Theses of Doctoral (PhD) Dissertation

THE EFFECTS OF DIETARY REGIMENS AND AMINO ACID COMPOSITION OF FEED ON THE mTOR SIGNALLING PATHWAY AND PRODUCTION TRAITS IN JAPANESE QUAIL *(Coturnix japonica)*

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1. Background, justification and aim of the study

Nutrient sensing governed by the mechanistic target of rapamycin (mTOR) is a key pathway in mediating nutritional cues to coordinate growth, reproduction, and lifespan (JOHNSON, 2018; KAPAHI et al., 2017). At a glance, high nutritional availability upregulates the mTOR signalling pathway, which promotes growth and reproduction, while suppressing cellular processes that uphold organismal and cellular homeostasis (such as autophagy and apoptosis) (PAPADOPOLI et al., 2019).

Quantitative and qualitative dietary manipulation, in terms of feed restriction, nutritional unpredictability, and micro- and macronutrient variability, is one of the strategies applied to understand the mechanism of the mTOR pathway. Feed restriction (FR) is a robust method used to investigate the impact of limited resources on molecular, physiological, and phenotypic plasticity. At a glance, FR downregulates mTOR through three mechanisms: 1) through reduces circulating IGF-1 levels (REGAN et al., 2020). 2) FR causes systemic amino acid deficiency, leading to cellular amino acid starvation, which consequently inhibits amino acid-dependent mTOR activation (KIM, 2009). 3) FR results in reduced systemic energy availability, thereby activating intracellular AMPK signalling, which inhibits mTOR activation at multiple points (XU et al., 2012). Additionally, feed restriction regulates the expression of genes encoding nutrient sensing pathways at the transcriptional stage (NDUNGURU et al., 2024; REDA et al., 2024a; REDA et al., 2024b).

The other dietary condition important to understanding molecular mechanisms is dietary unpredictability. Unlike constantly high or low feeding, conditions organisms could predict and prepare for, dietary unpredictability could increase stress levels due to perceived uncertainty, potentially leading to physiological fluctuations and metabolic imbalances. These processes could be governed by alterations in gene expression. Genes in the mTOR pathway could be responsible for sensing nutritional fluctuation, undergoing adaptive expression changes, and linking genotypes and molecular functions to fitness outcomes. However, many previously reported studies used constant ad libitum and restricted feeding, which did not consider the effect of unpredictable feeding.

Micro- and macronutrient manipulation is another intervention strategy to extract the mechanism of the mTOR pathway. Amino acids are proposed to be the key regulators of the mTOR pathway. Manipulating key essential amino acids only instead of part of the whole feed can effectively inhibit the pathway (JUNG et al., 2015). However, we have limited knowledge of what will happen if we supplement certain essential amino acids under restricted feeding conditions. We believe that feed restriction and manipulation of amino acids may be an effective intervention to separate the effect of total energy intake and specific amino acids on mTOR pathway genes. Additionally, macronutrients, such as carbohydrates, lipids, and proteins play a crucial role in regulating the mTOR pathway. However, while studies have reported the effect of total calorie restriction on mammalian models, the effect of metabolisable energy restriction under full feeding is unknown. Furthermore, proteins regulate the mTOR pathway in two ways: 1) by influencing the expression of the upstream effectors (HILL et al., 2017; LI et al., 2024) and 2) by acting as a source of essential amino acids, thereby affecting the mTOR pathway through amino acid signalling (LAMMING et al., 2015). However, less

information shows the regulatory role of protein restriction compared to full-feeding and feed-restricted conditions, mainly in birds.

Understanding the function of mTOR in birds is very important due to their distinctive physiology. By studying the regulation of the mTOR pathway in birds, we can better understand the fundamental mechanisms that control the physiological adaptations of this evolutionarily independent lineage. In this study, we employed feed restriction, dietary unpredictability, and dietary amino acid and micronutrient manipulation to explore the mechanism of mTOR pathway genes and some physiological traits. We used Japanese quails as an experimental avian model. Therefore, the overall objective of the study was to investigate the mechanisms by which different dietary states regulate the expression of mTOR pathway genes and the links with life-history traits, mainly body weight and reproduction. The study is carried out with the following specific objectives:

- To investigating the effect of gradients of feed restriction on expression of key mTOR pathway genes and their association with resource allocation among life-history traits.
- To assess the sex-specific molecular and physiological response towards different levels of feed restriction
- To explore the molecular and phenotypic responses of Japanese quails to dietary unpredictability and tissue and sex-specific responses
- To separate the role of specific amino acids (methionine and leucine) on the regulation of the key mTOR pathway genes and its connection with body weight as a primary fitness measure.
- To evaluate the expression of specific key mTOR pathway genes in response to dietary protein and metabolisable energy restriction compared to full feeding and equivalent feed restriction.

2. MATERIALS AND METHODS

2.1. Experiment 1: Effect of feed restriction treatment on molecular, physiological and production traits in Japanese quail

2.1.1. Experimental animals and housing

Japanese quail chicks (Coturnix japonica), aged four weeks, were acquired from Budai Fürjészet, a commercial quail breeder in Hungary. The chicks were then maintained in the Animal House of the Institute of Animal Science, Biotechnology, and Nature Conservation at the University of Debrecen in Hungary. The birds were kept in a controlled environment until they reached maturity, and then they were given an additional 4 weeks to adjust to the experimental settings. At the age of 8 weeks, 64 female and male birds with sex-specific similar body weights were chosen for acclimation and placed in individual cages.

During the acclimation period, the birds were provided with *ad libitum* access to food and drink for 7 days to ensure they were accustomed to the individual living and experimental room settings. The experiment room was kept at a temperature of 24 ± 3 °C, with relative humidity ranging from 60% to 75% and a daily photoperiod cycle of 12:12 h of light:dark. The basal diet for experimental quails consisted of a breeder quail ration with a crude protein content of 20% and a metabolisable energy content of 12.13 MJ/kg. The feed was prepared on a corn-soybean-wheat basis, according to NRC (1994).

2.1.2. Experimental design

At the onset of the trial, when the birds were 9 weeks old, male and female individuals were randomly assigned to four different nutritional treatments. The birds in each treatment group were given feed at 80% (FR20), 70% (FR30), and 60% (FR40) of their average individual feed intake, whereas the control group was given *ad libitum* (ADL) feed. In order to account for any possible minor differences in light intensity, the cage system was divided into eight groups based on its vertical position in the cage system's staircase. The birds were then assigned to these groups, which were considered an experimental block. Every block was comprised of an equivalent number of males and females from each treatment group. The experiment was conducted for 14 days.

2.1.3. Measurements and sample collection

We measured body weight and collected blood samples at the beginning (day 0) and on days 7 and 14 of the experiment using a digital balance with a precision of ± 0.1 g. Eggs were marked with bird identity, collected, and immediately weighed individually using a digital laboratory analytical balance to ± 0.01 g daily. Blood samples were obtained from the brachial vein through venepuncture using heparinised capillary tubes and transferred into 0.5 ml microcentrifuge tubes. The blood samples were centrifuged at a force of 9000 × g for 10 minutes. The plasma sample was then stored at a temperature of -80 ℃ until it was used for laboratory purposes. On the 14th day of the trial, all birds were euthanised with the help of professional veterinarians by cervical dislocation. The birds were then immediately dissected to sample liver tissues. The collected samples were frozen using dry ice, immediately transported to the laboratory, and maintained at -80 ℃ until the next assay.

2.2. Experiment 2: Effect of unpredictable and restricted feeding on expression of mTOR pathway genes and production traits in Japanese quails

2.2.1. Animals and housing conditions

At the age of six months, 48 male and female birds were selected from the stock based on their sex-specific uniform body weight and housed separately in individual cages. After separation, the birds were kept on an *ad libitum* feeding condition for seven days of acclimation and another seven days of feeding intake measurement. Additionally, body weight was measured at three-day intervals to assess any potential changes over time, but no significant directional change in body weight or feed intake was observed.

2.2.2. Experimental design

Birds of both sexes were weighed and distributed equally into one of the three dietary treatments (full fed, unpredictably fed, and 40% restricted groups) based on their body weight. This ensured that the average body weight was similar among all experimental groups, considering the sex difference. Control birds received daily feed equal to their average feed intake individually. The unpredictably fed birds (UNPR) received the same amount of total food during the experimental period as the controls but a randomly variable daily amount of feed between 30% and 170% of their respective daily feed intake. Birds in the third group received a constant 60% of their respective feed intake, hence a feed restriction of 40% (FR40). On the measurement and sampling days (days 0, 4, 8, and 16), the UNPR birds were given 100% of their respective individual intake to maintain consistency in feeding conditions with the control group and avoid short-term effects of under- or overfeeding (Figure 1). The trial was conducted for 16 days.

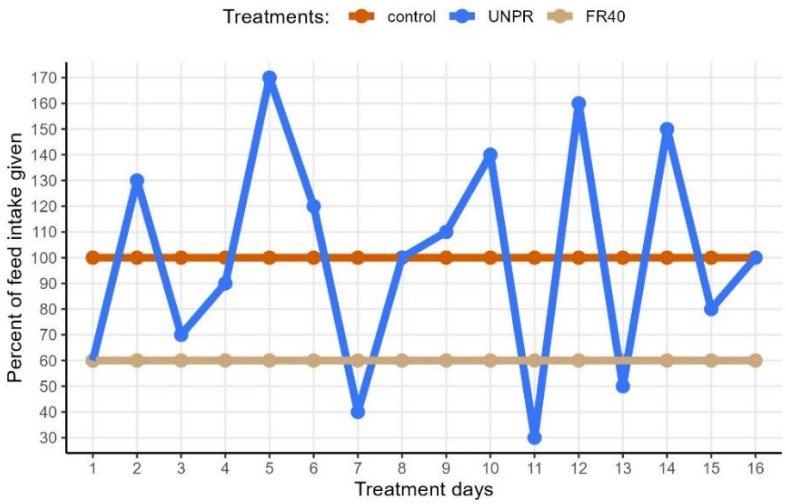

Figure 1. Experimental design for unpredictable feeding experiment. The 100% indicates feeding equivalent to the calculated feed intake during the acclimation period.

2.2.3. Measurements and sample collection

Body weight was recorded on days 0, 4, 8, and 16 during the treatment period. Eggs were marked with bird identity, collected into a quail egg box, and immediately weighed individually using a digital laboratory analytical balance to ± 0.01 g daily. On day 16, all the

birds were euthanised, and liver and breast muscle samples were promptly collected, rapidly frozen using dry ice, and stored at –80 ℃ until further assays.

2.3. Experiment 3: Effect of feed restriction, energy restriction or protein restriction and amino acid supplementation on expression of mTOR pathway genes and body weight in Japanese quail

2.3.1. Experimental animals, treatments and measurement and sampling

Eggs from Japanese quails kept for research purposes were incubated and hatched. After hatching, 340 newly hatched chicks were reared under a grower feeding for up to four weeks and breeder feeding for up to five weeks (Table 1). At the age of five weeks, we selected and allocated 56 female birds with similar body weights into an individual cage system for feed intake assessment and acclimation to the individual cage condition. They stayed for seven days under the ad libitum breeder feeding condition. At the age of 6 weeks, birds were randomly assigned to seven treatments, as described in Table 2. Therefore, each treatment group contains eight birds. To control for a slight difference in light intensity along the cage height levels, we divided it into 8 blocks with an equal number of birds from all treatments in each block. The ingredients and nutritional composition of each treatment feed are presented in Table 1. The trial was conducted for 14 days. Daily feed intake was monitored, and any increase in feed intake in the control birds was used to adjust the amount of feed given to the other treatment group.

Table 2. Treatment codes, their description and number of birds per treatments

No.	Treatment code	Description	Number of birds
1	Control	Full fed	8
2	FR	20% feed restriction	8
3	FR+L	FR + 20% Leucine	8
4	FR+M	FR + 20% methionine	8
5	FR+ML	FR + 20% methionine + 20% leucine	8
6	ER	20% metabolisable energy restriction	8
7	PR	20% protein restriction	8

Body weights were measured on days 0, 7, and 14 of the experimental period, with the same procedure as in Section 2.1.3. On the last day of the experiment (day 14), all birds were euthanised with the help of veterinary experts, and liver samples were collected, the same procedure as in Section 2.2.3.

Table 1. Ingredient composition and nutrient level of experimental feed

Ingredients	Growers' feed***	Experimental diets					
		Basal diet (control)	Energy restricted basal-20% (ER)	Protein restricted basal -20% (PR)	20% Feed restriction		
					Basal + 20% Leu (FR+L)	Basal + 20% Met (FR+M)	Basal+20% Met and Leu (FR+ML)
	Inclusion rate, %						
Corn	26.330	25.000	15.000	35.000	25.000	25.000	25.000
Soybean meal	28.410	24.860	17.380	11.800	25.480	24.960	25.570
Wheat	30.000	10.090	24.490	0.000	10.000	10.000	10.000
Wheat bran	0.000	10.000	10.000	10.000	9.170	9.960	9.030
Pea	0.000	13.020	25.000	25.000	12.390	12.900	12.300
Corn gluten meal	5.000	0.000	0.000	0.000	0.000	0.000	0.000
Fishmeal	5.000	0.000	0.000	0.000	0.000	0.000	0.000
Sunflower oil	2.780	9.070	0.000	8.840	9.270	9.120	9.300
Limestone	1.060	5.830	5.880	5.890	5.820	5.830	5.820
MCP	0.390	0.960	0.890	1.020	0.970	0.960	0.970
L-Lys	0.100	0.000	0.050	0.310	0.000	0.000	0.000
L-Met	0.000	0.190	0.200	0.250	0.190	0.300	0.300
DL-Met	0.070	0.000	0.000	0.000	0.000	0.000	0.000
L-Leu*	0.000	0.010	0.075	0.265	0.385	0.010	0.380
L-Ile*	0.000	0.005	0.037	0.133	0.193	0.005	0.190
L-Val*	0.000	0.005	0.037	0.133	0.193	0.005	0.190
L-Arg	0.000	0.000	0.000	0.230	0.000	0.000	0.000
L-Thr	0.130	0.070	0.080	0.200	0.070	0.070	0.070
L-Trp	0.000	0.000	0.000	0.040	0.000	0.000	0.000
Salt	0.230	0.380	0.380	0.380	0.380	0.380	0.380
Vit. and mineral premix**	0.500	0.500	0.500	0.500	0.500	0.500	0.500
Nutrient contents (analysed values)							
ME, MJ/kg	12.130	12.130	9.700	12.130	12.130	12.130	12.130
Crude protein, %	24.000	18.000 (18.20)	18.000 (18.10)	14.400 (14.40)	18.000	18.000	18.000
Lys, %	1.300	1.014 (1.25)	1.049 (1.32)	1.079 (1.13)	1.016	1.014	1.016
Met %	0.500	0.450 (0.30)	0.450 (0.52)	0.450 (0.31)	0.450	0.563	0.563
Thr, %	1.020	0.740	0.740	0.740	0.740	0.740	0.740
Trp, %	0.268	0.216 ()	0.207	0.190	0.216	0.216	0.217
Leu, %	2.122	1.420 (1.40)	1.422 (1.42)	1.420 (1.23)	1.775	1.420	1.775
Ile, %	1.013	0.770 (0.70)	0.780 (0.78)	0.720 (0.60)	0.950	0.770	0.950
Val, %	1.006	0.865 (0.76)	0.888 (0.80)	0.821 (0.57)	1.051	0.865	1.051
Arg, %	1.460	1.260	1.271	1.260	1.260	1.260	1.260
Leu/Ile/, ratio	2.095	1.840	1.820	1.972	1.870	1.840	1.870
Ca, %	0.800	2.500	2.500	2.500	2.500	2.500	2.500
P, %	0.572	0.608	0.610	0.579	0.602	0.607	0.602
non phytate P, %	0.323	0.350	0.350	0.350	0.350	0.350	0.350
Na, %	0.150	0.150	0.150	0.150	0.150	0.150	0.150

