Physiological costs of reproduction in female mice

by

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ABSTRACT

The hypothesis that the large demand of reproduction negatively impacts future reproduction and/or survival has become a major topic in many fields within biology. Although many assume negative linear correlations between characteristics associated with reproduction and those supporting self-maintenance (i.e., costs of reproduction), empirical studies do not always support this assumption. In this dissertation, I investigate the factors that contribute to costs of reproduction in female house mice by using multiple approaches. The overarching goal of this dissertation is to test the assumption that the increased demands of reproduction necessarily incur costs that negatively impact self-maintenance processes, which in turn affect future reproductive performance and/or survival of mothers and their young. To achieve this goal, I used a combination of data exploration and experimental studies to assess costs of reproduction in female wild-derived and laboratory house mice. First, I explored patterns of life-history and metabolic trait co-variation at the intraspecific level across strains of inbred mice with the goal of understanding the genetic architecture that may underlie constraints to reproductive performance. I then investigated how reproductive performance changes with maternal age and protein consumption, finding that performance is not static across age and that protein intake mediates age-specific reproductive strategies without a necessary cost to future reproductive bouts. I then explored the relationship between reproduction and immune defense in my next two chapters by assessing changes in maternal antibody responses as a function of reproductive demand, as well as the developmental effects of that immune challenge on offspring. These two chapters demonstrate that methodologies used to investigate the relationship between female reproduction and immune defense should take a female-centric approach and

incorporate responses in both mothers and their offspring. Together, my dissertation work contributes to the current literature by exploring gaps in our understanding and by testing implicit assumptions common among previous investigations.

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INTRODUCTION

Central to life history theory is the prediction that individuals have limited resources which must allocated to support self-maintenance, growth, and reproduction (Stearns 1992). Individual variation in maternal reproductive strategies are hypothesized to be shaped by trade-offs among competing physiological demands, and evolve through selection pressures on both the female and her offspring. Thus, the large demand of reproduction has long been assumed to impose proximate physiological (e.g. depressed immune function or decreased somatic maintenance) and ultimate costs to future fecundity and/or survival (Fisher 1930; Williams 1966; Reznick 1985). This partitioning of resources drives the evolution of key life history characteristics, including lifespan, age at first reproduction, maternal condition, and offspring size and number (Fisher 1930; van Noordwijk and de Jong 1986; Roff 2002). Together, these trade-offs comprise an organism's life-history strategy, which is dictated by the balance of allocating resources toward conflicting needs in a way that optimizes lifetime reproductive success (Williams 1966; Stearns 1992; Charnov 1997).

Although many assume negative linear correlations between characteristics associated with reproduction and those supporting self-maintenance (i.e., costs of reproduction), empirical studies do not always support this assumption (e.g., Skibiel et al. 2013). The lack of a consensus may be due to the dynamic nature of trade-offs themselves as well as methodological limitations to how we measure and interpret costs of reproduction. The overarching goal of this dissertation is to *test the assumption that the increased demands of reproduction necessarily incur costs that negatively impact self-maintenance processes, which in turn affect future reproductive performance and/or survival.*

My dissertation work expands on the current literature by exploring gaps in our understanding and by testing implicit assumptions common among previous investigations. Together, this body of work has four primary conclusions. First, although there are patterns of co-variation of life-history traits, costs of reproduction are only revealed under certain contexts, such as under resource limitation or during specific stressors (Reznick 1985). In my first chapter, I explore intraspecific patterns of life-history trait co-variation across strains of inbred mice to assess the genetic architecture that may underlie trade-offs among these traits. Here, I found evidence suggesting that these traits tend to co-vary along a fast-slow axis across strains of inbred mice, and that these traits are functionally integrated with metabolism. Importantly, despite the broad intraspecific range in reproductive strategies and performance, we did not find that reproduction necessarily comes at a cost to other traits (e.g., longevity) under benign laboratory conditions.

Second, the partitioning of limited resources among competing processes is not anticipated to be static throughout the lifetime of an organism. My second chapter uses wild-derived mice to investigate how reproductive performance changes with varying maternal protein intake and age. In this chapter, I found that an ecologically-relevant variation in dietary protein composition alters reproductive performance in female mice. Further, I found that females that consume a high protein diet employ different age-specific strategies for increasing their lifetime fitness than females consuming a low protein diet. These results suggest that investment current reproductive bouts do not necessarily come at a cost to future reproduction, and that protein acquisition and/or availability may mediate the expression of these costs.

Third, our methods to measure costs of reproduction must place female reproduction at the forefront because reproduction is central to all aspects of female physiology. Within the field

of ecological immunology (ecoimmunology, Sheldon and Verhulst 1996), evidence exists suggesting a trade-off between immune defense and paternal investment. Often, these findings are used to inform hypotheses on female trade-offs between reproduction and immune defense. Previous work on this topic within female rodents is inconsistent and contradictory, likely due in part to methodological limitations. In my third chapter, I investigate how the antibody response changes with varying reproductive demand and immune challenge. I found evidence that suggests reproduction itself may alter the antibody response curves, and that this curve deviates from the previous reports in male mice that are used to inform this method. This finding highlights the need to re-assess our methods for evaluating costs of reproduction so that they are adapted to female reproductive physiology.

Finally, inverse relationships between traits are not necessarily due to trade-offs between those traits. In my fourth chapter, I investigated how shifting maternal demands during lactation impacts offspring phenotype during development. Here, I administered an immune challenge to mothers of varying reproductive demands and measured offspring body and organ masses as well as gene expression for key genes involved in immune defense, stress response, and growth. Coupled with the previous chapter's findings that suggest an inverse relationship between reproductive status and serum antibody concentration, I found that investment in immune defense did not impact offspring mass. Despite no differences in body mass, I did find changes suggestive of developmental programming in response to maternal immune challenge. These findings demonstrate that mounting an immune response during reproduction does not always come at a cost to offspring quality, and that maternal immune challenge during this period may program

offspring physiology. This work demonstrates the need to investigate effects on offspring when assessing costs of reproduction in females.

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CHAPTER ONE

UNDERSTANDING PATTERNS OF LIFE-HISTORY TRAIT COVARIATION IN AN UNTAPPED RESOURCE, THE LAB MOUSE

Abstract. Artificial selection of the laboratory mouse was originally used to increase the expression of desirable traits during the domestication process of the house mouse. Though these strains are commonly-used models to investigate topics central to biomedicine, the present paper exploits the broad phenotypic variation across strains of mice to test ultimate, evolutionary hypotheses rooted in life-history theory. Previous interspecific analyses across taxa supports the existence of a fast-slow continuum along which species' life-history strategies tend to fall. More recently, emphasis has been placed on understanding not only the ecological and evolutionary factors underlying these strategies, but also the physiological and metabolic processes that support them. Yet, it remains unclear how these key traits scale across hierarchical levels, as ambiguous empirical support has been garnered at the intraspecific level. Such within-species investigations using wild populations have been thwarted by methodological constraints and environmental factors that may obscure the genetic architecture underlying the hypothesized functional integration of life-history and metabolic traits. In this heuristic analysis, we used the publicly-available Mouse Phenome Database to investigate the relationships among life-history (i.e., body size, reproductive performance, and lifespan) and metabolic (i.e., mass-specific metabolic rate and serum IGF-1 concentration) traits. Our findings revealed significant variation in reproductive strategies across strains of inbred mice, as well as

significant relationships among the investigated life-history and metabolic traits. Specifically, we found evidence for variation along the fast-slow life-history continuum, though the direction of some relationships among these traits deviated from interspecific predictions laid out in previous literature. Further, our results suggest that the strength of these relationships is age-dependent and tend to be stronger earlier in life.

INTRODUCTION

Variation in traits that effect growth, size, reproduction, and survival (i.e., life-history traits) are hallmarks of biodiversity, and tend to cluster along a "fast-slow" continuum that is linked to energetic expenditure (Harvey et al. 1989; Promislow and Harvey 1990a; Stearns 1992; Ricklefs and Wikelski 2002; Roff 2002). Promislow and Harvey (1990) found evidence for this pattern within Mammalia such that larger mammals lead slower lives characterized by longer lifespans, lower mass-specific metabolic rates, and less frequent production of small litters. In contrast, small mammals live fast lives with shorter lifespans, higher mass-specific metabolic rates, and more frequent production of large litters. Size, reproductive performance, and longevity also vary among individuals within species (Careau et al. 2010; Jimenez 2016), though it remains unclear whether the life-history patterns observed between species continue to govern performance within species. Identifying patterns of life-history trait covariation, as well as the proximate and ultimate factors that contribute to their variation, is fundamental to evolutionary biology particularly in relation to the fast-slow life-history continuum and the hypothesized metabolic traits that underlie the predicted patterns (Ricklefs and Wikelski 2002).

Several experimental paradigms (e.g., artificial selection experiments, sibling analysis, offspring-parent regression, etc.) have been used to evaluate genetic variation and covariation among life-history traits (Conner 2003). Studies using artificially selected organisms can directly answer whether traits co-evolve in response to selective pressures and can inform us of the architecture and strength of the genetic constraints that underlie life-history evolution (Conner 2003). We used life-history data collected from multiple

strains of inbred laboratory mice (*Mus musculus domesticus*) to explore intraspecific relationships among life-history and metabolic traits. All classic inbred strains of lab mice, including those used in the present study, have been inbred for at least 60 generations, at which point their genome is considered to be homozygous across individuals excluding spontaneous mutations (Silver 1995). This high homozygosity means that phenotypic variation among individuals within strains can be attributed to environmental factors such as maternal effects, differences in handling and husbandry practices, and an individual's social environment (Careau et al. 2012). In contrast, phenotypic variation across strains can be attributed not only to these effects, but also genetic and gene by environment effects (Careau et al. 2012). Phenotypic variation across strains or breeds that have been artificially selected is typically greater than the variation found in populations subject to natural selection (Grafen 1988; Conner 2003). This increase in variation should be particularly true for traits that play a large role in determining fitness, as selection pressures should remove phenotypes that decrease fitness in the wild. Therefore, comparing traits across strains of laboratory mice will capture a great breadth of co-varying traits than is expected in a natural population.

Both extrinsic and intrinsic constraints impose an upper limit to an individual's ability to maximize reproductive performance. Previous research has identified metabolic rate as a key determinant of reproductive performance, with metabolic rate impacting the amount of energy available to be allocated towards reproduction (Johnson et al. 2001). Lab mice are a valuable model for evaluating trade-offs associated with reproduction, as females display a remarkably high energetic demand during breeding. Lactating females have one of the highest sustained metabolic scopes identified in a vertebrate (Hammond and

Diamond 1997), and when given continuous access to a mate, they will spend most of their lives allocating resources to the pre- and post-natal care of offspring (Latham and Mason 2004). Given this exceptionally large demand, reproductive trade-offs are expected to be particularly pronounced in this species.

Here, we propose that data from lab mice can be used to identify the pleiotropic traits that contribute to intraspecific life-history variation. To achieve this goal, we used the publicly-available Mouse Phenome Database by the Jackson Laboratory (http://phenome.jax.org/; Bogue and Grubb 2004; Grubb et al. 2004; Bogue et al. 2018) to investigate correlations among life-history and physiological traits across strains of inbred lab mice. Because many phenotypes are included in this dataset and a large number of inbred strains are represented, the Mouse Phenome Database provides a unique opportunity for evaluating co-variation among life-history and metabolic traits within species. To measure reproductive performance, we assessed individual traits (i.e., age at first reproduction, number of lifetime litters, interbirth interval, and average number of pups per litter) and used a principle components analysis (PCA) to derive new variables, which we termed breeding frequency and reproductive intensity. Additionally, we were interested in other life-history traits such as average lifespan and age-specific body size (mass and length). Multiple metabolic traits were used, including oxygen consumption, carbon dioxide production, body heat production, food intake, caloric intake, heart rate, and serum concentration of insulin-like growth factor 1 (IGF-1).

Emphasizing these life-history, morphological, and metabolic traits (Table 1), we hypothesize that phenotypic variation among lab mouse strains will reveal correlations indicative of life-history trade-offs. Specifically, we predict that reproductive performance-

related variables will be negatively correlated with lifespan and body mass and body length, as previously described (Promislow and Harvey 1990). Further, body size, metabolic rate, and mean serum IGF-1 concentration were predicted to correlate with measures of reproductive performance and longevity. These results would indicate that either metabolism and/or IGF-1 play a role in mediating trait covariation. Together, these results will help elucidate the phenotypic and physiological correlates that accompany intraspecific life-history trait variation and will demonstrate that strains of inbred laboratory mice are a useful model for studying intraspecific variation.

METHODS

Data Collection and Variable Selection

The Mouse Phenome Database by the Jackson Laboratory (http://www.jax.org/phenome) is a collaborative database of mouse strain characteristics, allowing researchers to explore phenotypic data collected by different investigators (Bogue et al., 2018; Bogue and Grubb, 2004; Grubb et al., 2004). All data used in this synthesis has been previously published in peer-reviewed journals or was conducted by the Jackson Laboratory (Table 1). Data included and selection criteria following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines is outlined in Figure 1 (Liberati et al. 2009; Moher et al. 2009). Data were obtained from the Mouse Phenome Database, and individual records (i.e., variables) were screened to ensure that the measurements were taken from adult inbred female mice who were older than 6 weeks at the time of the measurement. Studies that included any experimental manipulations of endogenous or exogenous conditions (e.g., high-fat diet or pharmacological treatment) were excluded. Variables were then selected for their biological relevance to our question (e.g., behavioral analyses and histopathology were excluded). Variables were then excluded based on the within-strain sample size (inclusion criterion was $n>7$), exclusion of females in the data set, and number of strains the measurements were taken from. No duplicate parameters were included, and in the event of duplicated records, the data set with the highest within-strain sample size or greatest number of strains was used. All metabolic data (e.g., oxygen intake volume, carbon dioxide

output volume, body heat production, food intake, and caloric intake) had previously been adjusted to the individual's body mass before use in this data set.

Data from each variable represents the average measurement for a given strain; each parameter was treated as a discrete and independent variable, as raw data on individuals was not available for all of the records used. Each data point does not account for variance of individuals within the strain and thus, this analysis should be considered conservative. The number of strains that contribute data to each variable varies from 11 to 35 (Table 1). The within-strain sample sizes for each variable range from 7 to 265. For the reproductive variables (dam's age at the birth of her first litter, number of litters per dam, interbirth interval, and number of pups per litter), measurements were taken from the same continuously-bred females housed in a single colony until the females reached 40 weeks of age. For the remaining variables, data were collected from non-reproductive, nulliparous females. Although measurements for the same variable were taken at different time points, we treated each time point as being independent due to the large amount of time between measurements. Homogeneity of residuals was assessed, and data distribution across strains was tested for normality using the Shapiro-Wilk test. Three variables (number of pups per litter, and 3 and 12-month mass) were found to have distributions that deviated from normal. Normality was not significantly improved by transforming, and therefore remained untransformed.

Despite inbred strains of mice sharing a common evolutionary history, we did not take in to account their phylogenetic relatedness. Though the origins of many inbred strains are well-known, the origins and relatedness for some strains included in this study remain unclear and may include genetic contamination from other strains, leading to differences

in models of hypothesized phylogenetic relatedness that may obscure phylogenetic signal (Atchley and Fitch 1991). In one of the few previous comparative studies of inbred mouse strains, Rhodes et al. (2007) reported in their analysis of that the inclusion of phylogenetic signal across strains of inbred mice did not change the results as compared to conventional methods, like those employed here, that ignore the history of the strain development (but see Careau et al., 2012), who reported a mild improvement when incorporating phylogenetic signal in their model). Additionally, because many of variables are derived from few studies that each differ in number of data point contributing to each mean, phylogenetic signal is not expected due to low power of statistical methods under these conditions (Blomberg et al. 2003; Ashton 2004).

Statistical Analyses

All statistical analyses were performed on R (R Core Team 2013). Data from the reproduction variables were used in a principal component analysis (PCA). These variables (average litter size per dam, number of litters per dam, average interval between consecutive litters, and maternal age at the birth of her first litter; see Table 1 for list of variables) were selected so that each represents a different facet of reproductive performance. Data were centered and scaled before running this analysis to ensure that all variables had equal weights. Resulting principal components were retained after analyzing with a Scree plot. Further, these components were considered to be significant if they had an Eigenvalue > 1 and explained a large amount of the variance. Scores from these principal components were then extracted and used in further analyses. To explore the relationships among life history components, a correlation matrix was created using Pearson's product-moment correlation coefficient (r) to ensure that important variables and

relationships were not removed from the statistical model. Significance was established at $p \leq 0.05$.

RESULTS

Reproductive performance across strains of inbred mice

To better represent the multifactorial nature of reproductive performance, we used a principal component analysis (PCA) using variables that represent different aspects of reproductive performance (Table 1). Two principal components were identified, explaining approximately 75% of the variance when combined (Table 2). The first principal component (PC1) represents a continuum of *breeding frequency*, explains 40.6% of the variance, and was weighted heavily by the interval between consecutive litters and the average number of litters per dam for a given strain of mouse. Strains at negative values along this axis represent a reproductive strategy with fewer, less frequent litters, whereas strains with positive values have a strategy with more litters and a decreased interbirth interval between consecutive litters. Our second principal component explains 34.6% of the variance in the model. This axis represents *reproductive intensity*, as it is heavily negatively weighted by the dam's age at the birth of her first litter and positively by the average size of her litter. This axis represents a continuum with strains at negative values first reproducing at older ages, with an average of fewer offspring in each litter. Two other PCs were found but were excluded due to their low eigenvalues (> 1) and explanatory power for the model's variance.

The distribution of strains along the breeding frequency (PC1) and reproductive intensity (PC2) axes does not show a clear pattern (Figure 2), and suggests variation in reproductive strategies and performance across inbred strains of mice. When assessing the relationships among the individual variables, two significant relationships were revealed

(Figure 3). A significant positive relationship was found between the age of the dam at the birth of her first litter and the average number of litters per dam ($r = 0.40$, $p = 0.017$); that is, strains that first reproduce at older ages tend to have more litters per dam over the course of her reproductive lifespan. A significant negative correlation was found between the strain-average number of litters a female produced and the amount of time between consecutive litters ($r = -0.58$, $p < 0.001$).

Body size, reproduction, and other life-history characteristics

Data collected on body mass and body length (from tip of nose to base of tail) at 13 weeks (here after referred to as 3 months) and 6, 12, and 20 months was used to understand the impact of age on strength of trait co-variation. Body mass and length tended to be positively correlated with one another for the ages measured (Figure 3). Significant negative relationships were found between the average age of a female when she first gave birth and the average body mass and length at 3 months old ($r=-0.58$, $p=0.008$; $r=-0.57$, $p=0.03$, respectively), but not at 6, 12, nor 20 months of age (Figure 3). Body length at 3 months of age was negatively correlated with Breeding Frequency (PC1) ($r=-0.59$, $p=0.02$) and the average number of litters per dam ($r=-0.58$, $p=0.038$), but these relationships were not present at any other age nor with body mass at any age. Reproductive intensity (PC2) was positively correlated with body mass at 3 months of age ($r=0.47$, $p=0.03$), but not at any other time points nor with body length at any age. The average interbirth interval and litter size were not found to be related to body mass nor length at any of the time points.

The strains' average lifespans of females were significantly correlated with the average body masses at 3 ($r=-0.43$; $p=0.042$), 6 months ($r= -0.48$, $p=0.015$), and 12 months ($r=-0.41$, $p=0.03$ (Figure 3; Table 3). The average lifespan was not related to the average

body mass 20 months, nor with body length at any age. No significant relationships were found between the strains' average lifespans and any of the reproductive variables, including breeding frequency and reproductive intensity.

Physiological and metabolic traits associated with life-history characteristics

Metabolic rate is hypothesized to play a role in establishing patterns of life-history trait co-variation along the fast-slow continuum. Here, we explored relationships between mass-specific metabolic variables (average daily oxygen consumption, average daily carbon dioxide production, body heat production, food and caloric intake, and heart rate at 6, 12, and 20 months) and reproductive performance, lifespan, and body size. These metabolic variables were collected when the individuals were approximately 9 weeks old. Excluding heart rate, which did not appear to be related to any variables in this analysis, the variables representing metabolic rate tended to be highly correlated with one another; for example, daily oxygen intake volume and carbon dioxide production volume were tightly correlated ($r=0.98$, $p<0.0001$). Additionally, the daily mass-specific food and caloric intake volume was positively correlated with daily mass-specific oxygen intake ($r=0.63$, $p=0.02$; $r=0.63$, $p=0.02$, respectively) and carbon dioxide production volumes ($r=0.64$, $p=0.04$; $r=0.63$, $p=0.048$), respectively.

