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SYMPOSIUM

Methodological Considerations for Assessing Immune Defense in Reproductive Females

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Synopsis One of the key foci of ecoimmunology is understanding the physiological interactions between reproduction and immune defense. To assess an immune challenge, investigators typically measure an immune response at a predetermined time point that was selected to represent a peak response. These time points often are based on the immunological responses of nonreproductive males. Problematically, these peaks have been applied to studies quantifying immune responses of females during reproduction, despite the fact that nonreproductive males and reproductive females display fundamentally different patterns of energy expenditure. Previous work within pharmacological research has reported that the response to the commonly-used antigen keyhole limpet hemocyanin (KLH) varies among individuals and between females and males. In this heuristic analysis, we characterize antibody responses to KLH in females with varying reproductive demands (nonreproductive, lactating, concurrently lactating, and pregnant). Serum was taken from one animal per day per group and assessed for general and specific Immunoglobulins (Igs) G and M. We then used regression analysis to characterize the antibody response curves across groups. Our results demonstrate that the antibody response curve is asynchronous among females with varying maternal demands and temporally differs from the anticipated peak responses reflected in standardized protocols. 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.

Introduction

The expanding discipline of ecological immunology (or ecoimmunology) broadly aims to answer questions pertaining to the mechanistic, ecological, and evolutionary causes, as well as the consequences of natural variation in immune defense (Sheldon and Verhulst 1996; Demas and Nelson 2012). Of interest to many ecoimmunologists is the relationship between reproductive performance and immune defense, as developing, maintaining, and deploying a competent immune system is demanding and can exact fitness costs on the individual (Lochmiller and Deerenberg 2000; Bonneaud et al. 2003). Trade-offs between reproductive demand and immune defense have been observed across different taxa (reviewed in Klein 2000; Demas et al. 2012), though the ubiquity of such trade-offs remains unclear.

Many studies have documented interactions between reproductive effort and immune defense in birds (Deerenberg et al. 1997; Ilmonen et al. 2003; Ardia 2005), reptiles (French and Moore 2008; Cox et al. 2010), insects (Adamo et al. 2001), and mammals (Demas et al. 1997; Drazen et al. 2003; French et al. 2013). Yet, our understanding of how reproductive demand impacts immune defense of female mammals remains limited. Individual variation in maternal reproductive strategies is hypothesized to be shaped proximately by trade-offs among competing physiological demands and evolve through

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selection pressures on both the female and her offspring (Mousseau and Fox 1998). Thus, the enormous energetic demand associated with reproduction in females has long been assumed to impose both proximate physiological (e.g., depressed immune function) and ultimate costs (e.g., to future fecundity and/or survival) (Fisher 1930; Williams 1966; Trivers 1972; Reznick 1992). Of the scarce literature examining this topic in female mammals, investigations have focused on pregnant females (Xu et al. 2012) that have substantially lower energetic and nutritive demands during this phase than during lactation in most species (Gittleman and Thompson 1988; Speakman 2008, but see Drazen et al. 2003). Importantly, the relative concentration of nutrients and other bioactive factors in milk, including immunoglobulins (Igs), is reflective of the physiological consequences of balancing simultaneous physiological demands. As a result, milk plays a formative role in neonatal immune development, as the majority of maternal antibody transfer occurs via milk rather than *in utero* (Power and Schulkin 2016). Igs impart the mother's infection history via her antibody repertoire on to her offspring, allowing them to more effectively clear pathogens during this period (Boulinier and Staszewski 2008).

Paramount to the creation of a cohesive synthesis on the nature of reproductive-immune interactions in females is the use of appropriate experimental designs and methodologies that reliably reflect the proximate physiological changes thought to underlie these interactions. Experimental techniques for measuring immunity are often dependent on the administration of commonly-used immune challenges and the quantification of the resulting immune response after a standardized time lapse (Demas et al. 2011). Studies employing such techniques implicitly assume synchronous immune responses across individuals and between females and males. Recent work has challenged this assumption and demonstrated the importance of integrating the time progression of the immune response into future investigations (Zamora-Camacho 2019). Repeated sampling may be particularly important in studies evaluating the immune response in reproductive individuals, since patterns of energy use and physiological processes supporting reproduction differ dramatically between females and males (Hayssen and Orr 2017) and may, therefore, impact the progression of an immune response. Adopting inappropriate sampling times can obscure the true nature of the relationship between immunity and reproduction, leading to equivocal results and further complicating the creation of a cohesive framework.