Note: The nutrient levels of the restricted (FR) groups were the same as those of the control feed, except that the Leu and Met levels were raised by the level of feed intake restriction in order to have the same amino acid intake. ***** Leucine, isoleucine and valine were supplemented in a blended form as BCAA in a ratio of 2:1:1, respectively. Measured values are in parenthesis. ** 1 kg premix provided: 1000000 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-Pantothenate, 400 mg vitamin B6, 2 mg vitamin B12, 11 mg biotin, 2502 mg niacin, 60 mg folic acid, 300000 mg choline chloride, 13200 mg Zn, 1920 mg Cu, 9612 mg Fe, 13200 mg Mn, 180 mg I, 42 mg Se, 12 mg Co. *** Grower feed is the feed offered to the birds from hatching until the age of 4 weeks old.

2.4. Laboratory analysis

2.4.1. RNA isolation

For samples from the first experiment, a liver sample weighing 25-30 mg was disrupted using a D1000 handheld homogeniser (Benchmark Scientific, USA), and total RNA was isolated using the TRIzol reagent according to the manufacturer's protocol (Direct-zol™ RNA

MiniPrep, Zymo Research Corporation, USA). For samples from the second and third experiments, different RNA isolation and cDNA synthesis kits were used. Hence, total RNA was extracted using the peqGOLD Total RNA Kit based on the manufacturer's protocol (VWR Life Science, USA).

2.4.2. cDNA synthesis

For samples from the first experiment, cDNA synthesis was performed using the qScript cDNA synthesis kit, following the manufacturer's protocol (Quantabio Reagent Technologies, QIAGEN Beverly Inc., USA) in the PCRmax Alpha Thermal Cycler (Cole-Parmer Ltd., USA). For samples from the second and third experiments, cDNA synthesis was performed using the LunaScript® RT SuperMix Kit, following the manufacturer's protocol (New England Biolabs, Inc., USA). After synthesis, the cDNA samples were diluted 10-fold and stored at -20 °C until needed for Real-Time PCR analysis.

2.4.3. Quantitative real time polymerase chain reaction (qPCR)

Real-time qPCR was performed using HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX), 5X, containing HOT FIREPol® DNA polymerase, ultrapure dNTPs, $MgCl_2$, EvaGreen® dye, and ROX dye (Solis BioDyne, Estonia). Quail-specific primer pairs spanning introns were designed utilising Oligo7 software and acquired from Integrated DNA Technologies (BVBA-Leuven, Belgium). The data for target genes were normalised to a reference gene using the efficiency-corrected method (PFAFFL, 2004).

2.4.4. IGF-1 and Triglyceride measurements

Plasma IGF-1 levels were assessed using a competitive ELISA assay method developed and validated for birds, as detailed in MAHR et al. (2020). Plasma triglyceride levels were analysed using a photometric method with a half-automatic analyser according to the manufacturer's protocol (Lab-Analyse, Orvostechnika Ltd., Budapest, Hungary).

2.5. Statistical analyses

All data were analysed using R version 4.3.2, 'Eye Holes' (https://www.r-project.org/). Linear mixed-effects models were used to test the effect of dietary treatments on body weight, plasma IGF-1, and triglyceride levels across the treatment period. Linear models were employed to analyse the effect of treatments on total egg number and treatments and sex on gene expression. Molecular and physiological variables were transformed into natural logarithms to reduce skewness due to individual biological variability. We used a Gaussian polynomial mixed-effect model to analyse egg weight (DEMIDENKO, 2013). We used generalised linear mixed-effects models of the family logit using package `aod` v. 1.3.2 (LESNOFF and LANCELOT, 2012) to analyse binary response variable (daily egg laying). We employed Pearson correlation to analyse the association among expression of genes.

As expression genes showed a significant correlation, principal component analysis (PCA) was employed to test the overall association of gene expressions with body weight and egg production traits. We utilised the `ggbiplot` package (http://github.com/vqv/ggbiplot) to visually represent the relationship between the variables (gene expression) and the treatment groups. In addition, we employed Kaiser's criterion, as described by KAISER (1960), to determine which principal components to keep for further regression analysis.

Additionally, models were fitted to test how body weight, egg weight, and egg number explain variations in IGF-1 and triglyceride levels. Within-treatment centring was employed for body weight, egg number, and egg weight (subtracting treatment mean from each individual measurement) to disentangle the treatment-induced and the residual variation in these factors (VAN DE POL and WRIGHT, 2009).

3. RESULTS AND DISCUSSION

3.1. Experiment 1: Effect of feed restriction on morphological, molecular and physiological traits

3.1.1. Effect of feed restriction on body weight of Japanese quails

Feed restriction significantly affected body weight across time points in both sexes (treatment × week × sex: $F_{6,112} = 3.01$, $p = 0.009$). In females, birds grouped under all restriction levels showed a significant reduction in body weight compared to the *ad libitum*-fed birds (ADL) at both week 1 and week 2 (Figure 2). The FR40 also resulted in significantly lower body weight than the FR20 at both time points (week 1: $p = 0.039$, week 2: $p = 0.012$), while the other restricted groups did not differ significantly. All restricted groups showed significantly reduced body weight at both weeks compared to their respective initial body weights (week: $F_{2,112} = 3.01$, $p < 0.001$). The FR30 group showed a significant ($p = 0.035$) body weight reduction from week 1 to week 2, respectively, while the FR20 ($p = 0.332$) and FR40 ($p = 0.080$) groups showed no further significant variation from week 1 to week 2 restriction time points.

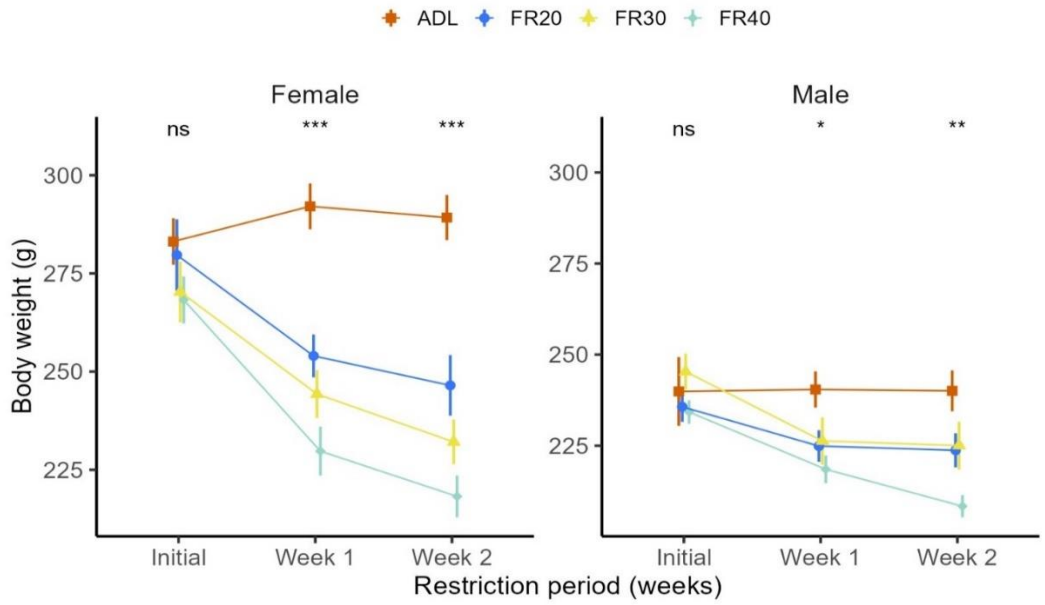

Figure 2. The effect of different feed restriction treatments on body weight of female and male quails at different time points. Data are represented by the mean ± SEM from 8 birds per group. Abbreviations: 'ns', not significant at $p < 0.05$; '*' significantly different at $p < 0.05$; '**' significantly different at $p < 0.01$; '***' significantly different at $p < 0.001$; ADL, *ad libitum*; FR20, 20% restriction; FR30, 30% restriction; FR40, 40% restriction.

In males, all quails from restricted groups showed a reduced trend in body weight compared to the quails from the ADL group (Figure 2). However, only quails from FR40 proved a statistically significant reduction in the first and second weeks (week 1: $p = 0.02$; week 2: $p < 0.001$). When compared to their initial body weight, all male quails from restricted groups showed significantly reduced body weight on both week 1 and week 2, whereas only males from FR40 groups showed further weight loss from week 1 to week 2 ($p = 0.050$).

3.1.2. Sex-specific effects of feed restriction on body weight

Over the period of restriction treatment, there were noticeable differences in the way males and females responded to feed restriction. This was evident from a significant interaction between sex, treatment, and restriction time (treatment × week × sex: $F_{6,112} = 3.01$, $p = 0.009$). In all treatment groups, males exhibited considerably lower initial body weight compared to females. This disparity was consistent throughout the experiment in the ADL fed and moderately restricted (FR20) groups. Nevertheless, the disparity in body weight between males and females ceased to exist by the second week in the FR30 group and by the first week in the FR40 group (Figure 3).

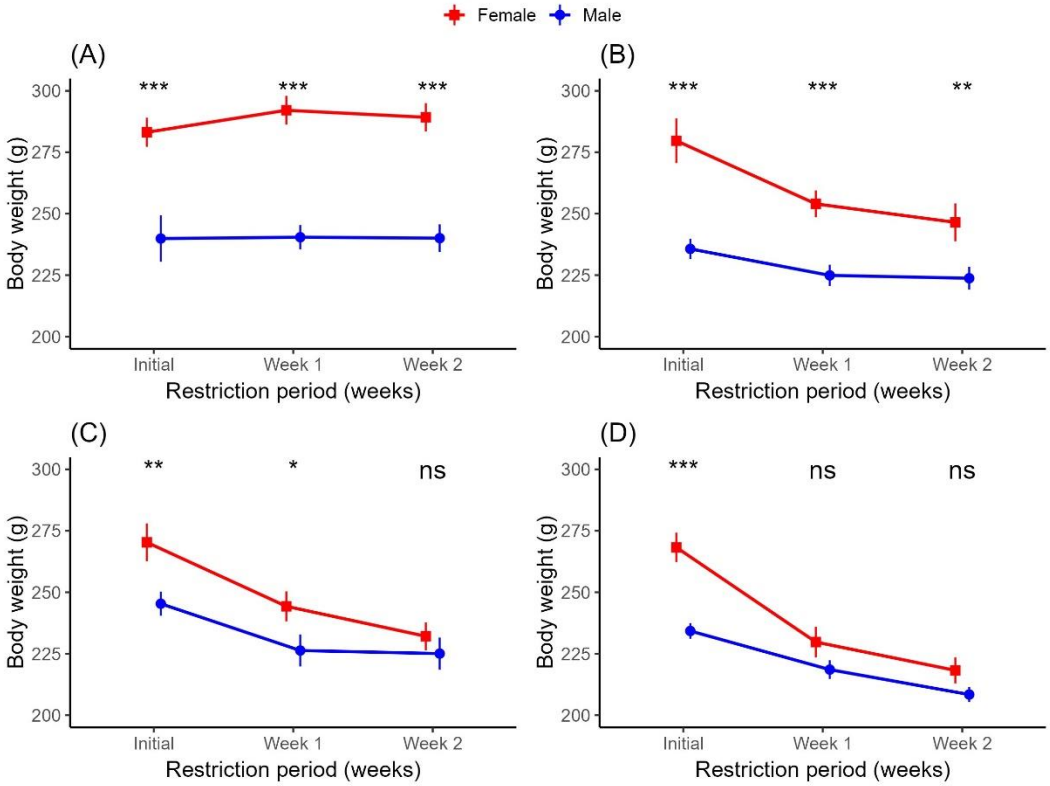

Figure 3. Comparing body weight of female and male Japanese quails in different feed restriction levels across restriction period. Different shaped dots and vertical bars represent the mean ± SEM from 8 birds per group, and data were analysed using ANOVA of linear mixed-effect model. Abbreviations from Figure 2.

As previously reported, FR substantially reduced body weight in both female and male birds, though the effect was more pronounced in females (REDA et al., 2024a; REDA et al., 2024b). Another study on growing quails also reported a similar trend: females subjected to 15% and 30% restriction for three weeks gained less weight than controls, while males were affected only by the 30% restriction (HASSAN et al., 2003). Females also lost body weight more than males in broiler chickens subjected to 30% restriction (TŮMOVÁ et al., 2022). Male and female birds exhibit different resource allocation strategies, driven by their distinct reproductive roles and selective pressures. Females tend to invest more resources in reproduction, while males focus on attracting mates and competing for access to females (MARN et al., 2022). Therefore, the disparate response between males and females to restriction

could arise from females having a greater egg production investment, which could be traded off against their body weight.

