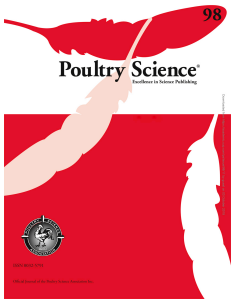

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Effects of genetically modified maize expressing Cry1Ab and EPSPS proteins on Japanese quail

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ABSTRACT

A 49-day feeding study was conducted to evaluate the effects of the genetically modified (GM) maize strain C0030.3.5 on Japanese quails (*Coturnix japonica*) in terms of body performance and egg quality. Furthermore, the bodily fates of transgenic proteins in the Japanese quails were investigated.

The results showed that the parameters of body weight, hematology, serum chemistry, relative organ weight and histopathological appearance were normal in male and female quails that consumed GM diets, and no differences could be attributed to the varying diets in regards to the laying performances or nutrient egg compositions between the groups. Furthermore, the transgenic Cry1Ab and EPSPS proteins were undetectable by Western blot in the blood, organ, fecal and whole egg samples of quails fed a diet containing GM maize. The results obtained after 49 days suggested that consumption of C0030.3.5 transgenic feed did not adversely affect quail health or egg quality, and there was no evidence of transgenic protein translocation to the blood, tissues, feces and eggs. Based on the different parameters assessed, C0030.3.5 transgenic maize is a safe food source for quails that does not differ in quality from non-GM maize.

Key words: insect-herbicide tolerance transgenic maize, C*oturnix japonica*, 49-day feeding study

INTRODUCTION

Since the first approval of commercial genetically modified (**GM**) maize in 1996, the extent of world GM maize cultivation has increased rapidly over the last 20 years, reaching 59.7 million hectares (32% of the global biotech area) in 2017. Many countries have approved the commercial releases of various events of GM maize for use in feeds, food and biofuels (ISAAA, 2018), and approximately 85% of GM maize and maize products are being used as feed material for animals (Flachowsky et al., 2012). At the same time, the increasing application of GM maize for livestock production has also raised safety concerns related to potential health effects (Guertler et al., 2010). One of the main concerns is the potential adverse effects of GM maize on animal performance and health. Other questions address resistance genes as unnecessary cloning consequences, the potential allergenicity of the newly produced proteins, or the possibility of transferring the transgene or protein from GM maize to animal organs. These debates about the application of GM maize to animals have slowed the adoption of GM crops in both developing and European countries.

In China, corn field environments are important feeding and loafing habitats for many bird species, such as *Alaudida arvensis*, *Pica pica*, and *Coturnix coturnix.* These birds play important roles in the spread of plant seeds and the biological control of pests as well as in the maintenance of ecosystem balance. During the growing seasons of genetically modified crops, birds can obtain necessary nutrients from fruits or seeds and may promote the dispersal of GM ingredients by seed or fecal distribution within their habitats (Liu et al., 2017); therefore it is important to consider

the potential health risks of GM crops to birds.

Several studies were conducted to assess the effects of GM crops on bird performance, mainly focusing on chickens (Taylor et al., 2005; McNaughtonet al., 2007; Scheideler et al., 2008; Lu et al., 2013; Ma et al., 2013; Halle and Flachowsky, 2014; Jacob et al., 2015; Zhan et al., 2019). Compared with chickens, quails are smaller in size and have shorter development times, and the physical characteristics of quail allow for experiments to be carried out more quickly and at a lower cost than comparable work with chickens (Huss et al., 2008). Japanese quail, as an excellent model species that was established during the domestication process, is quite resistant to various diseases, has a relatively short lifespan and is easily adaptable to various rearing conditions (Randall and Bolla, 2008; Jatoi et al., 2015; Ghayas et al., 2017; Mnisi et al., 2018). Birds are a popular animal model used in numerous fields of research, including toxicology, physiology and genetics (Huss et al., 2008; Agathe et al., 2012; Mahmoud et al., 2019). Recently, quail studies have included feeding studies to test the safety of GM food/products because the application of GM crops has led to great public concern; however, although laboratory tests have shown that the consumption of single-trait *Bt* (*Cry1Ab*) maize or *EPSPS* soya does not adversely affect quail body performance (Sartowska et al., 2012, 2015) or immune responses (Scholtz et al., 2010), little evidence regarding the safety of stacked GM plants for quails is available.

As a developing country, China has always attached great importance to the application of GM technology to improve agricultural productivity. After over 20

years of development, a large number of GM maize events and varieties have been obtained with important traits such as insect resistance, herbicide tolerance and high quality (Shen et al., 2016; Li et al., 2018). In recent years, some stacked GM maize varieties have also been bred. C0030.3.5 maize is genetically engineered to express the Cry1Ab toxin and CP4-EPSPS protein, which confers resistance to the Asian corn borer (*Ostrinia furnacalis*), *Mythimna separata* (Walker) and glyphosate herbicide. In the present study, C0030.3.5 maize as a feed source was tested for its effects on body performance, laying performance and egg quality in Japanese quails having received GM maize early in life, and the study was also designed to verify the possible transfer of transgenic protein to the blood, tissues, feces and eggs of quails after consumption of GM maize.