Mass-specific daily oxygen intake volume and mass-specific daily carbon dioxide production volume tended to be negatively correlated with body size (Figure 3; Table 3). Specifically, body mass at 3 months was negatively correlated with the mass-specific daily oxygen intake volume ($r=-0.78$, $p=0.005$), mass-specific daily carbon dioxide production ($r=-0.74$, $p=0.03$), and mass-specific daily food ($r=-0.72$, $p=0.02$) and caloric ($r=-0.72$, $p=0.02$) intake. Body length at 3 months was also negatively related to the mass-specific

daily food (r=-0.90, p=0.01) and caloric (r=-0.90, p=0.02) intake. Though not significant, a trend was present between mass- specific average daily oxygen consumption and reproductive intensity (PC2) (r=-0.51, p=0.065), where strains with higher mass-specific oxygen consumption tended to also reproduce at older ages and have smaller average litter sizes. Similarly, mass-specific average carbon dioxide production volume was negatively related to the average number of litters for a given strain (r=-0.62, p=0.05), indicating that a higher metabolic rate was correlated with fewer litters across the reproductive lifespan. Excluding these relationships, no other correlations were found between the metabolic variables and reproductive performance nor average lifespan.

The hormone IGF-1 has been identified as a putative link connecting pace of life and metabolic rate due to its pleiotropic effects (Ricklefs and Wikelski 2002). Our analysis reveals significant relationships between IGF-1 concentration and body size and lifespan. No significant relationships between IGF-1 and reproduction nor metabolism (other than IGF-1 concentration at other timepoints) were found. In general, the concentration of IGF-1 in serum was positively correlated with body mass and body length (Table 3). Concentrations of serum IGF-1 at the different timepoints tended to be highly positively correlated with one another. At 3 months of age, the strains' average serum concentration of IGF-1 was negatively correlated with the average lifespan for the strain (r = -0.40, p=0.027), but this relationship was not present at any other timepoints. We did not find any other significant correlations between concentration of IGF-1 and the reproductive nor metabolic variables included in this study.

DISCUSSION

At a cursory glance, insights gained from comparing inbred strains of laboratory animals typical of biomedical research may seem irrelevant in understanding natural populations. We argue the opposite: the high phenotypic diversity captured across the numerous strains of laboratory mice can be valuable for understanding topics central to evolutionary biology. Phenotypic diversity produced by artificial selection provides an excellent opportunity to evaluate the interactions among traits that would otherwise not persist in the wild (Grafen 1988; Conner 2003; Fuller et al. 2005; Swallow et al. 2009).

Interspecific analyses have identified body size as a key determinate of where species fall along the fast-slow life-history continuum (Promislow and Harvey 1990). Our intraspecific analysis revealed that body size correlates with multiple life history and metabolic variables, but these relationships are not static across the lifespan. The strongest correlations tended to appear earlier in life at 3 months of age. For many laboratory strains of mice, sexual maturity occurs at approximately 6-8 weeks old (Silver 1995). Specifically, we found that females from strains that were smaller at 3 months of age tended to start reproducing later, have fewer litters over the course of their reproductive lifespan, and have decreased overall reproductive performance (as indicated by our two principal components, breeding frequency and reproductive intensity) compared to their larger conspecifics. Likely, the decreased reproductive performance in these strains is due to shorter reproductive lifespans resulting from both delayed maturation and shorter lifespans, as body mass was not correlated with the amount of time between two consecutive litters. Previous intraspecific analyses, including comparisons across breeds of domesticated dogs

(Jimenez 2016), have found similar patterns, suggesting that body size plays a role in determining latency to sexual maturity and the number of offspring in each reproductive bout, rather than regulating the overall number of reproductive events (Frisch 1987; Chehab et al. 1997). Together, our results suggest that body size early in life, most likely a proxy for developmental rate, is linked to reproductive strategy and performance.

Despite finding relationships linking body size to both longevity and reproductive performance, we did not find any evidence for a trade-off between strain-average measurements of reproductive performance and longevity, despite the broad variation in reproductive performance across strains of inbred mice. However, although we did not find evidence for a direct trade-off between reproduction and lifespan, our results suggest that there is a mediating factor that links both traits; females from strains with smaller average masses tended to have decreased reproductive performance as well as longer lifespans. Likely, this body condition acts as the mediating factor between reproductive performance and longevity, as it has been widely documented that both traits are condition-dependent (Clutton-Brock 1984; Benton et al. 2008; Hamel et al. 2008, 2010). The notion that the large energetic demand of reproduction necessarily incurs costs to somatic investment, and therefore, longevity, is pervasive within the field of biology, despite empirical studies not always supporting this assumption (Zhang and Hood 2016). This finding demonstrates that reproductive performance does not necessarily trade-off with longevity, but rather persistent costs may only be revealed under certain environmental conditions not present in the benign environment of these laboratory populations.

At the proximate level, life-history strategies are mediated by physiological components, such as pleiotropic hormones and metabolic rate (Ketterson and Nolan 1992;

Hau et al. 2011; Garland et al. 2016) as a consequence of selection linking shared developmental and physiological networks (Davidowitz et al. 2012). Metabolic rate has been implicated as a mechanism underlying the observed relationships between body size and life history traits, such as longevity and reproductive performance. At both inter- and intraspecific levels, larger animals tend to have lower mass-specific metabolic rates when compared to smaller animals (Promislow and Harvey 1990; Careau et al. 2013; Jimenez 2016). This pattern was reflected in our results, as the mass-specific daily energy expenditure (i.e. mass-specific daily oxygen intake volume and mass-specific daily carbon dioxide output volume; DEE) tended to be negatively related to body size. DEE was not found to be related to longevity nor reproductive performance, similar to findings from previous work (Derting and McClure 1989; Earle and Lavigne 1990; Hayes et al. 1992). Thus, despite the presence of a relationship between DEE and body size, DEE does not appear to mediate the relationships between body size and other life history characteristics, or alternatively, may have a mediating role that only becomes apparent under specific conditions. The findings from these data, as well as previous work on domesticated animals (Jimenez 2016), may represent an uncoupling of the relationships between metabolic rate and life-history traits, since domesticated animals tend to have abundant resources and do not face the same challenges as their non-domesticated counterparts. It is also important to note that measurements for DEE were taken from non-reproductive females at approximately 9 weeks of age, which has the potential to impact the results.

Similar to the hypothesized mediating role of metabolism in inter- and intraspecific life history trait variation, pleiotropic hormones allow for coordinated responses in multiple tissue and organ systems. IGF-1 responds to the nutritional status of the individual and

exerts pleiotropic effects on metabolism, development, growth, body size and composition, onset of sexual maturity, reproduction, and lifespan (Bartke 2005; Yuan et al. 2012). This hormone has been found to putatively play a role in the inter- and intraspecific variation of life history traits in several different taxa, including mammals (Swanson and Dantzer 2014), passerine birds (Lodjak et al. 2018), and reptiles (Schwartz and Bronikowski 2016). For example, the negative relationship between IGF-1 and lifespan has been exploited in mutant strains of mice, such as the Snell, Ames, or growth hormone receptor knock out (GHRKO) mice; these strains have attenuated insulin/IGF-1 signaling as well as small body sizes and increased lifespans (Bartke and Brown-Borg 2004). Given the coordinating role of this hormone, it is feasible that IGF-1 underlies the observed relationships at the intraspecific level. Consistent with previous findings like those of Harper et al. (2003), our results indicate an age-dependent inverse relationship between lifespan and serum IGF-1 concentration at 6 months of age, but not at any other time points. The time frame of the age-dependent relationship between IGF-1 and lifespan mimics the relationships between body mass and lifespan and reproductive performance, providing further evidence of the importance of IGF-1 in the proximate regulation of life history strategies.

Taken together, this study suggests that intraspecific relationships among life history traits, and the physiological mechanisms behind those relationships, are not always consistent with predictions from interspecific studies. Differences between inter- and intraspecific patterns of life history trait covariation are most pronounced in the relationships between body size and longevity and reproductive performance. Despite the reversal in the direction of the relationships between body size and reproductive performance and lifespan, the overall relationships between mass-specific metabolic rate

and IGF-1 concentration match previous interspecific findings. It is not known whether these deviations from the predicted interspecific findings are caused by the domestication process, representing an uncoupling between metabolism and key life history traits, or whether these same deviations are present in non-domesticated populations. Given the importance of understanding physiological and metabolic mechanisms that support life history variation (Ricklefs and Wikelski 2002), this study highlights the importance of conducting intraspecific analyses of life history and metabolic trait patterns of covariation.

Table 1. Categories and Variables Used in this study. Categories and sources of the variables used in this analysis are shown here. The number of strains represented by each variable is noted in parentheses.

Category	Variables
Reproduction	Dam's age at the birth of her first litter (35)[1]
	Number of litters per dam (35)[1]
	Interval between consecutive litters (35)[1]
	Number of pups born per litter (35)[1]
Derived variables	Principal component 1 = Breeding Frequency (35)
	Principal component 2 = Reproductive Intensity (35)
Lifespan	Average lifespan (31)[2]
Body size	Body mass at 3 (30), 6 (19), 12 (26), and 20 (29) months[3]
	Body length at 3 (23), 6 (20), 12 (26), and 20 (18) months[3]
Metabolism	Daily mass-specific oxygen consumption (15)[4]
	Daily mass-specific carbon dioxide production (11)[4]
	Daily mass-specific body heat production (11)[4]
	Mass-specific daily food intake (13)[4]

Mass-specific daily caloric intake (13)[4]

Heart rate at 6 (23), 12 (24), and 20 (20) months[5]

Serum concentration of IGF-1 at 6 (29), 12 (27), and 1 (16) months[6]

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[1]The Jackson Laboratory, 2009

[2]Yuan et al., 2007a; Leduc et al., 2010; Yuan et al., 2009

[3]Center for Genome Dynamics, 2009

[4]Seburn, 2001

[5]Xing et al., 2008; Xing et al., 2009

[6]Yuan et al., 2007b; Yuan et al., 2012

Table 2. Breeding Frequency and Reproductive Intensity represent two separate axes of reproductive performance. Breeding frequency (PC1) accounts for 40.6% of the variance across strain averages and is positively weighted by the average number of litters per dam and negatively weighted by the average interval between consecutive litters. The second axis (PC2) represents reproductive intensity, which accounts for 34.6% of the variance, and is negatively weighted by the average age of a dam at the birth of her first litter and positively weighted by the average litter size for each dam of a given strain.

	PC1 Breeding frequency	**PC2 Reproductive intensity**	PC3	PC4
Overall:				
Percent variance	40.6	34.6	21.2	3.3
Eigenvalue	1.62	1.36	0.84	0.133
Individual variable weights:				
Litters per dam	0.74	-0.13	0.25	0.64
Litter size	0.06	0.57	0.63	-0.31
Inter-birth interval	-0.63	-0.37	0.47	0.56
Age at first birth	0.23	-0.72	0.56	-0.41

Table 3. Relationships between body size and reproductive performance, metabolism, and IGF-1 in inbred laboratory mice. Pearson's product-moment correlation coefficients (r) for body mass and body size versus several life-history and metabolic variables, and IGF-1 at three different ages. P-values given in parentheses and significant ($p \leq 0.05$) relationships are in bold.

			Fitness-related Variables		Metabolic Variables		Serum IGF-1 Conc. ([ng/mL])		
		Average lifespan (days)	Age at first birth	Reproductive intensity (PC2)	Daily O_2 (mL/kg/h)	Daily CO_2 (mL/kg/h)	6 months	12 months	18 months
Body mass (g)	3 months*	**-0.44 (0.02)**	**-0.61 (<0.001)**	**0.64 (0.001)**	**-0.76 (<0.001)**	**-0.74 (0.01)**	**0.73 (<0.001)**	0.40 (0.064)	0.36 (0.102)
	6 months	**-0.36 (0.05)**	**-0.57 (<0.001)**	**0.54 (0.011)**	**-0.74 (0.003)**	**-0.79 (0.007)**	**0.59 (0.008)**	**0.71 (0.001)**	**0.71 (0.001)**
	12 months	**-0.41 (0.03)**	-0.28 (0.23)	0.26 (0.27)	**-0.65 (0.02)**	**-0.82 (0.007)**	**0.53 (0.006)**	**0.54 (0.006)**	**0.72 (<0.001)**
	20 months	-0.17 (0.38)	-0.08 (0.74)	0.14 (0.57)	-0.47 (0.15)	-0.57 (0.11)	0.37 (0.121)	0.35 (0.146)	**0.55 (0.018)**

Body length (cm)	3 months*	·0.3 (0.14)	**-0.67 (<0.001)**	**0.72 (<0.001)**	**-0.67 (0.008)**	-0.59 (0.16)	**0.72 (<0.001)**	0.34 (0.198)	0.22 (0.419)
	6 months	-0.28 (0.12)	**-0.56 (<0.001)**	**0.57 (0.007)**	**-0.72 (0.008)**	**-0.78 (0.01)**	**0.78 (<0.001)**	**0.79 (<0.001)**	**0.73 (0.001)**
	12 months	-0.29 (0.13)	-0.35 (0.13)	0.37 (0.10)	-0.57 (0.07)	**-0.74 (0.03)**	**0.71 (<0.001)**	**0.75 (<0.001)**	**0.72 (<0.001)**
	20 months	-0.27 (0.15)	-0.12 (0.62)	0.11 (0.66)	**-0.76 (0.01)**	**-0.85 (<0.001)**	**0.66 (0.003)**	**0.67 (0.003)**	**0.81 (<0.001)**

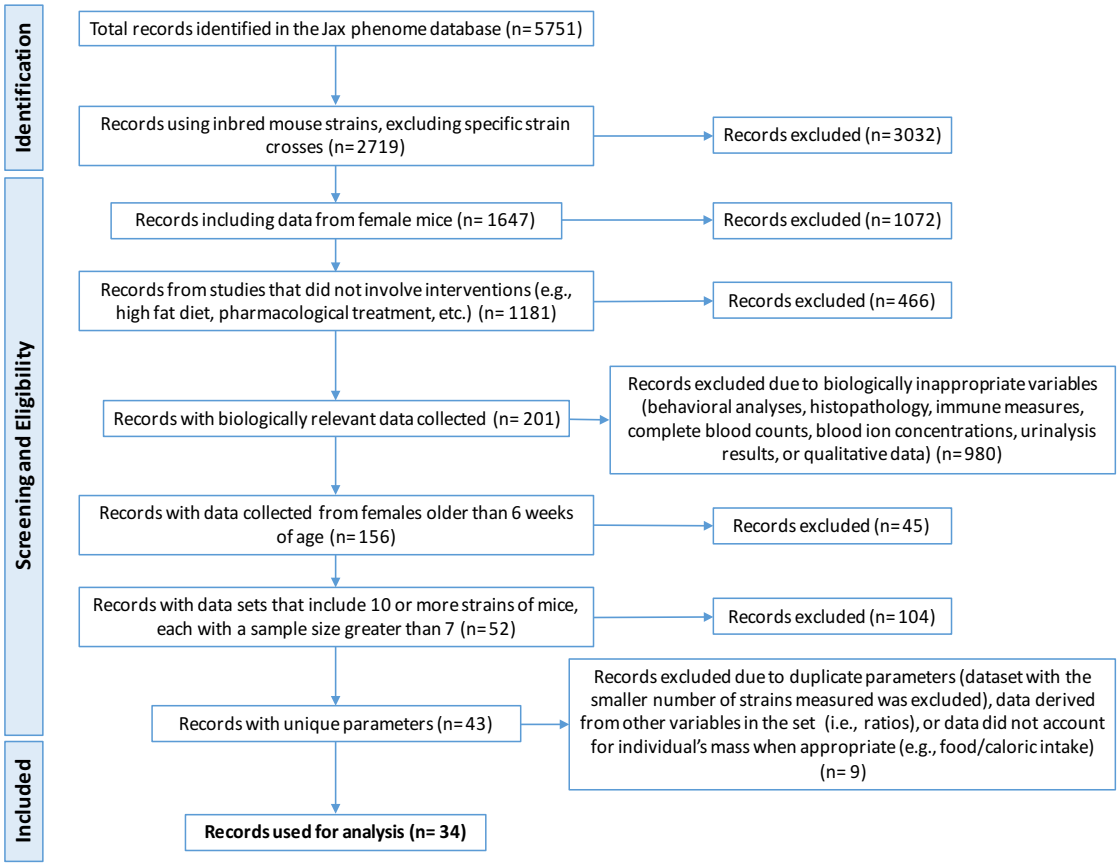

Figure 1. Guidelines for inclusion of data in the present study. Following the guidelines set by Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Liberati et al. 2009; Moher et al. 2009), we employed the above selection criteria to screen all available records (i.e., measurements of a variable from a study) on the Jackson Phenome Database.

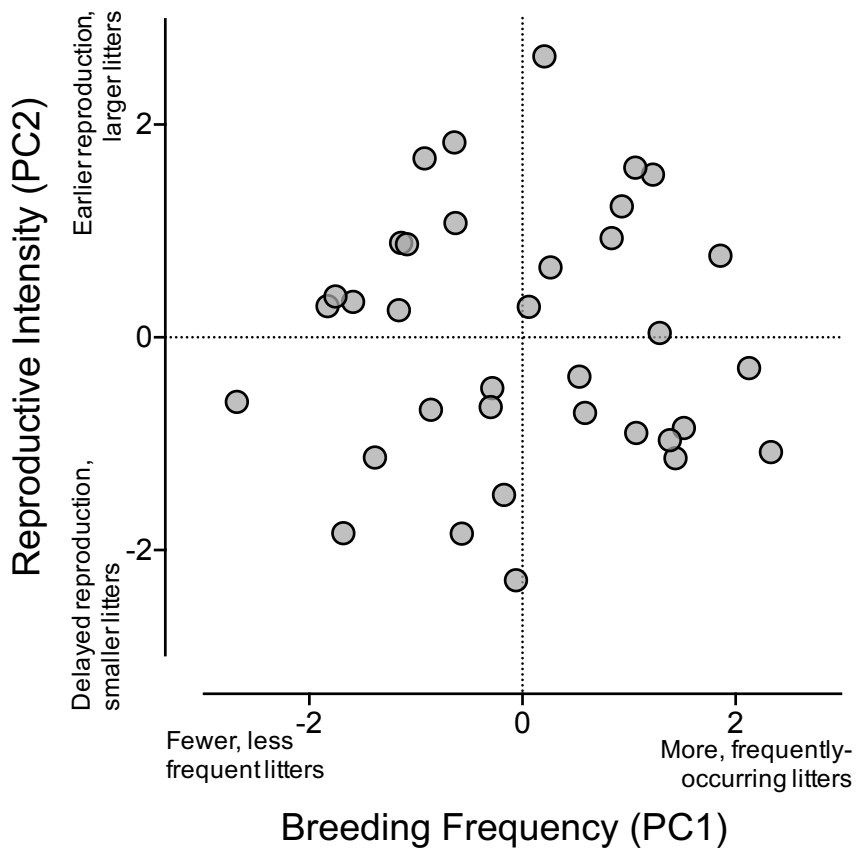

Figure 2. Variation in reproductive patterns across strains of inbred laboratory mice. Correlation analysis suggests that strains of inbred mice (n=35) do not display consistent relationship between the intensity and frequency of reproduction. X and Y variables are derived from the PCA analysis described herein. For both axes, negative values represent lower reproductive performance, whereas the higher values represent higher reproductive performance. Specifically, lower values for breeding frequency (PC1) represent fewer litters per dam and a longer time between each consecutive litter. Lower values for reproductive intensity (PC2) represent smaller litter sizes and a later age of first reproduction.

