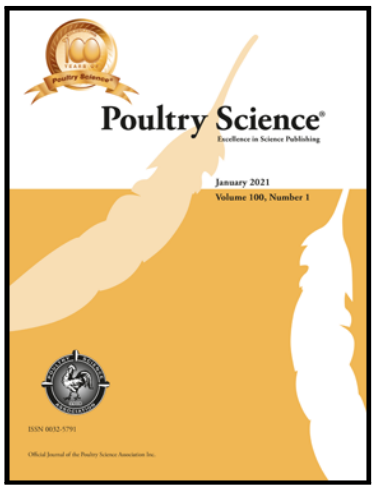

Mortality, growth, and egg production do not differ between non-transgenic and transgenic female chickens with ubiquitous expression of the 3D8 single chain variable fragment gene

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Short title: BIOMETRIC CHARACTERISTICS OF TG FEMALE CHICKENS

Mortality, growth, and egg production do not differ between non-transgenic and transgenic female chickens with ubiquitous expression of the 3D8 single chain variable fragment gene

Hyeon Yang, Sun A Ock, Seunghoon Lee, Mi-Ryung Park, Seokho Kim, Jingu No, Keon Bong Oh, Hayeon Wi, Sun Keun Jung, Yong Jin Jo, Bo Ram Lee, Minguk Lee, Sung June Byun[1]

Animal Biotechnology Division, National Institute of Animal Science, Rural Development Administration, Wanju-gun 55365, Republic of Korea

[1]Corresponding author:

Sung June Byun

Animal Biotechnology Division, National Institute of Animal Science, Rural Development Administration, 1500, Wanju-gun, 441-706, Republic of Korea

Tel: 82-63-238-7263, Email: pcs1778@korea.kr

Scientific Section

ABSTRACT

To date, many transgenic (TG) chicken lines have been developed, but few studies have performed a comparative analysis of their mortality, growth, and egg productivity. Previously, we reported the production of 3D8 scFv TG chickens showing antiviral activity. Here, we performed a biometric characterization of TG offspring female chickens. We selected 40 TG and 40 non-TG offspring female chicks among newly hatched chicks produced via artificial insemination of semen from heterotypic 3D8 scFv males into wild type female chickens. Serum was collected at 14 weeks of age, and serum concentrations of biochemical parameters, cytokines, and sex hormones were analyzed. Mortality and growth were monitored daily from 1 to 34 weeks, egg productivity was monitored daily from 20 to 34 weeks, and the weekly average values were used for analyses. Some serum parameters and cytokines were significantly different between non-TG and TG offspring female chickens. The levels of phosphorus (*PHOS*), total protein (*TP*), albumin (*ALB*), globulin (*GLOB*), and alanine aminotransferase (*ALT*) were significantly higher in non-TG chickens ($p<0.05$). The levels of alkaline phosphatase (*ALP*) and gamma-glutamyltransferase (*GGT*) were significantly higher in TG chickens ($p<0.05$). The levels of insulin growth factor-1 (*IGF-1*), interferon-gamma (*INF-γ*), interleukin-4 (*IL-4*), and *IL-8* were significantly lower in TG chickens ($p<0.05$). Despite these differences, the mortality rates, body weight, egg production rates, and egg weight were not significantly different in the experimental groups of non-TG and TG offspring female chickens ($p>0.05$). In conclusion, ubiquitous expression of the 3D8 scFv gene in TG offspring female chickens does not affect some biometric characteristics, including mortality, growth, and egg productivity.

Key words: chicken, transgenic, 3D8 scFv, biometric characterization

INTRODUCTION

Widespread transgenic (TG) chicken lines previously developed have been proven to be useful in specific disease resistance (Lyall et al., 2011) and especially in egg-based bioreactors (Sheridan, 2016). Recent studies reported that TG chickens for improving feed efficiency (Kim et al., 2020; Lee et al., 2020) and establishing host sterile surrogate systems were effective (Woodcock et al., 2019; Ballantyne et al., 2021).

