

# The pattern of duck sternal ossification and the changes of histological structure and gene expression therein

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**ABSTRACT** As the largest single bone, avian sterna are very different from those of mammals in terms of morphology and functions. Moreover, years of artificial selection in poultry led to incomplete sternal ossification at slaughter age, which may cause diseases, sternal injury, and restriction to breast muscle growth. However, in living birds, studies have rarely described the ossification pattern and underlying mechanisms of the sterna. Here, we examined the pattern (timeline, ossification centers, ossification directions, weekly changes of different parts, quantified differences in ossification degree among sexes and parts) and developmental changes (histological structure, gene expression) of post-natal duck sternal ossification. Direct observation and alcian blue and alizarin red staining of whole sterna samples revealed that, duck sterna mainly ossified during 5 to 9 wk old with five ossification centers. These centers and their ossification directions were different from and more complex than the previously studied birds. The

weekly changes of sterna and the quantitative analysis of ossification-related traits showed that ossifications in the three parts of duck sterna (sternum body, keel, posterolateral processes) were mutually independent in space and time, meanwhile, the male duck sterna were more late-maturing than the female. The results of hematoxylin-eosin, alcian blue, and toluidine blue stainings and the expression levels of *COL2A1*, *COL10A1*, *COL1A2*, and *CTSK* together supported that, duck sternal ossification was highly similar to typical endochondral ossification. Furthermore, continuously high expression of *MMP13* and *SPARC* and their significant ( $P < 0.05$ ) co-expression with *COL2A1*, *COL10A1*, *COL1A2*, and *CTSK* suggested the importance of *MMP13* and *SPARC* in duck sternal ossification. Taken together, our results may be helpful for the understanding of avian sternal ossification and the improvement of the performance and welfare of poultry from a new perspective.

**Key words:** duck, sternal ossification, phenotypic trait, histological staining, gene expression

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## INTRODUCTION

Skeleton plays an important role in the living of vertebrate species, including providing structural support, protecting internal organs, acting as a major source of inorganic ions, and withstanding muscular contraction (Sommerfeldt and Rubin, 2001; Wagner and Asperberg, 2011; Florencio-Silva et al., 2015). In the skeletal system that contains hundreds of bones, sternum, a part of the thoracic cage, is one of the heavily studied single bones.

Researches indicated that the sterna of mammals were segmented and small (Weaver et al., 2014; Eydt et al., 2015; Ateşoğlu et al., 2018), whereas avian sterna were unsegmented, generally keeled, and highly developed as the largest single bone (John et al., 2014; Zheng et al., 2014). This may be because avian sterna made much greater contributions to the operation of the ventilatory apparatus (Claessens, 2009) and the attachment of the massive pectoral muscles (Kudo et al., 2016). The sterna of different birds showed high morphological diversity. For example, the ratios of sternum width to its height were 1.56-1.95, 0.96-1.35, and 0.50-0.68 in swimming birds, flying birds, and walking birds, respectively (Düzler et al., 2006). Therefore, avian sterna are unique, important, changeable, and worthy of study. In general, the ossification process is thought to be responsible for the formation of bones. The anatomical structures of avian sterna have been extensively reported

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(Feduccia, 1972; Sathyamoorthy et al., 2012; John, et al., 2017). However, although several influential studies were done in enantiornithines (Zheng et al., 2012; O'Connor et al., 2015; Wang and Zhou, 2017; Knoll et al., 2018), present knowledge about the sternal ossification of modern birds is confined to the Galliformes (Maxwell, 2008a) and ratites (Maxwell and Larsson, 2009), and research on this subject is still lacking in other major clades.

Furthermore, years of artificial selection in poultry, which aimed to increase growth rate and breast muscle percentage, had led to developmental trade-offs. Two of these were the incomplete sternal ossification at slaughter age that related to ascites, pulmonary diseases, sternal injury, and welfare problems (Julian, 1988; Tickle et al., 2014) and the far slower growth rate of sternum area compared to breast muscle volume (Andrassy-Baka et al., 2003). The relative lag of sternal ossification also could be considered as a restriction for breast muscle development because of the close association between the sternum and breast muscle, including physical connection, mechanical interaction, phenotypic correlation (Siegfried, 1963), and endocrine link (Karsenty and Olson, 2016). Therefore, it is of particular significance to reveal the pattern and underlying mechanisms of poultry sternal ossification. Previous studies in chicken (Hogg, 1980; Atalgin et al., 2008; Tickle et al., 2014) and quail (Nakane and Tsudzuki, 1999; Nakamura et al., 2019; Pourlis and Antonopoulos, 2019) were mainly focused on the qualitative characteristics of sternal ossification, but there is a paucity of information regarding the quantitative characteristics and underlying mechanisms.

To our knowledge, no study reported the sternal ossification in domestic ducks as well as in the Anseriformes. An embryonic approach to the ossification sequence of the entire skeleton of domestic ducks indicated that the sternum was completely unossified during incubation (Maxwell, 2008b). Thus, this study was aimed to explore the pattern and potential mechanisms of duck sternal ossification after birth, which provides a foundation for future studies focusing on the specific aspects of sternal ossification in poultry.

## MATERIALS AND METHODS

### **Ethics Statement**

The experimental procedures and protocols that are applied in this study were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (Permit No. DKY-B20161709).

### **Animals and Sample Collection**

A total of more than 1,300 0-day-old Nonghua ducks, which with similar birth weight, were provided by the Sichuan Agricultural University Waterfowl Breeding Experimental Farm (Ya'an, Sichuan, China). These ducks were randomly divided into 10 replicates, resulting in 130 individuals per replicate (half male, half

female). Each replicate was reared in 1 of 10 equally matched semi-open house. Thereafter, ducks were tagged with a foot ring and given free access to feed and water. The entire experimental period was 9 wk. We have maintained feeding, water, and other conditions as similar as possible across the 10 replicates to minimize their effects on the growth of ducks.

