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Natural Antibodies Quantitative and Qualitative Profiles as Biomarkers to Differentiate Free-Range and Conventional Broiler Chicks

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Abstract

Naturally occurring antibodies (NAbs) are reliable biomarkers of baseline humoral immunity in poultry raised under diverse production housing systems. To validate and extend prior observations, we compared quantitative and qualitative profiles in IgM and IgY NAbs between free-range and conventional broiler chicks sampled at matched ages. Sera were collected from three farms (n = 144): conventional broiler (Ross 308) at 47 days (C-47), and free-range (Sasso) at 47 (FR-47) and 67 days (FR-67). NAb measurements were performed by indirect ELISA against actin, DNA, TNP–BSA, and LPS before and after dissociation assays (urea and heat-induced cryptic activity), alongside serum total IgM/Y class quantification. We found consistently higher IgM NAb levels in free-range broilers, and normalization to total IgM further confirmed these differences, supporting qualitative binding features between the two genotypes ($p < 0.001$). Persistent IgY patterns were antigen-specific: higher anti-LPS (~1.2-fold) and lower anti-DNA (~0.6-fold) in free-range broilers. While total IgM was higher in free-range groups, total IgY did not differ in concentration, although dissociation assays showed multiple qualitative differences. These findings further validate our previous observations and demonstrate that both, antibody levels and binding characteristics distinguish broiler genotypes, supporting the utility of NAbs as practical biomarkers for monitoring immune status and informing management decisions in commercial poultry production.

Keywords: Natural Antibodies; IgM and IgY antibodies; Innate immunity; Broilers; Poultry industry; Alternative housing systems; Free-range; Sasso; Ross 308

Introduction

Poultry production is among the most efficient systems for providing high-quality animal protein. However, increasing concern for animal welfare has led to the wider adoption of alternative housing systems, such as free-range, in addition to conventional intensive systems [1]. These contrasting environments generate different immunological pressures, while genotype choice critically modulates bird robustness and performance under each system [2]. As a result, there is growing interest among breeders and producers in identifying immune biomarkers that remain consistent across farm conditions and can inform both welfare and productivity [3].

Naturally occurring antibodies (NAbs) are germline-encoded, broadly polyreactive antibodies that are present in the absence of deliberate immunization and capable of binding both self- and non-self targets, contributing to early

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pathogen control and immune homeostasis [4]. In poultry and other species, NAbs are commonly measured against conserved endogenous and exogenous antigens, providing a practical means to assess baseline humoral immunity and antibody diversity [5]. In chickens, for example, NAb levels to keyhole limpet hemocyanin (KLH) are heritable and associated with survival, and have been considered as selection traits for enhanced disease resistance [6,7].

In our previous industrial-scale study, we analyzed circulating IgM and IgY NAbs against actin, DNA, 2,4,6-trinitrophenyl (TNP), and lipopolysaccharide (LPS) in conventional and free-range broiler chicks [8]. We reported consistently higher IgM NAb levels in free-range broilers along with genotype-specific IgY patterns, seasonal effects, and associations with both performance metrics and dietary supplementation. These findings suggest that NAbs, particularly IgM, can serve as effective biomarkers for distinguishing broiler genotypes in industrial settings and as practical tools for decision-making in the poultry industry [9].

The present study is a targeted follow-up to our previous work, addressing two specific questions that remain open. First, since earlier sampling was performed at genotype-specific slaughter ages (47 days for conventional and 67 days for free-range), we now collected samples from both breeds at the same time point to eliminate the confounding factor of sacrifice timing. Second, in addition to quantitative measurements, we investigated potential qualitative differences in NAb binding by measuring total IgM and IgY serum concentrations and applying standard protein dissociation treatments (heat, urea) within ELISA frameworks. Overall, our goal was to further support the use of NAbs as biomarkers under real industrial conditions with minimal methodological drift from our established protocol.

