Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X 2021 / v.23 / n.4 / 001-006

http://dx.doi.org/10.1590/1806-9061-2021-1468

Original Article

Effect of Supplementary Methionine on Feather Growth and Related Indicators of Pigeon Squabs

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■Keywords

Feather growth, IGF-1, methionine supplementation, squabs, White King pigeon.

Submitted: 04/March/2021
Approved: 07/June/2021

ABSTRACT

We assessed the effect of health sand dietary supplementation with methionine (Met) on White King pigeons. Paired pigeons (n = 180) were fed one of five diets; group T1 received no added Met, while T2, T3, T4 and T5 received 30, 60, 90 and 120 g of supplemental DL-Met/kg, respectively. Each treatment was replicated three times with 24 pairs in each replicate. The results showed that supplementary Met had a minor effect on the length of the fourth primary wing feather in 28-day-old squabs (p>0.05), but the length of 14-day-old squabs in T2 was significantly longer (p=0.010). Dietary Met had a minor effect on Wnt-7a and fibroblast growth factor receptors-2 (FGFR-2) mRNA levels in 28-day-old squabs (p>0.05). The IGF-1 concentration in plasma was highest in T4 and lowest in T2 (p=0.012), but there was no difference between T1, T2 and T5 (p>0.05). In the chest muscle, the expression of *IGF-1* in T3 and T4 was higher than in T1 (p=0.172 and 0.015, respectively). In the leg muscle, *IGF-1* mRNA level was higher in T4 and T3, and lower in T2 (p>0.05). The results indicate that the optimal Met supplement for increasing fourth primary wing feather length was 30 g/kg Met in health sand, and the feathers were the longest in 14-day-old squabs. Adding 90 g/kg Met to health sand can improve the concentration of IGF-1, which is important for growth performance of pigeon squabs.

INTRODUCTION

In recent years, the White King pigeon has been a source of meat and eggs in human diet (Pomianowski *et al.*, 2009). Pigeon meat is easily digestible, richer in protein, also considered as medicine in China (Kabir *et al.*, 2013), and squabs are important for meat production (Sales & Janssens, 2003). Methionine (Met) is considered a major growth-limiting amino acid in poultry (Bunchasak, 2009), and an important nutrient for feather growth and protein synthesis. Met deficiency impairs growth and metabolism, while excess met can be toxic (Harper *et al.*, 1970). Met deficiency could impair the growth of broilers (Wu *et al.*, 2012), Met supplemented to the diets positively affected body weight gain, feed conversion and production index of broilers (Koreleski & Świątkiewicz, 2008). Feather growth is important for pigeons because it reflects carcass quality; optimum feathering is crucial in poultry production, and poor feathering results in downgrading of birds at slaughter (Hickling *et al.*, 1900; Zeng *et al.*, 2015). The width of the daily growth of the feathers has been used to assess the nutritional condition of the birds while the feathers were growing (Mallet-Rodrigues, 2012). Furthermore, Zeng *et al.* (2015) also revealed that dietary Met content can affect the feather growth in Peking duck; the optimal Met requirement for the fourth primary wing feather length was 0.404%. However, studies on the optimal dosage of Met for feather growth in pigeons has not been reported.

Feathers are epithelial appendages resulting from cell proliferation, cell differentiation and cell migration (Chuong & Edelman, 1985). The size of the feather tracts can be altered by experimental modulation of Wnt pathway members. Wnts are secreted signalling molecules involved in the axis determination in vertebrates (Moon *et al.*, 1997). The fibroblast growth factor (FGF) family of peptide growth factors is involved in cell proliferation and morphogenesis. FGF signals are mediated by high-affinity fibroblast growth factor receptors (FGFRs) (Matovinovic & Richman, 1997). The spacing of feather buds involves signals from the epidermis to the underlying mesenchyme, including Wnt-7a and FGFs (Wolpert, 1998), and feather growth is closely related to Wnts and FGFRs. Noji *et al.* (1993) pointed out that Wnt7-a and FGFR-2 are expressed in the posterior domain of feather buds (Choung *et al.*, 1990).