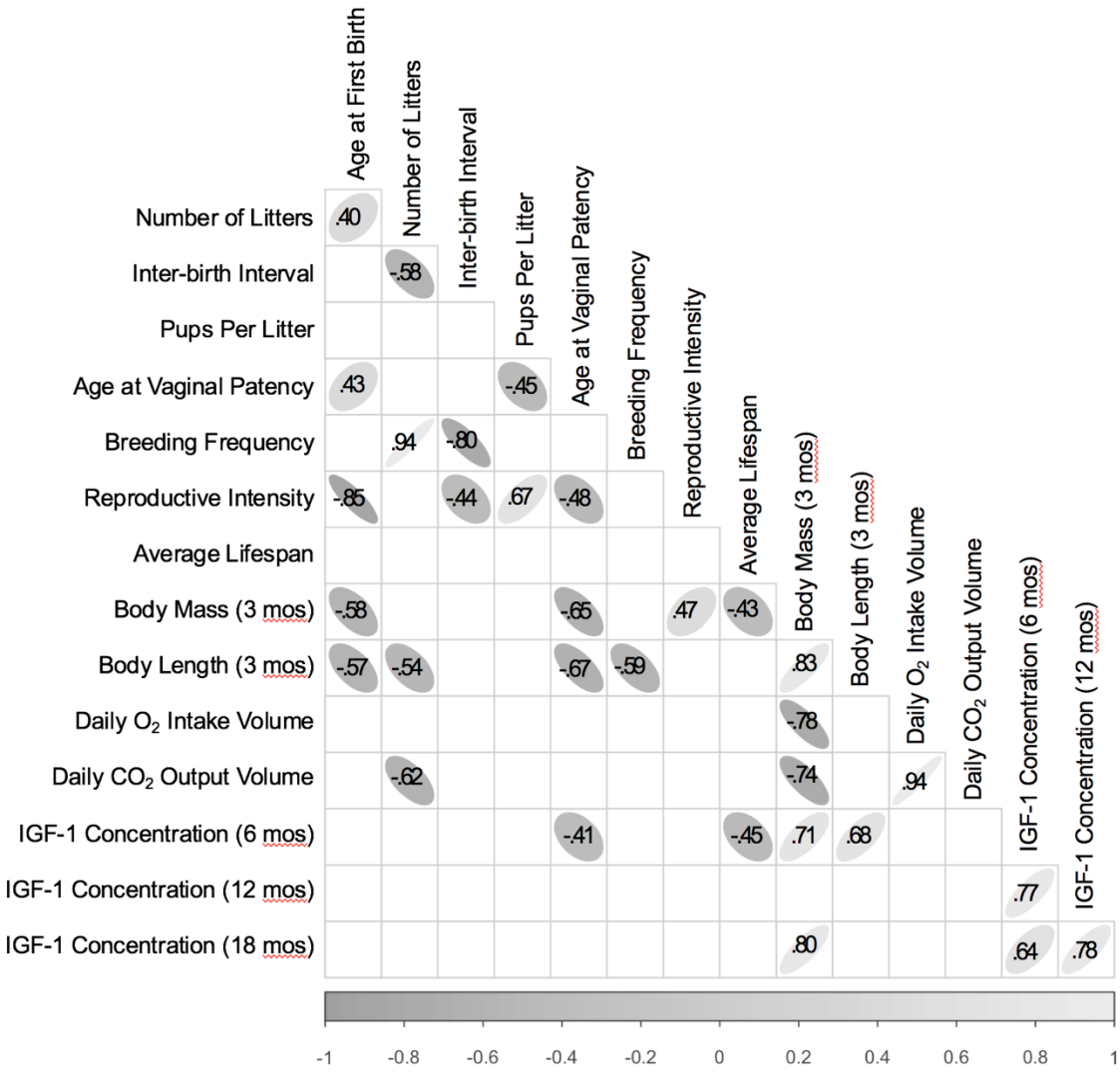

Figure 3. Correlations between reproduction, lifespan, body size, and metabolism in inbred laboratory mice. Significant ($p<0.05$) Pearson's product-moment correlation coefficients (r) are given. The oval under each value ranges from dark (more negative) to light (more positive); and narrow (strong correlation) to wide (weak correlation).

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CHAPTER TWO

PROTEIN INTAKE MEDIATES REPRODUCTIVE PERFORMANCE IN WILD-DERIVED MICE

Abstract. Both maternal age and diet are expected to impact reproductive performance, though it is not clear how these factors interact. This present work investigates reproductive performance over time in female wild-derived house mice who were given a high (20%) or low (10%) isocaloric protein diet. We found that reproductive performance was increased in females given a high protein diet relative to those on the low protein diet, as they produced more litters and more offspring. When looking at reproductive performance over time, we found that for mothers fed a high protein diet, as they aged, they tended to produce smaller, more numerous offspring in more frequent litters. In contrast, mothers on the low protein diet tended to produce larger, more numerous offspring in less frequent litters. Together, these findings represent alternative age-specific reproductive strategies, highlighting the mediating role of protein intake in determining lifetime reproductive performance.

INTRODUCTION

Fisher (1930) first posited that the high energetic demands of any given reproductive event should impose a cost on a female's future reproduction, longevity, and/or survival. However, the proximate trade-offs that underlie these costs of reproduction are largely contingent on environmental conditions, as well as environment by phenotype interactions, and may only be revealed when an organism is resource-limited or exposed to stressful conditions (Tuomi et al. 1983; Stearns 1992; Roff 2002). As such, reproductive performance has been widely documented as being condition-dependent (Clutton-Brock et al. 1983; Clutton-Brock 1984), as offspring production is contingent on the acquisition and allocation of resources (van Noordwijk and de Jong 1986; Reznick et al. 2000). Maternal body condition may therefore mediate individual variation in the degree to which costs of reproduction are expressed (Hamel et al. 2008). Tuomi et al. (1983) suggest that such variation exists because some animals are able to mitigate the energetic costs of reproduction by meeting the elevated demands of reproduction through increased intake rather than mobilizing endogenous resources at a cost to self-maintenance processes.

Consequently, maternal diet is expected to impact reproductive output. Experimental evidence across taxa has supported this prediction (Bomford 1987; Therrien et al. 2008; Houslay et al. 2015). Yet, much of this work has focused on dietary quantity and/or caloric intake, as it is often assumed that the currency that is being partitioned among competing resources is energetic (but see Dobson and Kjelgaard 1985; Derrickson and Lowas 2007; Warner et al. 2007). However, caloric restriction may not be common in all

wild populations. For example, wild populations of house mice (*Mus musculus*) typically do not experience caloric limitations on their diets (Austad and Kristan 2003; White 2012).

The reproductive performance of animals varies as a function of experience, age, fluctuating resources, and parental condition (Pianka and Parker 1975; Tuomi et al. 1983; Reznick 1985; Reznick et al. 2000). Age-specific patterns of reproductive performance have been documented in many species, finding that many iteroparous mammalian species exhibit an initial increase in reproductive performance with age up until a plateau at intermediate ages, typically followed by a decline at the end of the lifespan (Angelini and Ghiara 1984; Clutton-Brock 1984; Broussard et al. 2003; Houslay et al. 2015). Within species, however, it remains unclear which factors contribute to individual variation in rates of reproductive senescence, although the interaction between maternal condition and cost of reproduction undoubtedly plays an important role (Reznick et al. 2000; Zhang and Hood 2016). For example, Hamel et al. (2008) found that low-quality reproductive females had higher costs to future reproductive bouts than their high-quality conspecifics in three ungulate species, and that these costs were amplified with increasing age.

Here we aim to examine the effects of maternal protein intake on reproductive performance across the lifespan in wild-derived house mice (*Mus musculus*) housed in semi-natural conditions. We chose to manipulate protein intake because of its ecological relevance to this omnivorous species; a high (~20%) protein diet similar to our experimental diet reflects an insect-heavy diet, whereas a low (~10%) protein diet reflects a grain-heavy diet (Bomford 1987; Tann et al. 1991; Smith et al. 2002). We measured whether protein impacted reproductive performance, as well as how performance changed across the lifespan. We predicted that lifetime reproductive performance would be reduced

in the low protein group relative to females fed a high protein diet. We also predicted that performance would decline with age, and that this decline would be potentiated by a low protein diet.

METHODS

All work described herein was approved by the Auburn University IACUC committee PRN 2012-2104. Wild-derived house mice (*Mus musculus*) were obtained from established breeding colonies at the University of Utah (maintained by W. Potts) in 2012 and were approximately 10 generations removed from the wild. Animals were maintained in our semi-natural facilities. Each of the ten enclosures used in this study were 1.4 m x 3.5 m and lined with aluminum flashing in order to prevent escapes. These enclosures also allow the animals to form social hierarchies similar to those found in nature, with animals placed in mixed-sex groups of 3 males to 7 females; deviations from a typical 1:1 sex ratio were employed so that male-male aggression was reduced, and no more than 10 adults were in an enclosure at a time. Adult mice were allowed to breed and compete for mates similarly to how they would in the wild. Each animal used in this experiment was given a subcutaneous PIT tag and a unique ear punch pattern for identification.

Mice were randomly assigned to enclosure and dietary treatment at weaning (post-natal day (PND) 28). Mice were given *ad libitum* access to either a high (20%) or low (10%) protein diet prepared by TestDiet (Purina Animal Nutrition, St. Louis, MO). Protein composition amounts were chosen to mimic the broad range of protein availability that wild house mice may face (Bomford 1987; Tann et al. 1991; Robbins 1993). Wild house mice consuming an entirely grain-based (corn or wheat) diet would consume a diet comprised of approximately 10-13% protein; our high protein group represents an insectivorous diet comprised of approximately 20% protein. Both treatment diets provided

3.9 kJ of energy per kg of chow, and cornstarch was used to increase the caloric deficit caused by the decreased protein in the 10% diet (see Table 1 for macronutrient and energy composition). In addition to being isocaloric, the diets were also similar in their concentrations of fat, fiber, minerals, and vitamins. Because animals were housed in enclosures with other individuals, we were not able to quantify the amount of chow consumed by each female.

All animals included in this study were born to parents housed under similar conditions (Mowry et al. 2016), but their home enclosure was changed at weaning. Female and male siblings from the same litter were not placed in the same enclosure to avoid inbreeding effects, and no more than two males and two females from the same litter were used in order to increase genetic diversity of animals used in this experiment. Sexual maturity in this species has been reported to occur between 6-8 weeks (Whittingham and Wood 1983), but can be delayed up to 12 weeks of age due to environmental conditions and/or the presence of available mates (Latham and Mason 2004). Typically, males reach sexual maturity sooner than females, though this is variable (Miller et al. 2000). In this experiment, males in each enclosure were older or the same age than the oldest female in each enclosure when the breeding colony was established. The breeding of these animals was monitored in each enclosure daily, and offspring were counted from birth (post-natal day (PND) 1) until weaning (PND 28-31), when they were sacrificed. Information on sex was collected after confirmation of the presence (or absence) of testes.

After approximately 8 months of breeding, the males were removed from each enclosure; females were able to reach approximately one year of age before all of the remaining females were euthanized. This lag in time between when the males and females

were removed was so that the mothers were able to complete any final reproductive bouts. Previous work indicates that the maximum lifespan of wild house mice typically is approximately 1 year, but is substantially reduced on average due to predation (Miller et al. 2000).

Parentage was determined based on behavioral observations (i.e., the number of days the mother was present on each nest of pups) during the daily husbandry routine, and then genetically confirmed. Microsatellite analysis was run using six different loci that have previously been used in the ancestors of these mice (Meagher and Potts 1997). Capillary electrophoresis was then used to determine the length of the microsatellites used, and the likelihood of parentage was determined using the program Cervus (Kalinowski et al. 2007).

Specifically, we assessed reproductive performance for each treatment by using a t-test to compare means for the following variables: the number of reproductive females per treatment (n), females' ages when they first gave birth (d), the total number of successful litters per female (n), number of pups per litter (i.e., litter size) at parturition and weaning (n), the total number of offspring born and weaned (n), and the percent of offspring per litter surviving from birth until weaning (%). To analyze how diet was impacted by maternal age, we used R (http://www.r-project.org/; R Core Team 2013) to create linear mixed effect models (nlme package). Our variables of interest were the time between two consecutive litters (i.e., interbirth interval, d), number of pups per litter at birth and weaning (i.e., litter size, n), the cumulative mass of each litter at weaning (g), and individual pup masses at weaning (g). Maternal identification was used as a random effect and was found to account for approximately 25-30% of the variance in each model. Additionally, because there was slight variation in the days at which offspring were weaned from their mother

(between post-natal days 28-31), all models that include offspring mass include the day of weaning as a random effect. Fixed effects used in each model were maternal age (as determined by her date of birth) protein diet group, and an interaction term. Models were compared and selected based on AIC criteria. Unless otherwise noted, data are presented as means ± standard error.

RESULTS

Impact of Diet on Reproduction

Of the 70 females included in this study, 16/34 (47.1%) in the high protein group were reproductive, compared to 20/36 (55.6%) of low protein group (Fig.1). A Fisher's exact test did not reveal a significant impact of protein intake on whether or not a female reproduced ($p = 0.63$). Additionally, diet did not appear to impact the age at which the female first gave birth ($t = 0.81$, $df = 3$, $p = 0.42$).

Of those females that reproduced during the study, the females in the high protein group had significantly more litters relative to those in the low protein group ($t=1.79$, $df=28.40$, $p=0.04$; Fig. 2). On average, females on the high protein diet produced 4.5 ± 0.7 litters during their reproductive lifespans, whereas females in the low protein group produced an average of 3.1 ± 0.5 litters. Diet did not appear to impact the average number of pups per litter at parturition ($p=0.52$) nor the average number of pups per litter at weaning ($p=0.57$). Females fed the high protein diet gave birth to 11.27 ± 5.3 more offspring over the course of their lifetime than did those on the low protein diet ($t=2.12$, $df=28.82$, $p=0.02$; Fig. 3A). Similarly, females fed the high protein diet weaned 7.6 ± 3.8 more pups than females in the low protein group ($t=2.00$, $df=24.85$, $p=0.03$; Fig. 3B), though the lifetime-average percent of offspring surviving to weaning was not impacted by diet ($p=0.33$).

Impact of Age and Diet on Litter Characteristics

The time between two consecutive litters was not impacted by maternal age nor protein intake alone. However, there was a significant interaction between these two variables ($p=0.05$; Fig.4) such that the latency between reproductive bouts increased over the course of their reproductive lifespans for females on the low protein diet, whereas females fed the high protein diet had a decrease in interbirth interval as they age (Fig. 4). Litter size at parturition significantly increased with increasing maternal age ($p=0.02$), but no difference between the diets was found (Fig.5). Though not significant, the same trend of an increase in litter size at weaning with increasing age was found ($p=0.07$), though protein intake did not appear to have an impact ($p=0.55$; Fig. 6).

The mass of individual offspring at weaning was significantly impacted by diet ($p < 0.001$) as well as an interaction between diet and maternal age ($p < 0.001$), though maternal age itself was not significant (Fig. 7). Females in the high protein group had smaller offspring as they aged, whereas females in the low protein group showed the opposite pattern. The total mass of each litter was not impacted by diet ($p=0.98$), maternal age ($p=0.23$), nor an interaction between age and diet ($p=0.55$). Table 3 summarizes our results.

DISCUSSION

In natural populations of small mammals, such as house mice, individuals are likely not calorically restricted, though the quality of available food may be highly variable and thus, food quality rather than quantity is predicted to constrain performance (Austad and Kristan 2003; White 2012). Protein demand is particularly high during gestation and lactation, as proteins transferred from mother to young are assimilated into the developing organs of the offspring both *in utero* and via milk (Derrickson and Lowas 2007). Thus, we predicted that when available protein is limited, the reproductive capacity of a female would also be limited and become more pronounced with age. In general, our findings (Table 3) supported the prediction that females on low protein diets would have reduced reproductive performance relative to females on high protein diets. However, contrary to our initial predictions, we did not find any evidence of reproductive senescence in either treatment group. Instead, we found a difference in age-specific reproductive strategies between the treatment groups, suggesting differential costs of reproduction between the high and low protein groups.

Maternal protein availability previously has been demonstrated to have an impact on life-history traits such as the age of first reproduction, reproductive performance, and litter size in wild rodent populations (Cole and Batzli 1978; Dobson and Kjelgaard 1985). In our experiment, however, protein intake did not impact the number of reproductive females per enclosure or the age at which each reproductive female first gave birth. In

contrast, a prior study of wild house mice on rice-based diets found that protein supplementation (from 8% to 11% dietary protein intake) increased the proportion of females in each dietary group who reproduced (Bomford and Redhead 1987). It is possible that our low protein group (~10%) was not protein-restricted enough to see these same effects, either due to the absolute protein intake not being low enough or increased demands in wild populations relative to ours housed in semi-natural enclosures. It has also been suggested that food availability, rather than the nutritive quality, drives variation in these specific traits (Becker et al. 1998).

Females given the high protein diet produced more offspring and produced more litters over the course of the study relative to those on the low protein diet, which is consistent with previously reported findings (Bomford 1987; Warner et al. 2007; Houslay et al. 2015). We believe this increase in lifetime reproductive output is driven by the frequency of reproductive bouts, particularly at older ages. This difference in the latency between consecutive litters was associated with high-protein females having overlapping reproductive bouts. House mice experience a brief period of postpartum estrus, allowing for concurrent gestation and lactation, which has been reported to occur in as much as 82% of reproductive females in a wild population (Bruce and East 1956). In this study, females in the high protein group had 21 occurrences of the interbirth interval being less than 19 days (the approximate lower range for nutritional independence in this species; Berry 1970), compared to 5 occurrences in the low protein group. Thus, females on higher protein diets appeared to have greater capacity to meet the demands of gestating during lactation.

As an alternative to the cost of reproduction hypothesis (Williams 1966) that suggests reproductive performance should decline with age, Pianka and Parker (1975)

predict that species with age-related declines in residual reproductive value should have an increase in reproductive effort with age. Our results demonstrate that reproductive output increases with age for females in both of the protein treatment groups, though this increase is achieved through different strategies. In this experiment, females in the high protein group had shorter interbirth intervals as they aged, whereas females in the low protein group reproduced less frequently as they aged.

Additionally, these age-specific changes were not accompanied by age nor diet impacts on the cumulative litter mass at weaning, further emphasizing the role of protein intake in determining optimal maternal strategies for partitioning resources within a reproductive bout. For the females in the high protein group, an increase in reproductive performance with age was achieved by producing smaller offspring in larger, more frequent litters. In contrast, females in the low protein group produced larger offspring in larger litters, but at a less frequent rate. In her work on house mice, König et al. (1988) hypothesizes that females can optimize their reproductive success by partitioning available resources among offspring in a way that supports the largest number of offspring that can be reared at an intermediate mass above which fitness benefits are minimal. Thus, mothers fed low protein diets may employ a strategy that emphasizes investment in individual offspring, whereas females belonging to the high protein group may favor a tactic that increases the number of offspring produced.

In summary, this work demonstrates that protein intake alters age-dependent reproductive strategies in wild-derived house mice. We found that high protein intake increased reproductive performance, and reproductive performance increased with age regardless of diet, though the high and low protein intake groups achieved this through

different strategies. The ability to modify reproductive strategy to match the environment likely has contributed to the success of this species as a generalist, as evidenced by their ability to thrive in a broad range of environments (Weber and Olsson 2008). Taken together, these results suggest that variation in protein intake does not constrain reproductive effort, but may mediate age-specific patterns of reproduction in order to optimize fitness given their environments.

Table 1. Macronutrient composition and caloric value of high and low protein diets. All animals were put on high or low protein diets at weaning. The protein composition of each diet was selected so as to reflect ecologically-relevant variation in dietary protein intake that wild populations of house mice may experience. The high protein group (n=16) mimics an omnivorous, but insect-heavy diet, whereas the low protein diet (n=20) mimics a diet heavy in grains.

	High protein diet	Low protein diet
Protein	20.6%	10.1%
Fat	16.4%	16.2%
Carbohydrate	63.0%	73.7%
Energy	3.88 kcal/g	3.95 kcal/g

Table 2. Significance of variables included in statistical models. Mixed-effects models were used to understand the relationships between variables. For each model, maternal age and dietary treatment were treated as fixed effects, and maternal ID was used as a random effect. For the two variables that included body mass (offspring mass at weaning, total litter mass at weaning), the age of the offspring (d) was used as a random effect. Significance was established at $p \leq 0.05$; significant p-values are italicized below.

Variable	Coefficient	SE	*d.f.*	*t*	P
Time between consecutive litters					
y-intercept	29.55	8.29	93.6	3.56	<0.001
Low vs. High protein	-9.46	11.95	92.2	-0.79	0.43
Maternal age	-0.04	0.04	70.0	-1.01	0.32
Interaction	0.11	0.06	82.8	2.01	*0.05*
Number of offspring at parturition					
y-intercept	3.65	1.05	129	3.49	<0.001
Low vs. High protein	1.48	1.48	129	1	0.32
Maternal age	0.014	0.006	129	2.41	*0.02*
Interaction	-0.01	0.008	129	-1.23	0.22
Number of offspring at weaning					
y-intercept	1.6	1.17	125	1.36	0.18
Low vs. High protein	0.993	1.67	119	0.59	0.55
Maternal age	0.011	0.006	129	1.78	0.07
Interaction	-0.007	0.009	128	-0.79	0.43
Offspring mass at weaning					
y-intercept	12.84	1.23	50.5	10.43	< 0.001
Low vs. High protein	-4.07	1.47	54.0	-2.77	*<0.001*
Maternal age	-0.004	0.006	45.8	-0.69	0.49
Interaction	0.002	0.008	50.2	2.626	*<0.001*
Total litter mass at weaning					
y-intercept	37.5	17.4	59.0	2.15	0.04
Low vs. High protein	0.54	21.3	59.0	0.03	0.98
Maternal age	0.11	0.09	59.0	1.21	0.23
Interaction	-0.07	0.11	59.0	-0.6	0.55

Table 3. Summary of results. Comparisons between the high and low protein groups, as well as the age-specific effects of diet on reproductive performance, are given below.

