Research Note: Morphology and immune function development of the jejunum and ileum in squab pigeons (*Columba livia*)

Qianqian Xu, Sasa Miao, Huafeng Jian, Xiaoting Zou, and Xinyang Dong[1]

Key laboratory for Molecular Animal Nutrition of Ministry of Education, Key Laboratory of Animal Feed and Nutrition of Zhejiang Province, Feed Science Institute, College of Animal Science, Zhejiang University (Zijingang Campus), Hangzhou, 310058, China

ABSTRACT The study was aimed to evaluate the morphology and immune function development of the jejunum and ileum in squab pigeons (*Columba livia*), by determining the villus ultrastructure, secretory IgA, and cytokines. Eight squabs were randomly selected and sampled on the day of hatch (**DOH**), d 7 (D 7), 14 (D 14), and 21 (D 21) post-hatch, respectively. The results showed that under transmission electron microscope, the enterocyte circumference in jejunum and ileum decreased with age. The tight junction involved in enterocyte circumference of jejunal villi plateaued from D 7, whereas that of ileal villi changed irregularly. The microvilli of jejunal and ileal villi was maximum at D 14. Under scanning electron microscope, the villus morphology of jejunum and ileum appeared finger-shaped at DOH. After D 7, the jejunal villi were still finger-shaped whereas the ileal villi were leaflike. The secretory IgA in jejunum was significantly increased at D 21. The TGF-β decreased linearly in jejunum and ileum. The anti-inflammatory cytokines increased linearly and proinflammatory cytokines decreased linearly in jejunum and ileum with age. In conclusion, the morphological changes of jejunal epithelium were concentrated at DOH−D 7 and ileal epithelium at DOH−D 14 mainly. The changes in mucus layer and immune-related factors of jejunum and ileum persisted for almost the entire period.

Key words: squab pigeon, intestine, morphology, scanning electron microscopy, transmission electron microscopy

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INTRODUCTION

The domestic pigeon (*Columba livia*) is one of the economically important farmed poultry, which is widely distributed in the whole world. Squab is the main product of meat pigeon breeding industry. As a representative of altrices, pigeon squab has a rather high relative growth rate and can reach sale weight at around three weeks of age. The small intestine, as the main organ functioning in digestion, absorption, and immunity, plays a crucial role in this process of rapid growth and development (Xu et al., 2020). Previous study has also clarified functional maturation of the small intestine involves both morphological and physiological variation and is the primary constraint to optimal early growth of birds (Konarzewski et al., 1990). Thus, identifying the normal development and functional maturation of the pigeon small intestine in the post-hatch period is necessary.

Previous study in our lab has explored the pattern of luminal morphological and digestion-absorption functional development in the small intestine of pigeon squab during early post-hatch growth (Dong et al., 2012). Gao et al. (2016) also found the growth rate of pigeon squab was closely related to the ability for nutrient absorption in the small intestine. However, the knowledge on the development of small intestinal immunity and ultrastructure in squabs is still scarce, which needs research to fill the blank. The mechanical barrier formed by intestinal epithelium and the cytokines secreted by immune cells are closely involved in the intestinal mucosal immune system (Malago, 2015). Considering that intestinal immunity is mainly concentrated in the middle and posterior intestinal segments, the objective of this study was to evaluate the morphology and immune function development of jejunum and ileum in squab pigeons (*Columba livia*), by determining the villus ultrastructure, secretory IgA (**sIgA**) and cytokines in the jejunum and ileum on the day of hatch (**DOH**), d 7 (**D7**), 14 (**D14**), and 21 (**D21**) post-hatch.

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[1]Corresponding author: sophiedxy@zju.edu.cn

MATERIALS AND METHODS

All experimental protocols involving animals were approved by the Animal Care and Welfare Committee of Animal Science College and the Scientific Ethical Committee of Zhejiang University (No. ZJU2013105002) (Hangzhou, China).