We previously reported the first production of TG chickens expressing 3D8 single chain variable fragment (3D8 scFv) gene (Byun et al., 2017). As overexpression of the 3D8 scFv gene induced degradation of host nucleic acids (Jang et al., 2009), the chicken beta-actin promoter in the TG chickens was used, and 3D8 scFv mRNA and the encoded protein, were detected in all organ tissues and in isolated chicken embryonic fibroblast cells. 3D8 scFv TG chickens showed antiviral activity against H9N2 low-pathogenic avian influenza virus (AIV), and further studies showed similar activity against infectious bronchitis virus (IBV), and Newcastle disease virus (NDV) (Lee et al., 2019; Byun et al., 2020). In addition, chickens fed *Lactobacillus* species expressing 3D8 scFv showed improved growth performance and immune homeostasis (Sureshkumar et al., 2019, 2020). However, whether whole body 3D8 scFv gene expression in TG chickens can affect biometric characteristics, such as mortality, growth, and productivity, is unknown.

Comparative analyses between TG and non-TG animals report results obtained through a relative simple analytical approach, such as differences in gene expression pattern. In addition, although many TG chicken lines have been developed, few studies have performed comparative analyses of TG and non-TG chickens. Thus, it is important to understand the effect of foreign gene expression on biometric characteristics. Here, we aimed to investigate serum biochemistry,

sex hormones, cytokines, mortality, growth, and egg productivity in non-TG and TG offspring female chickens with ubiquitous expression of 3D8 scFv gene.

MATERIALS AND METHODS

Animal Care and Use

Experimental chickens were produced via artificial insemination (AI) of semen from heterotypic 3D8 scFv male chickens and wild type (WT) female chickens purchased from a specific pathogen-free (SPF) facility of White Leghorn (WL) poultry flock. These heterotypic 3D8 scFv male chickens were transgenic progeny, and founders (G0) had been developed using the lentivirus and surrogate eggshell incubation system. At that time, two G0 roosters (Lohmann Brown; LB) had been found to be able to produce TG progeny. Subsequently, G1 and G2 3D8 scFv TG chickens had been crossbred with WT hens to generate 3D8 scFv TG offspring chickens (Byun et al., 2017). All experiments were performed based on an approved animal use protocol and in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the National Institute of Animal Science (NIAS-2020-419) in the Republic of Korea.

PCR Analysis

Serum from newly hatched chicks was collected, and was used to perform sexing and transgene detection using a Phusion Blood Direct Polymerase Chain Reaction (PCR) system (Thermo Scientific, Waltham, MA, USA). Information on the chicken W-chromosome- and transgene cassette-specific primers is shown in Table 1. The PCR conditions for sexing were as follows: 98°C for 5 min, followed by 40 cycles at 98°C for 1 s, 53°C for 10 s, 72°C for 15 s, and 72°C for 1 min. The conditions for amplification of the transgene cassette were as follows: 98°C for 5 min, followed by 40 cycles at 98°C for 1 s, 62°C for 5 s, 72°C for 20 s, and 72°C for 1 min. PCR

was performed using a SimpliAmp PCR system (Applied Biosystems, Foster City, CA, USA).

Study Population

Two hundred ten chicks were newly hatched via AI. After PCR analyses, one hundred (47.6%) were confirmed to be females, and 56 of the female chicks (56.0%) were 3D8 scFv positive, consistent with Mendelian inheritance. Then, forty non-TG and 40 TG offspring female chicks were randomly assigned into 5 experimental groups (n=5, each group with 8 chickens). As a chick in non-TG and TG offspring female chicks were dead at 7 and 13 weeks of age, respectively, serum of non-TG (n=39) and TG offspring female chickens (n=39) at 14 weeks of age was collected, and was stored at -20°C until use. The chicks were individually tagged, and they were collectively housed in wire cages (width: 90 cm x depth: 60 cm x height: 30 cm) with 20 chicks until 7 to 8 weeks of age and in wire cages (91 x 92 x 100 cm) with 10 chickens until 17 to 18 weeks of age. Subsequently, all chickens were separated into individual wire cages (33 x 41 x 55 cm). The lighting program was 24 h light per day at hatch, and then was weekly, gradually reduced to 16 h light per day.