	High Protein Diet	Low Protein Diet
Number of reproductive females	=	
Age at first reproduction	=	
Total number of litters	>	
Litter size at parturition	=	
Litter size at weaning	=	
Total offspring born	>	
Total offspring weaned	>	
Percent offspring surviving to weaning	=	
Interbirth interval	↓ with age	↑ with age
Litter size at parturition	↑ with age	↑ with age
Litter size at weaning	↑ with age	↑ with age
Total litter mass at weaning	=	=
Individual pup mass at weaning	↓ with age	↑ with age

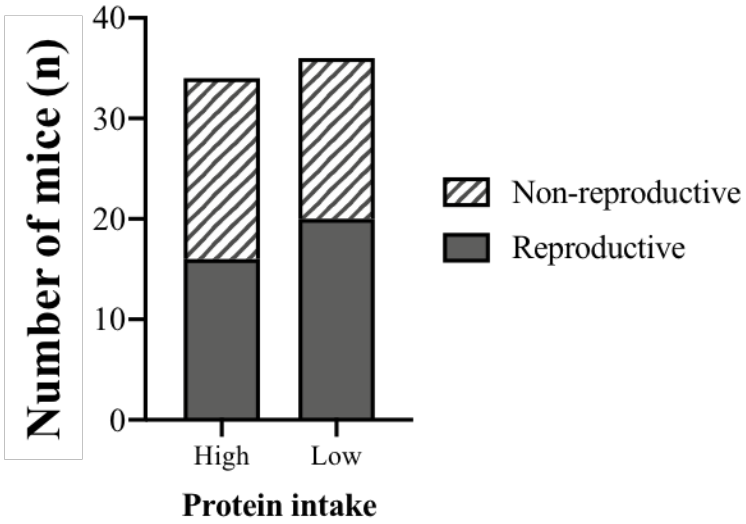

Figure 1. Number of reproductive females on high and low protein diets. Protein intake did not impact the probability of reproducing in females ($p = 0.63$). 16/34 (47.1%) of females given high protein chow reproduced compared to 20/36 (55.6%) of females on the low protein diet.

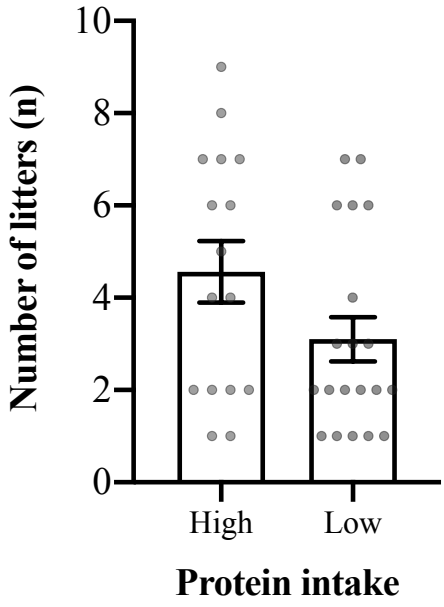

Figure 2. Impact of protein diet on number of litters per mother. Females in the high protein group (n = 16) had significantly more litters during their lifespans relative to those in the low protein group (n = 20) (t=1.79, df=28.40, p=0.04). Distribution of data is shown, with each of the grey points representing one individual. Bars represent the mean for each group. Error bars represent the standard error.

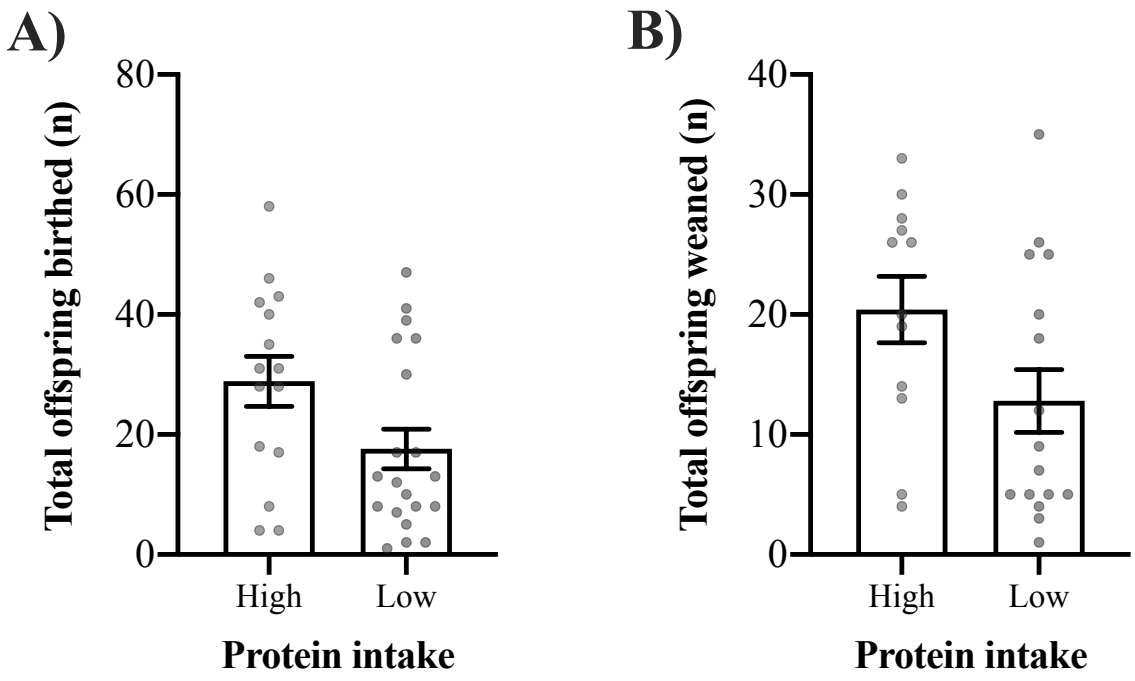

Figure 3. Cumulative number of offspring per mother at A) birth and B) weaning by protein diet. Females on the high protein treatment diet gave birth to and weaned more pups over the course of their reproductive lifespans (t=2.12, df=28.8, p=0.02; t=2.00, df=24.9, p=0.03, respectively). Distribution of data is shown with each of the grey points representing one individual. Bars represent the mean for each group. Error bars represent the standard error.

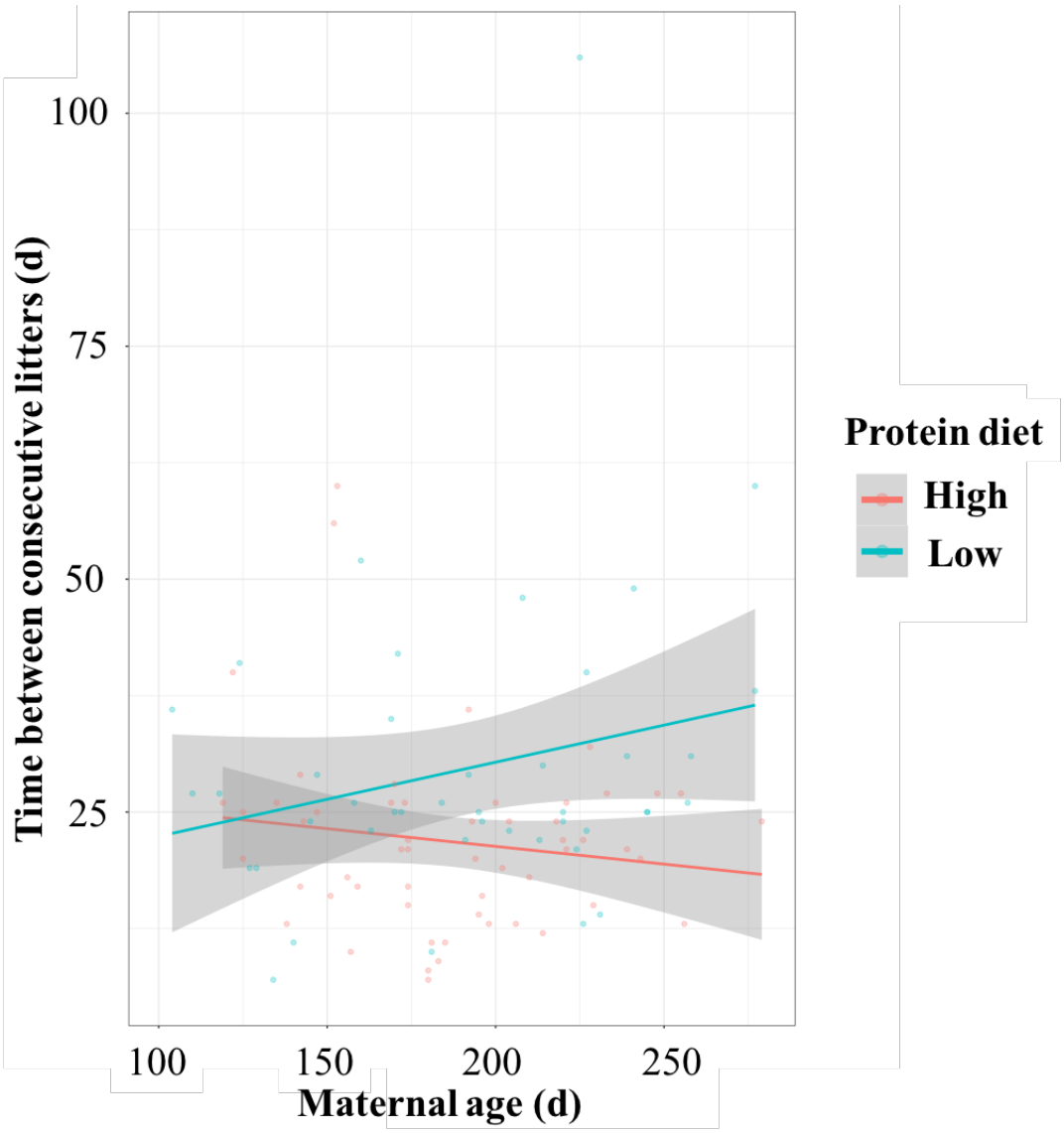

Figure 4. Interbirth interval (d) by maternal age (d) for females on high and low protein diets. A mixed effect model was used with protein diet and maternal age as fixed effects and maternal ID as a random effect. The time between two consecutive litters was not found to be impacted by maternal age (p=0.32) nor protein intake (p=0.43), though there was a significant interaction between these two variables (p=0.05). Individual data points for each litter are given, as is the 95% CI.

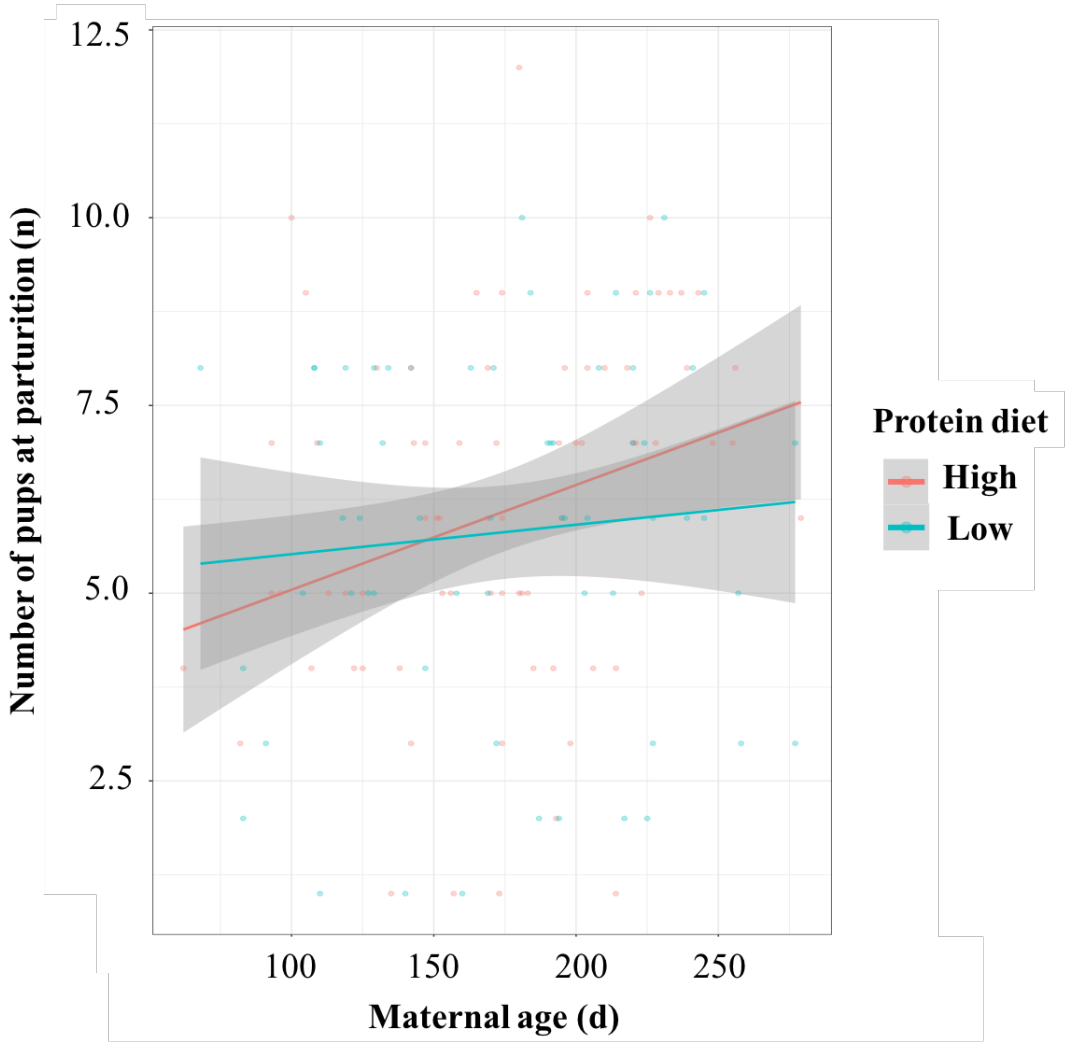

Figure 5. Litter size at birth by maternal age (d) for females on high and low protein diets. A mixed effect model was used with protein diet and maternal age as fixed effects and maternal ID as a random effect. The number of offspring produced in each litter was found to significantly increase with increasing maternal age (p=0.02), but was not impacted by protein intake (p=0.32). Individual data points for each pup are given, as is the 95% CI.

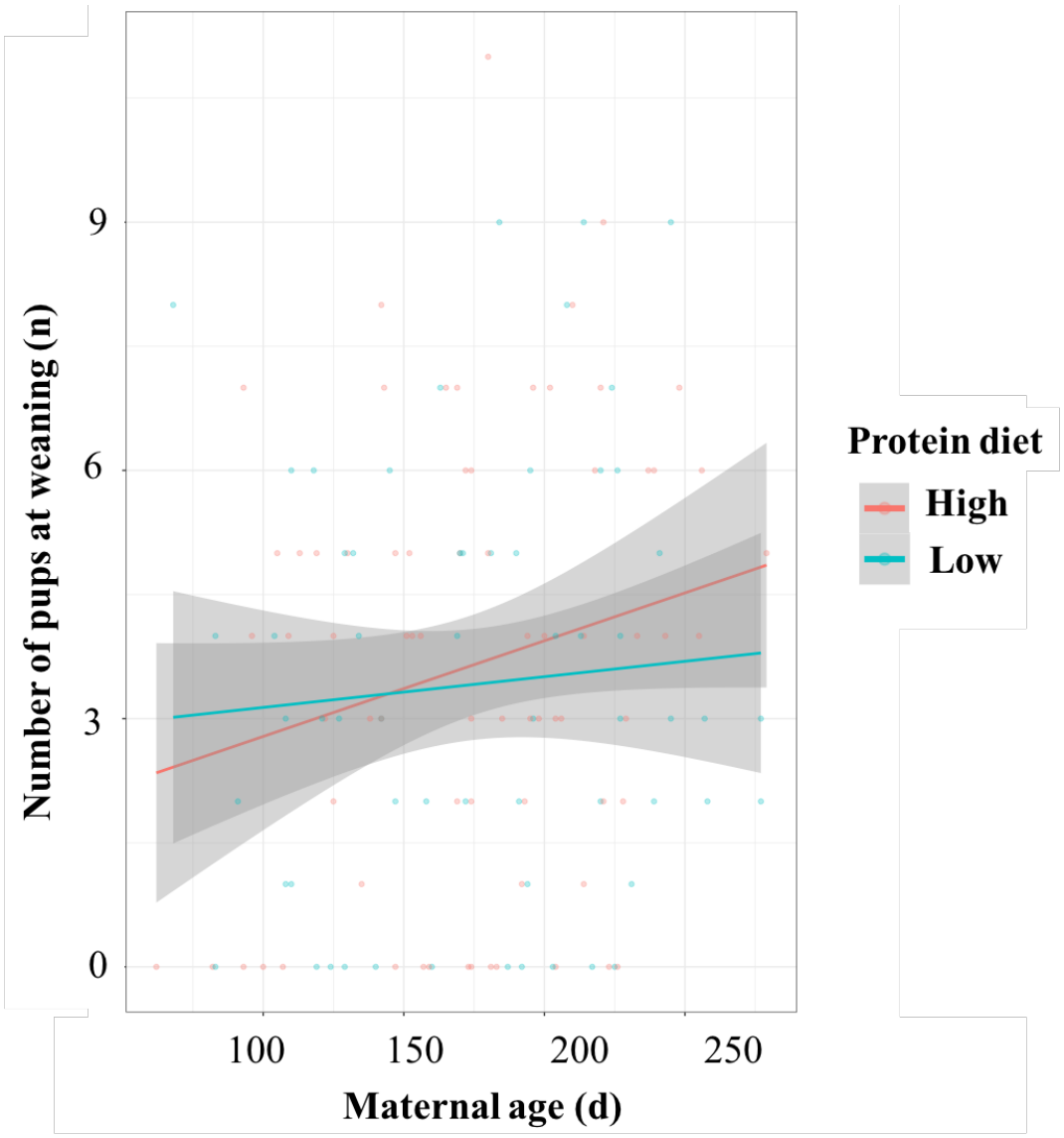

Figure 6. Litter size at weaning by maternal age (d) for females on high and low protein diets. A mixed effect model was used with protein diet and maternal age as fixed effects and maternal ID as a random effect. Age did not significantly impact the number of offspring at weaning (PND 28-31), though a trend exists (p=0.07). Diet was not found to impact litter size at weaning (p=0.55). Individual data points are given, as is the 95% CI.

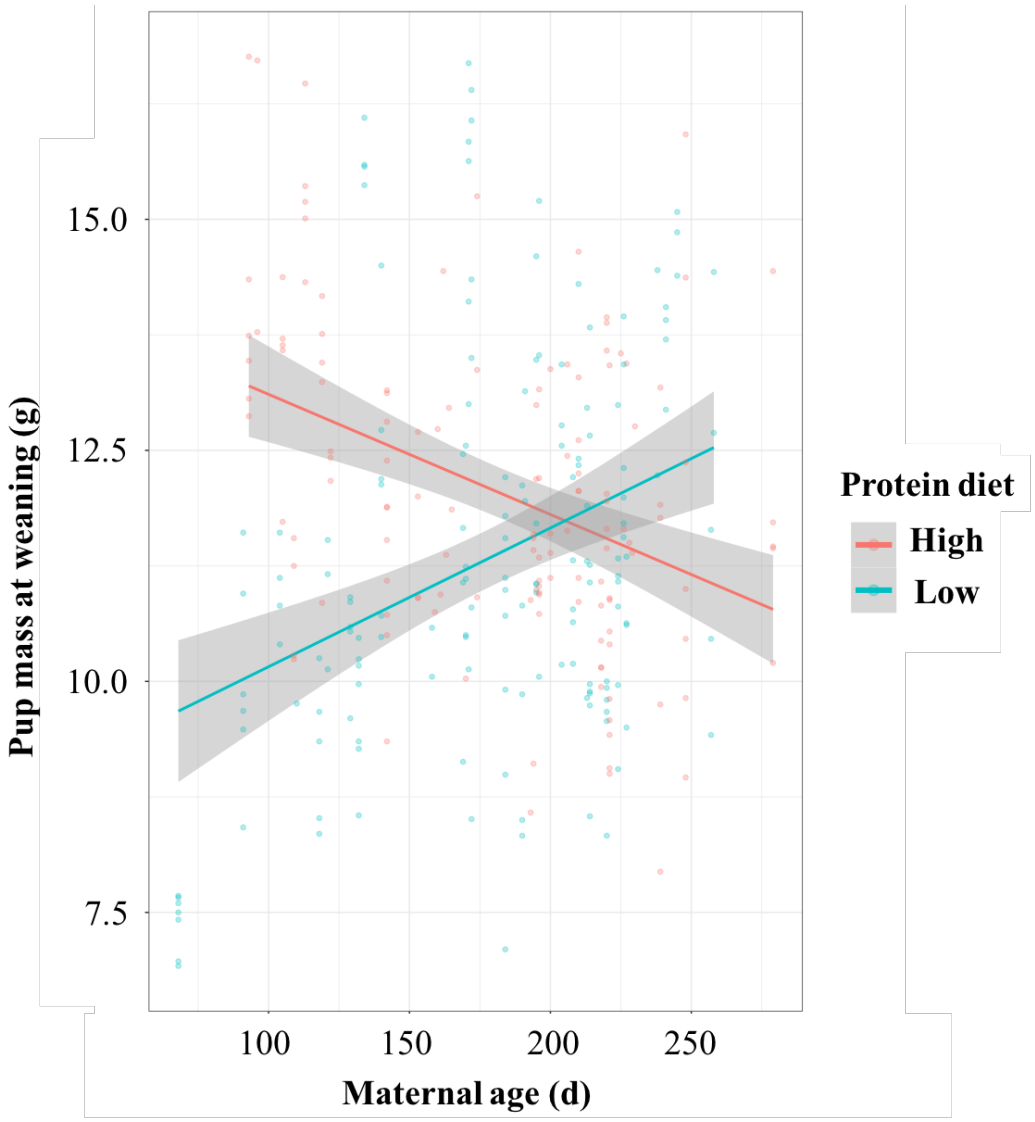

Figure 7. Individual offspring masses at weaning by maternal age (d) for females on high and low protein diets. A mixed effect model was used with protein diet and maternal age as fixed effects and maternal ID and offspring age (d) as random effects. Diet was found to impact offspring mass at weaning (PND 28-31) ($p < 0.001$), as was an interaction between maternal age and diet ($p < 0.001$). Maternal age was not found to have an effect on offspring mass ($p=0.49$). Individual data points for each pup are given, as is the 95% CI.