Materials and Methods

Study design

Fast-growing broiler chicks (Ross 308, Aviagen Group, Huntsville, Alabama, USA) raised in conventional/intensive (indoor) system and slow-growing broiler chicks (Sasso, Hendrix Genetics BV, The Netherlands) raised in free-range system (outdoor), provided by Agricultural Poultry Cooperative of Ioannina "PINDOS" (Greece) and raised under industrial scale systems as described previously [8].

In this study, we randomly analyzed 16 broilers from three different farms collected between September and October of 2023. Samples were collected for the conventional group (C) at 47th days of age (slaughter age) and for the FR group at 47th and 67th days of age (slaughter age). A total of 144 serum samples (48 per group) were collected and analyzed herein (**Table 1**). Blood samples were gathered by jugular vein-puncture and tubes centrifuged at 1.500 rpm for 10 min at 4 °C. Supernatants (serum) were stored at -80 °C until used.

Table 1: Broiler chicks charecteristics and study design.

Groups	Conventional (C)	Free-range (FR)
Housing	Indoors	Outdoors
Growth	Fast	Slow
Genotype	Ross 308	Sasso
Slaughter age	47d	67d
Herd	n = 3	n = 3
Birds/herd	n = 16	n = 16
Serum	47d	47d & 67d
Total	n = 48	n = 48 + 48 = 96

Measurement of IgM and IgY NAb levels by indirect ELISA

Measurement of NAb levels by ELISA were conducted as previously described, in order to investigate the reproducibility of our results. Briefly, actin (A3653) from bovine muscle, deoxyribonucleic acid sodium salt (native DNA) from Calf Thymus (D1501) and lipopolysaccharide (LPS) from *Escherichia coli* (L2880) were purchased from Sigma Aldrich (St. Louis, MO, USA). The hapten trinitrophenyl (TNP) coupled to bovine serum albumin (BSA) was prepared as previously described [18]. High binding ELISA microplates (Nunc-MaxiSorp, Roskilde, Denmark) were used for antigens' coating (10μg/ml). Sera were diluted 1:100, and alkaline phosphatase-conjugated secondary antibodies against chicken IgM (SAB3700239-1, Sigma Aldrich) or IgY (303-055-008, Jackson ImmunoResearch, Pennsylvania, USA) were used. Antibody binding was assessed with pNPP substrate (N2765, Sigma), and the optical density (OD) of the colored product was measured at 450nm (620nm reference) at a TECAN photometer (TECAN Spark Control Magellan V2.2, Grödig/Salzburg, Austria). Positive and negative controls were used in every plate for inter-assay normalization, which was set to less than 15%.

Quantification of Total Serum IgM and IgY by Sandwich ELISA

To enable total IgM and IgY quantification, we performed a pilot in-house sandwich ELISA to define the working range and the linear segment of the standard curve using serum-purified and quantified IgM and IgY control standards. ELISA plates were coated with anti-chicken IgM or IgY (1 μg/mL) overnight at 4 ^{0}C. The next day, plates were washed with PBS and blocked with PBS-BSA (1%) for 1h at 37^{0}C. For each assay, standards were serially diluted from 4 μg/ml through 11 two-fold dilutions down to 0.0019 μg/ml. The generated standard curves were used to identify linear range sample dilutions, and the optimal working dilution was set at 1:8000 for IgM and 1:150000 for IgY, which were applied to all sera (n = 144) measurements. In screening assays, samples were incubated for 2h at 37^{0}C in plates together with standards, both diluted in PBS-Tween (0.01%)- BSA(1%).

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After extensive wash, alkaline phosphatase conjugated secondary antibodies against chicken IgM (SAB3700239-1, Sigma Aldrich, St. Louis, MO, USA) or IgY (303-055-008, Jackson ImmunoResearch, West Grove, Pennsylvania, USA) were added for 0.5 and 0.1 μg/ml final concentrations, respectively, and incubated for 2 h at 37^{0}C. After final washes, antibody binding was assessed with pNPP substrate (N2765, Sigma), and OD was measured as mentioned above. IgM and IgY concentrations were obtained from the linear segment of the respective standard curve, and final serum concentrations were calculated in mg/ml by multiplying values with the dilution factor.