Serum Biochemistry

Serum from non-TG (n=39) and TG offspring female chickens (n=39) at 14 weeks of age was collected to analyze serum biochemical parameters, namely, the concentration of glucose (*GLU*), calcium (*CA*), phosphorus (*PHOS*), total protein (*TP*), albumin (*ALB*), globulin (*GLOB*), alanine aminotransferase (*ALT*), alkaline phosphatase (*ALP*), and gamma-glutamyltransferase (*GGT*). Concentrations were determined using an automated clinical chemistry analyzer (Hitachi Automatic Analyzer 7180, Tokyo, Japan).

Evaluation of Serum Sex Hormones and Cytokines by ELISAs

Enzyme-linked immunosorbent assays (ELISAs) were performed to determine the serum concentrations of sex hormones and cytokines including insulin growth factor-1 (*IGF-1*), interferon-gamma (*IFN-γ*), interleukin-1b (*IL-1b*), -4 (*IL-4*), -6 (*IL-6*), -8 (*IL-8*), and tissue necrosis factor-1 alpha (*TNF-1α*) in non-TG and TG offspring female chickens. The serum concentrations of sex hormones estradiol (n=39, 39 in non-TG and TG, respectively) and testosterone (n=39, 39) were determined using commercial chicken ELISA kits (Cusabio, Houston, Tx, USA). The serum concentrations of the *IGF-1* (n=39, 39), *IFN-γ* (n=36, 36), *IL-1b* (n=34, 29), *IL-4* (n=30, 39), *IL-6* (n=38, 33), *IL-8* (n=39, 36), and *TNF-1α* (n=17, 18) were determined using commercial chicken ELISA kits. The *IL-4* ELISA kit was purchased from Genorise Scientific (Glen Mills, PA, USA), the *IGF-1* kit was purchased from LSBio (Seattle, WA, USA), and the remaining kits were purchased from Abebio (Wuhan, Hubei, China).

Mortality, Growth, and Egg Productivity Measurements

The experimental groups of non-TG (n=5) and TG offspring female chickens (n=5) were used to analyze mortality, growth, and egg productivity. The mortality rates and body weight in the experimental groups of non-TG and TG offspring female chickens were monitored daily from 1 to 34 weeks of age. The egg production rates and egg weight in the experimental groups of non-TG and TG offspring female chickens were monitored daily from 20 to 34 weeks of age. The weekly average values in the experimental groups of non-TG and TG offspring female chickens were used for analyses.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism statistical software (GraphPad Prism 5.03 software, San Diego, CA, USA). Student's t test was used to compare serum biochemical parameters, cytokine and sex hormone levels in non-TG and TG offspring female chickens, and a p value of less than 0.05 was considered to indicate statistical significance. A mixed model ANOVA was used to compare mortality rates, body weight, egg production rates, and egg weight in the experimental groups of non-TG and TG offspring female chickens, and a p value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Serum Biochemistry

Serum biochemistry analysis of non-TG and TG offspring female chickens at 14 weeks of age showed significant differences in some parameters (Table 2). The mean concentration of *PHOS*, *TP*, *ALB*, *GLOB*, and *ALT* were significantly higher in non-TG chickens ($p<0.05$). In contrast, the mean concentrations of *ALP* and *GGT* were significantly higher in TG chickens ($p<0.05$). However, the mean concentrations of *GLU* and *CA* were not significantly different between non-TG and TG offspring female chickens ($p>0.05$).