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CHAPTER THREE

METHODOLOGICAL CONSIDERATIONS FOR ASSESSING ANTIBODY-MEDIATED IMMUNE DEFENSE IN REPRODUCTIVE FEMALES

Abstract. Within the field of ecoimmunology, many investigations characterizing the nature of the interactions between reproductive investment and immune defense have been completed. Among these experiments, studies focusing on males and avian models predominate. The results from such experiments are often used to inform subsequent investigations on these topics within the context of female physiology and maternal investment, despite fundamental differences in selection pressures, parental investment in offspring production, and physiological constraints during reproduction. Further, of the studies that have explored this topic, the pharmacokinetics are assumed to be the same across all treatment groups. Previous work within immunotoxicology, however, has reported differences in the pharmacokinetics in response to vaccination with the commonly-used antigen keyhole limpet hemocyanin (KLH). Here, we explore the assumption that trade-offs between reproduction and antibody-mediated immune defense are the same in males and females as it relates to the use of KLH. We highlight methodological inconsistencies that limit the synthesis of findings, and present alternative hypotheses for how differences in antibody responses could occur. These hypotheses were then tested in females with varying reproductive demand (non-reproductive, lactating, concurrently lactating and pregnant). These responses were compared with a fourth group who was lactating and given a control vehicle. Serum was taken from one

animal per day per group and assessed for general and non-specific IgG and IgM. We then used polynomial regression to assess the antibody response curves across groups. Our results demonstrate that the antibody response curve can differ among individuals with varying maternal demands. These findings highlight the importance of multiple sampling points across treatment groups for a more integrative assessment of how reproductive demand alters antibody responses in females beyond a single measurement that is often assessed from a more-is-better perspective.

INTRODUCTION

The intimate, bidirectional links between reproduction and immune defense have long been noted across several sub-disciplines within the field of biology. These interactions underlie the relatively new, but rapidly expanding discipline of ecological immunology (or ecoimmunology; Sheldon and Verhulst 1996), which broadly aims to answer questions pertaining to the mechanistic, ecological, and evolutionary causes, as well as the consequences of, natural variation in immune defense (Martin et al. 2011; Demas and Nelson 2012; Downs et al. 2014). Central to this discipline is the well-supported hypothesis that developing, maintaining, and deploying a competent immune system is demanding and incurs fitness costs (Lochmiller and Deerenberg 2000; Bonneaud et al. 2003; Lee 2006; Martin et al. 2008). As such, immune defense likely plays a key role in shaping an organism's life history strategy, as selection should act to maximize defense against pathogens while minimizing subsequent fitness costs (Viney et al. 2005).

An individual's immune defense strategy is optimized based on both the potential fitness gain of mounting an effective response, as well as the pool of resources available to be partitioned among competing demands (Viney et al. 2005). Accordingly, a decrease in the ability to effectively clear a pathogen (i.e., "immunocompetence") is hypothesized to be a cost of reproduction (Zera and Harshman 2001), and a trade-off between reproductive effort and immunity has been hypothesized, and subsequently demonstrated, in many taxa (Sheldon and Verhulst 1996; Demas et al. 1997; Lochmiller and Deerenberg 2000; Read and Allen 2000; Schmid Hempel 2011). However, immunocompetence is not monolithic and should not be assessed from a more-is-better perspective; rather, an

optimal defense strategy is one that dynamically balances conflicting physiological needs within the context of factors such as the individual's environment, life-history stage and current priorities, and competing physiological demands.

Briefly, we review the use of the antigen KLH in the field of ecoimmunology, highlighting methodological limitations and inconsistencies. We then present predictions on how reproduction may influence the antigen response curve, then experimentally test these predictions.

Complexities Of Evaluating The Antibody-Mediated Immune Response

In practical applications, limitations on the ability to measure immune compounds in wild, non-model organisms have complicated protocol standardization (Demas et al., 2011). Experimental techniques for measuring components of adaptive immunity are dependent on the administration of several commonly-used antigens and quantifying the resulting antibody response. We focus on the use of keyhole limpet hemocyanin (KLH) due its established impact on the energetic demands of exposed animals (Demas et al., 1997), use in immunotoxicological studies due to its immunostimulatory properties (Lebrec et al., 2014), and the broad availability of both the antigen and assay kits that measure the specific KLH antibody response. Yet, despite these advantages of KLH, interpreting and synthesizing results proves challenging due to methodological inconsistencies.

KLH is often used in studies investigating reproduction-immune interactions across various taxa, yet the ability to draw comparisons based on these experiments is limited by inconsistent methods, as summarized in Table 1. KLH is a novel, non-replicating antigen derived from the giant keyhole limpet (*Meagthura crenulata*) that stimulates a T-cell

dependent antibody response (Lebrec et al., 2014). Exposure to KLH is considered a mild immune challenge that is known to increase metabolic rate without inducing fever, inflammation, or sickness behavior (Demas et al., 1997; Dixon et al., 1966; Martin et al., 2003). One downside to using immune challenges, such as KLH, is that they are comprised of large amounts of dead, non-replicating antigens that are administered via routes that are inconsitant with natural pathogen exposure. Differences in the routes of administration across studies affects the degree of localization of the immune response, thereby influencing which modulatory immune cells are deployed, leading to downstream differences in local and systemic immune responses (Zhang et al., 2015). As Viney et al. (2005) points out, many of the doses administered in these investigations contain high amounts of antigen (e.g., 100-150 uL KLH used in a small rodent; Table 1). To put this dose in perspective, vaccine doses for humans tend to range from 5-100 ug of antigen, despite the obvious size difference between humans and the small rodents used in these studies. Previous immunotoxicological investigations on the impact of KLH dose on subsequent antibody production report that even small differences in dose may create large differences in functional outcomes (Lebrec et al., 2014). Differences with respect to the type (class) of antibody (i.e., IgM versus IgG) and the timing of that response are of particular importance, as they have large implications on the interpretation of experimental results.

The demands associated with developing a competent adaptive immune system are the largest investment in immune funcation that a vertebrate will make (Martin et al., 2007). The clonal selection hypothesis (Burnet, 1957; Jerne, 1955; Rajewsky, 1996) aims to explain the ability of the adaptive immune system to create antibodies against a wide

variety of antigens with minimal self-reactivity to the host. During organismal development, antibodies on naïve, inactivated T- and B-lymphocytes are semi-randomly produced, and undergo subsequent selection against self-reactivity during the initial stages of clonal selection (Janeway et al., 2004). Lymphocytes with a high affinity for self-antigens are destroyed, while those with low self-reactivity proliferate and create lineages of daughter cells. In chickens, more than 90% of these cells are estimated to be eliminated during this checkpoint before they reach peripheral circulation (Martin et al., 2007; Reynaud and Weill, 1996). Similarly, after exposure to a novel antigen, clonal selection and expansion (i.e., proliferation) occurs in order to select for antibodies with maximum avidity to the presented antigen. Measuring antibody titers in blood is therefore comparable to estimating the size of an iceberg based on the visible tip: measuring the bredth and complexity of the response is possible, though difficult, and the majority of the investment occurs before the response can be measured. This initial investment carries large demands, and is in part responsible for the lag between exposure to an antigen and when measurable amounts of antibodies are present.

Clonal selection and expansion of antibodies is important in understanding ways that trade-offs between investment in reproductive performance and immune defense may manifest. The vast majority of experiments using KLH to investigate these trade-offs operate on the implicit assumption that all individuals reach their peak antibody response at the same time, and hence, direct comparisons are made based on one sampling time point. Although the kinetics of the antibody response to KLH based on multiple longitudinal samples often are included in pharmaceutical and immunotoxicological studies (for review, see Lebrec et al., 2014), repeated sampling methods are rare within

the field of ecoimmunology. Adopting inappropriate sampling times can obscure the true nature of the relationship between immunity and reproduction, leading to equivocal results across studies, further complicating the creation of a cohesive synthesis.

Rather than interpreting the complex relationship between reproduction and immunity based on one timepoint, we propose adopting repeated measures, when feasible, to gain a holistic, integrated view of the adaptive immune response. Such integrative assessments are rare (but see Martin et al. 2007), but provide invaluable information. Three features that characterize the response curve can be measured: the lag in time between intial exposure and peak titer levels, the magnitude of the peak antibody titer, and the area under the curve (or integral). We propose that differences in these response curves can reveal how females adapt to the simultaneous demands of reproduction and mounting an antibody-mediated immune response (Fig. 1).

Under this framework, the predominating assumption that individuals reach their peak antibody response at the same time is represented by Fig. 1A. Assuming a tradeoff between reproduction and immune function is present, individuals with the highest reproductive demand would have the lowest antibody titers, whereas the highest would belong to those with the least reproductive demand. This scenario would most directly reflect a trade-off in resouce allocation towards deploying the adaptive response. If indeed all peak titers across are reached at the same time regardless of reproductive status, then interpreting reproductive-immune interactions based on one sample may be appropriate. However, though titers taken at one time point are informative, a more holistic view of the antibody response curve may help by parsing out where the costs to immune defense are occurring. Predictions on how a decrease in titer may impact disease

susceptibility and other functional outcomes are easier to make by narrowing down the specific phases in the adaptive immune response pathway that are most impacted by reproduction.

Negative relationships between the adaptive immune response and reproductive investment can also manifest in other ways. For example, differences in the timing of the peak (Fig.1B) would indicate that reproductive demand impacts how long the selection and expansion of antibodies occurs, potentially due to the large initial protein demand associated with the creation of multiple lineages (Janeway et al., 2004). Functionally, however, a delay in the timing of the peak response may not necessarily indicate a cost of reproduction if the peak response titer or overall response is not impacted by reproductive demand. Further, if the area under the antibody response curve were similar, the overall protein and energetic investment would likewise be similar for individuals with varying reproductive demands. Similar areas under the antibody response curve with changes in the peak response timing and magnitude (Fig 1C) could also occur as a result of reproductive demand limiting both the initial investment as well as the overall investment, indicating an overall dampening of the immune response due to decreased allocation of resources.

The class of antibody measured is another important consideration to make when designing experiments. All vertebrate taxa have five analagous isotypes of antibodies, termed immunoglobulins, each with different effector functions that can be characterized by the constant region of their heavy chains (Bengtén et al., 2000). Immunologically naive antibody-secreting B-cells initially can only express immunoglobulin types M (IgM) and D (IgG), however, once activated, the B-cell is able to produce daughter cells

with identical antigen-binding regions, but of different isotypes, such as IgG, IgE, or IgA (Janeway et al., 2004). In other words, IgM is the first immunoglobulin to appear after exposure to a novel antigen, but upon subsequent exposures, other isotypes with increased avidity predominate (Janeway et al., 2004). Previous studies utilizing KLH as an immune challenge have included measurements of IgM or IgG (Table 1), but rarely both. The source of each of these immunoglobulins differs, as IgG is only released from longer-lived plasma B-cells after class-switching has already occurred (Janeway et al., 2004), and therefore may be more informative for understanding the long-term functional implications of variation in antibody titer.

METHODS

Animals and experimental design

Sixty-four female adult ICR mice (Envigo, Prattville, AL) were housed from October to December 2017 under the approval of Auburn University's IACUC (PRN #2017-3167). All animals were housed in standard polypropylene rodent boxes (~29.2 x 19.0 x 12.7 cm^3) and given *ad libitum* standard rodent chow and water. The mice were maintained on a 12:12 light dark cycle at 24°C.

To induce an immune challenge, the experimental groups were injected with keyhole limpet hemocyanin (KLH). KLH is a non-replicating antigen that has been used in antibody-mediated immune response studies including previous studies on inbred laboratory mice (Demas et al., 1997; Dixon et al., 1966; Martin et al., 2003). KLH induces a mild immune challenge that increases metabolic rate without causing anorexia, fever, inflammation, or sickness behavior. In this study, all adult female mice received a 0.1ml intrascapular subcutaneous injection of either KLH (150μg/mouse) or phosphate buffered saline (PBS) vehicle. The KLH was suspended in sterile, pyrogen-free PBS without any adjuvants. Females received an injection the day after parturition on PND 2. This timing was selected so that the hypothesized peak maternal antigen titers occurred at or before peak lactation so that maternal reproductive demand and maternal immune demands were highest. Female mice were then assigned randomly to one of four following groups (n=16 per group) based on their reproductive demand and immune challenge: 1) control and lactating (Control-L); 2) immune-challenge and non-

reproductive (Immune-NR); 3) immune-challenged with KLH and lactating (Immune-L); and 4) immune-challenged with KLH and concurrently pregnant and lactating (Immune-PL).

The groups were monitored and checked daily for evidence of breeding; males housed with females in the lactating-only groups were housed with the female for two weeks after pairing. Males housed with females in the concurrently gestating and lactating group were removed two days after the birth of the first litter, as to allow for mating during post-partum estrus. Females in the non-reproductive group were pair housed. On post-natal day (PND) 2 (where PND1 is the day of parturition), the litter sizes were standardized to 8 pups per litter. One litter initially consisted of three pups and was subsequently removed from analyses. In two cases, the females had 7 pups; these groups were included in the analyses.

Serum collection and antibody response

Animals in the reproductive groups received either an injection of KLH or PBS (control) the day after parturition (post-natal day 2; PND 2) in order to ensure that the majority of sampling time points occur before peak lactation, which is approximately PND 14 for this species. Animals in each of the four experimental groups were randomly assigned to a time point between 5 and 20 days post-injection (with post-injection day 1 being the day the injection was given, meaning that blood sampling occurred between PND 6 and 21, respectively). Absolute serum concentrations of serum total IgM and IgG and anti-KLH IgM and IgG were measured using enzyme-linked immunosorbent assays

(ELISAs) obtained from Life Diagnostics (West Chester, PA), following the manufacturer's instructions.

The antibody response curves for each of the specific and non-specific antibodies were first modeled using polynomial least squares regression (Figs. 2 and 3, A and D) after comparing alternative models. Polynomial coefficients were examined in all models, and in all cases were found to differ across groups, and differences were examined based on the 95% confidence intervals of estimates from the polynomial regression equations. Additionally, potential coefficients (i.e., unadjusted litter size, mass of unadjusted litter size at PND2, average pup mass at PND 2) were explored by the creation of alternative models and comparing AIC criteria; these were not found to be significant and were therefore left out of the model. All parameters for the polynomial equations, along with the 95% C.I.s and estimates of goodness of fit of the models, are given in Table 2. To elucidate more specific trends within the data that may be obscured in the polynomial regression, smoothing splines were created (Figs. 2 and 3, B and E). Finally, the integral of the antibody response was taken (Figs. 2 and 3, C and F), giving an estimate of the total antibody response (i.e., area under the curve) at a given time point.

RESULTS

Total and anti-KLH IgM was expected to peak at approximately 5 days post-injection. The immunogenicity of KLH was confirmed by an increased serum concentration of both total and specific anti-KLH IgM (Fig. 2A-C, Fig. 3A-C) in the immune-challenged groups compared to the group that received PBS vehicle. We found that the antibody response curves were different among the groups; no group was found to peak 5 days post-injection. The total investment (integral or area under the antibody response curve) of total and specific anti-KLH IgM among the immune-challenged groups, did not appear to be different, though it did appear to be pronounced relative to individuals receiving PBS.

Similar to our data for serum concentration of total and specific IgM, immune-challenged females appeared to have greater serum concentrations of total and specific IgG (Fig. 2D-E, Fig. 3D-E). The antibody response curves for both total and non-specific IgG were also found to be different across groups (Table 1; Fig. 2D-E, 3D-E). The serum concentration of both total and non-specific IgG appeared to be increased in the immune-challenged non-reproductive group relative to the reproductive groups, as did the integral of the response curve (Figs. 2&3F). The expected peak for this immunoglobulin was expected to occur approximately 14 days after injection, which did not appear to occur in our data.

DISCUSSION

Taken together, our results indicate that the specific and non-specific IgM response to KLH is not impacted by reproductive demand, rather, the differences in serum immunoglobulin concentrations can be attributed to the antigenic challenge itself. For total and specific IgM, we found that immune challenge increased the serum concentration of these antibodies as we expected. IgM typically is found in low levels in circulation under normal conditions and increases in response to a novel antigen during the primary immune response (Janeway et al. 2004). The differences in the timing of the peak anti-KLH IgM titer, but not the magnitude nor total investment in anti-KLH IgM production, suggest that reproduction may slow the initial clonal selection process after exposure to a novel antigen without impacting the overall ability to mount a response. Total and specific IgG serum concentration was similarly decreased in the control groups relative to the immune-challenged groups, though reproductive status appeared to have a greater impact on the response curve. For these antibodies, the non-reproductive immune-challenged group appeared to have a more robust response to KLH than did the other immune-challenged groups. Thus, the ability to produce antibodies that could confer immunological memory may not be functionally impaired during reproduction, but the ability to recognize and clear the pathogen via IgG may be. In other words, the ability to recognize and remember a pathogen upon subsequent exposure may remain intact, but there may be variation in the efficiency of those subsequent exposures.

It may be tempting to interpret the increase in IgG serum concentrations in the non-reproductive group relative to the reproductive groups as resulting from a trade-off. However, this inverse relationship between serum antibody concentration and reproduction may occur as a result of physiological changes that accompany offspring production, and may not come at a cost to maternal survival or future reproduction. In other words, our data demonstrate that there may be a decrease in IgG serum concentration that accompanies reproduction, but it remains unknown how variation in reproductive demand impacts the IgG-mediated response to KLH. In mammals with hemochorial placentas such as rodents, IgG is the only antibody that is able to cross the placenta as a passive immune process that confers protection in the immunologically naïve neonate (Borghesi et al., 2014). Thus, an optimal immune defense strategy for reproductive females may be one that optimizes passive immune transfer while minimizing negative maternal fitness effects. Alternatively, changes in antibody-mediated immune defenses may not be related to fitness at all (i.e., changes may occur as "spandrels", or necessary byproducts of reproduction and may not have any adaptive benefit themselves; Gould & Lewontin 1979).

Most importantly, our findings highlight the need for careful consideration during experimental design. Our data demonstrate that the antibody-mediated response to KLH challenge differs based on reproductive status. Further, the anticipated peak responses for both IgM and IgG did not appear to be as expected. Differences in the pharmacokinetics of the response to KLH may impact our interpretation of results, as many studies implicitly assume that the response curves will be approximately the same in all individuals, regardless of sex. It is not well-understood which factors (e.g., sex,

reproductive demand, age, environmental conditions, etc.) contribute to variation in the response, nor the functional differences that may result from such variation.

For example, in addition to including females, experiments that characterize the antibody response curve should include a multitude of species, including wild populations. In a comparison of wild and laboratory populations of house mice, Abolins et al. (2011) reported that wild-caught mice had a higher concentration and avidity of anti-KLH antigens after immune challenge. Thus, it is possible that domestication and/or the constant laboratory environment may influence our experimental data. Further, similar response curves should be measured using an actively replicating pathogen in order to understand whether these observed negative correlations between investment in reproduction and antibody titer are truly trade-offs (i.e., the decrease in antibody titer results in a decrease in maternal survival and/or fitness) or whether they result from shared pathways (i.e., the decrease in antibody titer has no impact on fitness, and results from changes in proximate pathways that link reproduction and immunity).

Female mammals have traditionally been underrepresented as model organisms within the field of ecoimmunology, where much of the current literature comprises studies using males and/or bird models. Despite the large demand of reproduction, especially lactation, relatively little is known about how these demanding processes impact the antibody-mediated immune defense. Of the studies that have explored this topic, the pharmacokinetics are assumed to be the same across all treatment groups. Previous work within immunotoxicology, however, has reported differences in the pharmacokinetics in response to vaccination with KLH (Leduc et al. 2010). Our work suggests that the antibody response curve can differ among individuals with varying

maternal demands, potentially due to shared pathways that both allow for offspring production and alter the pharmacokinetics of an antibody response. Investigations into the relationships between female reproduction and immune defense should therefore reflect female reproductive physiology. Accordingly, methodological approaches to measuring costs of reproduction should be re-assessed from this perspective.