To illustrate this point, we focus on the use of keyhole limpet hemocyanin (KLH) due its established impact on the energetic demands of an individual (Demas et al. 1997), common use in immunological studies due to its immunostimulatory properties (Lebrec et al. 2014), and the broad commercial availability of both the antigen and assay kits for quantifying specific anti-KLH antibodies. Although pharmacological studies using KLH commonly include longitudinal samples to characterize the antibody response (for review, see Lebrec et al. 2014), repeated sampling methods are rare within the field of ecoimmunology (but see Martin et al. 2007) and the importance of the temporal progression of the response has received little attention. Hence, direct comparisons between groups of varying reproductive demands are often based on antibody titers at one time point. Problematically, the predominant protocol for measuring the antibody response to KLH is based on data collected from nonreproductive male mice (Dixon et al. 1967; Demas et al. 1997) and may therefore not be reflective of the progression of the antibody response in females. In this study, we characterized antibody responses to KLH over time in female mice of varying reproductive demands (nonreproductive, lactating, and simultaneously lactating and pregnant). Our goal was to challenge the assumption that individuals have synchronous antibody responses, regardless of the reproductive stage, and to understand how reproductive stage itself can impact the progression of the antibody response. We predicted that reproductive stage would temporally alter the antibody response to KLH, and that the timing of the peak antibody response would differ from time points used in standard protocols.

Methods

Animals and husbandry

All husbandry and experimental procedures were carried out between October and December 2017 under the approval of the Auburn University Institutional Animal Care and Use Committee (PRN 2017-3167). Eighty-two (64 female and 18 male) adult outbred ICR mice (Envigo, Prattville, AL) were used in this investigation; all animals were obtained at $\geq$35 g mass and 4–7 weeks of age and had no prior breeding experience. Upon arrival at our facility, individuals were allowed 48 h to acclimatize before handling. Mice were housed in standard polypropylene rodent boxes ($\sim$29.2 $\times$ 19.0 $\times$ 12.7 cm^3) on a 12:12 light:dark cycle at 24°C and given *ad libitum* access to standard rodent chow and

water. Females assigned to reproductive groups were housed with a single male; non-reproductive females were housed in pairs.

Experimental design

Females were equally and randomly divided into four groups ($n = 16$ for each) based on their reproductive demand and immune challenge: (1) control (phosphate buffered saline [PBS]) and lactating (Control-L); (2) immune-challenged and nonreproductive (Immune-NR); (3) immune-challenged and lactating (Immune-L); and (4) immune-challenged and concurrently lactating and pregnant (Immune-PL). Females were monitored and checked daily for evidence of breeding. Males housed with females in the lactating-only groups (Control-L and Immune-L) were housed with the female for 14 days after pairing. Males housed with females in the concurrently gestating and lactating group (Immune-PL) were removed 2 days after the birth of the first litter, as to allow for mating during post-partum estrus. Litter sizes were standardized to 8 pups/L the day after the birth of the mother's first litter, on post-natal day (PND) 2, with the date of birth identified as PND 1. In three cases, females had <8 pups. Grubb's tests were run to identify whether data from these individuals represented significant outliers. No outliers were identified and thus, we retained the data from these animals in all analyses. Offspring were housed with their mother until weaning at PND 21.

Females in the immune-challenged groups received a single 100 µL intrascapular subcutaneous injection of KLH (Cat. no. H7017, Sigma-Aldrich, St Louis, MO, USA) suspended in sterile, pyrogen-free PBS at a dose of 150 µg KLH/mouse (Dixon et al. 1967; Demas et al. 1997). Females in the Control-L group were given a PBS vehicle of the same volume. KLH is a commonly-used, nonreplicating antigen that induces a mild immune response and increases metabolic rate without causing anorexia, fever, inflammation, or sickness behavior (Dixon et al. 1967). To minimize stress, all females were injected when litter sizes were adjusted on PND2 (for females within the Immune-PL group, PND2 refers to the day after parturition of their first litter). This timing was selected so that the hypothesized peak maternal antigen titers occurred at or before peak lactation so that both maternal reproductive demand and maternal immune demands were highest (approximately PND 14 for this species; Knight et al. 1986).