3.1.3. Effect of feed restriction on egg production and egg weight

The level of FR and restriction period significantly explained the daily egg-laying probability. Restricted birds decreased their daily egg-laying probability compared to the ADL group (Figure 4A). Additionally, the overall probability of daily egg laying was significantly reduced across the restriction period (p = 0.004). Concerning the total number of eggs laid in the 14 days, treatment showed a significant effect ($F_{3,24}$ = 5.448, p = 0.045). The FR40 group laid significantly fewer eggs than the ADL group (t = 2.86, p = 0.039), while the FR20 and FR30 groups did not show significant variation compared with the ADL (Figure 4B). These results corroborate previous findings (Li et al., 2011; Mahrose et al., 2022), indicating that moderate restrictions had no significant impact on egg production at the expense of egg weight and body weight. Depending on the magnitude of the reduction in feed intake, birds had to face different trade-offs. At a low restriction level (FR20), individuals had to allocate more resources from a limited budget to reproduction, but they could do it without compromising egg size.

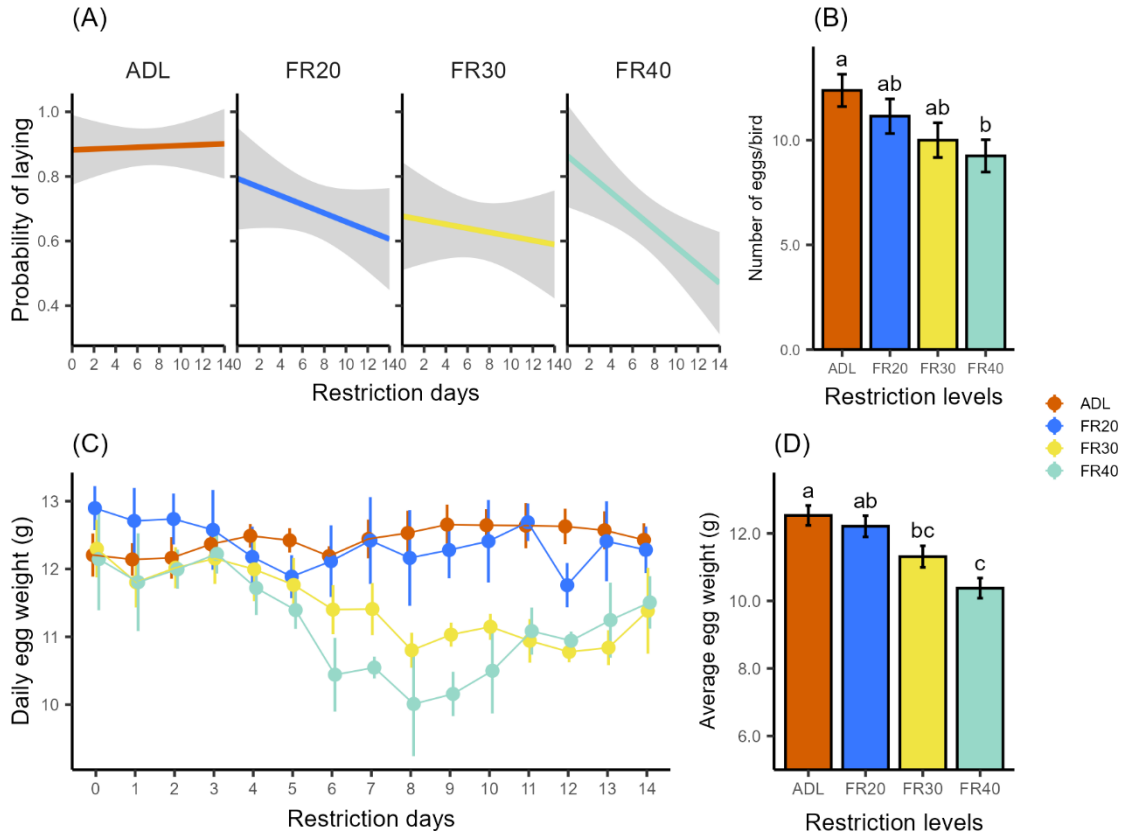

Figure 4. Effect of feed restriction on egg laying probability and egg number and egg weight. (A) Probability of daily egg laying, (B) total number of eggs, (C) daily egg weight (g), (D) average egg weight in the two weeks.

Restriction treatment and restriction period significantly affected egg weight (treatment: $F_{3,25.68}$ = 5.18, p = 0.006; day: $F_{2,309.91}$ = 24.89, p < .0001; treatment × day: $F_{6,309.83}$ = 14.85; p = <.0001). The time-dependent trend indicated that egg weight was significantly

reduced in FR30 and FR40 groups starting from day 5 (Figure 4C). Like the ADL group, egg weight from the FR20 group showed no change throughout the restriction period. On the last days of the experiment, egg weight from the FR30 and FR40 groups showed improvement. Average egg weight in the two weeks restriction period was significantly lower in the FR30 and FR40 groups compared to the ADL fed groups, while the FR40 group still showed significantly lower average egg weight than the FR20 (Figure 4D). These results corroborate previous findings (LI et al., 2011; MAHROSE et al., 2022), indicating that moderate restrictions had no significant impact on egg production at the expense of egg weight and body weight. Depending on the magnitude of the reduction in feed intake, birds had to face different trade-offs. Our analysis of resource allocation strategy (REDA et al., 2024b) supports the idea that birds invest in reproduction at moderate restriction (FR20), whereas they favour self-maintenance at more severe restriction (FR40) (Figure 8).

3.1.4. Effect of feed restriction on expression of hepatic mTOR pathway genes

Feed restriction significantly affected the targeted mTOR pathway genes, with a slight difference in females and males (Figures 5 and 6). Restriction treatment affected *GHR* gene expression ($p < 0.001$). Intriguingly, GHR showed significantly increasing and marginally increasing expression in females and males, respectively. Both the FR3 and FR40 showed significantly higher GHR expression than the ADL and FR20 in females. In males, both showed the same trend but no statistical difference among treatment groups (Figure 5A). Our result contradicted the previously reported findings in mammals and fishes, where nutritional deficit reduces *GHR* expression (DAUNCEY et al., 1994; WALOCK et al., 2014).

FR significantly decreased *IGF1* gene expression in both sexes ($p < 0.001$). All the restricted groups had significantly lower *IGF1* gene expression than the ADL-fed controls (Figure 5B). Despite a similar trend, *IGF1R* gene expression remained statistically undistinguishable among the four groups in females, while in males the FR40 showed significant reduction (Figure 5C). Although previous studies on birds are scarce, 20% restriction in normal mice showed no effect on *IGF1* gene expression (ROCHA et al., 2007), while 30% restriction increased *IGF1* expression (MASTERNAK et al., 2004), which is contrary to our finding on quails.

The *mTOR* gene expression showed a significant and gradual decrease across the FR levels ($p < 0.001$; Figure 5D). Although the specific mechanisms underlying the effect of FR on *mTOR* gene expression have not been thoroughly investigated, our findings shed light on the similarity between the effect of FR on gene expression and the previously studied effect on the abundance of activated mTORC1 (TULSIAN et al., 2018; VELINGKAAR et al., 2020). Interestingly, the expression of *RPS6K1* was increased in response to the treatments in both sexes ($p < 0.001$), showing an increasing trend with the severity of the restrictions in females (Figure 5E). We expected that downregulation of *mTOR* expression would have negative consequence on the expression of *RPS6K1* and subsequently reduce body weight (BAE et al., 2012). However, the result was contrary to our assumptions (REDA et al., 2024a; REDA et al., 2024b). Previous studies have reported reduced *RPS6K1* expression in the liver of overfed geese (HAN et al., 2015).

Furthermore, the restriction treatment significantly increased autophagy genes ($p < 0.001$). The *ATG9A* showed significantly higher expression in the FR40 compared to the other

groups in females, while in males, all the restricted groups showed significantly higher expression levels compared to the ADL group (Figure 5F). All restricted groups showed higher *ATG5* gene expression compared to the ADL groups; though treatments showed no statistical variation in the male groups (Figure 5G). The effect of FR is consistent with previous studies from other organisms (YANG et al., 2022). Under scarce resources (FR), the downregulation of mTORC1 allows the nuclear localisation and activity of Transcription Factor EB (TFEB) and upregulates autophagosome formation through coordinating the expression of genes involved in autophagy such as *ATG9A* and *ATG5* (MARTINA et al., 2012).

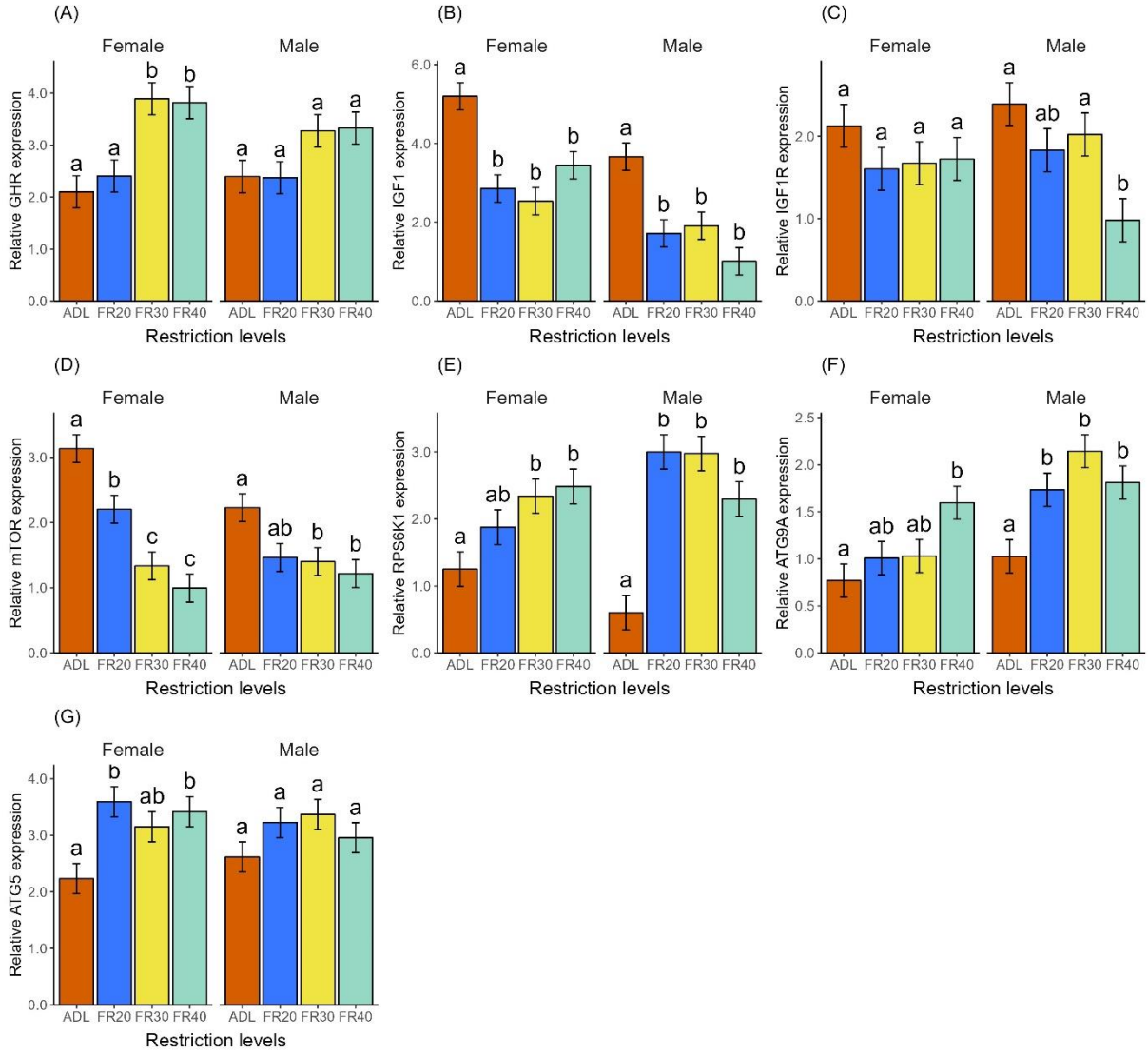

Figure 5. Effect of feed restriction on expression of certain mTOR pathways genes in female and male Japanese quails. (A) growth hormone receptor (*GHR*), (B) insulin-like growth factor 1 (*IGF1*), (C) insulin-like growth factor 1 receptor (*IGF1R*), (D) mechanistic target of rapamycin (*mTOR*), (E) ribosomal protein S6 kinase 1 (*RPS6K1*), (F) autophagy-related 9A (*ATG9A*), (G) autophagy-related 5 (*ATG5*). Relative gene expression was analysed in log fold change. Data are represented by the mean ± SEM from 8 birds per group. The emmeans adjusted for Tukey was used as *post hoc* test with $p < 0.05$ significance level. Means followed by a common letter are not significantly different at $p < 0.05$. Abbreviations: ADL, *ad libitum*; FR20, 20% restriction; FR30, 30% restriction; FR40, 40% restriction.

3.1.5. Sex difference in hepatic gene expression

Sex has a significant effect on the expression of most of the measured mTOR pathway genes. The *GHR* showed no significant variation between the female and male groups, and the trend of the effect of FR treatment is similar, though it was intensified in females (Figure 6A). Females showed higher *IGF1* expression at all restriction levels, and the response to FR was similar, though it showed slight divergence at the FR40 treatment (Figure 6B). Furthermore, the pattern of change in *IGF1R* gene expression was similar in both sexes up to the FR30 level, although males showed a higher reduction at the FR40 level (Figure 6C). Although little is known about the sex differences in the impact of FR on *IGF1* gene expression, previous studies examining circulating IGF-1 have suggested that both the level and the influence of IGF-1 exhibit sex-based differences in mammals (ASHPOLE et al., 2017) and in birds (BACON et al., 1993; TÓTH et al., 2022; TÓTH et al., 2018).