Table 1. Comparison of methods involving KLH. A comparison of experimental methods used in studies assessing female rodents' responses to immune challenge with KLH. When given in the original paper, the mass of the females or dosage of KLH relative to females' body masses is given.

Species	Sex	Reproductive state	Dose of KLH	Route of administration	Antibody class	Source
Peromyscus spp.	M, F	NR	150 ug	Intraperitoneal	IgG	(Martin et al. 2007)
Octodon degu	F	L	1 mg*	Intraperitoneal	IgG, IgA	(Becker et al. 2007)
Common vole	M, F	NR	5 ug/g animal**	Subcutaneous	IgG	(Devevey et al. 2008)
Siberian Hamster	M, F	NR	100 ug	Subcutaneous	IgG	(Bilbo and Nelson 2001)
Siberian Hamster	F	NR, P, L	100 ug	Subcutaneous	IgM	(Drazen et al. 2003)

Brandt's voles	F	P, L	100 ug	Subcutaneous	IgM, IgG	(Xu et al. 2012)

Notes: M = Male, F = Female; NR = Non-reproductive, P = Pregnant-only, L = Lactating-only; *mean body mass of female degust reported as 223g, approximately 9-10 times larger than other species listed here; **mean body mass at start of experiment reported as 27.5-28g.

Table 2. Estimates of coefficients, confidence intervals, and measurements on goodness-of-fit of polynomial regression equations. Results from our experiment assessing how the antibody-response curve differs in females of varying reproductive demands were modeled using polynomial regression techniques. Below, the coefficients (with 95% C.I.) are given for each of the polynomials in Figs. 2 (A,C) and 3 (A, C). Alternative models were explored and selected based on AIC criteria; as such, all equations given represent the best possible goodness-of-fits given the data.

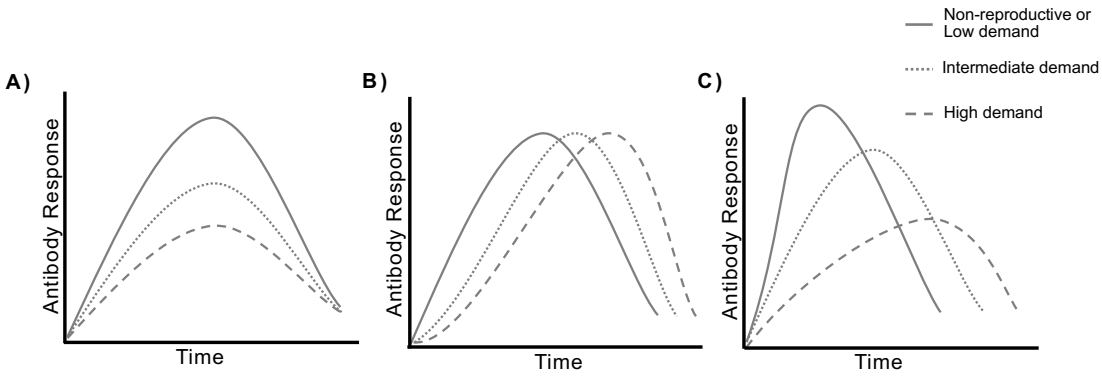
			Treatment Groups			
			Immune-NR	Immune-PL	Immune-L	Control-L
Total IgM	Best-fit polynomial coefficient estimates	B0	84.25	40.39	47.77	-13.85
		B1	-6.20	-0.18	-0.80	5.16
		B2	0.14	-0.07	-0.037	-0.215
	95% CI	B0	61.32 to 107.2	-3.40 to 84.19	29.08 to 66.46	-26.15 to -1.547
		B1	-10.19 to -2.212	-7.80 to 7.441	-4.05 to 2.50	3.02 to 7.30
		B2	-0.02 to 0.30	-0.38 to 0.23	-0.17 to 0.09	-0.30 to -0.13
	Goodness of Fit	D.f.	13	13	13	13
		R squared	0.8641	0.4998	0.7973	0.7106

		Sum of Squares	394.4	1439	262.1	113.5
Total IgG	Best-fit polynomial coefficient estimates	B0	-104.4	-28.15	-17.11	35.4
		B1	30.29	6.221	8.904	-4.492
		B2	-1.243	-0.1672	-0.3623	0.1656
	95% CI	B0	-176.2 to -32.49	-60.49 to 4.194	-57.51 to 23.29	11.11 to 59.69
		B1	17.79 to 42.79	0.5942 to 11.85	1.876 to 15.93	-8.717 to -0.2673
		B2	-1.736 to -0.7490	-0.3893 to 0.05488	-0.6397 to -0.08491	-0.001142 to 0.3324
	Goodness of Fit	D.f.	13	13	13	13
		R squared	0.6995	0.6675	0.3823	0.3099
		Sum of Squares	3876	784.8	1224	442.5

Anti-KLH IgM	Best-fit Values	B0	28.23	-7.898	-8.809	1.368
		B1	-1.807	3.332	4.711	-0.03566
		B2	0.0262	-0.1412	-0.2119	0.003849
	95% CI	B0	0.4321 to 56.02	-24.02 to 8.223	-21.44 to 3.825	-4.767 to 7.503
		B1	-6.643 to 3.028	0.5275 to 6.136	2.514 to 6.909	-1.103 to 1.032
		B2	-0.1647 to 0.2171	-0.2519 to -0.03053	-0.2986 to -0.1251	-0.03828 to 0.04598
	Goodness of Fit	D.f.	13	13	13	13
		R squared	0.44	0.3951	0.7569	0.04506
		Sum of Squares	579.6	195	119.8	28.23
		B0	-3.238	-0.1437	0.5657	0.1888

	Best-fit polynomial coefficient estimates	B1	0.8824	0.07944	-0.05151	-0.0324
		B2	-0.0296	-0.002606	0.003383	0.001364
Anti-KLH IgG	95% CI	B0	-18.87 to 12.40	-1.508 to 1.221	-1.168 to 2.299	0.06961 to 0.3080
		B1	-1.838 to 3.603	-0.1579 to 0.3168	-0.3531 to 0.2501	-0.05315 to -0.01169
		B2	-0.1370 to 0.07777	-0.01197 to 0.006762	-0.008521 to 0.01529	0.0005459 to 0.002183
	Goodness of Fit	Degrees of Freedom	13	13	13	13
		R squared	0.06091	0.07199	0.1624	0.5212

	Sum of Squares	183.4	1.396	2.254	0.01066



Peak response timing	No differences	Low < Intermediate < High	Low < Intermediate < High
Peak response height	High < Intermediate < Low	No differences	High < Intermediate < Low
Area under curve	High < Intermediate < Low	No differences	No differences
Interpretation	Most directly indicates a trade-off between reproductive and immune investment	Reproduction limits resources needed for clonal selection of antibodies without a change in magnitude of the response nor approximate total allocation of resources towards immune defense.	Reproduction limits resources needed for clonal selection of antibodies and magnitude of the response without a change in approximate total allocation of resources towards immune defense.

Figure 1. Trade-offs between reproductive demand and antibody-mediated immune defense. Trade-offs between reproduction and immunity can manifest in different ways. Above, we give alternative hypotheses for different ways, as well as an interpretation, of how reproductive demand could impact the antibody response to a novel antigen (e.g., KLH during a primary challenge).

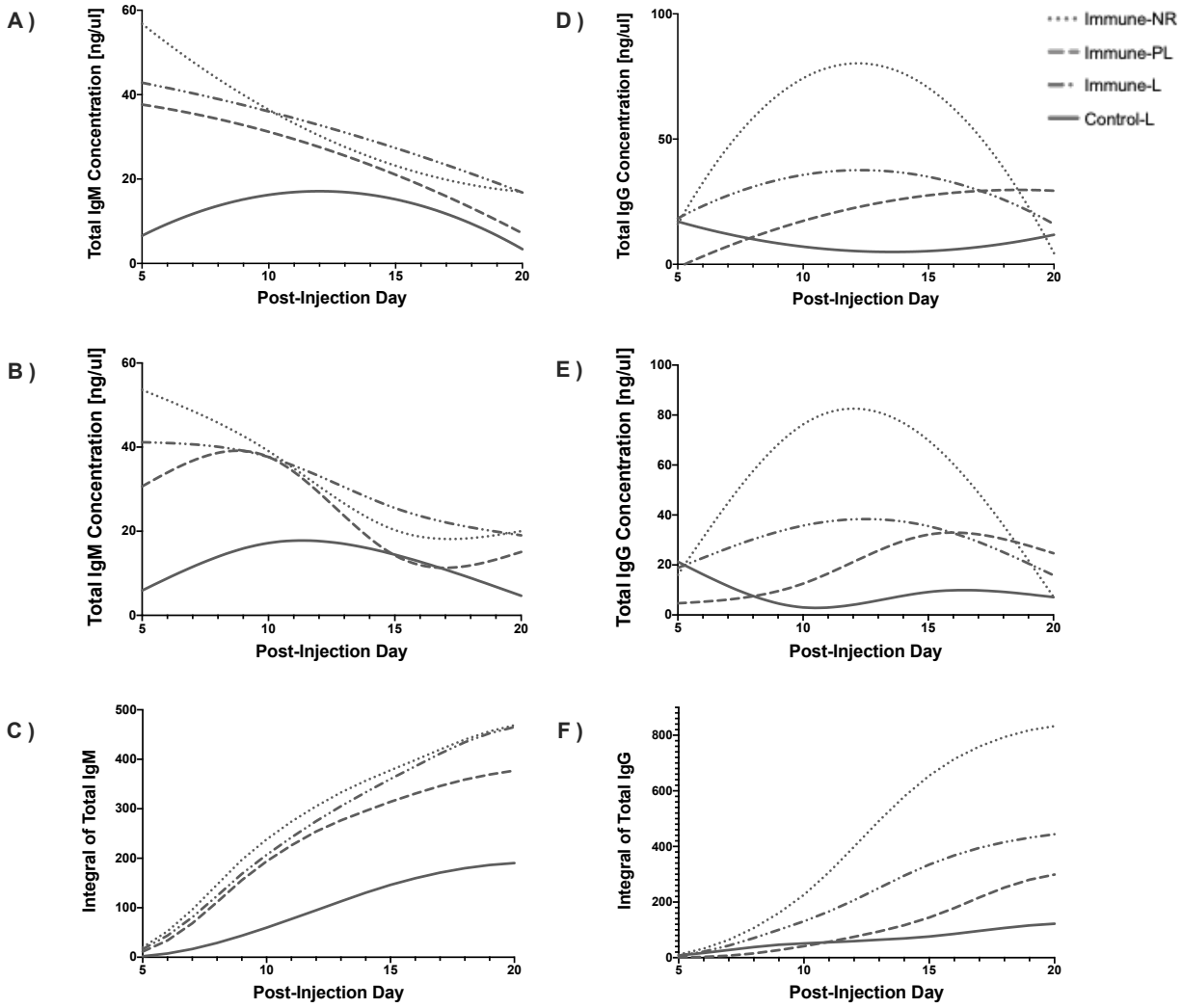


Figure 2. Total (non-specific) IgM and IgG responses during reproduction. The above graphs depict the changes in antibody concentration over time in the four different groups used in our study. A and D represent the best-fit polynomial regression (coefficient estimates given in Table 2) for the serum concentration of Total IgM (A) and Total IgG (D). Splines (B and E) were used to visualize specific trends we may have missed in polynomial models, though these specific trends were present (e.g., Immune-PL group in Fig.2B), overall, the polynomial model is still reflected. C and F give the additive integral by the number of days after injection; these represent the area under the

curve up until the number of days after injection and give an estimate for the total amount of antibodies produced during the response.

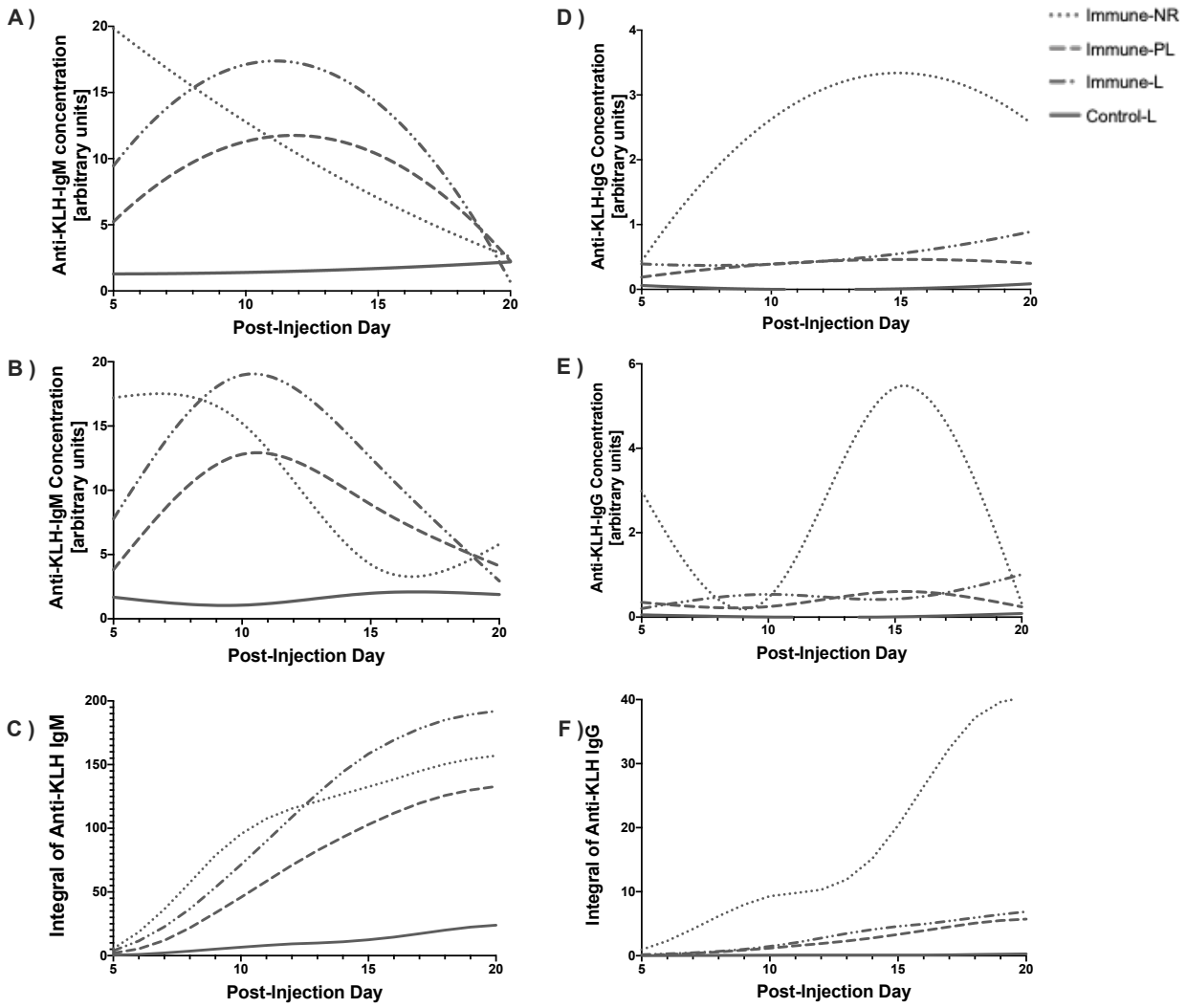


Figure 3. Specific Anti-KLH IgM and IgG responses during reproduction. The above graphs depict the changes in antibody concentration over time in the four different groups used in our study. A and D represent the best-fit polynomial regression (coefficient estimates given in Table 2) for the serum concentration of Anti-KLH IgM (A) and Anti-KLH IgG (D). Splines (B and E) were used to visualize specific trends we may have missed in polynomial models, though these specific trends were present (e.g., Immune-NR group in Fig.3E), overall, the polynomial model is still reflected. C and F give the additive integral by the number of days after injection; these represent the area

under the curve up until the number of days after injection and give an estimate for the total amount of antibodies produced during the response.

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CHAPTER FOUR

DEVELOPMENTAL EFFECTS OF A MATERNAL IMMUNE CHALLENGE IN *MUS MUSCULUS*

Abstract. Maternal passive transfer of antibodies is widely accepted to occur, though relatively little is known about how maternal investment in reproduction impacts this transfer. Previous work has demonstrated that an immune challenge during the neonatal period can program adult immune responses, stress responses, and body size. In this experiment, we tested the impact of varying maternal reproductive demand during an antigenic challenge with keyhole limpet hemocyanin (KLH) on offspring body and organ masses and gene expression of key pro-inflammatory cytokines (IL-1b, TNFa, and IL-6), glucocorticoid receptors (glucocorticoid and mineralocorticoid receptors), and somatotropic axis receptors (growth hormone receptors and IGF-1 receptors). Passive immune transfer was confirmed by the presence of specific anti-KLH antibodies in offspring of mothers administered KLH during lactation. Our results demonstrate that maternal antigenic challenge during lactation impacts offspring mass at weaning sex-specifically, relative thymus, spleen, and liver masses, and the hepatic expression of GR, IL-1b, TNFa, and growth hormone receptors. Increased maternal reproductive demand (concurrent pregnancy and lactation) did not appear to further impact these responses, though we did find that offspring weaned from these mothers had higher expression of hepatic IGF-1r, likely due to altered maternal physiology.

INTRODUCTION

Although ontogenesis is largely controlled by a genomic blueprint, the individual's phenotype remains malleable during development and is prone to regulation by the environment (Gluckman et al., 2007; McMillen and Robinson, 2005; Monaghan, 2008). Termed developmental programming (Lucas, 1991), the persistent responses that result from early-life conditions are hypothesized to provide an adaptive advantage by calibrating an individual's phenotype to the environment the young is likely to experience in adulthood (i.e., the predictive adaptive response; Bateson et al., 2014; Gluckman et al., 2007) or to match maternal condition (i.e., the thrifty phenotype hypothesis; Wells, 2007). Beyond providing passive immune protection during the early-life period, maternal antibodies impart maternal disease history and may serve to prime the offspring for the anticipated disease environment (Boulinier and Staszewski, 2008). Previous work in rodents has demonstrated that neonatal innate immune challenge programs offspring behavior, neurodevelopment, stress response, and immune defense (Bilbo and Schwarz, 2012, 2009; Spencer et al., 2010, 2006).

Despite the growing body of literature on developmental immune programming, few studies have investigated such programming as a result of antigenic immune challenge on neonates (Barrios et al., 1996) or their lactating mothers. A large proportion of the literature investigating maternal priming of offspring immunity via the antibody response has focused on avian species (Addison et al., 2010; Grindstaff, 2016; Grindstaff et al., 2003; Hasselquist and Nilsson, 2008) and has demonstrated that the advantages of maternal

transfer of antibodies extend beyond specific protection. In mammals, maternal immunoglobulin transfer is similarly predicted to prime the immune response of offspring (Lemke et al., 2012), and has been experimentally shown to increase growth and survival rates in mice (Gustafsson et al., 1994) and latency to sexual maturity in bank voles (Kallio et al., 2006). Yet despite the majority of maternal antibody transfer occurring via milk rather than *in utero* (Power and Schulkin, 2016), many studies within mammals have focused on understanding antibody transfer during gestation (but see Kallio et al., 2006).

Milk is reflective of the maternal physiological milieu that results from balancing simultaneous physiological demands. Variation in maternal investment has been found to impact offspring behavior, growth, and survival due to changes in components within milk (Hinde, 2013; Hinde and Capitanio, 2010; Skibiel and Hood, 2015). Although many studies have documented the interactions between parental effort and immune defense in birds (Ardia, 2005; Deerenberg et al., 1997; Ilmonen et al., 2003), reptiles (Cox et al., 2010; French and Moore, 2008), and male mammals (Demas et al., 1997), it remains unclear how immune challenge impacts maternal investment in mammals. Of the scarce literature investigating mammalian females, investigations have focused on pregnant females (Xu et al., 2012) who have significantly decreased reproductive demand relative to lactating females (Drazen et al., 2003).

Here, we examined how maternal reproductive demand and immune challenge during lactation impact offspring development. To test our hypothesis that variation in maternal demand during lactation impacts offspring development, a novel antigen (keyhole limpet hemocyanin; KLH) with an established large metabolic cost (Demas et al. 1997) was administered to females of varying reproductive demands the day after parturition (post-

natal day, PND, 2). Reproductive demand was increased by mating females immediately after parturition, when they can become pregnant while concurrently lactating (Bruce and East, 1956).