Serum collection and antibody response

Animals in each group 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). Sampling started 5 days post-injection in order to minimize stress and limit cannibalism. Individuals were sampled only once during this experiment, and all samples were collected between 12:00 and 16:00. Blood samples were obtained from the submandibular facial vein using a 5.5 mm lancet and collected into microcentrifuge tube. No more than a total of 100 uL of blood, or 10% of total blood volume, was collected from the animal (Hoff 2000). After sample collection, blood was allowed to clot at room temperature for 30–45 min, after which it was centrifuged, and serum was collected. The serum was stored at −80°C until the samples were processed ~4–5 months later.

Serum concentrations for four Igs (i.e., nonspecific IgM, specific [anti-KLH] IgM, nonspecific IgG, and specific [anti-KLH] IgG) were quantified. IgM is the predominant immunoglobulin class in the initial primary antibody response, whereas IgG is the most abundant and predominates during the later phases of the primary antibody response to an antigen (Janeway et al. 2004). Based on methods common in previous literature (Dixon et al. 1967; Demas et al. 1997; Drazen et al. 2003; Martin et al. 2007; Xu et al. 2012) as well as the manufacturer's instructions, anti-KLH titers are anticipated to peak at 5 (anti-KLH IgM) and 14 (anti-KLH IgG) days after injection with KLH. Total and anti-KLH IgM and IgG were measured using enzyme-linked immunosorbent assays obtained from Life Diagnostics (West Chester, PA, USA), following the manufacturer's instructions. All samples within each assay were run at the same dilution and were run in duplicate. Plates were read at 450 nm using a BioTek PowerWave XS plate reader. Each assay was validated using a serial dilution of pooled samples. Inter- and intra-assay variations were calculated to be <15%.

Statistical analyses

Statistical analyses and graphs were completed using R (R Core Team 2013) and GraphPad PRISM version 8.0 (GraphPad Software, San Diego, CA, USA) software. Antibody responses for each of the non-specific (total IgM and IgG) and specific (anti-KLH IgM and IgG) antibodies were modeled using the *a priori*

Table 1 Best-fit regression equations and relevant parameters

	Total IgM	Total IgG	Anti-KLH IgM	Anti-KLH-IgG
Regression equations				
Control-L	$y = -13.9 + 5.2x - 0.2x^2$	$y = 35.4 - 4.5x + 0.2x^2$	$y = 1.7 - 0.03x + 0.01x^2$	$y = 0.3 - 0.03x + 0.01x^2$
Immune-NR	$y = 84.3 - 6.2x + 0.1x^2$	$y = -104.4 + 30.3x - 1.2x^2$	$y = 28.2 - 1.8x + 0.03x^2$	$y = -1.3 + 0.25x - 0.01x^2$
Immune-L	$y = 47.8 - 0.8x - 0.04x^2$	$y = -17.1 + 8.9x - 0.4x^2$	$y = -9.81 + 4.71x - 0.22x^2$	$y = 0.6 - 0.1x + 0.01x^2$
Immune-PL	$y = 40.4 - 0.18x - 0.08x^2$	$y = -28.2 + 6.2x - 0.2x^2$	$y = -7.89 + 3.33x - 0.14\ x^2$	$y = -0.2 + 0.1x - 0.1x^2$
R^2 values				
Control-L	0.71	0.31	0.36	0.24
Immune-NR	0.86	0.70	0.44	0.33
Immune-L	0.80	0.39	0.76	0.14
Immune-PL	0.50	0.67	0.40	0.12
Time of peak response (days)				
Control-L	12.0	5.0	17.2	5.0
Immune-NR	5.0	11.9	6.9	16.2
Immune-L	5.0	12.6	10.5	19.8
Immune-PL	8.8	16.0	10.5	15.2
Titer maximum				
Control-L	17.8	21.3	2.8	0.11
Immune-NR	53.6	82.6	17.5	2.26
Immune-L	41.2	38.4	19.1	1.02
Immune-PL	39.1	33.0	12.9	0.70
Cumulative response (AUC)				
Control-L	195	124	32	0.88
Immune-NR	474	850	153	16.80
Immune-L	468	461	195	7.75
Immune-PL	377	305	135	7.34

Best-fit equations (Fig. 1) differed among treatment groups for each of the antibodies measured in this study. To characterize each pattern, the data were fit with splines and estimates for the timing of the peak antibody response, magnitude of that response, and the cumulative response (as estimated by the area under the curve) were taken. Values for maximum titer concentrations are given in (ng/μL) for total IgM and IgG and (arbitrary units) for specific IgM and IgG.

