoms (Gormally et al. 2022). For the female LPS treatment, females were captured from their home cages, weighed, and injected i.m. with LPS (2 mg/kg). They were then returned to their home cage for 1 hour. Non-injected females (in the male LPS and male control treatments) remained un-altered during this time. At 1h after injections, female quail were then moved into the experimental cages containing the males where they remained for 3 hours. Behavior was recorded remotely via security cameras. At the conclusion of this 3-hour period, blood samples were taken from male and female quail for a separate experiment and females were moved into new cages containing a new untreated male for an additional 3 hours. A blood sample from the second male was

also obtained. This male was removed at the end of this time, while the female remained in this cage. Eggs from all females were collected for the 5 days following the experiment.

To allow the embryos to develop sufficiently for DNA extraction (Bozkaya et al. 2013), the eggs were incubated for 5 days (Ovation 56 EX Egg Incubator, Brinsea, Titusville, FL) and then frozen at -80˚C. Albumen, yolk, and embryos were separated from thawed eggs and were frozen again at -80˚C for use in future assays. The number of fertilized eggs was obtained during this processing. Albumen at day 5 of incubation and even at later stages can be extracted from eggs of Japanese quail (Foote 1967). Albumen was clearly distinguishable from yolk during thawing (it thaws faster).

Behavior

To assess how untreated females behaved in the presence of healthy versus immune-challenged males, we focused on expression of avoidance and aggressive behaviors as female quail do not display a strong approach response to male sexual behavior (Gutiérrez and Domjan 2011). We therefore characterized female behaviors into three categories: *avoidance* was when the female was either walking (not crouching still) while the male attempted to mount her or when she was running away from the male; *chasing* was when the female was chasing away the male; and *pecking* was when the female was pecking at the male. The total time of each of these behaviors (except for pecking, where occurrences were counted) was coded using Behavioral Observation Research Interactive Software (BORIS; Friard and Gamba 2016) by coders who were blinded to treatment. The initial 20 minutes the female and the first male (LPS

or control treated) were together was analyzed, as it is the period where males are most interested in interacting and mating with females (Gormally et al. 2022).

DNA extraction

DNA was extracted from red blood cells from males and females in the male LPS treatment and in the male control treatment. DNA was also extracted from embryonic tissue (embryos collected from eggs laid between 2 and 5 days after treatment from all treatments). The Zymo Quick-DNA Miniprep Plus kit was used for all extractions with minor modifications for nucleated red blood cells and tissues (detailed in Supplementary Material).

Paternity

Females, males, and embryos in the male saline and male control treatments were genotyped at four microsatellite loci using fluorescently labeled primers. Primer sequences were obtained from (Kayang et al. 2002) and have been used in additional Japanese quail studies (Langen et al. 2017; Smith et al. 2018). Detailed information on primers used, sequencing and allele calling is provided in Supplementary Material.

Embryo molecular sexing

Molecular sexing of all embryos from eggs collected between days 2 and 5 after treatment was performed using published primers (2550F: 5'-GTTACTGATTCGTCTACGAGA – 3' and 2718R: 5'- ATTGAAATGATCCAGTGCTTG – 3') that flank an intron on the CHD1 gene (Fridolfsson and Ellegren 1999), a gene that

sits on the avian sex chromosomes (W and Z). Because there is a size difference in the region amplified based on whether the region comes from the W or Z chromosome, when run on an agarose gel, the PCR products show two bands for animals with a WZ genotype (females) and one band for animals with a ZZ genotype (males). Details of PCR conditions are contained in the Supplementary Materials.

Egg assays

To determine how the maternal infection risk environment affected egg physiology, we quantified a series of egg allocations. Corticosterone was quantified in albumen according to (Navara and Pinson 2010). While the yolk usually contains higher levels of corticosterone, corticosterone was quantified in albumen for several reasons: 1) in chickens, albumen corticosterone has been shown to strongly correlate with female plasma corticosterone (Downing and Bryden 2008); 2) yolk and albumen reflect accumulation of corticosterone over very different time scales (Cook 2012): corticosterone can accumulate in yolk over days while it accumulates in albumen over a period of hours, which means that albumen is a better representative measure of events that would have taken place after exposure to a male in our experimental set up; and 3) due to the high amounts of other steroids present in yolk, which cross-react with corticosterone antibodies, precise quantification of corticosterone in yolk can be problematic (De Baere et al. 2015). Albumen lysozyme activity was measured using a kinetic spectrophotometric assay according to (Horrocks et al. 2014). Albumen lysozyme was quantified because it is an antimicrobial protein (one of the egg's immune defenses) and it has been shown in birds to change both based on certain

environmental conditions (Horrocks et al. 2014) and based on mate attractiveness (D'Alba et al. 2010). The levels of ovotransferrin, another antimicrobial egg protein, were measured in egg albumen using previously published methods (Horrocks et al. 2011, 2014). In adults, circulating levels of both lysozyme and of ovotransferrin have been shown to increase after an immune-challenge or bacterial infection, which was another reason to assess their levels in eggs (Millet et al. 2007; Rath et al. 2009). The OXY-Adsorbent kit was used to measure antioxidant capacity in yolk samples using previously established methods (Costantini 2010). We quantified yolk antioxidant capacity because antioxidants protect the egg and the offspring from oxidative stress and also because in great tits (*Parus major*), it was shown that the level yolk antioxidants increased depending on the quality of the male females were socially mated to (Remeš et al. 2011). The detailed descriptions of these procedures can be found in the Supplementary Materials.

Statistical analysis

Statistical analysis were carried out in R v. 4.1.2 (R Core Team 2021). Residuals for none of the behaviors were normally distributed, so behaviors were analyzed using Wilcoxon Mann-Whitney Rank Sum tests (a non-parametric version of the t-test). Responses were modeled as a function of treatment. Boxplots for these results were produced using ggplot2 (Wickham 2016) and represent the median, the first and third quartiles (lower and upper hinges), and the smallest and largest values (lower and upper whiskers) no further than the interquartile range. Points beyond the whiskers are represented as black circles.

For any egg-based responses, including paternity, number of eggs, egg mass, fertilization, sex ratio, and egg components, the first egg laid (day 1) was excluded from analysis. The first egg (day 1) was not used because it is impossible to know the extent to which those eggs reflected an effect of treatment (this would depend on the position of the egg inside of the oviduct during treatment). Indeed, out of 38 eggs laid on day 1, only two were fertilized, suggesting that when females were paired with males their day 1 eggs were likely already at an advanced stage in the oviduct.

Paternity was obtained only for embryos of females in the male saline or male LPS treatments. For these females, the first male the female was mated with could be an LPS (male LPS treatment) or control treated male (male saline), while the second male was always untreated. For each female, the number of embryos sired by the first male was coded as the number of successes and the number of embryos sired by the second male as the number of failures. This response object was modeled as a function of female treatment, using binomial error variance.