MATERIALS AND METHODS

Ethics Statement

All animal care and protocols were approved by the Animal Core Facility and Nanjing Medical University. All procedures were carried out in strict accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China, and all efforts were made to minimize suffering.

Maize and Experimental Diets

Seeds derived from GM maize C0030.3.5 (GM maize thereafter) and the non-GM parental control DBN318 maize (non-GM maize thereafter) were used in this animal study. Seeds of both varieties were kindly provided by DaBeiNong (**DBN**) Technology Group Co., Ltd. (Beijing, China), and a double-antibody sandwich

89 enzyme-linked immunosorbent assay (**ELISA**) was used to determine the protein
90 expression levels of Cry1Ab and CP4-EPSPS in samples of GM and isogenic maize.
91 The results showed that the Cry1Ab and EPSPS expression levels in GM maize were
92 1.49 μg/g and 35.42 μg/g, respectively. All negative controls and non-GM maize
93 DBN318 were negative for Cry1Ab and EPSPS expression.

94 A basal diet was prepared and formulated to meet all nutrient requirements of
95 Japanese quail according to the NRC (1994). Both GM and non-GM diets were
96 formulated to contain 61.5% maize grain, and the ingredient compositions of the
97 experimental diets are shown in Table 1. During feed pelleting, the temperature was
98 kept below 60°C to maintain protein activity. After diet preparation, the feed was
99 vacuum-packed in plastic bags, labeled and kept at 4°C until used for feeding.

100 The nutrient components of both the non-GM diet and GM diet were analyzed, and
101 three samples were randomly selected from each diet type for nutritional proximate
102 analysis (Table 1). The moisture, ash, fat, crude protein and fiber contents were
103 determined in accordance with Chinese standard methods (GB/T6435-2014,
104 GB/T6438-2007, GB/T6433-2006, GB/T6432-1994 and GB/T6434-2006).

105 ***Feeding and Bird Management***

106 Ten-day-old Japanese quails (*Coturnix japonica)* of mixed sexes were sourced from
107 a farm in Nanjing. Prior to the feeding trial, the birds were fed a commercial quail
108 starter diet for a one-week adaptation period (Lemme and Mitchell, 2008; Uchewa
109 and Onu, 2012). Afterwards, 90 birds were randomly divided into three different
110 groups with 3 replicates of 10 birds each (5 female and 5 male). Birds in one control

group received a commercial compound feed, and those in the two experimental groups received the same amount of C0030.3.5 GM maize or its non-GM counterpart DBN318 maize. All birds were kept in wire cages under similar housing and management conditions, and feed was provided 2 times per day at a daily amount of 400 g (0900, 1400); residues and bird feces were collected every morning, and water was freely available. The room temperature was maintained constant at approximately 24 ± 2°C, with a 50%~70% relative humidity and a 12-h light/dark cycle.

Body Weight

During the feeding trial, quails were monitored daily for mortality and clinical signs of morbidity or toxicity, and the body weight of each bird was measured weekly before the morning feeding using a SE602F electronic balance (Ohaus).

Blood Analyses, Relative Organ Weight, and Histopathological Evaluations

At the end of the study, birds were fasted for 24 h and provided water ad libitum, and 6 birds (3 female and 3 male) from each cage were then taken. Heparinized blood samples were collected from the wing veins for hematological parameter analysis. Whole blood was used for hematological analysis, and serum samples were used for biochemical analysis. The white blood cell (**WBC**) count, red blood cell count (**RBC**), hemoglobin (**HGB**) concentration, hematocrit (**HCT**) level, mean corpuscular volume (**MCV**), mean corpuscular hemoglobin concentration (**MCHC**), hemoglobin distribution width (**HDW**), platelet count (**PLT**), and mean platelet volume (**MPV**) were measured by ADVIA 120 (Bayer Diagnostics, USA).

The serum chemistry parameters alanine aminotransferase (**ALT**), aspartate

aminotransferase (**AST**), total protein (**TP**), albumin (**ALB**), total bilirubin (**TBIL**), alkaline phosphatase (**ALP**), urea nitrogen (**BUN**), creatinine (**CREA**), cholesterol (**CHOL**) and triglyceride (**TG**) were analyzed with Dimension Xpand.