NB: It is possible that the actual peak times of these immunoglobulins occurred before our first sample time, which was 5 days after the females were injected with KLH.

hypotheses that the relationship between time and the serum concentration of each antibody would be quadratic and that the best fit equation would differ among the experimental groups. Both of these hypotheses were tested by comparing differences in Akaike's Information Criterion (AICc) between the hypothetical models and alternative models. The hypothesis that the antibody response to KLH over time would be quadratic (Siegel et al. 1984) was tested by comparing AICc values of using quadratic versus linear equations to describe antibody titer concentration over time for each treatment group. In 10/16 cases, the models were found to be significantly improved by using a quadratic function (i.e., ΔAICc was <2) rather than a linear function (Mazerolle 2006). Four of these models described the relationship between anti-KLH IgG titers over time, and thus, linear models were used (Table 1). Excluding these models, no models were found to have a ΔAICc of less than -2, which would suggest a strong preference for the linear model. Thus, for consistency, quadratic equations were used to describe the remainder of the relationships (Table 1). To understand the effect of reproductive status on the antibody response curves, AICc values were compared between global (i.e., one curve for all included treatment groups) and more parameterized (i.e., different curves for included treatment groups) models (Table 2). For all four antibodies, models were significantly improved by using different equations for each treatment group rather than one global model. To understand which treatment groups specifically differed from one another, we partitioned data based on treatment groups and compared the goodness-of-fit for global and

Table 2 Comparisons of models using different parameters

	Global AICc	Parameterized AICc	ΔAICc	Interpretation
IgM				
C-L + I-NR + I-L + I-PL	313.9	260.0	53.9	Different
I-NR + I-L + I-PL	205.1	202.9	2.2	Different
I-NR + I-L	116.1	114.0	2.1	Different
I-NR + I-PL	147.9	145.8	2.1	Different
I-L + I-PL	142.1	142.2	−0.1	Global
IgG				
C-L + I-NR + I-L + I-PL	406.1	327.3	78.8	Different
I-NR + I-L + I-PL	300.3	256.8	43.5	Different
I-NR + I-L	198.9	180.9	18	Different
I-NR + I-PL	212.9	178.1	34.8	Different
I-L + I-PL	161.7	151.1	10.6	Different
Anti-KLH IgM				
C-L + I-NR + I-L + I-PL	239.9	202.5	37.4	Different
I-NR + I-L + I-PL	168.6	166.3	2.3	Different
I-NR + I-L	118.1	117.3	0.8	Global
I-NR + I-PL	120.6	120.7	−0.1	Global
I-L + I-PL	95.43	91.8	3.6	Different
Anti-KLH IgG				
C-L + I-NR + I-L + I-PL	−35.66	−47.15	11.49	Different
I-NR + I-L + I-PL	−23.4	−29.2	5.8	Different
I-NR + I-L	−4.6	−7.3	2.7	Different
I-NR + I-PL	−5.1	−9.7	4.6	Different
I-L + I-PL	−64.5	−59.4	−5.1	Global

AICc values are given for the best-fit quadratic models explaining the relationship between time and antibody titer for different combinations of treatment groups. AICc values are given for global quadratic models (i.e., one curve for all included groups) and parameterized models (i.e., different curves for each included group) to describe the relationship between antibody titer and time. Each row represents a separate comparison and compares a different subset of data, as data were partitioned based on treatment group. Thus, each row includes data for the given treatment groups and compares the goodness-of-fit (ΔAICc) for models using a single function versus separate functions to describe the relationships between titer and time. A ΔAICc value with an absolute value >2 indicates a strong preference for a given model; values >2 indicate a strong preference for the more complex model (i.e., the parameterized model), whereas those less than −2 indicate a strong preference for the simpler global model. In cases where there was no indication of a strong preference, the simpler (global) model was chosen as a default.

parameterized models for different combinations of treatment groups. To elucidate more specific trends within the data that may be obscured in the regression, data were fit using smoothing splines with four knots and 64 segments. Using these curves, the timing and magnitude of the peak antibody responses were interpolated. Similar to Martin et al. (2007), we estimated the cumulative response for each antibody by calculating the total area under the regression curves using the integral of each equation.