Serum Sex Hormones and Cytokines

Serum sex hormones and cytokines concentrations of non-TG and TG offspring female chickens at 14 weeks of age were determined by ELISAs, and the mean concentration of serum estradiol was 161.1 ng/mL for non-TG chickens and 137.6 ng/mL for TG chickens. The mean concentration of serum testosterone was 2.0 ng/mL for non-TG chickens and 3.9 ng/mL for TG chickens. The sex hormones concentrations were not significantly different between non-TG and

TG offspring female chickens ($p>0.05$). The mean concentrations of some cytokines were significantly different between non-TG and TG offspring female chickens (Fig. 1). The mean concentrations of *IGF-1*, *IFN-γ*, *IL-4*, and *IL-8* were significantly higher in non-TG chickens ($p<0.05$). However, the mean concentration of *IL-1b*, *IL-6*, and *TNF-1α* were not significantly different between non-TG and TG offspring female chickens ($p>0.05$).

Mortality, Growth, and Egg Productivity

Mortality, growth, and egg productivity in the experimental groups of non-TG and TG offspring female chickens were analyzed with weekly average data. The first chicken death in the experimental groups of non-TG and TG offspring female chickens was observed at 7 and 13 weeks, respectively. In total, ten of the 40 (25.0%) non-TG chickens and 11 of the 40 (27.5%) TG chickens were confirmed dead by 34 weeks of age. (Fig. 2A). The mean body weight at 34 weeks of age was 1,670.0 g for non-TG chickens and 1,600.4 for TG chickens (Fig. 2B). Egg production in half of the chickens was initiated at approximately 23 weeks of age, and the total number of eggs produced by the experimental groups of non-TG and TG offspring female chickens from 20 to 34 weeks of age were 2,194 and 2,243, respectively. The mean egg production rates of TG chickens (84.9%) were slightly higher than those of non-TG chickens (69.7%) from 23 to 29 weeks of age, but the differences were decreased beginning at 30 weeks of age in the experimental groups of non-TG chickens (71.1%) and TG chickens (69.9%) (Fig. 2C). The mean egg weights in the experimental groups of non-TG and TG offspring female chickens at 23 weeks of age were 47.6 g and 45.9 g, and at 34 weeks of age were 56.7 g and 54.7 g, respectively (Fig. 2D). The cumulative mortality rates, and the mean of body weight, egg production rates, and egg weight during experimental period were not significantly different in

experimental groups of non-TG and TG offspring female chickens ($p>0.05$).

DISCUSSION

3D8 scFv exhibits nonspecific nuclease activity toward both DNA and RNA, and has been shown to have antiviral effects against classical swine fever virus in a porcine cell line (Jun et al., 2010). 3D8 scFv is also used in *Lactobacillus paracasei* (*L. paracasei*) for the use of this bacterium as feed additive (Hoang et al., 2015). TG chickens ubiquitously expressing 3D8 scFv gene was developed, and they were verified as avian models showing nuclease activity (Byun et al., 2017; Lee et al., 2019; Byun et al., 2020).

Random integration mediated by lentivirus may result in unfavorable and uncontrolled side effects in TG chickens, which have been well documented; for example, phenotypic problems of chickens expressing human erythropoietin driven by the cytomegalovirus promoter have been reported. TG chickens show signs of cytotoxicity, premature death, and other abnormalities (Koo et al., 2017). Accordingly, these techniques are not realistic to use if the resulting gene expression adversely affects biosafety in TG chickens and negatively affects phenotypic performance measures, such as mortality, growth, and productivity. Additionally, recent transgenesis techniques, especially CRISPR/Cas9 gene editing, may elicit concerns about safety and efficacy when the birds are used mainly for the mass production of meat and eggs (Zhang et al., 2015; Chira et al., 2017; Khwatenge and Nahashon, 2021). Therefore, it is important to understand the effect of genomic modifications, including foreign gene expression or deletion, on the biometric characteristics of TG chickens.