After blood collection, the same quails were weighed individually and killed by cervical dislocation. Major organs, including the heart, brain, liver, lungs, stomach, spleen, kidneys, small and large intestines, ovaries and testes, were removed, weighed individually, and fixed in 4% neutral buffered formalin. Tissue sections (4 ~ 6 μm thick) from these organs were cut and stained with hematoxylin and eosin (**HE**) for histopathological examination with a Nikon Eclipse E600. Relative organ weight is expressed as a percentage of the whole body weight (Zhang et al., 2012; Wang et al., 2013).

Eggs

At 4:00 pm, the egg numbers and weights were recorded, with the weights being measured on KERN 440-35N scales. After weighing, collected eggs were placed in plastic bags and stored at 4°C for nutrition analysis. At the end of the study, ten eggs from each replicate were collected, and the moisture, protein, fat, lecithin and cholesterol contents were measured according to the methods reported by previous studies (Leeson and Caston, 2003; An et al., 2013; Sun et al., 2013).

Western Blot Analysis of the Cry1Ab and EPSPS Proteins in Blood, Tissue, Fecal and Egg Samples

For Western blot analysis, blood, heart, liver, lungs, stomach, spleen, kidney, and small and large intestine tissue samples were carefully removed, washed with ice cold

water and then immediately stored at -80°C for protein extraction. Frozen tissues were homogenized and lysed with RIPA lysis buffer (Sigma, USA) for 30 min on ice. After centrifugation at 12,000 g for 15 min, supernatants were transferred into fresh tubes for further use. Whole egg samples were washed with water and disinfected with 75% ethanol to ensure that the egg samples were not contaminated with feed and feces. Then, 5 eggs from each group were homogenized, and 0.5 mL of whole egg liquid was used for protein extraction by RIPA lysis buffer according to the manufacturer's instructions. Fecal collection and protein extraction were performed according to the method reported by Scheideler et al. (2008). All sample supernatants were stored frozen at −80°C until analysis. The protein concentration was determined by the BCA assay (Pierce Biotechnology, Rockford, IL, USA).

Tissue, fecal and egg samples were used for immunoblot analyses. Approximately 50 μg of soluble protein from each sample was loaded onto a 10% SDS-polyacrylamide gel; the protein extract of GM maize C0030.3.5 was also used as a positive control. Immunoblots were performed according to the method of Jafari et al. (2009). Gels were electroblotted onto nitrocellulose membranes (Bio Rad), and free sites were blocked by Tris buffer saline with 0.1% Tween-20 (**TBST**) containing 5% nonfat dried milk at room temperature for 2 h. Monoclonal and polyclonal antibody (both diluted 1/2,000 in TBST) binding was detected by incubation with TRITC-conjugated goat anti-rabbit IgG or goat anti-mouse IgG+IgM (both diluted 1/4,000 in TBST); all antibodies were purchased from LI-COR (USA). The blots were then washed in TBST and scanned on a Licor Odyssey system. Protein bands were quantified using software

177 provided with the Licor Odyssey system.

178 ***Statistical Analysis***

179 SPSS 20.0 software was used for statistical analyses. One-way ANOVA was used
180 for group comparisons. Tukey's multiple comparison test was used to determine the
181 significance of differences between groups, which were considered significant at $P <$
182 0.05.

183 **RESULTS AND DISCUSSION**

184 ***Body Weight***

185 During the experiment, all the quails seemed pretty healthy, no behavioral changes
186 or adverse signs of toxicity or mortality were observed in the treated and control
187 groups. The mean body weights of male quails (Figure 1A) and female quails (Figure
188 1B) fed commercial control diets, GM diets and non-GM diets are shown in Figure 1.
189 The body weights were similar in all groups during the feeding trial, and there were
190 no significant differences in body weight between male and female quails fed
191 different diets ($P > 0.05$). The results of this study are consistent with those of
192 previous studies (Chen et al., 1996; Sartowska et al., 2012; Liu et al., 2017),
193 suggesting that GM meal consumption had no unintended effects on the body weights
194 of quails compared to those of quails fed the non-GM diet.

195 ***Blood Analyses, Relative Organ Weight, and Histopathological Evaluations***

196 In the current study, no significant differences were found in hematological
197 parameters (Table 2), relative organ weight (Table 4) or histopathological appearance
198 (Figure 3) between quails consuming the GM maize diet and those consuming the

non-GM maize diet. However, there was a significant difference in serum chemistry between the GM and non-GM groups regarding ALP and CHOL levels in male quails (Table 3). Such differences did not occur in the female group, and all the serum chemistry parameters were within the normal range and did not conform to a pattern indicative of organ dysfunction and were therefore not correlated with differences in relative organ weight or histopathology; thus, we considered that the differences in ALP and CHOL levels in male quails were not biologically significant. Therefore, GM maize diets did not adversely affect the hematological parameters, serum chemistry, relative organ weight or histopathological appearance. A similar conclusion was reported by Liu et al. (2017), who fed quails transgenic soybean ZZ-J9331 containing the CP4-EPSPS protein and suggested that some significant differences in the serum biochemistry, hematological and relative organ weight parameters of quails were not biologically significant; these differences were not related to transgenic soybean consumption, and this conclusion agrees with those presented herein.

