

# Effects of nutritional programming on growth and metabolism caused by albumen removal in an avian model

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## Abstract

In mammalian models of prenatal undernutrition the maternal diet is manipulated, exerting both nutritional and hormonal effects on the offspring. In contrast, in the chicken, strictly nutritional effects can be applied. Prenatal protein undernutrition in chickens was induced by partial replacement of albumen with saline during early embryonic development (albumen-deprived group) and results were compared with a sham-manipulated and a non-manipulated group. Body weight of the albumen-deprived hens was reduced throughout the entire experimental period (0–55 weeks). The reproductive capacity was diminished in the albumen-deprived hens as reflected in the reduced number of eggs and lower egg weight. The plasma triiodothyronine levels were increased in the albumen-deprived group compared with the non-manipulated hens, but not the sham-manipulated hens. An oral glucose tolerance test (OGTT) at 10 weeks of age revealed a decreased glucose tolerance in the albumen-deprived hens. During adulthood, an age-related loss of glucose tolerance was observed in the hens, leading to disappearance of treatment differences in the OGTT. The offspring of the albumen-deprived hens (PA chicks) had reduced body weight until at least 3 weeks of age. In addition, the PA chicks had a decreased relative residual yolk weight at hatching. An insulin tolerance test revealed increased sensitivity to insulin for the PA chicks compared with the offspring of the non-manipulated (PN) and sham-manipulated hens (PS). In conclusion, prenatal protein undernutrition by albumen removal caused long-term effects on body weight, reproductive performance, and physiology.

## Key Words

- ▶ prenatal protein undernutrition
- ▶ reproduction
- ▶ glucose homeostasis
- ▶ chickens

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## Introduction

Results of epidemiological studies in humans have indicated a correlation between *in utero* growth restriction and the subsequent development of adult degenerative diseases such as type 2 diabetes (Roseboom *et al.* 2011,

Hales & Barker 2001). Several mammalian models such as rat and sheep have been developed to study this relationship (Ozanne 2001). One of the most extensively studied models is the maternal low-protein-diet rat model

(Tarry-Adkins *et al.* 2010). In this model, offspring exhibited growth retardation (Shepherd *et al.* 1997) and alterations in body composition. It is known that during periods of poor maternal nutrition, the fetus adapts by diverting nutrients to critical organs such as the brain, at the expense of visceral organs such as the liver (Armitage *et al.* 2004). In the liver, both structural and functional changes were observed: offspring of animals fed a low-protein diet had larger hepatic lobules (Burns *et al.* 1997) and displayed changes in several hepatic enzymes important in glucose homeostasis (Desai *et al.* 1997), which could lead to the programming of adult offspring glucose tolerance. In young life, the offspring exhibited improved glucose tolerance compared with controls (Shepherd *et al.* 1997), whereas at 70 days of age, glucose intolerance with lower insulin response was observed (Dahri *et al.* 1991).

In the mammalian models discussed above, the fetus grows and develops inside the uterus of the mother, and is entirely dependent on the nutrients that are continuously supplied by the mother via the placenta. Maternal diet manipulation will not only induce nutritional alterations, but in addition maternal hormonal effects will also be involved. The combination of these nutritional and secondary hormonal changes can induce the developmental programming observed in the offspring. In birds, however, the embryo develops independently in the egg and all nutrients and signals are deposited in the egg at the time of laying. The egg components are readily accessible for manipulation, allowing investigation of strictly nutritional effects. As albumen is the main source of protein for tissue synthesis in the developing embryo (Freeman & Vince 1974), prenatal protein undernutrition can be induced by partial replacement of albumen with saline during early embryonic development (reviewed by Willems *et al.* (2014a)). The main advantage of the use of an avian model is thus the possibility of examination of the direct nutritional effects without inference of secondary maternal (e.g., hormonal) effects.

Long-term effects of albumen deprivation have been studied previously (Willems *et al.* 2013). During the rearing period, reduced body weight was observed, whereas during the laying period, catch-up growth was manifested resulting in increased body weight with reduced reproductive capacity, as reflected in the reduced number and weight of the eggs. Effects on egg composition were also apparent. The eggs from the albumen-deprived hens had a higher proportional yolk and lower proportional albumen weight. This led to the hypothesis that embryonic protein deprivation by albumen removal could result in

transgenerational effects, affecting not only the embryonic development directly but also the post-hatching performance of the offspring. Several studies on the effects of partial albumen removal have been published previously (Hill 1993, Finkler *et al.* 1998, Everaert *et al.* 2013, Willems *et al.* 2013, 2014b), but, to our knowledge, this is the first report examining potential transgenerational effects of albumen deprivation during embryogenesis.

Transgenerational effects of prenatal protein undernutrition have already been studied in mammalian models. The second-generation offspring (F<sub>2</sub>) of rat dams fed low-protein diets had higher birth weights, which remained over the whole experiment (6 months) at least for the male offspring (Pinheiro *et al.* 2008). Moreover, the F<sub>2</sub> offspring exhibited hyperglycemia accompanied by hyperinsulinemia and insulin resistance in both sexes (Pinheiro *et al.* 2008).

The objective of this study was to investigate the long-term effects of prenatal protein undernutrition due to albumen removal on body weight, body composition, hormones and metabolites, glucose tolerance and insulin resistance in laying hens and whether these effects can be transmitted to the offspring of these albumen-deprived hens. In addition to being a new model for prenatal low protein availability, this study can also contribute to understanding the importance of albumen proteins during embryonic development in the chicken.

## Materials and methods

### Ethics statement

All experiments were conducted in strict accordance with the European Communities Council Directive (2010/63/EC) and were approved by the Ethical Commission for Experimental Use of Animals of the KU Leuven (P002-2012).

### Experimental design

**Incubation and albumen removal** The set-up of this experiment has been described previously by Willems *et al.* (2013). Briefly, Isa Brown layer-type eggs were commercially obtained from a 48-week-old breeder flock (Vepymo, Poppel, Belgium) and randomly divided between the three treatments. The eggs were incubated in a forced-draft incubator (PAS Reform, Zeddam, The Netherlands) at a dry bulb temperature of 37.6 °C and a wet bulb temperature of 29.0 °C and were turned every hour over 90°. After 1 day of incubation, a hole was drilled in the egg, 3 ml of albumen was removed using a needle and syringe and replaced by

approximately the same volume of sterile saline, followed by sealing of the hole using a drop of paraffin (albumen-deprived group). A sham-manipulated group was mock treated by inserting the needle, similar to the albumen-deprived group, except for the actual albumen removal and saline injection. A third group received no treatment (non-manipulated group). After hatching, chicks were sexed on the basis of the colors of their down and only female chicks were used.