Fertilization and embryo sex ratio were analyzed in a similar way as paternity. For fertilization, per female, the number of eggs fertilized was coded as the number of successes and the number of eggs unfertilized as the number of failures. This response object was modeled as function of female treatment, using the quasi-binomial family (given that the same model using the binomial family showed overdispersion). For embryo sex ratio, the number of male embryos was coded as successes and the number of female embryos as failures. This response object was modeled as a function of female treatment, using binomial error variance. For models using binomial

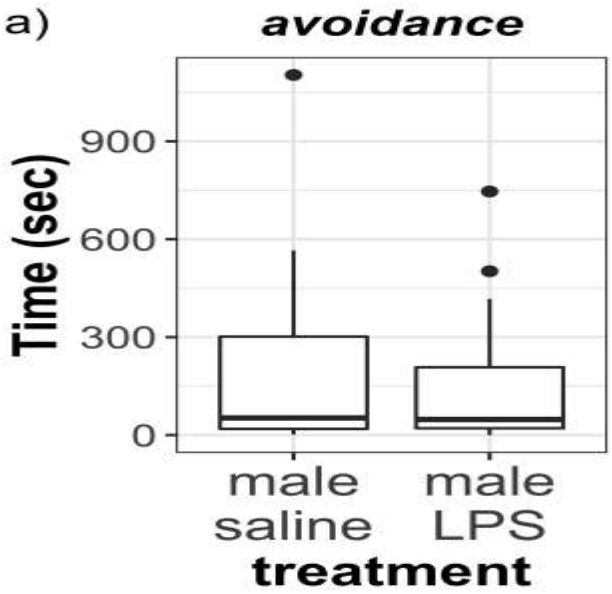
distribution, Chi square tests are reported. For models using quasi-binomial distributions, F tests are reported.

Number of eggs laid per female was modeled as a function of treatment, using the Poisson distribution. The effect of treatment was obtained by calling the drop1 function on this model and Chi square tests are reported. Egg mass, corticosterone, lysozyme, ovotransferrin, and antioxidant capacity were each modeled first as a function of the factor treatment, the covariate time (egg laying day), and an interaction of these variables, as well as a random effect of female ID, using the normal distribution. A possible relationship between embryo sex and albumen corticosterone was tested by modeling embryo sex (male or female) as a function of albumen corticosterone, with a random effect of female ID, using the binomial distribution. These mixed models were setup using the lmer function of the package lme4 (Bates et al. 2015). Likelihood ratio tests were then used to compare the full model (containing the interaction) to an additive model. When the interaction of treatment by day was non-significant (at $p < 0.1$ cutoff), it was dropped from the model and main effects for treatment and day are reported. Main effects were obtained by using the drop1 function on the additive model. When the interaction of treatment by day was significant ($p < 0.1$), it was retained in the model and main effects were not tested, so as to respect the principle of marginality. For mixed models using the normal distribution, F tests are reported and were obtained using the lmerTest package (Kuznetsova et al. 2017) in conjunction with drop1. For both ovotransferrin and for antioxidant capacity, one outlier value was detected that was over two times higher than one standard deviation of the mean. Those outlier values came from different animals (one from male saline treatment and one from male LPS

treatment) and were likely due to reading errors and removed from the analysis. Inspection of model residuals revealed non-normally distributed residuals for both corticosterone and ovotransferrin. Albumen corticosterone had to be square root transformed and ovotransferrin log transformed. Aside from behavioral responses (described above), the plots report estimated marginal means and 95 % confidence intervals, obtained using the ggemmeans function in the ggeffects package (Lüdecke 2018). Transformed data (corticosterone and ovotransferrin) were back-transformed to the response scale for plotting.

Results

We found no differences in the amount time avoiding males (W = 121, p = 0.98, d.f. = 1), chasing away males (W = 136, p = 0.43, d.f. = 1) nor in the number of pecks at males (W = 148.5, p = 0.18, d.f. = 1) in females paired with control males (male saline treatment) relative to females paired with immune-challenged (male LPS; Fig. 1).



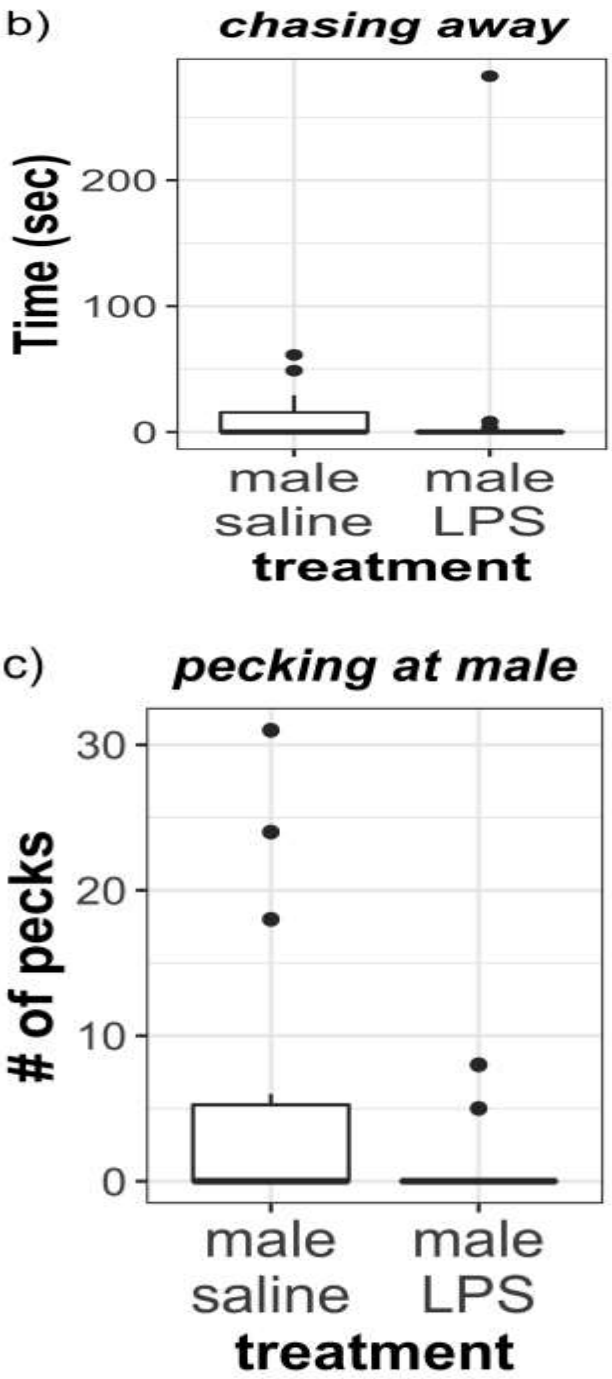


Fig. 1 – Behaviors of females when paired with either control (male saline) or immune-challenged males (male LPS). The time females spent actively moving away from males is shown in a), the time spent chasing away males is shown in b) and the number of pecks at males is shown in c).

Females in the male saline and male LPS treatments were exposed to two types of males during the course of the experiment: first, either an LPS-treated or a saline-treated male, and second, a healthy untreated male. There was no statistically significant difference due to male treatment in the proportion of embryos sired by the first male ($\chi^2 = 0.44$, $p = 0.51$, d.f. = 1; Fig. 2).