Experimental Birds and Conditions

The experiment was performed in a commercial pigeon farm (Hangzhou, China). A birdcage made of stainless wire equipped with a perch and a nest was provided for each pair of parent pigeons. Each pair laid 2 eggs in the nest. Eggs were picked out and transferred to an artificial incubator for 18-d incubation (55 ± 2% relative humidity and 38.1 ± 0.1°C). Meanwhile, 2 fake eggs which are similar in size, color, and shape to real eggs were placed into each nest to meet parent pigeons' brooding characteristics. At DOH, 320 hatched squabs with similar body weight were selected from the commercial farm, pair-matched and allocated into the nests of 160-pair parent White King pigeons (60-wk-old, male and female half) to replace the fake eggs. Each parent pair adopted 2 artificially hatched squabs. Parent pigeons were randomly divided into 8 replications, each of 20-pair pigeons. All parent pigeons were supplied with a cereal based diet. The based diet included corn, wheat, peas, sorghum, and mung beans, mainly containing 13.50% crude protein and 12.32 MJ/kg metabolizable energy. The pigeons were given water ad libitum and were fed twice daily (6:00 A.M. and 3:00 P.M.) throughout the experiment. The squabs were fed with crop milk by parent pigeons in a beak-to-beak manner. The crop milk which was secreted from the crop of parent pigeons mainly contained 52.68 to 58.47% crude protein and 18.94 to 32.77% crude fat (on a dry-matter basis). The ambient temperature was 18 to 26°C, and the relative humidity was 60 to 70%. The photoperiod was 12 h light−12 h dark throughout the total study period.

Sample Collection

On the DOH, D7, D14, and D21, eight squabs (1 squab from each replication) were selected randomly for sampling, respectively. The squabs from DOH were weighed and slaughtered within 2 h after hatch, but before feeding. The squabs at other ages were fasted for 12 h before weighing and slaughtering. The selected squabs were all killed by cervical dislocation (squabs with body weight over 250 g were sedated before cervical dislocation). Two segments of the small intestine (jejunum and ileum) were sampled free of adhering tissues, flushed with 0.9% saline to remove all the contents and processed for morphological examination. The mucosa of each intestinal segment was carefully scraped off with a glass slide, frozen in liquid nitrogen rapidly and then stored at −80°C for subsequent analyses.

Villus Morphology Under Scanning Electron Microscope

Approximately 3 × 3 mm samples of jejunum and ileum were collected. Samples were first fixed with 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.0) for more than 4 h and washed 3 times in the phosphate buffer (0.1 M, pH 7.0) for 15 min at each step. Then these samples were postfixed with 1% OsO_4 in phosphate buffer for 1 h and washed 3 times in the phosphate buffer (0.1 M, pH 7.0) for 15 min at each step. Next, the samples were first dehydrated by a graded series of ethanol (30, 50, 70, 80, 90, and 95%) for about 15 min at each step, and dehydrated by 100% alcohol 2 times for 20 min each time. After that the critical point drying was performed using a critical point dryer (Hitachi Model HCP-2). The dehydrated samples were coated with gold-palladium in the ion sputter (Hitachi Model E-1010) for 4 to 5 min and observed under scanning electron microscope (**SEM**) (Hitachi Model SU-8010).

Villus Morphology Under Transmission Electron Microscope

Approximately 1 × 3 mm samples of jejunum and ileum were collected and proceeded with double fixation which was the same as the processing procedures of SEM. Next, the samples were first dehydrated by a graded series of ethanol (30, 50, 70, 80, 90 and 95%) for about 15 min at each step, dehydrated by alcohol for 20 min, and transferred to absolute acetone for 20 min. The samples were placed in 1:1 mixture of absolute acetone and the final Spurr resin mixture for 1 h at room temperature then transferred to 1:3 mixture of absolute acetone and the final resin mixture for 3 h and to final Spurr resin mixture for overnight. These samples were placed in eppendorf contained Spurr resin and heated at 70°C for more than 9 h, after which the samples were sectioned in an ultramicrotome (LEICA EM UC7). The sections were stained by uranyl acetate and alkaline lead citrate for 5 to 10 min respectively and observed under transmission electron microscope (**TEM**) (Hitachi Model H-7650). The measurement of microvillus length, enterocyte circumference, and tight junction length was in line with Karcher and Applegate (2008).

