Theses of Doctoral (Ph.D.) Dissertation

EFFECTS OF NUTRIENT SUPPLY AND RAPAMYCIN TREATMENT ON GENE EXPRESSIONS OF mTOR SIGNALLING PATHWAY AND THE DEVELOPMENT OF JAPANESE QUAIL

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1. INTRODUCTION, JUSTIFICATION AND AIMS OF THE STUDY

Environmental factors, especially food availability, critically influence reproductive investment in animals by affecting nutrient intake needed for growth and reproduction (JARMAN *et al.*, 2024). Nutrient demands rise during reproduction, with mothers allocating vital resources like hormones and nutrients into eggs, impacting offspring development (GIORDANO *et al.*, 2014; WILLIAMS & GROOTHUIS, 2015). Habitat quality, social environment, and mate choice also influence resource allocation (DAROLOVÁ *et al.*, 2014; LAHAYE *et al.*, 2015). Nutritional deficiencies caused by environmental stress or feed restriction can impair embryonic development, delay sexual maturation, and reduce reproductive performance (ZUIDHOF *et al.*, 2014; AFROUZIYEH *et al.*, 2021), These challenges increase oxidative stress, which harms growth and reproduction, but targeted nutritional supplements can mitigate these effects by enhancing antioxidant capacity (RUAN *et al.*, 2018; ALAGAWANY *et al.*, 2021; OKE *et al.*, 2024).

Early nutrient supplementation during embryonic development can improve growth despite limited food intake, with amino acids playing key roles through growth pathways like GH/IGF-1 and mTOR (SENGUPTA *et al.*, 2010; SENGUPTA *et al.*, 2010; LODJAK & VERHULST, 2020). However, the molecular mechanisms linking embryonic amino acids to postnatal growth remain unclear. While rapid growth may boost reproduction, it can compromise immune function, which requires balancing energy between growth and defense involving cytokines that regulate inflammation (VAN DER MOST *et al.*, 2011; DINGEMANSE *et al.*, 2020; SONG *et al.*, 2021). Rapamycin, an mTOR inhibitor known for immunosuppressive and anti-inflammatory effects in mammals, may modulate this balance in birds, whose immune system differs from mammals (POWELL *et al.*, 2012; MORGAN, 2021; PHILLIPS & SIMONS, 2023).

The Japanese quail (*Coturnix japonica*), a rapidly growing and attainment of early sexual maturation just six to eight weeks after hatching, was used in the experiment (OTTINGER, 2001). The overall objective of the PhD Program was to investigate the effects of the amino acid composition of feed on the mechanisms controlling growth and ageing. The specific objectives of the doctoral study are to investigate:

- Effects of feed restriction and supplementation with leucine and methionine on the growth and development of ovaries and ovarian follicles during sexual maturation in Japanese quail
- Impact of predictable and unpredictable feed access on the proportions of egg components in Japanese quail.
- The influence of embryonic amino acids supplementation on growth and expression of mTOR signalling pathway genes in Japanese quail.
- Effects of rapamycin treatment on growth and immune-related genes in the mTOR signalling pathway in Japanese quail.

2. MATERIALS AND METHODS

All experiments that were conducted complied with the EU Directive on protecting animals used for scientific purposes. The Ethical Committee for Animal Use at the University of Debrecen, Hungary (Protocol No. 5/2021/DEMAB) secured the approval. We confirm that all procedures adhered to the relevant institutional standards and regulations, ensuring the welfare of the animals involved in the experiment. The experiments were conducted in the animal facility at the Institute of Animal Science, Biotechnology, and Nature Conservation, University of Debrecen, Hungary.

2.1. Experiment 1: Effects of feed restriction and amino acid supplementation on body weight changes and ovarian development during sexual maturation in Japanese quail

2.1.1. Experimental animal, housing, experimental design and measurements

The basal (control) grower feed was prepared according to the NRC (1994) recommendation for quails on a corn-wheat-soybean meal basis. The birds in the control group were full-feed (feed intake plus an extra 5% feed) and had free access to water. At the age of 5 weeks, we selected 40 female quails with similar body weight and kept them in individual cages in the same room with a controlled temperature of 23-25°C and humidity of 60-65%. Breeder birds were allowed free access to breeder feed and water for one week of the acclimation period. Individual weekly body weight changes and daily feed intake to the nearest (0.01 g accuracy) were measured.

A two-week experimental period began when the quails reached six weeks old. Birds were divided into five experimental groups (8 birds per group): ad libitum as a control feed, a 20% restricted feed (DR20) (i.e. given 80% of their average individual feed intake), a restricted feeding supplemented with 20% of the recommended amount of methionine (DR20+Met), a restricted feeding supplemented with 20% of the recommended amount of leucine (DR20+Leu) and a restricted feeding supplemented with 20% leucine and 20% methionine of the recommended amount (DR20+Leu+Met) based on the individual feed intake (Table 1).

Table 1. Composition and nutrient content of the experimental feeds

Ingredients %	Treatments				
	Control	DR20	DR20+Met	DR20+Leu	DR20+Met+Leu
Corn	26.33	26.33	26.14	25.53	25.34
Soybean meal	28.41	28.41	28.44	28.55	28.58
Wheat	30	30	30	30	30
Corn gluten meal	5	5	5	5	5
Fishmeal	5	5	5	5	5
Oil	2.78	2.78	2.84	3.02	3.08
Limestone	1.06	1.06	1.06	1.06	1.06
MCP	0.39	0.39	0.39	0.39	0.39
L-Lys	0.1	0.1	0.1	0.09	0.09
DL-Met	0.07	0.07	0.17	0.07	0.17
L-Thr	0.13	0.13	0.13	0.13	0.13
L-Leu	0	0	0	0.42	0.42
Salt	0.23	0.23	0.23	0.23	0.23
Premixture[a]	0.5	0.5	0.5	0.5	0.5
Nutrient content					
ME, MJ/kg	12.13	12.13	12.13	12.13	12.13
Crude protein %	24	24	24	24	24
Lys, %	1.3	1.3	1.3	1.3	1.3
Met %	0.5	0.5	0.6	0.5	0.6
Thr, %	1.02	1.02	1.02	1.02	1.02
Trp, %	0.27	0.27	0.27	0.27	0.27
Leu, %	2.12	2.12	2.12	2.54	2.54
Ile, %	1.01	1.01	1.01	1.01	1.01
Arg, %	1.46	1.46	1.46	1.47	1.47
Leu/Ile, ratio	2.1	2.1	2.1	2.51	2.51
Ca, %	0.8	0.8	0.8	0.8	0.8
P, %	0.57	0.57	0.57	0.57	0.57
non phytate P, %	0.32	0.32	0.32	0.32	0.3
Na, %	0.15	0.15	0.15	0.15	0.15

Control: Control feed (full-fed); DR20: 20% feed restriction; the amount of feed from the control reduced by 20% (Control-20%); DR20+Met: 20% feed restriction+20% methionine; DR20+Leu: 20% feed restriction+20% leucine; DR20+Met+Leu: 20% feed restriction + 20% methionine + 20% leucine.

[a]1 kg premix provided: 1000K NE vitamin A, 200 000 NE vitamin D3, 4900 mg/kg vitamin E, 200 mg vitamin K3, 150 mg vitamin B1, 500 mg vitamin B2, 1200 mg Ca-d-Pantothetane, 400 mg vitamin B6, 2 mg vitamin B12, 11 mg biotin, 2502 mg niacin, 60 mg folic acid, 300K mg choline chloride, 13200 mg Zn, 1920 mg Cu, 9612 mg Fe, 13200 mg Mn, 180 mg I, 42 mg Se, 12 mg Co.

2.1.2. Measurements

At the end of the experimental period, body weight was measured using digital scale VWR software version 6.02 with 0.01 g accuracy (Avantor, Radnor, PA, USA). Birds were sacrificed by cervical dislocation and collecting ovaries and follicles, then measured ovary weight using a digital balance (0.01 g accuracy), three biggest hierarchical follicle weights, the first (F1),

second (F2) and third (F3) to nearest 0.01 g accuracy, and follicle diameter to nearest 0.1 mm accuracy using vernier calliper and counted number of the first three biggest hierarchical follicles (F1, F2, and F3) (Figure 4). The ovary index is a proportional ovary weight expressed as a percentage of total body weight and was calculated using the following formula.

2.2. Experiment 2: Effects of feed restriction on the variation of egg components

2.2.1. Animals and housing

The four-week-old Japanese quail (*Coturnix japonica*) chicks were purchased from a commercial quail breeder (Budai Fürjészet, Hungary). The quail was kept in the animal house until it reached 8 weeks of age. In this experiment, two trials were conducted. In the first trial, 40 8-week sexually matured female quails with similar body weights (≈275.3±3.64 g) were selected and placed in individual cages. During the acclimation period, quails were fed ad libitum every morning, and their feed intake was measured at 24-hour intervals. This allowed us to calculate the average daily feed intake for each bird, and live initial and final body weights were recorded on a digital balance (± 0.1 g). After acclimation, quails were randomly distributed into four treatment groups consisting of 20% restriction ad libitum as a control feed, a 20% restricted feed (DR20: i.e. given 80% of their average individual feed intake), 30% restriction (DR30: i.e. given 70% of their average individual feed intake), 40% restriction (DR40: .e. given 80% of their average individual feed intake) and a control group that received full feed.

In the second trial, 24 sexually matured birds were placed in individual cages. The control group received ad libitum feed throughout the experimental period, which was equal to their daily individual feed intake. The unpredictable fed group (UNPR) received the same total amount as the control group throughout the experiment, but with daily variability ranging from the lowest amount, 30% per day, to 170% per day of their respective feed intake. The restricted group (DR40) received an average of 60% of their feed intake (Figure 1). The experimental house in both trials was maintained at 24 ± 3 °C, with relative humidity levels between 60% and 75%. A standardised lighting regimen was implemented with a 12:12 light-to-dark cycle controlled using timers. The basal diet for the quails was formulated based on corn, wheat, and

soybean meal to meet the specific nutrient requirements for the breeder quails, as outlined in NRC (1994).

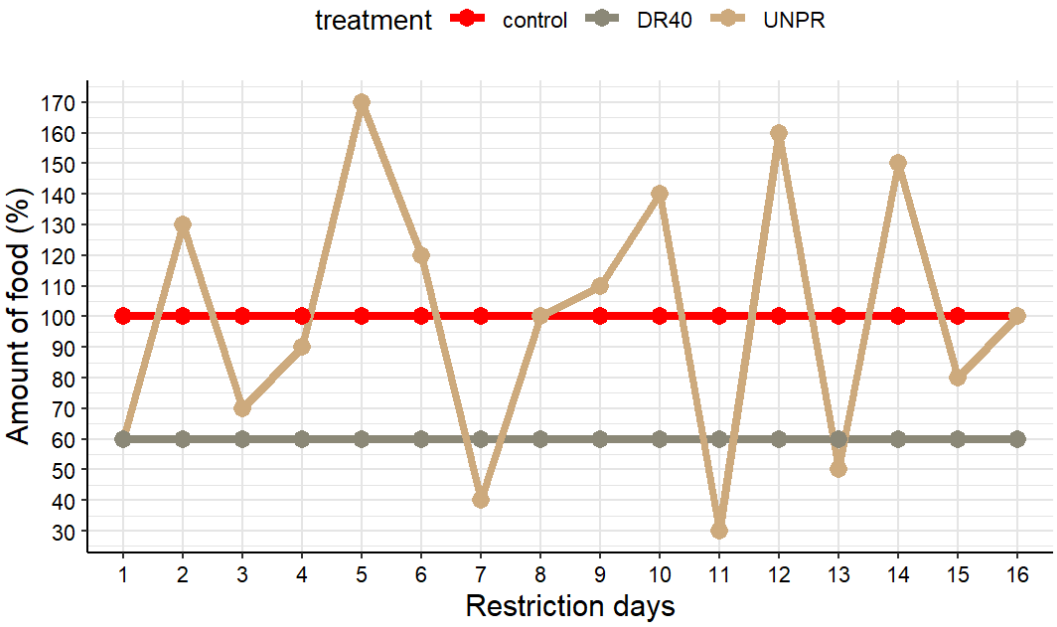

Figure 1. Dietary treatments over sixteen days of the experimental period. Each treatment is based on the birds' average daily feed intake under ad libitum conditions measured during acclimation (i.e. 100%)

2.2.2. Egg trait measurements

Eggs were collected daily, marked, and weighed using a digital laboratory analytical balance (± 0.01 g). Egg width (along the maximum breadth) and height (along the longitudinal axis) were measured using a 200-mm digital calliper. The egg shape index was expressed as the percentage of egg width to length. Egg volume was measured by the liquid displacement method in a graduated cylinder. Eggs were carefully broken with the blunt end of a knife, and the eggshells were washed, air-dried and weighed using an analytical balance (± 0.01 mg). The eggshell ratio was calculated as the percentage of eggshell weight to egg weight. The egg yolk and albumen were separated into a clean container, and the weight of both components was measured (± 0.01 g). Eggshell, yolk and albumen ratio, expressed in percentage in figures, were calculated as the ratios of the eggshell, yolk and albumen weights to the whole egg weight, respectively. The egg yolk to albumen ratio was calculated by dividing the yolk weight by that of albumen weight.