Eggs

In a two-generation study, Sartowska et al. (2012) reported some differences in the chemical compositions of breast muscle and egg yolk, but no clear effect of a genetically modified diet was observed on those parameters; feeding quails the GM herbicide-tolerant (**HT**) soybean meal and *Bt* maize did not negatively affect the laying performance or the nutritional value of the final product for consumers. Follow-up 10-generation feeding studies also found that the quail performance and egg yolk chemical compositions in the GM maize and GM soya groups did not differ

221 from those in the non-GM control group (Sartowska et al., 2015). Similar to the
222 studies described above (Sartowska et al., 2012; 2015), our results also did not show
223 any significant difference in egg production or egg weight for quails fed GM and
224 non-GM maize (Table 5), and the moisture, protein, fat, lecithin, cholesterol and VB2
225 components in eggs were compared between the non-GM or GM maize groups (Table
226 5). In addition, unlike previous studies (Sartowska et al., 2012, 2015) aimed at
227 assessing the long-term feeding safety of GM HT soybean and *Bt* maize in quails, our
228 feeding study mainly focused on the period from juveniles to young adults (10 to 49
229 days). Since quail body weight and organ development rapidly increase during this
230 period, chicks need to allocate their available energy between maintenance, growth
231 and maturation, and food availability consequently plays a crucial role during this
232 period (Wariboko et al., 2015). Noting changes in growth performance, organ
233 pathology and laying performance may provide an early warning of a biological effect;
234 thus, the findings of this study offer some assurance to consumers regarding the safety
235 of short-term exposure of quails to GM feed.

236 ***Western Blot Analysis of the Cry1Ab and EPSPS Proteins in Blood, Tissue, Fecal***
237 ***and Egg Samples***

238 One of the food and feed safety concerns of the public is the potential transfer of
239 GM proteins into animal tissues. In the current study, the widely used reference
240 protein β-actin was used as a loading control in Western blot analysis and was
241 detected in all samples (Figure 3), while no Cry1Ab or EPSPS protein was detected in
242 the blood, heart, liver, lung, spleen, small and large intestine, fecal or egg samples of

243 quails fed either the non-GM or GM-based diet (Figure 3). Using the ELISA detection
244 method, Jennings et al. (2003) reported that the Cry1Ab protein was not detectable by
245 ELISA in the breast muscles of chickens fed a diet containing *Bt* (MON 810) maize
246 for 42 days. Using the same detection method as Jennings et al. (2003), Ash et al.
247 (2003) also demonstrated that the whole egg, albumin, liver, and feces were all
248 negative for the CP4-EPSPS protein. Another study performed by Ma et al. (2013)
249 reported that the PhyA2 protein was not found in the blood, heart, liver, spleen, kidney,
250 or breast muscles of laying hens fed a phytase transgenic corn diet. Our findings and
251 those of previous studies suggest that no transgenic proteins are found in any organ or
252 tissue samples from animals fed GM plants, probably because the transgenic proteins
253 are readily degraded under simulated gastric digestion conditions (Okunuki et al.,
254 2002; Jennings et al., 2003); therefore, it is highly unlikely that transgenic proteins
255 would be present in tissue samples from chickens. Similar to transgenic proteins in
256 tissue samples, no recombinant DNA sequences have been found in any organ or
257 tissue sample from quails (Flachowsky et al., 2005; Korwin-Kossakowska et al.,
258 2013), broilers (Aeschbacher et al., 2005; Deaville et al., 2005; Świątkiewicz et al.,
259 2010) or laying hens (Ma et al., 2013) fed a GM diet. The studies mentioned above
260 suggest that the risk of GM ingredient transfer from food or feed to poultry organs is
261 low in feeding trials with different exposure times, which may also be the reason that
262 the consumption of GM feed does not adversely affect poultry health, organ pathology
263 or laying performance.

264 In conclusion, the results of the 49-day feeding experiment demonstrated that

C0030.3.5 transgenic maize had no adverse effects on quails in terms of body weight, hematology, serum chemistry (with the exception of the ALP and CHOL levels in male quails; however, this was not associated with organ histopathology), relative organ weight, histopathological appearance, laying performance or nutrient egg composition. No transgenic Cry1Ab or EPSPS protein was found in organ, fecal or whole egg samples, which was consistent with studies on previous birds fed different GM crop varieties (Kan et al., 2004; Taylor et al., 2005; Scheideler et al., 2008; Ma et al., 2013; Halle and Flachowsky, 2014; Jacobs et al., 2015) and suggests that consumption of C0030.3.5 transgenic feed does not adversely affect poultry health or eggs and does not increase potential health risks. In addition, it is worth noting that birds usually eat fresh grain in natural ecosystems, but GM crops are usually processed into feed in feeding experiments, and GM ingredients may not remain consistently stable during the processing stages (Chiter et al., 2000; Kharazmi et al., 2003); thus, unprocessed seed experiments to supplement the existing data are encouraged in future feeding trials.