**Rearing and laying period** A total of 47 non-manipulated, 32 sham-manipulated and 46 albumen-deprived female chicks were randomly assigned to floor pens (15–16 chicks/pen) located in an environmentally controlled room with wood shavings as litter. The different number of chicks per group was due to a difference in the number of set eggs and in hatching success per group (Willems *et al.* 2013). Body weight of the hens was individually measured each week starting from hatching. The room temperature was initially set at 34 °C and was gradually decreased to 20 °C at 5 weeks of age. This temperature was maintained until 55 weeks of age. In the beginning, a 23 h light cycle was provided and was gradually decreased to 10 h at 6 weeks. From 14 weeks, the light period was prolonged gradually to 15 h by 19 weeks of age in order to stimulate sexual maturation, according to the Isa Brown Management Guide (Hendrix Genetics, Boxmeer, The Netherlands). The hens received soy–wheat–corn-based diets, formulated based on the Isa Brown Management Guide (Research Diet Services, Wijk bij Duurstede, The Netherlands; dietary information is available in Willems *et al.* (2013)) and were allowed to feed *ad libitum*. At 18 weeks of age, 24 hens/treatment were placed together with two purebred Isa Brown parent roosters aged 28 weeks in floor pens to obtain fertilized eggs, while the remainder of the hens were kept for sampling. For the eggs collected for incubation at 25, 40, and 55 weeks, the different groups of hens (non-manipulated, sham-manipulated, and albumen-deprived) were placed both in another pen and with another pair of roosters to minimize paternal effects.

**Roosters** In total, six 28-week-old parental Isa Brown roosters were obtained from Hendrix Genetics. Body weight of the roosters started from 2507 ± 35 g at 28 weeks and increased to 3205 ± 81 g at 65 weeks of age. No significant differences in body weight, body composition, or plasma triiodothyronine (T<sub>3</sub>), thyroxine (T<sub>4</sub>), cholesterol, and corticosterone at 55 weeks between the three pairs of roosters were observed.

**Incubation of fertilized eggs to obtain offspring** Fertilized eggs were collected for 11 consecutive days around 25 and 40 weeks and for 15 consecutive days around 55 weeks of hen age, corresponding to the start of the laying period, the peak lay, and the end of the laying period respectively. Laying rate (%) is calculated at 25, 40, and 55 weeks as number of eggs divided by number of hens in a pen multiplied by 100. Eggs were collected daily, numbered, weighed, and stored at 12 °C until the start of incubation. The eggs were incubated as described above and hatched in individual hatching baskets. After hatching, chicks were individually numbered using a leg number (the same as the egg number) and weighed. Progeny of the non-manipulated hens are defined as PN, of the sham-manipulated hens as PS, and of the albumen-deprived hens as PA. Relative chick weight was calculated as chick weight relative to the egg weight before incubation. After 21.5 days of incubation, unhatched eggs were opened and checked for fertility. Hatchability (%) was calculated as the number of hatched chicks relative to the number of fertile eggs. At hatching, the residual yolk in the offspring was weighed for 12 (at 25 weeks) or 15 (at 40 and 55 weeks) chicks/group and the relative residual yolk weight to egg weight at setting was calculated.

**Rearing of offspring** At 25, 40, and 55 weeks of hen age, 45 chicks/treatment were divided over three pens (15 chicks/pen). Body weight was individually measured every 3 days post-hatching until 3 weeks of age. All floor pens were located in an environmentally controlled room. The room temperature was set at 34 °C and this was gradually decreased to 25 °C at 3 weeks of age. A 23 h light cycle was provided and this gradually decreased to 18 h at 3 weeks. The chicks received a soy–wheat–corn-based commercial diet (2920 kcal/kg metabolizable energy and 180.3 g/kg crude protein).

**Body composition** Body composition of the adult hens was determined at 25 and 40 weeks ( $n = 12$ , except for the sham-manipulated group ( $n = 8$ )). At 55 weeks of age, all remaining hens were sampled: 23 non-manipulated, 16 sham-manipulated, and 22 albumen-deprived hens. Water was provided until they were killed. The liver, breast muscle (only left side), digestive tract, abdominal fat (not at 25 weeks), ovary, oviduct, and heart weight were recorded. The digestive tract is defined as starting from the end of the esophagus, containing the proventriculus, the gizzard, the small and large intestine until the beginning of the cloaca. The digestive tract was not emptied before sampling, but the hens had fasted

overnight. Proportional weights relative to body weight (%) of all components were calculated. The number of large yellow follicles (weight > 1 g) in the ovary was also counted (Decuyper *et al.* 1993, Bruggeman *et al.* 2005).

**Blood sampling** Blood was sampled from the laying hens at hatching and at 25, 40, and 55 weeks of age. In the offspring, blood was sampled at hatching and at 7 and 21 days post-hatching. At hatching and on day 7, 0.5 ml of blood was taken from the vena jugularis. In chickens older than 1 week, 1 ml of blood was collected from the wing vein. First, disinfection with 70% ethanol was applied. Blood was sampled using a 1-ml syringe and a 27 G needle and collected in heparinized tubes (Sigma). The blood was separated by centrifugation at 1500 g for 10 min at 6 °C. The plasma samples were collected and stored at –20 °C for later analyses of T<sub>3</sub>, T<sub>4</sub>, corticosterone, cholesterol, and glucose. Cholesterol and corticosterone were not measured in the offspring. Plasma glucose was only measured at hatching both in the parent hens and offspring.

**Plasma hormones and metabolites** Plasma analysis was performed on eight (at hatching in the parent hens) or 12–15 samples/group per sampling day for each measurement. Plasma T<sub>3</sub> and T<sub>4</sub> concentrations were measured by RIA as described by Darras *et al.* (1996). The antisera for T<sub>3</sub> and T<sub>4</sub> were purchased from Byk-Belga (Brussels, Belgium). Intra-assay coefficient of variation (CV) values were 4.5 and 5.4% for T<sub>3</sub> and T<sub>4</sub> respectively. The level of corticosterone was measured with the Corticosterone Double Antibody – 125I RIA Kit for Animal Testing (07120103, MP Biomedicals, Carlsbad, CA, USA). The intra-assay CV for corticosterone was 7.1%. Plasma glucose concentration was determined using a commercially available kit (298-65701, WAKO Pure Chemical Industries Ltd, Osaka, Japan). The intra-assay CV for glucose was 2.2%. Total cholesterol was determined using a commercially available kit (RX Monza CH200, Randox Laboratories Limited, County Antrim, UK). The intra-assay CV values for cholesterol was 1.99%.