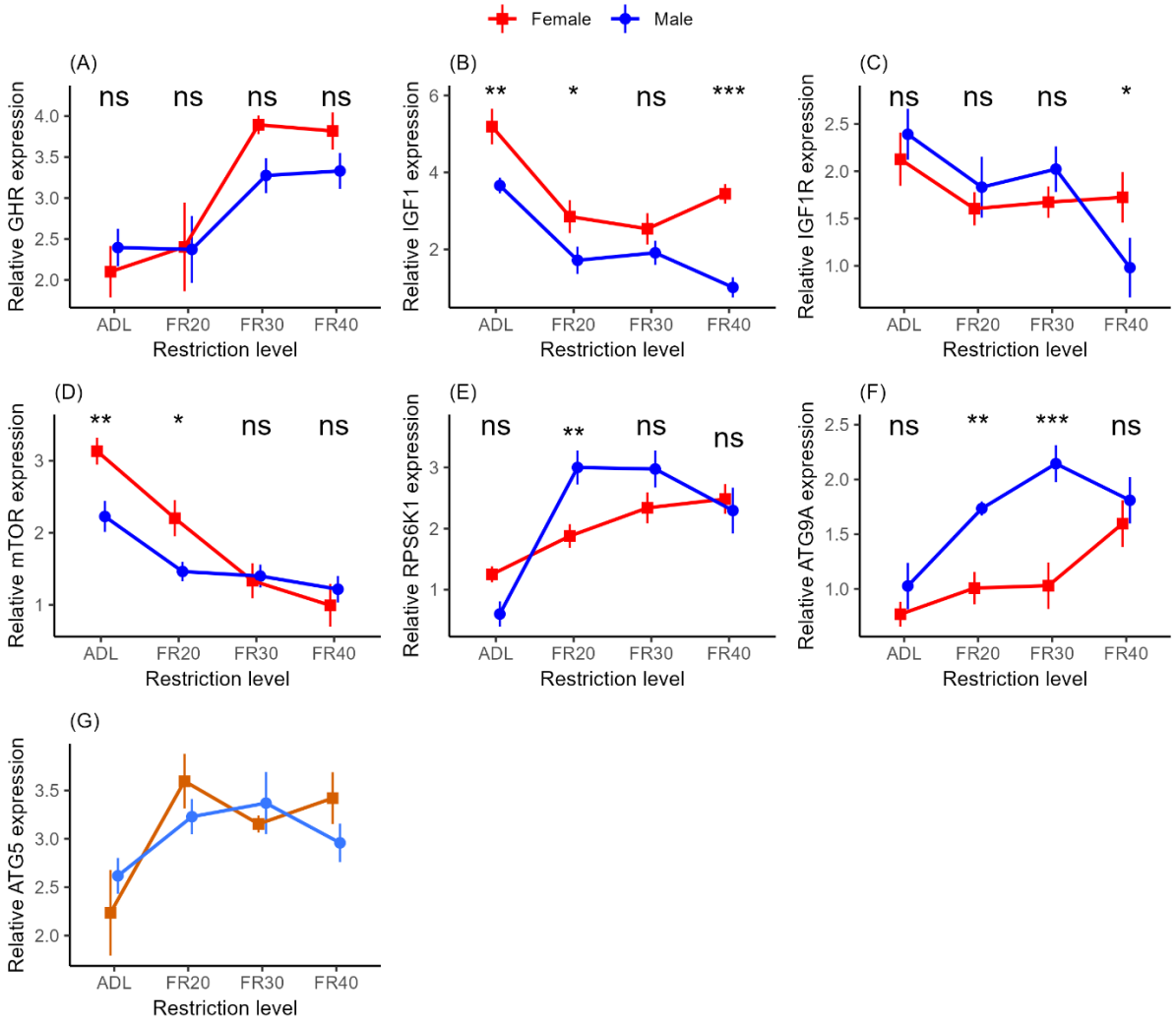

Figure 6. Sex-specific effects of dietary restriction in Japanese quails. Dots and vertical bars represent the mean ± SEM from 8 birds per group. Abbreviations from Figure 5.

Females showed higher *mTOR* expression than the males in the ADL and FR20 groups, while no significant difference was observed between the sexes in the severely restricted groups (Figure 6D). This pattern in response to FR is slightly different in females and males, whereas in females, the reduction is intensified with increased restriction levels (REDA et al., 2024a).

This indicates that similar to body weight (Figure 3), the effect of FR was stronger on female birds than on males, which may be due to physiological, morphological, and hormonal differences and reproduction strategies between the sexes (BENNETT-KEKI et al., 2023; PIEKARSKI et al., 2014). Females exhibited marginally higher *RPS6K1* expression in the ADL fed group, while lower in the FR20 and FR30 groups with no difference on FR40 groups (Figure 6E).

While there was no sexual difference in *ATG5* gene expression (Figure 6G), *ATG9A* showed lower expression in females than males (Figure 6F). The pattern of changes across restriction levels was different: males showed a pronounced increase at all restriction levels, while females showed a significant increase only at the severely restricted level, resulting in significant sex differences in the FR20 and FR30 groups (Figure 6F). At the expense of anabolic progressions and stress, FR is critical in maintaining pathways required to retain cellular function. The lower *mTOR* expression in both males and females may have a significant contribution to the upregulation of *ATG9A* expression. However, the pronounced increase in *ATG9A* expression, specifically in males, may contribute to the rapid recycling of cytoplasmic waste and supply it as energy and amino acids for other cellular activities. The process could potentially contribute to keeping the pace of *mTOR* expression through a positive feedback mechanism, as we observed sustained expression levels across all restriction groups and relatively lower body weight loss in males.

3.1.6. Overall associations using principal components

To disentangle the complex interplay of liver gene expression, we employed principal component analysis (PCA). The PCA indicated that the first two principal components have eigenvalues greater than 1 and thus were retained for further regression analysis. These two components explained 60.2% of the total variance.

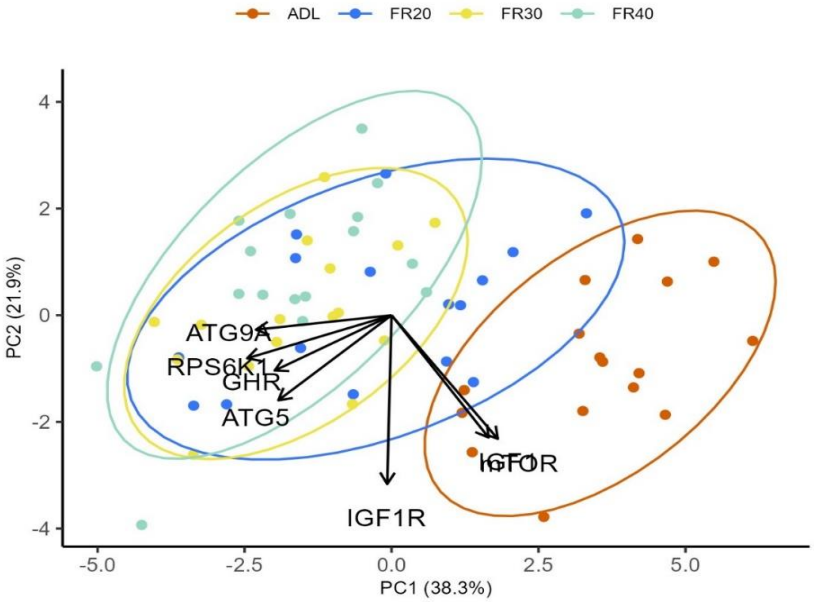

Figure 7. A biplot of PCA for the liver gene expression and Body weight in Japanese quails treated with different dietary restriction levels for two weeks. Clustering is based on dietary restriction levels and a dimensional indication of genes in line with the restriction levels. The ellipsoids are defined by the treatment groups. *IGF1* and *mTOR* are overlapped on the ellipse.

The PC analysis revealed that expression of all genes except *IGF1R* significantly contributed to PC1, influencing variation in different directions, while expression of *IGF1R* predominantly shaped PC2 (Figure 7). The biplot indicated a clustering of liver *mTOR* and *IGF1* expression around the control treatment and had positive influences on the PC1, while *RPS6K1*, *GHR* and autophagy genes expression clustered around the restricted treatments and showed negative influences on PC1 (Figure 7). Finally, PC1 significantly and positively explained body weight ($p < 0.001$), egg number ($p = 0.006$), and egg weight ($p = 0.002$), whereas PC2 significantly and negatively explained body weight ($p < 0.001$), while noticeably influencing egg weight ($p = 0.077$).

3.1.7. Resource allocation strategy

Based on resource availability, birds allocate resources toward body weight, reproduction, or self-maintenance (Figure 8). In week 1, the FR20 and FR30 groups increased relative reproductive investment (Figure 8C), while the FR40 re-allocated resources to body weight. The ADL controls did not deviate significantly from zero re-allocation, and all restriction groups had a similar strategy by the end of the second week (Figure 8D), where resources are re-allocated towards reproduction. At the end of the experiment, individual variation in allocation strategy was related to *mTOR* expression ($t = -3.118$, $p = 0.008$).

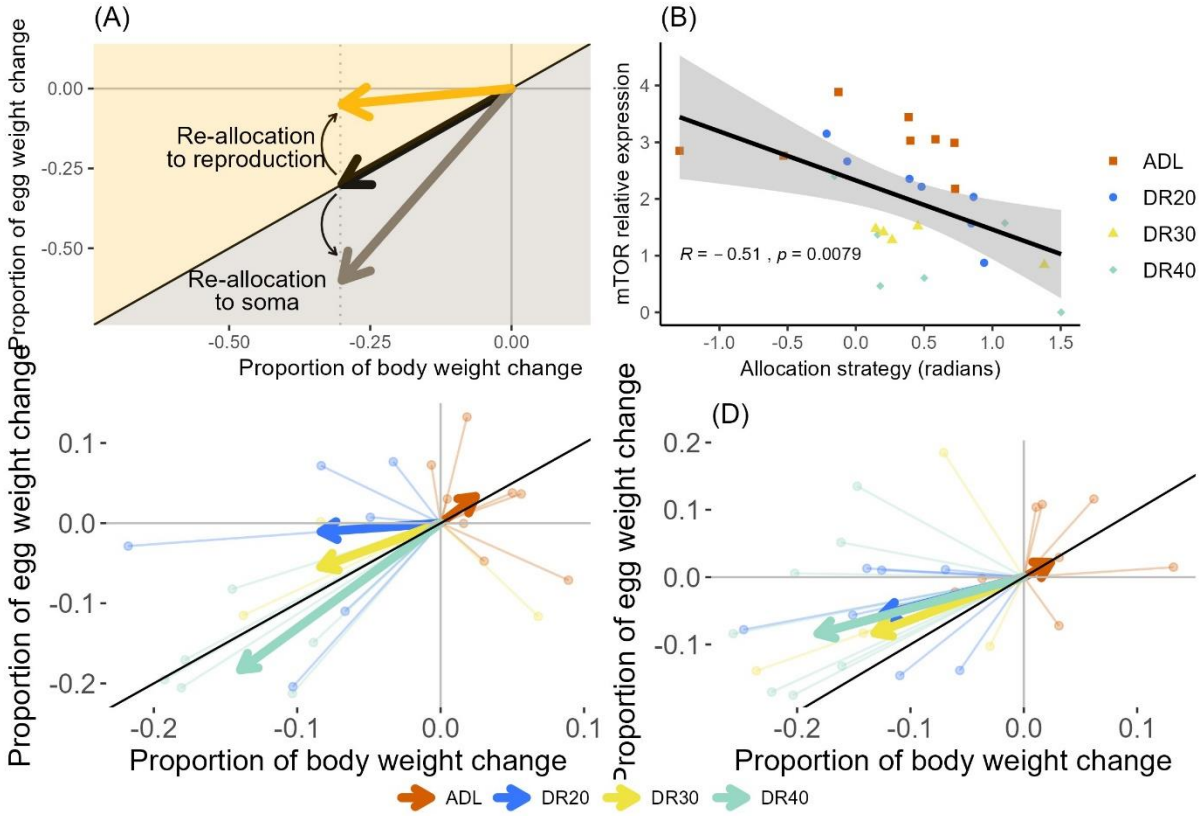

Figure 8. Effect of feed restriction on resource allocation decision. (A) A conceptual figure illustrating resource allocation decisions. The x- and y-axis show the proportional change in body weight and egg weight, respectively, during the experimental period (compared to the pre-treatment body weight and egg weight, respectively, thus, all vectors start from the origo). The angle of the vectors (radians) illustrates the allocation strategy. The solid black line shows

where y = x, i.e. when there is no re-allocation: a unit change in body weight is accompanied by the same amount of correction in egg weight. The space above the identity line indicates reproductive re-allocation: in response to a change of available resources, the individual allocates more to reproduction at the cost of self-maintenance. In contrast, the space under the identity line indicates re-allocation towards self-maintenance because the same change in body weight is associated with a proportionally larger reduction in reproductive investment. (B) Individual allocation strategy is related to mTOR expression. (C-D) The thin lines show individual data points, the thick vectors show the median response of the respective treatment group. In the first week (C), FR20 and FR30 groups invest more in reproduction under a loss of body weight, while FR40 group tends to re-allocate resources to self-maintenance. (D) Over two weeks, all restricted groups show reproductive re-allocation, albeit to different degrees. The longer vectors in the FR40 also illustrate that this treatment imposed a higher cost than in the other two groups (where the lengths of the vectors are similar). The ADL control group remains unchanged over time.

Irrespective of the treatment, individuals with lower *mTOR* values were more likely to invest proportionally more in reproduction than individuals with higher *mTOR* expression (Figure 8B). The resource re-allocation hypothesis suggests that organisms shift resources between reproduction and somatic maintenance when faced with limited resources (MOATT et al., 2020; REGAN et al., 2020), a process mediated by the mTOR pathway. When nutrition is limited, mTORC1 activity is downregulated, triggering alternative pathways (JOHNSON et al., 2013; LI et al., 2015). In our study, the higher resource re-allocation to reproduction at a lower individual *mTOR* expression (Figure 8B) may have triggered upregulation of the autophagy pathway and recycling of damaged cell contents as an energy substitution for the nutrient deficit (ADLER and BONDURIANSKY, 2014; CHUNG and CHUNG, 2019).

3.1.8. Effect of feed restriction and sex on IGF-1 levels

Dietary restriction and its interaction with sex and the restriction period (week) did not affect plasma IGF-1 levels (treatment × week × sex: $F_{6,111.19} = 1.53$, $p = 0.174$). Females exhibited higher plasma IGF-1 levels (64.7%) than males at all restriction levels (sex: $F_{1,49} = 15.67$, $p < 0.001$, Figure 9). Our findings are consistent with previous reports across different species, suggesting that IGF-1 exhibits sex-specific expression (ASHPOLE et al., 2017; BAÉZA et al., 2001). Our data also further revealed repeatable individual variation in IGF-1 levels across the restriction period with high repeatability (repeatability: 0.81). Despite the reducing effect of FR on body weight and *IGF1* gene expression in our study (REDA et al., 2024a; REDA et al., 2024b) and on IGF-1 in human, fly and worm models (ex. FONTANA et al., 2008; REGAN et al., 2020), we did not observe any directional change in circulating IGF-1 levels at any restriction levels in our adult quails (REDA et al., 2024c). The observed unmatched response of IGF1 gene expression and circulating IGF-1 levels might be due to regulatory mechanisms stretched from transcription until posttranslational modification (BUCCITELLI and SELBACH, 2020). Adult animals may boost local tissue production of IGF-1 to play autocrine and paracrine functions (LEE and KIM, 2018; WANG et al., 2021). For example, females produce local IGF-1 in the ovary, regulating follicular growth in an autocrine manner without requiring circulating endocrine IGF-1 (BERNARDI et al., 2021; ONAGBESAN et al., 2009).