Results

Model selection using differences in AICc values supported differences between treatment groups for all antibodies measured (Table 2). The best-fit quadratic equations for each group's antibody response over time are given in Table 1. The immunogenicity of KLH was confirmed by an increase in titers of all four antibodies in the immune-challenged groups relative to the control.

Our results suggest that reproduction impacts IgM titer over time. Goodness-of-fit comparisons between global and more parameterized models (Table 2) demonstrated that IgM titers over time in the non-reproductive group have a different best-fit curve than either of the immune-challenged reproductive groups (L and PL). Goodness-of-fit was not improved by using different curves for the reproductive groups (L and PL), suggesting that the total IgM

response to KLH does not differ as a result of increased reproductive effort. During the early phases of the response to KLH, when IgM is expected to predominate (Boes 2000), nonspecific IgM appeared to be higher (i.e., no overlap in 95% CI on statistical models) in mice belonging to immune-challenged groups than those in the control group (Fig. 1A and B). Among the immune-challenged groups, the maximum titer and cumulative non-specific IgM appeared to decrease with increasing reproductive demand (i.e., NR > L > PL) (Fig. 1A and B; Table 1). While serum anti-KLH IgM titers have been assumed to peak 5 days after immunization with KLH (Dixon et al. 1967), anti-KLH IgM titers at 5 days post-immunization were not significantly increased by KLH challenge (i.e., there was no difference between the Control-Lactating and the Immune-Lactating groups) (Fig. 1C). Instead, it appears that lactation delays when anti-KLH IgM peaks in circulation (Fig. 1C and D; Table 1), though we cannot rule out the possibility that the actual peak response occurred prior to 5 days post-injection. We also saw a trend suggesting that reproduction dampens the specific IgM response to KLH. Among the immune-challenged groups, both the maximum titer value and the cumulative anti-KLH IgM response is greatest in the lactating-only group, followed by the non-reproductive group and then the pregnant and lactating groups (i.e., L > NR > PL) (Table 1).

Both reproductive status and immune challenge significantly impacted total IgG concentrations over time (Tables 1 and 2). Goodness-of-fit was significantly improved in more parameterized models, suggesting that a model using different curves for each treatment group is better fit than models that combine treatment groups (i.e., reproductive status impacts total IgG titers; Table 2). Upon visual inspection of the resulting models, these differences are most evident during intermediate timepoints, when total IgG appears to be increased in all immune-challenged groups relative to the control (Fig. 1E, F) based on the 95% CI of the regression curves. Among the immune-challenged groups, non-reproductive animals had higher total IgG than the two reproductive groups. At Day 14 post-injection, when specific titers are expected to peak, total IgG was greater in the Immune-L group than the Immune-PL group; this difference is no longer present at D16 (PND14), when peak lactation for this species occurs (Fig. 1E and F). The timing and magnitude of peak concentrations of total IgG were different across groups, as was the area under the curve (Table 1). Among the immune challenged groups, reproductive status appeared to delay the peak of total IgG (Table 1). Our data suggest that non-reproductive individuals had greater peak concentrations and areas under the curve of total IgG. Between the Immune-L and Immune-PL group, a difference in both peak concentration and area under the curve was present, though it appears to be slight (Table 1; Fig. 1[E] and F).

Anti-KLH IgG is anticipated to peak at 14 days after immunization; at this time point, we did not find any evidence that the regression curves differed among the immune-challenged groups based on the overlap of their 95% CI (Fig. 1G and H). Comparisons of goodness-of-fit of global and parameterized models (Table 2) suggests that titers of anti-KLH IgG are impacted by reproduction (i.e., the best-fit curves for the reproductive and nonreproductive groups differ), but not by reproductive effort (i.e., the best-fit curves for the L and PL groups do not differ). At later timepoints, however, we observed a trend suggesting the Immune-NR group had increased titers relative to the Immune-L and Immune-PL groups, which did not differ from one another (Fig. 1G, H). Similar to our findings for anti-KLH IgM, titers for anti-KLH IgG peaked later than anticipated (Table 1). The cumulative response for anti-KLH IgG was increased in the Immune-NR group relative to the reproductive groups, which were similar to one another (Table 1).