Secretory IgA and Cytokine Analysis

The homogenates of intestinal mucosa were prepared with PBS for sIgA and cytokine analyses. The sIgA concentration and the concentrations of tumor necrosis factor-α (**TNF-α**), transforming growth factor-β (**TGF-β**), interleukin-1β (**IL-1β**), IL-4, IL-6, IL-8, and IL-10 were measured with commercial ELISA kits (Beijing Sino-uk Institute, Beijing, China) because these cytokines are often assayed to determine intestinal immune function in poultry in previous research (Xu et al., 2020). First, add 100 μL Antibody to each well and coated for 2 h. Discard Liquid, dry by swing, add

washing buffer to every well, still for 30 s then drain, repeat 5 times, dry by pat. Second, add 200 μL sealing liquid to each well, mix and let stand at room temperature for 30 min. Discard Liquid, dry by swing, and repeat the above washing steps 5 times. Third, add 25 μL coating solution with 25 μL specimen or 50 μL standard to each well and mix. Add 100 μL HRP to each well, mix, and let stand at room temperature for 60 min. Discard liquid, dry by swing, and repeat the above washing steps 5 times. Next, add 100 μL color solution to each well, mix and develop color for 15 min. Finally, add 100 μL stop solution to each well to stop the reaction and read absorbance at 450 nm within 15 min. SIgA and cytokine concentrations were expressed as units per mg of protein.

Statistical Analysis

The data were subjected to a one-way analysis of variance in SPSS 24.0 (SPSS Inc., Chicago, IL) for Windows. The differences between means were tested by Tukey's multiple range test. The effect of age was determined using orthogonal polynomials for linear and quadratic effects. The level of significance was chosen at $P < 0.05$. Plotting was performed with GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA).

RESULTS AND DISCUSSION

The integrity of the intestinal epithelium barrier is maintained by the multiple epithelial junctional complexes which tether epithelial cells to each another (Knight and Hansbro, 2018). An important constituent of the framework is the tight junction. The evaluation of the tight junction as a percentage of the epithelial cell in squabs was in accordance with Karcher and Applegate (2008). In this study, the ratio of tight junction length to enterocyte circumference (**TJL/EC**) of jejunal villi in squabs stabilized from D 7, whereas that of ileal villi has an uneven presence with age (Figure 1D). The results implied that tight junction structure matured as jejunal villi developed to ensure the integrity of the villi epithelium. However, the tight junction of ileal villi is still in unstable development. Microvilli are membrane extensions on the apical surface of polarized epithelia. In this study, the microvillus length in jejunum and ileum both responded to increasing age in linear (both $P < 0.001$) and quadratic ($P < 0.001$ and $P = 0.008$, respectively) manner, and their maximal response was observed in D14 (Figure 1A). The growth post-hatch might be influenced by the microvillus length along the intestinal villi dictating nutrient uptake (Karcher and Applegate, 2008). The results suggested that squabs at D 14 might have stronger capacity in nutrient absorption. The enterocyte circumference in jejunum and ileum decreased gradually in squabs with age (Figure 2B), which was dissimilar in chicken whose enterocyte circumference increased at DOH to 3 d post-hatch (Karcher and Applegate, 2008). Research on enterocyte circumference of poultry post-hatch in longer periods was limited, nothing but study on cell apical surface area in enterocytes. The apical surface area increased in jejunum (1 d to 2 wk) but did not change in ileum in chicken (Ferrer et al., 1995). Therefore, we hypothesized that enterocytes in squabs should have different developmental patterns from those in chicken.