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435

436 **Table 1.** Ingredients and nutritional components of the non-GM and GM diets.

	non-GM diet	GM diet
Ingredients		
Maize (w/w)	61.50%	61.50%
Soybean meal (w/w)	25.00%	25.00%
Sunflower seed kernel cake (w/w)	4.00%	4.00%
Fish meal (w/w)	2.00%	2.00%
Calcium carbonate (w/w)	5.70%	5.70%
Dicalcium phosphate (w/w)	1.27%	1.27%
Vitamin premix (w/w)[1]	0.25%	0.25%
Mineral premix (w/w)[2]	0.10%	0.10%
Salt (w/w)	0.18%	0.18%
Nutritional components		
Moisture (%)	11.26 ± 2.03	11.59 ± 0.26
Ash (%)	10.20 ± 0.26	10.26 ± 0.33
Crude protein（g/kg）	173.97 ± 7.09	171.45 ± 11.52
Crude fat (%)	4.71 ± 0.51	4.54 ± 0.26
Crude fiber (%)	4.43 ± 0.50	4.71 ± 0.73

437 [1]The vitamin premix supplied per kilogram of diet: vitamin A, 11,000 IU; vitamin E,

438 30 IU; vitamin D3, 4,000 IU; vitamin B12, 0.015 mg; vitamin B1, 1.40 mg; vitamin

439 B2, 4 mg; vitamin B6, 3 mg; vitamin K, 4.5 mg; folic acid, 1 mg; choline, 1,000 mg;

nicotinic acid, 30 mg; and pantothenic acid, 10 mg.

[2]The mineral premix supplied per kilogram of diet: manganese, 80 mg; zinc, 84.70 mg; iron, 50 mg; copper, 10 mg; iodine, 1 mg; and selenium, 0.20 mg.

P values < 0.05 were deemed statistically significant as determined by t-tests.

444 **Table 2.** Serum chemistry in male and female Japanese quails fed commercial,
445 non-GM and GM diets.

	Commercial diet	Non-GM diet	GM diet
Male			
WBC ($\times 10^9$/L)	803.91 ± 47.07[a]	761.07 ± 13.52[a]	707.73 ± 84.38[a]
RBC (10^9/L)	2.84 ± 0.10[a]	2.84 ± 0.24[a]	2.76 ± 0.25[a]
HGB (g/dL)	12.37 ± 1.72[a]	12.03 ± 2.61[a]	10.13 ± 0.97[a]
HCT (%)	31.1 ± 3.03[a]	27.3 ± 1.80[a]	28.63 ± 2.41[a]
MCV (fL)	109.16 ± 6.99[a]	105.87 ± 4.12[a]	103.93 ± 2.01[a]
MCH (pg)	43.43 ± 4.52[a]	39.47 ± 1.36[a]	36.83±1.07[a]
MCHC (g/dL)	39.70 ± 1.73[a]	37.93 ± 0.95[ab]	35.43 ± 0.45[b]
CHCM (g/dL)	35.76 ± 0.76[a]	35.03 ± 1.27[a]	34.06 ± 0.51[a]
HDW (g/dL)	9.86 ± 0.44[a]	9.76 ± 0.42[a]	9.76 ± 0.29[a]
PLT ($\times 10^9$/L)	312.00 ± 79.95[a]	288.00 ± 128.36[a]	300.33 ± 41.10[a]
MPV (fL)	40.40 ± 6.56[a]	34.67 ± 5.92[a]	37.63 ± 1.15[a]
Female			
WBC ($\times 10^9$/L)	759.60 ± 47.01[a]	843.56 ± 36.71[a]	655.26 ± 79.55[a]
RBC (10^9/L)	3.04 ± 0.19[a]	3.15 ± 0.41[a]	2.59 ± 0.54[a]
HGB (g/dL)	13.77 ± 1.52[a]	12.3 ± 0.78[ab]	10.13 ± 1.70[b]
HCT (%)	33.93 ± 2.39[a]	33.8 ± 1.92[a]	28.73 ± 6.39[a]
MCV (fL)	117.10 ± 3.65[a]	110.36 ± 4.27[a]	110.50 ± 2.65[a]
MCH (pg)	42.53 ± 1.38[a]	42.06 ± 1.38[a]	39.46 ± 2.02[a]

MCHC (g/dL)	37.20 ± 0.82[a]	37.33 ± 1.17[a]	35.73 ± 2.02[a]
CHCM (g/dL)	34.23 ± 0.49[a]	34.73 ± 1.05[a]	35.13 ± 1.49[a]
HDW (g/dL)	10.14 ± 0.35[a]	10.24 ± 0.66[a]	10.03 ± 0.27[a]
PLT ($\times 10^9$/L)	335.33 ± 108.87[a]	362.33 ± 139.53[a]	400.00 ± 267.26[a]
MPV (fL)	35.16 ± 2.55[a]	39.63 ± 4.78[a]	34.73 ± 8.93[a]

Note: WBC, leukocyte; RBC, erythrocyte; HGB, hemoglobin; HCT, hematocrit value; MCV, mean corpuscular volume; MCH, average red blood cell hemoglobin content; MCHC, mean corpuscular hemoglobin concentration; HDW, hemoglobin distribution width; PLT, thrombocyte; MPV, mean platelet volume.

Means in the same row with different letters are significantly different at the a=0.05 level as determined by one-way ANOVA followed by Tukey's test; data are expressed as the mean ± SD (n=9).

453

454 **Table 3.** Blood biochemistry in male and female Japanese quails fed commercial,
455 non-GM and GM diets.

	Commercial diet	Non-GM diet	GM diet
Male			
ALT (U/L)	4.98 ± 0.27[a]	4.90 ± 0.31[a]	4.94 ± 0.32[a]
AST (U/L)	270.67 ± 14.57[a]	274.33 ± 19.60[a]	310.33 ± 43.61[a]
TP (g/L)	28.97 ± 0.29[a]	26.07 ± 0.89[a]	25.60 ± 3.08[a]
ALB (g/L)	13.27 ± 0.29[a]	11.13 ± 0.50[b]	10.23 ± 0.38[b]
TBIL (μmol/L)	6.70 ± 0.57[a]	5.00 ± 2.64[a]	5.30 ± 1.15[a]
ALP (U/L)	164.00 ± 3.46[a]	147.00 ± 4.00[b]	156.67 ± 4.04[a]
BUN (mmol/L)	0.83 ± 0.35[a]	0.73 ± 0.21[a]	1.17 ± 0.15[a]
CREA (μmol/L)	8.33 ± 0.58[a]	9.67 ± 1.53[a]	7.00 ± 1.00[a]
CHOL (mmol/L)	5.04 ± 0.75[ab]	5.17 ± 0.27[a]	4.03 ± 0.02[b]
TG (mmol/L)	1.13 ± 0.37[a]	1.47 ± 0.22[a]	2.27 ± 1.68[a]
Female			
ALT (U/L)	5.57 ± 1.02[a]	5.12 ± 1.06[a]	4.58 ± 0.50[a]
AST (U/L)	333.00 ± 52.42[a]	344.67 ± 177.65[a]	194.67 ± 70.44[a]
TP (g/L)	37.03 ± 11.99[a]	33.57 ± 11.49[a]	26.17 ± 5.06[a]
ALB (g/L)	15.47 ± 4.82[a]	13.43 ± 4.15[a]	10.73 ± 2.06[a]
TBIL (μmol/L)	7.00 ± 0.17[a]	8.00 ± 0.17[a]	6.00 ± 2.64[a]
ALP (U/L)	172.00 ± 23.64[a]	146.00 ± 10.00[a]	154.00 ± 27.05[a]

BUN (mmol/L)	1.67 ± 0.57[a]	1.33 ± 0.95[a]	0.80 ± 0.36[a]
CREA (μmol/L)	5.67 ± 2.31[a]	8.33 ± 0.58[a]	6.67 ± 2.08[a]
CHOL (mmol/L)	4.78 ± 0.62[a]	4.67 ± 1.75[a]	3.58 ± 0.61[a]
TG (mmol/L)	5.46 ± 0.73[a]	2.50 ± 1.98[a]	4.44 ± 3.57[a]

Note: ALT, alanine aminotransferase; TP, total protein; ALB, albumin; TBIL, total bilirubin; ALP, alkaline phosphatase; AST, glutamyl transpeptidase; GLU, blood glucose; BUN, blood urea nitrogen; CREA, creatinine; CHO, cholesterol; TG, triglyceride. Means in the same row with different letters were significantly different at the a=0.05 level as determined by one-way ANOVA followed by Tukey's test; data are expressed as the mean ± SD (n=9).

462

463 **Table 4.** Relative organ weights in male and female Japanese quails fed commercial,

464 non-GM and GM diets.

	Commercial diet	Non-GM diet	GM diet
Male			
Brain	0.66 ± 0.12[a]	0.56 ± 0.04[a]	0.58 ± 0.07[a]
Heart	0.97 ± 0.10[a]	0.93 ± 0.09[a]	1.00 ± 0.28[a]
Liver	1.93 ± 0.58[a]	1.81 ± 0.52[a]	1.64 ± 0.26[a]
Lung	1.07 ± 0.33[a]	1.19 ± 0.22[a]	1.19 ± 0.44[a]
Spleen	0.03 ± 0.006[a]	0.05 ± 0.02[a]	0.03 ± 0.005[a]
Thymus	0.57 ± 0.21[a]	0.72 ± 0.27[a]	0.74 ± 0.16[a]
Testis	1.86 ± 0.83[a]	2.41 ± 1.46[a]	2.43 ± 0.36[a]
Female			
Brain	0.52 ± 0.01[a]	0.51 ± 0.07[a]	0.49 ± 0.04[a]
Heart	0.59 ± 0.03[a]	0.82 ± 0.08[a]	0.73 ± 0.16[a]
Liver	2.55 ± 0.83[a]	2.29 ± 0.37[a]	1.84 ± 0.37[a]
Lung	0.84 ± 0.04[a]	0.92 ± 0.15[a]	0.77 ± 0.09[a]
Spleen	0.03 ± 0.001[a]	0.04 ± 0.02[a]	0.05 ± 0.03[a]
Thymus	0.83 ± 0.07[a]	0.72 ± 0.04[a]	0.80 ± 0.21[a]
Ovary	0.52 ± 0.01[a]	0.17 ± 0.11[b]	0.23 ± 0.04[ab]

Means in the same row with different letters are significantly different at the a=0.05 level as determined by one-way ANOVA followed by Tukey's test; data are expressed as the mean ± SD (n=9).

468 **Table 5.** Laying performance and nutrient egg composition of Japanese quails fed
469 commercial, non-GM and GM diets.

	Commercial diet	Non-GM diet	GM diet
Laying performance			
Egg production (%)	57.14 ± 14.28[a]	48.57 ± 2.85[a]	48.23 ± 5.26[a]
Egg weight (g)	9.51 ± 0.25[a]	10.23 ± 0.34[a]	9.96 ± 0.17[a]
Nutrient composition			
Moisture (%)	70.71 ± 0.64[a]	70.94 ± 1.59[a]	70.61 ± 0.64[a]
Protein (%)	9.17 ± 0.92[a]	9.92 ± 0.68[a]	10.50 ± 0.69[a]
Fat (%)	13.61 ± 1.20[a]	13.03 ± 1.62[a]	12.8 ± 0.95[a]
Lecithin (%)	814.67 ± 44.71[a]	786.20 ± 39.14[a]	781.4 ± 35.05[a]
Cholesterol (μg/100 g)	13.26 ± 0.70[a]	13.1 ± 0.17[a]	12.26 ± 1.04[a]
VB2	0.73 ± 0.04[a]	0.76 ± 0.03[a]	0.79 ± 0.04[a]

470 Note: Values in the same row with different superscripts represent significant
471 differences ($p < 0.05$); values in the same row with the same superscripts are not
472 significantly different ($p > 0.05$); data are expressed as the mean ± SD (n=10).

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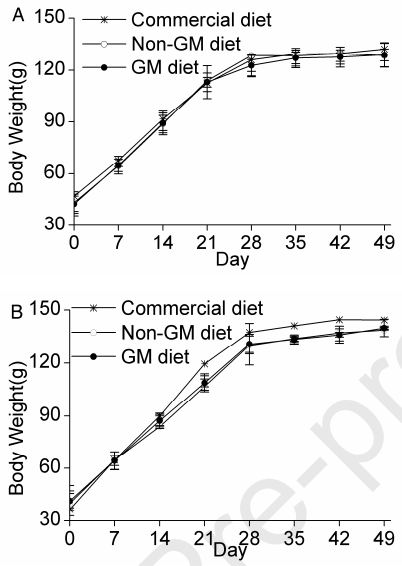

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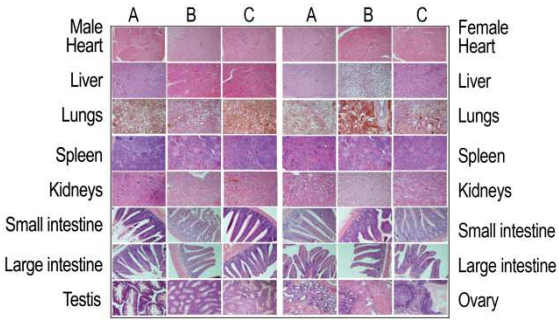
478 **Figure 1**. Mean weekly body weights of Japanese quails fed commercial, non-GM

479 and GM diets for 49 days. A, male; B female.