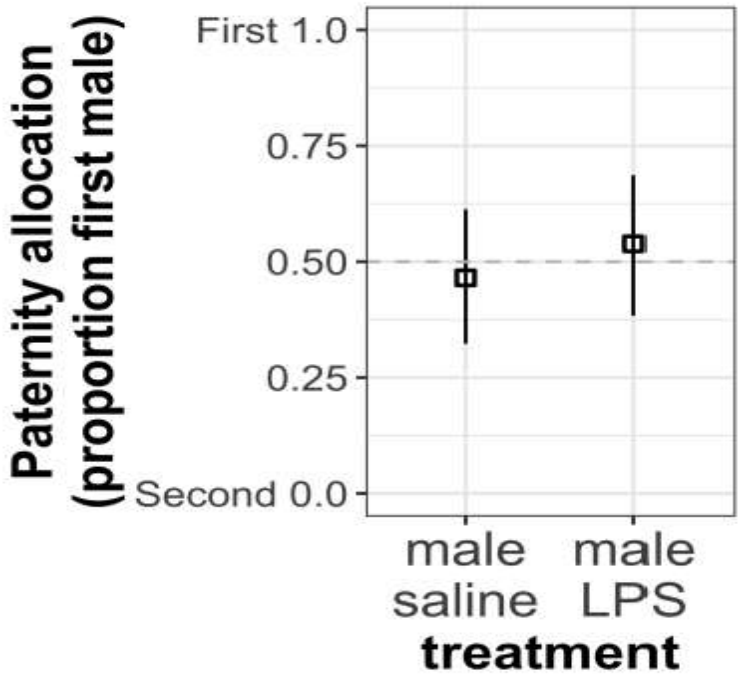
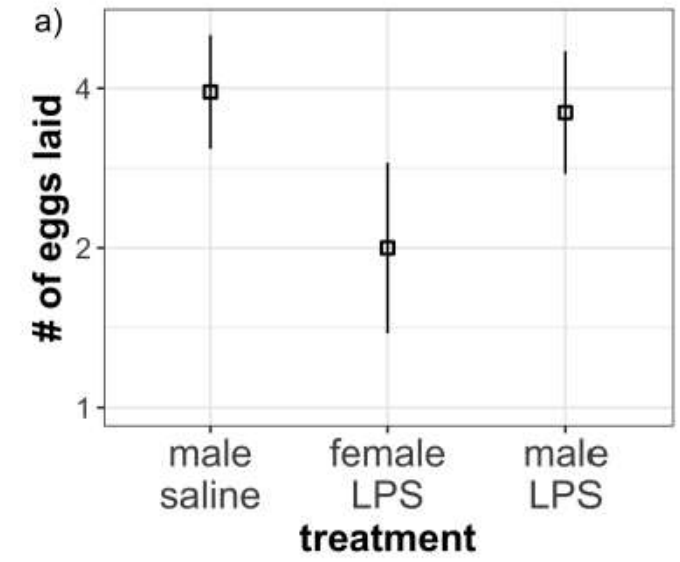
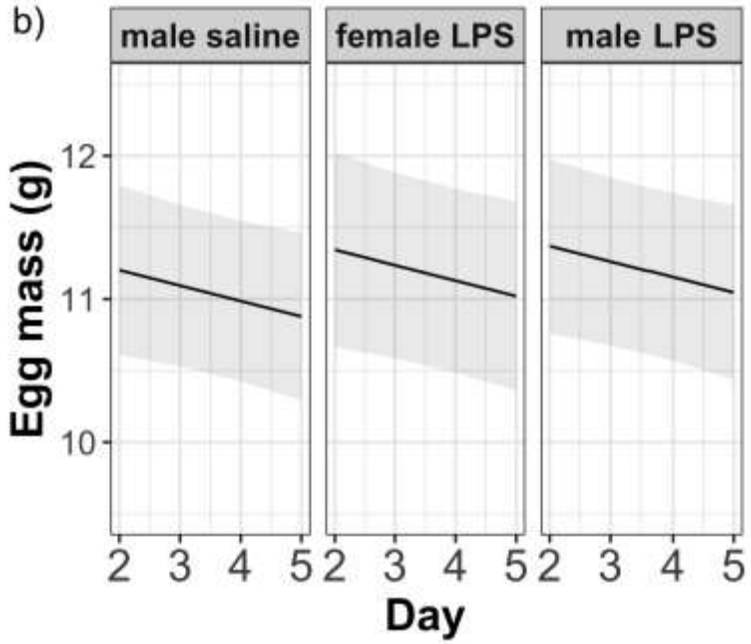


Fig. 2 – Predicted probability of embryo being sired by the first male that females were paired with. Under the male saline treatment, the first male was saline treated, and under the male LPS treatment, the first male was LPS treated. Second males were untreated. The dotted line represents even allocation.

Females treated with LPS and paired with saline males (female LPS treatment) laid fewer eggs than females in other treatments ($\chi^2 = 10.53$, $p < 0.0052$, d.f. = 2) (Fig.

3a). The mass of the eggs was not affected by either treatment ($F = 0.10$, $p = 0.90$, d.f. = 2) nor time after treatment ($F = 2.88$, $p = 0.093$, d.f. = 1) (Fig. 3b). There was no difference between treatments for the number of eggs fertilized ($F = 0.53$, $p = 0.59$, d.f. = 2) (Fig. 3c). There was also no difference due to treatment in terms of embryo sex ratio ($\chi^2 = 0.31$, $p = 0.86$, d.f. = 2) (Fig. 3d).