Given that TEM would not allow the surface image of the villi to be observed, SEM was performed subsequently. Obviously in Figure 1F, the jejunal villi develop faster than the ileal villi visually under SEM. The shape of villi can be divided into several types which include tongue-shaped, finger-shaped, ridged-shaped, convoluted, and foliate villi. In this study, the villus morphology of jejunum and ileum was relatively similar at DOH, both appearing stubby finger-shaped, but the ileal villi are smoother and more compact than the jejunal villi. After D 7, the jejunal villi were still finger-shaped whereas the ileal villi were short, broad, leaflike, and their apical surfaces were flattened. The morphological characteristics of the villi in the small intestine of squabs distinguish them from other domestic fowl. In chicks, the intestinal villi appear as zigzag ridges (Lim and Low, 1977). In this study, the SEM micrographs of the 14- and 21-day-old squabs displayed epithelial crevices and disruptions in jejunum villus surface, which was similar in day-old chicks (Karcher and Applegate, 2008). The crevices on the villi surface may be similar in function to the mammalian counterparts in the neonate allowing for the passage of undigested nutrients. However, the adverse effects of epithelium discontinuities could include either rapid transfer of enteric pathogens or food antigens into circulation of squabs. Interestingly, the sIgA concentration in jejunum responded to increasing age in linear ($P = 0.008$) and quadratic ($P = 0.041$) manner with the maximal response observed in D 21, whereas there was no significant ($P > 0.05$) difference in ileal sIgA concentration among 4 ages (Figure 2A). The sIgA is a vital immune effector molecule on the intestinal mucosal surface. The rise of jejunal sIgA secretion might be a response to the morphological characteristics of jejunal villus surface, enhancing the 'immune exclusion' ability (Xu et al., 2020).

In addition to the mechanical barrier, the cytokines are also tight participants in the intestinal mucosal immune system (Malago, 2015). Cytokines are important mediators and regulators of host against foreign antigens. TNF-α, IL-1β, IL-6, and IL-8 are known as proinflammatory cytokines, whereas IL-4 and IL-10 are classified as anti-inflammatory cytokines (Xu et al., 2020). In this study, the change trend of cytokine content in jejunum and ileum was similar, displaying anti-inflammatory cytokines increased linearly and proinflammatory cytokines decreased linearly with age (Figure 2). The interaction between anti-inflammatory and proinflammatory cytokines modulates the inflammatory response. This indicates that as squabs grow and develop after hatch, their ability to inhibit inflammation is gradually enhanced with increasing exposure outside. The balance between inflammatory cytokines