**Glycogen determination** At hatching, liver samples were collected for determination of the hepatic glycogen content ( $n=8$  in hens,  $n=12$  in offspring). A method adapted from that of Dreiling *et al.* (1987) was used and this has been previously described in Willems *et al.* (2014b).

**Oral glucose tolerance and insulin tolerance test** At 10, 40, and 55 weeks of age of the laying hens and at 3 weeks of age in the offspring (obtained at 25, 40, and 55 weeks), an

oral glucose tolerance test (OGTT) was performed. After an overnight fast, a mean glucose load of 2 g/kg body weight, dissolved in milliQ, was administered by intubation in the crop using a plastic tube and 5 or 10 ml syringe (Terumo, Tokyo, Japan), depending on the age of the chicken. The administered dose was selected based on data published in the literature (Buyse *et al.* 1990). The volume of the administered solution was kept at a minimal level, based on the solubility of glucose. At 10 weeks of age ( $n=11$  per treatment), blood was taken from the hens before and 30, 60, and 120 min after the glucose load. At 40 and 55 weeks and in the offspring ( $n=8$  per treatment), blood was taken before and 20, 40, and 60 min after administration.

At 51 weeks of age for the laying hens and at 3 weeks of age for the offspring obtained at 55 weeks, an insulin tolerance test (ITT) was performed ( $n=8$  per treatment). After an overnight fast, insulin from bovine pancreas (I4011, Sigma–Aldrich) was intramuscularly injected into the thigh muscle using a 1 ml syringe (Terumo) and 27G needle (Neolus, Terumo) at a mean concentration of 30 µg/kg body weight. The administered dose and injection site were selected based on results published in the literature (Simon *et al.* 2000). In the laying hens, blood was taken before injection and 30, 90, 240, 360, and 480 min after insulin administration. In the offspring, blood was sampled before and 30, 90, 240, and 360 min after insulin injection.

Blood samples were taken as described previously and plasma glucose concentrations were determined on all samples.

### Statistical analysis

All data were processed using the statistical software package SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA). In all analyses, a hierarchical model-building strategy was followed, starting with the most complex model including main effects (maternal age, maternal treatment (non-manipulated, sham-manipulated, and albumen-deprived), cockerel pair, and progeny age) and interactions, and removing non-significant effects to obtain the final model.

**Hens** Body weight was analyzed after a log-stabilizing transformation using repeated measurements in the linear mixed effects model, containing age, treatment, and their interaction as variables. Leg numbers were used for identification. The laying rate was analyzed using a logistic regression with treatment and age (25, 40, and 55 weeks) as variables. Body composition parameters, egg weight, plasma T<sub>3</sub> and T<sub>4</sub>, corticosterone, and cholesterol were

analyzed using a general linear model with treatment and age (25, 40, and 55 weeks) and their interaction as variables. At hatching, plasma hormones and metabolites and hepatic glycogen concentration were analyzed using a one-way ANOVA with treatment as a variable. The OGTT and ITT results were analyzed separately per age, and the plasma glucose concentrations from these tests were analyzed using a repeated measurement in the linear mixed effects model, containing time after glucose administration, treatment, and their interaction as variables.

**Offspring** Hatchability of the offspring was analyzed using a logistic regression model with maternal treatment and maternal age (eggs collected at 25, 40, and 55 weeks) as variables. Absolute and relative chick weight, absolute and relative residual yolk weight, plasma glucose, and hepatic glycogen at hatching were analyzed using a general linear model with maternal treatment and maternal age (25, 40, and 55 weeks) and their interaction as variables. Body weight was analyzed using repeated measurement in the linear mixed effects model, containing age of the offspring (every 3 days from hatching to 3 weeks of age), maternal treatment and maternal age (25, 40, and 55 weeks), and their interaction as variables. Leg numbers were used for identification. Plasma  $T_3$  and  $T_4$  were analyzed using the general linear model with the age of the offspring (at hatching, and 1 and 3 weeks post-hatching), maternal treatment, maternal age (25, 40, and 55 weeks), and all their interactions as variables. The results of the OGTT and ITT were analyzed using repeated measurements in the linear mixed effects model, including maternal treatment, maternal age (25, 40, and 55 weeks), time after glucose or insulin administration, and all their interactions as variables. Leg numbers were used for identification.

When there was a significant effect of treatment, or interaction with age, means were further compared using *post-hoc* Tukey's test. For all parameters, significance was set at the 5% level. All values were expressed as means, and when possible with their S.E.M.

**Table 1** Body weight (g) of the non-manipulated ( $n=47$ ), sham-manipulated ( $n=32$ ), and albumen-deprived laying hens ( $n=46$ ) at 25, 40, and 55 weeks of age. Data are expressed as mean  $\pm$  S.E.M.

	Non-manipulated	Sham-manipulated	Albumen-deprived	P value treatment	P value age	P value interaction
Body weight (g)	<sup>a</sup>	<sup>b</sup>	<sup>c</sup>	<0.001	<0.001	NS
Hatching	42.4 $\pm$ 0.5	43.5 $\pm$ 0.8	41.2 $\pm$ 0.5			
25 weeks	1888 $\pm$ 22	1901 $\pm$ 44	1745 $\pm$ 26			
40 weeks	2145 $\pm$ 33	2185 $\pm$ 42	2013 $\pm$ 33			
55 weeks	2247 $\pm$ 46	2202 $\pm$ 59	2054 $\pm$ 47			

<sup>a,b,c</sup>Treatment means with different superscripts are significantly different ( $P<0.05$ ). P values of effects of treatment, age, and their interaction are listed in separate columns.

