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Effects of *Pax3* and *Pax7* expression on muscle mass in the Pekin duck (*Anas platyrhynchos domestica*)

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ABSTRACT. This study aimed to investigate whether the differential expression of muscle development-related genes is one of the reasons why muscle development differs between Pekin, Jianchang, and Heiwu ducks, which are all domesticated duck breeds (*Anas platyrhynchos domestica*) breeds. At 2 weeks of age, the RNA expression of paired box 7 (*Pax7*), paired box 3 (*Pax3*), myogenic differentiation antigen (*MYOD*), and myogenin (*MYOG*) genes were measured by quantitative polymerase chain reaction, and *Pax3* and *Pax7* protein levels were detected by western blot assay. Myofiber morphology was investigated using paraffin-embedded muscle sections. At 8 weeks of age, 30 ducks of each breed were slaughtered for meat quality determination. The results revealed that *Pax3* and *Pax7* expression levels at both the RNA and protein levels were high in the Pekin duck. In addition, *MYOG* expression levels in the Jianchang duck were significantly higher than in the other two duck breeds ($P < 0.05$). There were no significant differences in *MYOD* expression levels between the breeds ($P > 0.05$).

Myofiber diameter and cross-sectional area were the largest in the Pekin duck and the smallest in the Heiwu duck. There were significant differences in slaughter data between these breeds, and muscle content was greatest in the Pekin duck. The results indicate that the muscle content of three different duck breeds is associated with the expression of satellite-cell marker genes.

Key words: *Pax3*; *Pax7*; Muscle content; Pekin duck; Jianchang duck; Heiwu duck

INTRODUCTION

Skeletal muscle is an important factor in animals of economic significance. Skeletal muscle fibers are formed during embryogenesis, after which the amount of muscle fiber will not change (Du et al., 2010). The process of muscle development can be divided into several distinct phases (Abou-Khalil et al., 2009). During embryonic myogenesis, mesoderm-derived structures generate the first muscle fibers of the body, after which additional fibers are generated along these template fibers (Tajbakhsh, 2009). During the perinatal phase, muscle progenitors firstly proliferate diffusely, but decrease when the number of myonuclei reaches a steady state and myofibrillar protein synthesis reaches a maximum (Davis and Fiorotto, 2009). Once the muscle has matured, these progenitors will enter quiescence, and henceforth stay within it as satellite cells. It is now generally accepted that satellite cells are closely related to the progenitors of somatic origin (Schienda et al., 2006; Lepper and Fan, 2010). Adult skeletal muscle, like all renewing organs, relies on a mechanism to maintain tissue homeostasis (Schmalbruch and Lewis, 2000). This kind of myogenesis depends on the activation of satellite cells that have the potential to differentiate into new fibers (Charge and Rudnicki, 2004). When mature muscle is damaged, the majority of satellite cells increase mitosis and differentiate to repair the tissue and reestablish homeostasis (Kuang et al., 2008).

Satellite cells are also responsible for postnatal muscle growth, hypertrophy, and regeneration. In mature muscle, most satellite cells are in a quiescent state, but they can activate and begin proliferating in response to extrinsic signals (Wozniak and Anderson, 2007). Following activation, their proliferating progeny, the skeletal myoblasts, express the paired-box transcription factors Pax7 and Pax3, as well as the myogenic regulatory factors Myf5 and MYOD. Once committed to differentiation, myoblasts stop cycling and cease Pax7, Pax3, and Myf5 expression. Differentiating muscle cells will then align and fuse to form multinucleated myofibers. MRF4 is required for the hypertrophy of the new fibers (Le Grand and Rudnicki, 2007). Some of the satellite cells' progeny return to a quiescent state during the process of self-renewal (Collins et al., 2005; Montarras et al., 2005). This process is tightly regulated by growth factors, and by the expression of key transcriptional regulators such as Pax7, Pax3, and myogenic regulatory factors (Bentzinger et al., 2012, 2013; Yin et al., 2013).

Current opinion is that all satellite cells express the transcription factor Pax7 (Seale et al., 2000; Kuang et al., 2006). Extensive analyses of mice carrying null mutations in Pax7 have confirmed the progressive loss of satellite cells in skeletal muscle, resulting in severe muscle atrophy and death. Recent studies have demonstrated that Pax7 is a crucial requirement for satellite cell function in adult skeletal muscle. Following Pax7 deletion, satellite cells and myoblasts exhibit cell-cycle arrest and the dysregulation of myogenic regulatory factors.