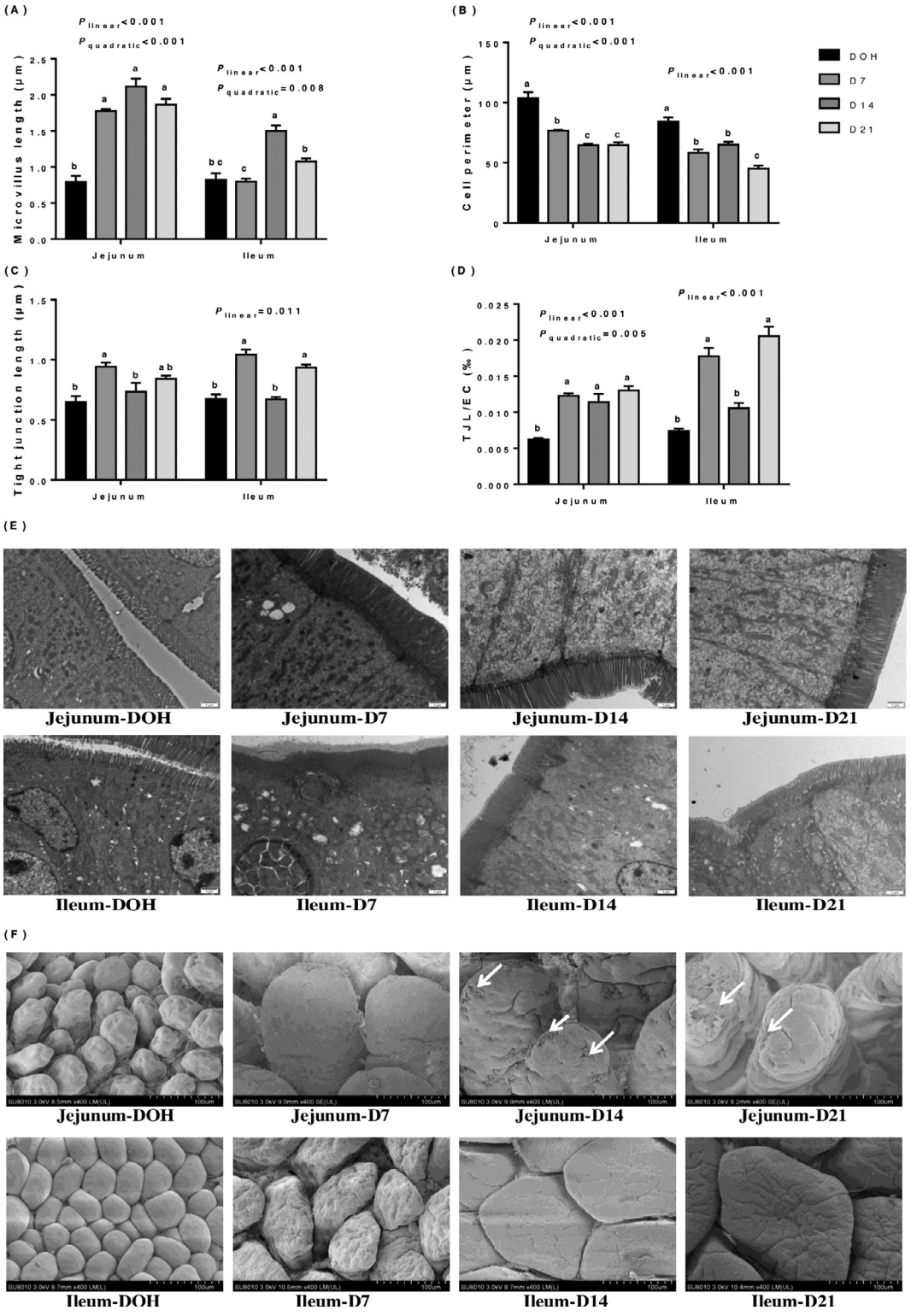

Figure 1. Morphological development changes of jejunoileal villi in domestic pigeon squabs under transmission electron microscope and scanning electron microscope. (A) Microvillus length, (B) enterocyte circumference, (C) tight junction length, (D) the ratio of tight junction length to enterocyte circumference (TJL/EC), (E) uranyl acetate and alkaline lead citrate staining of jejunum and ileum. Bar = 1 μm. (F) jejunum and ileum observed under scanning electron microscope. The arrow points to villous damaged part. Bar = 100 μm. DOH = day of hatch; D 7 = day 7 post-hatch; D 14 = day 14 post-hatch; D 21 = day 21 post-hatch. Values are means with their standard errors of eight squabs, n = 8. $^{a-c}$ Means w ith unlike letters are significantly different (Tukey test, $P < 0.05$).

is an essential factor for intestinal immunological homeostasis. The change of cytokines at each age might be a gradual process toward the equilibrium of immune response. Besides, the TGF-β controls the development and homeostasis of most tissues in metazoans (Massagué, 1998). In this study, TGF-β content decreased linearly both in jejunum and ileum. We hypothesized that as squabs grow, the need for TGF decreased