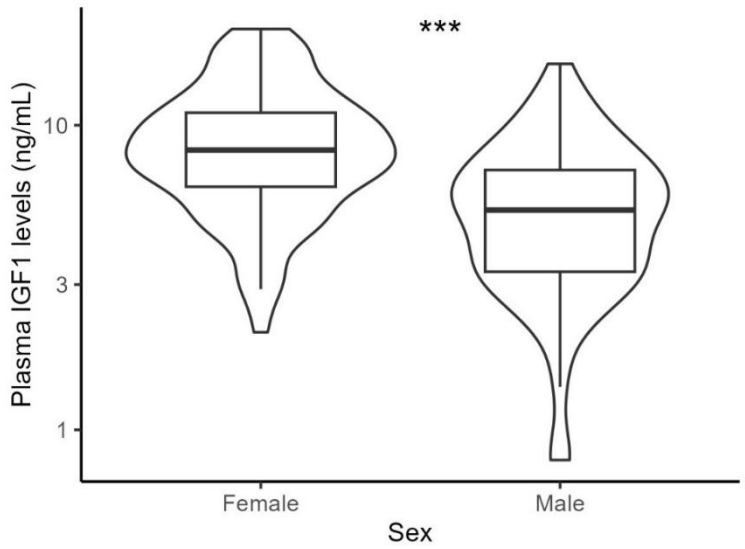

Figure 9. Sex difference on plasma IGF-1 levels. Data were analysed from 32 birds per sex group in three time points. Data were collected on day 0 (initial), week 1 and week 2 periods of the experiment.

3.1.9. Effect of feed restriction on plasm triglyceride levels

Treatment and its interaction with the restriction period showed a significant effect (treatment: $F_{3,52} = 11.45$, $p < 0.001$; treatment × week: $F_{6,120} = 4.10$, $p = 0.001$). Comparing treatment groups at different time points, all the restricted female groups showed significantly lower triglyceride levels than the control group in week 2 (Figure 10). However, in the first treatment week, FR20 and FR40 treatments imposed a reduction in the levels of plasma triglycerides, while FR30 treatment showed significant reduction compared to the ADL-fed group ($p = 0.020$). In males, all restricted groups showed significantly lower plasma triglyceride levels than the ADL group in weeks 1 and 2 (Figure 10). Previous evidence from different species suggests that dietary restriction can reduce triglyceride levels (TEOFILOVIĆ et al., 2022; ZHAN et al., 2007). Nutritional limitations forced birds to use body fat reserves and, in the meantime, be exposed to a substantial decrease in circulating triglyceride levels (LANDYS et al., 2005). Furthermore, plasma triglycerides are biomarkers of obesity and cardiovascular disease (LYU et al., 2022). In broiler chickens, death due to cardiovascular disease is a common cause of loss on farms (CHERIAN, 2007), and dietary restriction is suggested as a primary solution to prevent such morbidity (OLKOWSKI, 2007).

Additionally, we tested the effect of restriction time and found that in both females and males, all restricted groups showed reduced value compared to their baseline plasma triglyceride levels at both week 1 and week 2 (week: $F_{2,120} = 46.48$, $p < 0.001$; Figure 10). In case of the trend from week 1 to week 2, FR30 and FR40 of female groups showed a significant reduction in week 2 (FR30: $p = 0.027$. FR40: $p = 0.017$), while all other groups in both sexes showed no significant reduction. Furthermore, sex has a significant effect on plasma triglyceride levels at all-time points (sex: $F_{1,52} = 1305.07$, $p < 0.001$), with females on average, having more than six-fold higher triglyceride levels than males (Figure 10). The observed difference in sexual trends may be attributed to reproductive investment. Females might compromise their weight to maintain triglyceride levels, ensuring a steady supply for egg

production, as de novo triglycerides are mostly deposited into eggs (CHERIAN, 2015; VANDERKIST et al., 2001).

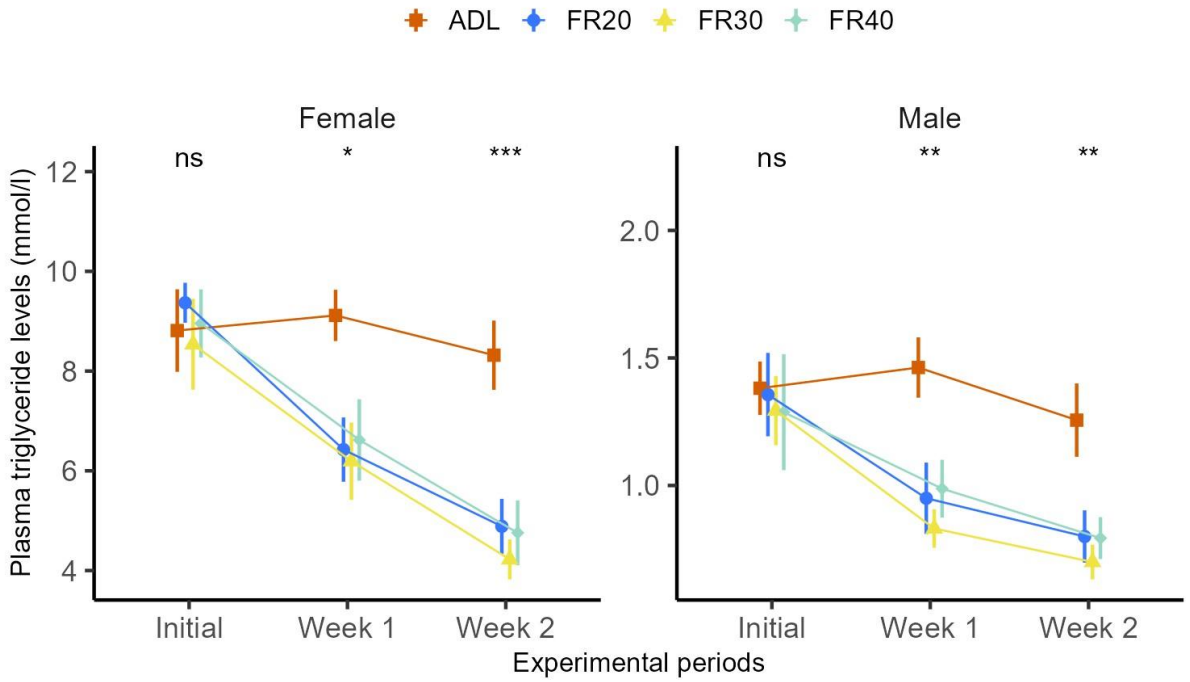

Figure 10. Effect of feed restriction on plasma levels of triglycerides across the two weeks restriction period in females and males Japanese quails. Data are represented by the mean ± SEM and were statistically analysed from the linear mixed-effect model. Note the different scales for the sexes, reflecting the significant disparity in female and male triglyceride values.

3.2. Experiment 2: Effect of unpredictable and restricted feeding on expression of mTOR pathway genes and production traits in Japanese quails

3.2.1. Effect of unpredictable feeding on body weight

Treatment, sex, and time had a significant interaction effect on body weight ($F_{6,126}$ = 2.95, p = 0.10). The result revealed that unpredictable feeding (UNPR) has sex-specific effects. In females, although the control and unpredictably fed groups had equivalent average feed intake at the end of the experiment relative to their initial feed intake, unpredictable feeding significantly reduced body weight compared to the control group on day 16 (p = 0.029, Figure 11). In males, unpredictability feeding did not affect body weight at all-time points. The 40% restricted group (FR40) exhibited lower body weight than the control group at all treatment time points in females (day 4: p = 0.015; day 8: p < 0.001; day 16: p < 0.001). In FR40 males, a significant reduction in body weight was observed only on day 16 (p = 0.019). Furthermore, when comparing the UNPR and FR40 treatments, the FR40 treatment showed significantly lower body weight on day 16 only (p = 0.029) in females, while it tended to be lower on day 8 (p = 0.092). In males, the FR40 treatment resulted in lower body weight than the UNPR group on day 16 (p = 0.006).

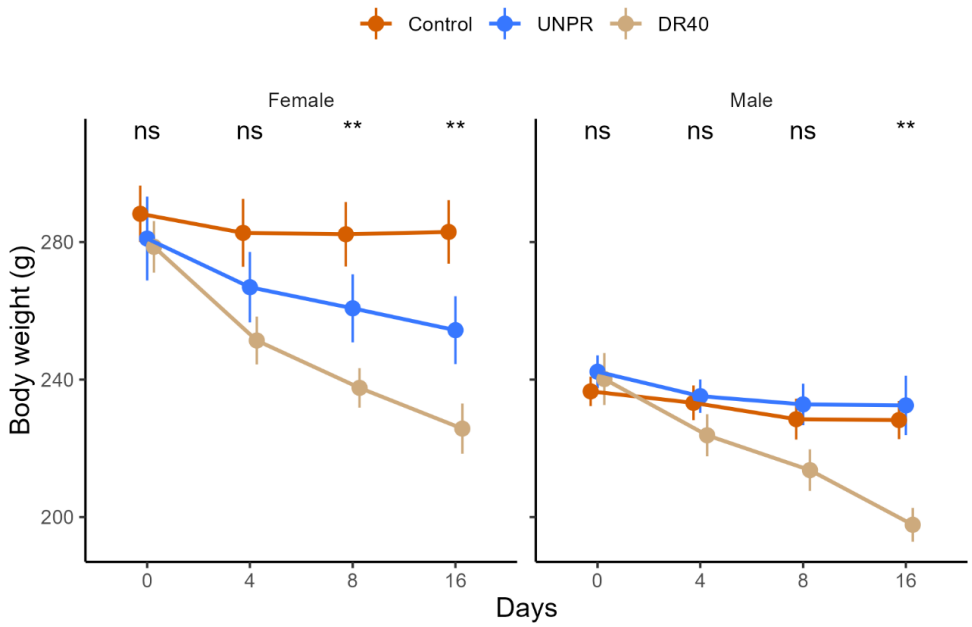

Figure 11. Effect of feed treatment on body weight of female and male quails across 16 days treatment period**.** Data are represented by the mean ± SEM from 8 birds per group. Abbreviations: 'ns', not significant at $p < 0.05$; '*' significantly different at $p < 0.05$; '**' significantly different at $p < 0.01$; Control, birds received equals to their daily feed intake; UNPR, unpredictable feeding; FR40: 40% restriction.

3.2.2. Effect of unpredictable feeding on egg production

The temporal variation in feed supply (UNPR) had no significant effect on the total number of eggs laid during the 16-day treatment period ($p = 0.783$, Figure 12B) or the probability of daily egg laying ($p = 0.856$, 17A). However, the 40% restriction (FR40) treatment significantly reduced both the total number of eggs ($p < 0.001$, Figure 12B) and the probability of daily egg laying ($p < 0.001$, Figure 12A). Furthermore, the UNPR treatment did not significantly affect egg weight, nor did it interact with linear ($p = 0.656$) and quadratic ($p = 0.508$) terms of treatment time. However, the FR40 significantly reduced the average egg weight in the 16 treatment days ($p = 0.007$, Figure 12D), and demonstrated a significant interaction with both the linear ($p < 0.001$) and quadratic ($p < 0.001$) terms of restriction time (Figure 12C). Even though female birds in the unpredictable feeding showed reduced body weight, all egg parameters were kept similar to the control group. Therefore, this outcome was contrary to our hypothesis.

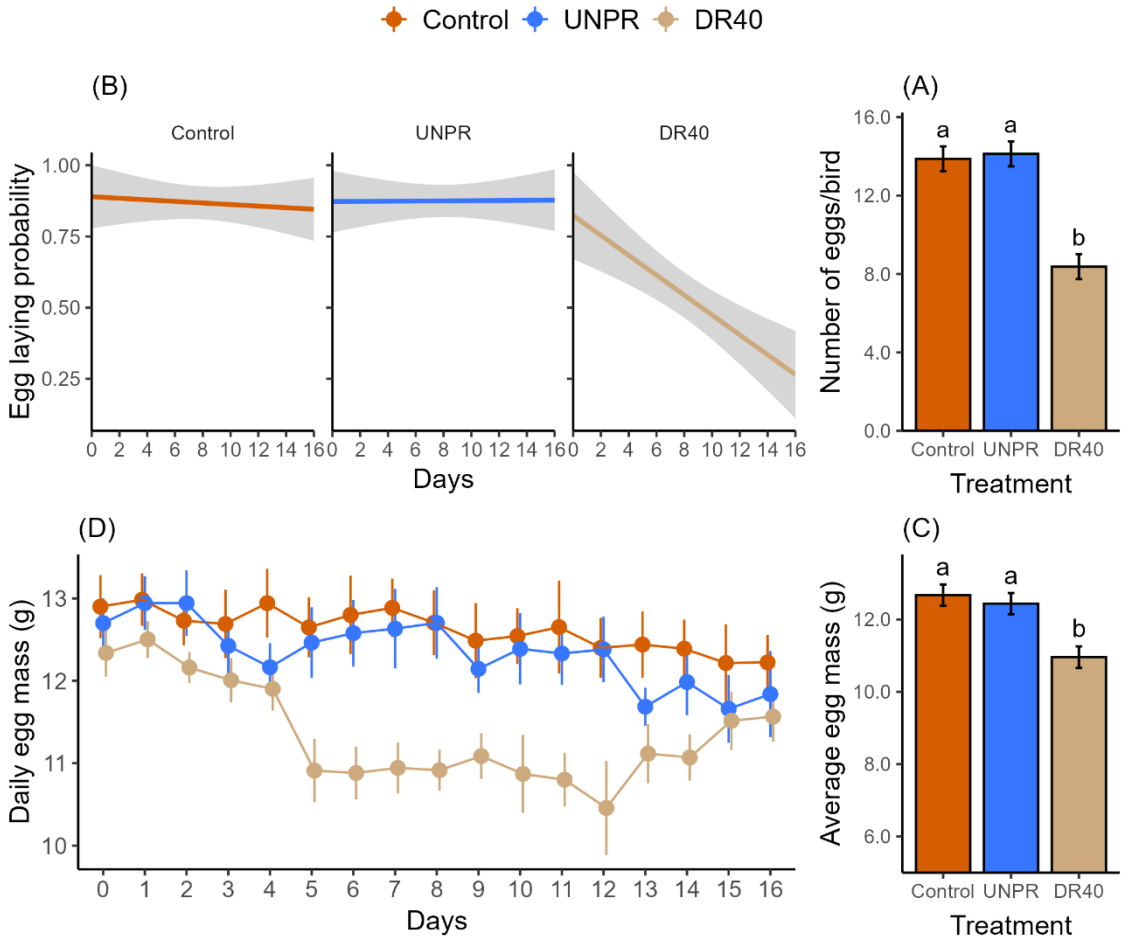

Figure 12. Effect of unpredictable feeding on egg number and egg weight. Abbreviations from Figure 11.