Discussion

Despite the large demand of lactation (Gittleman and Thompson 1988) and importance of maternal antibodies to the survival of the immunologically naïve neonate (Boulinier and Staszewski 2008), relatively little is known about how lactation impacts the antibody-mediated immune defense. Much of the current literature investigating the relationship between reproductive effort and immune defense in mammals is conducted within the context of male reproduction. As a result, the mammalian female perspective (Orr et al. 2020) has not been fully integrated into theoretical works within ecoimmunology (but see Cox 2014). Our heuristic analysis suggests that the antibody response curve differs among individuals with varying maternal demands and that peak responses differ temporally from standard protocols for KLH (Dixon et al. 1967). Of the scarce literature exploring this topic in mammalian females, investigations have yielded contradictory results (Drazen et al. 2003; Xu et al. 2012), potentially due to only sampling at one standardized time point. Our results demonstrate that immune responses are

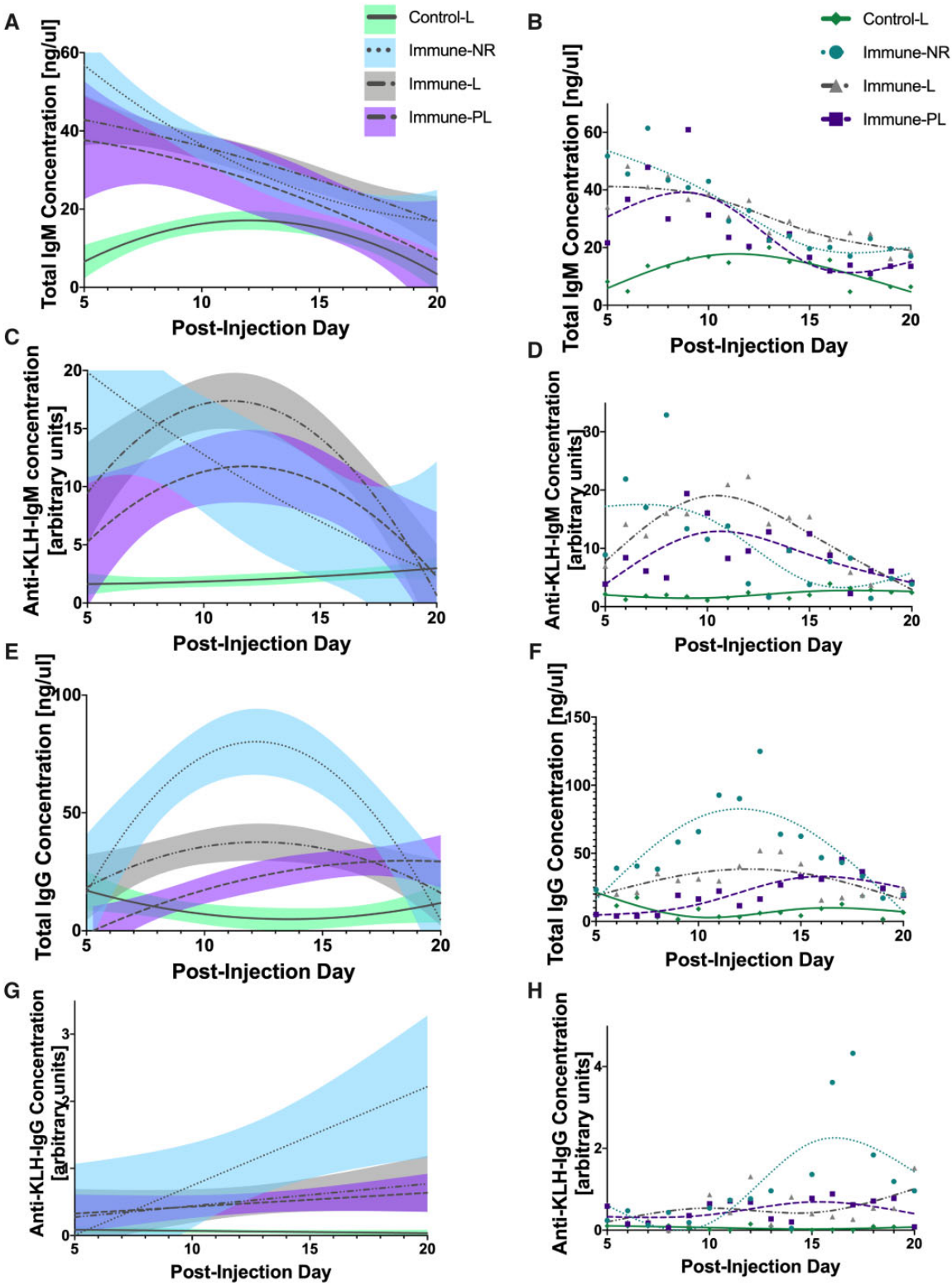

Fig. 1 Total (**A** and **B**) and specific (**C** and **D**) IgM and total (**E** and **F**) and specific (**G** and **H**) IgG responses in female mice with varying reproductive demands. Serum concentrations of antibodies over time were modeled using linear or quadratic regression (left panels; Table 1 for equations). Confidence bands are given for 95% CI. To more easily visualize and measure patterns that might otherwise be obscured by regression, cubic spline curves were generated and individual data points are shown (right panels).

variable along a gradient of reproductive demand. Importantly, this variation is great enough that had we sampled at only one time point, we would have completely lost the complexity of these relationships.

Our results suggest that reproduction attenuates the antibody response to immune challenge with KLH, and that this change is more apparent in IgG than in IgM. Previous studies utilizing KLH as an immune challenge have included measurements of either IgM or IgG, but rarely both (Bilbo and Nelson 2001; Drazen et al. 2003; Martin et al. 2007). IgM typically is found in low levels in circulation under normal conditions and increases during the early stages of an immune response to a novel antigen (Boes 2000). In contrast, IgG is only released from longer-lived plasma B-cells that confer immunological memory (Janeway et al. 2004). Functionally, clinical correlates suggest that the serum concentration of specific IgG, but not IgM, is important in determining the likelihood of protection upon subsequent exposures to an antigen after vaccination (Plotkin 2010). IgG is of particular importance during mammalian reproduction, as it can be transferred across the placenta and via milk through passive immune processes that confer protection in the immunologically naïve neonate (Boulinier and Staszewski 2008; Borghesi et al. 2014). Taken together, IgG therefore may be more informative for understanding the long-term functional implications of variation in antibody titer.