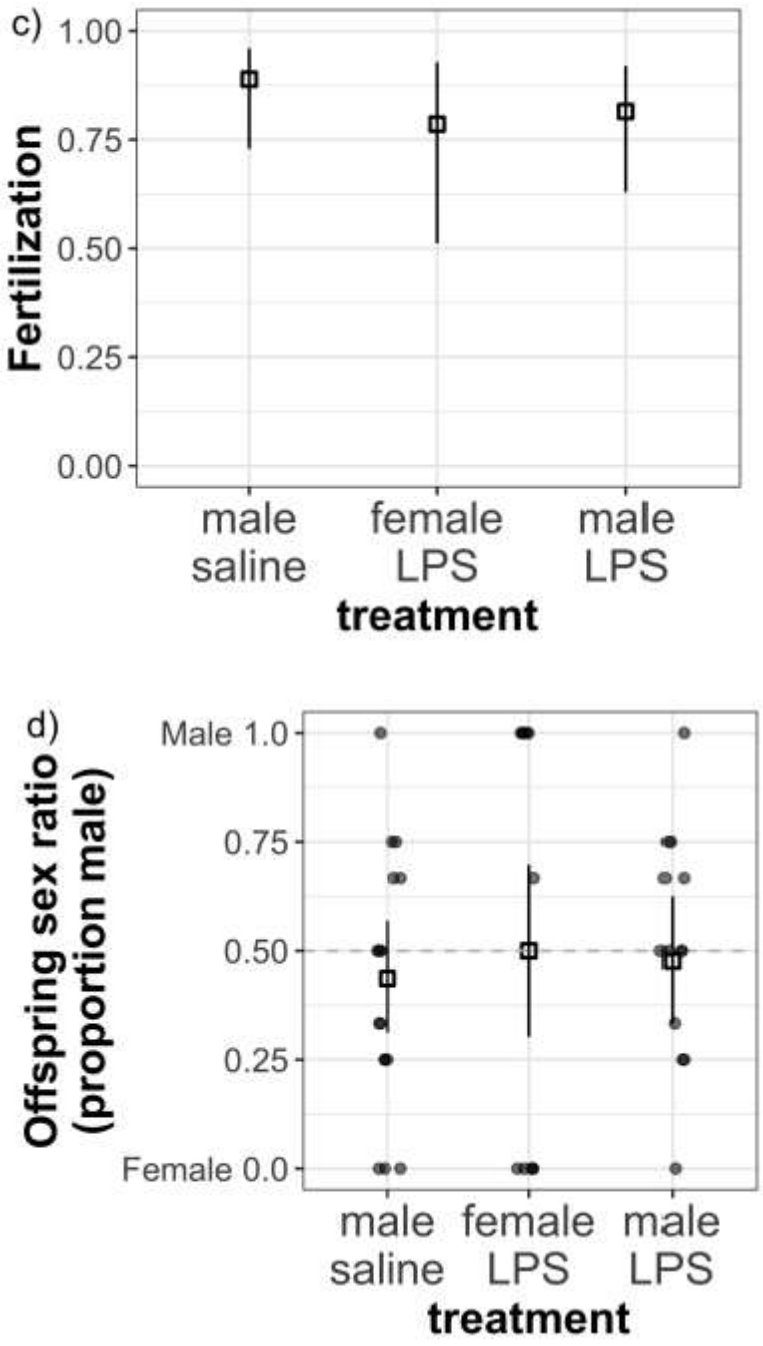


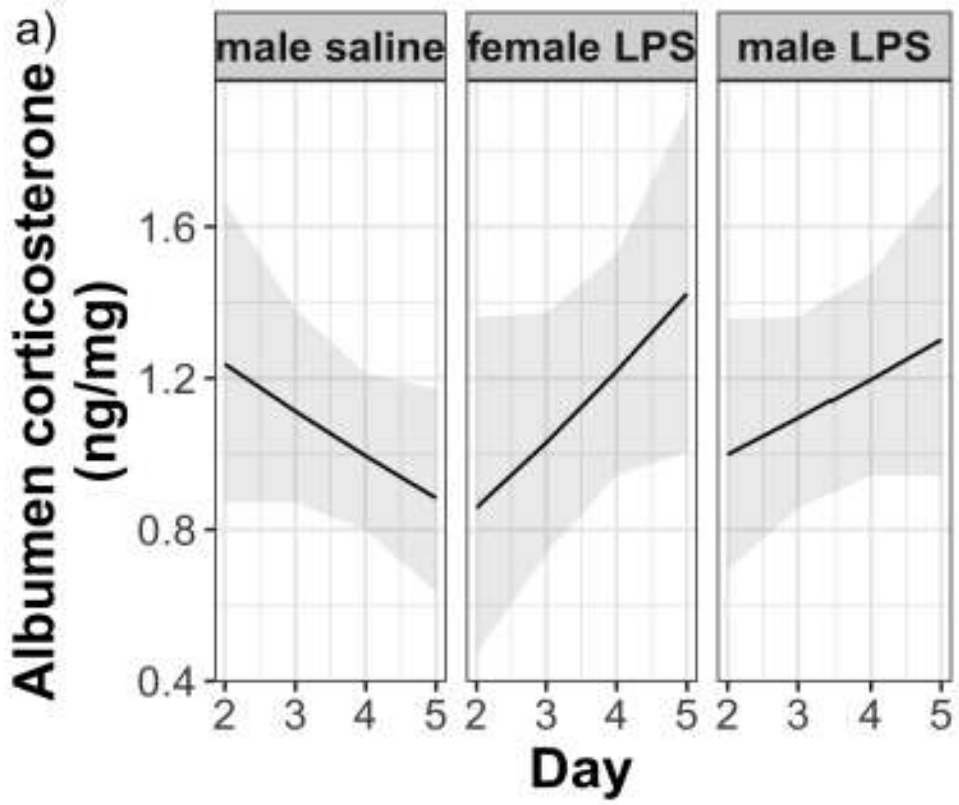
Fig. 3 – Estimated marginal means of number of eggs laid (a) and mass of eggs (b); predicted probability of egg being fertilized (c) and of embryo being male (d) as a function of treatment. Egg mass is also represented as a function of time (day; b). For offspring sex ratio, we added a dotted line representing even sex ratio and we overlayed

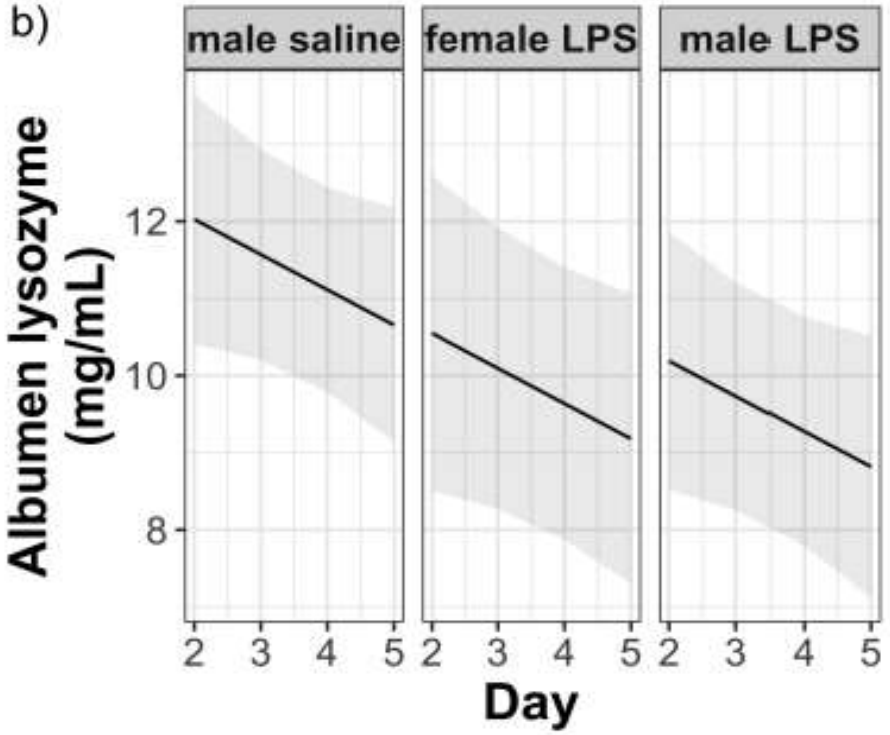
clutch sex ratios (circles) for visualization purposes. Treatments: Male saline consists of un-injected females exposed to saline-injected males; female LPS consists of LPS-injected females exposed to untreated males; and male LPS consists of un-injected females exposed to LPS-injected males.