We then systematically evaluated the impact of these competing demands on offspring weaned from mothers from each of the three experimental groups by comparing body and organ masses, specific anti-KLH antibodies, and expression of genes associated with immune defense, the hypothalamic-pituitary-adrenal axis, and the somatotropic axis. Specifically, we looked at differences in expression of 1) key regulatory pro-inflammatory cytokines (interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha; IL-1b, IL-6, and TNF-a, respectively); 2) glucocorticoid receptors (GR) and mineralocorticoid receptors (MR); and 3) receptors for IGF-1 (IGF-1r) and growth hormone (GHr). We predicted that if we assume a trade-off between reproduction and immunity, then maternal demand during lactation would be inversely related to offspring body mass at weaning (i.e., KLH+PL < KLH+L < PBS+L). We also hypothesized that there would be an accompanying change in somatotropic axis receptors (GHr and IGF-1). Additionally, we predicted that maternal immune challenge would increase the relative mass of immune organs (i.e., spleen and thymus) as well as the hepatic gene expression of the identified pro-inflammatory cytokines and glucocorticoid receptors.

METHODS

Animals and Experimental Design

Forty-eight nulliparous female adult ICR mice (Envigo, Prattville, AL) were housed from October to December 2017 under the approval of Auburn University's IACUC (PRN #2017-3167). All animals were housed in standard polypropylene rodent boxes (~29.2 x 19.0 x 12.7 cm^3) and given *ad libitum* standard rodent chow and water. The mice were maintained on a 12:12 light dark cycle at 24°C.

Female mice were randomly assigned to one of the three following groups (n=16 per group) based on their reproductive demand and immune challenge: 1) control and lactating (PBS+L); 2) immune-challenged with KLH and lactating (KLH+L); and 3) immune-challenged with KLH and concurrently pregnant and lactating (KLH+PL). The groups were monitored and checked daily for evidence of breeding; males housed with females in the lactating-only groups (PBS+L and KLH+L) were housed with the female for two weeks after pairing. Males housed with females in the concurrently gestating and lactating group (KLH+PL) were removed two days after the birth of the first litter, as to allow for mating during post-partum estrus. On post-natal day (PND) 2 (where PND1 is the day of parturition), the litter sizes were standardized to 8 pups per litter. One litter initially consisted of three pups and was subsequently removed from analyses. In two cases, the females had 7 pups; these groups were included in the analyses.

To induce an immune challenge, the experimental groups were injected with keyhole limpet hemocyanin (KLH), a benign protein derived from the giant keyhole

limpet (*Megathura crenulata*). KLH is a non-replicating antigen that has been used in antibody-mediated immune response studies including previous studies on inbred laboratory mice (Demas et al., 1997; Dixon et al., 1966; Martin et al., 2003). KLH induces a mild immune challenge that increases metabolic rate without causing anorexia, fever, inflammation, or sickness behavior. In this study, all adult female mice received a 0.1ml intrascapular subcutaneous injection of either KLH (150μg/mouse) or phosphate buffered saline (PBS) vehicle. The KLH was suspended in sterile, pyrogen free phosphate buffered saline without any adjuvants. Females received an injection the day after parturition on PND 2. This timing was selected so that the hypothesized peak maternal antigen titers occurred at or before peak lactation so that maternal reproductive demand and maternal immune demands were highest.

Pup masses were collected on PND 2, 12 and 21 between the hours of 1200-1400 to ensure consistency in data collection among groups. All pups from the first litters were removed and euthanized at weaning on PND 21, and pups from the second litters (in the KLH-PL group only) were counted, weighed, and removed at PND 2. Two males and two females for each litter were randomly selected for tissue collection. Sex was confirmed by presence/absence of testes. All dissections were performed between 1200 and 1800, and the brain, thymus, spleen, kidneys, heart, and liver were removed and weighed. Trunk blood was collected and allowed to clot at room temperature before centrifugation to collect serum. Prior to weighing, each organ was blotted as to remove any excess blood. Of the four offspring dissected, livers were retained from one male and one female. The livers were flash frozen and used to quantify gene expression. All liver and serum samples were kept at -80C until analysis.

RNA Extraction, cDNA synthesis, and qPCR

Livers were homogenized in TRIzol (Invitrogen, Waltham, MA) and a phase separation was performed using chloroform to isolate RNA. Each sample was then treated with Turbo DNAse (Ambion Inc., Austin, TX). RNA was then used as input for cDNA synthesis using SuperScript Double-Stranded cDNA Synthesis kit (ThermoFisher Scientific, Waltham, MA) according to manufacturer's instructions.

We then used qPCR to determine the hepatic gene expression of our seven genes of interest (IL-1b, IL-6, TNFa, GR, MR, IGF-1r, and GHr) relative to a constitutively-expressed housekeeping control, GADPH (see Table 1 for primer sequences). qPCR was performed similarly to RT-PCR from (Layé et al., 1994). Briefly, 200ng cDNA was added to a PerfeCTa SYBR Green FastMix (ThermoFisher Scientific, Waltham, MA) reaction containing gene-specific primers (see Table 1 for primer sequences) at a concentration of 1uM. qRT-PCR was performed on an Eppendorf RealPlex2 (Hamburg, Germany) with the following conditions: 2min 95C, (1min 95C, 1min 60C, 1min 72C) x 40 cycles, followed by melt curve analysis to ensure the correct product was amplified. Expression of all genes were determined relative to GAPDH expression using the deltaCT method. Two replicates were performed per sample.

Antibody Measurements

To assess passive transfer of specific anti-KLH antibodies, offspring serum anti-KLH IgG concentrations were assayed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Life Diagnostics, West Chester, PA) according to the

manufacturer's instructions. Serum samples were randomly selected from each group (n=8 for PBS-L, n=6 for KLH-L, and n=6 for KLH-PL) and plated in duplicate. Positive control samples (pooled serum from five mice previously determined by our lab to have high levels of anti-KLH antibody using the same methods) and negative control samples (pooled serum from five mice who are immunogenically-naïve to KLH) were run in duplicate. The OD was determined for each well using a plate reader (BioTek PowerWave HT, Winooski, VT) equipped with a 405-nm wavelength filter and was expressed as a percentage of the OD for the plate-positive control. Samples were determined to be negative for anti-KLH IgG if the sample's OD value fell below 10% of the plate-positive control. There were no cases in which the sample duplicates gave conflicting results. The impact of maternal treatment on whether the offspring were positive for serum anti-KLH IgG was evaluated using a Fisher's exact test.

Statistical Analyses

All statistics were performed with GraphPad Prism (GraphPad Prism version 8.2 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com) or R (Team, 2013). Shapiro-Wilks tests were performed to test for normality of the data, and Levene's test was used to test for homogeneity of variances. Data were not transformed. Grubbs' outlier test was performed; no outliers were removed. All organ masses were corrected for body mass; therefore, organ mass reflects the size of the organ relative to the pups' total body mass. Unless otherwise noted, we used two-way repeated measures analyses of variance (ANOVAs) to understand the impact of treatment and sex on

offspring physiology and development while controlling for potential effects due to the mother's identity. Significance was established at $\alpha = 0.05$.

RESULTS

Offspring and Organ Masses

Average pup mass was not found to differ across treatment groups before adjusting the litter size to eight pups at PND2 (Table 2). The average mass of the pups retained was also similar between groups after the litters were adjusted on PND2 and at mid-lactation on PND 12. A two-way ANOVA with interaction terms of treatment group and sex was used to test for effects on mean pup mass on PND21, when pups were sacrificed. Though neither treatment nor sex were found to have a significant effect, though a significant interaction between sex and treatment was observed. We found a significantly increased mass at weaning in males belonging to the KLH+L group compared to the PBS+L group ($p=0.02$). Females belonging to the PBS+L group, however, were significantly larger in the PBS+L treatment compared to females in the KLH+L ($p=0.002$) and the KLH+PL group ($p <0.001$) (Figure 1).

No significant effects of treatment, sex, nor treatment*sex were observed for brain, heart, nor kidney masses relative to the individual's body mass (Table 2). Treatment had a significant effect on liver mass ($F_{2,135}=10.03$, $p<0.001$), but this effect did not vary with pup sex ($F_{1,135}=0.01$, $p=0.908$; Table 2; Figure 2A). When comparing relative liver mass between the two lactating-only groups, liver mass was lower in offspring whose mothers were challenged with KLH (KLH+L; 0.050g/g ± 0.001) compared to the PBS-treated group (PBS+L; 0.051 g/g ± 0.001). However, liver masses were found to be higher in the KLH+PL group (0.056g/g ± 0.001) relative to the PBS+L

group. A significant difference in mean liver mass was also found between the KLH+L and KLH+PL groups.

Offspring from the PBS+L group had significantly smaller spleens (0.0068 g/g± 0.0004) compared to the KLH+L (0.0089 g/g± 0.0006) and KLH+PL (0.0092 g/g± 0.0007), which did not significantly differ from one another (Figure 2B). A significant main effect of treatment ($F_{2, 131}$=4.96, p<0.01) and a simple effect of sex ($F_{1, 131}$=6.02, p=0.01) was found for thymus mass relative to the individual's mass. We found that males tended to have lower relative thymus masses than females, and offspring belonging to immune-challenged groups tended to have larger masses, with a significantly lower average thymus masses in offspring in the PBS+L group (0.0058g/g ± 0.0002) relative to both the KLH+L (0.0068g ± 0.0002) and the KLH+PL (0.0069g/g ± 0.0003) groups (Figure 2C). Treatment had a significant main effect on relative spleen mass of offspring at PND21 (Table 2), though no sex or interaction effects were found.

Stress Response-Associated Receptors

Basal levels of glucocorticoids are associated with mineralocorticoid receptors (MR), whereas glucocorticoid receptors (GR) generally mediate the physiological changes necessary to restore homeostasis after a stressful event (de Kloet et al., 1998). Gene expression of GR and MR was measured and is given as a relative value to the constitutively-expressed gene GADPH (Table 2). For GR, significant effects of treatment group ($F_{2, 31}$=35.2, p<0.001), offspring sex ($F_{1, 31}$=13.8, p=0.001), and mother ($F_{31, 31}$=6.008, p<0.001) were found, though the interaction between offspring sex and treatment group was not found to be significant (p=0.72 Fig.3,). Relative to the PBS+L

group, both groups whose mothers received KLH (KLH+L and KLH+PL) had significantly greater mean GR expression ($p < 0.001$), but these two groups did not significantly differ from one another ($p=0.554$). Within the KLH+L group, females had significantly greater expression of GR relative to the males within that same group ($p=0.01$); these sex differences in expression were not found in any other group.

MR expression relative to GADPH was not significantly impacted by treatment ($F_{2, 31}=2.96$, $p=0.067$) or offspring sex ($F_{1, 31}=3.65$, $p=0.007$), though a trend was present. The interaction between sex and treatment was also not significant ($F_{2, 31}=2.55$, $p=0.09$). Though no differences across treatment groups were found, a near-significant increase in MR expression was observed in the KLH+PL group relative to the KLH+L group ($p=0.07$). When looking at just the female offspring from the KLH+PL group, females had significantly greater MR expression relative to females from the KLH+L group ($p=0.04$) and a near-significant increase in MR expression relative to the males in their same treatment group ($p=0.08$).

Growth Hormone/IGF-1

Gene expression of GH receptor was significantly impacted by maternal treatment group ($F_{2,29}=3.81$, $p=0.03$), and offspring from the PBS+L group had significantly greater expression than the KLH+L group ($p=0.032$), but not the KLH+PL group ($p=0.32$)(Table 2). Mother was found to be a significant term ($F_{29, 29}=2.051$, $p=0.028$). No sex differences were observed ($p=0.26$).

IGF-1 receptor expression was not significantly affected by offspring sex ($p=0.98$) or mother ($p=0.53$), but a significant effect of treatment was found ($F_{2,55}=3.20$,

p=0.05). No significant difference in mean IGF-1 receptor expression was evident between the control (PBS+L) group and the groups receiving an immune challenge (KLH+L and KLH+PL). Among these two immune-challenged groups, offspring whose mothers were concurrently lactating and pregnant had significantly fewer IGF1-r compared to offspring whose mothers were only lactating. Together, these results suggest only simultaneous gestation, but not immune-challenge, appears impacts offspring hepatic IGF-1 receptor expression.

Immune Molecule Expression

No effect of treatment or sex was found in our analysis of hepatic gene expression of IL-6 (Table 2). Hepatic IL-1b gene expression relative to GADPH was found to be significantly impacted by treatment ($F_{2,31} = 4.90$, p=0.02); hepatic expression was elevated in offspring in the KLH+L group relative to the PBS+L group (Table 2; Figure 5). No effect of sex was found. Differences in TNFα expression was assessed both qualitatively (i.e., whether any TNFα was detected in the sample) and quantitatively. Treatment was found to have a significant effect on the expression of TNFα relative to GADPH ($F_{2,31} = 3.32$, p=0.05), though there were no sex effects nor a sex*treatment effect (Table 2; Figure 6A). Treatment also impacted the probability that TNFα could be measured in the sample (Chi square: 27.44, df = 2, p <0.01; Figure 6B). The proportion of samples positive for TNFα was 4/24 (16.7%) of the PBS+L group, 20/30 (66.7%) of the KLH+L group, and 14/14 (100%) of the KLH+PL group.

Maternal immune challenge was found to significantly impact the presence of anti-KLH IgG in serum; 0/8 pups belonging to the PBS+L group were found to have

these antibodies, whereas 5/6 and 4/6 of the offspring from the KLH+L and KLH+PL groups were found to be positive, respectively. Fisher's exact test revealed these proportions to be significantly different ($p=0.001$; Figure 7).

DISCUSSION

Immune challenge during the neonatal period can have persistent effects on an individual's immune defense strategy, growth and development, stress response, and behavior (Barrios et al., 1996; Bilbo and Schwarz, 2012, 2009; Spencer et al., 2006). The passive transfer of maternal antibodies impacts neonatal growth and survival, and may similarly act to program developing offspring's immune systems (Addison et al., 2010; Boulinier and Staszewski, 2008; Grindstaff, 2016). The presence of specific (anti-KLH) antibodies in the offspring of immune-challenged mothers confers previous findings of passive antibody transfer via milk. In mammals, passive transfer of antibodies through mammary secretions far exceeds transplacental transfer (Barrios et al., 1996). Yet much of the work investigating the role of passive transfer in offspring development focuses on prenatal transfer and literature investigating this phenomenon during lactation are scarce. To the best of our knowledge, this study is the first to examine the effects of competing maternal physiological demand during lactation on the integrated offspring phenotype.

Together, our results from offspring body and organ mass demonstrate that maternal immune challenge as well as reproductive demand has the potential to alter offspring phenotype at the organismal level. We hypothesized that variation in maternal demands during lactation would reduce offspring body and key organ mass at weaning relative to reproductive demand (i.e., KLH-PL < KLH-L < PBS-L). Contrary to our initial predictions, treatment alone did not impact offspring mass. Interestingly, however, we did find an interaction between maternal treatment and sex such that males in the

KLH+PL group were larger at weaning than males in the PBS+L group. When looking at just female offspring, our initial prediction was supported, and the largest offspring were born to the PBS+L group. This finding suggests that mothers trying to meet the concurrent demands of lactation, gestation, and immune challenge invest more in sons than daughters. It is possible that under these constraints, mothers may employ a reproductive strategy that produces the best quality sons given the circumstance (Charnov, 1991), particularly because size at weaning is an important determinant of adult size and reproductive success.

Offspring belonging to mothers who were immune-challenged, regardless of maternal reproductive demand, had significantly larger thymuses at weaning. Likely, this finding is due to slowed thymic involution, rather than thymic hypertrophy. Age-related thymic involution is a normal developmental process found in almost all vertebrates characterized by a regression of the thymus size and related anatomical changes (Janeway et al., 2004). Although the thymus is fully developed at birth (Janeway et al., 2004), size increases in the perinatal period due to the production of T-lymphocytes that will populate the offspring's lymphoid tissues; once a population of T-cells has been established, the thymic compartment shrinks (Janeway et al., 2004). The increase in thymus mass and differences in qualitative TNF-a expression in these groups indicates the presence of developmental immune priming; if assumed to be an adaptive response, maternal signals may communicate the need for upregulation of the immune system in a pathogen-heavy environment in order to calibrate offspring physiology to the environment they are likely to face as adults (i.e., a predictive adaptive response; Bateson et al., 2014; Gluckman et al., 2007)

The vertebrate stress response, as mediated by the hypothalamic-pituitary-adrenal (HPA) axis is highly conserved and has pervasive effects throughout the organism. The reciprocal relationship between the glucocorticoid-mediated stress response and the immune system has been well-documented. For example, about 20% of the human leukocyte transcriptome is affected by glucocorticoids (Galon et al., 2002), and almost all tissues in the body have a glucocorticoid receptor (Ballard et al., 1974). The two subtypes of glucocorticoid-binding receptors have different implications on the organismal stress response. The glucocorticoid receptor (GR) has a lower affinity for glucocorticoids and is associated with playing a regulatory role when glucocorticoid levels are high, such as in the acute stress response; in contrast, the mineralocorticoid receptor (MR) is implicated in the diurnal basal fluctuation in glucocorticoids (Joels and DeKloet 1992). Our results suggest that maternal immune challenge during lactation increases GR expression in the liver, and that female offspring may be more vulnerable to these effects. Previous work has found that hepatic GR signaling impacts the sex-specific differences that occur during inflammatory processes (Quinn and Cidlowski, 2015), further emphasizing the importance of GR in regulating inflammation. Aside from the stress-related effects of glucocorticoid receptors, they are also implicated in regulating metabolic pathways and gluconeogenesis, particularly in the liver (Picard et al., 2014). Maternal glucocorticoid exposure in late gestation in rats has been found to also increase hepatic GR expression and alter the individual's metabolism by increasing gluconeogenesis during exposure to corticosterone (Nyirenda et al., 1998).

Metabolic rate plays a key role in mediating life-history trade-offs, as it determines the amount of endogenous resources or energy that can be allocated among

competing processes, thereby potentially constraining the degree of phenotypic plasticity an individual is capable of achieving (Ricklefs and Wikelski, 2002). In addition to the regulatory role of glucocorticoids on metabolism, the somatotropic axis acts to regulate both growth and metabolism. Growth hormone (GH), also known as somatotropin is a tissue-specific mitogen that stimulates cellular reproduction and regeneration in cells with the appropriate GH receptor (Brooks and Waters, 2010). Our results indicated that with lactating-only mice, immune challenge decreased hepatic GHr expression, giving a possible mechanistic link behind the observed decrease in body mass at weaning. GHr expression mediates individual's responsiveness to the hormone, and a decrease in GHr expression restricts the growth rate for a given tissue (van Kerkhof et al. 2003). GH can also exert indirect effects on metabolism via its interactions with IGF-1, as the majority of the effects associated with GH occur primarily as a result of this mediating hormone that is secreted in response to GH (Bartke, 2005).

Immune challenge itself did not seem to impact hepatic IGF-1r expression, as no significant difference between the control-lactating and the immune-challenged lactating group was found. However, when comparing the two immune-challenged groups, offspring belonging to mothers who were concurrently lactating and pregnant had significantly reduced expression. This result indicates that the decrease in IGF-1r in these animals is likely due intrinsically to fluctuations in the maternal hormonal milieu needed to support pregnancy. Indeed, maternal serum IGF-1 concentrations increases during gestation, and a decrease in lactating offsprings' expression may occur as a compensatory response (Merimee et al., 1984).

Our results demonstrate that immune challenge during lactation does not necessarily impose a cost on offspring quality. Rather, immune challenge during lactation may prime offspring immune defenses, potentially conferring an adaptive advantage in offspring. Specifically, we demonstrated that a single maternal antigenic immune challenge during lactation has the potential to alter offspring development and physiology. Given that developmental programming is hypothesized to confer an adaptive advantage in offspring if there is environmental matching between the anticipated adult environment and the actual environment (Bateson et al., 2014; Gluckman et al., 2007), follow up experiments should directly test whether offspring have fitness benefits as a result of these changes.

Table 1. qPCR Primer Sequences for Hepatic Gene Expression. Primer sequences used to measure hepatic gene expression in developing house mice are given below.

Gene	Function	Nucleotide Sequence (5' to 3')	Source
GADPH	Housekeeping (control) gene; catalyzing enzyme used in glycolysis	F: CGGCCGCATCTTCTTGTG R: GTGACCAGGCGCCCAATAC	(Labaka et al. 2017)
Interleukin-1β (IL-1β)	Pro-inflammatory cytokine	F: TTGACGGACCCCAAAAGATG R: AGAAGGTGCTCATGTCCTCA	(Layé et al. 1994; Cribb et al. 2017)
Interleukin-6 (IL-6)	Pro-inflammatory cytokine	F: GTTC TCTGGGAAATCGTGGA R: TGTACTC CAGGTAGCTATGG	Layé et al. 1994
Tumor Necrosis Factor α (TNFα)	Pro-inflammatory cytokine	F: TCTCATCAGTTCTATGGCC R: CGGGAGTAGACAAGGTACAAC	Layé et al. 1994

Glucocorticoid Receptor (GR)	Low-affinity intracellular glucocorticoid receptor; implicated in the mammalian stress response (Lattin et al. 2012)	F: CCCATGGAGGTAGCGATTGT R: TGTAAAGGCTGCCCAATGTGT	Labaka et al. 2017
Mineralocorticoid Receptor (MR)	High-affinity intracellular glucocorticoid receptor; implicated in basal diurnal fluctuations in glucocorticoids (Lattin et al. 2012)	F: ACCTGCAGAGAGGACCAATGA R: GGAGTAATTCGTGTTTTTCTTTGCT	Labaka et al. 2017

Insulin-like Growth Factor 1 receptor (IGF-1r)		F: TTGTGTTGTTCGTCCGGTGTG R: ATCTCCAACCCAGGGCAAAT	(Hjortebjerg et al. 2017)
Growth Hormone receptor (GHr)		F: CCACCCAATGCAGATGTTCT R: CTGGATATCTTCTTCACATGCTTCC	Hjortebjerg et al. 2017

Table 2. Significance of variables in ANOVA analyses. Significance was established at $p \leq 0.05$, and differences between groups found to be significant after post-hoc analysis with Tukey's HSD are denoted by an asterisk.