2.3. Experiment 3: Effects of embryonic methionine on early postnatal growth and development via the mTOR signalling pathway

2.3.1. Experimental design

Freshly laid Japanese quail (*Coturnix japonica*) eggs were collected and kept at room temperature between 16°C and 18°C for 1-5 days. On the day of laying, eggs were weighed on a digital scale (±0.01 g accuracy). A total of 200 eggs were weighed and randomly selected; half of them were injected with 1 mg L-methionine dissolved in 50 µl saline solution, while the remaining eggs received physiological saline solution as a control group.

2.3.2. Preparation and in ovo injection of methionine

A batch of the amino acid solution was prepared by dissolving crystalline L-methionine (CAS No. 63-68-3, Sigma Aldrich, BioUltra, > 99.5%) in 0.9% physiological saline solution (Sigma Aldrich). On the broad end site, 50 µl of either the L-methionine or saline solution was injected into the egg yolk before incubation using a 50 µl ethanol-sterilised Hamilton syringe, and eggs were sealed with candle wax. Immediately after the injections, eggs were transferred to the automatic turning incubator (WQ-63 Model 2021 Version 2, AGROFORTEL, Budapest Hungary). The set incubation temperature at 37.8 ± 0.5°C and relative humidity at 50-60% until 14 days. On day 8 of embryonic development, eggs were candled with a flashlight and removed from those where embryonic development had not started or had stopped. On day 14 of the incubation, eggs were transferred from the incubator tray to the hatching tray. The hatching tray was portioned based on the treatment groups to separate the eggs and avoid mixing of chicks during hatching. The temperature was reduced to 35.5 °C, while the relative humidity was increased to 65–70%.

2.3.3. Rearing the experimental chicks

The hatched chicks were transferred from the incubator to the chick-rearing cages (40 cm long × 50 wide × 40 cm high) and reared for three weeks (21 days). Chicks were provided with ad libitum feed and water as recommended (NRC, 1994). During the experimental chick-rearing period, body weight was measured using an electronic digital scale (± 0.01 g), and

tarsal, head, and wing lengths were measured using a vernier calliper with ± 0.01 mm accuracy on days 1, 3, 5, 7, 14, and 21.

2.3.4. Sample collection

After recording body weight, a random sample of 8 quail chicks from each experimental group was selected for blood and tissue sampling on day 1 and 21 post-hatching. After sampling, the blood samples were immediately centrifuged in 1000g, separated then plasma from red blood cells using a Hamilton syringe and stored at -20 °C for further laboratory analyses. Liver samples collected were snap-frozen in dry ice and then stored at -80 °C for further laboratory analysis.

2.4. Experiment 4: Effects of embryonic leucine on early postnatal growth and mTOR signalling pathway

2.4.1. Experimental design

Freshly laid eggs with similar weights were selected for incubation to reduce the effects of egg weight differences. The eggs were divided into two experimental groups (50 eggs each) and incubated in an automatic turning incubator (WQ-63-WQ-98 Model 2021 Version 2, AGROFORTEL, S.R.O., Budapest, Hungary). The set incubation temperature was 37.8 ± 0.5°C, and relative humidity was 50-65% until 14 days. After transferring the eggs to the hatching tray, the incubation temperature was reduced to 35.5 °C, and relative humidity increased to 65–70%.

2.4.2. Preparation and in ovo injection of leucine

The leucine solution (50 mg leucine/ml saline solution) was prepared by dissolving crystalline L-leucine (reagent grade; Sigma Aldrich, purity > 98%) in a 0.9% physiological saline solution (Braun Melsungen, Germany). We injected 50 µl into each egg, with 2.5 mg/egg of the leucine solution into the amniotic fluid using a Hamilton syringe on embryonic day ten (ED10). Then, the second group received 50 µl of the physiological saline solution (Braun Melsungen, Germany), each serving as a control group. After the injection, the hole was sealed with candle wax, and the tube was transferred to the incubator to resume incubation.

2.4.3. Rearing experimental hatchlings

Hatched quail chicks from each experimental group were immediately transferred to cages (40 cm long × 50 wide × 40 cm height) and reared for an average of three weeks (21 days) in groups of their treatments. Chicks were provided with free access to water and feed (Table 2). Post-hatch body weight was recorded using an electronic digital scale with a precision of 0.01g, V.W.R. software version 6.02 (Avantor, U.S.A.). Wing, tarsus, and head lengths were also measured using a vernier calliper (to the nearest 0.01 mm) on days 1, 3, 5, 7, 14, and 21.

Table 2. Composition and nutrient level of the basal diet of the quail chicks

Feed ingredients	**Composition %**
Corn	23.69
Wheat	30
Soybean meal (46% CP)	34.85
Fishmeal	5
Sunflower oil	4.09
Limestone	1.01
MCP	0.37
Salt	0.24
DL-Methionine	0.1
L-Threonine	0.13
Vitamin and mineral premix[a]	0.5
Nutrient composition	
Metabolisable energy M.J./kg	12.13
Crude protein	24
Calcium	0.8
Available phosphorus	0.3
Sodium	0.15
Methionine	0.45
Lysine	1.34
Threonine	1.02
Tryptophan	0.29

[a] *1 kg premix provided: 10^6 NE vitamin A, 20^5 NE vitamin D_3, 4900 mg/kg vitamin E, 200 mg vitamin K_3, 150 mg vitamin B_1, 500 mg vitamin B_2, 1200 mg Ca-d-Pantothetane, 400 mg vitamin B_6, 2 mg vitamin B_{12}, 11 mg biotin, 2502 mg niacin, 60 mg folic acid, 30^5 mg choline chloride, 13200 mg Zn, 1920 mg Cu, 9612 mg Fe, 13200 mg Mn, 180 mg I, 42 mg Se, 12 mg Co.*

2.4.4. Sample collection

Eight chicks (One and 21 days old) from each experimental group were randomly selected for sample collection after body weight recording. Birds were sacrificed due to

cervical dislocation. Then, liver samples were collected, snap-frozen in dry ice, and stored at −80 °C for further gene expression assay.

2.5. Experiment 5: Effects of rapamycin treatment on body weight changes, relative gene expression, haematological, histomorphology

2.5.1. Experimental design

In this experiment, 40 5-week-old quails with similar body weights (average weight of females: 154.6 ± 5.2 g; males: 180.43 ± 7 g) were assigned into two experimental groups: the control ((ethanol with isotonic saline solution) and the rapamycin-treated groups (99% (J62473.MC, Thermo Fisher Scientific, Waltham, MA, USA). Birds were provided with free access to feed and water ad libitum, and the basal diet for quails was formulated as a breeder quail ration (24% CP; 12.13 MJ/kg ME) based on corn, soybean, and wheat (NRC, 1994). Each experimental group consisted of 20 birds, 10 females and 10 males. Then, quails were injected subcutaneously with 1 mg/kg body weight rapamycin every second day for two weeks (7 times, once daily at 10:00 hours). The purity of rapamycin powder was 99% (J62473.MC, Thermo Fisher Scientific, Waltham, MA, USA).

2.5.2. Growth measurement and relative spleen weight

The body weight was measured at the beginning of the experiment (day 1) and on days 3, 5, 7, 10, 12, and 14 before the injections and at the end of the experiment (day 15) using an electronic scale (± 0.01 g). At the end of the experiment, the birds were sacrificed by cervical dislocation. The whole spleen was aseptically excised, collected and transferred to the liquid nitrogen and stored it at -80°C for later RNA isolation. The relative spleen weight was also measured as the percentage of the total live weight of each bird

2.5.3. Measurement of intestinal histomorphology

The stained intestinal segments by hematoxylin-eosin staining on 20 samples (n = 5 birds from each sex per treatment) were examined using an Olympus BX61 light microscope paired with a DP71 camera (Olympus Corporation, Shinjuku, Tokyo, Japan) to capture images.

The images were examined using cellSens Entry imaging software and the arbitrary line measurement tool. Villus height was measured from the tip of the villus to the crypt-villus junction. Crypt depth was defined from the base of the crypt up to the villus-crypt axis, and total mucosa thickness was determined from the top of the villus to the wall of the intestine, involving villus height, crypt depth, and muscular mucosa. The 12 ileal villi, crypts, and mucosa on the segments were measured.

2.5. Laboratory analyses

2.5.1. Total antioxidant capacity analyses

The total antioxidant capacity (TAC) of quail serum samples was quantified using the Antioxidant Assay Kit (Sigma-Aldrich, Merck, KGaA, Darmstadt, Germany) following the manufacturer's instructions. The absorbance of standards and samples were measured in duplicate at 570 nm using a microplate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments Inc., Winooski USA). The concentrations of the TAC were calculated as Trolox equivalent antioxidant capacity (TEAC) and expressed in millimole Trolox equivalents per volume (mmol TE/L).

2.5.2. Enzyme-Linked Immunosorbent Assay (ELISA)

The circulating plasma IGF-1 levels were measured by a competitive enzyme-linked immunosorbent assay (ELISA) as described previously (MAHR *et al.*, 2020). The samples were analysed in duplicate on a single plate (Figure 6). Non-sterile Nunc™ 96-Well Polypropylene MicroWell™ Plates (Thermo Scientific™) were used. The optical density was measured at 450 nm (reference at 620 nm) and absorbance (OD) was read using a Magellan™ microplate reader for F50 software (TECAN Trading AG, Switzerland).

2.5.3. Total RNA isolation

In experiment 3, total RNA was extracted from frozen liver tissue using TRIzol and Direct-zol RNA MiniPrep kits, including a DNA digestion step. RNA integrity was verified via agarose gel electrophoresis, and concentration and purity were measured spectrophotometrically. In experiments 4 and 5, RNA was isolated using the peqGOLD Total

RNA Kit and peqGOLD DNase Digestion Kit. Concentration and purity were again measured spectrophotometrically, while RNA integrity was assessed using the Qubit RNA IQ Assay and Qubit 4 Fluorometer.