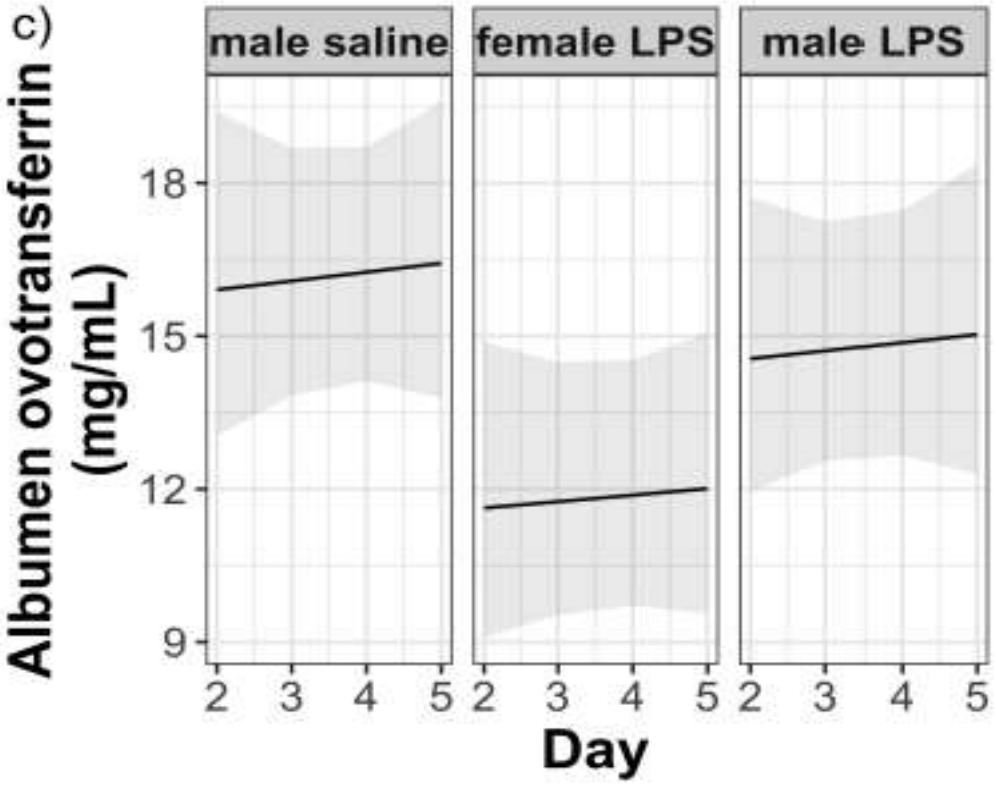
In terms of egg contents, for albumen corticosterone the best fit model included an interaction between treatment and time after treatment ($F = 2.73$, $p = 0.071$, d.f. = 2). While albumen corticosterone decreased over time after treatment in the male saline treatment, it increased in the other two treatments (Fig. 4a). For albumen lysozyme, neither treatment ($F = 2.08$, $p = 0.14$, d.f. = 2) nor time ($F = 2.59$, $p = 0.11$, d.f. = 1) affected this metric (Fig. 4b). Albumen ovotransferrin levels were not affected by time after treatment ($F = 0.066$, $p = 0.8$, d.f. = 1), but were affected by treatment (Fig. 4c; $F = 3.32$, $p = 0.04$, d.f. = 2), with eggs produced by females in the female LPS treatment having the lowest levels. In addition to the interaction between treatment and time for corticosterone, an interaction between these terms was also found for yolk antioxidant capacity ($F = 5.3$, $p = 0.0067$, d.f. = 2; Fig. 4d). There were no interactions between these terms for any other responses ($p > 0.1$). Whereas the trend was for yolk antioxidant capacity to increase over time after treatment in the male saline treatment (slope estimate = 0.58), it decreased in the female LPS treatment (slope estimate = -0.71) and showed a slower increase in the male LPS treatment (slope estimate = 0.20) (Fig. 4d).

Even though treatment did not affect embryo sex ratio, it is interesting to note, however, that all embryos of each female in the female LPS treatment tended to be of a

single sex (either all male or all female) (Fig. 3d), a pattern not observed for the other treatments. Because maternal corticosterone has been shown to affect embryo sex ratio (Pike and Petrie 2006), we tested for a relationship between embryo sex and albumen corticosterone. We did not find a significant relationship between these variables (χ^2 = 2.34, p = 0.13, d.f. = 1), but the pattern obtained followed the one found by the Pike and Petrie (2006) study, with increased concentrations of albumen corticosterone being associated with more female biased sex ratios (Supplemental Fig. 1).







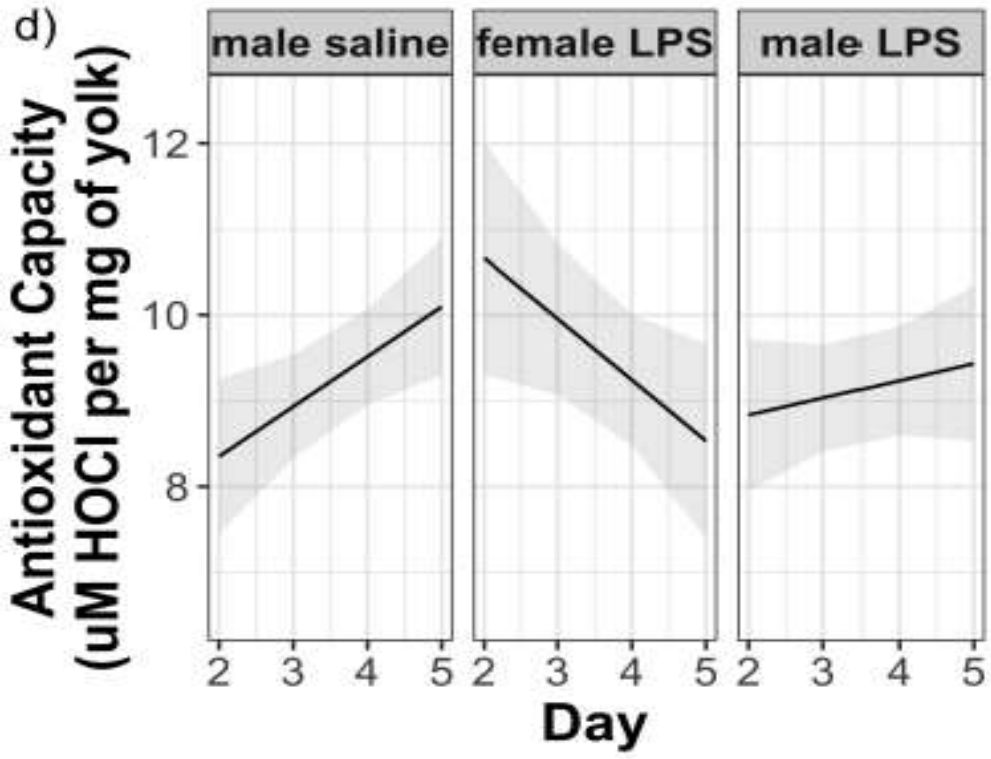


Fig. 4 – Estimated marginal means of albumen corticosterone (a), albumen lysozyme (b), albumen ovotransferrin (c) and yolk antioxidant capacity (d) as a function of treatment and time after treatment (Day). Treatments: Male saline consists of un-injected females exposed to saline-injected males; female LPS consists of LPS-injected females exposed to untreated males; and male LPS consists of un-injected females exposed to LPS-injected males. Shaded areas represent 95 % confidence intervals.