Maintenance of Pax7 deletion through continuous tamoxifen administration prevents the regrowth of Pax7-expressing satellite cells and a serious muscle regeneration deficit (Seale et al., 2000). Primary myoblasts, in which Pax7 has been deleted, exhibit a reduction of almost 75% in the levels of Myf5 mRNA, a 25% reduction in MYOD expression, and no change in MYOG expression levels (Zammit et al., 2006). Many studies have shown that Pax7 is essential for regulating the expansion and differentiation of satellite cells during adult myogenesis. Pax3 is expressed in a subset of quiescent satellite cells, and can be detected after activation in most skeletal myoblasts. However, its role in muscle regeneration is unclear, because Pax3-null mice die in utero, and no conditional mutation of Pax3 in adults has been described (Relaix et al., 2006).

There is a large difference in the production properties of the Pekin, Jianchang, and Heiwu duck breeds (*Anas platyrhynchos domestica*). Muscle content is highest in the Pekin duck and lowest in the Heiwu duck. Skeletal muscle development and growth may be related to satellite-cell marker genes (Pax3 and Pax7) and myogenic regulatory factors (MYOD and MYOG), which are affected by Pax3 and Pax7. This study aimed to investigate the association between the expression levels of the above genes and muscle development in different breeds of the domestic duck.

MATERIAL AND METHODS

Animals and sampling

Ducks were obtained from the Sichuan Agricultural University Waterfowl Breeding Experimental Farm, and maintained under conditions that complied with the Beijing Animal Welfare Committee. Three ducks of each breed were randomly selected and slaughtered by rapid bloodletting at 2 weeks of age. The leg muscle of each individual was removed and weighed. Some of the samples were fixed in 4% formalin to generate paraffin sections, and the other tissue samples were frozen in liquid nitrogen for RNA and protein extraction. Thirty ducks of each breed were randomly selected and slaughtered for meat quality determination at 8 weeks of age.

Histological analysis

The fixed samples were dehydrated by a series of ethanol dilutions, from a low concentration (75%) to a high concentration (100%), treated with xylene, and then embedded in paraffin. Paraffin blocks were sectioned (6 μm) along the horizontal axis of the sample. The sections were then stained with hematoxylin and eosin (H&E) and observed through a microscope (100X) (Nikon, Tokyo, Japan). Sections were obtained from three individuals of each breed, and at least five photographic images of each section were taken.

RNA extraction, cDNA synthesis, and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using a TRIzol reagent (TaKaRa, Otsu, Japan) following the manufacturer protocol. A 4-μL aliquot of total RNA was used to synthesize first-strand cDNA using a reverse transcription kit, following the manufacturer protocol (TaKaRa). The newly synthesized cDNA product was immediately stored at -20°C for later analysis.

Expression levels of *MYOD*, *MYOG*, *Pax3*, and *Pax7* were detected by qPCR using an IQ™ 5 System (Bio-Rad, USA). β-actin and glyceraldehyde 3-phosphate dehydrogenase were used as internal reference genes. The primers are listed in Table 1. The qPCR consisted of 3 min of pre-denaturation at 95°C, 40 cycles of denaturation at 95°C for 30 s, and 60°C for 40 s. Each sample was repeated three times. The relative expression levels of all of the genes of interest were calculated using GeNorm algorithms, which were based on the geometric means of the two reference genes.

Table 1. Sequence of primers designed for real-time polymerase chain reaction.

Gene	Primer sequence (5'→3')	Product length (bp)	Annealing temperature (°C)	GenBank accession No.
Pax3	Forward: GTCAATCAGCTCGGAGGAGT Reverse: TCTCCTGGTACCTGCAGAGA	133	59	JQ070187
Pax7	Forward: GAGTTCAGGTGTGGTTCAGCA Reverse: GAAATGGTGGTGGTTGGGTAG	169	60	JQ070188
MYOD	Forward: GCAACGCCATCCGCTACAT Reverse: GCAATCAAGGCTGGAAACAACA	85	55	FJ374143
MYOG	Forward: CGGATCACCTCCTGCCTGA Reverse: CGTCCTCTACGGCGATGCT	87	60	GQ303573
β-actin	Forward: GCTATGTCGCCCTGGATTTC Reverse: CACAGGACTCCATACCCAAGAA	168	55	EF667345
GAPDH	Forward: AAGGCT GAGAATGGGAAAC Reverse: TTCAGGGACTTGTCATACT TC	254	50	AY436595