We compared serum biochemistry between non-TG and TG offspring female chickens at 14 weeks of age. To the best of knowledge, this is the first report characterizing and comparing the serum biochemical parameters between non-TG and TG offspring female chickens. Zhang et

al. (2018) and Guerrini et al. (2022) reported serum parameters in laying hens at 28 and 17 weeks of age, respectively. The serum levels of *GLU*, *TP*, and *ALB* in this study may be acceptable to avoid abnormalities, but the levels of *ALT* and *ALP* were different, which are likely due to the differences in the breed, age, dietary, and health status of the experimental chickens (Ognik and Krauze, 2016; Tang et al., 2017; Guo et al., 2021). The serum parameters, *ALT* and *ALP* mainly existed in the liver, and high levels in blood means liver damage. In the current study, the levels of *ALT* and *ALP* were observed conflicted between non-TG and TG offspring female chickens, non-TG chickens showed relatively higher serum level of *ALT* than that of TG chickens, but the level of *ALP* was higher in TG than in non-TG chickens. Therefore, further study is still necessary to investigate the effect of whole body 3D8 scFv gene expression on hepatic function.

We found that the *IFN-γ*, *IL-4*, and *IL-8* concentrations were significantly reduced in the TG chickens. Sureshkumar et al. (2019, 2020) reported significantly reduced levels of the cytokines *IL-4*, and *IL-4*, *IL-8*, *TNF-1α* in chickens fed with *L. reuteri* and *L. salivarius* expressing the 3D8 scFv gene, respectively. This substantial reduction in cytokines is consistent with the findings of this study, but clear evidences that reduced cytokines were directly involved with whole body 3D8 scFv gene expression were not confirmed. In the feeding studies of the Sureshkumar (2019, 2020), serum level of *IGF-1* was decreased, and growth performance was improved. However, the level of *IGF-1* was not decreased or increased in groups fed with WT *L. salivarius* and *L. reuteri* (Sureshkumar et al. 2021, 2022). In the current study, TG chickens showed reduced serum level of *IGF-1*. However, growth performance in the experimental groups of non-TG and TG offspring female chickens was not different, consistent with the observation in 3D8 scFv transgenic mice (Lee et al., 2014). Therefore, we concluded that effect of whole

body 3D8 scFv gene expression, without feeding *Lactobacillus* species expressing 3D8 scFv, may not be lead to improvement of growth performance.

Alagawany and Mahrose (2014) reported that the body weight of LB laying hens at 34 weeks of age was approximately 1.8 kg. Additionally, Berghof et al. (2015) reported that the mean body weight of dams in the population of WL chickens was 1.61 kg at 35 to 40 weeks of age and that the mean egg weights at 17 to 34 and 35 to 56 weeks of age were 53.7 and 58.7 g, respectively. Although non-TG and TG offspring female chickens in this study were produced via AI using semen from TG males and WT WL females, the mean body and egg weight ranges of the chickens at 34 weeks of age may be acceptable to avoid abnormalities. The trends in the egg production curves, weeks of peak egg production, and age of hens at the peak in non-TG and TG offspring female chickens were consistent with those in a previous study, in which selected and nonselected purebred WL lines were used (Savegnago et al., 2012).

In conclusion, our results confirmed the lack of differences in mortality, growth, and egg productivity in the experimental groups of non-TG and TG offspring female chickens. These results are meaningful because this is the first comparative analysis between non-TG and TG offspring female chickens, and these findings provide evidence that the health status and productivity were not impaired by whole body 3D8 scFv gene expression.

FUNDING

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DECLARATION OF COMPETING INTEREST

The authors have no conflicts of interest to declare.