## Results

### Body weight and composition of the hens

The albumen-deprived hens weighed significantly less than the non-manipulated ( $P<0.001$ ) and the sham-manipulated hens ( $P<0.001$ ). The non-manipulated hens differed from the sham-manipulated hens ( $P=0.005$ ) at all ages measured (Table 1). The body composition data are summarized in Table 2. The albumen-deprived group exhibited a lower absolute breast muscle and ovary weight when compared with the non-manipulated group ( $P=0.007$  and  $P=0.008$  respectively). The sham-manipulated group was not different from the two other groups. The albumen-deprived group had less abdominal fat than the sham-manipulated group ( $P=0.042$ ) and tended to have less abdominal fat than the non-manipulated group ( $P=0.079$ ), which were similar in this respect. The albumen-deprived group displayed a trend towards lower absolute digestive tract weight when compared with the non-manipulated group ( $P=0.070$ ) and the sham-manipulated group ( $P=0.078$ ), which were similar. The albumen-deprived group had a trend towards a higher proportional liver weight than the non-manipulated group, but not the sham-manipulated group. The number of large yellow follicles ( $>1$  g) present was significantly affected by treatment ( $P=0.045$ ), age ( $P=0.004$ ), and almost significantly affected by their interaction (0.076). The non-manipulated hens had a trend toward more follicles ( $6.5 \pm 0.4$ ) when compared with the sham-manipulated ( $5.7 \pm 0.5$ ) and the albumen-deprived hens ( $5.5 \pm 0.5$ ).

### Plasma hormones and metabolites and hepatic glycogen concentration of the laying hens

At hatching, no differences in plasma glucose,  $T_3$ ,  $T_4$ , total cholesterol and corticosterone were observed (data not shown). No effect of treatment was detected on the hepatic glycogen content of newly hatched chicks,

**Table 2** Body composition of the laying hens of the non-manipulated, sham-manipulated, and albumen-deprived group at 25, 40, and 55 weeks ( $n=32-47$ ). Data are shown as mean  $\pm$  S.E.M.

	Non-manipulated	Sham-manipulated	Albumen-deprived	P value treatment	P value age	P value interaction
Absolute weight (g)						
Breast muscle (left)	80.4 $\pm$ 1.8 <sup>a</sup>	77.6 $\pm$ 2.1 <sup>ab</sup>	74.9 $\pm$ 1.6 <sup>b</sup>	0.010	<0.001	NS
Liver	35.1 $\pm$ 0.9	35.1 $\pm$ 1.2	35.4 $\pm$ 1.1	NS	<0.001	NS
Heart	7.7 $\pm$ 0.2	7.9 $\pm$ 0.3	7.6 $\pm$ 0.2	NS	<0.001	NS
Ovary	8.4 $\pm$ 0.2 <sup>a</sup>	7.7 $\pm$ 0.3 <sup>ab</sup>	7.4 $\pm$ 0.3 <sup>b</sup>	0.009	<0.001	NS
Oviduct	64.1 $\pm$ 1.6	65.7 $\pm$ 2.3	61.1 $\pm$ 2.3	NS	<0.001	NS
Digestive tract	171.4 $\pm$ 2.7	172.3 $\pm$ 3.5	163.2 $\pm$ 3.2	0.039	0.005	NS
Abdominal fat	90.4 $\pm$ 6.2 <sup>ab</sup>	94.5 $\pm$ 8.3 <sup>a</sup>	71.9 $\pm$ 4.4 <sup>b</sup>	0.029	NS	NS
Proportional weight to body weight (%)						
Breast muscle (left)	4.00 $\pm$ 0.12	3.96 $\pm$ 0.16	3.99 $\pm$ 0.13	NS	<0.001	NS
Liver	1.73 $\pm$ 0.05	1.79 $\pm$ 0.07	1.91 $\pm$ 0.07	0.063	<0.001	NS
Heart	0.38 $\pm$ 0.01	0.39 $\pm$ 0.01	0.39 $\pm$ 0.01	NS	<0.001	NS
Ovary	0.41 $\pm$ 0.01	0.39 $\pm$ 0.01	0.39 $\pm$ 0.01	NS	NS	NS
Oviduct	3.15 $\pm$ 0.08	3.32 $\pm$ 0.12	3.13 $\pm$ 0.11	NS	0.005	NS
Digestive tract	8.46 $\pm$ 0.14	8.74 $\pm$ 0.25	8.66 $\pm$ 0.19	NS	<0.001	NS
Abdominal fat	4.24 $\pm$ 0.23	4.44 $\pm$ 0.33	3.61 $\pm$ 0.19	NS	NS	NS

<sup>a,b,c</sup>Treatment means with different superscripts are significantly different ( $P<0.05$ ).  $P$  values of effects of treatment, age and their interaction are listed in separate columns.

although the albumen-deprived chicks ( $0.64 \pm 0.22$  ng/mg liver) had numerically a lower hepatic glycogen content than the non-manipulated chicks ( $1.29 \pm 0.24$  ng/mg liver) and the sham-manipulated chicks ( $1.15 \pm 0.67$  ng/mg liver). At 25, 40, and 55 weeks, no differences in plasma corticosterone concentrations could be detected, and only an effect of age ( $P<0.001$ ) could be observed on the plasma  $T_4$  and cholesterol concentrations (data not shown). The plasma  $T_3$  concentrations were affected by group ( $P=0.030$ ) and age ( $P=0.005$ ), but not by the interaction. The albumen-deprived hens had higher plasma  $T_3$  concentrations ( $0.60 \pm 0.06$  ng/ml) than the non-manipulated hens ( $0.43 \pm 0.04$  ng/ml) ( $P=0.025$ ), whereas the sham-manipulated hens had intermediate values ( $0.53 \pm 0.05$  ng/ml).

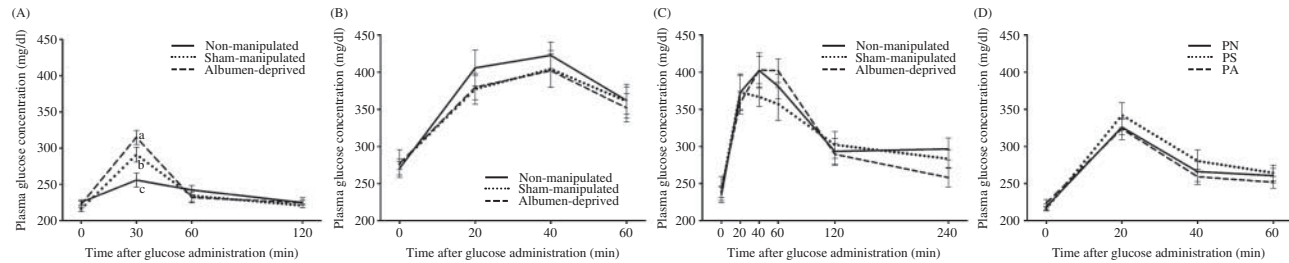
### OGTTs and ITT

The OGTT and ITT results are presented in Figs 1 and 2 respectively. The basal glucose level in the plasma of the hens at 10 weeks was approximately 220 mg/dl, whereas in adult hens this level was increased to 250 mg/dl. The maximal plasma glucose levels after a glucose load were approximately 400 mg/dl at 40 and 55 weeks. At 10 weeks of age, however, the glucose levels did not rise to this level. The glucose concentration measured after an OGTT performed at 10 weeks of age exhibited an effect of time after glucose load ( $P<0.001$ ), nearly of treatment

