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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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ACTB	beta-actin
Akt	protein kinase B
AMPK	adenosine monophosphate-activated protein kinase
ATG9A	autophagy related gene 9A
ATG5	autophagy related gene 5
Deptor	DEP-domain-containing mTOR-interacting protein
FASN	fatty acid synthase
FGF21	fibroblast growth factor 21
FOXO1	forkhead box O1
GHR	growth hormone receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IGF-1	insulin-like growth factor 1
IGF1	insulin-like growth factor 1 gene
IGF-1R	insulin-like growth factor 1 receptor
IGF1R	insulin-like growth factor 1 receptor gene
mLST8	mammalian homolog of protein Lethal with Sec13 protein 8
mSin1	mammalian stress-activated protein kinase interacting protein 1
mTORC1	mechanistic target of rapamycin complex 1
mTORC2	mechanistic target of rapamycin complex 2
PRAS40	proline-rich AKT substrate of 40 KD
Protor	protein observed with Rictor-1
Raptor	regulatory-associated protein of mTOR
Rictor	rapamycin-insensitive companion of mTOR
RPL19	ribosomal protein L19
RPS6K1	ribosomal protein S6 Kinase 1 gene
RN18s	18S ribosomal RNA
S6K1	ribosomal protein S6 Kinase 1
SOD2	superoxide dismutase 2
SDHA	Succinate dehydrogenase
Tel2	telomere maintenance 2
Tti1	Tel2-interacting protein 1
ULK1	Unc-51-like autophagy-activating kinases
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
4EBP1	eukaryotic initiation factor 4E-binding proteins

1. INTRODUCTION

Nutrition is a crucial factor in molecular and physiological responses and, consequently, has a significant impact on important processes such as growth, development, reproduction, and immune defence. An insufficiency in the entire meal or certain vital nutrients affects an organism's physiology and its fitness (BUCHANAN et al., 2022; LEE et al., 2008; MAGWERE et al., 2004). The presence of these vital nutrients in the environment exhibits significant variation, contingent upon elements such as climate, season, and the existence of other creatures (competitors and predators) (BIRNIE-GAUVIN et al., 2017; KUMAR et al., 2022). Additionally, feed cost constitute the majority of expenses in commercial farms (TANDOĞAN and ÇIÇEK, 2016; WONGNAA et al., 2023). Therefore, organisms must have strategic mechanisms to cope with the deficit. Depending on the availability of feed resources, organisms must allocate the resources to competing life-history traits, including growth, body maintenance, reproduction, and immunity (CARLSSON et al., 2021; FLATT et al., 2013; REDA et al., 2024b). Animals prioritize current reproduction above somatic maintenance and future reproduction when feed is freely accessible (ENGLISH and BONSALL, 2019; KOOIJMAN and LIKA, 2014; PONTZER and MCGROSKY, 2022). The inclination towards reproductive investment could entail physiological costs, including oxidative stress and lowered immune capacity (CHANG VAN OORDT et al., 2022; METCALFE and MONAGHAN, 2013). These consequences could then affect the organism's health span and future reproductive performance (HASSAN et al., 2003; MAHROSE et al., 2022; PICK et al., 2019). Under severe nutritional scarcity, animals have to shift their energy budget from reproduction to somatic maintenance (CARLSSON et al., 2021; FLATT et al., 2013).

Nutrient-sensing pathways mediate these processes. Two of the most important nutrient-sensing pathways are the inter-connected insulin-like growth factor-1 (IGF-1) and mechanistic target of rapamycin (mTOR) (JOHNSON, 2018; KAPAHI et al., 2017). High nutritional availability upregulates the IGF-1/mTOR signalling pathway, which, in turn, promotes growth and reproduction while suppressing cellular processes that uphold organismal and cellular homeostasis (such as autophagy and apoptosis) (MONTOYA et al., 2022; PAPADOPOLI et al., 2019). Previous studies on regulatory function of mTOR pathway have been focussed on mammals and humans, ignoring avian system in this process.

Understanding the function of mTOR in birds is very significant due to their distinctive physiology. Compared to mammals, birds possess elevated metabolic rates and require significant energy stores to support activities such as flight, reproduction, and the maintenance of a high body temperature (HOLMES and OTTINGER, 2003; SATOH, 2021). By studying the regulation of the mTOR pathway in birds, we can gain a deeper understanding of the fundamental mechanisms that control the physiological adaptations of this evolutionarily independent lineage.

Dietary manipulation, in terms of feed restriction, nutritional unpredictability, and micro- and macronutrient manipulation, 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. However, Previous studies on FR have primarily focused on model organisms other than birds, understanding the mechanism of mTOR signalling in avian biology remains largely unclear. Studies conducted in mammals have indicated that a higher nutritional intake and metabolic rate upregulate the mTOR pathway, leading to a subsequent downregulation of autophagy (ESCOBAR et al., 2019). This, in turn, exposes organisms to oxidative damage and cellular senescence. However, FR without malnutrition has been found to mitigate these effects (CARROLL and KOROLCHUK, 2018). On the contrary, birds have higher metabolic rates, circulating glucose levels, and body temperature than mammals, while they live twice as long as size-matched mammals (BARJA, 1998). Despite the fact that a higher metabolic rate contributes to oxidative and glycoxidative damage, birds suffer less oxidative stress and related damage compared to mammals at a given body size (JIMENEZ et al., 2019). This metabolic paradox may be due to a different diet-fitness relationship in birds, which can be elucidated using dietary manipulation experiments. Effects can also be sex-specific due to the differential reproduction strategy and sexually dimorphic nature of organisms such as Japanese quails.

The other dietary condition important to understand molecular mechanisms is dietary unpredictability. Organisms often encounter unpredictable fluctuations in feed availability, which can significantly affect their biological functions even without an overall feed shortage. This is because organisms need to anticipate feed availability to adjust their physiology accordingly. They adapt to these unpredictable conditions through heritable variation, phenotypic plasticity, and life-history decisions (REED et al., 2010; RIPA et al., 2010; TARAZONA et al., 2019; TUFTO, 2015). As a result, unpredictability can

drive changes in growth, reproduction, immunity, and lifespan mechanisms, leading to diverse physiological and behavioural responses (BOTERO et al., 2015; CORNELIUS RUHS et al., 2019; FRANCH-GRAS et al., 2017). However, many of the previously reported studies used constant *ad libitum* and restricted feeding, which did not consider the effect of unpredictable feeding. Unlike constantly high or low feeding, a condition 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. One of the important biological mechanisms for regulating dietary conditions involves altering gene expression (LÓPEZ-MAURY et al., 2008). Genes in the mTOR pathway could be responsible for sensing nutritional fluctuation, undergoing adaptive changes in expression that link genotypes and molecular functions to fitness outcomes. Due to their sexually dimorphic nature (BALL and BALTHAZART, 2010), organisms may respond sex-specifically to dietary unpredictability, and the expression of genes may mediate this process. Depending on the target function, a gene may have a tissue-specific differential expression (BAAR et al., 2016). Therefore, tissue- and sex-specific responses are also of interest to biologists.

Manipulating micro- and macronutrients is another intervention strategy to extract the mechanism of the mTOR pathway. Amino acids are the building blocks of proteins and serve as regulators of the mTOR pathway. Manipulating amino acids only, instead of the whole dietary intake, can effectively inhibit the pathway (JUNG et al., 2015; ZHOU et al., 2016). However, we have limited knowledge of what will happen if we supplement certain essential amino acids under restricted feeding conditions. Under laboratory conditions, feed restriction and manipulation of amino acids could separate the effect of total energy intake and specific amino acids on mTOR pathway genes. Methionine and leucine are essential amino acids that play a crucial role in the growth and development of birds (FAGUNDES et al., 2020). Methionine is often considered the first limiting amino acid in poultry diets and is tested to activate mTOR signalling (GU et al., 2017; HU et al., 2020). Leucine is another essential amino acid required for protein synthesis and is known to be a potent activator of the mTOR signalling pathway (DODD and TEE, 2012; WOLFSON et al., 2016). Supplementing methionine, leucine, or both under feed restriction conditions could be crucial for understanding these amino acids' specific roles in regulating mTOR pathway genes. It is also important to study the effect of the concurrent or separate supplementation of methionine and leucine on mTOR pathway genes. Supplementation

of these essential amino acids could also result in a negative result due to an amino acid imbalance (GRANDISON et al., 2009).

Additionally, macronutrients, such as carbohydrates, lipids, and proteins, have an indispensable role in regulating the mTOR pathway. However, while studies have reported on the effect of total calorie restriction on mammalian models, the effect of metabolisable energy restriction under full feeding is yet to be studied. Furthermore, less information is available showing the regulatory role of protein restriction compared to full feeding and feed-restricted conditions, mainly in birds.

We employed feed restriction, dietary unpredictability, amino acid supplementation, and energy or protein restriction to explore the mechanism of mTOR pathway genes and some physiological traits in response to nutritional cues. We used Japanese quails as an experimental avian model. The Japanese quail is a well-known avian model for studying molecular, physiological, and phenotypic fitness in response to treatments. The domesticated Japanese quail is heterogeneous, retaining several experimentally suitable characteristics, such as a smaller body size compared to other domesticated species and robustness in both health and reproduction. They have quick generational turnover and early sexual maturity, which make them crucial for research purposes. Japanese quails are size-dimorphic birds, with adult females being heavier than males (BALL and BALTHAZART, 2010; OTTINGER, 2007; OTTINGER et al., 2005).

1.1 Aims of the Doctoral Study

The objective of my doctoral work was to investigate the mechanisms by which different dietary conditions affect the expression of mTOR pathway genes and their links with life-history traits, mainly body weight and reproduction. In this doctoral study we conducted three consecutive experiments:

1. Effect of feed restriction treatment on molecular, physiological and fitness traits in Japanese quail
2. Effect of unpredictable feeding on expression of mTOR pathway genes and fitness traits in Japanese quail
3. Amino acid supplementation on top of restricted feeding and macronutrient restriction in Japanese quail

The doctoral work was applied with the following specific objectives:

- To investigate the effect of gradients of feed restriction on the 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 regulation of the key mTOR pathway genes and its connection with body weight primary fitness measure.
- To evaluate the expression of key mTOR pathway genes in response to dietary protein and metabolisable energy restriction compared to full feeding and equivalent feed restriction.

2. LITERATURE REVIEW

2.1. The Mechanistic Target of Rapamycin (mTOR) Signalling Pathway

One of the key signalling pathways monitoring nutrient availability is the mechanistic target of rapamycin (mTOR) (LAPLANTE and SABATINI, 2012; REGAN et al., 2020). This pathway comprehends a series of cross-talking genes at different stages of cellular functioning. mTOR is an intracellular serine/threonine protein kinase that plays a crucial role in protein synthesis, cell growth, differentiation, and subsequent organismal growth and reproduction (KENNEDY and LAMMING, 2016). In response to growth hormones and energy availability, mTOR receives signal transduction from extracellular growth factors, mainly insulin and IGF-1 binding to its receptor (IGF-1R) at the plasma membrane (Figure 1), which, in turn, activates downstream effectors by phosphorylation (PAPADOPOLI et al., 2019; SANDRI et al., 2013). Nutrients (intracellular amino acids) also directly regulate mTOR activity (GOLLWITZER et al., 2022; HARA et al., 1998). The mTOR is then responsible for activating and inhibiting several transcription and translation factors and binding proteins, ultimately affecting gene and protein expression (LAPLANTE and SABATINI, 2013).

The functions of mTOR exist in two protein complexes, the mechanistic target of rapamycin complex 1 (mTORC1) and complex 2 (mTORC2) (HEITMAN et al., 1991). Both complexes share the mTOR, DEP-domain-containing mTOR-interacting protein (Deptor) (PETERSON et al., 2009), the mammalian homolog of protein Lethal with Sec13 protein 8 (mLST8) (KIM et al., 2003), and the Tti1/Tel2 complex (INOKI et al., 2006). Besides, mTORC1 also contains proline-rich AKT substrate of 40 KD (PRAS40) (OSHIRO et al., 2007) and regulatory-associated protein of mTOR (Raptor) (HARA et al., 2002). Whereas mammalian stress-activated protein kinase (SAPK) interacting protein 1 (mSin1) (SAXTON and SABATINI, 2017), protein observed with Rictor-1 (Protor) (PEARCE et al., 2007), and rapamycin-insensitive companion of mTOR (Rictor) (SARBASSOV et al., 2004) are specific to mTORC2 (Figure 1) (HRESKO and MUECKLER, 2005).

Compared to mTORC2, mTORC1 is relatively well studied in model organisms other than birds. Once the mTORC1 is activated by growth factors and amino acids, there are a number of downstream targets either activated or inhibited (Figure 1). Proteins involved in mRNA translation are the best-studied downstream targets of mTORC1 (FONSECA and PROUD, 2009; MA and BLENIS, 2009). These include the

ribosomal protein S6 kinases 1 (S6K1) (CHAUVIN et al., 2014; ISOTANI et al., 1999) and the eukaryotic initiation factor 4E-binding proteins (4EBP1) (PAUSE et al., 1994).

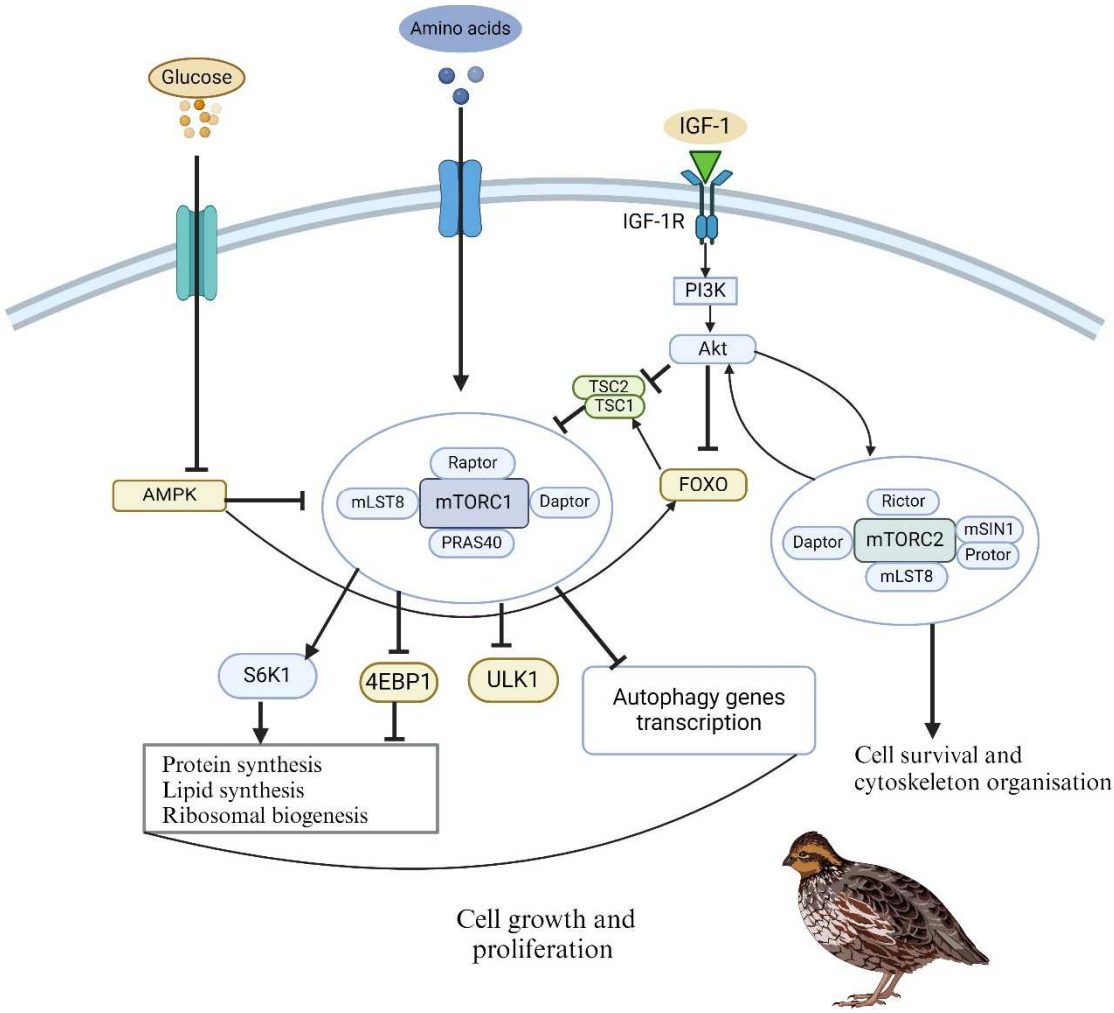

Figure 1. Simple schematic representation of mechanistic target of rapamycin signalling pathway. Both mTORC1 and mTORC2 receive signal transduction from IGF-1throught protein kinase B (Akt). mTORC1 further receives signals from energy availability through adenosine monophosphate-activated protein kinase (AMPK). Additionally, mTOR senses cellular amino acid availability. The activated mTORC1 phosphorylates a ribosomal protein S6 Kinase 1 (S6K1), which further facilitates the initiation of ribosomal translation. Furthermore, mTORC1 phosphorylates eukaryotic initiation factor 4E-binding proteins (4EBP1) and dissociates from eukaryotic initiation factor 4E (eIF4E), which otherwise would delay translation. Activated mTORC1 also phosphorylates ULK1 and autophagy transcription factors, which downregulates autophagy in response. This entire process upregulates growth and reproduction. In response to activation by Akt, mTORC2 facilitates cell survival and cytoskeleton organisation.

The S6K1 is the most important signalling hub in the mTORC1 pathway. The mTORC1-phosphorylated S6K1 activates ribosomal activity by activating other factors

or by direct phosphorylation of the ribosomal unit (FENTON and GOUT, 2011; MEYUHAS and DREAZEN, 2009). The S6K1 inhibits the eukaryotic elongation factor 2 kinase (eEF2K), the inhibitor of eEF2, which is indirectly involved in the elongation of the polypeptide in the ribosome (REDPATH et al., 1996; TAHA et al., 2020). The phosphorylated S6K1 also activates eukaryotic initiation factor 4B (eIF4B) which is also important for polypeptide translation (SHAHBAZIAN et al., 2006). The activated S6K1 stimulates carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD), thereby stimulating pyrimidine synthesis (ROBITAILLE et al., 2013). The 4EBP1 binds eukaryotic initiation factor 4E (eIF4E) to delay translation. The mTORC1, therefore, inhibits 4EBP1 to activate eIF4E and initiate polypeptide elongation (protein synthesis) (SHOWKAT et al., 2014; SIDDIQUI et al., 2012). Activated mTOR phosphorylates kinases such as Unc-51-like autophagy-activating kinases (ULK) and inhibits localisation to phagophores, thereby, downregulates autophagy (KIM et al., 2011; ZOU et al., 2022). Furthermore, transcription factors like transcription factor EB (TFEB), the transcription factors for the autophagy genes, signal transducer and activator of transcription 3 (STAT3), a transcription factor for a number of genes, including fatty acid synthase (FASN), and Specificity Protein 1 (Sp1), a transcription factor for Superoxide Dismutase 2 (SOD2), are phosphorylated by the active mTORC1. As a result, these proteins' nuclear localization is either halted or facilitated, and the expression of certain genes is suppressed or enhanced (CHANG et al., 2017; NAPOLITANO et al., 2018; WANG et al., 2020).

Furthermore, FOXOs, the evolutionarily conserved transcription factors, play an antagonistic role by mediating catabolic processes in response to nutritional limitations. Positioned downstream of IGF-1, FOXOs govern the expression of genes that manage metabolic and stress responses, thereby influencing cell proliferation and viability (JIANG et al., 2020; NOWAK et al., 2018). During nutritional and oxidative stress conditions, FOXO proteins are more likely to be present in the nucleus, facilitating transcription of metabolic homeostasis genes (DU and ZHENG, 2021). The interplay of these pathways is examined in short-lived lab animals (BLACKWELL et al., 2019; LIU et al., 2020; YANG et al., 2023). However, it remains unknown in birds due to their unique physiological and metabolic characteristics compared to mammals (JIMENEZ et al., 2019; MUNSHI-SOUTH and WILKINSON, 2010).

At a glance, insulin regulates the mTOR signalling pathway through activation of the PI3K-Akt cascade. Under ample nutritional availability, insulin binds insulin receptor

at the plasma membrane and activates the PI3K-Akt, the upstream activator of mTORC1 (NAKAMURA et al., 2022). Dysregulation of mTOR has been found to be strongly linked with insulin resistance (MENON et al., 2014; PANWAR et al., 2023). However, in avian species, the mTOR signalling pathway is less dependent on insulin due to natural insulin resistance (SATOH, 2021), with IGF-1 signalling playing a more prominent role. This adaptation supports avian metabolic demands, especially in energy-intensive activities (SWEAZEA, 2022).

2.2. Feed Restriction in Study of Molecular Mechanisms

Feed restriction (FR) is a reduction in the calorie intake or manipulation of macronutrients within an ad libitum feeding that has been successfully applied in evolutionarily distant model organisms (MOATT et al., 2017; SPEAKMAN and MITCHELL, 2011). In yeast, primates, flies, mice, and worms, FR extends lifespan by reducing age-related diseases and delaying ageing (BORDONE and GUARENTE, 2005; CHUNG and CHUNG, 2019; COLMAN et al., 2009; MA et al., 2015a). It is a 10–40% intake reduction of a nutritious basal diet, considered the robust non-genetic mechanism to extend health span and lifespan (FONTANA et al., 2010b; GOLDBERG et al., 2015; MATTISON et al., 2012b). FR delays ageing and age-related problems, including susceptibility to infection, body condition abnormality, and decline in the metabolic system (MOATT et al., 2019; PONTON et al., 2011; SOLON-BIET et al., 2014), allowing an organism to reproduce later (KIRKWOOD and SHANLEY, 2005).

FR is a widely used strategy in commercial poultry production, mainly applied to breeder layers. This approach involves either the withdrawal of a portion of the daily feed intake (quantitative restriction) or the manipulation of specific macro- or micronutrients (qualitative restriction) (ANENE et al., 2023; EBEID et al., 2022; LU et al., 2021; WALSH and BRAKE, 1997). FR is applied to slow down the rapid growth of specialised breeders and prevent excessive fat accumulation, thereby reducing metabolic disorders, increasing reproductive lifespan, and improving ovarian morphology and overall productivity (CARNEIRO et al., 2019; HASSAN et al., 2003; MCDANIEL et al., 1981). FR can have varying effects on egg production and quality. Moderate FR may initially boost egg production as hens efficiently allocate available resources. Moderately feed-restricted birds have higher total and settable egg production, fewer defective or damaged eggshells and higher fertility and hatchability than those fed *ad libitum* (HOCKING et al., 2002). However, severe and prolonged restriction can lead to declining egg production (ANENE

et al., 2023; MAHROSE et al., 2022; OLAWUNI et al., 1992). Additionally, FR can impact the interior and exterior characteristics of eggs, resulting in altered shape and size. As this process implicates a complex resource allocation trade-off among egg production/quality and other life-history traits, balancing the degree and duration of FR is vital in improving egg production and quality. Early feed restriction (up to 30%) on Japanese quails showed no reduction in egg production and quality through increased feed conversion efficiency while body weight is reduced (FIDAN and KAYA, 2014; GEBHARDT-HENRICH and MARKS, 1995; HASSAN et al., 2003). Studies on Japanese quails also indicated that moderate restriction tended to increase egg production and quality, while severely restricted quails reduced most egg traits (MAHROSE et al., 2022). Birds can also have resource allocation strategy among different components of the egg.

2.2.1. Feed restriction and the mTOR pathway at a glance

The effect of FR was found to be related to genes encoding specific molecular mechanisms, that respond to nutrient intake (KAPAHI et al., 2017). FR, through lowering IGF-1/mTOR signalling, the major promoter of cellular and organismal growth, extends the lifespan and responds to other downstream effects of the pathway (FONTANA and PARTRIDGE, 2015; FONTANA et al., 2010b). FR inhibits mTORC1 by downregulating the insulin/IGF-1 pathway (DEMIDENKO and BLAGOSKLONNY, 2009) and activation of the adenosine monophosphate-activated protein kinase (AMPK) (JIANG et al., 2008; XU et al., 2012). FR can also directly downregulate mTORC1 via amino acid starvation (POWERS et al., 2006; SANCAK et al., 2010).

Furthermore, FR has a complex impact on mTORC2 activity. It has been observed that FR downregulates mTORC2 by inhibiting insulin/Akt signalling (SAXTON and SABATINI, 2017; YU et al., 2019). On the other hand, there is evidence to suggest that FR can upregulate mTORC2 through the activation of the adenosine monophosphate-activated protein kinase (AMPK) pathway and inhibition of the mTORC1 pathway (FU and HALL, 2020; TULSIAN et al., 2018). Inhibition of mTORC1 downregulates the inhibitory effect of S6K1 on mTORC2 (RAGUPATHI et al., 2024).

2.2.2. Effect of feed restriction on gene expression

The activity of mTOR and its downstream effectors is further regulated through transcriptional regulation/mRNA expression and post-translational modifications (DENG et al., 2014; MIERZIAK et al., 2021; ROLLINS et al., 2019). While post-translational

modifications and specific amino acid availability induce mTOR activation, general resource availability is responsible for adaptive changes in gene expression (EFEYAN et al., 2015; MIERZIAK et al., 2021; SANDRI et al., 2013). Although the final activity of mTOR is influenced by multiple factors, higher expression of the genes can potentially increase the pool of available proteins for activation, while lower gene expression leads to reduced protein production and availability (BUCCITELLI and SELBACH, 2020). Studies on model organisms have predominantly focused on the post-translational activation of mTOR and its downstream effectors (LAPLANTE and SABATINI, 2012; PAPADOPOLI et al., 2019). However, the impact of FR on the differential expression of mTOR pathway genes and their role in mediating fitness remain to be fully elucidated.

2.2.3. Effect of feed restriction on circulating IGF-1

One physiological marker of nutritional availability affecting life history is the insulin-like growth factor 1 (IGF-1). IGF-1 is an essential peptide hormone that exerts pleiotropic effects on cellular growth, proliferation, metabolism and survival in almost all developmental stages. IGF-1 regulates life-history traits, essentially, growth, development, reproduction and lifespan across diverse taxa (LODJAK et al., 2018; LODJAK and VERHULST, 2020; REGAN et al., 2020). In response to growth hormone and nutritional availability, IGF-1 is primarily produced in the liver, transported by IGF-1 binding globulins, and exerts its effects by binding to and activating its tyrosine kinase membrane receptors on cell surfaces. This initiates signal transduction to the intracellular signalling cascades, further activating intracellular kinases, including the mechanistic target of rapamycin (mTOR), which facilitates protein synthesis, cell growth and differentiation (ALLARD and DUAN, 2018; LARON, 2001; OLDHAM and HAFEN, 2003). From a production point of view, IGF-1 is considered a key modulator of postnatal development, sexual maturation, and embryonic follicular growth, which are important for growth and reproduction (FRANCOEUR et al., 2023; OGUNPAIMO et al., 2021). From an ecological perspective, IGF-1 is viewed as a regulator of life-history trade-offs (LEWIN et al., 2017; LODJAK et al., 2018). Variations in IGF-1 levels among individuals can lead to variations in growth rates and sizes within a population, potentially influencing natural selection and evolutionary processes. For example, individuals with higher IGF-1 levels may grow larger or mature faster, thereby affecting their survival and reproductive success (LODJAK et al., 2017; LODJAK et al., 2014; REGAN et al., 2020). Differences in IGF-1 levels among individuals of the same or different species can influence competitive outcomes,

especially in contexts where size or growth rates determine access to resources or mates (ODDIE, 2000; RIDENOUR et al., 2023). Within a given species, IGF-1 levels are positively correlated with body weight, while IGF-1 deficiency is associated with dwarfism due to reduced growth rates and a slower metabolism (BERRYMAN et al., 2008; STUART and PAGE, 2010). Contrarily, across species, IGF-1 level has been reported to negatively correlate with body weight, which is also proposed to be a function of longevity (LODJAK and VERHULST, 2020; STUART and PAGE, 2010).

Studies on model organisms, ranging from yeast to mammals, have revealed that FR decreases insulin/insulin-like signalling (BONKOWSKI et al., 2006; FONTANA et al., 2010a; KLEMENT and FINK, 2016). However, the effect of FR is specific to species, sex, age and mode of intervention (BACON, 1986; KEWAN et al., 2021; LUNA-CASTILLO et al., 2022; MELLOUK et al., 2018). For instance, in mammal models and humans, long-term FR reduces circulating IGF-1 levels (FONTANA et al., 2008; LETTIERI-BARBATO et al., 2016; MITCHELL et al., 2016). However, other studies on rats indicate no significant effect of FR on circulating IGF-1 (HANJANI et al., 2021), and it also does not alter *IGF1* gene expression in the brain (MARSH et al., 2008) and liver (MASTERNAK et al., 2005) of mice. Furthermore, the effect of FR on plasma IGF-1 appears to diminish with age in mammals (BREESE et al., 1991; RAMSEY et al., 2000). Therefore, the nature of relationship between dietary restriction and IGF-1 levels is context-dependent, which needs further study in other model organisms, such as Japanese quails among birds.

2.2.4. Effect of feed restriction on triglyceride levels

Another critical physiological marker of nutritional status is the level of circulating triglycerides. They have been proposed as the most reliable indicators of nutritional state (MASELLO and QUILLFELDT, 2004; PERES et al., 2014). The concentration of triglycerides reflects an organism's accessibility to feed sources in its environment. For instance, species with a high intake of fatty foods may exhibit elevated plasma triglyceride levels, suggesting adaptation to a particular nutritional niche (JACKSON et al., 2023; SMITH et al., 2021). Triglyceride levels have a strong correlation with body weight and predict reproductive success (JENNI-EIERMANN et al., 2002; MASELLO and QUILLFELDT, 2004). Triglycerides are excellent indicators of fat metabolism in birds, as they function as primary energy reserves (ROCCIO et al., 2021; SMITH and MCWILLIAMS, 2009). The levels of triglycerides in their blood are influenced by nutrient availability

(FOKIDIS et al., 2012). FR has been shown to reduce triglyceride levels (KUDCHODKAR et al., 1977; SOLON-BIET et al., 2015; ZHU et al., 2004), which is a sign of increased use of energy reserves. In mammals, FR elicits conflicting effects on triglyceride levels: restricted males show lower, while females exhibit higher levels than their unrestricted counterparts (MATTISON et al., 2012a).

2.2.5. Feed restriction and resource allocation among life-history traits

Resource availability is a key driver of resource allocation decisions that shape life-history trade-offs (NG'OMA et al., 2017; ZERA and HARSHMAN, 2001). When feed is abundant, animals allocate resources towards current reproduction against somatic maintenance and further reproduction (ENGLISH and BONSALL, 2019; KOOIJMAN and LIKA, 2014; PONTZER and MCGROSKY, 2022). The bias for reproductive investment may be accompanied by physiological costs, including oxidative stress and a reduced immune potential (CHANG VAN OORDT et al., 2022; METCALFE and MONAGHAN, 2013), which in turn affects future reproductive performance and the health span of the organism (HASSAN et al., 2003; MAHROSE et al., 2022; PICK et al., 2019). This phenomenon changes under limited resources when organisms must divert energy from reproduction to somatic maintenance (CARLSSON et al., 2021; FLATT et al., 2013).

Reducing calorie intake antagonistically affects the investment in self-maintenance, growth, and reproduction (MCCRACKEN et al., 2020; REGAN et al., 2020). The classical resource allocation theory predicts that FR should lead to a linear decrease in reproduction in favour of self-maintenance (SHANLEY and KIRKWOOD, 2000). However, modest FR can maintain or even improve reproductive performance by activating cell-recycling mechanisms such as apoptosis and autophagy (ADLER and BONDURIANSKY, 2014; MAHROSE et al., 2022). When the level of FR becomes more severe, the organism must shift energy away to meet basal energetic requirements; thus, the reproduction rate will decline (MOATT et al., 2016; OTTINGER et al., 2005; SHANLEY and KIRKWOOD, 2000). The key mediators of this process are the nutrient-sensing pathways governed by insulin-like growth factor-1 (IGF-1) and mechanistic target of rapamycin (mTOR) (JOHNSON, 2018; KAPAHI et al., 2017). The IGF-1/mTOR signalling pathway is activated by high nutrient availability and triggers growth and reproduction while downregulating cellular processes that maintain organismal and cellular homeostasis (e.g., apoptosis and autophagy) (MONTOYA et al., 2022; PAPADOPOLI et al., 2019).

2.2.6. Sex-specific effect of feed restriction on molecular and physiological variables

Exploring the distinction between male and female phenotypes and genes that show sex-specific expression has long been the interest of biologists. Recent studies identified somatic genes differentially expressed across different tissues in response to treatment in males and females in mammal and fly models (BAZHAN et al., 2019; BENNETT-KEKI et al., 2023; KHODURSKY et al., 2022; MCDONALD et al., 2021). In mammals, theoretical and empirical evidence shows strong sexual differences in the mTOR-mediated life history regulation (BRONIKOWSKI et al., 2022; BROOKS and GARRATT, 2017; MITCHELL et al., 2016; PARIHAR et al., 2020). For instance, in mice, a moderate feed restriction (20% DR) improves health span for both males and females, while a severe restriction (40% FR) is detrimental for females but still increases lifespan in males due to divergent physiological and molecular responses. In fruit flies, the effects of dietary restriction on lifespan and mortality rates also differ between sexes, with females showing a peak in lifespan at higher food concentrations and a more pronounced response to restriction (MAGWERE et al., 2004). Another experiment in the same species revealed that dietary restriction-mediated sex differences in fitness are associated with sex-specific effects on the expression of genes mediating the mTOR pathway (BENNETT-KEKI et al., 2023; MCDONALD et al., 2021). The proposed explanation for these differences is rooted in sexual variations in nutrient requirements and energy allocation. Divergent reproductive strategies, the modulating function of sex hormones and specific optimal diets for reproduction are among the suggested reasons for the difference in the expression of genes in males and females (CAMUS et al., 2019; GEGENHUBER et al., 2022). Because of these inherent physiological and reproductive differences, the response to FR could be sex-specific (BROOKS and GARRATT, 2017; MAKLAKOV et al., 2008; SATOH, 2021). However, despite the theoretical and empirical evidence in other taxa, the sex differences in mTOR pathway response to FR in birds remain unexplored.

2.3. Effect of Dietary Unpredictability

2.3.1. Dietary unpredictability at a glance

Nutritional unpredictability is a crucial factor affecting the physiology and subsequent health and longevity of organisms (FOKIDIS et al., 2012; LYNN et al., 2023). The availability of nutrients in the natural environment can vary widely, depending on

factors such as weather, season, and other natural or anthropogenic disturbances (BIRNIE-GAUVIN et al., 2017; KUMAR et al., 2022). Similarly, there is a temporal variation in feed quantity and quality in commercial and backyard animal production systems. Hence, organisms often face an unpredictable recurrent fluctuation in dietary availability, which can substantially affect biological function, even if there is no overall shortage of feed. This is because organisms should be able to anticipate when feed will be available so as to adjust their physiology accordingly. Organisms make adaptive responses to unpredictability through phenotypic plasticity, heritable variation, and life-history decisions (REED et al., 2010; RIPA et al., 2010; TARAZONA et al., 2019; TUFTO, 2015).

Unpredictability may therefore force animals to change the mechanism of growth, reproduction, immunity, and lifespan, and it is evolutionarily important as they undergo divergent physiological and behavioural responses (BOTERO et al., 2015; CORNELIUS RUHS et al., 2019; FRANCH-GRAS et al., 2017). For instance, studies on two small marsupian mice *Sminthopsis macroura* (LESLIE et al., 2015) and *Sminthopsis crassicaudata* (MUNN et al., 2010) reported that daily unpredictable feed supply increases the frequency of torpor use and duration of torpor-bouts. Additionally, studies on a new-world songbird, the curve-billed thrasher (*Toxostoma curvirostre*) (FOKIDIS et al., 2012) and zebra finches (*Taeniopygia guttata*) (LYNN et al., 2023) reported certain physiological variables, body weight, and feeding behaviours. Birds received the same amount of feed but with unpredictable daily quantities. They showed increased baseline and reduced stress-induced corticosterone levels, decreased body weight, and increased rate of feed consumption compared to *ad libitum* and constantly fasted birds. Therefore, understanding animals' molecular, physiological, and morphological mechanisms of adaptation to unpredictable changes in dietary availability is an essential question in evolutionary biology.

2.3.2. Effect of dietary unpredictability of gene expression

One of the important biological mechanisms for regulating environmental/dietary conditions involves altering gene expression (LÓPEZ-MAURY et al., 2008). Organisms may respond to unpredictability through altering patterns of expression of certain genes (FEIGE-DILLER et al., 2022). Cells have the ability to acclimate to typical alterations within their native habitat by sensing environmental factors (EFEYAN et al., 2015; SUNG et al., 2023). This adaptation is always moderated through initiating specialized regulatory networks that have evolved to establish adaptive gene expression profiles

(DWORKIN and LOSICK, 2001; HAMANN et al., 2021; MARTÍNEZ SOSA and PILOT, 2023). Genes in the nutrient-sensing pathway, including insulin-like growth factors (*IGFs*), mechanistic target of rapamycin (*mTOR*), Forkhead box Os (*FOXOs*), autophagy-related genes (*ATGs*) and their downstream effectors, are therefore responsible for sensing nutritional availability and undergo adaptive changes in gene expression and link genotypes and molecular functions to the fitness outcomes (BENNETT-KEKI et al., 2023; EFEYAN et al., 2015; NOURMOHAMMAD et al., 2017).

2.4. Amino Acid Regulation of the mTOR Pathway

Amino acid regulation of mTORC1 is an evolutionarily conserved signalling independent of growth hormone signalling pathway (KOGAN et al., 2010). Amino acids signalling that regulate mTORC1 activity are initiated inside the cells. The proteolipid membrane layer in the cell surface is a selective barrier to signals and nutrients; hence, numerous receptors and transporters are embedded in cell membrane and participate in signalling and nutrient transportation (BROER, 2008; STEIN, 2012). Dietary amino acids are expected to be transported into cells and apply for their regulatory roles. Transporters are responsible for exchanging amino acids between extracellular and intracellular mediums of cells (BROER, 2008; ZHENG et al., 2016).

Amino acids activate mTORC1 on the Rag-GTPases, Ragulator complex, and the vacuolar H+–adenosine triphosphatase (v-ATPase) dependent manner, which promotes translocation of mTORC1 to the membrane-bound lysosome to be activated by Ras homolog enriched in brain (RHEB) (JONES and PEARCE, 2017; SANCAK et al., 2008). There are four Rag protein families (RagA, RagB, RagC, and RagD), of which RagA and RagB, and RagC and RagD are 90% and 81% similar, respectively, and are functionally redundant (SEKIGUCHI et al., 2001). Amino acids, mainly leucine and arginine, promote the bounding of RagA/B with GTP and RagC/D with GDP, which leads to the interaction of mTORC1 with the Rag protein heterodimers on the Raptor component of the mTORC1 complex that serves as a docking site for mTORC1 on the lysosomal surface. The RagGTPases bind to the lysosomal surface with the help of Ragulator complexes (LAMTOR1-5) (SANCAK et al., 2010). In amino acids deprived cells, RagA/B bound GDP while RagC/D bound to GTP then mTOR can be found dispersed throughout the cytoplasm (ZHU and WANG, 2020). Therefore, the addition of amino acids rapidly localizes mTORC1 into its functioning spot, the lysosomal surface, by triggering the increased activation of Rag and mTORC1 (MENON et al., 2014; SANCAK et al., 2008).

Contrary to this, other report suggested that mTORC1 could be differentially activated by specific amino acids independent of the RagGTPases (JEWELL et al., 2015).

While the role of amino acids in mTOR signalling is receiving more research attention, the mechanism of every type of amino acid and their interactions in the regulation of mTORC1 is not well understood. A study proposed that specific amino acids in assorted compartments activate different mTORC1 components, which means the mTOR pathway has evolved to sense some particular amino acids (GOBERDHAN et al., 2016). The branched-chain amino acid leucine is a direct acting nutrient signal for activating mTORC1 signalling and subsequent protein synthesis (KIMBALL et al., 1999). Leucine rich diets are shown to activate the mTORC1 signalling across diverse organisms (CRUZ et al., 2019). A heterodimeric bidirectional amino acid transporter SLC7A5/SLC3A2 regulates the simultaneous efflux of intracellular L-glutamine out of cells and transport of extracellular branched chain and aromatic amino acids, mainly L-leucine into cells (JEWELL et al., 2015). Supplementation of L-leucine also increases the concentration of L-glutamine produced by the cells to regulate the uptake of L-Leucine into the cell and rapidly decreases with the efflux from the cell via SLC7A5/SLC3A2 and influxes L-leucine (NICKLIN et al., 2009).

Methionine is another critical essential amino acid in the regulation of mTOR. Via its metabolite *S*-adenosylmethionine (SAM), methionine disrupts the SAMTOR-GATOR1 complex by binding directly to SAMTOR protein, thereby activating mTORC1 by inhibiting the action of GATOR 1 (GU et al., 2017; MOTA-MARTORELL et al., 2020). Hence, when the action of Sestrin 2 and CASTOR 1/2 on GATOR 2 and the action of SAMTOR on GATOR1 disrupted, GATOR1 (the negatively regulator of mTORC1) will be inhibited so that mTORC1 activated (HESKETH et al., 2020).

The effect of dietary methionine on mTOR pathway in birds is not well studied. A study implemented by supplementing 20% higher methionine on the basal diet show higher upregulation effect on mTOR and an inhibitory effect on 4EBP1of broiler birds (WEN et al., 2014). Supplementation of methionine in the form of DL-methionine or DL-2-hydroxy-4-(methylthio) butanoic acid show no change in the expression of genes or relative phosphorylation of mTOR, S6K and 4EBP1 in all the starter, grower and finisher stage of broilers fed on wheat–soya bean meal-based basal diets (ZEITZ et al., 2019). A research conducted on fast growing and slow growing broiler chicks also shown no change in gene expression of mTOR and its downstream effectors with increase in 20% dietary methionine above optimum (WEN et al., 2017). In bovine mammary glands, higher

activation of mTOR pathway observed with the addition of up to 15% methionine on the breeders recommended level (YANG et al., 2015). This shows inconsistent results of the effect of amino acids on mTOR and its downstream effectors in birds. All the experiments were also done on group of birds based, where it has ambiguity in studying molecular parameters. Therefore, it needs further approval by intensifying experiments on individual birds and diverse laboratory analysis methods.

2.5. Conclusions and insights into avian system

The mTOR pathway is a signalling pathway that underpins a form of adaptive plasticity when individuals encounter constraints in their dietary availability. Feed restriction (FR) is a robust intervention used to study the effect of resource limitation on the mTOR pathway and subsequent life-history traits. Additionally, organisms often face an unpredictable recurrent fluctuation in dietary availability, which can substantially affect biological function. This may force organisms to change the mechanisms of growth, reproduction, immunity, and lifespan. Specific micro- and macronutrients have also proved to regulate the mTOR pathways and life history in mammalian models.

Energy metabolism and hormonal regulation are markedly different in birds compared to mammals, and physiological responses to dietary components may show different patterns. Birds exhibit substantially higher metabolic rates and maintain higher glucose levels while having more than twice as long lifespans as size-matched mammals. This observation seems counterintuitive to the general rule that animals with higher metabolic rates usually have shorter lifespan (HOLMES et al., 2001; SATOH, 2021). This paradox in birds may arise from their variable physiological response to nutritional availability, which differs from what is observed in mammals. Therefore, conclusions drawn from mammalian nutritional physiology cannot be directly applied to avian systems. Additionally, the physiological response to dietary variables may vary among avian species. Other studies have reported that FR increased plasma IGF-1 levels in female canaries (*Serinus canaria*) (HARGITAI et al., 2022), broiler chicken hens (HOCKING et al., 1994), and male and female bearded reedlings (*Panurus biarmicus*) (TÓTH et al., 2022). These mixed results indicate that, based on the existing evidence, it is difficult to draw a general conclusion about how changes in feed availability affect the IGF-1/mTOR system.

Given the paradoxical observations in birds, it becomes essential to investigate the mTOR pathway across gradients of dietary availability for a comprehensive understanding of the distinct patterns of energy metabolism and hormonal regulation in avian models under laboratory conditions. This is crucial for life history insights that cannot be extrapolated solely from mammalian studies. Recognizing mTOR pathway as a key proxy for growth and reproduction, as well as a regulator of trade-offs, examining its changes under different dietary conditions provides valuable insights into how organisms respond to different nutritional states and the subsequent impact on their physiological functions. Furthermore, the majority of bird studies have focused on juvenile/nestling stages, overlooking processes in adult subjects.

3. MATERIALS AND METHODS

All experiments were conducted in accordance with the EU Directive "Legislation for the protection of animals used for scientific purposes" and received approval from the Ethical Committee for Animal Use of the University of Debrecen, Hungary (Protocol No. 5/2021/DEMAB). We affirm that all the procedures were conducted in accordance with the applicable institutional standards and regulations.

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

3.1.1. Experimental animals and housing

Japanese quail chicks (Coturnix japonica), aged four weeks and comprising both males and females, 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 8 weeks old, 64 female and male birds with similar sex-specific body weights were chosen for acclimation and placed in individual cages.

During the acclimation period, the birds were provided with *ad libitum* access to feed and drink for 7 days. 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), as detailed in Table 1.

3.1.2. Experimental design

Before starting the experimental treatment, we conducted a 7-day assessment of the daily feed intake. The feed was provided in a plastic feeder with a capacity of 200 g, specifically designed to prevent any spillage of the feed. Each bird's live body weight was assessed at the beginning and end of the acclimatization phase to assess if there were significant changes in body weight. The objective was to initiate the experimental treatment after the birds had completed their growth phase.

Table 1. Ingredient composition and nutrient level of standardised basal feed for experimental quails

Feed ingredients	Inclusion rate, %
Corn	30.37
Wheat	20.00
Soybean meal (46% CP)	34.88
Sunflower oil	6.79
Limestone	5.64
MCP	1.29
Salt	0.38
DL-Methionine	0.15
Vitamin and mineral premix*	0.50
Nutrient content**	
Metabolisable energy MJ/kg	12.13 (13.93)
Crude protein	20.00 (17.8)
Methionine	0.45 (0.28)
Methionine + cysteine	0.75
Lysine	1.08 (1.12)
Threonine	0.74 (0.64)
Leucine	1.59 (1.40)
Isoleucine	0.86 (0.69)
Arginine	1.33 (0.89)
Tryptophan	0.25 (0.54)
Calcium	2.50
Available Phosphorus	0.35
Sodium	0.15

* 1 kg premix provided: 1000000 NE vitamin A, 200 000 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, 300000 mg choline cloride, 13200 mg Zn, 1920 mg Cu, 9612 mg Fe, 13200 mg Mn, 180 mg I, 42 mg Se, 12 mg Co. ** Measured values are in parenthesis.

At the onset of the trial, when the birds were 9 weeks old, both male and female individuals were assigned randomly 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. The average daily feed intake during the acclimation period for the ADL, FR20, FR30, and FR40 was 30.41 ± 1.07, 29.70 ± 1.11, 30.08 ± 1.14, and 29.76 ± 0.51, respectively, for females and 19.36 ± 1.39, 19.10 ± 1.46, 21.27 ± 0.79, and 19.31 ± 1.63 for males, respectively. 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. Each block comprised an equivalent number of males and females from each treatment group. The experiment was conducted for a duration of

14 days. The daily feed provided to the ADL group was quantified and analysed to detect any notable variation in temporal consumption. However, no significant changes were observed.

3.1.3. Measurements and sample collection

Body weight was assessed 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 (Figures 2A and 2B). Eggs were marked with bird identity, collected, and immediately weighed individually using a digital laboratory analytical balance (± 0.01 g, Figures 2A and 2C) on a daily basis. Blood samples were also collected on days 0, 7, and 14 of the study period (Figure 2A). When the automatic light system turned on, all the feeders were removed from all birds to ensure consistent feeding conditions (empty gut) between the ADL and restricted birds during measurement and sampling. To simplify the process of blood sampling and minimize the overlap between simultaneous samplings, a staggered sampling approach was implemented by initiating the experiment and blood sampling on consecutive days. This allowed ample collection from only 16 birds per day, with two birds from each of the four treatment groups and eight birds from each sex. No significant impact on IGF-1 ($p = 0.450$), triglyceride levels ($p = 0.489$), or body weight ($p = 0.469$) was observed due to staggering. In order to reduce handling time, multiple individuals bled 2 to 3 birds at the same sampling session on each sampling day. The median time it took from when the door of the experiment room opened to complete the bleeding process was 155.5 seconds. There was no noticeable change in IGF-1 ($p = 0.495$) or triglyceride levels ($p = 0.750$) caused by the handling time. Following the first blood sampling session, the experimental room remained undisturbed for one hour before proceeding with another sampling session from the remaining birds using the same method. The bleeding session had no significant impact on the levels of IGF-1 ($p = 0.694$) or triglycerides ($p = 0.607$).

Blood collection was performed every day from 8:00 a.m. to 10:00 a.m., after which proceeded to feed the birds and clean the room. Afterwards, the birds were not disturbed for the rest of the day. The 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 9000 × g for 10 minutes. The plasma sample was then stored at a temperature of −80 °C until it was used for laboratory purposes. On the 14th day of the trial, all birds were euthanized with the help of professional veterinarians by cervical dislocation (Figure 2D). The birds were then

immediately dissected to sample liver tissues. The liver tissue samples were promptly placed in a collection tube, quickly frozen using dry ice, immediately transported to the laboratory, and maintained at –80 °C until the next assay.

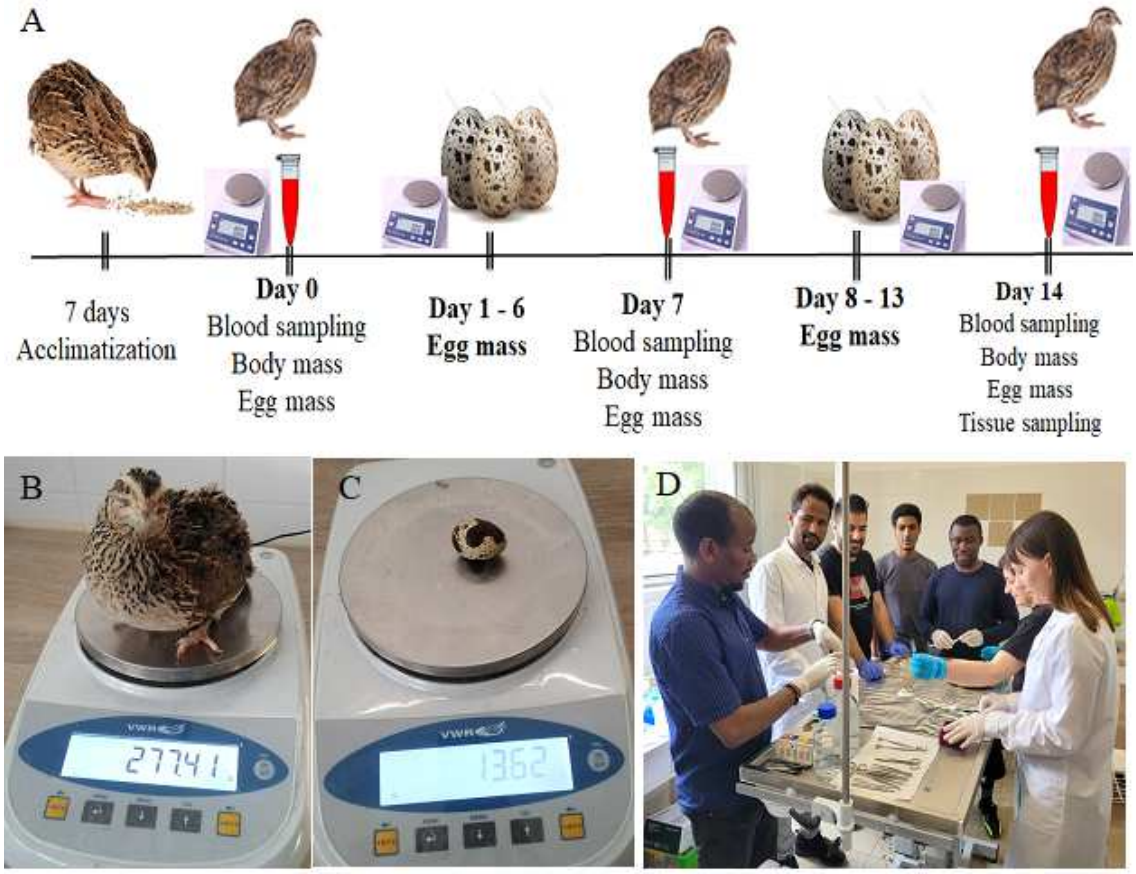

Figure 2. Measurement and sampling. (A) Series of activities in the experimental period; (B) live weight measurement; (C) egg weight measurement; (D) Tissue sampling.

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

3.2.1. Animals and housing conditions

Japanese quail chicks, hatched for research purposes in the Animal House of the Institute of Animal Science, Biotechnology, and Nature Conservation at the University of Debrecen (Hungary), were initially kept in a group of 10 birds in a room with constant temperature (25±2°C) under a 12:12 light:dark photoperiod. At the age of six months, 48 male and female birds were selected from the stock based on their uniform body weight (average weight for males: 249.14±12.49 g; females: 268.98±17.77 g) 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. The quail breeder basal feed was formulated using a soybean-corn-wheat

basis, as reported in Table 1 (NRC, 1994). During the seven-day feed intake measurement, daily feed intake was determined by calculating the difference between the amount of feed offered and the amount left unconsumed. 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.

3.2.2. Experimental design

The acclimated 48 birds of both sexes were weighed and distributed into one of the three dietary treatments (control-fed, unpredictably fed, and 40% restricted groups) based on their body weight, ensuring that the average body weight was similar among all experimental groups. Control birds received a daily amount of feed equal to their average feed intake individually. The unpredictably fed birds (UNPR) received the same amount of total feed 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 3). The trial was conducted for 16 days, and every morning, immediately when the light was on, feeders for all birds were removed before birds started eating. We measured the left feed and replenished it with a new feed.

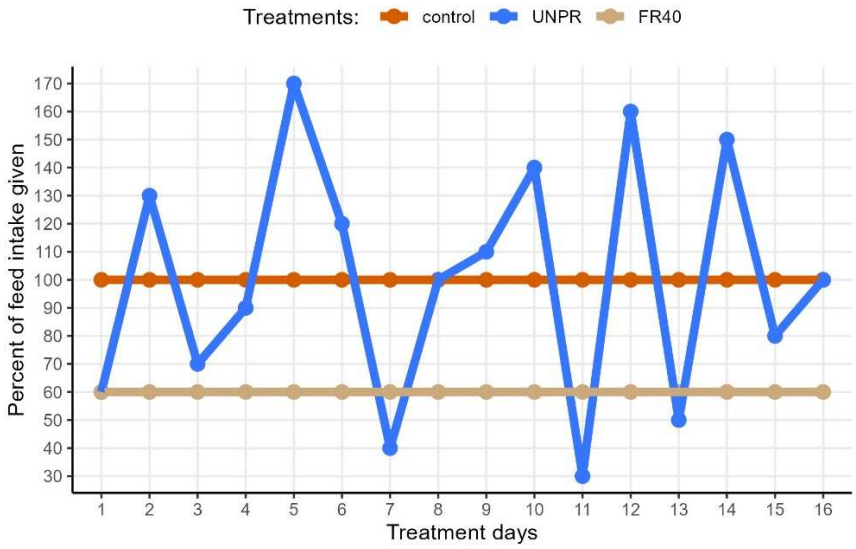

Figure 3. Experimental design for unpredictable feeding experiment. The 100% indicates feeding equivalent to the calculated feed intake during the acclimation period.

3.2.3. Measurements and sample collection

Initial body weight was recorded on day 0 (the beginning of the experiment) and subsequently measured on days 4, 8, and 16 during the treatment period. To measure egg traits, similar procedures to those mentioned in Section 3.1.3 above were employed. On day 16, all the birds were euthanised. Immediately after, liver and breast muscle samples were collected, then rapidly frozen on dry ice and stored at –80 °C until further assays.

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. Experimental animals, housing and treatments

Japanese quail eggs were incubated and hatched. After hatching, 340 newly hatched chicks were reared under grower feeding up to four weeks and breeder feeding up to five weeks (Table 3). At the age of five weeks, we selected and placed 56 female birds with similar body weights into individual cages for feed intake assessment and acclimation to the experimental conditions. Birds stayed for seven days under the *ad libitum* breeder feeding condition. During this period, we carefully monitored each individual's feed intake, which was used to calculate the amount of feed given to each bird according to its respective treatment. 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 positional effects (e.g., slight differences in light intensity along the cage height levels), 8 blocks with an equal number of birds from all treatments in each block were used.

Table 2. Treatment codes, their description, and the 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

Table 3. Composition and nutrient content of the experimental diets

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.

The quail basal feed was formulated using soybean, corn, pea, and wheat meal in the range of nutrient requirements of breeder quails (NRC, 1994). L-methionine and L-leucine were purchased from Vital-Trend Kft., Debrecen, Hungary. We added 20%

methionine, 20% leucine, or both 20% methionine and 20% leucine on top of the recommended levels and thoroughly mixed each. We controlled for branched-chain amino acid (BCAA) imbalance to the recommended level. The recommended range of ratio BCAA to poultry a breeder including Japanese quails is 1.8:1:1 to 2.2:1:1 for Lue:ILeu:Val (CARVALHO et al., 2022; PASTOR et al., 2013). Each supplemented diet was formulated and mixed separately. The experimental diets were formulated using a low-cost formulation program (WinFeed 2.8, WinFeed Limited, Cambridge, UK). When adding certain amount of specific amino acids, the programme adjust the amount of other ingredients proportionally to keep the consistent nutrient profile. By preparing technical mixtures of the added amino acids, and using all together 8 minutes mixing time, we ensured homogenous mixing. The metabolisable energy and protein restriction treatments were formulated by manipulating dietary ingredients.

The low-energy diet was achieved by reducing the levels of carbohydrate- and lipid-containing ingredients (corn, soybean meal, and oil) and increasing the levels of wheat and pea. The energy-restricted group received a feed with 9.7 MJ/kg metabolisable energy. The low-protein feed was achieved by reducing the amount of wheat and soybean meal and increasing the levels of corn and pea. Therefore, the protein content of the feed given to the protein-restricted group was 14.4% of the total feed. Our study does not assess the effects of fat and carbohydrate composition of feed on the mTOR pathway, which could be explored in future research. The ingredients and nutritional composition of each treatment feed are presented in Table 3. The trial was conducted for 14 days, and every morning, immediately after lights on, we removed the feeders before birds started eating, measured the remaining feed, and replenished the feeders with the pre-determined amount and type of feed. 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.

3.3.2. Measurements and sampling

Body weight was measured on days 0, 7, and 14 of the experiment, with the same procedure as in Section 3.1.3. On the last day of the experiment (day 14), all birds were euthanised by veterinary experts. Euthanised birds were dissected, the liver tissue of each bird removed and weighed, and a portion of it was sampled into a cryotube and rapidly frozen in the dry ice. Samples were then stored at -80°C until the following assay.

3.4. Laboratory analysis

3.4.1. RNA isolation

For samples from the first experiment, total RNA was isolated from liver tissue using the TRIzol reagent according to the manufacturer's protocol (Direct-zol™ RNA MiniPrep, Zymo Research Corporation, USA). The protocol includes a DNase digestion step to prevent DNA contamination. In summary, a sample weighing 25 - 30 mg was disrupted using a D1000 handheld homogenizer (Benchmark Scientific, USA) in 600 µl of TRIzol reagent. The resulting mixture was centrifuged at 16,000 g for 30 seconds at 4 °C. Next, the supernatant was transferred into an RNase-free tube and thoroughly mixed with an equivalent volume of ethanol (95 –100%). Next, the mixture was transferred onto a Zymo-Spin™ IIC column placed on a collecting tube and subjected to centrifugation at a force of 16,000 g for 30 seconds. The flow-through was discarded, and the RNA pellet was rinsed with 400 µl of RNA wash buffer, repeating the centrifugation procedure. Subsequently, a DNA digestion procedure was performed by adding 5 µl of DNase I (6 U/µl) and 75 µl of DNA digestion buffer and incubating at room temperature for 15 minutes. Following the addition of 400 µl of Direct-zol™ RNA PreWash, the RNA solution was centrifuged for 30 seconds and repeated the step. Next, 700 µl of RNA wash buffer was added for the final wash and centrifuged for 2 minutes to ensure the removal of all reagents. Ultimately, we obtained the purified RNA by adding 50 µl of DNase/RNase-Free water for subsequent RNA quality and quantity assessment. The RNA concentration and purity were evaluated using the HTX Synergy Multi-Mode Microplate Reader spectrophotometer (Agilent BioTek, BioTek Instruments Inc, USA). A 1% agarose gel electrophoresis was used to assess RNA integrity.

For samples from Experiment 2 and 3, 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). The RNA isolation protocol included a DNA digestion step using the peqGOLD DNase Digest Kit. Briefly, frozen liver (~25 mg) and muscle (~30 mg) tissues were excised and disrupted using mortar and pistol, and homogenised in 700 µL TRK Lysis Buffer. After centrifugation in 13,000 × g for 5 minutes, the lysate transferred to the peqGOLD RNA Homogenizer Column inserted into a 2 mL Collection Tube and centrifuged again at 13,000 x g for 1 minute. Equal volume 70% ethanol was added to the filtrate and vortexed to mix thoroughly. Then the sample was transferred to a peqGOLD RNA Mini Column

and centrifuged at 10,000 × g for 1 minute. After adding 250 µL RNA Wash Buffer I and centrifuged at 10,000 × g for 1 minute, the DNA digestion step followed by adding 75 µL DNase I stock solution and incubation for 15 minutes at room temperature. Nest, 250 µL RNA Wash Buffer I was centrifuged at 10,000 x g for 1 minute. Then, the washing step was applied by adding 500 µL of 80% ethanol and centrifuging at 10,000 × g for 1 minute, and this step was repeated. Finally, the RNA pellet was dried via maximum centrifugation for 2 minutes, and the RNA was eluted using 60 µL Nuclease-free Water. The RNA concentration and purity were measured using HTX Synergy Multi-Mode Microplate Reader spectrophotometer (Agilent BioTek, BioTek Instruments Inc, USA). RNA integrity was assessed using Qubit™ RNA IQ Assay according to the assay kit protocol (Life Technologies Corporation, Netherlands) using Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, US).

3.4.2. cDNA synthesis

For samples from Experiment 1, cDNA synthesis was performed using the qScript cDNA synthesis kit, following the manufacturer's protocol (Quantabio Reagent Technologies, QIAGEN Beverly Inc., USA) in PCRmax Alpha Thermal Cycler (Cole-Parmer Ltd., USA). To synthesise 20 µl of cDNA from 200 ng of total RNA, we utilised a reaction mix that included qScript cDNA SuperMix. This mix contained a 5X reaction buffer with optimized concentrations of MgCl2, dNTPs, recombinant RNase inhibitor, qScript reverse transcriptase, random primers, oligo(dT) primer, and stabilizers. We added RNase/DNase-free water to fill the final volume of the reaction mix. The thermal cycling conditions for cDNA synthesis were set as follows: initial priming at 25 °C for 5 minutes 1 cycle, reverse transcription at 42 °C for 30 minutes 1 cycle, and inactivation at 85 °C for 5 minutes 1 cycle. After synthesis, the cDNA samples were diluted 10-fold and stored at −20 °C until the next qPCR.

For samples from Experiment 2 and 3, cDNA synthesis was performed using the LunaScript® RT SuperMix Kit, following the manufacturer's protocol (New England Biolabs, Inc., USA). To synthesise 20 µL of cDNA, a 4 LunaScript® RT SuperMix reaction mix containing a random hexamer and oligo-dT primers, dNTPs, Murine RNase Inhibitor, and Luna® Reverse Transcriptase was used. 200 ng of total RNA was added and filled with RNase/DNase-free water to reach the 20 µL mix. The thermal cycling conditions for cDNA synthesis were set as follows: primer annealing at 25 °C for 2 minutes, cDNA synthesis at 55 °C for 10 minutes, and heat inactivation at 95 °C for 1

minute. After synthesis, the cDNA samples were diluted 10-fold and stored at -20 °C until needed for Real-Time PCR analysis.

3.4.3. 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). The qPCR was performed using the following thermal conditions: 95°C for 12 min (initial activation of the polymerase), followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and elongation at 72°C for 20 s. At the end of each run, the amplification specificity of each product was confirmed by melting curve analysis. Amplification, melting curve analysis, and monitoring were performed using the Agilent AreaMx Real-Time PCR System (Agilent Technologies, USA). Quail-specific primer pairs spanning introns were designed utilizing Oligo7 software and acquired from Integrated DNA Technologies (BVBA-Leuven, Belgium).

To verify target specificity, Primer-Blast software from the National Centre for Biotechnology Information (NCBI) was employed (https://www.ncbi.nlm.nih.gov/). The primer sequences and their characteristics are shown in Table 4. The average of each technical replicate of a sample was used for subsequent analysis. The data for target genes were normalized to a reference gene using the efficiency-corrected method (PFAFFL, 2004). Stable reference genes were selected using the NormFinder, BestKeeper, and deltaCt algorithms (MENG et al., 2019). The natural logarithm of the ratio of expressions was utilized for statistical analysis of the relative mRNA expression, henceforth referred to as gene expression. In order to account for differences between plates, certain samples were repeated across the plates for calibration purposes. For each gene, samples from both males and females (in experiments I and II), as well as from all treatment groups, were placed on the same qPCR plate.

Table 4. Primer sequences and their characteristics

Gene transcript	Sequence	Annealing temp, °C	Product size, bp	Accession No.
Target genes				
GHR_F	GGC ACT GGT CTG TGT GAA TGA CT	57.93	89	XM_032441512.1
GHR_R	CCA GCT CAG GTG ATC TGC ACT T	57.58		
IGF1_F	CAC TAT GCG GTG CTG AGC TGG TT	55.8	118	
IGF1_R	ATC CCC TTG TGG TGT AAG CGT CT	55.4		XM_015867574.2
IGF1R_F	TAC AAC TAC CGC TGC TGG ACC AC	56.0	107	
IGF1R_R	AGG CAC TCA GGA TGG CAA CAC	55.0		XM_015873184.2
mTOR_F	CCG AAG CAT TGA ATT GGC CCT	53.5	116	
mTOR_R	CAT CTC TCA AAG GCA GCG GAC C	55.2		XM_015882433.2
RPS6K1_F	AGG CAG GAA CCC TCC GTG CAA	58.8	106	
RPS6K1_R	AAG CTC AAA CTG CGA AGG GTC GG	56.9		XM_015883670.2
4EBP1_F	ACC AGC CCA ATT GTG GAG GAG TT	59.29	120	XM_015883175.1
4EBP1_R	CTC AGG GCA CGT GCT TTA GAT GT	58.03		
FASN_F	TCA GCC CGA ACC TCC GCC AT	61.24	72	XM_015879647.2
FASN_R	ATG CCT GCA ATC ACC ACG TCT	57.67		
FOXO1_F	AAGGGCGACAGTAACAGTTCAGC	58.21	102	
FOXO1_R	CTTCCCTGTGCCCTCATTCTGC	58.47		XM_015851898.1
ULK1_F	ACAGACTCTGCTGGGCAAGGA	58.47	105	
ULK1_R	ACCAAGTAGACAGAATTTGCCACCTC	58.14		XM_015877646.2
ATG9A_F	CAA CGC CCT CAG GAT CCC CAT	56.8	69	
ATG9A_R	ACG ATG CGG GCC TGT ACC TCC	59		XM_015868966.2
ATG5_F	ATA GTG GAT TTC GGT ACA TCC CA	54.03	95	XM_015858736.2
ATG5_R	TCC TCC AGA AGC AAT TGG TCG	55.34		
SOD2_F	ACA GCA AAC ACC ACG CCA CCT	60.38	100	XM_015858046.1
SOD2_R	AGC GAC ACC TGA GCT GTA ACA T	56.99		
Reference genes				
ACTB_F	CCC CTG AAC CCC AAA GCC AAC	56.2	114	
ACTB_R	ACC AGA GGC ATA CAG GGA CAG C	56.1		XM_015876619.1
GAPDH_F	GCA CTG CGC CAC CTT CTC ACT	57.5	116	
GAPDH_R	TGA CCA GGC GGC CAA TAC GG	57.5		XM_015873412.2
RN18s_F	CCC TGC CGG AGC GTC GAG AA	59.4	102	
RN18s_R	CCG GTA ATG ATC CTT CCG CAG GT	56.1		XR_006936397.1
RPL19_F	CATCGGTAAGAGGAAGGGT	50.8	162	
RPL19_R	ACGTTGCCCTTGACCTTCAG	55.54		XM_015885843.1
SDHA_F	GCG CAG CCT GGC TAA GGG A	59.93	83	XM_015854268.1
SDHA_R	AGT GGA AGT TGC GTG CAA GAG T	27.95		
YWHAZ_F	AGA CTG AGC TAA GAG ACA TCT GC	54.62	91	XM_015856086.1
YWHAZ_R	AAA ACT TTG CTT TCT GCT TGC G	54.40		

* *GHR*, growth hormone receptor; *IGF1*, insulin-like growth factor 1; *IGF1R*, insulin-like growth factor 1 receptor; *mTOR*, mechanistic target of rapamycin; *RPS6K1*, ribosomal protein S6 kinase 1; *4EBP1*, Eukaryotic translation initiation factor 4E-binding protein 1; *FASN*, fatty acid synthase; *FOXO1*, forkhead box O1; *ULK1*, Unc-51 like autophagy activating kinase 1; *ATG9A*, autophagy-related gene-9A; *ATG5*, autophagy-related 5; *SOD2*, superoxide dismutase 2; *ACTB*, beta-actin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *RN18s*, 18S ribosomal RNA; *RPL19*, ribosomal protein L19; *SDHA*, Succinate dehydrogenase A; *YWHAZ*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

3.4.4. IGF-1 Enzyme-linked immunosorbent assay (ELISA)

Plasma IGF-1 levels were assessed using a competitive ELISA assay method developed and validated for birds, as detailed in MAHR et al. (2020). Briefly, Nunc™ 96-Well Polypropylene MicroWell™ microtiter plates (Thermo Scientific™) except blank

wells were coated overnight with 100 µL of polyclonal anti-serum. On the next day, unbound capture antibodies were removed, and the wells were washed using 250 µL of washing buffer. Subsequently, 80 µl of biotinylated IGF-1 tracer solution was pipetted into each well. Following this, 20 µL of assay buffer was added to the blank and total binding wells, while 20 µL of standard solution and 10 µL of plasma sample along with 10 µL of assay buffer were added to the standard and sample/reference wells, respectively. The plate was then incubated at room temperature for 2 hours. Post-incubation and further washing, 100 µL of streptavidin-horseradish peroxidase (HRP) enzyme conjugate dilution was added to each well and incubated for 30 minutes. After removing unbound enzymes through washing, 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) chromogenic substrate (Acros Organics) was added to each well and incubated at room temperature for 30 minutes. The enzymatic reaction was halted by adding 100 µL of 1M H2SO4 solution to each well, and absorbance was measured at 450 nm (with reference at 620 nm) using a Magellan F50 spectrophotometer plate reader (Fornax Technologies GmbH, Bühlinger Straße 56, Germany).

3.4.5. Triglyceride measurements

Plasma triglyceride levels were analysed using a photometric method with a half-automatic analyser (Lab-Analyse, Orvostechnika Ltd., Budapest, Hungary) according to the manufacturer's protocol. Briefly, 5 µL plasma sample was added into a unit of triglyceride reagent (pH = 7.20 of 50 mmol/L PIPES buffer, 5 mmol/L Mg++, 4 mmol/L 4-chlorophenol, 2.0 mmol/L ATP, 2000 U/L lipoprotein lipase, 400 U/L glycerol kinase, 1500 U/L glycerol-3-phosphate oxidase (GPO), 2000 U/L peroxidase and 0.4 mmol/L 4-aminoantipyrine). Sample was added to the reagent, thoroughly mixed, and incubated for 5 minutes, then measured in the photometric device at a wavelength of 505 nm. Triglyceride reagent was used as a blank before measurement.

3.5. Statistical Analyses

All data were analysed using R v. 4.3.2, 'Eye Holes' (https://www.r-project.org/). The `ggplot` function to process all graphs and the `ggsave` function to save the graphs at a resolution of 300 DPI were employed from the `ggplot2` v.3.4.3 package (WICKHAM, 2016). The `lmer` function from the `lme4` package (BATES et al., 2015) was used to estimate model parameters for both fixed and random effects. Additionally, the `lmerTest` v. 3.1-3 package (KUZNETSOVA et al., 2017) was employed to compute p-

values in the ANOVA and model summary tables. For mean comparison, the `emmeans` function adjusted for Tukey was used as a posthoc test with a $p < 0.05$ significance level (LENTH et al., 2018).

The best-supported models were selected using Akaike's Information Criterion corrected (AICc) as (BURNHAM and ANDERSON, 2010). 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. The structure of the initial saturated models included dietary treatments, treatment period, sex, and their interactions as fixed factors. Individual bird identity (to control for the effect of repeated measures) was nested within experimental blocks (in the first and third experiments) as random variables (HARRISON et al., 2018). Linear models were employed to analyse the effect of treatments on total egg number and treatment and sex on gene expression. Molecular and physiological variables were transformed into natural logarithms to reduce skewness due to individual biological variability. To analyse egg weight, we used a Gaussian polynomial mixed-effect model (DEMIDENKO, 2013) to incorporate the polynomial linear pattern of the variables across days. We used generalised linear mixed-effects models of the family logit using package `aod` v. 1.3.2 (LESNOFF and LANCELOT, 2012) to analyse the binary response variable (daily egg laying). To analyse association among expression of different genes, we employed Pearson correlation using the `corr.test` function in the `psych` package v. 2.3.3 (REVELLE, 2023). To control for the family-wise error rate, the function automatically performs p-value adjustments using Bonferroni correction (WEISSTEIN, 2013).

As expression of different genes showed significant correlation, principal component analysis (PCA) was employed to test the overall association of gene expressions with body weight and egg production traits. This process transformed the original correlated gene expression variables into a new set of linearly uncorrelated principal components. The `prcomp` function from the `stats` package was utilized for this purpose. Principal Component Analysis (PCA) was employed to mitigate the issue of multicollinearity that may arise among the predictor variables (gene expressions). We utilized 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).

4. RESULTS AND DISCUSSION

4.1. Experiment 1: Effect of feed restriction on morphological, molecular and physiological traits in Japanese quails

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

While there is no significant initial body weight difference among groups in both sexes, feed restriction significantly affected body weight across time points (Table 5). 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 4, Appendix Table 1). The FR40 treatment also resulted in significantly lower body weight than the FR20 at both time points (week 1: $p = 0.039$, week 2: $p = 0.012$, Appendix Table 1), 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 (Appendix Table 1). The FR30 showed a significant body weight reduction from week 1 to week 2 ($p = 0.035$), 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 (Appendix Table 2).

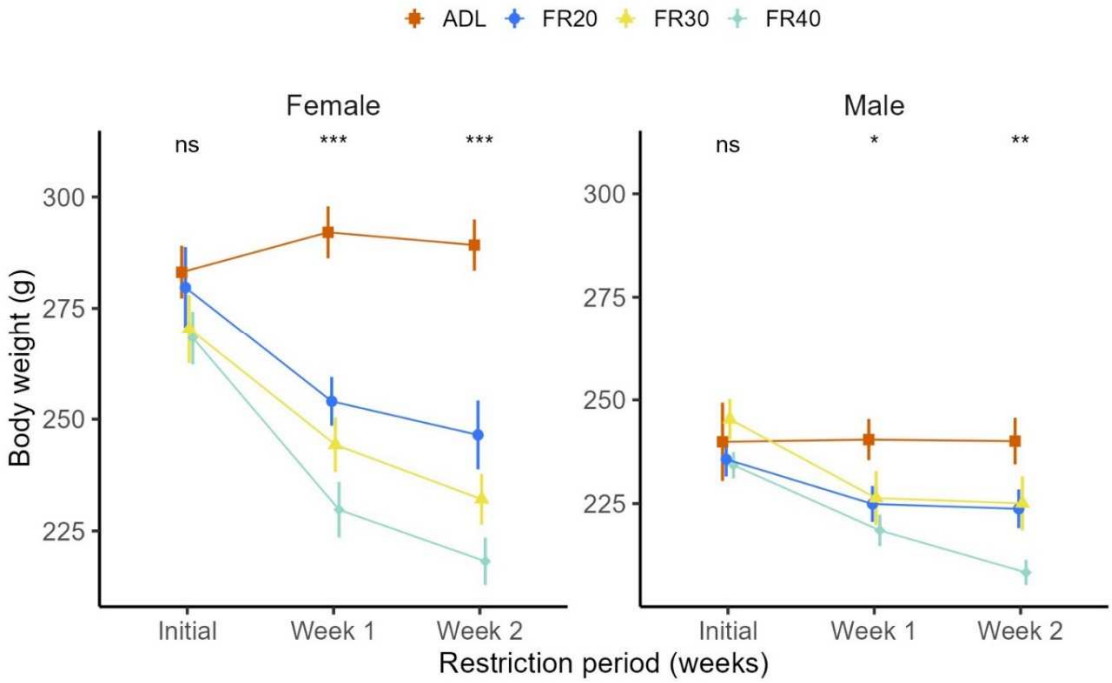

Figure 4. The effect of different feed restriction treatments on body weight of female and male quails at different time points. Data are represented by the mean ± SEM from 8 birds per group. Abbreviations: 'ns', not significant at $p < 0.05$; '*' significantly different at $p < 0.05$; '**' significantly 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 lower body weight trend compared to the ADL group quails (Figure 4). However, only quails from FR40 proved a statistically significant reduction in the first and second weeks (week 1: $p = 0.02$; week 2: $p < 0.001$; Appendix Table 1). 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 the FR40 group showed further weight loss from week 1 to week 2 ($p = 0.050$; Appendix Table 2).