It may be tempting to interpret our findings (e.g., increased titer concentrations in non-reproductive females relative to reproductive females) as resulting from a trade-off between reproductive effort and immune defense. 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 or reproductive stage, and competing physiological demands (Viney et al. 2005). We, therefore, caution against assigning valence to our findings without investigating the functional impact of these differences on both the mother and her offspring. Proximately, the observed inverse relationship between titer values and reproductive stage may occur as a result of necessary physiological changes that accompany offspring production, as reproduction itself is an inflammatory process and requires restructuring of the immune system in order to sustain a pregnancy and passively transfer antibodies (Clancy 2013). Thus, an optimal immune defense strategy for reproductive females may be one that optimizes passive immune transfer while minimizing negative fitness effects. Shared proximate pathways linking immune defense and reproductive investment in males have been proposed (Hill 2011; Koch et al. 2017), though these hypotheses are framed within the context of sexual selection and maintenance of honest signals and cannot be generalized to include female physiological processes. Understanding the relationship between reproduction and immune defense in females, therefore, requires that we place female reproductive physiology at the center of such investigations because reproduction is central to all aspects of female physiology (Hayssen and Orr 2017).

Most importantly, our findings highlight the need for careful consideration during experimental design and interpretation of results. Our data demonstrate that the antibody-mediated response to KLH is asynchronous and differs based on reproductive stage. Further, we found that the actual peak responses for both IgM and IgG differed from the anticipated peak responses (Days 5 and 14 post-injection, respectively) used in standard protocols for KLH (Dixon et al. 1967; Demas et al. 1997; Martin et al. 2007). 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. Similar to findings from Zamora-Camacho (2019), our results provide evidence that time progression of an immune response is an important variable to measure and may provide valuable information beyond the magnitude of a response.

Conclusion and future directions

Here, we provide evidence that reproductive status temporally impacts the immune response to an antigenic immune challenge in females. 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 immune response. More comprehensive views can aid us in understanding the dynamic ways in which immune responses may change over reproductive stages, and help us parse out which, if any, aspects of the adaptive immune response may be linked to fitness.

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