In the present study, we measured the length of the fourth primary wing feather in pigeon squabs, examined *Wnt-7* and *FGFR-2* mRNA levels in feathers, and determined growth indices including IGF-1 concentration in plasma and *IGF-1* expression in chest and leg muscles, after supplementary Met was added to health sand.

MATERIALS AND METHODS

Animals and management

A total of 180 paired White King pigeons (180 males and 180 females) were obtained from the Tangshan Cuigu pigeon industry (Nanjing, China), and each pair fed two squabs (d1). Squabs with similar body weight (16.78 ± 2.15 g) were divided into five groups, with six replicates in each group, and 12 pigeon squabs per replicate. Dietary treatment 1 (T1) contained no added Met, whereas treatments T2, T3, T4 and T5 contained 30 g, 60 g, 90 g and 120 g of supplemental DL-Met/kg in health sand, respectively (Table 1). All groups were fed the same basal diet (Table 2), the health sand was fed separately, and water and food were available *ad libitum*. The birds were housed in pairs and raised under natural illumination over 31 days. The pigeons were raised in accordance with local farming practices.

Feather growth

At d14 and 28, 6 squabs per treatment were randomly selected for the analysis of feather growth, measuring the length of the fourth primary wing feather. The fourth primary wing feather length was measured using a ruler with a minimum scale of 0.01 mm.

Table 1 – Composition and nutrient levels of the health sand (%).

Ingredients	Content
$CaHPO_4$	17
Shell meal	17
Medium coarse sand	38
NaCl	5
Premix[1]	20
Strong complement vitamin[2]	2
Cod-liver oil[3]	1
Total	100
Nutrient levels[4]	
Ca	10.84
TP[5]	3.87
Lys	0.07

[1]Premix provided the following per kg of health sand: vitamin A, 250000 IU; vitamin D_3, 82500 IU; vitamin E, 300 IU; vitamin K, 40 mg; vitamin B_1, 20 mg; vitamin B_2, 170 mg; pantothenic acid calcium, 1000 mg; nicotinic acid, 650 mg; vitamin B_6, 160 mg; vitamin B_{12}, 100 mg; biotin, 40 mg; Fe, 1200 mg; Cu, 160 mg; Zn, 66 mg; Mn, 1320 mg; Se, 6 mg; I, 20 mg.

[2]Strong complement vitamin provided the following per kg of health sand: vitamin A, 640000 IU; vitamin D_3, 60000 IU; vitamin E, 130 IU; vitamin K_3, 50 mg; vitamin B_1, 30 mg; vitamin B_2, 60 mg; pantothenic acid calcium, 300 mg; nicotinic acid, 700 mg; vitamin B_6, 40 mg; vitamin B_{12}, 0.1 mg.

[3]Cod-liver oil provided the following per kg of health sand: vitamin A, 300000 IU; vitamin D_3, 30000 IU; vitamin E, 300 IU; vitamin C, 1 g.

[4]Nutrient levels were calculated value.

[5]Total phosphorus.

Table 2 – Composition and nutrient levels of the basal diet (air-dry basis) (%).

Ingredients	Content
Corn grain	57.9
Soybean meal	35
Wheat bran	7.1
Total	100
Calculated nutrient[1]	
Metabolizable energy (MJ/kg)	12.69
Crude Protein (%)	14.77
Calcium(%)	0.06
Phosphorus (%)	0.27
Lysine (%)	0.62
Methionine (%	0.40

[1]Nutrient levels were calculated value.

Tissue collection

In total, 30 birds (on d28) were selected randomly from the five groups (six squabs from each group) and the feather pulp was isolated from the fourth primary wing feather. Chest and leg muscles were collected at the same time of day, immediately frozen in liquid nitrogen, and stored at -80°C until analysis. Nine squabs from each group were selected and blood samples (0.5 to 0.8 mL per sample) were collected from 0900 to 1000 h. Blood was collected, immediately transferred to tubes containing 2 µL heparin sodium (0.8 M), and

centrifuged at 3000 × *g* for 5 min at 4°C. Plasma samples were stored at -20°C until further analysis.

Plasma IGF-1 concentration assay

The concentration of IGF-1 in plasma was measured using a commercial ELISA kit (Nanjing Jiancheng Co. Ltd., Nanjing, China) according to the manufacturer's protocol. Each sample was measured in triplicate, and concentrations are represented as ng/mL in plasma. The lowest limit of detection was 3 ng/mL. Intra-assay and inter-assay coefficients of variation were lower than 10.0% and 12.0%, respectively.

Real-time PCR (RT-PCR) analysis

Total RNA was extracted using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol and reverse transcribed using a Fast Quant RT Kit (catalogue numbers DP405 and KR106, respectively; TIANGEN Biotech Co., LTD, Beijing, China). Expressions of *IGF-1*, *Wnt-7a* and *FGFR-2* were measured by RT-PCR using primers designed based on the coding region of the target genes (Table 3). SuperReal PreMix (SYBR Green; catalogue number FP204; TIANGEN Biotech Co., Ltd.) was used to detect expression. Reactions (20 μL) contained 10 μL 2× SuperReal Premix, 0.4 μL 50× ROX Reference Dye, 0.6 μL of each primer, 1 μL cDNA, and 7.4 μL ddH_2O. Thermal cycling involved cDNA denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 10 s and 60°C for 32 s. Assays were repeated independently three times.

Table 3 – Primer sequences of target genes and GAPDH gene.

Gene	Sequence (5′- 3′)	Length (bp)
GAPDH	F:CTCTACTCATGGCCACTTCCG R:ACAACGTATTCAGCACCAGC	138
Wnt-7a	F:TCCCAGAAGAAACGCTGACGA R:GTCAGTGCTGCTTGCTTTAACCA	172
FGFR-2	F:TGCCTTCACAGAGACCGAC R:TCATCACCTGAAGAACACGAG	157
IGF-1	F:ATGGCACATTCATTCTTCGTT R:CACCTAAATCTGCACGCTCT	161

Statistical analysis

Data are expressed as means ± standard error and analysis was performed by one-way ANOVA using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The significance of differences between groups was evaluated by the least significant difference post hoc multiple comparisons test. The significance level was set at $p<0.05$.

RESULTS

Feather growth

The effect of dietary Met supplementation on feather growth of squabs is presented in Table 4. Although the length of the fourth primary wing feather of 28-day-old squabs supplemented with dietary Met was longer than the control treatment, there were no between-treatment differences ($p=0.973$). Compared with the control treatment, the fourth primary wing feather length of 14-day-old squabs in T2 was significantly longer than T5 ($p=0.010$). However, with increasing supplemental dosage, the fourth primary wing feather of squabs decreased, and T5 had the shortest length ($p<0.001$). These results suggested that dietary Met supplementation had no effect on the fourth primary wing feather length of 28-day-old squabs, however, it affected the feather length of squabs aged 14 days.

Table 4 – Feather growth of squabs fed varying concentrations of dietary Met at 14d and 28 d age.

Item	T1	T2	T3	T4	T5	SEM
14d	52.49 ^{a}	56.19 ^{b}	52.14 ^{a}	52.58 ^{a}	48.99^{C}	0.98
28d	129.16	129.78	129.39	129.64	129.43	1.18

Values marked with different lower letters in row are significantly different ($p<0.05$), different capital letters in row are very significantly different ($p<0.01$).

Analysis of Wnt-7a and FGFR-2 expression

The effects of dietary Met on the expression of *Wnt-7a* and *FGFR-2* were examined by RT-PCR (Fig. 1). Expression of *Wnt-7a* was highest in T4 and lowest in T5, but there were no differences between these treatments ($p>0.05$). *FGFR-2* mRNA levels were higher in T4 than in other groups, but there were no differences among different treatments ($p>0.05$). Thus, dietary Met had a minor effect on *Wnt-7a* and *FGFR-2* mRNA levels, which related to the feather growth.

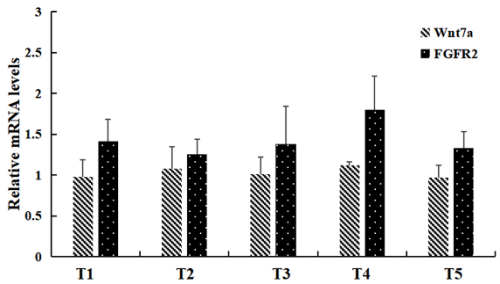

Figure 1 – The expressions of Wnt-7a and FGFR-2 in feathers under different dietary Met. Values marked with different letters on the bars are significantly different ($p<0.05$).

IGF-1 concentration in plasma

Determination of the IGF-1 concentration in plasma (Table 5) showed that it was highest in T4 and lowest in

Table 5 – The concentration of IGF-1 of squabs fed varying concentrations of dietary Met (ng/mL).

Item	T1	T2	T3	T4	T5	SEM
IGF-1	42.06[a]	41.67 [a]	54.07 [b]	56.70[b]	48.07[a]	2.82

Values marked with different lower letters in row are significantly different (p<0.05).

T2 (p=0.012), but there were no differences between T1, T2 and T5 (p>0.05). Although the concentration of IGF-1 was higher in T4 than in T3, there was no difference (p=0.963), which indicated that the concentration of dietary Met in T4 and T3 increased the IGF-1 concentration.

Analysis of IGF-1 expression in chest and leg muscle

Expression of *IGF-1* in the chest and leg muscle of squabs under different dietary Met treatments is shown in Fig. 2. In the chest muscle, *IGF-1* mRNA level was higher in T4 than in T3, but there was no significant difference (p=0.101). Expression of *IGF-1* in T1 was lower than in T3 and T4 (p=0.172 and 0.015, respectively), but there were no differences among T1, T2 and T5 (p>0.05). In the leg muscle, *IGF-1* mRNA levels were higher in T4 and T3, and lower in T2, but there were no differences among different treatments (p>0.05). The results indicated that dietary Met affected the expression of *IGF-1* in the chest and leg muscles.

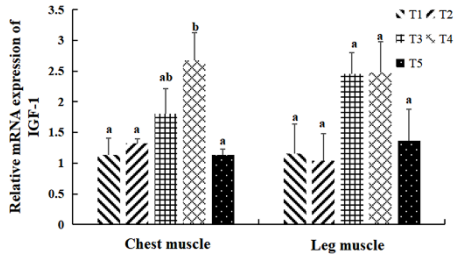

Figure 2 – The expressions of IGF-1 in chest muscle and leg muscle under different dietary Met. Values marked with different letters on the bars are significantly different (p<0.05).

DISCUSSION

The NRC (1994) recommendation for Met in the diet of chickens is 0.5% at 21 days and 0.38% at 42 days. In the present study, the fourth primary wing feather length was significantly longer in pigeon squabs supplied with 30 g/kg Met in health sand than in other treatments at 14 days of age, and there was a significant decrease in birds receiving 120 g/kg Met, which is in accordance with Agostini *et al.* (2016), who suggested that a lower Met requirement in the starter phase should be administered. Our results indicate that supplementary Met affects feather growth in starter (day 1 to 14) pigeon squabs, and adding 30 g/kg Met to health sand can induce feather growth, Guo (2011) also found that supplementary Met in 1 to 21 d of age Pekin duck's diet increased feather growth. Although feather growth was greater in the 30 g/kg Met treatment than in the other treatments at 28 days of age, there were no differences among different treatments. Zhang *et al.* (2014) indicated that a deficiency of Met can lead to impaired feather growth of Pekin ducks, which suggested that adequate Met didn't affect feather growth. Furthermore, Urdaneta-Rincon & Leeson (2004) demonstrated that feather growth of broilers from 1 to 21 d of age were more influenced by dietary CP per se than by levels of animo acid.