2.5.4. cDNA synthesis and real-time polymerase chain reaction (RT-PCR)

The study conducted reverse transcription using the qScript cDNA synthesis kit with 200 ng of RNA, following the manufacturer's protocol on a PCRmax Alpha thermal cycler. Intron-spanning primers specific to quail were designed using Oligo 7 and validated with NCBI's Primer-BLAST (Table 3). Quantitative PCR was performed on an Agilent AriaMx Real-time PCR system using HOT FIREPol® EvaGreen® Mix, with reactions run in duplicate. Gene expression levels were normalized against the RPL19 gene, identified as the most stable reference among six housekeeping genes using three different algorithms (delta Ct, BestKeeper, and NormFinder). Data were analyzed with Aria AgilentMx software, and relative expression of target genes (*mTOR, RPS6K1, and IGF1*) was calculated using the double-ΔCT method, with the sample showing the highest delta Ct value used as the calibrator.

Table 3. Primer sequence of the target genes (*IGF1, IGFR, mTOR, RPS6K1, FOXO1, IL-1β, myD88, NF-κB, STAT3*) and Reference gene (*RPL19*)

Gene	Gene name	Primer sequences (5' → 3') (forward/reverse)	NCBI GenBank	Fragment size (bp)
RPL19	ribosomal protein L19	F: CATCGGTAAGAGGAAGGGT	XM_015885843.1	163
		R: ACGTTGCCCTTGACCTTCAG		
mTOR	mechanistic target of rapamycin	F: CCGAAGCATTGAATTGGCCCT	XM_015882433.2	116
		R: CATCTCTCAAAGGCAGCGGACC		
RPS6K1	ribosomal protein S6 kinase 1	F: AGGCAGGAACCCTCCGTGCAA	XM_015883670.2	106
		R: AGCTCAAACTGCGAAGGGTCGG		
IGF-1	insulin-like growth factor-1	F: CACTATGCGGTGCTGAGCTGGTT	XM_015867574.2	118
		R: TCCCCTTGTGGTGTAAG CGTCT		
IGF1R	Insulin-like growth factor 1 receptor	F: TACAACTACCGCTGCTGGACCAC	XM_015873184.2	107
		R: AGGCACTCAGGATGGCAACAC		
FOXO1	forkhead box O1	F: TGAGCGAGATCTGCGAGTTCAT	XM_015851898.1	102
		R: AGGAAGCTCCCGTTGTCGAACA		
IL-1β	Interleukin-1 beta	F: CGTGCTGGAGTCACCCACACA	XM_015882931.2	98
		R: CACGGGGACGGTACAGAGCGAT		
myD88	Myeloid differentiation primary response 88	F: CTGGGCCGTCACGATGTGCT	XM_015852905.2	119
		R: CTGTCTACCGCCGGGACCTG		
NF-κB	Nuclear factor-kappa B	F: TGCATCGTTTGAGAGCTCCGGTT	XM_015860720.2	77
		R: CTTTACGCAGACGCACAGCTT		
STAT3		F: GGTCATCAAAACCGGTGTGCAGT	XM_015885854.1	120

	Signal transducer and activator of transcription 3	R: GCTGCAACATCACCAGAGTCCT

2.6. Statistical analyses

All statistical analyses were performed using R in the RStudio version 4.3.3 'Angel Food Cake' (http://www.r-project.org/). Graphs (images) were visualized using the *ggplot* function provided by the 'ggplot' package version 3.4.3.

The effects of amino acid supplementation on top of dietary restriction on body weight change, final body weight, growth rate, number of hierarchical follicles and TAC with the function *lm* using a linear model (KUZNETSOVA *et al.*, 2017). Yolk weight, albumen weight, and shell weight, a polynomial mixed-effect model of the Gaussian family (DEMIDENKO, 2013), with the function lmer from the lme4 package v. 1.1.31 (BATES *et al.*, 2015). For yolk-to-albumen ratio, a Generalised Additive Mixed-Effects Model (GAMM) from the Gaussian family, using the gamma function from mgcv package v. 1.8.42. For *IGF-1*, *mTOR* and *RPS6K1 and* response variables, linear model levels as a treatment and days as independent variables. Body weight and morphological traits (wing, head, tarsal, and feather lengths) across the days (1, 3, 5, 7, 14, and 21) as response variable were analysed using a linear mixed model, with treatment and days as fixed factors and individual bird identity as a random factor. The p-values were calculated using the Tukey HSD test (NANDA *et al.*, 2021). The effects of rapamycin on body weight were analysed by a linear mixed model with bird identity as a fixed factor across the days, while treatment and sex were fixed factors. Histomorphology parameters were analysed using a linear model. The selected reference genes the *RPL19* was finally considered the most stable reference gene. We calculated the relative gene expression levels using the $2^{-\Delta\Delta Ct}$ methods described previously (SCHMITTGEN & LIVAK, 2008). The linear model was also fitted to analyse the effects of treatment on the relative gene expression of *IGF1R, mTOR*, *IGF1*, *RPS6K1* and *FOXO1, IL-1β, NF-κβ, STAT3, MyD88.* A one-way Analysis of Variance (ANOVA) was used to assess the statistical significance among the treatment groups in all analysed parameters where only one factor was involved. A two-way ANOVA was used to determine the statistical difference among the treatments involving two factors. Means for

treatment groups were computed using the emmeans (SEARLE *et al.*, 1980), adjusted for Tukey with $p < 0.05$ significance level.

3. RESULTS

3.1. Experiment 1: Effects of feed restrictions and amino acid supplementation on body weight changes and ovary development during sexual maturation in Japanese quail

3.1.1. Effects of feed restriction and amino acid supplementation on body size

The initial body weight was similar among the treatment groups ($p = 0.884$, Table 4). Dietary restriction treatment significantly reduced the final body weight ($p = 0.009$, Table 4). However, supplementation of leucine, methionine and their interaction on top of restricted feeding restored the final body weight similar to the control group ($p > 0,05$ for all, Table 4).

Body weight changes were affected by dietary treatments (Table 4). The dietary restriction showed a significant negative change in body weight compared to the control group ($p < 0.001$; Table 4). The multiple comparison showed a significant positive change in the body weight compared to the control group by supplementing methionine or leucine individually or in combination within a restricted diet (DR20+Met: $t = 4.394$, $p = 0.001$; DR20+Leu: $t = 4.501$, $p < 0.001$; DR20+Leu+Met: $t = 3.143$, $p = 0.027$, Table 4). All other pairwise comparisons were not significant ($p > 0.05$, Table 4). The dietary restriction and amino acid supplementation significantly affected growth rate; the results were similar to body weight changes ($p < 0.001$, Table 4).

Table 4. Comparative effects of amino acid supplementation on initial body weight, final body weight, change in body weight and growth rate in Japanese quails reared on restricted feeding

Parameters	Treatments				
	Control	DR20	DR20+Leu	DR20+Met	DR20+Leu+Met
Initial body weight (g)	233.98±7.78	232.14±10.04	241.24±5.99	239.48±5.25	233.59±5.61
Final body weight (g)	265.94±9.34^{a}	225.65±7.15^{b}	244.11±6.97^{a}	244.01±5.47^{a}	245.24±15.95^{a}
Body weight change (g)	31.96±5.28^{a}	-6.49±5.30^{b}	2.89±5.17^{ab}	4.54±2.12^{ab}	11.66±3.14^{ab}
Growth rate (g/day)	2.28±0.38^{a}	-0.46±0.38^{b}	0.21±0.37^{ab}	0.32±0.15^{ab}	0.83±0.22^{ab}

Values are in mean values ± standard error of the mean (SE) from sample size (n) = 8 for each treatment group. Means with common superscripts are not significantly different ($P < 0.05$).

3.1.2. Effects of feed restriction and amino acid supplementation on ovary weight

Dietary restriction significantly reduced ovary weight compared to the control ($p < 0.001$; Figure 2A). The supplementation of Leu or combination of leucine and methionine with the dietary restriction significantly restored ovary weight similar the control group ($p > 0.05$). Multiple comparison showed ovary weight significantly increased compared to the restricted group with amino acid supplementation (DR20+Leu: $t = -2.913$, $p = 0.047$, DR20+Leu+Met: $t = -2.939$, $p = 0.045$, Figure 2A). All other compared groups were like a control group ($p > 0.05$, Figure 3). Dietary restriction marginally reduced the ovary index compared to the control group ($p < 0.001$, Figure 2B). The supplementation of Leu or combination of leucine and methionine with the dietary restriction marginally restored ovary index similar to the control group ($p = 0.655$, Figure 2B).

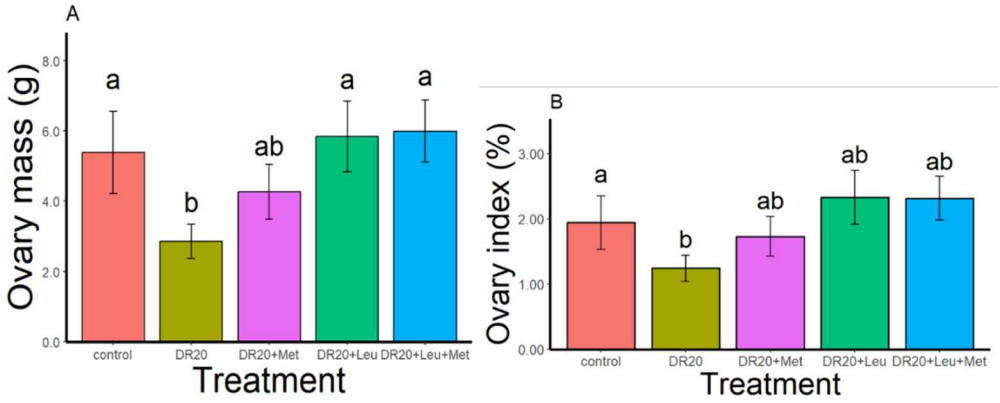

Figure 2. Effects of feed restriction and amino acid supplementation on A: Ovary weight. B: Ovary index of Japanese quails at the age of 8 weeks. Control: full-fed; DR20: 20% feed restriction (Control-20%); DR20+Met: 20% feed restriction + 20% methionine; DR20+Leu: 20% feed restriction+20% leucine; DR20+Met+Leu: 20% feed restriction + 20% methionine + 20% leucine. Bars represent the mean ± standard error mean (SE) from 8 birds per group. Means without a common superscript differ significantly ($p < 0.05$).

3.1.3. Effect of feed restriction on the number of hierarchical follicles

The average number of hierarchical follicles (F1, F2, and F3) exhibited a significant response to dietary treatment (Figure 3). Interestingly, dietary restriction and leucine supplementation did not show a significant difference in follicle counts compared to the control group ($p = 0.654$). In contrast, supplementation with methionine, whether administered alone or in combination with leucine within a restricted diet, resulted in a significant increase in follicle numbers ($p < 0{,}001$). Multiple comparison showed a significant difference compared to the

restricted group in methionine and combination with leucine (DR20+Met: $t = 8.216$, $p < 0.001$; DR20+Leu+Met: $t = 6.390$, $p < 0.001$, Figure 3), but not leucine supplementation alone (DR20+Leu: $t = 0.913$, $p = 0.887$, Figure 3).