( $P=0.066$ ) and of the interaction between treatment and time ( $P<0.001$ ). From 30 min following glucose administration, the albumen-deprived group had a higher level of plasma glucose than the non-manipulated ( $P<0.001$ ) and the sham-manipulated groups ( $P=0.017$ ). The level for the sham-manipulated group was also increased compared with the non-manipulated group ( $P<0.001$ ). The OGTT performed at 40 and at 55 weeks and ITT at 51 weeks were only affected by time after administration ( $P<0.001$ ) and not by group or the interaction.

### Incubation of the progeny

The albumen-deprived hens had a reduced laying rate compared with both the non-manipulated and sham-manipulated groups throughout the entire laying period ( $P<0.001$  for all). At 40 and 55 weeks of age, the sham-manipulated hens also exhibited a decreased laying rate compared with the non-manipulated group ( $P=0.009$  and  $P<0.001$  respectively). Only an effect of age ( $P<0.001$ ) could be detected on the hatchability ( $81.4 \pm 4.0\%$ ). At 25 and 40 weeks of age, the albumen-deprived hens produced eggs with lower egg weight compared with the non-manipulated ( $P<0.001$  and  $P=0.027$  respectively) and the sham-manipulated groups ( $P<0.001$  and  $P=0.024$  respectively). At 55 weeks, however, no differences were observed (Table 3). At hatching, the PA chicks had a decreased absolute and relative chick weight (Table 3)



**Figure 1**

Oral glucose tolerance test performed at (A) 10, (B) 40, and (C) 55 weeks of age of the laying hens of the non-manipulated, sham-manipulated, and albumen-deprived groups ( $n=8-11$ ) and (D) after 22 days of age in the progeny of the non-manipulated (PN), sham-manipulated (PS), and albumen-deprived laying hens (PA) originating from eggs laid by hens at

25, 40, and 55 weeks of age ( $n=8$  per group per incubation). A glucose load of 2 g/kg body weight was intubated in the crop and blood was taken at several time points after administration. Glucose in the plasma (mg/dl) was measured. Data are expressed as mean  $\pm$  S.E.M. a, b, and c refer to differences between treatment groups ( $P<0.05$ ) per time point.

compared with the PS chicks originating from eggs from 25- ( $P<0.001$  and  $P<0.001$  respectively), 40- ( $P<0.001$  and  $P=0.008$  respectively), and 55-week-old hens ( $P=0.002$  and  $P<0.001$  respectively) and compared with the PN chicks originating from eggs from 25- ( $P<0.001$  and  $P<0.001$  respectively) and 40-week-old hens ( $P<0.001$  and  $P=0.004$  respectively). In eggs produced by 55-week-old hens, the relative chick weight tended to be lower for the PA compared with the PN chicks ( $P=0.098$ ) and the PN chicks were also absolutely and relatively less heavy than the PS chicks ( $P=0.013$  and  $P<0.001$  respectively). The PA chicks tended to have a decreased residual yolk weight (Table 3) compared with the PS chicks, whereas the PN chicks had an intermediate value. The PA chicks had a decreased relative residual yolk weight (Table 3) compared with the PS chicks ( $P=0.036$ ), whereas the PN were not different from the other groups.

### Body weight of the progeny

The body weight of the offspring measured until 3 weeks of age (Fig. 3) was affected by breeder age ( $P<0.001$ ), treatment ( $P<0.001$ ), age of the progeny ( $P<0.001$ ), and interaction effects between breeder age and treatment ( $P<0.001$ ), breeder age and age of the progeny ( $P<0.001$ ), and between treatment and age of the progeny ( $P<0.001$ ), but no three-way interaction effect among treatment, breeder age, and age of the progeny. The PA chicks weighed less than the PN and PS chicks at 25 ( $P<0.001$  and  $P<0.001$  respectively) and 40 weeks ( $P=0.005$  and  $P<0.001$  respectively) over the entire 3-week rearing period. In addition, the PN chicks had an increased body weight compared with the PS chicks at 25 weeks ( $P<0.001$ ). At 55 weeks, no difference in body weight between the different groups could be detected.

### Plasma hormones and metabolites and hepatic glycogen concentration of the progeny

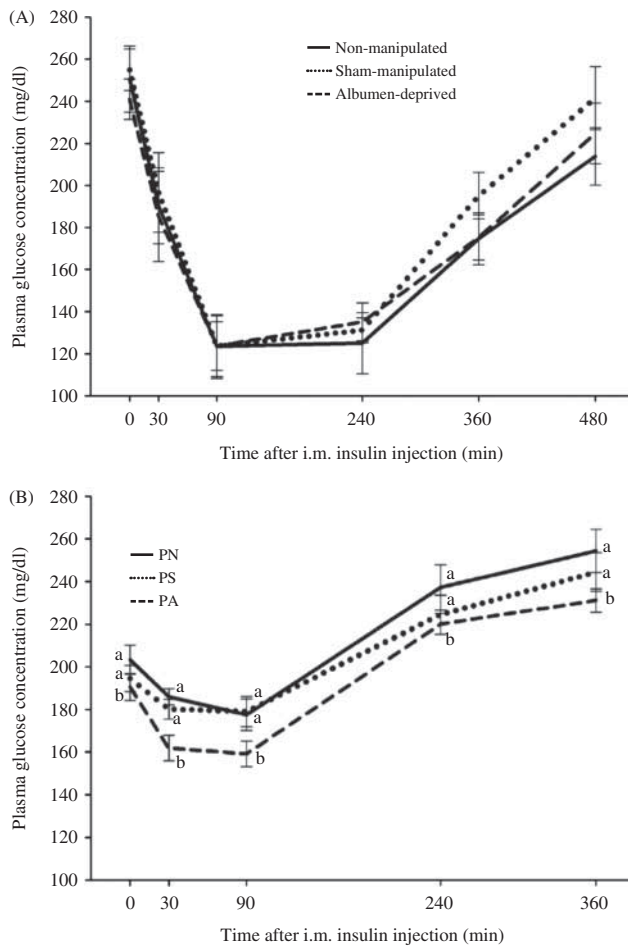
At hatching, the progeny plasma glucose concentrations were only affected by breeder age ( $P<0.001$ ) and not by treatment or the interaction (data not shown). The plasma  $T_3$  concentrations were not affected by treatment, but only by breeder age ( $P<0.001$ ), age of progeny ( $P<0.001$ ), and their interaction ( $P<0.001$ ) (data not shown). The plasma  $T_4$  concentrations were affected by the interaction among breeder age, progeny age, and treatment ( $P<0.001$ ), but no significant treatment effects were found on the different progeny and breeder ages (data not shown). The glycogen concentrations at hatching were not influenced by treatment and breeder age, but were almost significantly influenced by the interaction ( $P=0.071$ ). The hepatic glycogen content of the PA was  $0.66 \pm 0.10$  ng/mg, PN  $1.02 \pm 0.17$  ng/mg and PS chicks  $0.77 \pm 0.14$  ng/mg.