Discussion

This study was aimed at assessing whether exposure to infection risk via interaction with immune-challenged males changed female behaviors towards those males, and to assess whether offspring paternity is skewed under these circumstances. No differences in behavior or paternity allocation were found. We also aimed to

understand whether infection risk had effects at the level of embryos or egg contents. Overall, we found that immune-challenged females but not females exposed to immune-challenged males, laid fewer eggs, with an almost bimodal distribution of sex ratio for embryos. Exposure to immune-challenged males led to increased deposition of albumen corticosterone over time, which paralleled the response in eggs from the female LPS treatment but contrasts the pattern in the male saline treatment (where corticosterone decreased over time). This latter result suggests that healthy female Japanese quail may be able to physiologically respond to immune-challenged males, but that more intense female responses may require additional modalities of male sickness symptoms, exposure to more pronounced symptoms or more prolonged exposure to sick males.

The lack of difference in the behavioral responses to immune-challenged relative to healthy males could be due to the fact that males are generally good at hiding or reducing behavioral symptoms of infection when presented with mating opportunities (Lopes 2014). In a separate study (Gormally et al. 2022), we found that LPS-treated male Japanese quail paired with females showed similar amounts of copulatory behaviors as saline-treated males, and that LPS-treated males paired with females rested less than LPS-treated males kept in isolation (resting is a sickness behavior). Still, there could be additional behaviors or cues (such as changes in odors or coloration of males) that would allow females to detect disease status in the current study. However, females did not demonstrate behavioral avoidance of these males. Disease detection based on stronger or additional symptoms, such as changes to physical

appearance [e.g., the presence of lesions (Love et al. 2021)] may therefore be more reliable indicators of sickness and females may be more attuned to those.

Paternity allocation was evenly distributed between the two males that each female was mated with, regardless of male treatment. Contrary to this finding, last male sperm precedence (i.e., the last male to mate has higher fertilization success) has been found in several bird species (Birkhead and Møller 1992). However, a previous study in Japanese quail using successive matings within short time intervals between the two males found no difference in paternity allocation between the two males (Singh et al. 2011), which fits with our findings. It has been proposed that female Japanese quail have some control over paternity (i.e., that some degree of post-copulatory female choice takes place) (Adkins-Regan 1995). While we would have predicted that, if indeed females can control paternity, they would skew paternity towards the healthy male, it may be that a male that is undergoing an infection and can still perform copulatory behaviors is perceived as a higher quality male. In the blue-footed booby (*Sula nebouxii*), the female partners of immune challenged (diphtheria–tetanus vaccine) males laid eggs earlier and their earlier clutches had larger volumes than those of females paired with control males (Velando et al. 2014). In that study, male foot coloration (a sexually attractive signal) was enhanced after the immune challenge and clutch volume was positively correlated with foot color. It is possible that, in certain species, infections lead to increased investment in sexual signals and mating effort by males, as a form of terminal investment. If this is the case, knowledge of infection risk from a mate may have different consequences relative to knowledge of infection risk from other sources.

The reduced egg production by females treated with LPS could be due to a disruption of the reproductive axis (Tomaszewska-Zaremba and Herman 2009) and to a reallocation of energetic resources needed for egg production into the production of a robust immune response (Sheldon and Verhulst 1996; Norris and Evans 2000). While the terminal investment hypothesis would predict an increase in reproductive investment under deteriorating conditions (an infection could be a cue of low residual reproductive value) (Williams 1966; Clutton-Brock 1984), this may not always be possible during the period when animals are acutely sick. For example, female house sparrows (*Passer domesticus*) treated with LPS experienced decreased reproductive success for the brood that coincided with LPS administration, but produced second-broods that were more successful than broods of controls (Bonneaud et al. 2003). Similarly, female house sparrows that were immune-challenged with the Newcastle disease virus vaccine after completion of their first clutch and had that clutch removed, were more likely to produce a replacement clutch and a larger clutch than saline treated controls (Bonneaud et al. 2004). In both studies, the second clutch was laid at a later time point after manipulation, when the females were likely no longer experiencing the acute effects of immune activation. This is interesting, because it indicates that females with information about parasitism risk but no longer experiencing an infection showed increased reproductive investment. In our experiment, females exposed to LPS-treated males did not show increased egg laying, at least during the five-day period of the study. This result could be due to a ceiling effect, given that both females exposed to saline males and to LPS males were laying close to the maximum amount of eggs

possible (the majority of females of this species lays about one egg per day, but it is possible to oviposition two; Woodard and Mather 1964).

Egg mass was not affected by LPS-treatment (or any other treatment), a result that matches previous findings obtained from Japanese quail treated with two different antigens, including LPS (Grindstaff 2008). In the blue-footed booby (*Sula nebouxii*), when females mated to an immunized male (diphtheria–tetanus vaccine) laid eggs at a date close to male manipulation, they had clutches with larger egg volume than those of controls (Velando et al. 2014). This is an indication that egg size can be changed as a response to disease risk cues, but we did not observe this at the level of egg mass.

While neither infection risk nor LPS treatment affected the mean sex ratio of embryos, for any given LPS-treated female, the eggs laid over the 2–5-day period after treatment tended to be composed of either all female or all male embryos. This is an intriguing observation. A study that manipulated maternal corticosterone in Japanese quail found that this manipulation reduced the proportion of male embryos produced and that offspring sex ratio was correlated with fecal corticosterone concentrations (Pike and Petrie 2006). LPS treatment is known to activate the hypothalamic-pituitary-adrenal axis, leading to increased production of glucocorticoids (Beishuizen and Thijs 2003). Plasma corticosterone levels in response to LPS treatment tend to increase fast (within 1 h), but the peak and duration of corticosterone elevation is dose dependent. For example, in chicken, a lower dose of LPS (0.5 mg/kg) can lead to elevation of corticosterone for a period of less than 24 h (Wang et al. 2021), while a higher dose (8 mg/kg) can lead to elevated corticosterone even at 10 days post administration (Shini et al. 2008). It is thus possible that the number of male and female embryos produced was

related to female plasma corticosterone levels on the day of egg-laying. In our experiment, we quantified albumen corticosterone because albumen corticosterone has been shown in chickens to covary with female plasma corticosterone (Downing and Bryden 2008), thus providing a non-invasive way to track plasma corticosterone. We did not find a significant relationship between embryo sex and albumen corticosterone. This might mean that albumen deposited to the yolk is more relevant for sex ratio allocation, which would be relevant to study further to better understand the proximate factors underlying sex allocation during an infection.

Albumen lysozyme concentrations appeared to modestly decrease over time, but this effect was not significant. Egg lysozyme concentrations have been previously shown to vary with laying sequence. For instance, similar to our results, in barn swallows (*Hirundo rustica*), the first eggs in a clutch contained higher lysozyme concentrations than eggs laid later (Saino et al. 2002). Saino et al. (2002) proposed that this pattern may reflect exhaustion of maternal lysozyme over the course of the clutch production. However, contrary to Saino et al. (2002) and our results, in the Great Cormorant (*Phalacrocorax carbo*) lysozyme increased with laying order (Cao et al. 2015). The underlying reason for these differences in patterns is not known but may be a reflection of investment of maternal resources in ways that either promotes survival of the first laid eggs or promotes survival of the entire clutch (discussed in Cao et al. 2015), which may depend on the species' reproductive strategy.