Western blot

Tissue proteins were extracted from the leg muscle using a tissue protein extraction kit (BestBio, China). Proteins were separated on 10% SDS-PAGE at 100 V for between 1.5 and 2 h, and were then transferred to a nitrocellulose membrane (Beyotime, China) at 25 V for between 20 and 30 min. The membrane was incubated in a blocking solution (Beyotime) for 2 h at 37°C, and stained with a primary antibody at a dilution of 1:1000 at 4°C for 12 h, before being washed four times with TBS-Tween for 10 min. Finally, the membrane was incubated with a secondary antibody at a dilution of 1:1000 at 37°C for 2 h, washed four times with TBS-Tween for 10 min, and twice with TBS for 5 min. An anti-β-Tubulin antibody (Bioss, China) was used as a control reference. Immune-reactive proteins were observed using a 3,3'-diaminobenzidine tetrahydrochloride horseradish peroxidase color development kit (Beyotime).

Statistical analysis

All of the statistical results are reported as means ± SE. The data were subjected to analyses of variance, and significant differences between the means were assessed using the Duncan test. All of the statistical analyses were conducted in SPSS (version 20).

RESULTS

Gene expression at the RNA level

There was no significant difference in *MYOD* expression levels between the three duck breeds ($P > 0.05$; Figure 1). *MYOG* expression levels in the Jianchang duck were significantly higher than those in the Pekin or Heiwu ducks ($P < 0.05$). The expression levels of *Pax7*

were significantly higher in the Pekin and Jianchang ducks than in the Heiwu duck ($P < 0.05$), and the expression levels of *Pax3* were significantly higher in the Pekin duck than in either the Jianchang or Heiwu ducks ($P < 0.05$).

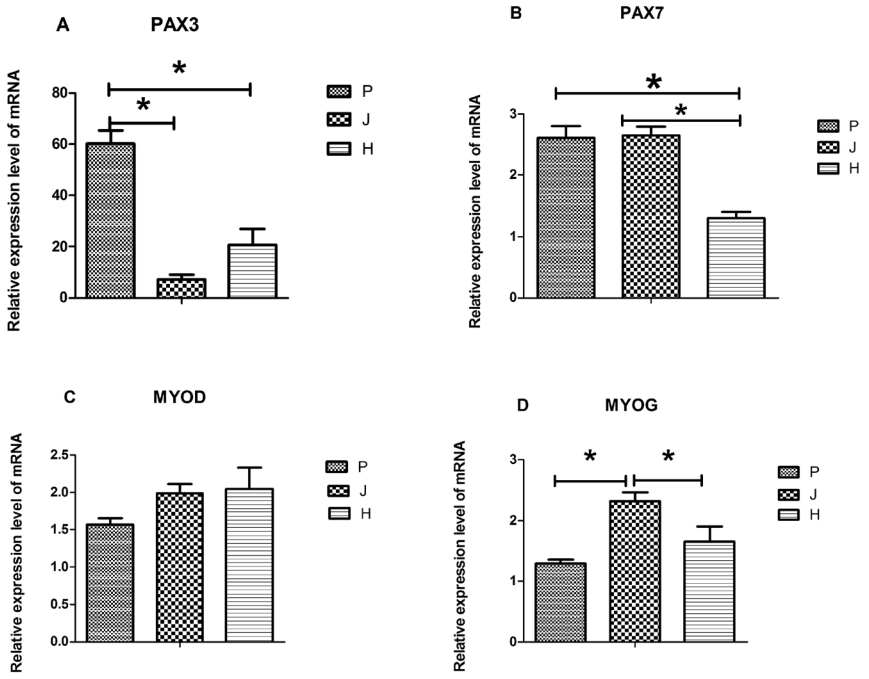

Figure 1. Leg muscle expression levels of *Pax3* (**A**), *Pax7* (**B**), *MYOD* (**C**), and *MYOG* (**D**) mRNA in three domestic duck breeds at 2 weeks of age. The mRNA expression patterns of *Pax3*, *Pax7*, *MYOD*, and *MYOG* were analyzed using quantitative polymerase chain reaction. Data are reported as means ± SE. *Significant difference between groups ($P < 0.05$). P = Pekin; J = Jianchang; H = Heiwu.