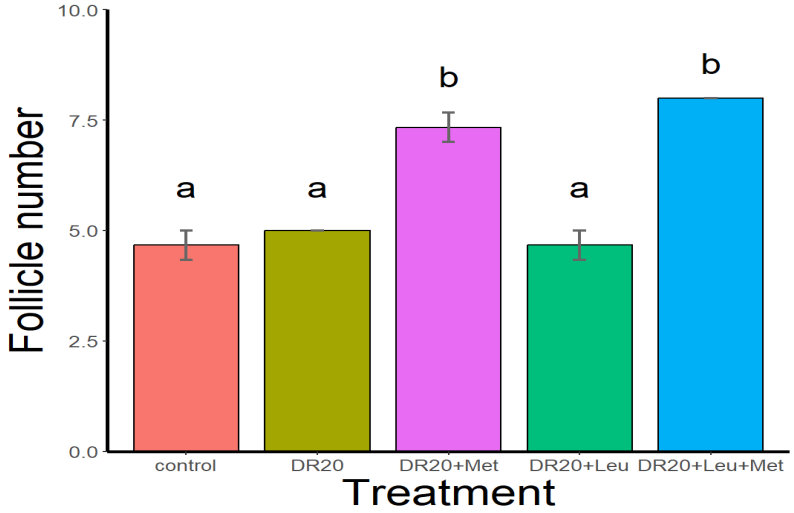

Figure 3. Average number of hierarchical follicles in 8-week-old Japanese quails across different treatments. Groups: Control (full-fed), DR20 (20% feed restriction), DR20+Met (20% feed restriction + 20% methionine), DR20+Leu (20% feed restriction + 20% leucine), and DR20+Met+Leu (20% feed restriction + 20% methionine + 20% leucine). Data are shown as mean ± SE from 8 birds per group. Different superscripts indicate significant differences ($p < 0.05$).

3.1.4. Effects of feed restriction on plasma total antioxidant capacity

Dietary treatments affected total antioxidant capacity levels in a similar way to body weight (Figure 4). Dietary restriction significantly reduced antioxidant capacity compared to the control group ($p = 0.021$; Figure 4). However, supplementing methionine or leucine individually or in combination show no significant difference ($p = 0.442$). Multiple comparison showed all amino acid supplemented groups within a restricted diet were not significantly different compared to the dietary-restricted group ($p > 0.05$ for all, Figure 4).

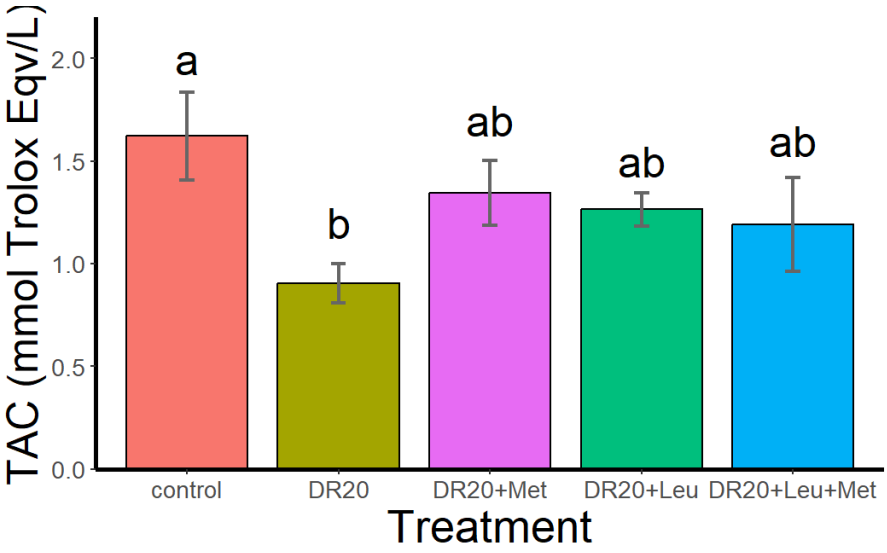

Figure 4. Effects of amino acid supplementation on top of restricted feeding on total antioxidant capacity (TAC) in Japanese quails at the age of 8 weeks. control: Full-fed; DR20: 20% feed restriction; DR20+Met: 20% feed restriction + 20% methionine; DR20+Leu: 20% feed restriction+20% leucine; DR20+Met+Leu: 20% feed restriction + 20% methionine + 20% leucine. Bars represent the mean ± standard error of the mean (SE) from sample size (n =7 for each group). Means without a common superscript differ significantly ($p < 0.05$).

3.2. Experiment 2: Effects of predictable and unpredictable feed access on variability egg components

3.2.1. Effects of predictable feed restriction on weights and proportions of egg components

Egg yolk weight significantly varied across the duration of the treatment period ($p < 0.001$, Figure 6A). However, the overall egg yolk weight showed no significant difference among the treatment groups (Figure 6B). Treatment and its interaction with the treatment period significantly affected albumen weight throughout the restriction period ($p < 0.001$). The DR40 showed the lowest overall albumen weight compared to the control ($p = 0.002$), DR20 ($p = 0.011$), and UNPR ($p = 0.016$), while the other groups showed no significant response to the dietary treatment (Figure 6C).

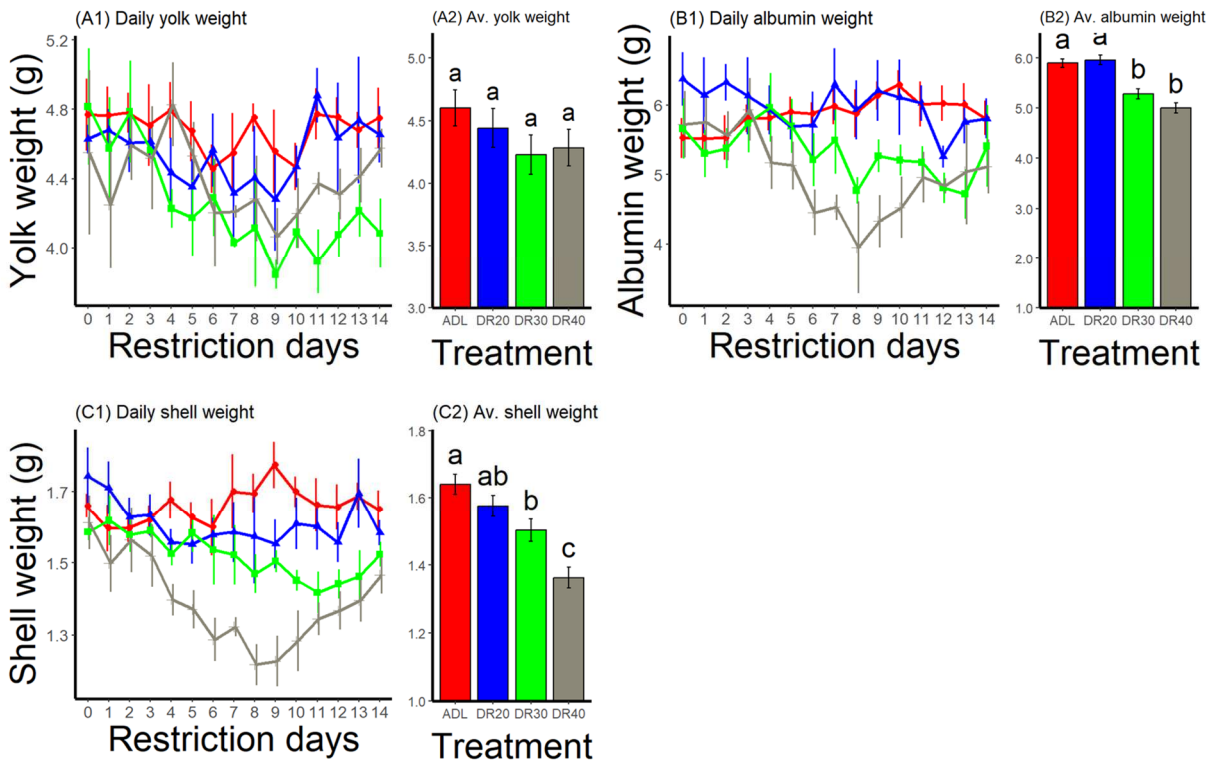

Figure 5. Effects of feed restriction on egg components of 10-week-old Japanese quails over 14 treatment days. Parameters include daily and average weights of yolk (A1, A2), albumen (B1, B2), and shell (C1, C2). Bars represent 14-day average values for each variable. Tukey's test was used to compare adjusted marginal means at a significance level of $p < 0.05$. Means sharing a common letter are not significantly different. Groups include: Control (full feeding), DR20 (20% restriction), DR30 (30% restriction), and DR40 (40% restriction.

Proportions of egg components relative to the egg weight showed varied trends with levels and duration of dietary restriction treatments (Figure 6A). Dietary restriction treatments and their interaction with the treatment period significantly affected the yolk ratio (Figure 6B). The yolk ratio of the DR40 group was comparable to the control group ($p = 0.316$) but significantly different from the DR20 group ($p = 0.045$). There were no significant differences in yolk ratio among the other treatment groups ($p > 0.05$ for all groups). In contrast, the albumen ratio was significantly reduced in the severely restricted groups (Figure 6B). DR40 scored the lowest albumen ratio compared to the control ($p < 0.039$) and DR20 ($p < 0.011$), while no difference was observed with other treatment groups compared to the control ($p > 0.05$ for all groups). The shell ratio was significantly reduced in DR40 compared to the control group ($p < 0.007$) and DR30 ($p < 0.039$, Figure 6C). Treatment significantly affected the yolk-to-albumen ratio ($p < 0.001$). The DR40 group exhibited a significantly high yolk-to-albumen ratio value

compared to the control groups ($p < 0.001$), DR20 ($p = 0.002$), and DR30 ($p = 0.005$), while there was no significant difference observed among the other groups ($p > 0.05$; Figure 6D).

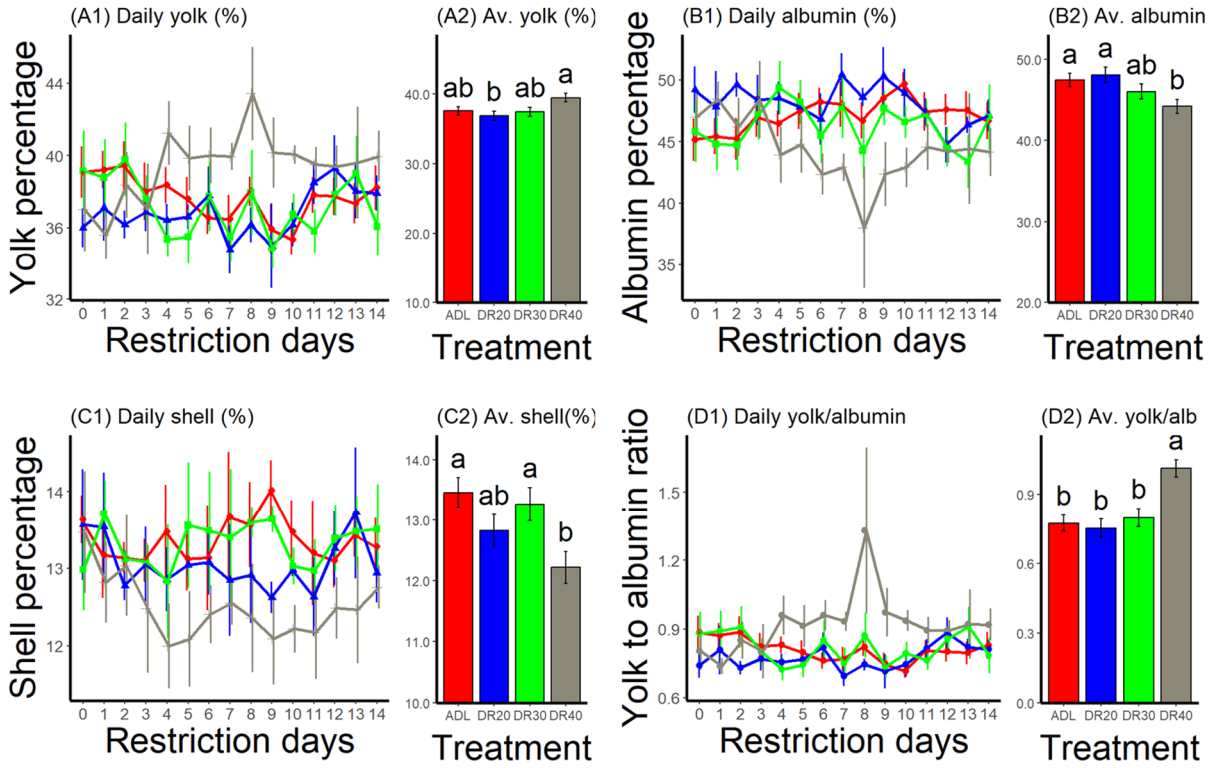

Figure 6. Proportional variations in egg components of 10-week-old Japanese quails under different feed restriction treatments over 14 days. Parameters include daily and average ratios of yolk (A1, A2), albumen (B1, B2), shell (C1, C2), and yolk-to-albumen ratio (D1, D2). All ratios, except yolk-to-albumen, are expressed as percentages of total egg weight for clarity. Bars represent the 14-day average for each variable. Tukey's test was used to compare adjusted marginal means ($p < 0.05$). Means sharing a common letter are not significantly different. Treatments: Control (full feeding), DR20 (20% restriction), DR30 (30% restriction), DR40 (40% restriction).,

3.2.2. Effects of unpredictable feed access on weights and proportions of egg component

Egg yolk weight showed significant variation across the treatment period ($p < 0.001$) but did not differ significantly between treatment groups (Figure 7A). Both treatment and duration significantly affected albumen weight (treatment: $p < 0.001$; day: $p < 0.001$), with no interaction effect; the DR40 group had significantly lower albumen weight than the control ($p = 0.017$), while the UNPR group did not differ from control ($p = 0.728$) (Figure 7B). Eggshell weight was significantly influenced by treatment, duration, and their interaction ($p < 0.001$). The DR40 group had significantly lower shell weight compared to the control ($p < 0.017$) and

UNPR groups ($p < 0.015$), with no significant difference between UNPR and control ($p = 0.998$) (Figure 7C).