Females treated with LPS had the lowest levels of albumen ovotransferrin. While this finding was initially surprising, since we expected an increase in this egg defense, a previous study showed that ovotransferrin mRNA expression is decreased in the

magnum (a portion of the oviduct important for protein production) of LPS-treated chicken at 24 h post-injection (Hallquist and Klasing 1994).

Finally, both albumen corticosterone and yolk antioxidant capacity showed an interaction between treatment and time after treatment. While the slopes for yolk antioxidant capacity levels over time for eggs in both the male saline and the male LPS treatments were positive, the slope for the female LPS treatment was negative. Activation of an immune response is often associated with increased oxidative stress (Costantini and Møller 2009), which could explain why yolk antioxidant capacity was initially higher in LPS-treated females and decreased over time. Since in great tits (*Parus major*) it was shown that male quality could affect yolk antioxidant deposition (Remeš et al. 2011), one could expect reduced yolk antioxidant capacity for eggs of females exposed to LPS-treated males, but this was not the case in our study. The temporal pattern of albumen corticosterone levels in eggs from the male LPS treatment better matched the pattern seen in eggs from the female LPS (positive slopes) than the one seen in eggs from the male saline treatment (negative slope). This result could be an indication that female Japanese quail do adjust egg contents in response to immune-challenged males and should be investigated further either by experiments prolonging the exposure to immune-challenged males (over several days) or by treating males with a parasite or immune-challenge that triggers additional disease symptoms (e.g., conjunctivitis). We predict that, given that females of several species make mating decisions (in terms of whom to mate with) in very short periods of time, length of exposure is likely less important than sickness cue type. It will be interesting for future

studies to determine what are the important cues that lead to physiological responses to parasitism risk and how these vary depending on taxa and ecological context.

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Ethics statement

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.

Data availability

Data are available at: https://doi.org/10.6084/m9.figshare.19424231

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Authorship

PCL devised the experiments, helped collect data, analyzed data, and wrote the initial draft of the manuscript. BMGG ran the experiment, collected data, optimized, and ran all the egg assays except corticosterone, wrote the methods section, and edited the manuscript. AM, CL, and DS helped code the behavioral data. AM and DS helped optimize and run the egg assays, except for the corticosterone assay. UB ran the corticosterone assays in LMR's lab, and both edited the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

Bibliography

Adkins-Regan E. 1995. Predietors of fertilization in the Japanese quail, Coturnix japonica. *Anim Behav* 50:1405–15.

Adkins-Regan E, Banerjee SB, Correa SM, Schweitzer C. 2013. Maternal effects in quail and zebra finches: Behavior and hormones. *Gen Comp Endocrinol,* 10th International Symposium on Avian Endocrinology 190:34–41.

Adkins–Regan E, MacKillop EA. 2003. Japanese quail (Coturnix japonica) inseminations are more likely to fertilize eggs in a context predicting mating opportunities. *Proc R Soc B* 270:1685–89.

Agrawal AA, Laforsch C, Tollrian R. 1999. Transgenerational induction of defences in animals and plants. *Nature* 401:60–63.

Bates D, Maechler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. *J Stat Softw* 67:1–48.

Beishuizen A, Thijs LG. 2003. Endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis. *J Endotoxin Res* 9:3–24.

Birkhead TR, Møller AP. 1992. Sperm competition in birds. Evolutionary causes and consequences. Academic Press, London, UK.

Bonneaud C, Mazuc J, Chastel O, Westerdahl H, Sorci G. 2004. Terminal Investment Induced by Immune Challenge and Fitness Traits Associated with Major Histocompatibility Complex in the House Sparrow. *Evolution* 58:2823–30.

Bonneaud C, Mazuc J, Gonzalez G, Haussy C, Chastel O, Faivre B, Sorci G. 2003. Assessing the cost of mounting an immune response. *Am Nat* 161:367–79.

Bozkaya F, Gürler S, Yertürk M, Aydılek N. 2013. Isolation of DNA from embryo and chorio-allantoic membranes and sexing by PCR in Japanese quail. *Br Poult Sci* 54:106–11.

Bozler J, Kacsoh BZ, Bosco G. 2020. Maternal Priming of Offspring Immune System in Drosophila. *G3 (Bethesda)* 10:165–75.

Cao J, Li J, Wang W, Yang F, Li Z, Li L. 2015. Maternal lysozyme concentrations in the eggs of the Great Cormorant (Phalacrocorax carbo) in relation to breeding density and laying order. *Avian Res* 6:21.

Clutton-Brock TH. 1984. Reproductive Effort and Terminal Investment in Iteroparous Animals. *Am Nat* 123:212–29.

Cook NJ. 2012. Review: Minimally invasive sampling media and the measurement of corticosteroids as biomarkers of stress in animals. *Can J Anim Sci* 92:227–59.

Correa SM, Horan CM, Johnson PA, Adkins-Regan E. 2011. Copulatory behaviors and body condition predict post-mating female hormone concentrations, fertilization success, and primary sex ratios in Japanese quail. *Horm Behav* 59:556–64.

Costantini D. 2010. Complex trade-offs in the pigeon (Columba livia): egg antioxidant capacity and female serum oxidative status in relation to diet quality. *J Comp Physiol B* 180:731–39.

Costantini D, Møller AP. 2009. Does immune response cause oxidative stress in birds? A meta-analysis. *Comp Biochem Physiol A: Mol Integra Physiol* 153:339–44.

Cremer S, Armitage SAO, Schmid-Hempel P. 2007. Social immunity. *Curr Biol* 17:R693–702.

Curno O, Behnke JM, McElligott AG, Reader T, Barnard CJ. 2009. Mothers produce less aggressive sons with altered immunity when there is a threat of disease during pregnancy. *Proc R Soc B* 276:1047–54.

D'Alba L, Shawkey MD, Korsten P, Vedder O, Kingma SA, Komdeur J, Beissinger SR. 2010. Differential deposition of antimicrobial proteins in blue tit (Cyanistes caeruleus) clutches by laying order and male attractiveness. *Behav Ecol* Sociobiol 64:1037–45.

De Baere S, Rosendahl Larsen T, Devreese M, De Backer P, De Neve L, Fairhurst G, Lens L, Croubels S. 2015. Use of LC–MS–MS as an alternative to currently available immunoassay methods to quantitate corticosterone in egg yolk and albumen. *Anal Bioanal Chem* 407:4351–62.

Downing JA, Bryden WL. 2008. Determination of corticosterone concentrations in egg albumen: A non-invasive indicator of stress in laying hens. *Physiol Behav* 95:381–87.

Foote FM. 1967. Weights of component parts of the incubating egg of the Japanese quail {Coturnix cotumix japonica). *Trans Illinois State Acad Sci* 60:371–74.

Friard O, Gamba M. 2016. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods Ecol Evol* 7:1325–30.

Fridolfsson A-K, Ellegren H. 1999. A Simple and Universal Method for Molecular Sexing of Non-Ratite Birds. *J Avian Biol* 30:116–21.

Gallagher JD, Siva-Jothy MT, Evison SEF. 2018. Social cues trigger differential immune investment strategies in a non-social insect, Tenebrio molitor. *Biol Lett* 14:20170709.

Gormally BMG, Bridgette K, Emmi A, Schuerman D, Lopes PC. 2022. Female presence does not increase testosterone but still ameliorates sickness behaviors in male Japanese quail. *R Soc Open Sci* 9: 220450.