	Mean +/- SE							Comparisons		
	PBS+L	KLH+L	KLH+PL	Term	d.f.	F	P	PBS+L v. KLH+L	PBS+L v. KLH+PL	KLH+L v. KLH+PL
Offspring Mass (g)										
PND2 mass (unadjusted; g)	2.06 ± 0.06 (16)	1.99 ± 0.04 (16)	2.00 ± 0.04 (15)		2, 44	0.43	0.65	n.s.	n.s.	n.s.
PND2 mass (adjusted; g)	2.05 ± 0.06 (16)	2.00 ± 0.04 (16)	2.03 ± 0.05 (15)		2, 44	0.18	0.83	n.s.	n.s.	n.s.
PND12 (g)	9.36 ± 0.21 (16)	9.23 ± 0.14 (16)	9.06 ±0.15 (15)		2, 44	0.78	0.46	n.s.	n.s.	n.s.
PND21 (g)	16.28 ± 0.31 (16)	16.14± 0.30 (16)	16.11 ± 0.24 (14)	Treatment	2, 344	0.57	0.56	n.s.	n.s.	n.s.
				Sex	1, 344	0.32	0.57			
				Interaction	2, 344	12.72	<0.01*			
Relative Organ masses (mg/g)										

Brain	26 ± 0.4 (47)	25 ± 0.3 (57)	24 ± 0.5 (50)	Treatment	2, 138	1.95	0.15	n.s.	n.s.	n.s.
				Sex	1, 138	0.32	0.99			
				Interaction	2, 138	0.46	0.46			
Heart (mg/g)	6.1 ± 0.3 (47)	6.3 ± 0.2 (57)	6.5 ± 0.2 (50)	Treatment	2, 137	0.69	0.50	n.s.	n.s.	n.s.
				Sex	1, 137	1.63	0.21			
				Interaction	2, 137	0.20	0.82			
Thymus (mg/g)	5.8 ± 0.2 (40)	6.8 ± 0.2 (57)	6.9 ± 0.3 (40)	Treatment	2, 131	4.96	<0.01*	*	*	n.s.
				Sex	1, 131	6.02	0.01*			
				Interaction	2, 131	0.65	0.53			
Spleen (mg/g)	6.8 ± 0.4 (46)	8.9 ± 0.6 (57)	9.2 ± 0.7 (40)	Treatment	2,137	5.10	<0.01*	*	*	n.s.
				Sex	1, 137	1.35	0.24			
				Interaction	2, 137	0.18	0.84			

Kidneys (mg/g)	10 ± 0.2 (45)	14 ± 0.4 (57)	14 ± 0.3 (40)	Treatment	2, 136	0.48	0.62	n.s.	n.s.	n.s.
				Sex	1, 136	0.38	0.54			
				Interaction	2, 136	1.57	0.21			
Liver (mg/g)	51 ± 1 (46)	50 ± 0.7 (57)	56 ± 0.9 (38)	Treatment	2, 135	10.03	<0.01	n.s.	*	*
				Sex	1, 135	0.01	0.91			
				Interaction	2, 135	0.09	0.92			
Gene Expression (relative to GADPH)										
IL-1β	0.009 ± 0.001 (24)	0.021 ± 0.003 (30)	0.017 ± 0.03 (14)	Treatment	2, 31	4.90	*0.01**	* *(0.01)*	* (0.02)	n.s. (0.64)
				Sex	1, 31	0.10	0.75			
				Interaction	2, 31	0.02	0.98			
				Mother ID	31, 31	1.42	0.17			
IL-6	0.009 ± 0.001 (24)	0.012 ± 0.0022 (30)	0.014 ± 0.004 (14)	Treatment	2, 31	1.06	0.36	n.s.	n.s.	n.s.

				Sex	1, 31	0.22	0.64			
				Interaction	2, 31	1.45	0.25			
				Mother ID	31, 31	1.15	0.35			
TNFα	0.00004±0.00002 (24)	0.013 ± 0.003 (30)	0.012 ± 0.006 (14)	Treatment	2, 31	3.32	0.05*	*	n.s.	n.s.
				Sex	1, 31	3.44	0.07			
				Interaction	2, 31	0.98	0.39			
				Mother ID	31, 31	5.64	<0.001*			
GR	0.45 ± 0.036 (24)	1.04 ± 0.046 (30)	0.94 ± 0.039 (14)	Treatment	2, 31	35.23	<0.001*	**	**	n.s.
				Sex	1, 31	13.81	<0.001*			
				Interaction	2, 31	0.33	0.73			
				Mother ID	31, 31	6.01	<0.001*			
MR	0.16 ± 0.021 (24)	0.11 ± 0.013 (30)	0.20 ± 0.037 (14)	Treatment	2, 31	2.96	0.07	n.s.	n.s.	n.s.

				Sex	1, 31	3.65	0.065			
				Interaction	2, 31	2.55	0.09			
				Mother ID	31, 31	1.46	0.15			
GHr	0.16 ± 0.022 (24)	0.09 ± 0.011 (28)	0.11 ± 0.013 (14)	Treatment	2, 29	3.81	0.03*	*	n.s.	n.s.
				Sex	1, 29	1.34	0.26			
				Interaction	2, 29	3.01	0.07			
				Mother ID	29, 29	2.05	0.03*			
IGF-1r	0.027 ± 0.004 (24)	0.037 ± 0.005 (28)	0.019 ± 0.004 (14)	Treatment	2, 55	3.20	0.05*	n.s.	n.s.	*
				Sex	1, 55	<0.01	0.98			
				Interaction	2, 55	0.64	0.53			

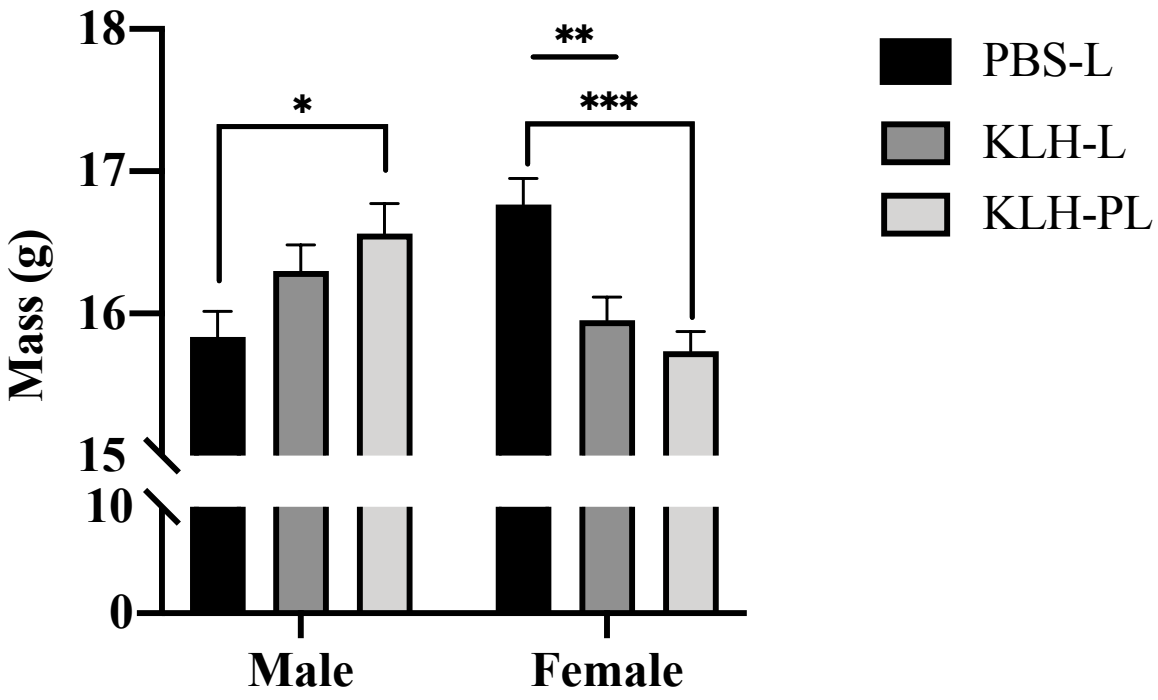

Figure 1. Offspring Body Mass at Weaning (PND21). Males are represented in black bars, while females are given in grey bars. Error bars are given as the standard error. "∗ = $p < 0.1$, ∗∗ = $p < 0.05$, ∗ ∗ ∗ = $p < 0.01$.

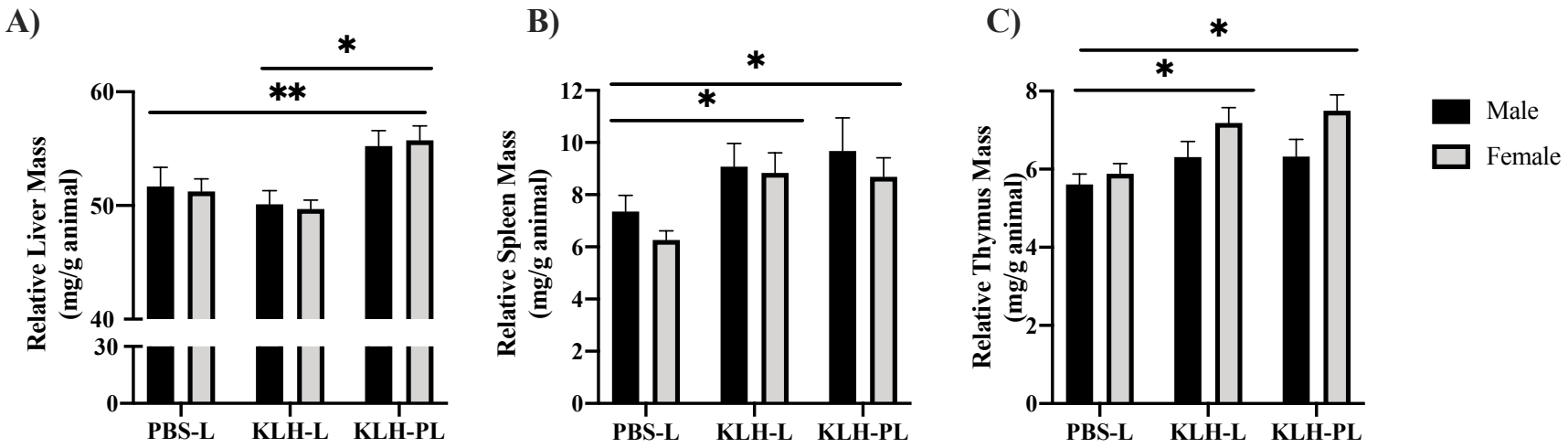

Figure 2. Relative A) Liver, B) Spleen, and C) Thymus masses (mg organ/g animal) in male and female offspring across treatments. Males are represented in black bars, while females are given in grey bars. Error bars are given as the standard error. "∗ = p < 0.1, ∗∗ = p < 0.05, ∗ ∗ ∗ = p < 0.01.

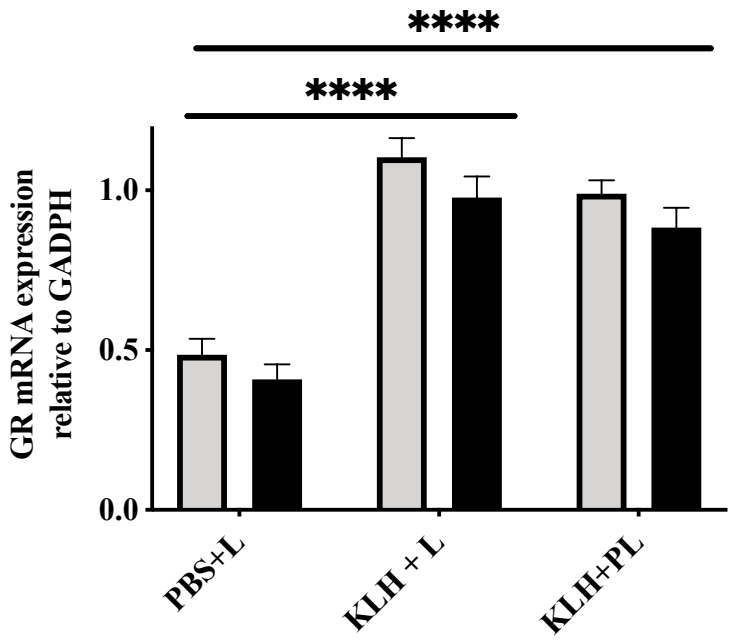

Figure 3. Hepatic Glucocorticoid Receptor (GR) expression relative to GADPH in male and female offspring across treatment groups. Males are represented in black bars, while females are given in grey bars. Error bars are given as the standard error. "∗ = $p < 0.1$, ∗∗ = $p < 0.05$, ∗ ∗ ∗ = $p < 0.01$, ∗ ∗ ∗ ∗ = $p \leq 0.0001$.

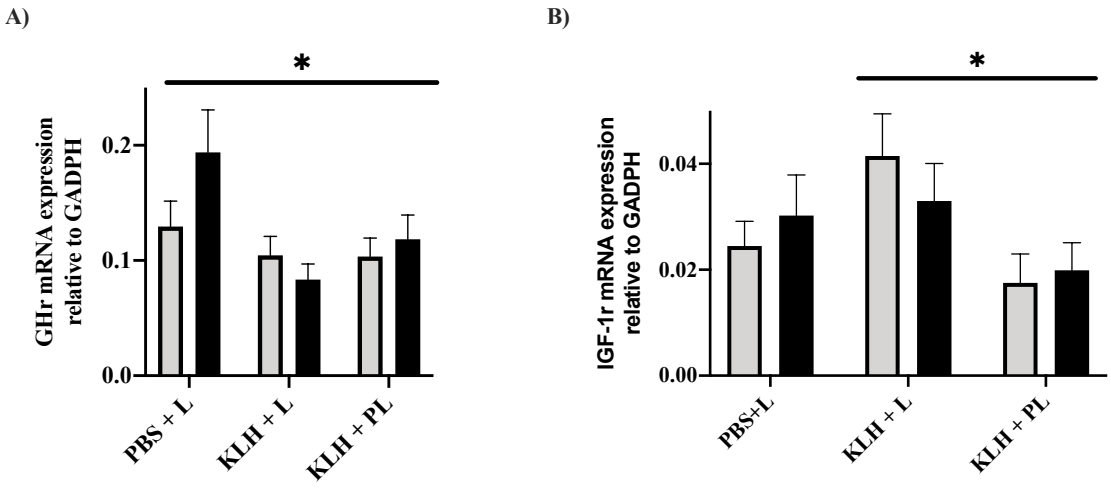

Figure 4. Hepatic A) Growth Hormone receptor (GHr) and B) IGF-1 receptor expression relative to GADPH in male and female offspring across treatment groups. Males are represented in black bars, while females are given in grey bars. Error bars are given as the standard error. "$*$ = $p < 0.1$, $**$ = $p < 0.05$, $***$ = $p < 0.01$

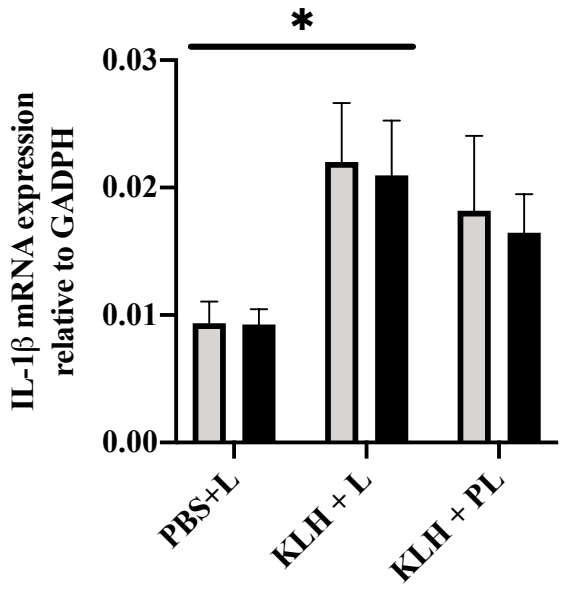

Figure 6. Hepatic IL-1β expression relative to GADPH in male and female offspring across treatment groups. Males are represented in black bars, while females are given in grey bars. Error bars are given as the standard error. "∗ = $p < 0.1$, ∗∗ = $p < 0.05$, ∗ ∗ ∗ = $p < 0.01$

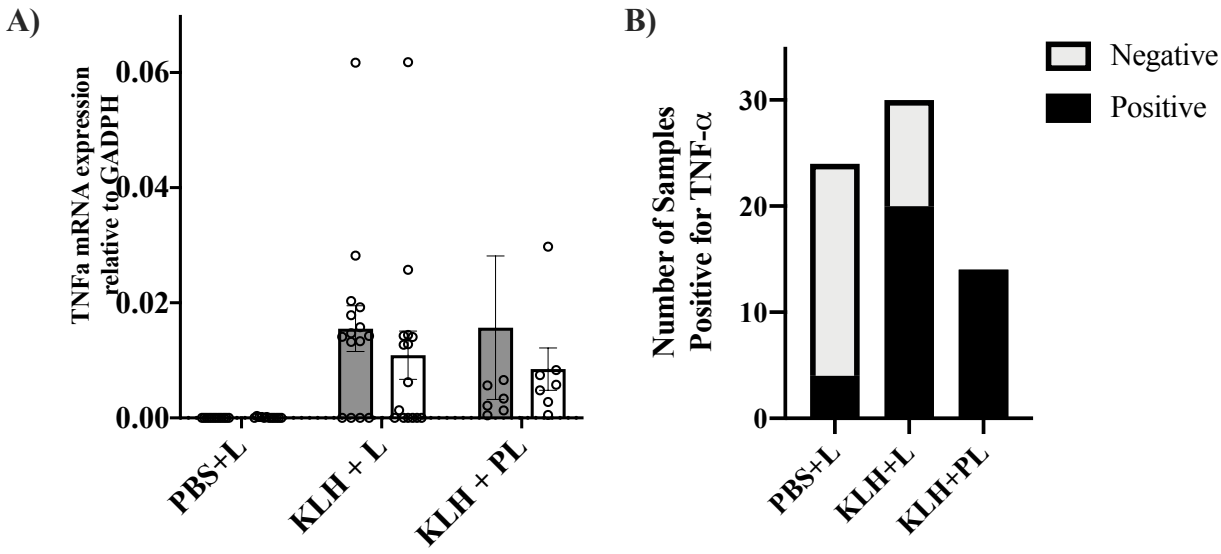

Figure 6. Quantitative (A) and qualitative (B) differences in hepatic TNFα gene expression. Results on gene expression relative to GADPH are given in A, along with the distribution of data. Our results showed a dichotomous response (B) on whether we were able to detect any TNFα mRNA in the samples, which we found to be significant.

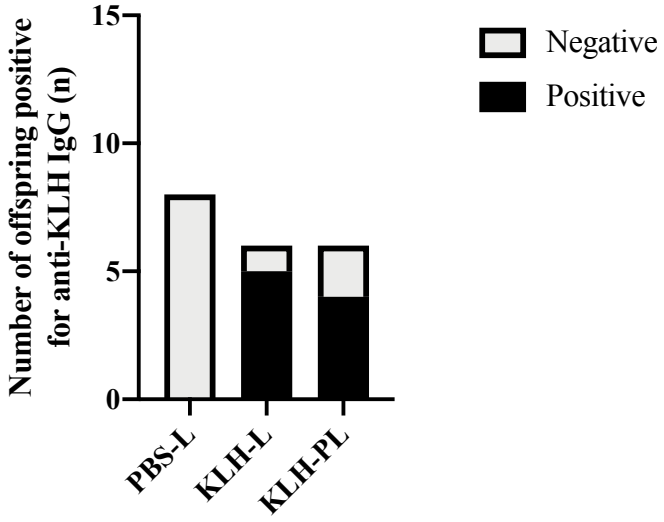

Figure 7. Proportion of samples positive for anti-KLH IgG after maternal treatment with KLH. We found a significant difference between groups in the proportion of offspring with detectable specific antibodies against KLH. These results demonstrate the passive transfer of maternal antibodies though milk following immune challenge with KLH.

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