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TABLES

Table 1. Primer information

Name	Primer sequences
Sexing F	5`-AGA ATG AGA AAC TGT GCA AAA CAG- 3`
Sexing R	5`-CTA TCA GAT CCA GAA TAT CTT CTG C-3`
CBA promoter F	5`-CCT CTG CTA ACC ATG TTC ATG CCT TC-3`
CBA-3D8 scFv R	5`-GCT AGT GAA TGT GTA TCC AGA AGC CTT-3`

Table 2. Serum biochemistry in non-TG and TG offspring female chickens

Parameters[1]	non-TG	TG	P value
GLU, mg/dL	232.62 ± 13.98[2]	227.87 ± 14.91	0.3455
CA, mg/dL	12.93 ± 1.20	13.91 ± 1.30	0.0560
PHOS, mg/dL	8.54 ± 1.48[a]	5.01 ± 0.92	< 0.0001
TP, g/dL	6.46 ± 1.09[a]	5.06 ± 0.59	0.0003
ALB, g/dL	2.58 ± 0.45[a]	1.93 ± 0.23	0.0002
GLOB, g/dL	3.88 ± 0.66[a]	3.13 ± 0.40	0.0010
ALT, U/L	40.87 ± 15.38[a]	23.84 ± 9.12	0.0015
ALP, U/L	95.59 ± 43.92	168.08 ± 62.59[a]	< 0.0001
GGT, U/L	5.18 ± 5.88	11.67 ± 5.84[a]	0.0103

Serum collected from non-TG (n=39) and TG offspring female chickens (n=39) at 14 weeks of age was used to analyze serum biochemical parameters, namely, [1]*GLU* = glucose, *CA* = calcium, *PHOS* = phosphorus, *TP* = total protein, *ALB* = albumin, *GLOB* = globulin, *ALT* = alanine aminotransferase, *ALP* = alkaline phosphatase, *GGT* = gamma glutamyltransferase

[2]Data are presented as the mean ± SD values.

[a] Means values in the same row with no common superscript differ significantly.

FIGURE CAPTIONS

Fig. 1.