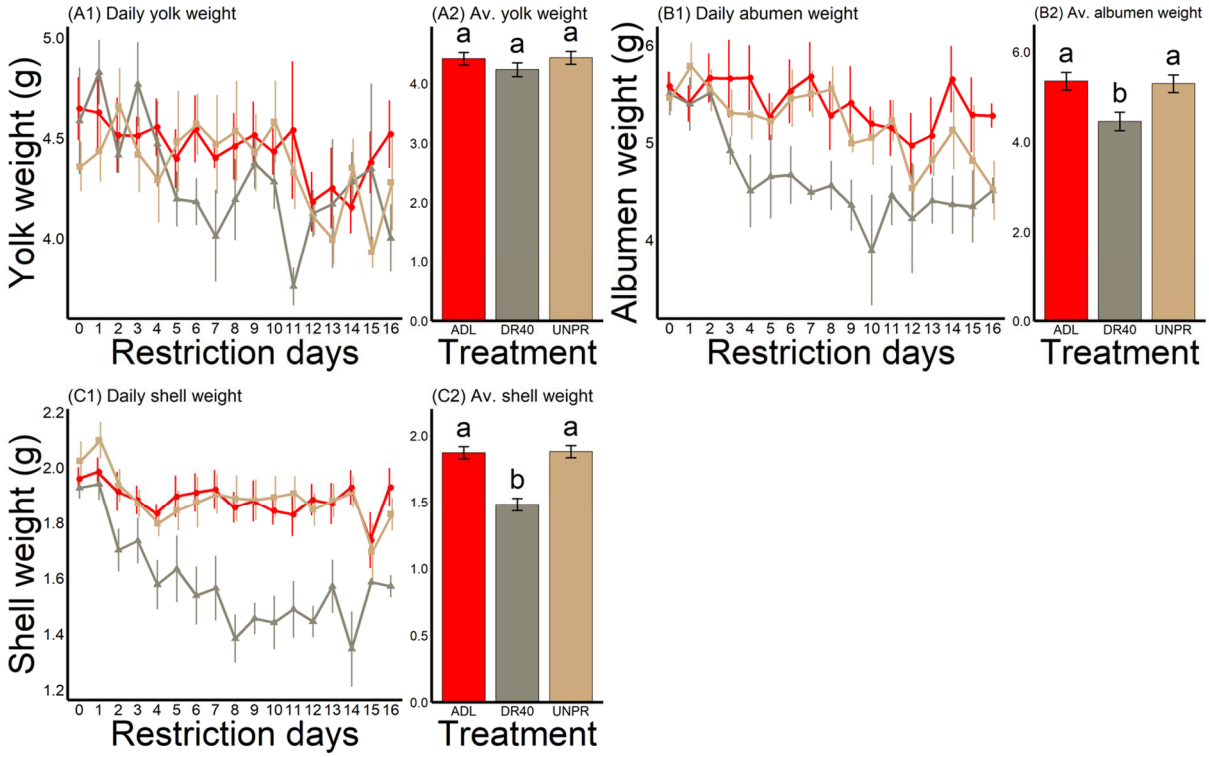

Figure 7 Variations in egg component weights of 10-week-old Japanese quails under unpredictable feed access over 16 days. Parameters include daily and average weights of yolk (A1, A2), albumen (B1, B2), and shell (C1, C2). Bars represent 16-day averages. Tukey's test compared adjusted marginal means at $p < 0.05$; means sharing a common letter are not significantly different. Groups: Control (full feeding), DR40 (40% restriction), and UNPR (unpredictable feed access).

Dietary restriction treatments significantly affected the yolk ratio but not the restriction period (Figure 8A). The yolk ratio of the DR40 group significantly differed from the control ($p = 005$) and UNPR ($p = 0.025$). There were no significant differences in yolk ratio between the control and UNPR treatment groups ($p = 0.799$, Figure 8A). The albumin ratio was affected by the duration of the dietary restriction (Figure 8b). In contrast, there was no significant difference in albumin ratio between the dietary treatment groups ($p > 0.05$, Figure 8B). Dietary treatment and treatment period significantly affected the eggshell ratio (Figure 8C, Table 10.). The eggshell ratio was significantly reduced in DR40 compared to the control group ($p < 0.045$) and UNPR ($p = 0.020$, Figure 8Cc). There was no significant difference in eggshell ratio between the UNPR and the control group ($p = 0.886$). Both dietary treatment and duration of treatment and their interactions significantly affected the yolk-to-albumen ratio ($p < 0.001$,

Figure 8D). The DR40 group exhibited a significantly high yolk-to-albumen ratio value compared to the control ($p < 0.045$) and UNPR ($p = 0.020$) groups. However, there was no significant difference in the yolk-to-albumen ratio between the UNPR and the control group ($p = 0.886$, Figure 9D).

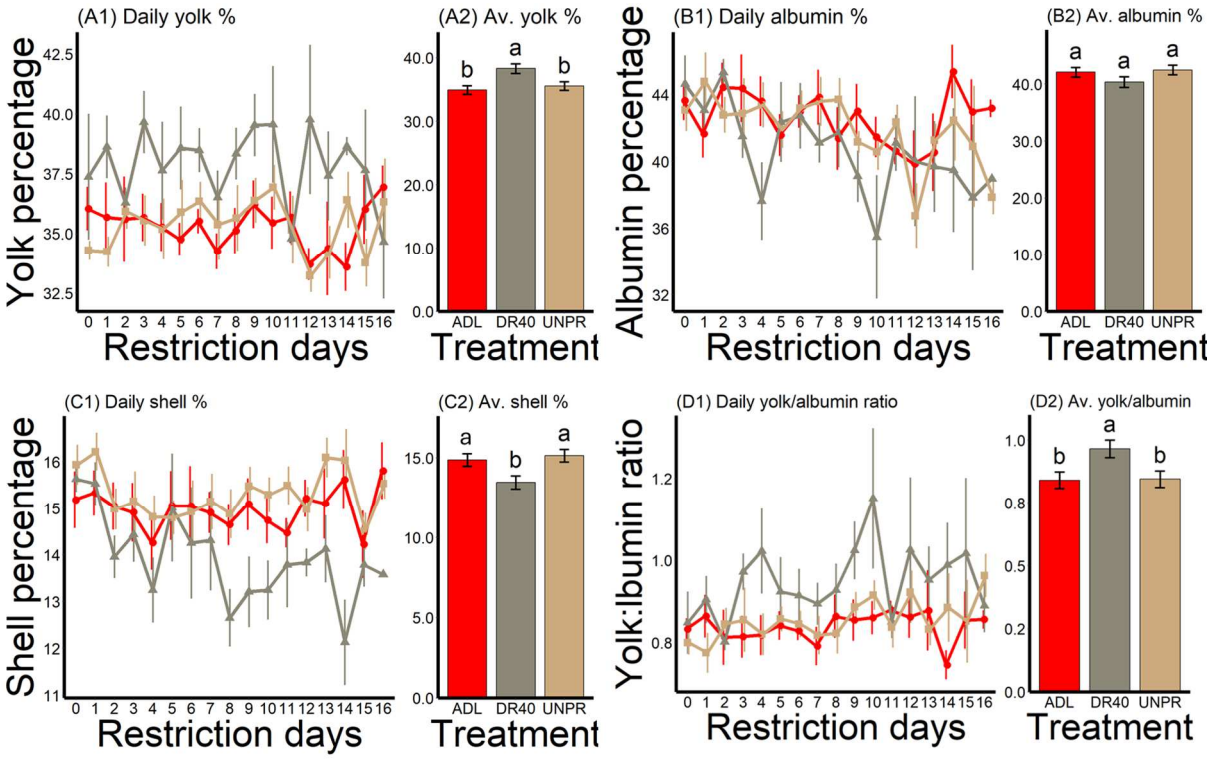

Figure 8. Variations of Japanese quail egg component proportions at 10-week age as affected by unpredictable feed restriction treatments across 16 treatment days. A1: daily yolk ratio, A2: average yolk ratio, B1: daily albumen ratio, B2: average albumen ratio, C1: daily shell ratio, C2: average shell ratio D1: daily yolk-albumen-ratio, D2: average yolk-albumen-ratio. Ratios other than yolk-albumen-ratio are calculated against the total egg weight and are presented as percentages for clarity in the presentation of proportions. Bars indicate the average value of the 16-day records of each variable. Tukey's test was applied to compare adjusted marginal means at $p < 0.05$ significance level. Means followed by a common letter are not significantly different at $p < 0.05$. Abbreviations: control: full feeding; DR40: 40% restriction; UNPR: Unpredictable feed access.

3.3. Experiment 3: Effects of embryonic methionine on early postnatal growth and development of Japanese quail

3.3.1. Effects of embryonic methionine on the hatchability of Japanese quail eggs

Hatchability tended to be lower in the methionine-injected group compared to the control group. Only 31.15% of quail chicks hatched in the methionine-injected group and 51.47% in the control group. The Chi-square analysis indicated no significant difference in

hatchability (χ^2 = 1.80, df =1, p = 0.176). Similarly, injection of the 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL methionine in chicken eggs decreased hatchability with increasing concentration compared to the control group, and it caused detrimental effects resulting in toxic effects that induced embryonic mortality (FARIAS *et al.*, 2023).

3.3.2 Effects of embryonic methionine body weight changes and morphological traits

The body weight at hatching through day 5 was not different between treatment groups. However, chicks in the L-methionine-injected group grew faster (treatment × age interaction, p < 0.001), starting from day 7; their body weight was consistently higher than controls till day 21 (Table 5). Wing, head, and tarsus length did not differ between treatments (p = 0.1148, p = 0.234, p = 0.372, respectively) and increased significantly with the age of chicks (p < 0.001, Table 6).