480



481 **Figure 2**. Histopathological results of the main organs of Japanese quails fed a
482 commercial diet, non-GM diet and GM diet. (A) Commercial diet group; (B)
483 Non-GM diet group; (C) GM diet group.

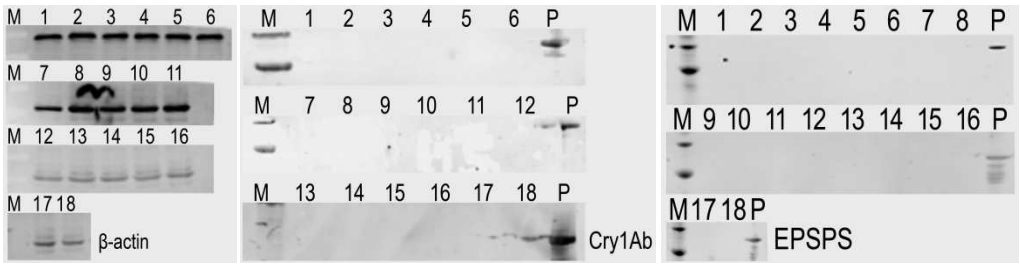
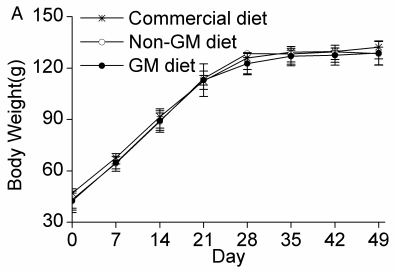
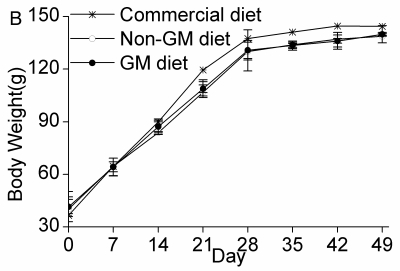
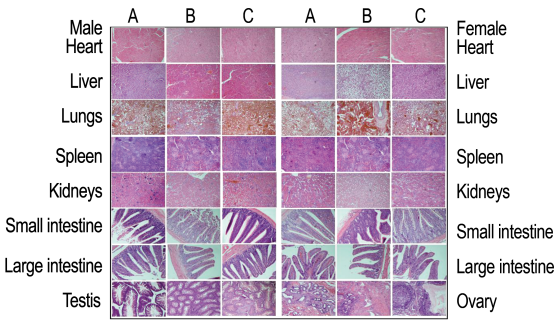
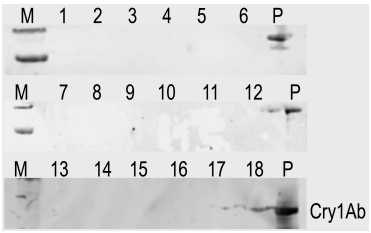


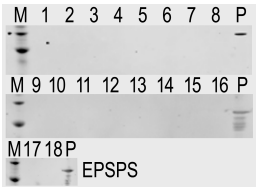
Figure 3. Western blot analysis of transgenic Cry1Ab and EPSPS proteins in the organs of Japanese quails fed a non-GM diet or GM diet. (β-actin) M, marker; nontransgenic group: lane 1, blood; lane 3, heart; lane 5, liver; lane 7, lung; lane 9, spleen; lane 11, small intestine; lane 13, large intestine; lane 15, feces; and lane 17, eggs. Transgenic group: lane 2, blood; lane 4, heart; lane 6, liver; lane 8, lung; lane 10, spleen; lane 12, small intestine; lane 14, large intestine; lane 16, feces; lane 18, eggs; (Cry1Ab) and (EPSPS) M, marker; nontransgenic group: lane 1, blood; lane 3, heart; lane 5, liver; lane 7, lung; lane 9, spleen; lane 11, small intestine; lane 13, large intestine; lane 15, feces; and lane 17, eggs. Transgenic group: lane 2, blood; lane 4, heart; lane 6, liver; lane 8, lung; lane 10, spleen; lane 12, small intestine; lane 14, large intestine; lane 16, feces; lane 18, eggs; p, positive control (GM maize).

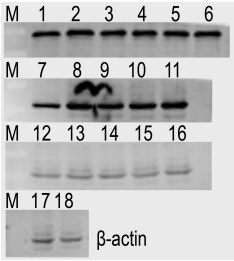












Conflict of Interest Statement

Dear Editors and Reviewers:

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the manuscript entitled "Effects of genetically modified maize C0030.3.5 on the body and laying performance and fate of transgenic proteins in Japanese quails after 49 days of feeding" (manuscript number: PSJ-D-20-00624).

The authors declare that there is no conflict of interest.

Best wishes,

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