3.2.3. Effect of unpredictable feeding on expression of hepatic mTOR pathway genes

Dietary treatment showed a significant effect on the expression of nutrient-sensing genes in both liver and muscle tissues of both sexes (Figures 13 and 14). Compared to the control group, unpredictable feeding (UNPR) and 40% restricted (FR40) groups showed a decreasing trend in liver *IGF1* gene expression in females (Figure 13A). While maintaining a similar pattern of changes, there was no significant treatment effect on the muscle *IGF1* expression of females or both tissues of males (Figures 13A and 14A). In the liver, neither of the treatments showed a significant effect on *IGF1R* in both male and female groups (Figure 13B). In contrast, in the muscle both, UNPR and FR40 significantly reduced *IGF1R* gene expression in females, but there was no effect in male groups (Figure 14B). Liver *FOXO1* gene expression showed an increasing trend in both the UNPR and FR40 groups compared to the control group, with significantly higher expression observed in the FR40 group (p = 0.046, Figure 13C). In the muscle, FR40 in the female groups showed higher *FOXO1* gene expression compared to both control (0.058) and UNPR (0.014) (Figure 14C).

The UNPR and FR40 showed an observable reduction in *mTOR* gene expression in females compared to the control group in both liver (UNPR: p = 0.021, FR40: p = 0.033, Figure 13D) and muscle (UNPR: p = 0.081, FR40: p = 0.003, Figure 14D). Although showing a similar

pattern, neither of the treatments showed a significant impact on both tissues in males. Additionally, liver *RPS6K1* gene expression showed variable response to treatments on females and males, with UNPR showing higher expression in females and FR40 showing higher expression in males (Figure 13E). In the muscle tissue, the trend due to dietary manipulation has changed, showing a reducing trend, although it was not statistically significant (Figure 149E). In the liver, *4EBP1* showed no significant effect of treatments in both females and males (Figure 13F). In the muscle, FR40 treatment increased *4EBP1* gene expression compared to both control and UNPR groups in both females and males, although the increase was statistically marginal (Figure 14F).

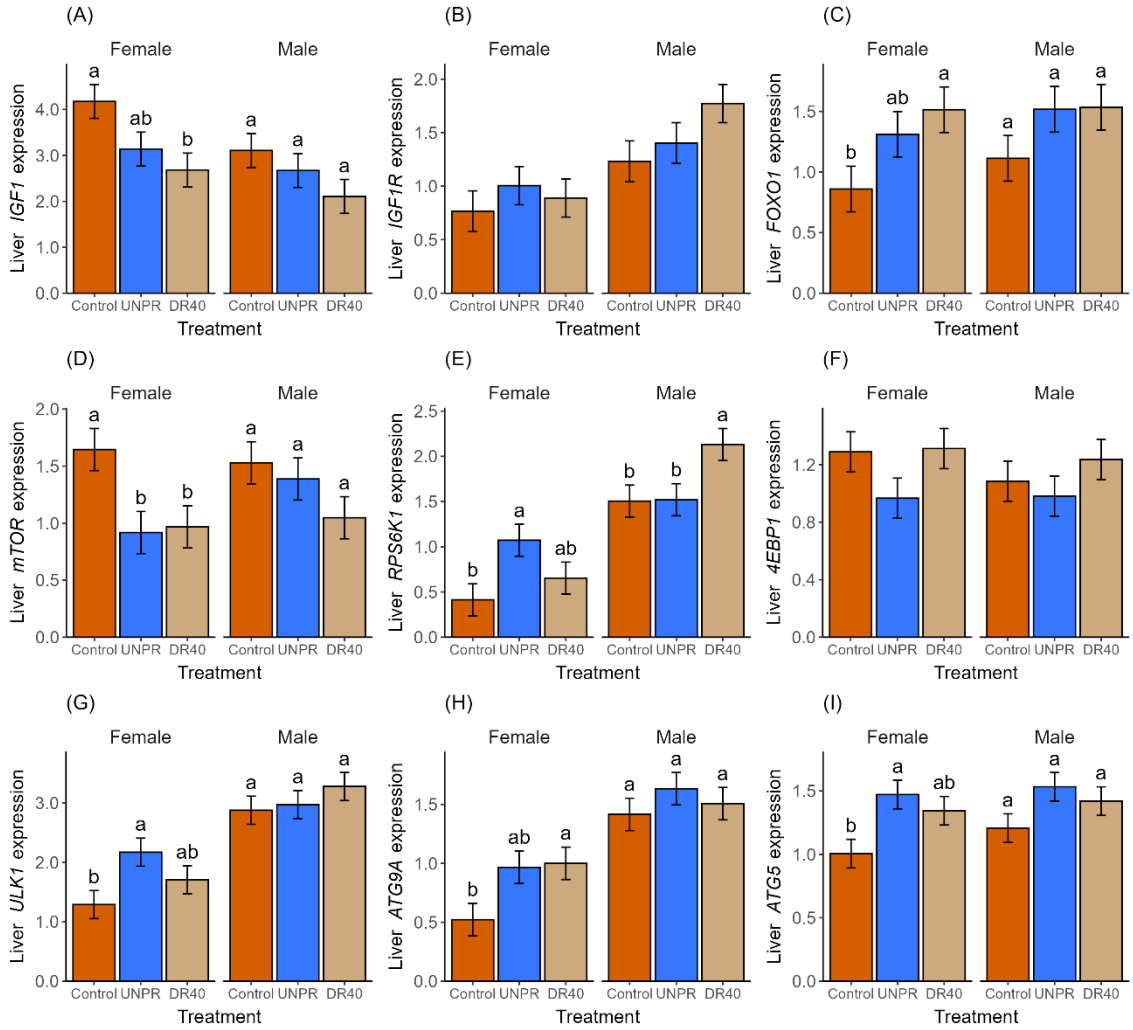

Figure 13. Effect of variable dietary treatment on expression of liver nutrient sensing genes. (B) *IGF1*, insulin-like growth factor 1; (B) *IGF1R*, insulin-like growth factor 1 receptor; (C) *FOXO1*, forkhead box O1; (D) *mTOR*, mechanistic target of rapamycin; (E) *RPS6K1*, ribosomal protein S6 kinase 1; (F) *4EBP1*, Eukaryotic translation initiation factor 4E-binding protein 1; (G) *ULK1*, Unc-51 like autophagy activating kinase 1; (H) *ATG9A*, autophagy-related gene-9A; (I) *ATG5*, autophagy-related 5. Data are represented by the mean ± SE from 8 birds per group. Means followed by a common letter with in sex are not significantly different at $p < 0.05$. Abbreviations: Control, birds received equals to their daily feed intake; UNPR, unpredictable feeding; FR40: 40% restriction.

Moreover, the control group exhibited lower expression values of liver *ULK1*, *ATG9A*, and *ATG5* gene expression in females while showing a similar trend but no significant variation in males (Figure 13G,H,I). In the muscle, while *ULK1* gene expression showed no change across all treatments in both sexes (Figure 14G), *ATG9A* showed an increasing trend in UNPR and FR40 groups with significant differences in females (Figure 14H). The weak response to the dietary treatment in males' body weight was coupled with the weak response in gene expression.

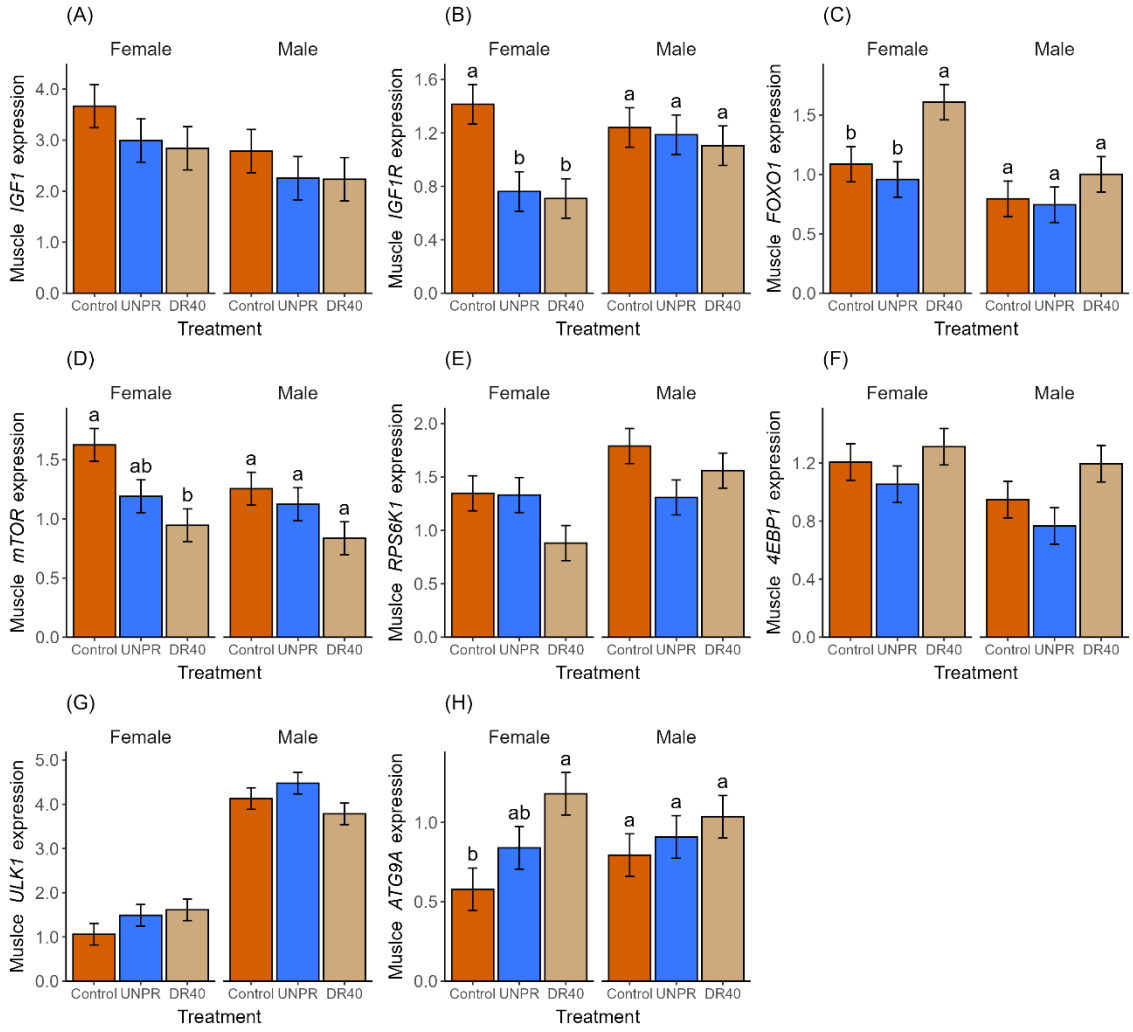

Figure 14. Effect of unpredictable dietary availability on expression of mTOR pathway genes in the muscle. Descriptions and Abbreviations from Figure 13

3.3. Experiment 3: Effect of feed restriction, energy restriction or protein restriction and amino acid supplementation on expression of mTOR pathway genes and body weight in Japanese quail

3.3.1. Effect of Leucine and methionine supplementation on top of restricted feeding

3.3.1.1. Effect on body weight

Treatment and its interaction with time significantly affected body weight ($F_{8,70} = 6.37$, $p < 0.001$). Initially, birds had similar body weights, and during the experiment, they showed

treatment-dependent divergent growth rates (Figure 15). At the end of the first week (day 7), treatments did not show a significant difference (Figure 15A). However, at the end of the second week, the FR and FR+M groups showed significantly lower body weight than the control group (FR: $p = 0.005$; FR+M: $p = 0.048$). The FR+L and FR+ML groups showed no significant difference from the control group. The restricted and all the amino acid-supplemented groups did not show significant differences among each other.

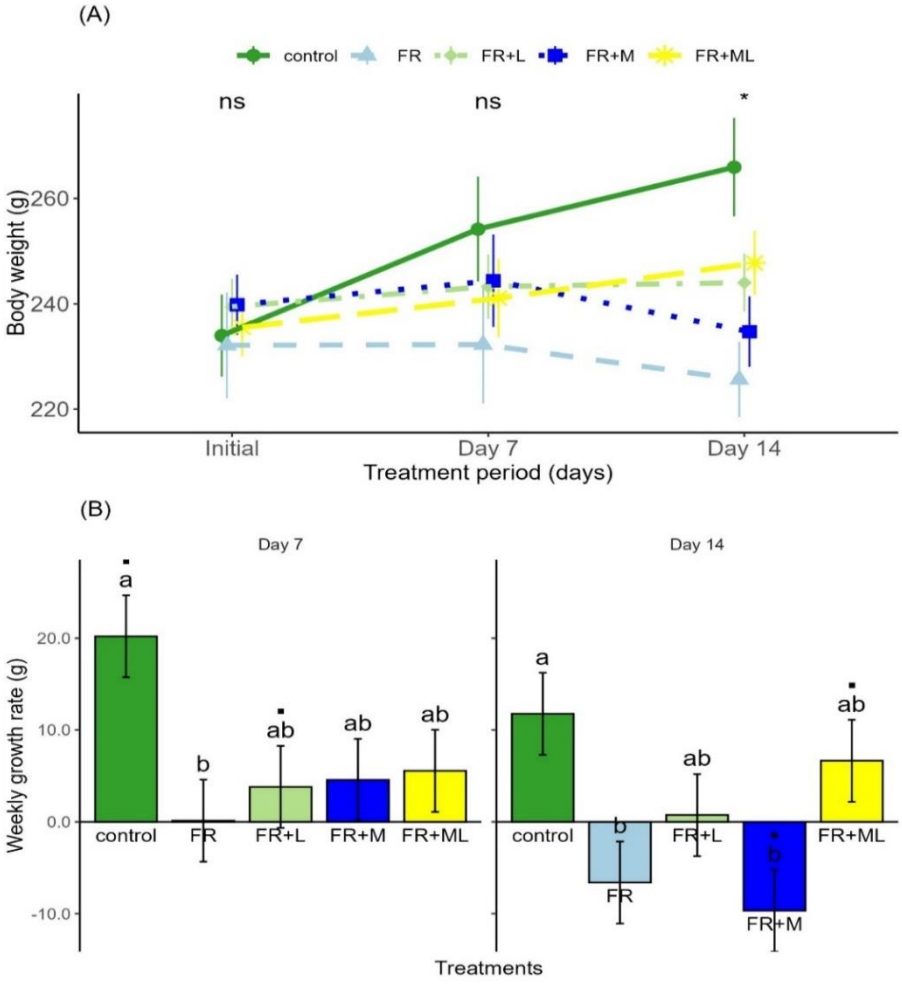

Figure 15. Effect of amino acid supplementation on top of restricted feeding on (A) body weight (B) body weight gain of Japanese quails. (A) Data are represented by the mean ± SEM from 8 birds per group across three time points. Abbreviations: 'ns', not significant at $p < 0.05$; '*' significantly different at $p < 0.05$. (B) Weekly body weight gain was determined by subtracting Day 1 weight from initial weight and Day 14 weight from Day 7 weight. Data are represented by the mean ± SEM from 8 birds per group. The emmeans adjusted for Tukey was used as *post hoc* test with $p < 0.05$ significance level. Means followed by a common letter are not significantly different at $p < 0.05$. Abbreviations: Control, birds received full of their daily feed intake; FR, 20% restriction; FR+L, 20% leucine supplemented on top of 20% restriction; FR+M, 20% leucine supplemented on top of 20% restriction; FR+ML, 20% methionine and leucine supplemented on top of 20% restriction

We also analysed the trend of body weight across treatment time points. Birds in the control treatment significantly increased their body weight compared to their initial body weight in the first week ($p < 0.001$) compared to the initial body weight. Additionally, there was a significant increase from the first to the second week ($p = 0.029$). The FR and FR+M

groups did not show significant change across all time points. The FR+L group showed a marginal increase in the second week ($p = 0.086$). Compared to the initial body weight, the FR+ML group significantly increased body weight in the second week while showing no significant change in the first week.

Furthermore, we compared the weekly body weight gain in the first and second weeks by subtracting the first week body weight from the initial and the second week body weight from the first week body weight (Figure 15B). Therefore, we found that in the first week, the control group showed significantly higher body weight gain (20.20 g) than the FR (0.12 g; $p = 0.018$). The body weight gain on the second week was significantly lower on the FR (-6.61 g; $p = 0.034$) and FR+M (-9.67 g; $p = 0.010$) compared to the control group (11.76 g). At this time point, the FR+ML showed higher weekly body weight gain (6.65 g) than the FR+M ($p = 0.084$; Figure 15B). The result suggested that supplementation with 20% of the recommended amount of the essential amino acids under dietary limitations could slightly mitigate the effect of nutritional scarcity on body weight.

3.3.1.2. Effect of amino acid supplementation on hepatic gene expression

Treatments did not show a significant effect on liver *GHR* gene expression ($p = 0.127$); there is a reducing trend in response to FR (Figure 16A). However, with the current effect size ($f = 0.495$), treatments could have a significant effect on *GHR* gene expression at a slightly higher sample size ($n = 11$) with 0.8 statistical power. The amino acid-supplemented groups showed an insignificant but increasing trend compared to the FR group. Treatment had a significant effect on liver *IGF1* gene expression ($p = 0.045$). The FR treatment showed significantly lower *IGF1* gene expression than the control group ($p = 0.025$). Interestingly, the amino acid-treated groups had no significant difference with either the control or the FR groups (Figure 16B). All the supplemented groups showed an increasing trend compared to the unsupplemented restricted. Treatments had no significant effect on liver *IGF1R* gene expression ($p = 0.665$; Figure 16C).

Feed restriction posed a significant reduction in liver *mTOR* gene expression compared to the control group ($p = 0.020$). It was interesting that adding leucine and methionine separately and together on top of the restriction groups raised *mTOR* expression to a level in the middle of the control and FR (Figure 16D). However, the increment was not statistically significant compared to both the control and the FR. As the trend of change was clearly visible, we compared each supplemented group separately with FR group and showed that all amino acid-supplemented groups had significantly higher *mTOR* gene expression than the FR group. The separate comparison of the supplemented groups with the control group did not have statistical significance. There was no difference between the FR+L and FR+M groups ($p = 0.999$). Intriguingly, the FR+ML group showed no difference compared to the FR+L and FR+M groups (Figure 16D), suggesting no additive effect. Treatments did not show a significant effect on liver *FASN* gene expression, though the FR group showed a reducing trend ($p = 0.447$; Figure 16E).

Methionine supplementation on top of restricted feeding showed an observable increase in liver *SOD2* gene expression compared to the other groups ($p = 0.030$, Figure 16F). Other treatment groups did not show significant differences among each other. Feed restriction significantly increased liver *ATG9A* gene expression compared to the other groups (control: p

= 0.041, FR+M: p = 0.029, FR+L: p = 0.073, FR+ML: p = 0.092). Interestingly, supplementation of leucine, methionine or both on the top of restricted feeding downregulated *ATG9A* gene expression to the level of the control group (Figure 16G). Treatments had no significant difference in liver *ATG5* gene expression (Figure 16H).

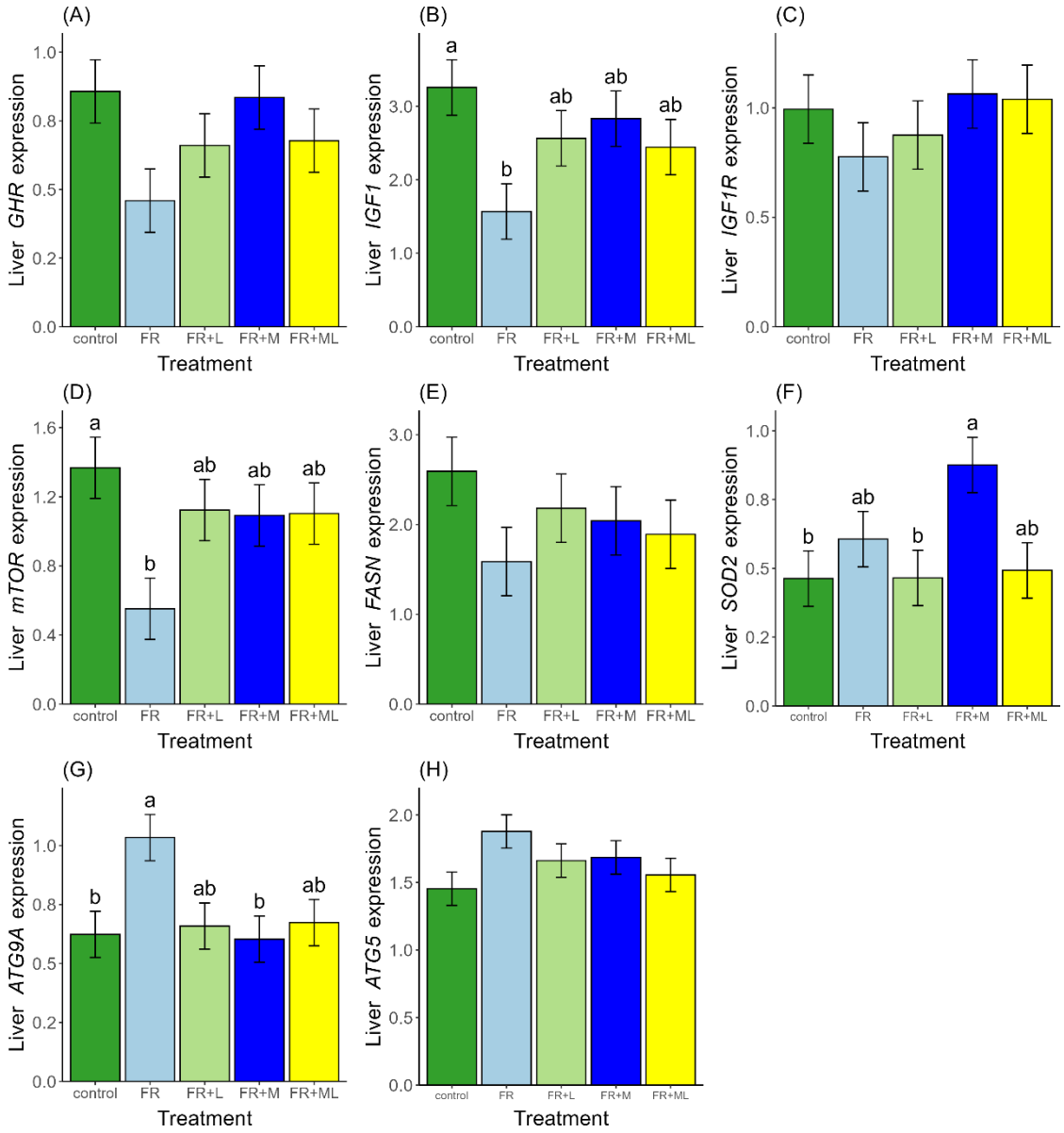

Figure 16. Effect of amino acid supplementation on the top of feed restriction on expression of mTOR pathway genes. (A) growth hormone receptor (*GHR*), (B) insulin-like growth factor 1 (*IGF1*), (C) insulin-like growth factor 1 receptor (*IGF1R*), (D) mechanistic target of rapamycin (*mTOR*), (E) Fatty acid synthase (*FASN*), (F) Superoxide dismutase 2 (SOD2), (G) autophagy-related 9A (*ATG9A*), (H) autophagy-related 5 (*ATG5*). Relative gene expression was analysed in log fold change. Data are represented by the mean ± SEM from 8 birds per group. The emmeans adjusted for Tukey was used as *post hoc* test with $p < 0.05$ significance level. Means followed by a common letter are not significantly different at $p < 0.05$. Abbreviations: Control, birds received full of their daily feed intake; FR, 20% restriction; FR+L, 20% leucine supplemented on top of 20% restriction; FR+M, 20% leucine supplemented on top of 20% restriction; FR+ML, 20% methionine and leucine supplemented on top of 20% restriction

3.3.2. Effect of energy and protein restriction

3.3.2.1. Effect on body weight

Treatment by time interaction has a significant effect on body weight ($F_{6,56} = 7.54$, $p < 0.001$). Body weight was significantly lower on the energy restriction (ER) feeding compared to the control and protein restriction (PR) feedings (Figure 17). In the first week, the trend showed observable variation among treatments (Figure 17A). However, Tukey's HSD adjustment for family-wise comparisons revealed no significant differences among the treatments.

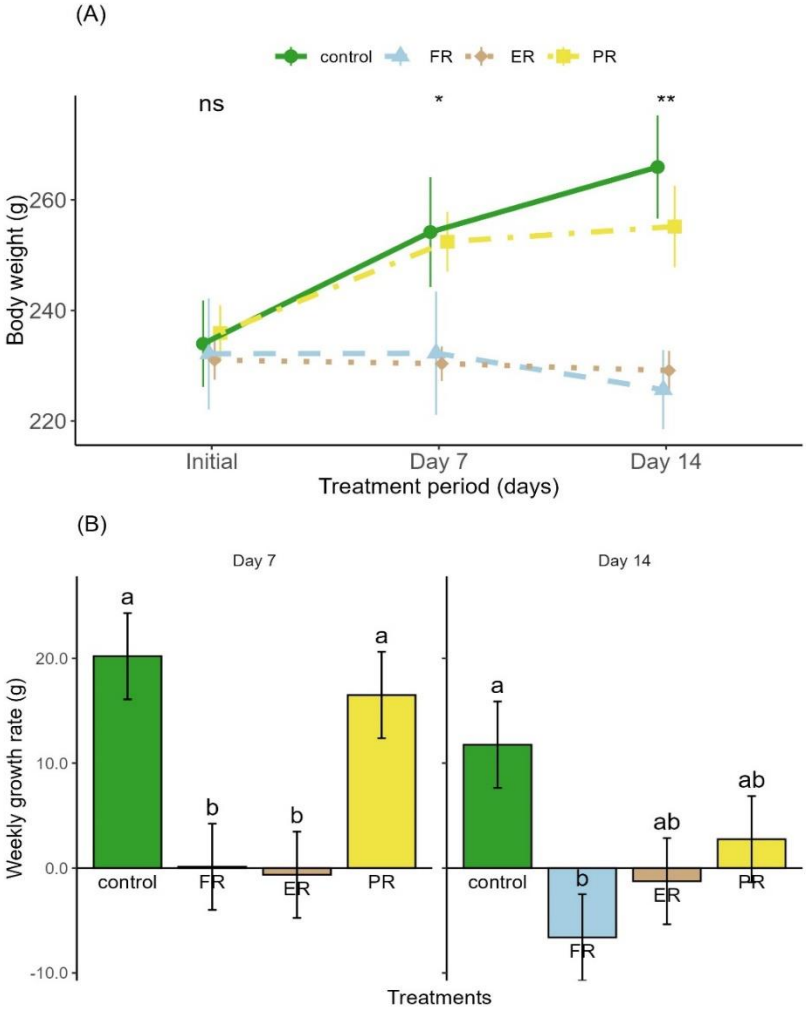

Figure 17. Effect of energy and protein restriction on (A) body weight and (B) weekly body weight gain. Abbreviations: Control, birds received full of their daily feed intake; FR, 20% restricted feeding; PR, 20% protein restriction; ER, 20% metabolisable energy restriction. Other explanations see from Figure 15.

In the second week, the mean comparison from the balanced model showed that ER was significantly lower than the control ($p = 0.007$) and tended to be lower than the PR ($p = 0.081$) groups. At all-time points, the ER group showed no difference with the FR group (Figure 17A). Intriguingly, PR feeding did not have a difference with the control group at all-time points while having a significantly higher body weight compared to the FR group in the

second week (p = 0.038). This is in contradiction with previous studies in chickens (ABDEL-HAFEEZ et al., 2016; URBAN et al., 2018). While the PR-fed groups showed increasing body weight with time, the ER-fed group did not show any increment (Figure 17). The weekly body weight gain analysis also indicated that the PR group had higher body weight gain compared to the FR and ER groups. The ER group also showed lower body weight gain than the control group (Figure 17B). This finding revealed that a 20% metabolisable energy reduction from standard feeding (NRC, 1994) is enough to attenuate the growth of maturing birds. Previous studies reported that 10% and 20% metabolisable energy restriction significantly reduced weekly body weight gain in grower chickens (LU et al., 2023). The study suggested that mild energy restriction is considered a means of improving flock uniformity in poultry production.

3.3.2.2. Effect on hepatic gene expression

Neither the energy restriction nor the protein restriction showed significant differences in the expression of all the anabolic genes compared to the control group (Figure 18A-E), except that the ER showed a noticeable reducing trend in mTOR gene expression (separate comparison: p = 0.069; Figure 18D). Unlike the effect on body weight (Figure 17), energy restriction did not show a significant effect on mTOR pathway anabolic genes.

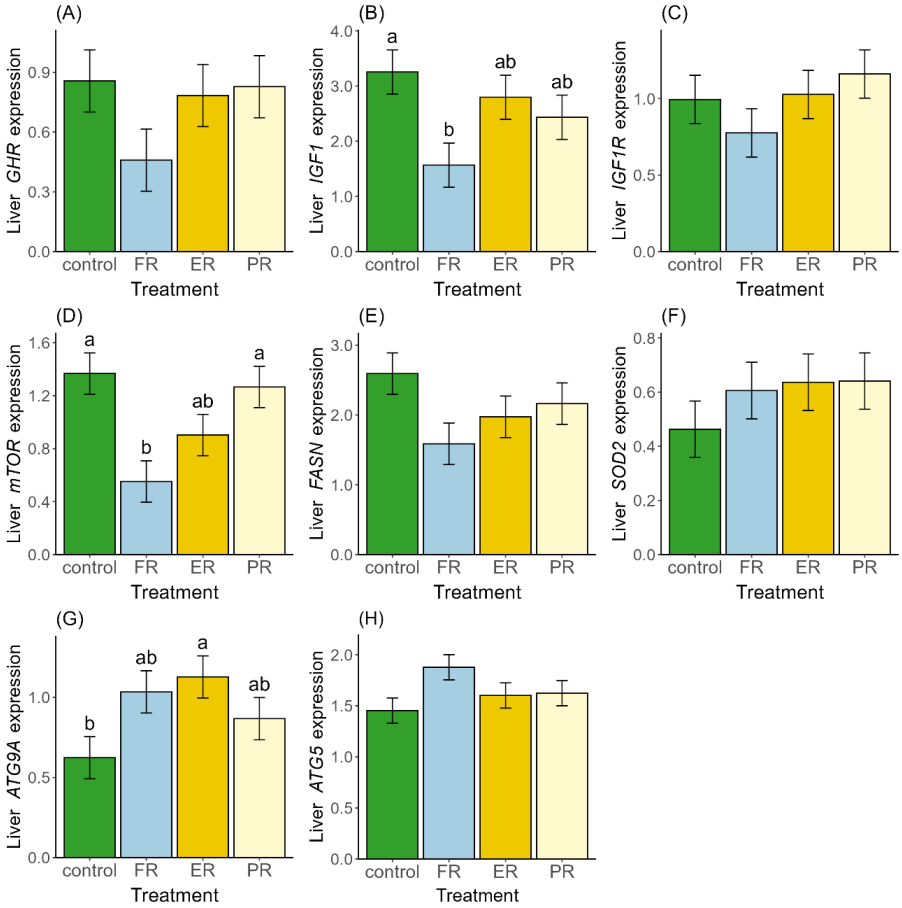

Figure 18. Effect of energy and protein restrictions on expression of mTOR pathway genes. Abbreviations: from Figure 17. Other explanations see from Figure 16.

The energy restriction treatment did not imitate the effect of total feed restriction on gene expression. However, the ER treatment significantly increased liver *ATG9A* gene

expression compared to the control groups ($p = 0.05$), which was also a similar effect with the FR-treated group (Figure 18G). The exceptional effect on *ATG9A* gene expression may contribute to an increased autophagosome formation, which is essential for recycling cellular debris and in turn fuels the transcriptional process of anabolic genes. The reduced body weight due to 20% energy restriction, without noticeable effects on genes other than ATG9A, could be attributed to a resource reallocation to traits prioritised over body weight. Similar to the effect on body weight, 20% protein restriction did not significantly affect expression of all genes compared to the control group (Figure 18), whereas it showed significantly higher *mTOR* gene expression compared to the FR group (Figure 18D).

4. CONCLUSIONS

The overall message of the study revealed that the mTOR pathway is a crucial pathway for future research in birds' development, reproduction, and ageing. Dietary limitation, dietary unpredictability, and specific nutrient manipulation had remarkable effects on the mTOR pathway. FR induces a lower expression of *mTOR* and *IGF1* and a higher expression of *RPS6K1,* GHR, *ATG9A,* and *ATG5* differently at different restriction levels and leads to overall lower fitness values. The gene expression pattern exhibited coordinated variation with levels of FR and was directly linked to production traits and resource allocation. Birds showed variable resource re-allocation strategies depending on the availability of the resource. We also witnessed that quails can be maintained under a moderate (up to 20%) restriction level without significantly affecting egg production or egg weight, whereas severe restriction affects most egg weight.

The study revealed that the fine-scale regulation of the mTOR pathway is sex-specific, as seen in the differential expression of most of the genes studied. Female birds exhibited a higher body weight and more intensive weight loss than males and demonstrated an intensified reduction in *mTOR* gene expression with increasing restriction levels. These findings align with females' larger body size and reproductive investment. In contrast, males exhibited a more pronounced upregulation in *ATG9A* gene expression, potentially aiding their ability to avoid severe body weight loss. It is also evident from the study that there is a notable disparity between females and males in the levels of IGF-1, with females exhibiting 64.7% higher compared to males, supporting the notion of sex-specific IGF-1 secretion patterns. Furthermore, FR reduced plasma triglyceride levels in both females and males. However, females displayed more than a six-fold higher level than males, suggesting that females require more level pf triglycerides to maintain a circulating energy source and support egg production. These sex-specific responses shed light on the intricate interplay between nutrient availability, gene expression, physiological variables and body size, highlighting the importance of considering sexual dimorphism in studies of dietary restriction, and animal physiology in general.

Regarding the unpredictable feeding treatment, only females showed a noticeable reduction in body weight. Birds kept laying consistent numbers and weights of eggs throughout the dietary fluctuation, which may be at the expense of their body weight. The unpredictable feeding has shown an observable effect on gene expression in females, with a reducing trend in liver *IGF1* and *mTOR* genes and an increasing trend in liver *FOXO1*, *RPS6K1, ULK1*, *ATG9A,* and *ATG5* genes. In the muscle, *IGF1R* and *mTOR* in females and 4EBP1 in males showed a reduced trend due to unpredictable feeding, while only *ATG9A* showed an increasing trend in females. The result indicated that genes showed tissue- and sex-specific expression intensities in response to dietary unpredictability. Overall, we can conclude that the two weeks of daily unpredictable feeding have an observable impact on female quails, while the effects on males were weak. The difference between females and males may be attributed to reproduction and physiological mechanisms.

Furthermore, methionine and leucine supplementation on top of restricted feeding showed an interesting effect on body weight and the expression of mTOR pathway genes. Supplementing combined methionine and leucine on top of restricted feeding improved the

weight gain of quails, while leucine supplementation maintained the initial body weight. The unsupplemented restricted feeding showed a decreasing trend during the two-week trial. However, supplementing with 20% methionine alone did not tend to maintain body weight. Supplementing with methionine, leucine or both have shown an observable increasing trend in expression of liver *GHR*, *IGF1,* and *mTOR* genes, while showed reducing trend in expression of *ATG9A*. Interestingly, methionine supplementation remarkably increased *SOD2* expression; a gene encoded antioxidant superoxide dismutase that handles reactive oxygen species. The study has witnessed that supplementing with leucine and methionine together did not have the additive molecular effects of both amino acids but rather aligned with the effect of one of them. Overall, the result supports our hypothesis that dietary supplementation of these essential amino acids partially fill the gap of feed restriction in regulating expression of mTOR pathway genes, and the mechanism was connected to fitness.

Energy restriction also showed an observable effect on the body weight of quails, almost similar to the effect of restricted feeding. However, the effect was not coupled with an effect on most of the expression of mTOR pathway genes, except for a slight reduction in *mTOR* gene expression. Energy restriction showed a remarkable increase in *ATG9A* gene expression, which is essential for recycling cellular debris and in turn fuels the transcriptional process of the anabolic genes. The non-significant effect of a 20% protein restriction on all variables was fascinating. Neither body weight nor expression of the mTOR pathways was affected. The result was contrary to our assumption and predicted that birds at the maturity stage might be more sensitive to protein restriction than energy restriction. The result suggested that Japanese quails could tolerate 20% protein restriction without showing significant consequences for molecular and phenotypic traits.

5. NEW SCIENTIFIC RESULTS

1. The egg production of 9-11-week-old Japanese quails was reduced by 25% at the 40% restriction level, while egg weight was reduced at the 30% and 40% restriction levels. The 20% restriction level did not show a noticeable effect on any egg traits during the two-week experiment.
2. In response to feed restriction, *mTOR* expression showed a restriction-level-dependent reduction and was proportional to body weight loss in females. The *IGF1* in females and *mTOR* and *IGF1* in males were downregulated equally under all restricted groups compared to the control group. Expression of the *RPS6K1* gene was upregulated equally at all restriction levels in both sexes. Expression of *ATG9A* in males and *ATG5* in females increased at all levels of restrictions. Expression of *GHR* in both sexes showed an increasing trend. Expression of *IGF1R* in males was reduced, and expression of *ATG9A* in females increased only in the 40% restricted groups.
3. Birds under the 20% and 30% feed restriction re-allocate resources towards reproduction with greater proportional loss in body weight, whereas birds in the 40% restriction re-allocate resources to body maintenance with greater proportional loss in egg weight.
4. Daily unpredictable feeding, ranging from 30% to 170% of their daily feed intake, reduced body weight of female Japanese quails by 10.25%, whereas there was no such effect on males.
5. Expression of mTOR pathway genes responds to daily unpredictable feeding in a tissue- and sex-specific manner. Expression of liver *mTOR* and *IGF1* genes was downregulated, while expression of *FOXO1, RPS6K1, ULK1, ATG9A,* and *ATG5* was upregulated in response to daily unpredictable feeding compared to control treatment in females.
6. Supplementation of 20% leucine (basal 14.2 g/kg) or leucine and methionine (basal 4.5 g/kg) together on top of restricted feeding reduces the loss of body weight due to the feed restriction. The treatment also reduced the magnitude of 20% feed restriction-imposed downregulation of anabolic mTOR pathway genes and upregulation of catabolic mTOR pathway genes.
7. A 20% protein restriction (basal 180 g/kg) did not affect either body weight or expression of mTOR pathways genes, while 20% metabolisable energy restriction (basal 12.13 MJ/kg) reduced body weight similar to 20% feed restriction.

6. PRACTICAL RESULTS

1. The results could be the basis of further investigations with broiler breeder hens or pullets to determine the better dietary regimen (UNPR, FR, ER, or PR) and magnitude of restriction to optimise reproduction.
2. The result could be served as a basis for optimising the high-energy and low-protein diet during heat stress conditions.
3. The study could initiate manipulation of mTOR activation to regulate growth and reproduction in the poultry industry.
4. Supplementation of leucine and methionine showed to rescue the effect of restricted feeding on growth and expression of key mTOR genes. Therefore, the poultry sector could consider specific amino acid manipulations to regulate metabolism and improve growth and reproduction.

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Registry number: DEENK/1/2025.PL
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List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (2)

1. **Reda, G. K.**, Ndunguru, S. F., Csernus, B., Lugata, J. K., Knop, R., Szabó, C., Czeglédi, L.: Individual cage housing affects feed intake and induces sex-specific effects on body weight in Japanese quails.
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2. **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.
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Foreign language scientific articles in international journals (3)

3. **Reda, G. K.**, Ndunguru, S. F., Csernus, B., Gulyás, G., Knop, R., Szabó, C., Czeglédi, L., Lendvai, Á. Z.: Dietary restriction and life-history trade-offs: insights into mTOR pathway regulation and reproductive investment in Japanese quails.
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4. **Reda, G. K.**, Ndunguru, S. F., Csernus, B., Knop, R., Lugata, J. K., Szabó, C., Czeglédi, L., Lendvai, Á. Z.: Dietary restriction reveals sex-specifc expression of the mTOR pathway genes in Japanese quails.
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7. **Reda, G. K.**: Nutrient-sensing genes expression mediate resource allocation in Japanese quails.
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8. **Reda, G. K.**, Lendvai, Á. Z.: A takarmányfelvétel hatást gyakorol az mTOR útvonalban részt vevő gének expressziójára a japán fürjnél = Feed intake regulates transcriptional regulation of mTOR pathway genes in Japanese quails.
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List of other publications

Foreign language scientific articles in Hungarian journals (1)

9. Lugata, J. K., Ozsváth, X. E., Ndunguru, S. F., **Reda, G. K.**, Knop, R., Angyal, E., Oláh, J., Szabó, C.: Effect of genotype on the hematological parameter of TETRA-SL and Hungarian Partridge coloured chickens at young age.
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10. Ndunguru, S. F., **Reda, G. K.**, Csernus, B., Knop, R., Lugata, J. K., Szabó, C., Lendvai, Á. Z., Czeglédi, L.: Embryonic Leucine Promotes Early Postnatal Growth via mTOR Signalling in Japanese Quails.
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J. Comp. Physiol. B-Biochem. Syst. Environ. Physiol. 194, 179-189, 2024. ISSN: 0174-1578.
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DOI: http://dx.doi.org/10.1038/s41598-024-54891-3
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13. Lugata, J. K., Ndunguru, S. F., **Reda, G. K.**, Ozsváth, X. E., Angyal, E., Czeglédi, L., Gulyás, G., Knop, R., Oláh, J., Mészár, Z. M., Varga, R., Csernus, B., Szabó, C.: Methionine sources and genotype affect embryonic intestinal development, antioxidants, tight junctions, and growth-related gene expression in chickens.
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DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

Total IF of journals (all publications): 24,8
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