Table 5. Injecting methionine into the eggs enhanced postnatal body weight gains in Japanese quail chicks

Parameter	Age (day)	Treatment		Estimate	SE	df	t.ratio	p.value
		Control (emmean ±se)	Methionine (emmean ±se)					
Body mass (g)	1	9.124 ± 0.79 (16)	9.406 ± 1.17 (35)	-0.283	1.418	96.672	-0.200	0.842
	3	12.192 ± 1.61 (8)	11.571 ± 1.62 (8)	0.621	2.292	125.690	0.271	0.787
	5	19.729 ± 1.61 (8)	21.484 ± 1.62 (8)	-1.754	2.292	125.690	-0.765	0.446
	7	25.804 ± 1.61 (8)	31.396 ± 1.62 (8)	-5.592	2.292	125.690	-2.439	0.016
	10	39.229 ± 1.61 (8)	45.346 ± 1.62 (8)	-6.117	2.292	125.690	-2.668	0.008
	14	60.042 ± 1.61 (8)	69.484 ± 1.62 (8)	-9.442	2.292	125.690	-4.119	<0.001
	21	104.242 ±1.61 (8)	110.084 ± 1.62 (8)	-5.842	2.292	125.690	-2.548	0.012
Head length (mm)	1	21.151 ± 0.22 (16)	20.612 ± 0.33 (35)	0.539	0.399	80.909	1.350	0.181
	3	22.771 ± 0.44 (8)	22.323 ± 0.45 (8)	0.448	0.625	124.124	0.716	0.475
	5	24.521 ± 0.44 (8)	24.086 ± 0.45 (8)	0.435	0.625	124.124	0.696	0.488
	7	26.596 ± 0.44 (8)	26.573 ± 0.45 (8)	0.023	0.625	124.124	0.037	0.971
	10	29.421 ± 0.43 (8)	29.986 ± 0.45 (8)	-0.565	0.625	124.124	-0.903	0.368
	14	33.734 ± 0.43 (8)	33.823 ± 0.45 (8)	-0.090	0.625	124.124	-0.143	0.886
	21	39.196 ± 0.44 (8)	39.886 ± 0.45 (8)	-0.690	0.625	124.124	-1.103	0.272
Tarsus length (mm)	1	15.668 ± 0.69 (16)	15.919 ± 0.72 (35)	-0.250	0.362	122.525	-0.691	0.491
	3	17.158 ± 0.78 (8)	17.257 ± 0.78 (8)	-0.099	0.599	129.790	-0.165	0.869
	5	21.270 ± 0.78 (8)	21.194 ± 0.78 (8)	0.076	0.599	129.790	0.127	0.899
	7	24.020 ± 0.78 (8)	24.543 ± 0.78 (8)	-0.523	0.599	129.790	-0.872	0.385
	10	27.470 ± 0.78 (8)	28.519 ± 0.78 (8)	-1.049	0.599	129.790	-1.751	0.082
	14	31.258 ± 0.78 (8)	32.069 ± 0.78 (8)	-0.811	0.599	129.790	-1.354	0.178
	21	36.408 ± 0.78 (8)	37.694 ± 0.78 (8)	-1.286	0.599	129.790	-2.147	0.034
Wing length (mm)	1	13.703 ± 0.67 (16)	14.538 ± 0.99 (35)	-0.835	1.202	98.458	-0.694	0.489
	3	16.183 ± 1.37 (8)	15.434 ± 1.38 (8)	0.750	1.948	125.887	0.385	0.701

	5	24.671 ± 1.37 (8)	25.296 ± 1.38 (8)	-0.625	1.948	125.887	-0.321	0.749
	7	35.021 ± 1.37 (8)	32.771 ± 1.38 (8)	2.250	1.948	125.887	1.155	0.250
	10	48.221 ± 1.37 (8)	44.434 ± 1.38 (8)	3.787	1.948	125.887	1.944	0.054
	14	65.346 ± 1.37 (8)	63.684 ± 1.38 (8)	1.662	1.948	125.887	0.853	0.395
	21	86.358 ± 1.37 (8)	84.184 ± 1.38 (8)	2.175	1.948	125.887	1.116	0.266

Abbreviations: Estimated marginal mean and standard error (emmean+se) in specific days and treatments. Tukey's test was applied to compare adjusted marginal means at $p < 0.05$ significance level, standard error (SE) of the two compared treatments within a day. Test statistics (t.ratio), degree freedom (df), and statistical value to measure the difference (p.value) at the significance level of $p < 0.05$. Numbers in brackets are sample sizes (n)

3.3.3. Effects of embryonic methionine on circulating IGF-1 levels

IGF-1 levels increased with the age of birds but in a treatment-specific manner. IGF-1 levels on day 1 did not differ significantly between the L-methionine-treated and control chicks ($p = 0.830$, Figure 9). Contrarily, three weeks later, at 21 days, while all chicks had higher IGF-1 levels than after hatching ($p < 0.001$), L-methionine-treated birds significantly increased their IGF-1 levels more than the control ($p < 0.001$, Figure 9).

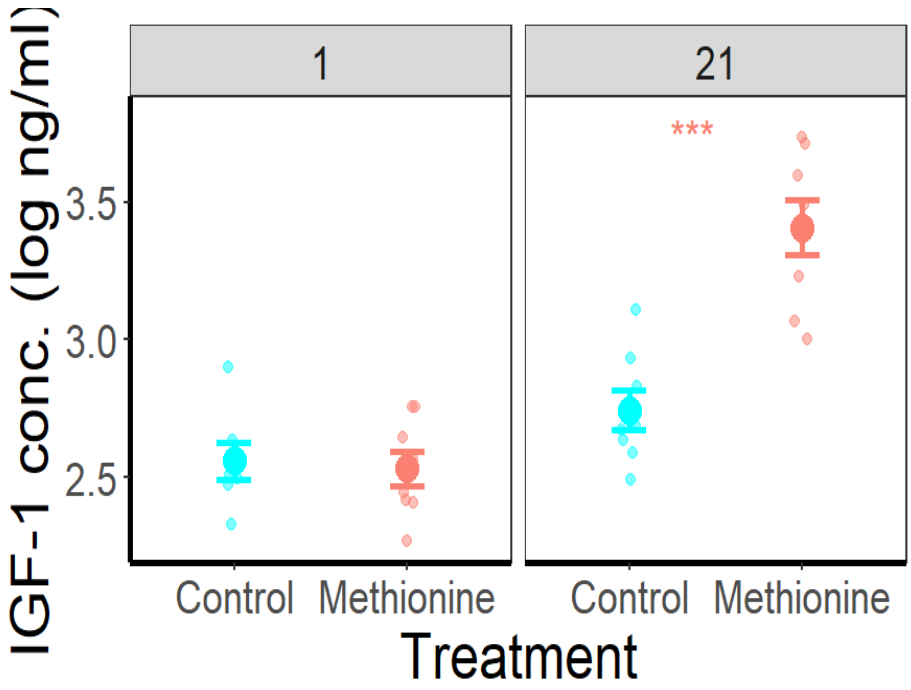

Figure 9. Injecting methionine in eggs increased the postnatal IGF-circulating levels in Japanese quail chicks (n = 8 in each treatment group). The asterisks denote the significant difference between the treatment groups at $p < 0.001$. Numbers at the top of the panels indicate the chick's age in days (1-day-old and 21-day-old chicks). The big circles indicate the mean, and the error bars indicate a standard error, mean ± standard error. Small circles indicate the individual measurement for each bird

3.3.4. Effects of embryonic methionine on mTOR pathway relative gene expression

The relative mRNA expression of *IGF1, mTOR, and RPS6K1* was influenced by methionine treatment in quail chicks. On day-old chicks, relative mRNA expression of *IGF1* significantly increased in a methionine-treated group than in the control group (p = 0.024); on reaching day 21, the relative mRNA expression of *IGF1* was equal among the groups (p = 0.835, Figure 10A). Further, on day-old chicks, the relative mRNA expression of *mTOR* significantly increased in the methionine-treated group than in the control group (p = 0.001), while on reaching day 21, the relative mRNA expression of mTOR disappeared (p = 0.763, Figure 10B). However, the relative mRNA expression of *RPS6K1* on day-old chicks did not differ significantly between the methionine-treated and control group (p = 0.083), whereas on 21 days old chicks, significantly increased in methionine-treated than in the control group (p < 0.001, Figure 10C).

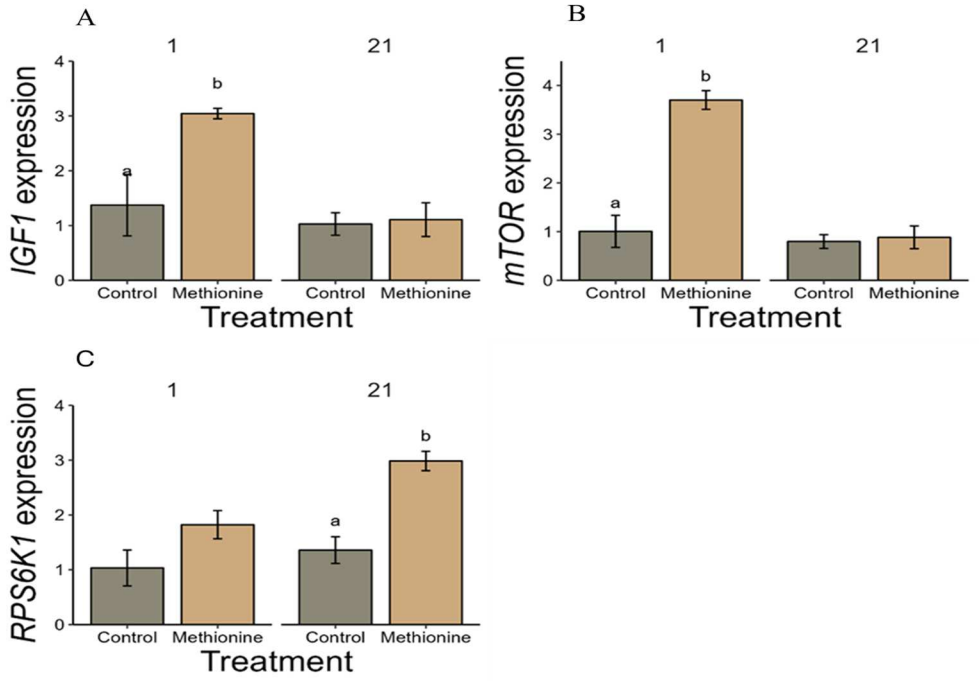

Figure 10. Fold-change gene expression patterns influenced by methionine in the nutrient pathway. Panels show relative expression of (A) Insulin-like growth factor 1 (IGF1), (B) mechanistic target of rapamycin (mTOR), and (C) ribosomal protein serine 6 kinase 1 (RPS6K1). Numbers above bars represent chick ages (1-day-old and 21-day-old). Bars indicate mean ± standard error (mean + SE). Tukey's test compared adjusted marginal means at $p < 0.05$; means with different letters are significantly different

3.4. Experiment 4: Effects of embryonic leucine on early postnatal growth and development of Japanese quail

3.4.1. Effects of embryonic leucine on hatchability of Japanese quail eggs

Similar to methionine, leucine treatment did not significantly affect the hatching success compared to the control group ($\chi2 = 0$, df = 1, $p = 0.59$). However, a noteworthy trend indicated the increasing hatching success in the embryonic leucine-supplemented group (65.1%) compared to the control placebo group (50%). Similar results were reported when the blend of branched-chain (BCAA) leucine, valine and isoleucine were injected into the broiler chicken eggs on day 7 or 14, showing no significant difference in hatchability compared to the control group (BHANJA & MANDAL, 2005).

3.4.2. Effects of embryonic leucine on body weight changes and morphological traits

We found that one-day-old chicks had no difference in body weight after hatching between the treatment groups. However, we observed a significant interaction between the treatment and the age of the individuals on body weight ($p < 0.001$). On reaching three days old, body weight increased consistently in the leucine-injected compared to the control group on all days until 21 days old (Table 6). We also found the interaction between treatment and age on the head ($p < 0.001$), wing ($p < 001$) and tarsus ($p < 0.001$, Table 6) lengths. While tarsus length did not differ in 1-day old individuals, it increased consistently high starting from 3 days old chicks in leucine injected group (Table 6), with no difference in head length on day and two days old chicks but differed starting five days (Table 6), and wing length differed from 7 days old chicks until 21 days (Table 6).