### OGTTs and ITT of the progeny

The glucose concentrations after administration of a glucose load were not affected by treatment, but only by breeder age ( $P<0.001$ ), time after intubation ( $P<0.001$ ), and the interaction ( $P<0.001$ ) (Fig. 1). In contrast, the ITT of the progeny originating from 55-week-old laying hens revealed that the plasma glucose concentrations were affected by treatment ( $P<0.001$ ) and age ( $P<0.001$ ), but not by the interaction (Fig. 2). The PA chicks had a lower plasma glucose concentration at all sampling times compared with both the PN ( $P<0.001$ ) and the PS chicks ( $P=0.008$ ).

### Discussion

Previously, the long-term effects of prenatal protein undernutrition by albumen removal in laying hens have



**Figure 2** Insulin tolerance test (ITT) (A) in the non-manipulated, sham-manipulated, and albumen-deprived laying hens at 51 weeks and (B) in the progeny of the non-manipulated (PN), sham-manipulated (PS), and albumen-deprived hens (PA) at 22 days of age originating from eggs laid by hens at 55 weeks of age ( $n=8$  per group). An insulin dose of  $30 \mu\text{g}/\text{kg}$  body weight was injected intramuscularly and blood was taken at several time points after administration. Glucose (mg/dl) was measured in the plasma. Data are expressed as mean  $\pm$  S.E.M. a and b refer to differences between maternal treatment groups ( $P < 0.05$ ) per time point.

been demonstrated on the body weight and reproductive performance (i.e., reduced number and weight of the eggs). In addition, these eggs had a higher proportional yolk and lower proportional albumen weight (Willems *et al.* 2013), leading to the hypothesis that transgenerational effects influencing the offspring could be present. The objective of this study was to investigate the long-term effects of prenatal protein undernutrition by albumen removal on body weight, body composition, hormones and metabolites, glucose tolerance, and insulin resistance, and whether these effects can be transmitted to the offspring of these albumen-deprived hens.

### Long-term physiological effects of prenatal protein undernutrition due to albumen removal

The reduced body weight of the albumen-deprived hens is in agreement with results from several mammalian studies. Body weight was reduced until at least 3 weeks of age in offspring of low-protein-diet-fed dams in mink (Matthiesen *et al.* 2010a) and in rat (Desai *et al.* 1996). When the low-protein maternal diet was maintained during both gestation and lactation, the reduced body weight was noticeable until at least 110 days of age in rats (Zambrano *et al.* 2005). The reduced body weight is in contrast with our previous results, where body weight was only reduced during the rearing period, but, during the laying period, catch-up growth resulted in a higher body weight of the albumen-deprived hens (Willems *et al.* 2013). Both experiments had the same experimental set-up during the rearing period and thus these results concur. In the laying period, however, the hens from our previous study were kept in laying battery cages with limited possibility for exercise, resulting in higher body weight of the albumen-deprived hens compared with their non-manipulated and sham-manipulated counterparts (Willems *et al.* 2013). In this study, however, hens were kept in floor pens in the presence of roosters, with thus more possibility for exercise and with more social interactions between the hens that might be stressful. In addition, when the roosters were first introduced the hens displayed clear signs of stress and anxiety, which might also have influenced the body weight. However, no effects of treatment on plasma levels of corticosterone, the major glucocorticoid hormone involved in stress, were observed. Although the plasma  $T_4$  concentrations did not differ, the plasma  $T_3$  concentrations were increased in the albumen-deprived hens. This hormone is positively associated with the metabolic rate. Previously, the plasma  $T_3$  levels tended to be reduced, resulting in a higher ratio of  $T_4$  to  $T_3$  in the albumen-deprived chicks at 3 weeks (E Willems, Y Wang, A Koppenol, L Franssens, E Decuypere, J Buyse and N Everaert unpublished results). The total plasma cholesterol concentrations did not differ in this study. In contrast, Lucas *et al.* (1996) reported reductions in total plasma cholesterol concentrations of the male adult offspring of the low-protein-diet rat model. However, few authors have evaluated cholesterol concentrations in low-protein models (Armitage *et al.* 2004).

The body composition was altered at adult age by embryonic albumen removal. The reduction in absolute breast muscle, digestive tract, ovary, and abdominal fat weight is most probably explained by the decreased total body weight. In other animal models involving a low-protein diet, fat mass is rarely measured (Armitage *et al.* 2004).



**Table 3** Laying rate, egg weight ( $n=136$ – $310$ ), chick and relative chick weight ( $n=96$ – $230$ ); residual yolk and relative residual yolk weight ( $n=12$ – $15$ ) of the progeny of the non-manipulated (PN), sham-manipulated (PS), and albumen-deprived laying hens (PA) at 25, 40, and 55 weeks. Data are expressed as mean  $\pm$  S.E.M.