Gene expression at the protein level

The protein expression levels of *Pax3* and *Pax7* were similar. Protein expression levels were highest in the Pekin duck and lowest in the Heiwu duck (Figure 2). But there were no significant differences ($P > 0.05$).

Leg muscle morphology

The paraffin sections revealed myofiber hypertrophy at 2 weeks of age (Figure 3A). There were no significant differences in myofiber diameter (MFD), cross-sectional area (CSA), or the number of myofibers per μm^2 (MFN) between the three duck breeds (Figure 3B, C, and D), but the MFD and CSA values tended to be highest in the Pekin duck and lowest in the Heiwu duck. The MFN values obtained were inversely proportional to the MFD and CSA values.

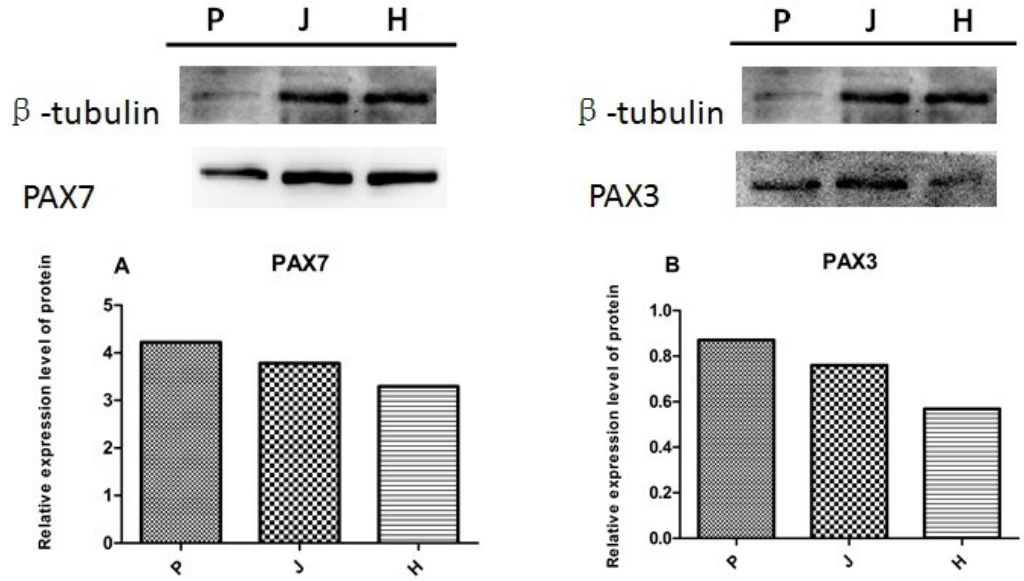

Figure 2. Western blot analysis of leg muscle *Pax7* (**A**) and *Pax3* (**B**) in three domestic duck breeds at 2 weeks of age. β-tubulin was used as a reference protein for the protein expression levels of *Pax3* and *Pax7*. P = Pekin; J = Jianchang; H = Heiwu.

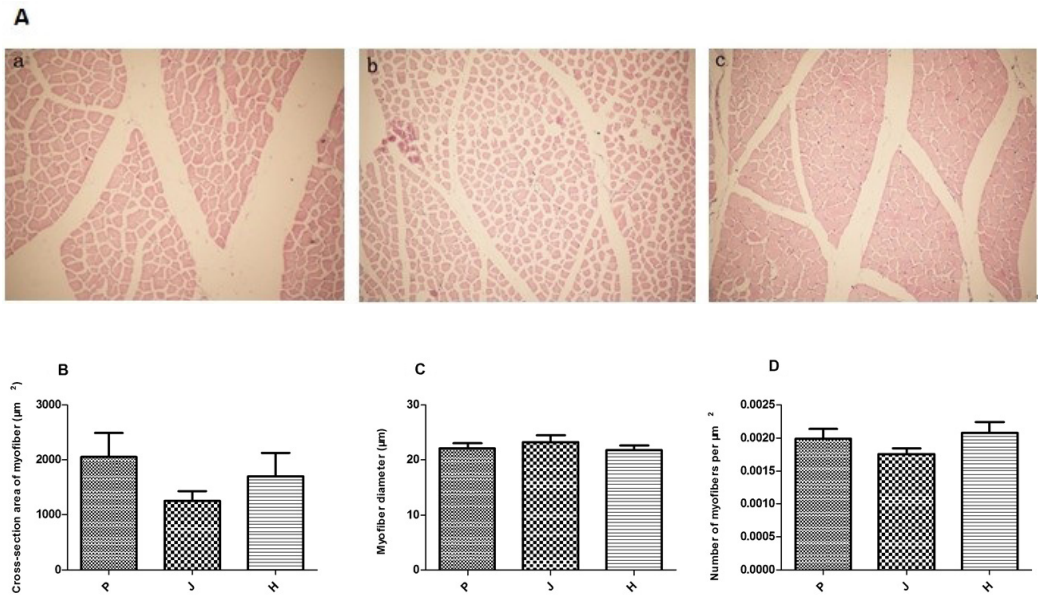

Figure 3. Muscle fiber sections from the leg muscle of three domestic duck breeds. **A.** Muscle section stained with H&E 2 weeks post-hatching: **a** is a cross-section from the Pekin (P) duck, **b** is a cross-section from the Jianchang (J) duck, and **c** is a cross-section from the Heiwu (H) duck (10 x 10). **B.** Myofiber cross-sectional area. **C.** Muscle fiber diameter in the leg muscle. **D.** Number of myofibers per μm^2.

Slaughter trait data in three duck breeds at 8 weeks

There were significant differences at slaughter traits between the three duck breeds at 8 weeks of age ($P < 0.05$; Table 2).

Table 2. Slaughter trait data of three domestic duck breeds at 8 weeks of age.

	P	J	H
Live weight (kg)	2.97 ± 0.05[a]	2.51 ± 0.01[b]	1.62 ± 0.03[c]
Dressed weight (kg)	2.65 ± 0.04[a]	2.22 ± 0.01[b]	1.32 ± 0.03[c]
Dressed percentage (%)	89.27 ± 0.51[a]	88.32 ± 0.20[a]	86.01 ± 0.73[b]
Half-eviscerated weight (kg)	2.42 ± 0.04[a]	2.03 ± 0.10[b]	1.20 ± 0.29[c]
Percentage of half-eviscerated weight (%)	81.91 ± 0.43[a]	81.30 ± 0.17[a]	76.43 ± 1.41[b]
Eviscerated weight (kg)	2.20 ± 0.039[a]	1.84 ± 0.009[b]	1.09 ± 0.24[c]
Percentage of eviscerated weight (%)	74.80 ± 0.49[a]	73.76 ± 0.15[a]	70.87 ± 1.39[b]
Leg muscle weight (g)	239.65 ± 4.50[a]	214.95 ± 2.03[b]	133.78 ± 3.54[c]
Breast muscle weight (g)	309.11 ± 7.79[a]	209.93 ± 1.65[b]	122.67 ± 5.42[c]
Percentage of leg muscle (%)	10.88 ± 0.16[b]	11.63 ± 0.10[a]	12.21 ± 0.32[a]
Percentage of breast muscle (%)	14.01 ± 0.25[a]	11.34 ± 0.08[b]	10.97 ± 0.37[b]

Data are reported as means ± SE. [a,b,c]Different letters in the same row indicate a significant difference ($P < 0.05$). P = Pekin; J = Jianchang; H = Heiwu.

DISCUSSION

The slaughter data confirmed that the muscle content differed significantly between the three duck breeds. The paraffin sections showed that the myofiber diameter and cross-sectional area were largest in the Pekin duck and smallest in the Heiwu duck, but were not significantly different. We found a difference in myofiber hypertrophy at post-hatching, and the leg muscle morphology results were in accordance with the slaughter trait data for all three duck breeds.

Recent reports have shown that satellite cells play a major role in muscle hypertrophy. These cells represent the oldest known adult stem cells, and are involved in the normal growth of muscle and the regeneration process (Ropka-Molik et al., 2011). Quiescent satellite cells express *Pax3* and *Pax7*, and after activation *Pax7* and *MYOD* genes are co-expressed. The activated cells ($Pax7^+/MYOD^+$) then proliferate, differentiate, and finally integrate with myotubes or form new myotubes (Zammit et al., 2002; Patruno et al., 2008). Proliferating cells that express *Pax7* but lack the *MYOD* gene ($Pax7^+/MYOD^-$) return to a quiescent state. Furthermore, *MYOD* and *MYOG*, which belong to a family of muscle-regulatory factors, play a major role in muscle growth and development (Tapscott, 2005; Knapp et al., 2006). The functions of *Pax3*, *Pax7*, *MYOD*, and *MYOG* are closely connected. Therefore, they are considered candidate genes for meat production traits.

The functions of the above genes have been revealed through studies on mutant mice. Mice with a knockout *Pax7* gene have a low number of satellite cells and exhibit poor muscle regeneration. In addition, knockout *Pax7* mice have lower muscle mass after birth, and are smaller and have only a few, small myofibers compared to controls. *Pax3*, a paralog of *Pax7*, is critical for the delamination and migration of somatic muscle progenitor cells to the limbs, as *Pax3* mutant mice lack limb muscles (Tajbakhsh et al., 1997). Analogously, mice lacking *MYOD*, *Myf5*, or *Myf6* do not have skeletal muscle at birth (Kassar-Duchossoy et al., 2004). *MYOG* is best known for regulating skeletal muscle development during the embryonic and fetal stages, and is required for embryonic myoblast differentiation. *MYOG*-null mice die at birth, because of severe skeletal muscle deficiency (Flynn et al., 2010).

We found that the muscle phenotypes of Pekin, Jianchang, and Heiwu ducks were significantly different. Our results show that *Pax7* and *Pax3* were highly expressed in the Pekin duck at the mRNA level, and its muscle mass was the highest among the three duck breeds. *MYOD* expression at the mRNA level was not significantly different between the ducks, but *MYOD* mRNA expression exhibited a decreasing trend, from a peak in the Heiwu duck to intermediate levels in the Jianchang duck to the lowest levels in the Pekin duck. This result suggests that high *Pax7* expression and low *MYOD* expression in the Pekin duck is associated with the presence of a large number of quiescent satellite cells (Ropka-Molik et al., 2011), and is consistent with the view that muscle growth is largely attributed to a population of cells that are resident in adult skeletal muscle, and are referred to as satellite cells (Dhawan and Rando, 2005). Previous studies have reported that mice that do not have *Pax7* exhibit a paucity of satellite cells and exhibit reduced postnatal muscle growth; this may provide a possible explanation for the fact that the Pekin duck, which exhibited high expression levels of *Pax7* in skeletal muscle, had higher muscle mass than the Jianchang and Heiwu ducks. In our study, *MYOG* mRNA expression levels in the Jianchang duck were much higher than those in the other two duck breeds ($P < 0.05$); this expression pattern did not overlap with the *Pax7* transcript level profile. *MYOG* is required for skeletal muscle development during embryonic and fetal life, but its role in adult skeletal muscle is unclear. *MYOG* plays a critical role as a high-level transcriptional regulator, and controls energy balance in adult skeletal muscle (Flynn et al., 2010). In light of the results of our study and previous studies, it is possible that *MYOG* is important for regulating exercise capacity rather than for muscle development in adult skeletal muscle.

At the protein level, the expression levels of *Pax3* and *Pax7* followed a similar pattern: they were highest in the Pekin duck, intermediate in the Jianchang duck, and lowest in the Heiwu duck. The fact that the expression levels of *Pax3* and *Pax7* at both the mRNA and protein levels were high in the Pekin duck suggests that *Pax3* and *Pax7* are highly expressed in breeds with a high muscle content. The increases in *Pax3* and *Pax7* mRNA and protein expression levels in the Pekin duck may provide a possible mechanism for the greater increase in muscle content in this breed compared to that in Jianchang and Heiwu ducks. However, *Pax3* expression levels at the protein and mRNA levels exhibited different patterns. The inconsistency of this gene's mRNA and protein expression levels suggests that the relationship between mRNA and protein expression levels is not strictly linear. Different regulation mechanisms that act on both synthesized mRNA and synthesized protein, such as synthesis and degradation rates, affect the amounts of the two molecules differently.

In conclusion, the expression levels of satellite-cell marker genes (*Pax3* and *Pax7*) and *MYOG* in the leg muscle were significantly different between the three duck breeds at 2 weeks of age. Further studies of gene expression in different duck breeds could reveal candidate genes associated with muscle development. Until now, only a few studies on the expression levels of *Pax3*, *Pax7*, *MYOD*, and *MYOG* in different duck breeds have been conducted. Investigating the expression patterns of satellite-cell marker genes and major myogenic regulatory factors may further our knowledge of the genetic basis of the postnatal myogenesis process, and its effects on muscle development. We suggest that *Pax3*, *Pax7*, *MYOD*, and *MYOG* may be contributing factors in the adult-specific muscle properties of these three duck breeds. High expression levels of *Pax3* and *Pax7*, in particular, may be one of the reasons why the Pekin duck has a higher muscle mass than other breeds.

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