Grindstaff JL. 2008. Maternal antibodies reduce costs of an immune response during development. *J Exp Biol* 211:654–60.

Guibert F, Richard-Yris M-A, Lumineau S, Kotrschal K, Guémené D, Bertin A, Möstl E, Houdelier C. 2010. Social Instability in Laying Quail: Consequences on Yolk Steroids and Offspring's Phenotype. *PLOS ONE* 5:e14069.

Gutiérrez G, Domjan M. 2011. Conditioning of sexual proceptivity in female quail: Measures of conditioned place preference. *Behav Processes* 87:268–73.

Hallquist NA, Klasing KC. 1994. Serotransferrin, ovotransferrin and metallothionein levels during an immune response in chickens. *Comp Biochem Physiol B* 108:375–84.

Horrocks NP, Hine K, Hegemann A, Ndithia HK, Shobrak M, Ostrowski S, Williams JB, Matson KD, Tieleman BI. 2014. Are antimicrobial defences in bird eggs related to climatic conditions associated with risk of trans-shell microbial infection? *Front Zool* 11:49.

Horrocks NPC, Irene Tieleman B, Matson KD. 2011. A simple assay for measurement of ovotransferrin – a marker of inflammation and infection in birds. *Methods Ecol Evol* 2:518–26.

Kayang BB, Inoue-Murayama M, Hoshi T, Matsuo K, Takahashi H, Minezawa M, Mizutani M, Ito S. 2002. Microsatellite loci in Japanese quail and cross-species amplification in chicken and guinea fowl. *Genet Sel Evol* 34:233.

Kuznetsova A, Brockhoff PB, Christensen RHB. 2017. lmerTest Package: Tests in Linear Mixed Effects Models. *J Stat Soft* 82:1–26.

Langen EMA, Engelhardt N von, Goerlich-Jansson VC. 2017. Social environment during egg laying: Changes in plasma hormones with no consequences for yolk hormones or fecundity in female Japanese quail, Coturnix japonica. *PLOS ONE* 12:e0176146.

Lopes PC. 2014. When is it socially acceptable to feel sick? *Proc R Soc B* 281:20140218.

Lopes PC. 2020. We Are Not Alone in Trying to Be Alone. *Front Ecol Evol* 8:172.

Lopes PC, French SS, Woodhams DC, Binning SA. 2021. Sickness behaviors across vertebrate taxa: proximate and ultimate mechanisms. *J Exp Biol* 224.

Lopes PC, French SS, Woodhams DC, Binning SA. 2022. Infection avoidance behaviors across vertebrate taxa: patterns, processes, and future directions. In: Ezenwa V, Altizer S, Hall R, editors. Animal Behavior and Parasitism Oxford University Press. DOI: 10.1093/oso/9780192895561.003.0014.

Love AC, Grisham K, Krall JB, Goodchild CG, DuRant SE. 2021. Perception of infection: disease-related social cues influence immunity in songbirds. *Biol Lett* 17:20210125.

Lüdecke D. 2018. ggeffects: Tidy Data Frames of Marginal Effects from Regression Models. *J Open Source Softw* 3:772.

Meunier J. 2015. Social immunity and the evolution of group living in insects. *Philos Trans R Soc B Biol Sci* 370:20140102.

Millet S, Bennett J, Lee KA, Hau M, Klasing KC. 2007. Quantifying and comparing constitutive immunity across avian species. *Dev Comp Immunol* 31:188–201.

Navara KJ, Pinson SE. 2010. Yolk and albumen corticosterone concentrations in eggs laid by white versus brown caged laying hens. *Poult Sci* 89:1509–13.

Norris K, Evans MR. 2000. Ecological immunology: life history trade-offs and immune defense in birds. *Behav Ecol* 11:19–26.

Persaud KN, Galef Jr. BG. 2005. Eggs of a female Japanese quail are more likely to be fertilized by a male that she prefers. *J Comp Psychol* 119:251–56.

Pike TW, Petrie M. 2006. Experimental evidence that corticosterone affects offspring sex ratios in quail. *Proc R Soc B* 273:1093–98.

R Core Team. 2021. A Language and Environment for Statistical Computing Vienna, Austria: R Foundation for Statistical Computing.

Rath NC, Anthony NB, Kannan L, Huff WE, Huff GR, Chapman HD, Erf GF, Wakenell P. 2009. Serum ovotransferrin as a biomarker of inflammatory diseases in chickens. *Poult Sci* 88:2069–74.

Remeš V, Matysioková B, Klejdus B. 2011. Egg yolk antioxidant deposition as a function of parental ornamentation, age, and environment in great tits Parus major. *J Avian Biol* 42:387–96.

Rutkowska J, Adkins-Regan E. 2009. Learning enhances female control over reproductive investment in the Japanese quail. *Proc R Soc B* 276:3327–34.

Saino N, Dall'ara P, Martinelli R, Møller AP. 2002. Early maternal effects and antibacterial immune factors in the eggs, nestlings and adults of the barn swallow. *J Evol Biol* 15:735–43.

Schaller M, Miller GE, Gervais WM, Yager S, Chen E. 2010. Mere visual perception of other people's disease symptoms facilitates a more aggressive immune response. *Psychol Sci* 21:649–52.

Sheldon BC, Verhulst S. 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol Evol* 11:317–21.

Shini S, Kaiser P, Shini A, Bryden WL. 2008. Differential alterations in ultrastructural morphology of chicken heterophils and lymphocytes induced by corticosterone and lipopolysaccharide. *Vet Immunol Immunopathol* 122:83–93.

Singh RP, Sastry KV., Pandey NK, Shit N, Singh R, Mohan. 2011. Sperm competition in Japanese quail (Coturnix japonica): Last male precedence is declined in two

successive matting with two different male-Indian Journals. *Indian J Poult Sci* 46:130–31.

Smith S, Fusani L, Boglarka B, Sanchez-Donoso I, Marasco V. 2018. Lack of introgression of Japanese quail in a captive population of common quail. *Eur J Wildl Res* 64:51.

Tomaszewska-Zaremba D, Herman A. 2009. The role of immunological system in the regulation of gonadoliberin and gonadotropin secretion. *Reprod Biol* 9:11–23.

Velando A, Beamonte-Barrientos R, Torres R. 2014. Enhanced male coloration after immune challenge increases reproductive potential. *J Evol Biol* 27:1582–89.

Wang H, Yang F, Song Z, Shao H, Bai D, Ma Y, Kong T, Yang F. 2021. The influence of immune stress induced by Escherichia coli lipopolysaccharide on the pharmacokinetics of danofloxacin in broilers. *Poult Sci* 101:101629.

Wickham H. 2016. Ggplot2: Elegant Graphics for Data Analysis. 2nd ed, Use R! Springer International Publishing.

Williams GC. 1966. Natural Selection, the Costs of Reproduction, and a Refinement of Lack's Principle. *Am Nat* 100:687–90.

Woodard AE, Mather FB. 1964. The Timing of Ovulation, Movement of the Ovum Through the Oviduct, Pigmentation and Shell Deposition in Japanese Quail (Coturnix coturnix japonica). *Poult Sci* 43:1427–32.