Table 6. The effects of embryonic leucine on the growth and development of morphological traits in Japanese quail

Parameter	Age (days)	Treatment		Estimate	SE	df	t.ratio	p.value
		Control (emmean±se)	Leucine (emmean±se)					
Body mass (g)	1	9.212 ± 1.90 (16)	9.885 ± 1.71 (20)	-0.673	2.558	61.563	-0.263	0.794
	3	11.628 ± 2.72 (8)	17.324 ± 2.19 (11)	-5.696	3.498	91.795	-1.628	0.107
	5	16.384 ± 2.89 (8)	25.483 ± 2.38 (9)	-9.099	3.752	98.442	-2.425	0.017
	7	25.800 ± 2.99 (8)	39.532 ± 2.49 (8)	-13.731	3.824	100.189	-3.591	<0.001

	10	30.867 ± 2.89 (8)	50.219 ± 2.49 (8)	-19.352	3.824	100.189	-5.061	<0.001
	14	40.250 ± 2.89 (8)	68.819 ±2.49 (8)	-28.569	3.824	100.189	-7.471	<0.001
	21	63.122 ± 3.10 (8)	93.407 ± 2.49 (8)	-30.285	3.977	103.713	-7.616	<0.001
Head length (mm)	1	20.075 ± 0.42(16)	19.730 ± 0.38 (20)	0.345	0.573	57.603	0.602	0.549
	3	22.133 ± 0.62 (8)	22.934 ± 0.49 (11)	-0.802	0.774	89.645	-1.036	0.303
	5	23.497 ± 0.64 (8)	25.128 ± 0.53 (9)	-1.631	0.827	96.805	-1.971	0.052
	7	24.197 ± 0.64 (8)	27.387 ± 0.54 (8)	-3.191	0.843	98.676	-3.787	<0.001
	10	27.830 ± 0.64 (8)	30.899 ± 0.55 (8)	-3.070	0.843	98.676	-3.643	<0.001
	14	28.797 ± 0.64 (8)	32.575 ± 0.55 (8)	-3.778	0.843	98.676	-4.484	<0.001
	21	29.847 ± 0.68 (8)	33.725 ± 0.55 (8)	-3.878	0.875	102.425	-4.434	<0.001
Tarsus length (mm)	1	16.175 ± 0.44 (16)	16.015 ± 0.40 (20)	0.160	0.594	58.856	0.269	0.789
	3	17.801 ± 0.63 (8)	19.901 ± 0.51 (11)	-2.100	0.805	90.373	-2.608	0.011
	5	20.737 ± 0.67 (8)	22.398 ± 0.55 (9)	-1.662	0.862	97.372	-1.929	0.057
	7	21.170 ± 0.67 (8)	23.664 ± 0.57 (8)	-2.494	0.878	99.204	-2.841	0.005
	10	24.870 ± 0.67 (8)	28.176 ± 0.57 (8)	-3.306	0.878	99.204	-3.766	<0.001
	14	26.703 ± 0.67 (8)	31.001 ± 0.57 (8)	-4.298	0.878	99.204	-4.896	<0.001
	21	28.863 ± 0.71 (8)	32.976 ± 0.57 (8)	-4.113	0.912	102.880	-4.511	<0.001
Wing length (mm)	1	15.250 ± 1.35 (16)	15.320 ± 1.21 (20)	-0.070	1.811	86.453	-0.039	0.969
	3	17.128 ± 2.02 (8)	17.795 ± 1.61 (11)	-0.667	2.584	101.051	-0.258	0.797
	5	22.546 ± 2.17 (8)	25.702 ± 1.77 (9)	-3.156	2.803	104.398	-1.126	0.263
	7	25.346 ± 2.17 (8)	31.806 ± 1.87 (8)	-6.460	2.867	105.365	-2.253	0.026
	10	38.879 ± 2.17 (8)	50.531 ± 1.87 (8)	-11.652	2.867	105.365	-4.064	<0.001
	14	46.229 ± 2.17 (8)	61.256 ± 1.87 (8)	-15.027	2.867	105.365	-5.241	<0.001
	21	57.485 ± 2.35 (8)	70.244 ± 1.87 (8)	-12.759	3.007	107.542	-4.244	<0.001

Abbreviations: Estimated marginal mean and standard error (emmean+se) in specific days and treatments. Tukey's test was applied to compare adjusted marginal means at $p < 0.05$ significance level, standard error (SE) of the two compared treatments within a day. Test statistics (t.ratio), degree freedom (df), and statistical value to measure the difference (p.value) at the significance level of $p < 0.05$. Numbers in brackets are sample sizes (n)

3.4.3. Effects of embryonic leucine on mTOR pathway relative gene expression

We found no significant interaction between treatment and age of chicks on *IGF1* gene expression ($p = 0.702$). However, we observed a significant difference in *IGF1* gene expression between the groups in both 1-day-old chicks ($p = 0.023$) and 21-day-old chicks ($p = 0.042$). The *IGF1* gene expression increased significantly in the leucine-injected group compared to the control group (Figure 11A). We found a marginally significant interaction between treatment and the age of chicks in *IGF1R* gene expression ($p = 0.071$). We also found a significant difference in *IGF1R* gene expression in day-one-old chicks between the two groups ($p = 0.026$). *IGF1R* gene expression was significantly higher in the leucine-injected group than in the control group (Figure 11B). There was no significant difference in *IGF1R* gene expression between the groups in 21-day-old chicks ($p = 0.435$). We found a significant interaction between treatment and age on *mTOR* gene expression ($F = 3.292$, $p = 0.021$). In 1-

day-old chicks, we observed a significant difference in *mTOR* gene expression between the two groups (p = 0.009; control: n = 8, leucine: n = 8). The mTOR gene expression increased significantly in the leucine-injected group than in the control group (Figure 11C). We observed no difference in *mTOR* gene expression between the two groups in 21-day-old chicks (p = 0.579). We found no interaction effects between treatment and age on *RPS6K1* gene expression (p = 0.251). We observed the difference in *RPS6K1* gene expression in day-old chicks between the two groups (p = 0.022). The *RPS6K1* increased markedly in one-day-old chicks in the leucine injected group compared to the control group (Figure 11D). The maximum *RPS6K1* gene expression was not different between the two groups in 21-day-old chicks (p = 0.977). We detected no significant interaction between the treatment and age on *FOXO1* gene expression (p = 0.665). Similarly, neither 1-day-old chicks (p = 0.702) nor 21-day-old chicks (p = 0.432) had a significant difference in *FOXO1* gene expression (Figure 11E).

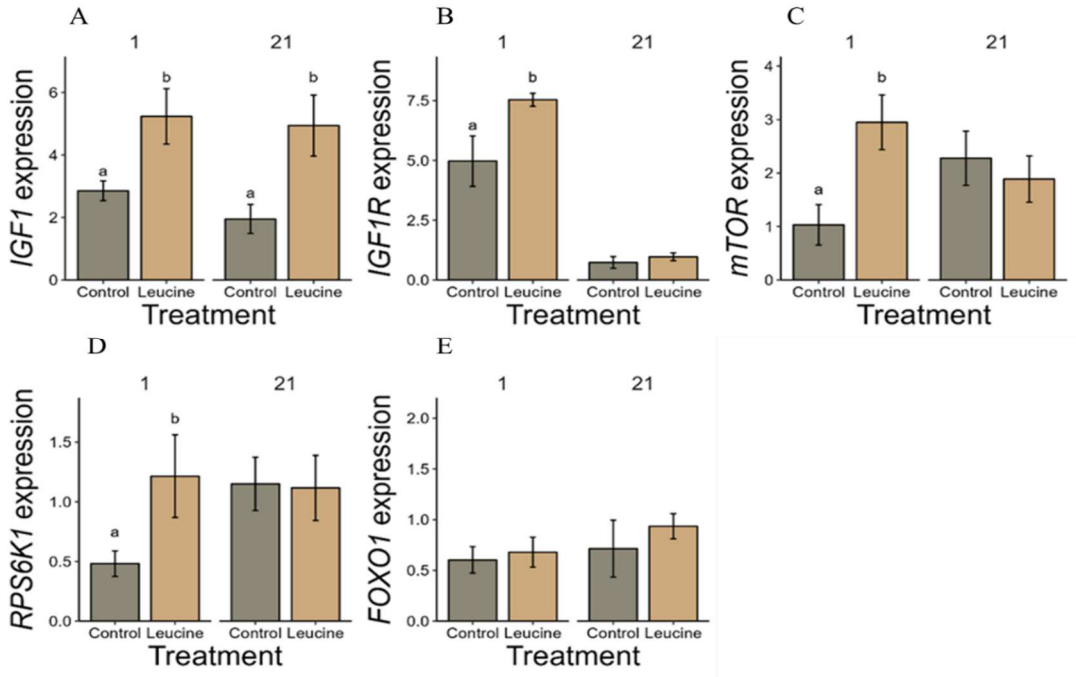

Figure 11. Gene expression patterns in fold change affected leucine treatment in the nutrient-sensing pathway.A: Insulin-like growth factor 1 (*IGF1*), B: Insulin-like growth factor 1 receptor (*IGF1R*), C: mechanistic target of rapamycin (*mTOR*), D: ribosomal protein serine 6 kinase 1 (*RPS6K1*) and E: Forkhead box protein O1 (*FOXO1*). The numbers on top of the bars indicate the age of the chicks (1-day-old and 21-day-old chicks). The bars and the error bars indicate the mean and standard error of the mean (Mean+SE). Tukey's test was applied to compare adjusted marginal means at $p < 0.05$ significance level. Means followed by distinct letters are significantly different at $p < 0.05$.

3.5. Experiment 5: Effects of rapamycin treatment on body weight changes, gene expression, haematological and histomorphology profiles

3.5.1. Effects of rapamycin treatment on body weight and relative spleen weight

The rapamycin treatment did not significantly affect body weight throughout all days nor at the end of the experiment (Table 7). There was no significant effect on body weight gain (Table 7). However, we observed the sex differences in spleen weight where female quails grew faster and increased body weight compared to males. Relative spleen weight was significantly higher in females than in male birds, and the rapamycin treatment resulted in significantly lower spleen values than the control group (Table 8).

Table 7. Body weights of the quails on injection days

Body weights	Treatment				Effect (P-value)		
	Control		Rapamycin		Treatment	Sex	Interaction
	female	male	female	male			
Day 1	153.1±1.5	178.8±1.9	156.4±1.7	182.0±2.4	0.099	<0.001	0.9649
Day 3	169.9±2.4	192.5±2.6	172.1±5	196.6±8.6	0.18628	<0.001	0.70517
Day 5	183.7±2.9	200.5±2.9	184.1±7.8	203.9±3.5	0.50514	<0.001	0.60545
Day 7	188.6±2.3	201.4±2.7	191.3±2.3	207.9±3.9	0.11999	<0.001	0.51245
Day 10	202.6±2	204.6±2.5	203.5±3.4	213.5±4.9	0.16035	0.07	0.24971
Day 12	216.4±3.6	211.1±2.7	216.7±5	217.9±4.7	0.37304	0.69265	0.42512
Day 14	229.2±3.9	212.4±3	225.5±5.9	217.4±4.8	0.88691	0.0096	0.34281

The results are represented as the mean and standard errors (n = 10/sex/treatment). The treatment and treatment × sex interaction did not result in significant differences. A significant difference was noted between the sexes.

Table 8. Effect of rapamycin on growth and relative spleen in Japanese quail

Parameters	Control		Rapamycin		Effect (P-value)		
	Female	Male	Female	Male	Treatment	Sex	Interaction
Body weight (at the end of the experiment)	234.5 ± 13	214.6 ± 9	224.7 ± 20	219 ± 14	0.566	0.009	0.135
Body weight gain (g/day)	5.43 ± 0.9	2.381 ± 0.6	4.552 ± 1.4	2.464 ± 0.8	0.206	<0.001	0.128
Relative spleen weight (g)	0.065 ± 0.012	0.044 ± 0.027	0.048 ± 0.017	0.031 ± 0.026	0.036	0.009	0.737

The mean and standard errors (n = 10/sex/treatment). The treatment and treatment × sex interaction did not result in significant differences. A significant difference was noted between the sexes in all variables.

3.4.2. Effects of rapamycin treatment on relative gene expression

The rapamycin treatment did not significantly affect *mTOR* expression compared to the control birds (p = 0.401); none of the sex effects (p = 0.129) or interaction (p = 0.903, Table 9). Rapamycin treatment significantly decreased *IGF1* in male quails compared to the

expression of male control birds (p = 0.038; Table 9), with an interaction effect between treatment and sex (p = 0.036). Interleukin-1β (IL-*1β*) expression did not significantly differ between the treatment groups (p = 0.088), and no sex effect was observed (p = 0.922) or interaction (p = 0.752; Table 9). *MyD88* expression was significantly higher in rapamycin-injected males compared to the control male group (p < 0.011) with an interaction effect (p = 0.0005; Table 9). However, *NF-κβ* expression did not significantly differ between rapamycin-treated and control groups (p = 0.851), and no sex effect was observed (p = 0.149) or interaction (p = 0.208, Table 9). Rapamycin did not significantly affect *STAT3* expression (p = 0.377), and no sex effects (P = 0.239) or interaction effects (Table 9).

Table 9. The relative gene expression in Japanese quails treated with 1 mg/kg body weight rapamycin.

Gene	Control		Rapamycin		Effects (P-values)		
	female	male	female	male	Treatment	Sex	Interaction
mTOR	1± 0.1	1± .06	0.84 ± 0.5	0.8 ± 0	0.401	0.129	0.903
IGF1	1 ± 0	1± 0.1	0.8 ± 0	0.5 ± 0	0.021	0.038	0.036
IL-1β	1 ± 0	1 ± 0	1.3 ± 0	1.3 ± 0	0.088	0.922	0.752
MyD88	1 ± 0	1 ± 0	0.5 ± 0	3 ± 0.2	<0.001	0.011	<0.001
NF-Kβ	1 ± 0	1 ± 0	0.8 ± 0	1.2 ± 0	0.851	0.149	0.329
STAT3	1± 0	1± 0.1	1.2 ± 0.1	1.2 ± 0	0.377	0.239	0.411

Abbreviations: mechanistic target of rapamycin mTOR; insulin-like growth factor 1 (IGF1): interleukin-1β (IL-1β); myeloid differentiation primary response gene 88 (MyD88); nuclear factor kappa beta (NF-κβ); signal transducer and activator transcription 3 (STAT3). Values are Means and standard errors of the mean (n = 20 per treatment group).

3.4.3. Effects of rapamycin treatment on intestinal morphology

However, crypt depth was higher in rapamycin-injected male quails than in control male birds (p = 0.001), but females showed overall higher crypt depths than male birds (p = 0.004, Figure 15A). Neither the rapamycin treatment (p =0.298), sex ($F_{1,181}$ = 1.471; p = 0.226), nor their interaction (p = 0.248) affected ileal villus height (Figure 15B). The rapamycin injection decreased the VH: CD ratio (p = 0.002) in treated males compared to the control male

birds, and we also observed a higher VH: CD ratio in male birds compared to females (p = 0.026, Figure 15C). Total mucosa was thicker in females (p = 0.016, Figure 15D), but the rapamycin treatment did not alter the mucosa thickness compared to the control group.

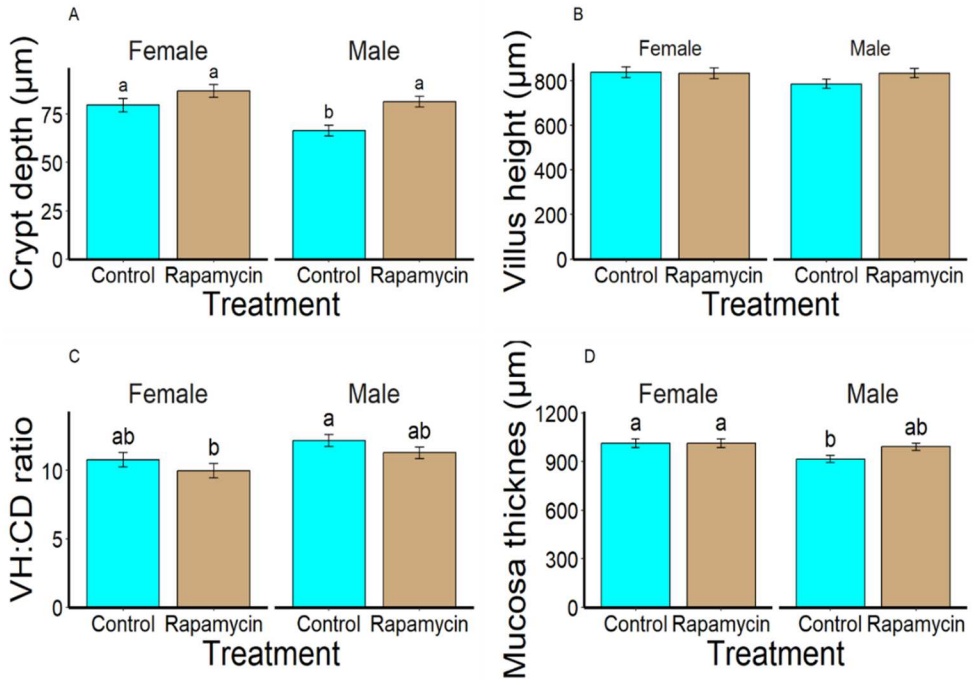

Figure 12. Intestinal histomorphology traits in the Japanese quail (Coturnix japonica) treated with 1 mg/kg body weight rapamycin. Error bars represent means ± standard errors of the mean (n = 5 birds of each sex per treatment). Groups with distinct letters are significantly different from each other at p > 0.05

4. CONCLUSIONS

Amino acid supplements can counteract the negative effects of feed restriction on birds. Specifically, supplementing a bird's diet with leucine or methionine can improve its body weight, reproductive health, and total antioxidant capacity, even during a 20% feed restriction. This suggests that the right nutritional composition, especially essential amino acids, is key to reversing the adverse effects of limited food intake on bird growth and reproduction.

The research also found that the duration and timing of feed restriction impact egg quality. While a 20%, 30%, or 40% reduction in food intake negatively affected egg components, an unpredictable feed access did not. This unpredictable feeding schedule maintained the quality of the yolk, albumin, and eggshell and kept the yolk-to-albumin ratio stable, suggesting that this method could be a viable strategy to maintain egg quality and improve overall productivity in the poultry industry.

Additionally, the study explored the effects of supplementing a bird's diet with amino acids during the embryonic stage. A 1 mg dose of methionine or a 2.5 mg dose of leucine during this period stimulated the nutrient-sensing pathway, leading to increased postnatal growth. These findings indicate that early nutritional intervention with specific amino acids can improve growth performance, suggesting a link between embryonic nutrition and a bird's later development.

Finally, the research looked at the effects of rapamycin, a drug often used to suppress the immune system. The study found that while rapamycin didn't significantly affect body weight, it did reduce spleen weight, which is associated with a more stable immune response. The drug also had sex-specific effects, with male birds showing a decrease in a growth-related gene (IGF1) and an increase in an immune-related gene (MyD88) compared to females. This indicates that rapamycin could be used to modulate the immune system in birds.

5. NEW SCIENTIFIC RESULTS

1. Two-week-long (from 6 to 8 weeks of age) restricted feeding (80%) of diets supplemented with 20% (4.2 g/kg) leucine or with 20% (0.9 g/kg) methionine above the recommended level (21.2 g/kg leucine and 4.5 g/kg methionine) or the combined treatment significantly increased final body weight of female Japanese quails compared to the restricted feeding (80%) of diets supplemented with the required amount of leucine and methionine. Furthermore, the +20% methionine treatment increased the number of hierarchical follicles, while the +20% leucine treatment increased ovary mass, and the combined treatment of the two amino acids increased both parameters compared to the restricted feeding (80%) of diets supplemented with the required amount of leucine and methionine.
2. The 20% continuous feed restriction of quails for 14 days did not significantly affect egg yolk, albumen, and eggshell weights compared to the ad libitum-fed control group. In contrast, the 30% and 40% continuous feed restrictions resulted in significant reductions of albumen and eggshell weight compared to the control group. The unpredictable daily feed supply ranging from 30% to 170% of the daily feed intake of quails for 16 days did not significantly affect yolk weight, albumen weight, eggshell weight or their proportions.
3. In comparison with the control group, the *in ovo* feeding of 1 mg (≈ 2%) methionine above the average methionine concentration of eggs (48.72 mg/egg) on E0 into the egg yolk significantly increased postnatal body weight of Japanese quails from day 7 to day 21 as well as the gene expression of hepatic IGF1 and mTOR on day 0, RPS6K1 on day 21, and plasma IGF1 concentration on day 21.
4. In comparison with the control group, the *in ovo* feeding of 2.5 mg (≈ 2%) leucine above the average leucine concentration of eggs (127.6 mg/egg) on E10 into the allantoic fluid significantly increased postnatal body weight of Japanese quails from day 5 to day 21 as well as the gene expression of hepatic IGF1 on day 1 and 21, mTOR and RPS6K1 on day 1.
5. Subcutaneous treatment of 1 mg/kg body weight of rapamycin every second day for two weeks (7 times, once daily at 10:00 hours) resulted in significantly lower relative spleen

weight of male and female, and in higher crypt depth of male Japanese quails but did not influence the body weight of the experimental animals.

6. Subcutaneous treatment of 1 mg/kg body weight of rapamycin every second day for two weeks (7 times, once daily at 10:00 hours) significantly decreased the splenic gene expression of IGF1 while increased the expression of MyD88 in male Japanese quails, and did not affect the gene expression of mTOR, NF-κβ, IL-1β and STAT3 compared to the control birds.

6. PRACTICAL RESULTS

1. The approach of using 20% leucine (14.2 g/kg) or 20% methionine (4.5 g/kg) of basal diet or their combination, on top of 20% feed restriction during the sexual maturation stage, can serve as a basis for further investigations on other poultry species, such as broiler breeder hens or pullets where feed restriction is part of the feeding technology to optimize the reproductive performance.
2. The unpredictable daily feed access preserved the proportions of the egg components, indicating that these findings in the Japanese quail model species may be a foundation for more applied research in pullets and broiler breeders under practical production conditions.
3. Provision of embryonic methionine and leucine feeding imitates maternal nutrient transfer in Japanese quail. This strategy can enhance postnatal growth performance by activating the IIS/mTOR pathway, which regulates the expression of growth-related genes. This approach is crucial for understanding the mechanisms that control growth through nutrient stimulation in other species.

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List of publications related to the dissertation

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1. Reda, G. K., **Ndunguru, S. F.**, Csernus, B., Lugata, J. K., Knop, R., Szabó, C., Czeglédi, L.: The effect of different dietary manipulations on haematological properties in Japanese quail. *Acta agrar. Debr. 2*, 35-41, 2024. ISSN: 2416-1640. DOI: http://dx.doi.org/10.34101/actaagrar/2/15082

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Foreign language scientific articles in Hungarian journals (3)

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