	Breeder age (weeks)	PN	PS	PA	P value treatment	P value breeder age	P value interaction
Laying rate (%)	25	89.0 $\pm$ 0.1 <sup>a</sup>	89.4 $\pm$ 0.3 <sup>a</sup>	65.5 $\pm$ 0.3 <sup>b</sup>	<0.001	<0.001	<0.001
	40	83.0 $\pm$ 0.3 <sup>a</sup>	73.5 $\pm$ 0.3 <sup>b</sup>	53.0 $\pm$ 0.4 <sup>c</sup>			
	55	91.6 $\pm$ 0.2 <sup>a</sup>	63.3 $\pm$ 0.2 <sup>b</sup>	43.2 $\pm$ 0.2 <sup>c</sup>			
Egg weight (g)	25	58.9 $\pm$ 0.3 <sup>a</sup>	59.6 $\pm$ 0.3 <sup>a</sup>	55.7 $\pm$ 0.4 <sup>b</sup>	<0.001	<0.001	<0.001
	40	65.0 $\pm$ 0.3 <sup>a</sup>	65.0 $\pm$ 0.3 <sup>a</sup>	63.7 $\pm$ 0.4 <sup>b</sup>			
	55	65.3 $\pm$ 0.3	65.9 $\pm$ 0.4	64.8 $\pm$ 0.4			
Chick weight (g)	25	42.0 $\pm$ 0.2 <sup>a</sup>	42.8 $\pm$ 0.2 <sup>a</sup>	39.0 $\pm$ 0.4 <sup>b</sup>	<0.001	<0.001	<0.001
	40	46.9 $\pm$ 0.3 <sup>a</sup>	46.9 $\pm$ 0.3 <sup>a</sup>	45.3 $\pm$ 0.3 <sup>b</sup>			
	55	47.0 $\pm$ 0.3 <sup>b</sup>	48.2 $\pm$ 0.3 <sup>a</sup>	46.4 $\pm$ 0.3 <sup>b</sup>			
Relative chick weight (%)	25	71.2 $\pm$ 0.2 <sup>a</sup>	71.6 $\pm$ 0.2 <sup>a</sup>	70.0 $\pm$ 0.3 <sup>b</sup>	<0.001	<0.001	0.024
	40	72.2 $\pm$ 0.2 <sup>a</sup>	72.2 $\pm$ 0.2 <sup>a</sup>	71.2 $\pm$ 0.3 <sup>b</sup>			
	55	72.2 $\pm$ 0.1 <sup>b</sup>	73.2 $\pm$ 0.2 <sup>a</sup>	71.7 $\pm$ 0.2 <sup>b</sup>			
Residual yolk weight (g)		6.9 $\pm$ 0.2	7.3 $\pm$ 0.2	6.6 $\pm$ 0.2	0.075	<0.001	NS
Relative residual yolk weight (%)		10.9 $\pm$ 0.3 <sup>ab</sup>	11.5 $\pm$ 0.4 <sup>a</sup>	10.5 $\pm$ 0.3 <sup>b</sup>	0.046	<0.001	NS

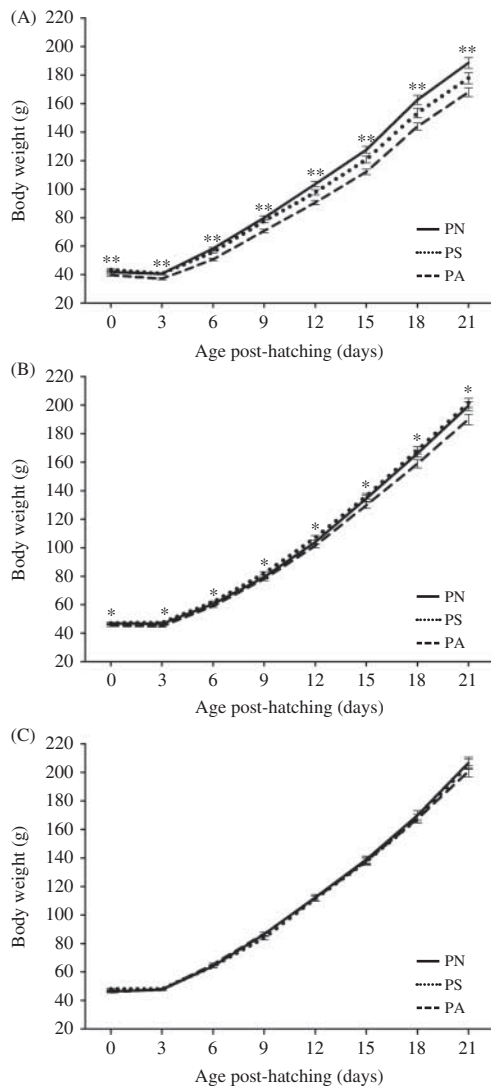
<sup>a,b</sup>Treatment means with different superscripts are significantly different ( $P < 0.05$ ). Laying rate (%) = number of eggs laid divided by number of hens  $\times$  100. Relative chick weight (%) = (chick weight at hatching/egg weight at setting)  $\times$  100. Relative residual yolk weight (%) = (residual yolk weight at hatching/egg weight at setting)  $\times$  100.

However, Shepherd *et al.* (1997) reported decreased adult fat depots and decreased body weight in the offspring of protein-restricted rat dams. The albumen-deprived hens tended to have an increased proportional liver weight, although this was not significant. Malnutrition at a critical period of fetal development can lead to a disproportionate growth of organs, which might alter their metabolism or structure or both (Armitage *et al.* 2004). Indeed, in the offspring of a low-protein-diet rat model, Desai *et al.* (1996) found growth retardation, combined with selective changes in organ growth at 21 days of age, including a reduced proportional liver weight. In the strictly nutritional model of chickens, liver weight was reduced at embryonic day 15 (Miller *et al.* 2002), but did not differ in near-term embryos (Finkler *et al.* 1998) or at hatching (Miller *et al.* 2002). The reduced ovary weight is in agreement with reduced number of large follicles and the diminished reproductive capacity, as observed from the reduced laying rate and egg weight, although the latter disappeared at 55 weeks of age. Deteriorated reproductive performance in the albumen-deprived hens is in agreement with our previous results (Willems *et al.* 2013) and similar to results from a study in sheep (Rae *et al.* 2002), in which the offspring of ewes fed a low-protein diet exhibited a decreased ovulation rate.

At hatching, no difference in plasma hormones or metabolites could be detected, although the hepatic glycogen content was numerically reduced in the albumen-deprived chicks, in agreement with the results of

Willems *et al.* (2014b). The large variation in the hepatic glycogen content of the latter study was attributed to a difference in hatching time, but, in this study, chicks were sampled within 2 h after hatching. Most probably, as the biological difference in glycogen content between individual chicks is high and the power of the current sample size was too small, significance was not reached.