Assessment of Serum IgY NAb Binding after Dissociating Treatments by ELISA

Indirect ELISAs were performed as described above, and run in parallel without any treatment (Control). Paired samples were placed in the same ELISA microplate, processed identically except for the dissociating treatment step. The use of heat to unmask binding populations and reveal "cryptic" activity, as well as the use of chaotropic agents such as urea, for ELISA-based avidity determinations is well established; different formats are reported (pre-incubation vs. post-binding wash), and temperature can modulate stringency [10,11]. We adhered to the guidance to keep readings within the assay's dynamic range, ensuring valid indices.

Heat exposure ("cryptic activity"): To probe heat-labile complexes masking antibody binding, serum samples for IgY measurements were incubated at 56 °C for 30 min, cooled to RT, then diluted in PBS- Tween (0.01%)- BSA(1%) (1/100) and added to coated wells (100 μL/well, 2 h, 37 °C). A heat index (HI) was calculated per sample as: HI = OD_heated / OD_untreated.

Urea treatment (avidity): To assess binding stability (functional avidity), sera for IgY ELISA were diluted in 6 M urea [prepared in PBS- Tween (0.01%)- BSA(1%)] and pre-incubated for 30 min at RT before loading (100 μL/well, 2 h, 37 °C). In preliminary trials using 3, 6, and 8 M urea, we selected 6 M because it provided robust discrimination without additional signal loss relative to 8 M; washing the plate with urea alone (post-binding) was less effective in our hands, so we standardized on serum pre-incubation in urea. An avidity index (AI) was calculated per sample as: AI = OD_urea / OD_untreated.

Statistical analysis

The d'Agostino-Pearson omnibus normality test was used to assess the normality of the data distribution. For two independent group comparisons the t-Test or Mann–Whitney U test was used. One way ANOVA or the non-parametric Friedman test was used to detect differences between groups. In all cases, the significance level was set at 5%, the tests were two sided and a result was considered significant if the estimated P-value (p) was less than the significance level. Statistical analysis was performed and graphs were made in GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, California USA).

Results

Evaluation of IgM and IgY NAb levels.

Levels of circulating IgM NAbs were measured by indirect ELISAs against actin (ACT), DNA, TNP–BSA, and LPS for FR at day 47 and 67 (n=48/time point) compared to C (n=48) at day 47, as shown in **Figure 1**. FR group exhibits significantly higher levels of IgM NAbs against the four antigens studied, for both time points ($p<0.001$). Compared to C group, FR group at day 47 exhibited 1.6- and 1.4-fold higher levels for endogenous targets actin and DNA, respectively ($p<0.001$). Similarly, for exogenous antigens FR group exhibited 1.5 and 2.4 fold higher levels for TNP and LPS, respectively ($p<0.001$).

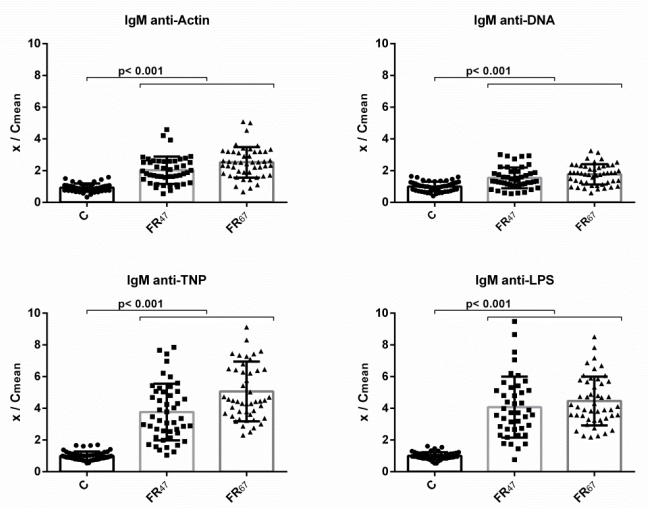

Figure 1: Comparison of IgM NAbs levels between conventional (C) group at 47 days, and free-range (FR) group at 47 and 67 days: IgM NAb levels were estimated by the binding capacity of serum NAbs to Actin, DNA, TNP and LPS. On Y axis, values are given as a ratio of individual NAb levels and the mean NAb levels of the control group [y= OD(x)/meanOD(C)]. Data are shown as mean ± SEM with individual values overlaid. Significance level was set at 5%.

Levels of circulating IgY NAbs for FR group at day 47 and 67 (n=48/time point) compared to C group (n=48) at day 47 are shown in **Figure 2**. FR group exhibited significantly higher IgY NAb levels only for LPS at both time points, estimated of about 1.2 fold higher at day 47 when compared to C group ($p<0.001$). A significant 1.2 fold elevation in anti-TNP NAb levels was observed for FR group at 47 day time point compared to C group, while no differences were observed for anti-Actin NAb levels. Interestingly, similarly to our previous observations, levels of NAbs targeting DNA

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were significantly lower, about 0.6 fold, for FR group for both time points in comparison to C group ($p<0.001$).

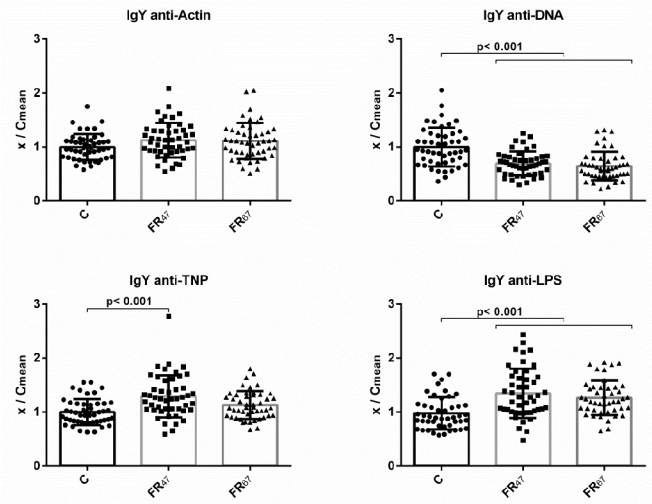

Figure 2: Comparison of serum IgY NAbs levels between conventional (C) group at 47 days, and free-range (FR) group at 47 and 67 days: IgY NAb levels were estimated by the binding capacity of serum NAbs to Actin, DNA, TNP and LPS. On Y axis, values are given as a ratio of individual NAb levels and the mean NAb levels of the control group [y= OD(x)/meanOD(C)]. Data are shown as mean ± SEM with individual values overlaid. Significance level was set at 5%.

Analysis of total IgM and IgY serum concentrations

Total serum IgM concentration differed across groups as shown in **Figure 3A**. Mean values were 1.00 ± 0.38 mg/mL (C), 1.50 ± 0.65 mg/mL (FR-47) and 1.50 ± 0.27 mg/mL (FR-67). Pairwise comparisons were statistically significant, with notably higher IgM concentrations in the FR groups compared to C ($p<0.001$). For total serum IgY, mean values were 4.8 ± 2.1 mg/mL (C), 4.1 ± 2.8 mg/mL and 4.5 ± 3.0 mg/mL (FR-67). In **Figure 3B**, no statistically significant differences are observed among groups for total serum IgY.

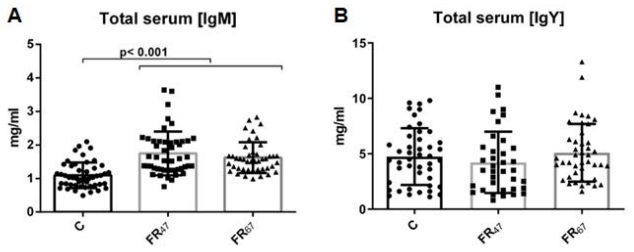

Figure 3: Serum total IgM (A) and IgY (B) concentrations. Ig concentrations were estimated for conventional (C) group at 47 days, and for free-range (FR) group at 47 and 67 days. On the Y axis, values are given in mg/ml, as calculated from calibrated standard curves. Data are presented as mean ± SEM with individual values shown. The significance level was set at 5%.

To further investigate the impact of total Ig concentrations on serum NAb levels, we normalized NAb values to the corresponding total Ig concentration per sample. Normalizing NAb signals to total IgM, as shown in **Figure 4**, did not abolish between-group differences, indicating that genotype-associated differences in IgM NAbs are not solely attributable to total IgM abundance. Since total IgY showed no significant variation among groups, differences in IgY NAbs reported elsewhere are qualitative rather than quantitative; accordingly, normalization to total IgY was not necessary.

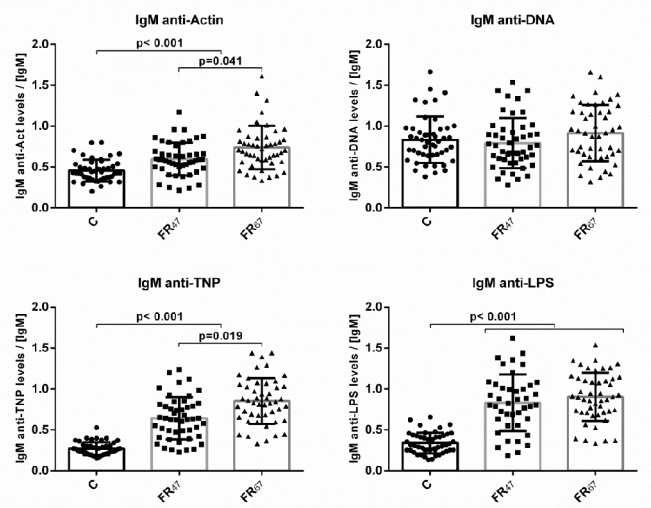

Figure 4: Comparison of serum IgM NAbs levels between fast- and slow- growth broilers: IgM NAb levels were estimated by the binding capacity of serum NAbs to Actin, DNA, TNP and LPS. On the Y axis, values are given as the ratio of individual NAb levels to the coresponding total IgM concentration of each sample (y= OD(x)/[IgM](x)). Data are presented as mean ± SEM with individual values shown. The significance level was set at 5%.

Effect of Destabilizing Factors on IgY NAb Levels

Indirect ELISAs were performed against actin (ACT), DNA, TNP–BSA, and LPS on paired samples processed in parallel with and without treatment. Groups: C-47 (47 d), FR-47 (47 d), FR-67 (67 d); n = 48 per group/time point.

To assess binding stability, sera were pre-incubated in 6 M urea and an Avidity Index (AI) was calculated as AI = OD_urea / OD_untreated (**Figure 5**, blue). At the matched 47-day time point, actin showed the clearest separation along the avidity axis, with mean values ≥1 and a significant difference between C-47 and FR-47 ($p=0.034$). Across antigens, DNA exhibited the lowest AI in all groups, whereas LPS showed a higher AI in FR-47 compared to C-47 ($p=0.01$). IgY anti-TNP activity displayed no significant differences between groups.

To probe masked binding, sera were heated at 56 °C for 30 min, and a Cryptic Activity/Heat Index (HI) was calculated as HI = OD_heated / OD_untreated (**Figure 5**, orange). Actin

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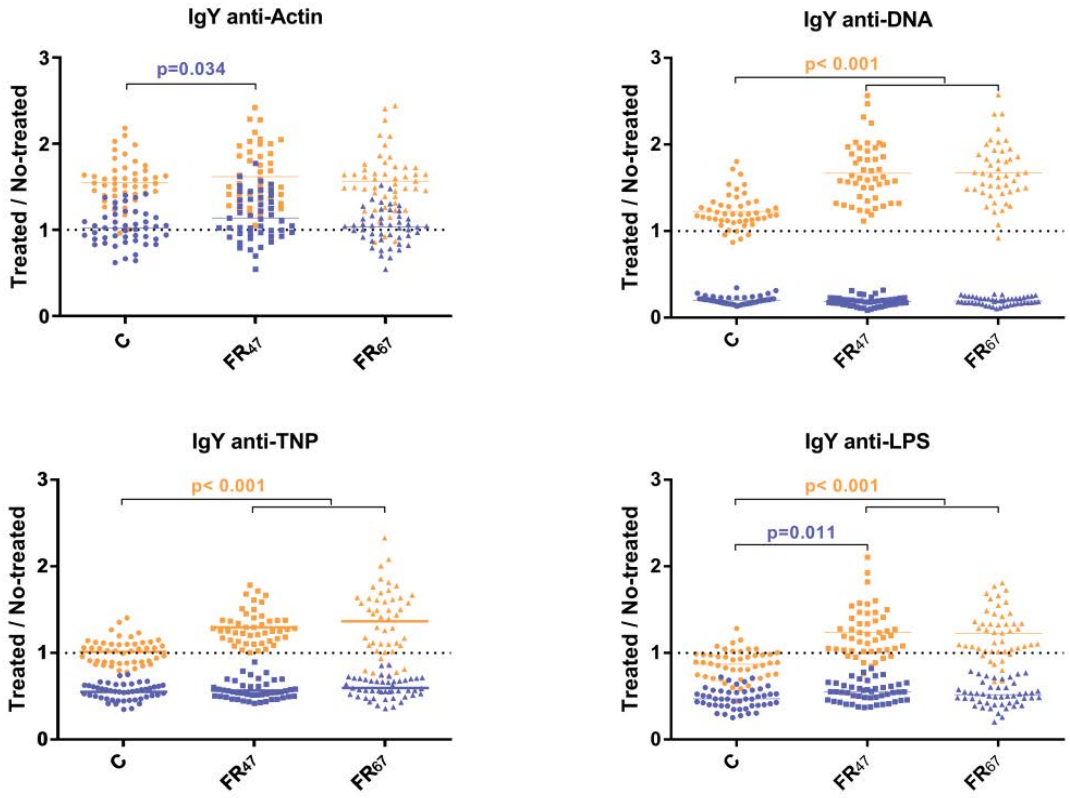

Figure 5: Effect of dissociating factors on serum IgY NAb levels. Blue bars show the Avidity Index (AI) calculated as AI = OD_urea / OD_untreated after pre-incubation of sera in 6 M urea for 30 min at room temperature; orange bars show the Heat (Cryptic Activity) Index (HI) calculated as HI = OD_heated / OD_untreated after 56 °C incubation for 30 min. Data are shown as mean ± SEM with individual values overlaid. Significance level was set at 5%.

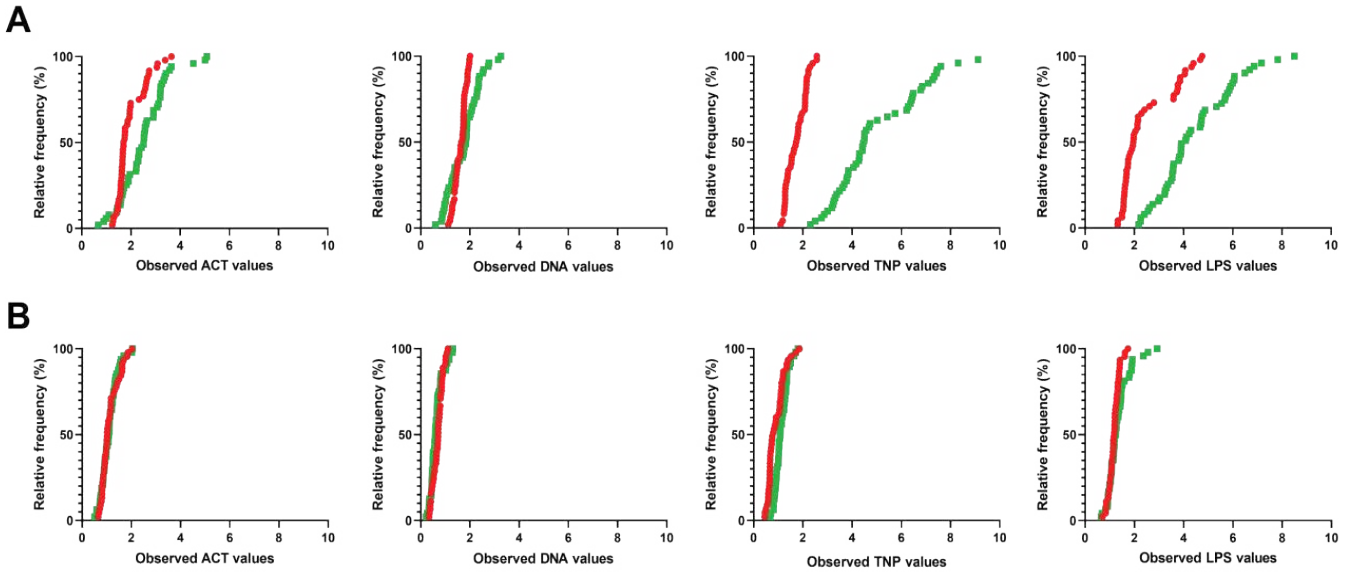

Figure 6: Relative frequency of NAb levels between measurements. Relative-frequency plots (percentage of samples per bin) show the distributions of normalized ELISA signals for IgM (A) and IgY (B) and each antigen (ACT, DNA, TNP, LPS) in the current dataset compared with our prior industrial-scale dataset at the final breeding step. Values were plate-normalized as y = OD(x) / meanOD(Control) to enable inter-plate comparisons. Groups: C-47, FR-47, FR-67 (n = 48 per group). Overlap of distributions illustrates the reproducibility of NAb measurements between studies.

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showed no significant differences in HI among groups. In contrast, for DNA, LPS, and TNP, significant differences in HI were observed between C-47 and FR-47 and between C-47 and FR-67, indicating antigen- and genotype-specific unmasking that is consistent across both free-range time points ($p<0.001$).

Reproducibility of ELISA-Based NAb Measurements

To validate the reproducibility of our measurements, we compared the current data with our previous results. (Figure 6) A high degree of reproducibility was observed across most measurements; however, for IgM NAbs against TNP and LPS, the FR group showed relatively higher values than the C group, in line with our earlier findings, with increases reaching up to fourfold in some cases.

Discussion

In this study, we re-examine NAbs as candidate biomarkers in an industrial context, focusing on two key questions: whether genotype-linked differences persist when sampling is matched by age, and whether these differences reflect both quantitative and qualitative binding characteristics. Using standardized ELISAs together with dissociation-based metrics (Avidity Index with urea; Heat Index for cryptic activity), we demonstrate that the NAb compartment can be assessed in a manner that is both operationally simple and biologically informative, supporting its potential use in practice. Our findings are consistent with, and extend, our previous industrial-scale analysis that identified robust differences in IgM and IgY NAbs between fast-growing and slow-growing broiler chicks [8]. By sampling both genotypes at the same age and incorporating dissociation assays, we minimize confounding effects on NAbs due to timing and add mechanistic insight (binding stability and unmasking activity) to previously observed differences in absolute levels, thereby reinforcing the utility of NAbs as biomarkers in commercial settings.

More specifically, in our previous work, fast-growing and slow-growing broiler chicks were sampled at their typical slaughter ages (≈47 d vs. ≈67 d), so age could have contributed to the observed differences. Here, by collecting both genotypes at 47 days and additionally at the slaughter age (67 days) of slow-growing broiler chicks as a reference, we disentangled genotype from age effects. Higher IgM NAb levels in free-range broilers and antigen-specific IgY patterns were already present at 47 days, indicating that these NAb patterns are not driven by age alone. Consistently, normalization to total serum IgM preserved the IgM NAb differences, while total IgY did not differ across groups or time points, pointing instead to qualitative regulation of IgY.

Two observations from the dissociation assays deserve emphasis. First, the Avidity Index after treatment with 6 M urea was frequently ≥ 1 for actin, indicating that denaturation can increase apparent binding and expose cryptic activities, as previously described [12]. Similar urea-dependent signal enhancements have been reported when antigen unfolding exposes previously hidden epitopes, and chaotropes are widely used to probe such qualitative features in ELISA-based avidity formats [13,14]. Second, although absolute anti-DNA IgY levels were higher in C-47 under native conditions, heat treatment reversed this pattern, with FR sera showing a higher Heat Index, consistent with masked anti-DNA activities that become detectable upon dissociation. Heat-induced unveiling of autoantibody reactivity has been previously documented, supporting the idea that mild denaturation can reveal binding otherwise hidden in immune complexes [15]. A concise explanation is that higher IgM NAb levels in FR contribute to the masking of IgY specificities (e.g., via immune complexes or Ig–Ig interactions), with dissociation restoring IgY access, an interpretation aligned with established evidence that IgM can modulate IgG availability and epitope access in mice [16]. Together, the urea and heat results indicate that genotype differences in the NAb compartment are not only quantitative but also reflect differences in binding architecture (epitope exposure, complexing/ complex formation), particularly for actin and DNA specificities, which have been extensively studied and correlated with muscle reconstruction upon tissue stress or damage [17-19].

Overall, the persistence of higher IgM NAb levels in free-range broiler chicks at 47 days together with antigen-specific IgY patterns, suggests a genotype-linked NAb architecture. Because these readouts derive from standard ELISAs combined with simple pre- treatment steps, they are readily transferable to routine settings. In practice, combining NAb IgM levels with IgY cryptic binding activity provides a compact panel capable of differentiating among broiler lines [20]. This validates our previous findings in a more rigorous context and supports the potential of NAbs as immunotools for monitoring and benchmarking in commercial flocks. Because NAbs are heritable and associated with robustness, routine profiling can facilitate genotype selection adapted to specific farm conditions, such as heat stress, stocking density, and lighting regimes [21]. Additionally, NAb profiling can support long-term selection goals related to welfare indicators, facilitate intervention quality control, and enable benchmarking of feeding additives or management strategies, consistent with our previous industrial data [22].

Future studies should evaluate our NAb target antigen panel in additional broiler genotypes and management settings (conventional, free-range, organic), with explicit sampling across all seasons, stocking densities, heat loads, feed regimens, and vaccination windows. Longitudinal designs (starter to slaughter) and multi-site ring trials will be important for harmonizing ELISA workflows and establishing reference intervals. Low-cost pooled-pen sampling can enable routine surveillance, while targeted stress or challenge models could

test responsiveness. Finally, applicability should be assessed in other poultry species (layers, turkeys, ducks, quail) and, where appropriate, in non-avian livestock by adapting isotype readouts (e.g., IgG in mammals). This breadth of testing will clarify generalizability and define where NAbs best support decision-making in the poultry industry and other sectors.

Author Contributions

Conceptualization: P.L. I.S; methodology: P.L. I.S; software: I.S. and C.T.; validation: P.L., V.T. (V. Tsiouris) and D.T.; formal analysis: I.S. and C.B.; investigation: C.B.; resources: A.P., V.T. (V. Tsiouris) and P.L.,; data curation: I.S.; P.L., A.P. and V.M. (V. Moussis); writing—original draft preparation: I.S. and P.L.; writing—review and editing: E.F., V.M. (V. Moulasioti), and V.T. (V. Tsiouris); visualization: I.S. C.B.; supervision: P.L.; project administration: V.T. (V. Tsikaris) and D.T.; funding acquisition: V.M. (V. Moussis). All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The samples were collected from broiler farms under field conditions; experimental animals were not used. Therefore, there was no need for ethical approval of the study.

Informed Consent Statement: Not applicable.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

The authors declare no conflicts of interest.

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