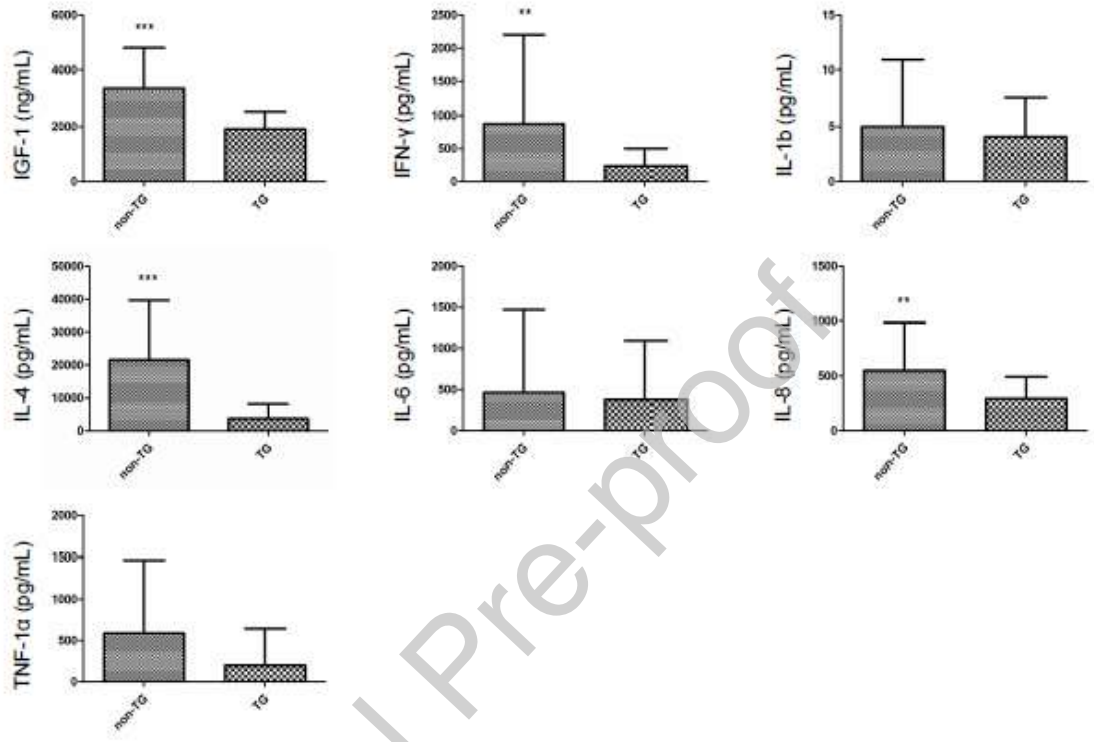

Fig. 1. Serum cytokines in non-TG and TG offspring female chickens.

Serum collected from non-TG and TG offspring female chickens at 14 weeks of age was used to analyze serum cytokine concentrations. The serum concentrations of the *IGF-1* (n=39, 39 in non-TG and TG, respectively), *IFN-γ* (n=36, 36), *IL-1b* (n=34, 29), *IL-4* (n=30, 39), *IL-6* (n=38, 33), *IL-8* (n=39, 36), and *TNF-1α* (n=17, 18) were determined by ELISAs. *IGF-1*, insulin growth factor-1; *IFN-γ*, interferon-gamma; *IL-1b*, interleukin-1b; *IL-4*, interleukin-4; *IL-6*, interleukin-6; *IL-8*, interleukin-8; *TNF-1α*, tissue necrosis factor-1alpha. The results are expressed as the mean ± SD values. ** $p<0.01$, *** $p<0.001$.

Fig. 2.

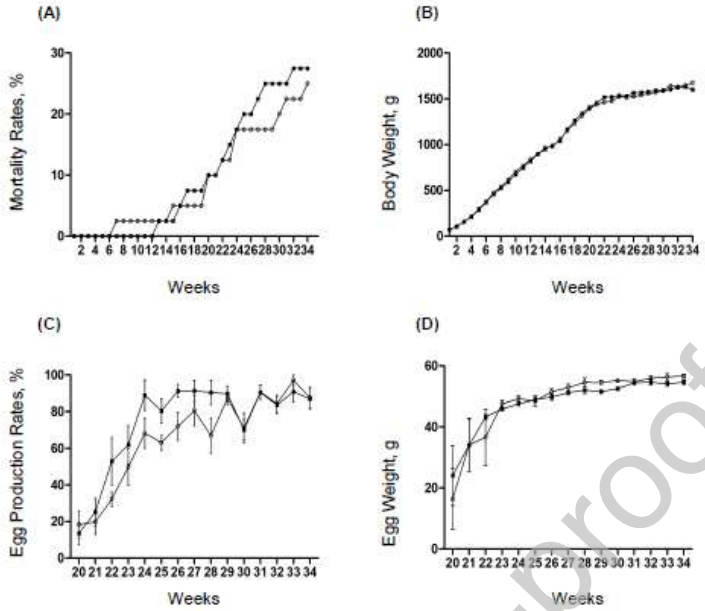

Fig. 2. Mortality, growth, and egg productivity in the experimental groups of non-TG and TG offspring female chickens.

Mortality rates, body weight, egg production rates, and egg weight in the experimental groups of non-TG (n=5) and TG offspring female chickens (n=5) are shown. The weekly average values in the experimental groups of non-TG (empty circle) and TG offspring female chickens (black square) were used for analyses. (A) Cumulative mortality rates in the experimental groups of non-TG and TG offspring female chickens are displayed as percentages of the mean. (B) Body weight in the experimental groups of non-TG and TG offspring female chickens is displayed in grams of the mean. (C) Egg production rates in the experimental groups of non-TG and TG offspring female chickens are displayed as the percentage of the mean ± SEM values. (D) Egg weight in the experimental groups of non-TG and TG offspring female chickens is displayed in grams of the mean ± SEM values.

Declaration of interests

☑ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: