Journal of Animal and Veterinary Advances 12 (9): 932-936, 2013
ISSN: 1680-5593

Effects of Age and Parental Sex on Digestive Enzymes, Growth Factors and Immuneglobulin of Pigeon Squab

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Abstract: In the current study, dynamic changes of digestive enzymes' activities, concentrations of growth factors and immnueglobulin of pigeon squabs under different ages and parental sex were investigated. Totally 90 squabs at post-hatching day 2, 4, 6, 8 and 10 were sampled. The result showed that the specific activities of amylase, trypsin and lipase were all increased significantly with the increase of age while the total enzymes' activities showed a different pattern. Parental sex has no effect on the activities of digestive enzymes. The concentrations of EGF and IGF-1 in crop milk of squabs were decreased from 2-8 days. Both of the two under the breeding of male parent pigeon had a sharp increase at 10 days and were significant higher than that under the breeding of female parent pigeon. No significant changes were observed in IgG level under the breeding of male and female parent pigeon over 10 days. Like mammals, there was probably a compensatory mechanism in crop milk which would be promotion for the development of squabs in the early stage.

Key words: Pigeon, crop milk, age, parental sex, mammals

INTRODUCTION

Pigeons (*Columba livia domestica*) are widely kept for production of meat, sports and experimental animals in biomedical research (Sales and Janssens, 2003). In the East of China, the breeding stock is increasing year by year which made it became the fourth biggest source of poultry-product around the whole country. However, knowledge concerning nutrient requirements and relevant biological mechanism of this species is very limited and so far, NRC has not provided any information on its nutrient standard. This is no doubt restrictive for the development of the whole industry.

Unlike chickens and other poultry, newly hatched squabs are fed pigeon milk, called crop milk from parents for nearly 28 days. Crop milk is composed of sloughed off squamous epithelial cell from pigeon crop. This cheesy, semi-solid substance is similarly produced by flamingos and male normal penguins. Pigeon seems to be at least structurally unrelated to mammals that have the traditional lactation. In the first 5 days after hatch, high concentrations of fat (9-11%) and protein (9-13%) but extreme deficiency of carbohydrates found in crop milk (Shetty *et al*., 1992) was ever considered to be the major reason for squabs' much higher growth rate than that of broiler, quail and ostrich (Sales and Janssens, 2003). Meanwhile, there were already data supported that nutrients including protein, fat, fatty acids and minerals in the crop milk showed dynamitic changing patterns during the beginning week of pigeon lactation (Shetty *et al*., 1990, 1992; Shetty and Hegde, 1991). However, the subsequent research found that artificial replicated diet referred to crop milk would make squab gain poor growth performance or even die which suggested that components such as digestive enzymes, growth factors and immuneglobulin other than the common nutrients in crop milk were probably very important for pigeon squabs.

Therefore, the objective of the present study was to determine the effect of age and parental sex on digestive enzymes, growth factors and immuneglobulin in the crop milk of squabs. The analysis of changes of these important components may contribute to a better understanding of altricial avian physiology which could in turn develop useful strategies for pigeon breeding.

MATERIALS AND METHODS

Bird husbandry: The experiment was conducted in accordance with Chinese guidelines for animal welfare and was approved by the animal welfare committee of Poultry Institute, Chinese Academy of Agricultural Sciences.

A total of 180 pairs of (60 weeks of age) adult White King pigeons (180 males and 180 females) were obtained from a commercial pigeon farm (Jiangyin city, China) and allocated to breeding pairs. Each pair was housed in a man-made aviary equipped with a nest and a perch. All the parent pigeons were chosen to keep the same

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oviposition interval. A total of 180 fertile eggs were hatched by parent pigeons and only one squab was assigned to be raised by each one pair of parent pigeon. In order to alleviate the stress responses of pigeons, the pairs were separated in the night when the squabs were one old age. The baby pigeon was only fed by one parent pigeon and the other one was discarded and transferred into other pigeonry. Finally, 90 male pigeons and female pigeons were chosen to be left, respectively in the original nests. During the study, caged birds were housed in a room under a 16L:8D lighting cycle. The mean daily temperature was 24±4°C. Feed in pellet form, sand and water were provided freely. Compositions of the formulated diet are shown in Table 1.

Collection of pigeon milk: Two groups, total 90 of squabs (fed by male and female parent pigeon, respectively) at post-hatching day 2, 4, 6, 8 and 10 were randomly selected and weighed. Pigeon milk was collected by the method as described by Shetty and Hegde (1991). Slits were made on the crop of squabs surgically which were closed by double sutures after extraction of the milk. The samples were quickly frozen in the liquid nitrogen and transferred to be stored at -80°C until use. Pigeon milk was homogenized in 4 volume of ice-cold 0.01 M PBS buffer (contained 0.15M NaCl) and centrifuged at 10,000×g for 10 min. The supernatants were stored for the measurement of activities of digestive enzyme, concentrations of growth factors and immuneglobulin.