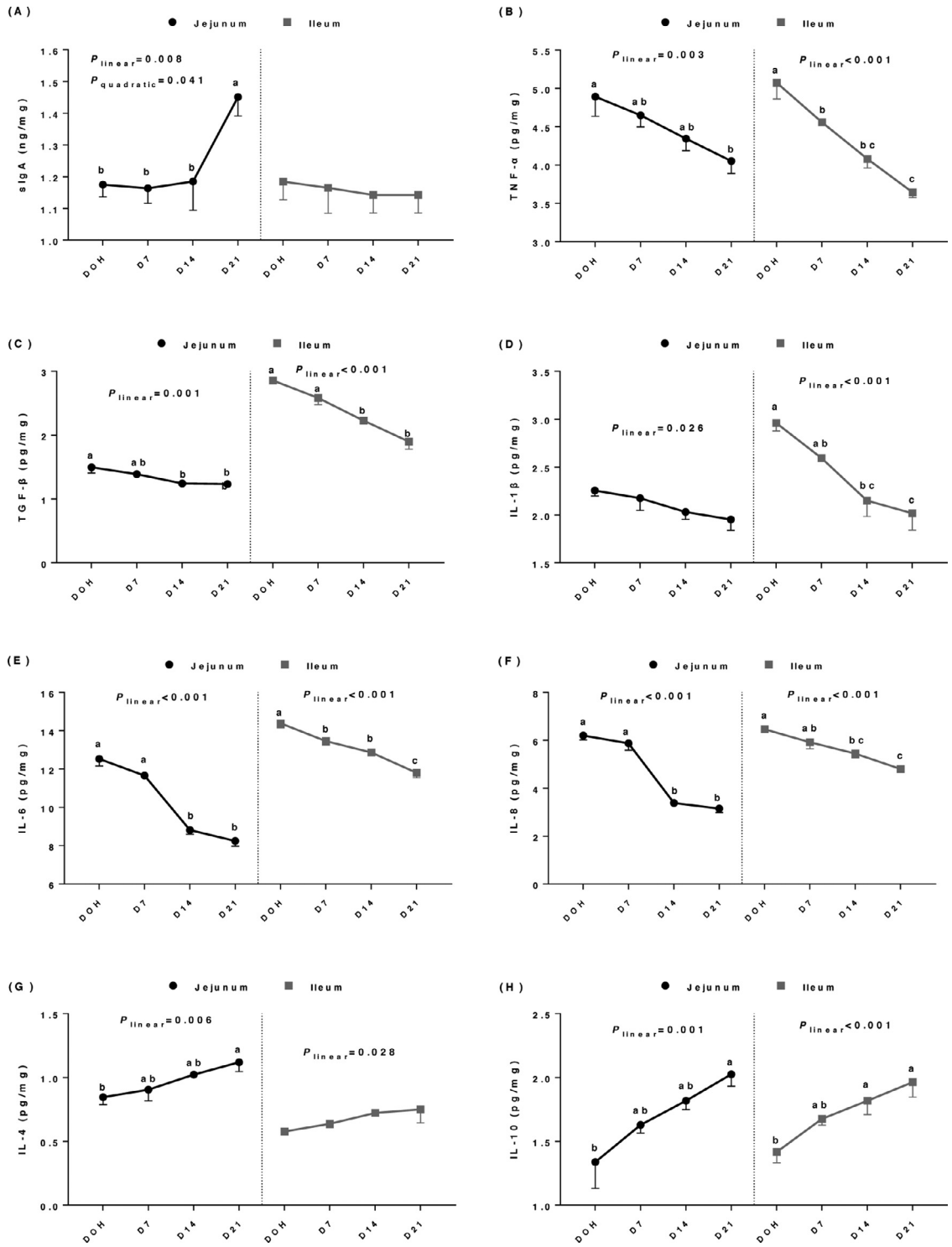

Figure 2. Jejunoileal secretory IgA (sIgA) and cytokine concentrations in domestic pigeon squabs at the day of hatch (DOH) and days 7 (D 7), 14 (D 14), 21 (D 21) post-hatch. (A) sIgA, (B) TNF-α, (C) TGF-β, (D) IL-1β, (E) IL-6, (F) IL-8, (G) IL-4, (H) IL-10. Values are means with their standard errors of eight squabs, n = 8. $^{a-c}$ Means with unlike letters are significantly different (Tukey test, $P < 0.05$).

gradually because the role of other cytokines was gradually sufficient for the maintenance of intestinal immune homeostasis.

In conclusion, the phase from DOH to D 14 was the critical period for the development of small intestinal mucosal immune system in squabs. The morphological changes of jejunal epithelium were mainly concentrated at DOH−D 7 and ileal epithelium at DOH−D 14 principally. The changes in the immune-related factors of jejunum and ileum persisted for almost the entire period reflected by increased anti-inflammatory cytokines and decreased proinflammatory cytokines.

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DISCLOSURES

The authors declare no conflict of interest.

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