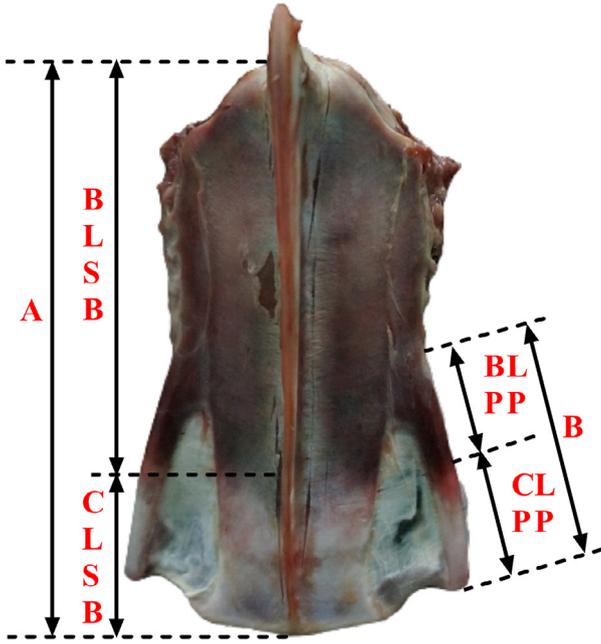
At weeks 5, 6, 7, 8, and 9, 240 healthy ducks (120 male and 120 female) with similar weight were selected from the 10 replicates and humanely killed by cervical dislocation after fasting. Subsequently, the whole sterna of ducks were collected and carefully cleared (wiping off breast muscle and other adherent tissues). At each age, 6 sterna (3 male and 3 female) were fixed in 90% ethanol at room temperature for skeletal staining, and the bone and cartilage samples of another 6 sterna (6 female) were collected, snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  for determining ossification-related genes expression. At week 7, some parts of 3 sterna (3 female) were dissected and fixed in 4% paraformaldehyde at room temperature for different histological stainings. The other sterna of each age were stored at  $4^{\circ}\text{C}$  for direct observation and the determination of ossification-related phenotypic traits. Additionally, at weeks 0, 1, 2, 3, and 4, 20 healthy ducks (10 male and 10 female) were selected for the pre-experiment.

### **Rough Assessment and Whole-mount Skeletal Staining**

To examine the appearance of sternal OC and the replacement of cartilage by bone, duck sterna obtained during the age of 5 to 9 wk were roughly assessed by direct observation and touch. Then, 6 representational sterna of each age (3 male and 3 female) were selected to perform alcian blue and alizarin red staining on the whole-mount samples. The detailed methods of staining have been previously described (Tickle and Codd, 2009; Rigueur and Lyons, 2014). Besides, all duck sterna obtained during the age of 0 to 4 wk were also assessed by direct observation and touch as the pre-experiment.

### **Determination of Ossification-related Phenotypic Traits**

The ossification degrees of duck sterna were quantized by determining and calculating six ossification-related phenotypic traits at weeks 7, 8, and 9. The traits are cartilage length of sternum body (CLSB), bone length of sternum body (BLSB), the percentage of BLSB (PBLSB) (BLSB divided by the total length of sternum body), cartilage length of posterolateral processes (CLPP), bone length of posterolateral processes (BLPP), and the percentage of BLPP (PBLPP) (BLPP divided by the total length of posterolateral processes). All measurements were taken according to Figure 1 by using an electronic vernier caliper. The data was obtained from the left half of each sternum and measured by the same operator to minimize error. The



**Figure 1.** Diagram of measuring ossification-related traits in duck sterna. Abbreviations: A, total length of sternum body; BLSB, bone length of sternum body; CLSB, cartilage length of sternum body; B, total length of posterolateral processes; BLPP, bone length of posterolateral processes; CLPP, cartilage length of posterolateral processes.

ossification degree of the keel was not analyzed, because its ossification extended in two directions and failed to be expressed by a length or proportion.

### Different Histological Stainings

Based on the results of above mentioned whole-mount skeletal staining, time point of histological staining was set as 7th wk to get a full and consecutive view of histological changes during duck sternal ossification process by sampling both the ossified and non-ossified regions of a sternum. Concretely, the paraffin sections of both the

cartilaginous zone and chondro-osseous junctional region of the sternum body were prepared from 3 female ducks at the age of 7 wk. The sections from the cartilaginous zone were detected using hematoxylin-eosin (HE), alcian blue, and toluidine blue stainings. The sections from the chondro-osseous junctional region were detected using HE staining. Thereafter, micrographs were taken using a microscope (Olympus, Tokyo, Japan) at magnifications of 100 × and 200 ×. Additionally, whole-slide images of HE sections from the chondro-osseous junctional region were taken using a digital scanning system (3DHISTECH, Budapest, Hungary).

### Determination of Ossification-related Genes Expression

By real-time PCR, the mRNA expression levels of four marker genes (*COL2A1*, *COL10A1*, *COL1A2*, *CTSK*) and three functional molecules (*COMP*, *MMP13*, *SPARC*) were detected during the age of 5 to 9 wk. These genes were selected due to their important roles in the different phases of ossification (Mackie et al., 2008). At each age, both the cartilage and bone samples were obtained from the sternum body of sterna of 6 female ducks. Total RNAs were isolated using the Trizol reagent (Takara, Dalian, China), and first-strand cDNAs were synthesized by using a reverse transcript system (Takara). Then, the mRNA expression levels were determined by a CFX96™ real-time system (Bio-Rad, Hercules, USA) using the Takara Ex Taq™ reagents (Takara). All reactions were repeated three times, and the identity of the amplified products was confirmed by sequencing (Huada Gene, Beijing, China). All raw data were normalized to *β-actin* (GenBank: EF667345) and *GAPDH* (GenBank: AY436595) by using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001). The primers were synthesized by Huada Gene (Beijing, China), and the primer information was listed in Table 1.

**Table 1.** Primer sets used for real-time PCR.

Gene	Primer sequence (5'-3')	Product size (bp)	Genbank accession number
<i>β-actin</i> <sup>1</sup>	Forward: GCTATGTCGCCCTGGATTTC Reverse: CACAGGACTCCATACCCAAGAA	98	EF667345
<i>GAPDH</i> <sup>1</sup>	Forward: GATGCTGGTGCTGAATACG Reverse: GGAGATGATGACACGCTTAG	102	AY436595
<i>COL2A1</i>	Forward: AGAAAGCCCTCCTCATCC Reverse: ATCCACGCCAAACTCCTG	218	XM_005012043.2
<i>COL10A1</i>	Forward: CAGGCAGCAACACTATGACC Reverse: TTGTAGAGTGCAACCCAAACA	117	XM_005025230.2
<i>COL1A2</i>	Forward: CTGGTAAAGATGGTCGCAATG Reverse: TGTTCAATGTTTTTCAGAGTGGC	234	XM_005010933.2
<i>CTSK</i>	Forward: GGTCTGTGTGTCGCCACG Reverse: GCCACCTCATCCGCCTCA	120	XM_005026764.2
<i>COMP</i>	Forward: CTGTTCCCTGGAGTGG Reverse: GGACCCGTTGCCTGAATAA	85	XM_013108759.1
<i>MMP13</i>	Forward: CCAGACTATGGAGGAGACGC Reverse: TTTAGGATGTTTTGGGTTTCG	261	XM_005010500.2
<i>SPARC</i>	Forward: CCATTTCTTCGCCACCAA Reverse: CGTTCTTCAGCCAGTCCC	152	XM_005010534.2

<sup>1</sup>In this study, *β-actin* and *GAPDH* are housekeeping genes for normalizing gene expression.

## Statistical Analysis

Statistical analyses were performed with SPSS 19.0 software (SPSS Inc., Chicago, USA), and the differences were considered to be significant at  $P < 0.05$ . Data were shown as the mean and SEM. By applying two-way ANOVA, differences in both the ossification-related phenotypic traits and ossification-related genes expression were analyzed. The following linear model was used:  $Y_{ijk} = \mu + a_i + b_j + (a \cdot b)_{ij} + e_{ijk}$ , where  $Y_{ijk}$  is the value of the analyzed trait or gene expression level,  $\mu$  is the overall mean of the analyzed trait or gene expression level,  $a_i$  is the effect of  $i$ -th age,  $b_j$  is the effect of  $j$ -th sex (for the trait) or tissue type (for the gene expression),  $(a \cdot b)_{ij}$  is the interaction between age and sex (for the trait) or tissue type (for the gene expression), and  $e_{ijk}$  is the random error. As a post hoc test, LSD test was employed to separate significantly different means. Furthermore, the correlations between *COMP*, *MMP13*, and *SPARC* mRNA expression levels and ossification-related genes expression were evaluated by the Pearson correlation coefficient.

## RESULTS

### Three Ossification Stages, Five OC, Ossification Directions, and Weekly Changes of Duck Sterna

As shown in Figure 2A, duck sterne kept unossified before the age of 5 wk. Direct observation and touch divided the sternal ossification into three stages (Figure 2B): unossified stage (0-5 weeks old), ossification start stage (5-6 weeks old), and ossification completion stage (6-9 weeks old).

Alcian blue and alizarin red staining results (Figure 3) had verified the above-mentioned three ossification stages. Moreover, Figure 3 showed five independent sternal OC and their fusion along with age. Two lateral longitudinal OC were found in the sternum body and located next to the sternal ribs, while another two OC were located at the proximal ends of posterolateral processes (Figure 3). One medial cranial OC was found in

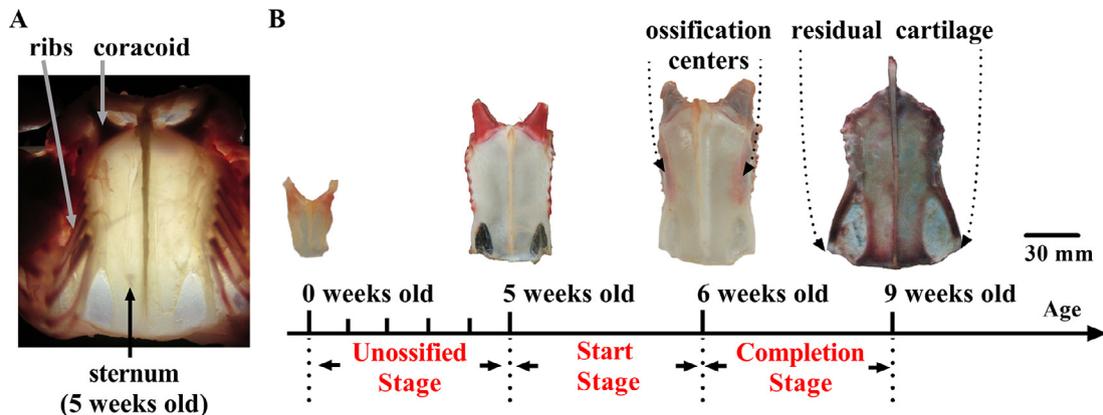
the dorsal part of the keel (Figures 3 and 4), and the two unossified regions of the keel (Figure 4) suggested that its ossification developed not only from cranial to caudal but also from dorsal to ventral.

By combining Figures 2-4 and observations in all sterne, we described the ossification directions of duck sterne based on the above-mentioned five sternal OC (Figure 5A) and summarized weekly changes in the three parts of sternum (sternum body, keel, posterolateral processes) during duck sternal ossification (Figure 5B).

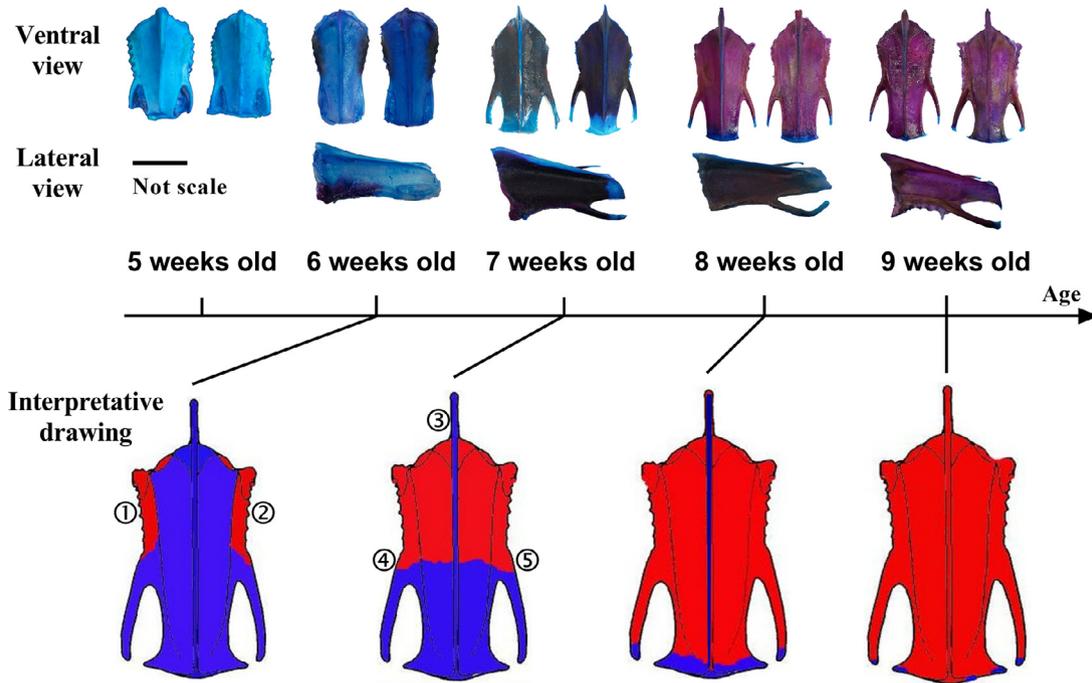
### Duck Sternal Ossification Differences Among Sexes and Different Parts of the Sternum

At each age, duck sterne could be divided into two or three types with different ossification degrees (Figure 6A). The individual difference was obvious at the age of 6 to 8 wk, but 82% male and 93% female duck sterne belonged to the same type at the age of 9 wk (Figure 6B). Compared with female ducks, much more sterne belonged to the types with lower ossification degrees in 6-8 wk old male ducks (Figure 6B). And this gender difference was greatly reduced at the age of 9 wk (Figure 6B).

Further quantitative analysis was performed by measuring six ossification-related phenotypic traits. As shown in Table 2, age, sex, and the interaction of age with sex all had extremely significant ( $P < 0.01$ ) effects on each trait. Compared with female ducks, the sterne of 7 and 8 weeks old male ducks had significant ( $P < 0.05$ ) lower ossification degree (PBLSB, PBLPP) and higher ossification potential (CLSB, CLPP) in both the sternum body and posterolateral processes (Table 2). And at the age of 9 wk, this gender difference was reduced (CLPP) or became insignificant (PBLSB, PBLPP, CLSB) (Table 2). Additionally, Table 2 also showed that at each age the ossification degrees of the sternum body (PBLSB) were higher than the posterolateral processes (PBLPP). PBLSB reached 99.48% and



**Figure 2.** Postnatal sternal ossification in ducks. (A) A 5-week-old duck sternum under white light. The coracoid and ribs are opaque, whereas the whole sternum is transparent. (B) The 3 stages of duck sternal ossification.



**Figure 3.** Alcian blue and alizarin red staining of duck sterna at the age of 5 to 9 wk. The ventral view and lateral view are displayed above the horizontal axis. The cartilage is stained blue and bone is stained purple-red. Meanwhile, corresponding interpretative drawings are displayed below the horizontal axis (blue indicates cartilage and red indicates bone). The sequence numbers in interpretative drawings represent the five independent sternal OC.

99.75% in male and female ducks at the age of 9 wk, whereas PBLPP was just 80.45% and 82.48%.

### Duck Sternal Developmental Changes in Histological Structure and Gene Expression

The histological changes that happened during duck sternal ossification were shown in Figure 7. Figure 7A-C displayed the single, small, and flat chondrocytes and uniformly dyed cartilage matrix. Figure 7D displayed the large, rounded, and closely arranged hypertrophic chondrocytes and marrow cavity with blood cells. Figure 7E showed the emergence of osteoblasts, osteoclasts, osteocytes, and pink-dyed bone matrix. Figure 7F showed the reduction of hypertrophic chondrocytes and cartilage matrix as well as the increase of osteocytes and



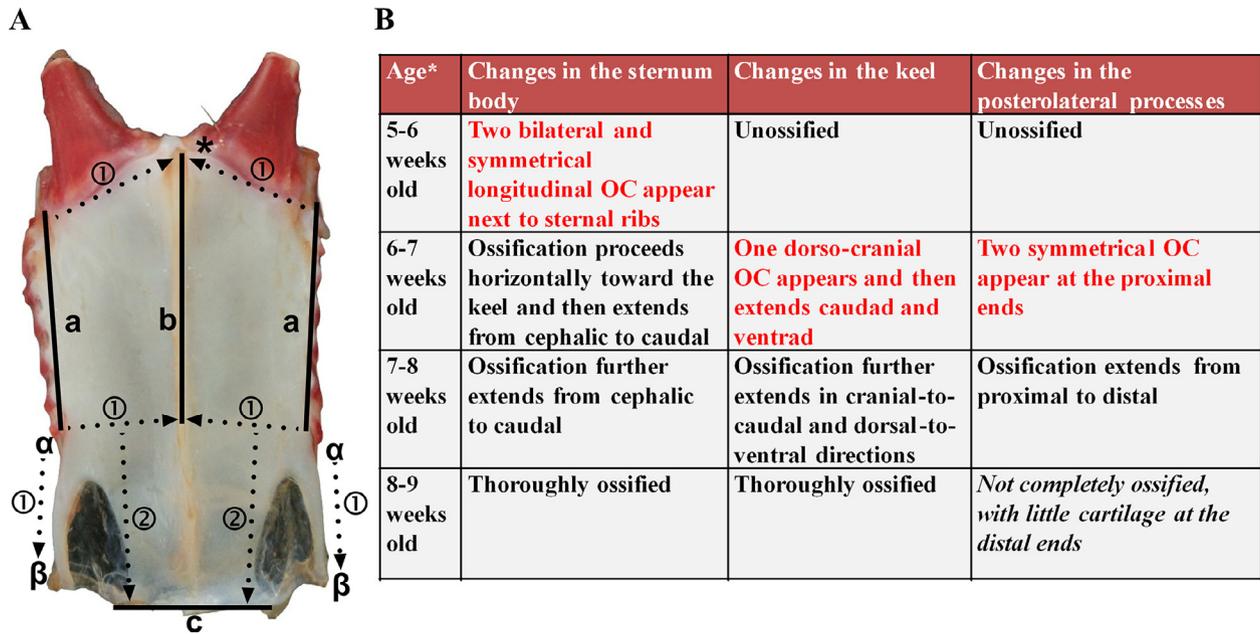
**Figure 4.** Alcian blue and alizarin red staining of a 7-wk-old duck sternum. \* indicates cartilage in the ventral part. \*\*\* indicates cartilage in the caudal end.

bone matrix. Figure 7G showed the forming of bone trabecula, which accompanied by the disappearance of hypertrophic chondrocytes and cartilage matrix. These histological changes could be found by comparing the panoramic image of the cartilaginous zone with that of the chondro-osseous junctional region (Figure 7H).

As shown in Figure 8, the mRNA expression levels of four marker genes (*COL2A1*, *COL10A1*, *COL1A2*, *CTSK*) and three functional molecules (*COMP*, *MMP13*, *SPARC*) were detected in the cartilage and bone samples obtained from female duck sterna. *COL2A1* and *COMP* were selectively and highly expressed in 5-8 weeks old sternal cartilage (Figure 8). *COL10A1* was predominantly expressed in 7 and 8 weeks old sternal cartilage and at the same time was moderately expressed in 5 and 6 weeks old sternal cartilage (Figure 8). *COL1A2* and *CTSK* were selectively and highly expressed in 7-9 weeks old sternal bone (Figure 8). *MMP13* and *SPARC* were continuously expressed in both the cartilage and bone (Figure 8). Moreover, further analysis found no significant correlation between *COMP* expression and other genes expression, and demonstrated that *MMP13* was co-expressed with *COL2A1* ( $r = 0.581$ ), *COL10A2* ( $r = 0.841$ ), and *CTSK* ( $r = 0.836$ ) in sternal bone, and *SPARC* was co-expressed with *COL2A1* ( $r = 0.730$ ) in sternal cartilage and was co-expressed with *COL1A2* ( $r = 0.760$ ) in sternal bone (Table 3).

## DISCUSSION

In this study, we explored the pattern of duck sternal ossification in both qualitative and quantitative ways



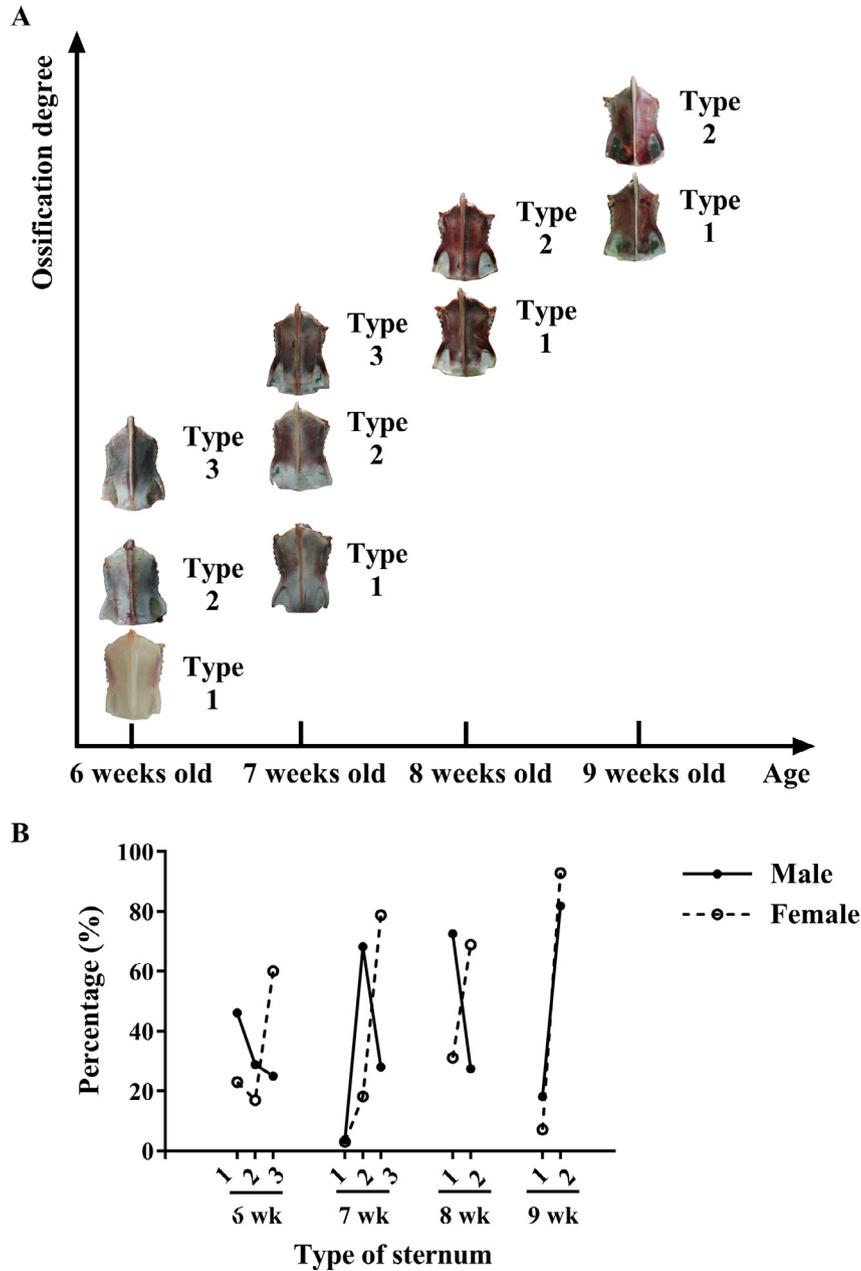
**Figure 5.** The ossification directions and weekly changes of duck sternal ossification. (A) Ossification directions in the sternum body, keel, and posterolateral processes. For the sternum body and posterolateral processes, arrows and sequence numbers indicate ossification directions and their order. \* represents that the keel ossifies along both the cranial-to-caudal and dorsal-to-ventral directions simultaneously. a, the lateral margins of sternum body; b, the midline of sternum body; c, the caudal end of sternum body;  $\alpha$ , the proximal ends of posterolateral processes;  $\beta$ , the distal ends of posterolateral processes. (B) Weekly changes in the sternum body, keel, and posterolateral processes. The appearances of OC are shown in red. \* represents that the changes at each age are on behalf of most sterna and at variance with the others.

and analyzed the sternal developmental changes in histological structure and gene expression. The earliest ossification region of duck sterna was observed at the age of 6 wk, then duck sternal ossification rapidly completed during 6-9 weeks old (Figures 2 and 3). Different from ducks, sternal ossification of chicken and quail (excluded the special rib-derived ossification) began just before hatching (Tickle et al., 2014; Poulis and Antonopoulos, 2019). Researchers have suggested that ontogenetic rate, either precocious or altricial, and external mechanical pressure were the influencing factors of skeletal ossification in birds (Blom and Lilja, 2004; Zheng et al., 2014). Chicken, quail, and duck were precocious and grew fastly, but duck sternum was much broader (Düzler et al., 2006) and thus able to reduce the external mechanical pressure during the early postnatal development. Meanwhile, the beginning of duck sternal ossification (5-6 wk) was close to the inflection point of breast muscle cumulative growth curve (male: 6.305 wk, female: 5.994 wk) (unpublished data), which was determined in the same breed. These results supported the hypothesis that duck sternal ossification may be promoted by the mechanical stress from breast muscle. Moreover, considering the known high correlation between breast muscle weight and keel length, we boldly assume that the indirect selection of sternal ossification degree at a fixed time point could contribute to more precocious breast muscle in duck. Further studies are needed to prove the assumptions.

From the qualitative perspective, our results found five independent OC of duck sterna and revealed their ossification directions during the age of 5 to 9 wk (Figures 3-5). The two symmetrical lateral OC in the

sternum body and their ossification directions in duck were consistent with ratites, but the appearance of another OC in the keel did not happen in ratites (Maxwell and Larsson, 2009). The sternum body and keel were ossified from different OC in duck, whereas they were developed from a single midline OC in the Galliformes (Atalgin et al., 2008; Poulis and Antonopoulos, 2019). Interestingly, OC in the keel of duck had unreported ossification directions as cranial-to-caudal and dorsal-to-ventral. The Chilean tinamou, a flighted and keeled paleognath, had three OC in the sternum body and keel, which were similar to those in duck sterna (Vega-Jorquera et al., 2019). However, the ossification direction of the keel of Chilean tinamou was simple, just from anterior to posterior (Vega-Jorquera et al., 2019). Meanwhile, the appearances of OC in the posterolateral processes did not happen in ratites (Maxwell and Larsson, 2009). The ossification of posterolateral processes extended from proximal to distal in duck, but in the Galliformes, it extended from the middle to both ends (Atalgin et al., 2008; Poulis and Antonopoulos, 2019). But, the three pairs OC in the posterolateral processes of Galliformes were driven by the ossification of ribs and were not true sternal ossification (O'Connor et al., 2015). These comparisons suggested that duck sternal ossification is different from and more complicated than the previously studied living birds.

From the quantified perspective, our results showed gender and regional differences in the ossification degrees of duck sterna (Figures 5 and 6, Table 2). The male duck sterna were more late-maturing than the female. This was in line with the law of individual development in poultry. Notably, ossifications in the



**Figure 6.** Ossification differences in duck sterna. (A) Different types of sterna and their ossification degrees. (B) The proportions of different types of sterna in male and female ducks. “Type 1”, “Type 2”, and “Type 3” in this figure are equivalent to the types showed in panel A.

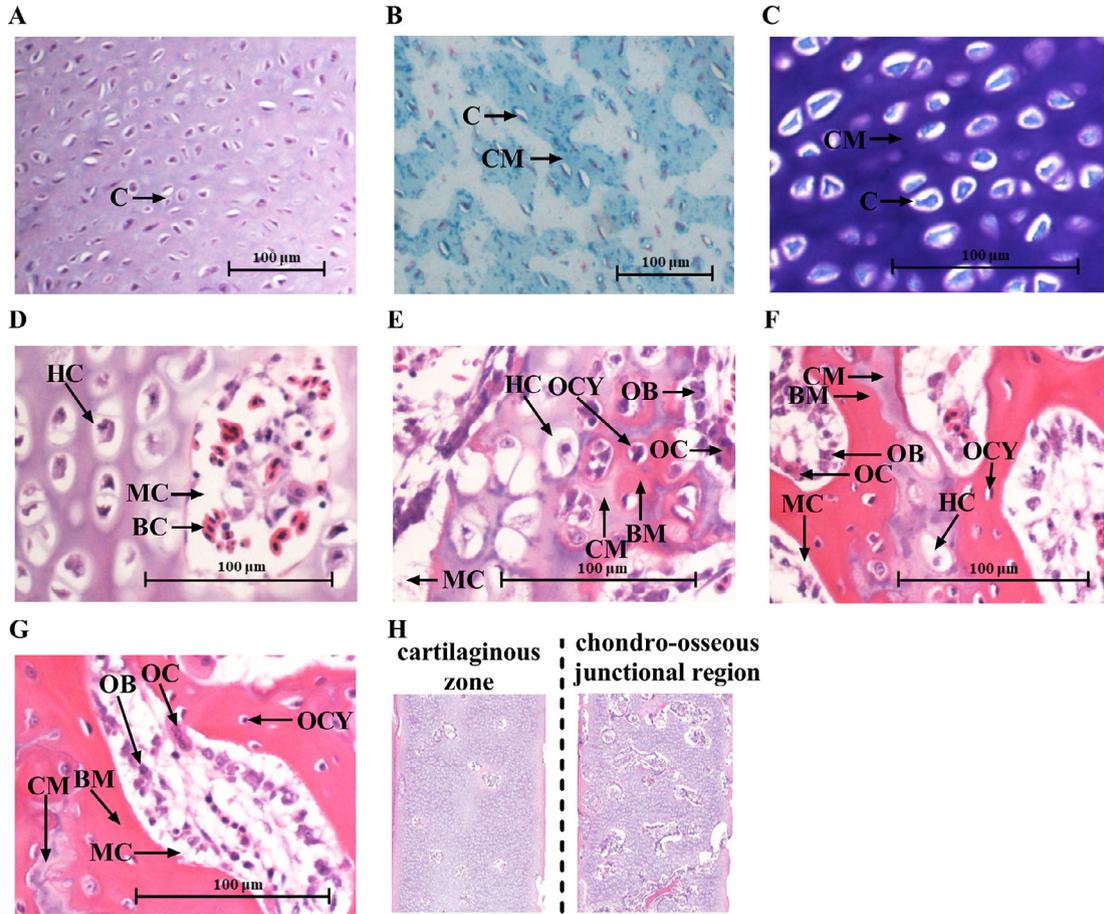
**Table 2.** Effect of age and sex on the ossification-related traits of duck sterna.

Item	7-week-old		8-week-old		9-week-old		SEM	P value		
	Male	Female	Male	Female	Male	Female		Age	Sex	A*S <sup>1</sup>
CLSB (cm)	2.87 <sup>a</sup>	1.83 <sup>b</sup>	0.58 <sup>c</sup>	0.17 <sup>d</sup>	0.08 <sup>d</sup>	0.03 <sup>d</sup>	0.11	<0.001	<0.001	<0.001
BLSB (cm)	9.93 <sup>e</sup>	10.36 <sup>d</sup>	13.07 <sup>b</sup>	12.66 <sup>c</sup>	13.92 <sup>a</sup>	12.99 <sup>b,c</sup>	0.13	<0.001	0.004	<0.001
PBSLB (%)	77.48 <sup>d</sup>	84.56 <sup>c</sup>	95.73 <sup>b</sup>	98.67 <sup>a</sup>	99.48 <sup>a</sup>	99.75 <sup>a</sup>	0.94	<0.001	<0.001	0.001
CLPP (cm)	2.29 <sup>a</sup>	1.76 <sup>b</sup>	1.41 <sup>c</sup>	1.16 <sup>d</sup>	0.79 <sup>e</sup>	0.65 <sup>f</sup>	0.03	<0.001	<0.001	<0.001
BLPP (cm)	1.37 <sup>c</sup>	1.72 <sup>d</sup>	2.49 <sup>c</sup>	2.51 <sup>c</sup>	3.22 <sup>a</sup>	3.07 <sup>b</sup>	0.04	<0.001	0.005	<0.001
PBLPP (%)	37.15 <sup>e</sup>	49.18 <sup>d</sup>	63.86 <sup>c</sup>	68.42 <sup>b</sup>	80.45 <sup>a</sup>	82.48 <sup>a</sup>	0.87	<0.001	<0.001	<0.001

<sup>a-f</sup>Means within a row with no common superscript differ significantly ( $P < 0.05$ ).

<sup>1</sup>A\*S represents the interaction of age and sex.

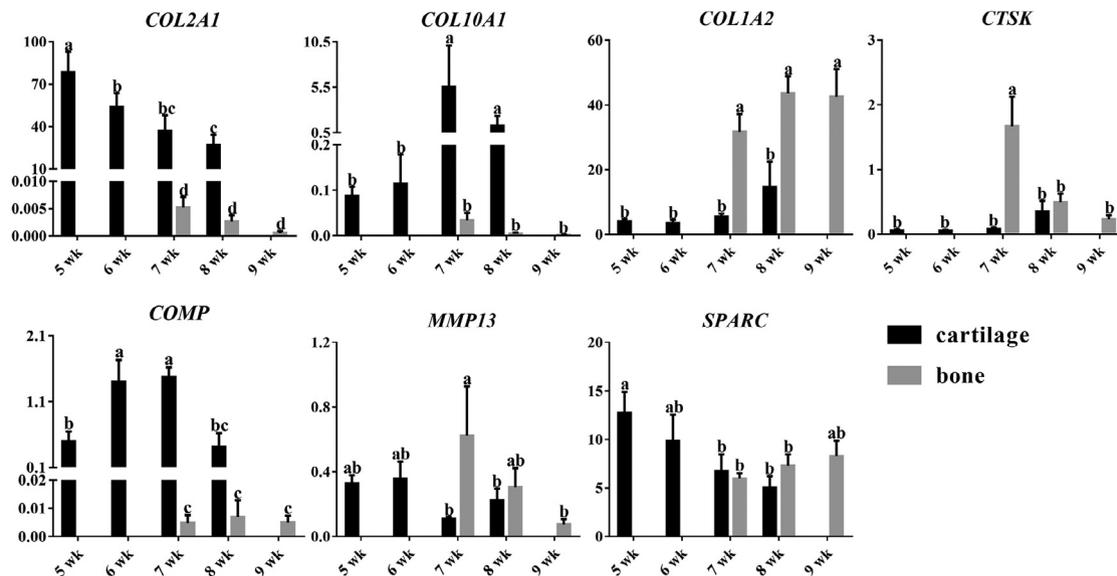
Abbreviations: CLSB, cartilage length of sternum body; BLSB, bone length of sternum body; PBSLB, the percentage of BLSB (divided by the total length of sternum body); CLPP, cartilage length of posterolateral processes; BLPP, bone length of posterolateral processes; PBLPP, the percentage of BLPP (divided by the total length of posterolateral processes).



**Figure 7.** Histological changes during duck sternal ossification. (A–C) Hematoxylin-eosin (HE), alcian blue and toluidine blue stainings of the cartilaginous zone. (D–G) HE stainings of the chondro-osseous junctional region with gradually increasing ossification degrees. (H) Panoramic images of HE stainings of the cartilaginous zone and chondro-osseous junctional region. Abbreviations: C, chondrocytes; CM, cartilage matrix; HC, hypertrophic chondrocytes; MC, marrow cavities; BC, blood cells; OB, osteoblasts; OC, osteoclasts; OCY, osteocytes; BM, bone matrix.

three different parts of duck sterna (sternum body, keel, posterolateral processes) were mutually independent in space and different in time. This was in line with the origin of avian sterna from several homologous elements (Zheng et al., 2012) and suggested

functional differences among the three parts. Also, these results supported that future improvements on the ossification degrees of the sternum body, keel, and posterolateral processes should be implemented independently.



**Figure 8.** Relative mRNA expression levels of seven ossification-related genes in duck sterna. Data are presented as mean value  $\pm$  SEM ( $n = 6$ ). Values without the same letter (a-d) represent statistically significant differences ( $P < 0.05$ ).

**Table 3.** The correlation of *COMP*, *MMP13*, and *SPARC* expression with other genes expression in duck sterna.

Samples	Genes	Pearson correlation coefficients		
		<i>COMP</i>	<i>MMP13</i>	<i>SPARC</i>
Sternal cartilage (n = 24)	<i>COL2A1</i>	-0.088	0.195	<b>0.730*</b>
	<i>COL10A1</i>	0.091	-0.258	-0.294
	<i>COL1A2</i>	-0.295	0.125	0.001
	<i>CTSK</i>	-0.330	0.152	-0.279
	<i>COMP</i>	1	0.215	-0.122
	<i>MMP13</i>	0.215	1	0.241
	<i>SPARC</i>	-0.122	0.241	1
Sternal bone (n = 18)	<i>COL2A1</i>	0.208	<b>0.581*</b>	-0.138
	<i>COL10A1</i>	-0.073	<b>0.841*</b>	-0.195
	<i>COL1A2</i>	0.232	-0.018	<b>0.760*</b>
	<i>CTSK</i>	-0.144	<b>0.836*</b>	-0.287
	<i>COMP</i>	1	-0.239	-0.118
	<i>MMP13</i>	-0.239	1	-0.101
	<i>SPARC</i>	-0.118	-0.101	1

\*Represents the Pearson correlation coefficients are significant ( $P < 0.05$ ).

There are two ways of ossification, one is intramembranous ossification and another is endochondral ossification. In mammals, it was confirmed that the sternum was formed according to the endochondral ossification (Eydtt et al., 2015). Histological results in this study (Figure 7) also demonstrated that duck sternal ossification was conformed to canonical endochondral ossification, which including changes in the cells, extracellular matrixes, and marrow cavities (Mackie et al., 2008). At the molecular level, we detected the expression of four marker genes (*COL2A1*, *COL10A1*, *COL1A2*, *CTSK*) and three functional molecules (*COMP*, *MMP13*, *SPARC*) in the sternal cartilage and sternal bone samples throughout 5-9 weeks old (Figure 8, Table 3). *COL2A1*, *COL10A1*, *COL1A2*, and *CTSK* were considered as markers of proliferative cartilage, hypertrophic chondrocytes, osteoblasts, and osteoclasts, respectively (Rossert et al., 2000; Rieman et al., 2001; Riemer et al., 2002; McAlinden et al., 2008). Similarly, our results showed that *COL2A1* selectively expressed in sternal cartilage, *COL10A1* predominantly expressed in 7 and 8 weeks old sternal cartilage and moderately expressed in 5 and 6 weeks old sternal cartilage, and *COL1A2* and *CTSK* selectively expressed in sternal bone. This again demonstrated that duck sternal ossification was endochondral ossification. Our results also showed that *MMP13* was co-expressed with *COL2A1*, *COL10A2*, and *CTSK* (in bone), and *SPARC* was co-expressed with *COL2A1* (in cartilage) and *COL1A2* (in bone). The low expression levels of *COL2A1* and *COL10A2* in sternal bone were not meaningful. Matrix metalloproteinases and cysteine proteases were two major classes of proteases to finish the degradation of extracellular matrix during ossification process (Hohenester and Engel, 2002; Lecaille et al., 2008). *CTSK* encoded cathepsin K represented 98% of the total cysteine protease activity, and *MMP13* encoded peptidase was one of the main types (Hohenester and Engel, 2002; Lecaille et al., 2008). Their co-expression suggested functional cooperation of *MMP13* and *CTSK* in duck sternal ossification. *SPARC* was required for the calcification of

collagens, the synthesis of extracellular matrix, and the changes of cell shape in endochondral ossification (Brekken and Sage, 2000). And, previous studies have reported the interaction of *SPARC* with both *COL2A1* and *COL1A2* (Termine et al., 1981; Sage et al., 1989). Therefore, *MMP13* and *SPARC* may be particularly important in duck sternal ossification. Further studies could be designed to establish phenotypic correlations between this two genes and sternal ossification in poultry, which provides basis for eliminating the inadequate sternal ossification and related disease.

In conclusion, for the first time, the present study revealed the pattern of duck sternal ossification, including its timeline, the location and ossification directions of OC, the weekly changes of different parts of the sternum, and quantified differences in ossification degree among sexes and parts. This ossification occurred under the histological and gene expression changes that were highly similar to those of typical endochondral ossification, and it may be closely related to the expression of *MMP13* and *SPARC*. These results would provide useful information for further researches on avian sternal ossification and underlying mechanisms, and provide a new perspective for improving the productive performance and welfare of poultry.

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## DISCLOSURES

The authors declare no conflict of interest regarding the publication of this article.

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