Table 1: Composition (%) and nutrient level (%) of formulated diet

Items	Values
Ingredients	
Corn	58.90
Pea	5.00
Soybean meal (48.0% CP)	20.00
Wheat	10.00
Soybean oil	1.00
Dicalcium phosphate	1.50
Limestoner	2.00
Salt	0.30
Premix[1]	1.00
Lysine	0.11
Methionine	0.07
Zeolite powder	0.12
Nutritional level[2]	
CP	16.74
Lysine	0.89
Methionine	0.31
Calcium	1.26
Avaible phosphorus	0.47

[1]Premix provided the following per kilogram; vitamin A, 4,000 IU; vitamin D_3, 1,725 IU; vitamin E, 24 mg; vitamin K_3, 1 mg; vitamin B_1, 3 mg; vitamin B_2, 13 mg; vitamin B_6, 2 mg; vitamin B_{12}, 2.5 mg; nicotinic acid, 15 mg; folic acid, 0.55 mg; pantothenic acid, 7.5 mg; biotin, 0.12 mg; choline chloride, 200 mg; Cu, 10 mg; Fe, 35 mg; Mn, 55 mg; Zn, 35 mg; I, 0.2 mg; Se, 0.25 mg; [2]Values were calculated from data provided by Feed Database in China (2010)

Digestive enzyme estimation: The activities of amylase, trypsin and lipase were measured with diagnostic kits (Nanjing Jiancheng Bioengineering Institute, China) according to the instructions of the manufacture. They were all was measured by spectrophotometer (Unico UV-2000, USA) under 660, 253 and 420 nm, respectively. One unit of amylase was defined as 10 mg of starch hydrolyzed per 30 min at 37°C. One unit of trypsin activity was defined as an absorbance increase of 0.003 per min at pH 8.0 at 37°C. One unit of lipase activity was defined as 1 μmol of substrate consumed per min at 37°C. The protein concentrations were determined using the Coomassie Brillant Blue G-250 reagent with BSA as a standard. Total enzyme activities were expressed as units per gram of pigeon milk. Specific enzyme activities were expressed as units per gram or milligram of protein.

Concentration of growth factors and immuneglobulin: Concentration of Epidermal Growth Factor (EGF) and Insulin-like Growth Factor-1 (IGF-1) in pigeon milk were measured by using ELISA Method (Boster Biological Technology, China) under 450 nm. Immuneglobulin G (IgG) was analyzed by diagnostic kit (Nanjing Jiancheng Bioengineering Institute, China) under 600 nm based on the method of immunosuppression.

RESULTS AND DISCUSSION

Digestive organs in bird is still an immature organ at hatch and the expression of nutrient transporters are therefore used as representatives of many physiological processes that need posthatch development (Ding and Lilburn, 2002). As the food of squabs, crop milk is known as its high concentration of protein and lipid, the immature digestive function means a potential restrictive factor for the nutrient absorption. So, it's reasonably speculated that digestive enzymes in crop milk are probably existed for the supplements of squab digestion. Hegde and Neelakantan (1970) had reported the presence of amylase, trypsin and lipase in crop milk. In the present study, researchers investigated the dynamitic changes of three enzymes, amylase, trypsin and lipase under different ages and parental sex. The result showed that the specific three enzymes' activities were all increased significantly with the increase of age ($p<0.05$) while the total enzymes' activities showed a different pattern (Table 2, Fig. 1). In addition, parental sex has no effect on the activities of digestive enzymes ($p>0.05$) and there was also no interactions between age and sex for these parameters evaluated. Although, total digestive enzymes' activity in crop milk of squab at 2 day were not changed much compared with that at 10 day especially trypsin its total

Table 2: The effects of age and parental sex on activities of amylase, trypsin and lipase in crop milk of pigen squabs

Items	Total amylase activity ($U\ g^{-1}$ protein)	Specific amylase activity ($U\ g^{-1}$ protein)	Total trypsin activity ($U\ g^{-1}$ pigeon milk)	Specific trypsin activity ($U\ g^{-1}$ pigeon milk)	Total lipase activity ($U\ g^{-1}$ pigeon milk)	Specific lipase activity ($U\ g^{-1}$ protein)
Age						
2	0.025	81.500^{a}	45.560^{a}	162.080^{a}	0.260^{a}	25.640^{a}
4	0.027	164.560^{ab}	19.800^{b}	148.540^{a}	0.330^{b}	59.700^{b}
6	0.026	247.450^{bc}	13.120^{b}	139.360^{a}	0.430^{c}	103.520^{c}
8	0.027	330.320^{cd}	16.120^{b}	265.290^{b}	0.160^{d}	56.340^{b}
10	0.030	353.150^{d}	15.760^{b}	228.380^{b}	0.280^{a}	97.090^{c}
Sex						
Male	0.028	214.910	24.520	170.860	0.300	67.050
Female	0.026	215.740	23.770	179.630	0.310	70.000
P-value						
Age	0.813	<0.001	<0.001	<0.001	<0.001	<0.001
Sex	0.397	0.596	0.972	0.740	0.429	0.762
Age×sex	0.169	0.468	0.955	0.099	0.223	0.229

$^{a-d}$Data in the same row with different small letter superscripts mean significant difference ($p<0.05$) while with the same and no small letter superscripts mean no significant difference ($p>0.05$)

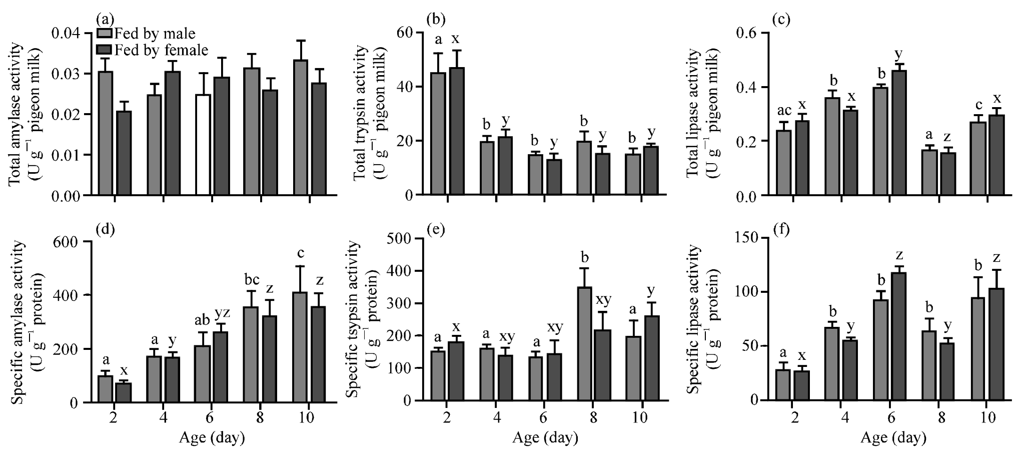

Fig. 1: The effects of age and parental sex on activities of a, d) amylase; b, e) trypsin and c, f) lipase in crop milk of pigen squabs. Bars represent means±SE (n = 9). $^{a-c}$and $^{x-z}$Bars with differing letters are significantly different ($p<0.05$)

activity was even much higher than that at other 4 ages. Calculation of specific activities of enzymes needs to use protein concentration and the earlier data showed that protein fraction in crop milk decreased from 12.1-8.4% in the first 5 days after hatching and the pure milk other than grains only accord for 14% in the whole sampled crop milk at 7 day (Shetty *et al*., 1992) so, the different changing pattern between two expressions of similar enzyme could be well explained. Among the three enzymes, the growth ratio of amylase activity in squabs increased much higher than other two enzymes. A sharp increase, 4 fold in specific amylase activity at 10 days was found in crop milk compared to that at 2 days. Earlier study showed that the concentration of carbohydrate in crop milk would grow up with the age while protein and lipid decreased dramatically after the 1st week (Leash *et al*., 1971; Shetty *et al*., 1992). So, researchers think that amylase activity in crop milk could be well in concert with the uptake of carbohydrate. Dong *et al*. (2012) had found that both amylase activity in pancreas and disaccharidase activity in small intestine had a sharp increase at 8 days after hatching which could be attribute to the increase of the grain content in pigeon crop. From this study, it is also intriguing that lipase activity reached maximum at 6 days after hatching. The rapid increase of expression of fatty acid transporter genes in pigeon intestine indicated that this specie possibly had excellent tolerance to fat and was more competent to absorb fatty acids in the early development than other birds (Xie *et al*., 2012, 2013). So, the role of lipase in crop milk during the development of the digestive function of squabs is need to be further investigated.

It has been proved that pigeon milk has a capability of growth-stimulating property for cell *in vitro* and the growth stimulation by 1% pigeon milk was approximately equal to that by 2% Foetal Bovine Serum (FBS) when CHO cells were used. Early research showed that the proliferation of crop glands could be brought about by the synergistic action of prolactin, epidermal growth factor and proinsulin (Anderson *et al*., 1987). Bharathi *et al*. (1993a, b) also speculated that crop tissue of pigeon could accumulate growth factors, expecially

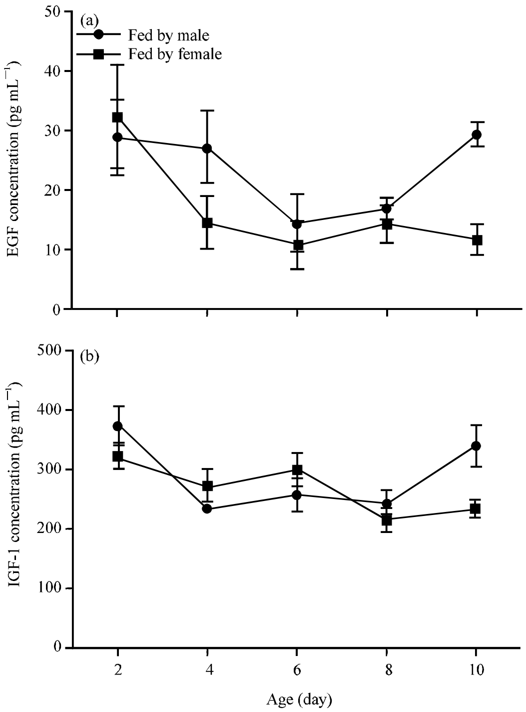

Fig. 2: The effects of age and parental sex on concentrations of a) Epidermal Growth Factor (EGF) and b) Insulin-like Growth Factor-1 (IGF-1) in crop milk of pigeon squabs. Data are means±SE (n = 9)

during incubation and brooding which was similar to the mammals both mammary tissue and milk possess growth factors (Connolly and Rose, 1988). In the present study, researchers used Elisa Method to measure the concentration of EGF and IGF-1. The results (Fig. 2) showed that both two growth factors had the surprising same changing pattern during 10 days after hatching under the breeding of male and female parent pigeon. The concentrations of EGF and IGF-1 in crop milk of squabs were decreased from 2-8 days. The former reached the minimum value at 6 days and the latter one was at 8 days. The interesting finding was that both EGF and IGF-1 concentration under the breeding of male parent pigeon had a sharp increase at 10 days and were significant higher than that under the breeding of female parent pigeon. Milk secreted by mammals contained higher concentration of EGF and IGF-1 and both of them decreased subsequently in the later period of lactation (Donovan *et al.*, 1994; Xu, 1996; Li *et al.*, 2001). The results were accordant with these findings basically. The concentration of EGF showed a negative relationship with the newborn weight and it may be attributed to the natural

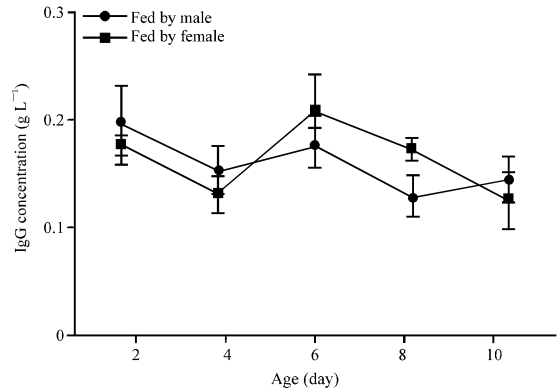

Fig. 3: The effects of age and parental sex on concentrations of Immuneglobulin G (IgG) in crop milk of pigen squabs. Data are means±SE (n = 9)

compensatory mechanism which would be promotion for the development of immature parts including gastrointestinal organs.

Like mammalian milk components, relevant factors in pigeon crop milk may play a role in immune development of the immune system directly by delivering immune molecules such as immunoglobulins and cytokines (Wagstrom *et al.*, 2000; Stelwagen *et al.*, 2009) and indirectly by influencing the microbiota through prebiotics (Chichlowski *et al.*, 2011). Both IgA and IgG were involved in the crop milk (Goudswaard *et al.*, 1979; Engberg *et al.*, 1992).

CONCLUSION

In the study, the IgG concentration (0.13-0.21 g L^{-1}) measured by Elisa Method was closed to the data (0.34 g L^{-1}) reported by Engberg *et al.* (1992). There was a decline tendency of IgG level under the breeding of male and female parent pigeon over 10 days but no significant changes were observed in the two results (Fig. 3). However, in mammals, immuneglobulin concentration maintained a high level in colostrums and then declined dramatically (Ogra and Ogra, 1978; Guo and Luo, 1999). Speciation may be an important factor for this diversity.

ACKNOWLEDGEMENTS

Researchers thank all the members in the institute for their generous technical advice. This research was supported by Independent Innovation of Agricultural Sciences in Jiangsu Province (CX(12)3073) and Programs for Agricultural Science and Technology development in Yangzhou City (yz2011050).

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