Some challenges were designed based on results for chickens published previously (Buyse *et al.* 1990, Simon *et al.* 2000) to examine the hens for signs of glucose intolerance or insulin resistance, as has been observed in mammalian models. At 10 weeks of age, the albumen-deprived hens had an increased glucose concentration at 30 min after administration of a glucose load compared with the non-manipulated and the sham-manipulated hens. The decreased ability to clear glucose from the blood might indicate decreased insulin production or insulin resistance. In mammalian models of prenatal protein undernutrition, glucose intolerance combined with diminished insulin secretory response was observed from 70 days of age and this was retained until adulthood (Dahri *et al.* 1991). This difference between treatments disappeared in our study when the test was repeated at adult age. Indeed, when comparing the tests performed at later ages, it is clear that the fasting and challenged glucose levels were much higher during adulthood and that the time required to clear the glucose from the blood and reach basal levels increased. With aging, the fasting glucose levels



**Figure 3**

Body weight measured every 3 days from hatching to 3 weeks of age of the progeny of the non-manipulated (PN), sham-manipulated (PS), and albumen-deprived laying hens (PA) originating from eggs incubated at (A) 25, (B) 40, and (C) 55 weeks of age ( $n=45$  per group per incubation). Data are expressed as mean  $\pm$  S.E.M. \*Per time point, the PA chick differs significantly ( $P<0.05$ ) from PN and PS chicks. \*\*Per time point, the PA chick differs significantly ( $P<0.05$ ) from PN and PS chicks. Additionally, the PN differ significantly ( $P<0.05$ ) from the PS chicks.

increased from 220 to 250 mg/dl, whereas the maximal glucose levels were up to 400 mg/dl in adulthood, while it did not increase above 250 mg/dl at 10 weeks of age in the non-manipulated group. At 10 weeks of age, glucose was cleared from the blood after 60 min, whereas at 55 weeks it took more than 4 h. This is indicative of an age-related loss of glucose tolerance and this might have caused the differences in glucose tolerance between the different treatment groups to disappear with age. No difference in

response to insulin injection in the different treatment groups was apparent. In contrast, in the rat model, isolated adipocytes or muscle strips of low-protein-diet-fed offspring had increased insulin-stimulated glucose uptake at 3 months of age (Ozanne *et al.* 1996, 1997).

Differences between the non-manipulated and the sham-manipulated group were observed (e.g., body weight). Indeed, the sham manipulation is a treatment *per se* and can therefore induce differences. Previously, post-hatching differences have been demonstrated as a result of puncturing a hole through the air cell during incubation (Molenaar *et al.* 2010). In our previous studies, effects from the sham-manipulated group were also observed (Everaert *et al.* 2013, Willems *et al.* 2013, 2014b), indicating the importance of a study design including all three groups.

### Transgenerational effects of prenatal protein undernutrition due to albumen removal

Fertilized eggs were incubated in order to obtain offspring from each treatment group. Hatchability was 81% and not affected by maternal treatment. However, the albumen-deprived hens produced eggs with reduced weight and therefore also lower newly hatched chick weight of the offspring. Interestingly, the relative chick-to-egg weight was also reduced, meaning that the body weight of the PA chicks was even more decreased than would be expected based on the reduced egg weight. In contrast, in mammalian models of low-protein diet, when nutritional and hormonal effects are combined, the  $F_2$  offspring had increased weight at birth both in mink (Matthiesen *et al.* 2010b) and rat (Dahri *et al.* 1995). The lower relative chick weight could be due to a different egg composition at laying. Results from our previous study indicated that the eggs from the albumen-deprived hens had a proportionally higher yolk and proportionally lower albumen weight. Albumen is composed of approximately 88.5% water and 10.5% solid compounds, mainly proteins (Romanoff & Romanoff 1949). Reduced amounts of albumen could cause decreased water availability and therefore reduced chick weight by lowering the water content of the chick. Another possibility is that a reduced amount of albumen proteins present would lead to decreased synthesis of body tissue (Romanoff & Romanoff 1949) and therefore reduced chick weight. In this context, it is also interesting that the offspring of the albumen-deprived hens tended to have a decreased absolute and relative residual yolk weight and hepatic glycogen content, which is in agreement with the results for the albumen-deprived hens themselves (Willems *et al.* 2013). The effect of offspring hatching weight might

be explained by altered egg composition and indicate nutritional programming.

The body weight of the progeny, obtained at 25 and 40 weeks of age of the albumen-deprived hens, was reduced until at least 3 weeks of age. It could be proposed that this reduced chick weight is only a direct effect of reduced hatching weight. The correlation coefficient ( $R^2$ ) between egg weight and hatching weight was 0.934, meaning that indeed the egg weight largely determined the subsequent hatching weight. However, the body weight at 3 weeks of age only had a correlation coefficient ( $R^2$ ) of 0.16 to egg weight and 0.17 to chick weight at hatching, meaning that other factors such as the embryonic programming and post-hatching environment were likely to be involved. No differences in glucose tolerance in the progeny of the different maternal treatments were observed. In the offspring of low-protein-diet-fed rat dams,  $F_2$  males had lower plasma glucose levels 30-min after glucose load than the control males (Benyshek *et al.* 2006), whereas no differences were observed in the females. Increased insulin sensitivity was observed in the PA chicks as indicated by the lower glucose concentration after an i.m. insulin injection throughout the entire challenge. However, the glucose concentration of the PA chicks before injection was also significantly different from those for the PN and PS offspring, but this difference was not observed in the OGTT. In contrast, in mammalian models of prenatal protein undernutrition, the  $F_2$  generation exhibited insulin resistance (Pinheiro *et al.* 2008).

In conclusion, prenatal protein undernutrition due to albumen removal led to decreased glucose tolerance during the juvenile phase. Moreover, long-term effects resulted in reduced body weight and reproductive performance. Decreased egg weight and decreased body weight until at least 3 weeks of age were observed in the offspring. The existence of transgenerational effects needs to be further explored.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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#### Author contribution statement

N E, J B, E D, and E W conceived and designed the experiment; A K, Y W, L F, and E W performed the experiments and collected the data. E W and B D

analyzed the data. E W and N E prepared and edited the manuscript before submission. All authors agree with the final format of the manuscript.

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