In this study, we measured mRNA levels of *FGFR-2* and *Wnt-7a*, since FGFs are known to induce new feather buds (Jung *et al.*, 1998), and *Wnt-7a* is involved in anterior-posterior patterning (Hammerschmidt & McMahon, 1997). Widelitz *et al.* (1999) demonstrated that overexpression of *Wnt-7a* caused feather buds to stop elongating and to become plateau-shaped appendages. *FGFR-2* is expressed in the feather placode epithelium (Orr-Urtreger *et al.*, 1991), and overexpression of *FGFR-2* in the skin of chicken embryos results in the complete failure of feather formation (Mandler & Neubüser, 2004). Wnt-7a and FGFR-2 play key roles in feather growth in birds. In the present work, although the expressions of *Wnt-7a* and *FGFR-2* at 28 days of age was in accordance with the rhythm of feather growth, adding Met had little effect on expressions of these genes, which may be related to the fact that the primary wing feather has already completed growth, furthermore, squabs were sold in the market at the age of 28 days. Met supplement may be effective at the growing period of squabs.

The expression of *IGF-1* in chest muscle was higher in the 90 g/kg Met treatment, but there were no differences between treatments in the leg muscle in our study. However, Del Vesco *et al.* (2013) previously reported higher levels of *IGF-I* mRNA in the liver of broilers receiving Met supplementation, while *IGF-I* expression in the muscle tissue was not affected. The concentration of IGF-1 in plasma was also higher in the 90 g/kg Met treatment, in accordance with Takenaka *et al.* (2000). Carew *et al.* (2003) suggested that the

IGF-1 concentration was affected by restriction of Met and the level of Met deficiency. Chickens with a high growth rate display higher circulating levels of IGF-1 (Beccavin *et al.*, 2001), and Guernec *et al.* (2003) reported that *IGF-I* mRNA levels are important in muscle growth rate during development. IGF-1 can be used as an indicator of growth rate in birds. Thus, our results suggest that supplementing 90 g/kg Met in health sand improved the growth performance of pigeon squabs. Feather growth is also an important aspect of the growth performance of squabs. Thus, further studies are needed to explore the molecular mechanism by which supplementary Met impacts feather growth in starter (day 1 to 14) and grower (day 15 to 28) pigeon squabs.

In conclusion, in 14-day-old squabs, the optimal Met supplement level for feather growth was 30 g/kg Met in health sand, but this had little effect on 28-day-old squabs. Additionally, adding 90 g/kg Met to health sand can improve the content of IGF-1 in pigeon squabs, which may improve the growth performance of pigeons.

ACKNOWLEDGMENTS

This study was funded by Jiangsu Modern Agricultural Industrial Technology System, Nanjing Jiangning Cuigu Promotion Demonstration Base (JATS [2019] 017), Jiangsu Overseas Visiting Scholar Program for University Prominent Young and Middle-aged Teachers and Presidents and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD; Jiangsu, China).

AUTHORSHIP CONTRIBUTION STATEMENT

Ying Wang, Haiming Yang and Zhiyue Wang contributed to the overall design of the study. Ying Wang, and Jun Meng collected data. Ying Wang, Zhenyu Guo, Xiaoshuai Chen and Zhengfeng Yang contributed to sample collection. Ying Wang participated in the manuscript writing and revision. All authors approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare no conflict of interest. The fundings had no role in the design, execution, interpretation, or writing of the study.

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