Table 5. Output of the linear mixed-effects model showing the effect of feed restriction treatment on body weight across the time points (week) in female and male Japanese quails

Fixed factors	***NumDF***	***DenDF***	***F value***	***p-value***
treatment	3	56	14.70	<0.001
week	2	112	94.06	<0.001
sex	1	56	58.41	<0.001
treatment × week	6	112	16.50	<0.001
treatment × sex	3	56	3.74	0.016
week × sex	2	112	9.88	<0.001
treatment × week × sex	6	112	3.01	0.009

Abbreviations: NumDF, numerator degree of freedom; DenDF, Denominator degree of freedom

Feed restriction is widely applied in broiler breeders and layer chickens to reduce fast early growth, delay sexual maturation, prevent metabolic disorders, and increase reproduction lifespan (CARNEY et al., 2022; LU et al., 2023; RENEMA and ROBINSON, 2004). In other model organisms, feed restriction is applied to study physiological and molecular markers of aging and health span (MCCRACKEN et al., 2020; MOATT et al., 2020; TAORMINA and MIRISOLA, 2014). Consistent with previous studies on different organisms (KITAYSKY et al., 2001; TÓTH et al., 2022; ZHANG et al., 2018), FR showed a strong effect on body weight. In the current study, the FR20, FR30, and FR40 regimens showed a strong effect on body weight compared to ADL-fed quails. We demonstrated that the 20% feed restriction is sufficient to sense scarcity in female quails, and only the 40% restricted group showed a tendency to further reduce body weight from week 1 to week 2. Hence, a one-week restriction is enough to demonstrate the relevant body weight change under the prelisted FR levels (REDA et al., 2024b). Most FR studies on chickens are applied at an early age and effectively affect late-life performance (DEMIR et al., 2004; FONTANA et al., 1992; ZHAN et al., 2007).

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

Throughout the restriction treatment, there were noticeable differences in how males and females responded to feed restriction. This was evident from a significant interaction between sex, treatment, and restriction time, as shown in Table 5. 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 5, Appendix Table 3).

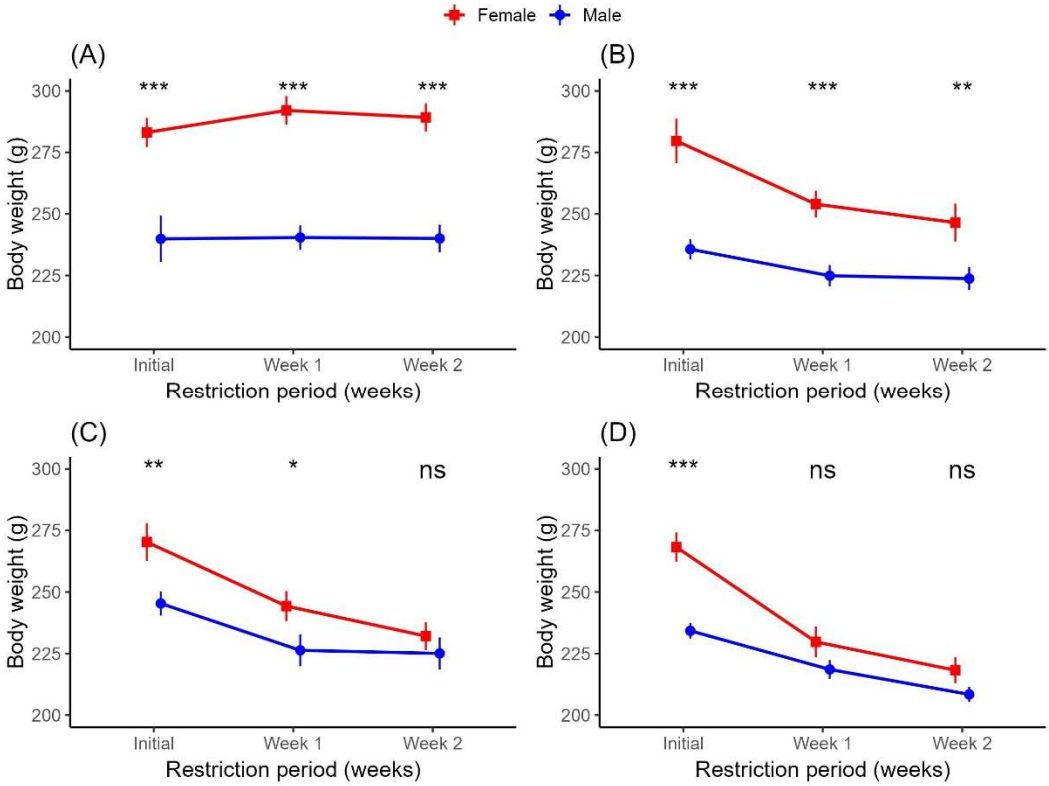

Figure 5. Comparing body weight of female and male Japanese quails in different feed restriction levels across restriction period. Different shaped dots and vertical bars represent the mean ± SEM from 8 birds per group, and data were analysed using ANOVA of linear mixed-effect model. Abbreviations: 'ns', not significant at $p < 0.05$; '*' significantly different at $p < 0.05$; '**' significantly different at $p < 0.01$; '***' significantly different at $p < 0.001$. Panels represent (a) Sex difference of body weight at (A) *ad libitum* group, (B) 20% restriction (C) 30% restriction, (D) 40% restriction.

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 (HORVÁTHOVÁ et al., 2012; 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. Alternatively, these results may occur due to sexual differences in digestive physiology. Males may experience a longer gut transit time, facilitating effective nutrient absorption to compensate for nutritional deficiencies. Such effects have been documented in sexually dimorphic species (MARKMAN et al., 2006; SANTIAGO-QUESADA et al., 2009), including the Japanese quail (NÓBREGA et al., 2020). Longer intestines and higher gut transit times may result in a higher assimilation efficiency in males, and they may even possess higher plasticity to grow intestines (NILAWEERA and SPEAKMAN, 2018; VAN GILS et al., 2008) in response to feed restriction, although this possibility awaits further research in quails.

4.1.3. 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 6A, Table 6). 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 6B). 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.

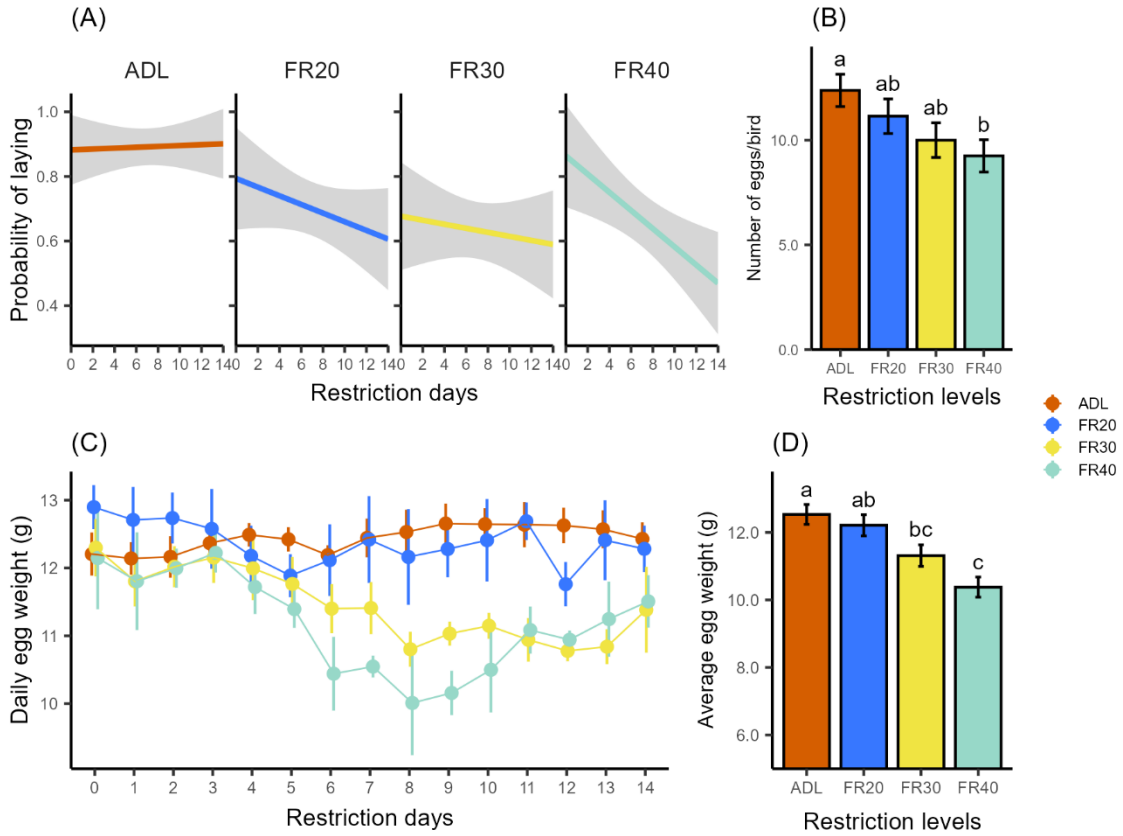

Figure 6. Effect of feed restriction on egg laying probability and egg number and egg weight. (A) Probability of daily egg laying, (B) total number of eggs, (C) daily egg weight (g), (D) average egg weight in the two weeks.

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 < .001; treatment × day: $F_{6,309.83}$ = 14.85; p < .001). The time-dependent trend indicated that egg weight was significantly reduced in the FR30 and FR40 groups starting from day 5 (Figure 6C). 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-week 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 6D). 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 12).

Table 6. Output of the generalised linear mixed-effect model of the family logit to predict the probability of daily egg laying as a function of feed restriction levels and restriction days.

Predictors	Estimates	SEM	*z value*	*p-value*
Intercept	2.93	0.54	5.37	<0.001
FR20	-1.38	0.67	-2.06	0.0391
FR30	-1.71	0.67	-2.55	0.0108
FR40	-1.60	0.66	-2.41	0.0158
Day	-0.08	0.03	-2.82	0.0048
Random effects				
birdID Variance	1.186			

N = 480; Abbreviations: birdID, individual bird identity; SEM, standard error of mean

4.1.4. 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 7 and 8). Restriction treatment showed a significant effect on *GHR* gene expression (Table 7). Intriguingly, *GHR* showed significantly increasing gene expression, respectively. Both the FR3 and FR40 showed significantly higher *GHR* expression than the ADL and FR20 in females. Both groups showed the same trend in males but with no statistical difference among treatment groups (Figure 7A). Our result contradicted the previously reported findings in mammals and fishes, where nutritional deficit reduces *GHR* expression and causes growth hormone resistance, consequently limiting *IGF1* expression (DAUNCEY et al., 1994; MAES et al., 1991; WALOCK et al., 2014; WANG et al., 2003).

FR significantly decreased *IGF1* gene expression in both sexes (Table 7). All the restricted groups had significantly lower *IGF1* gene expression than the ADL-fed controls (Figure 7B). 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 7C, Table 7). The *IGF1* and its receptor (*IGF1R*) are the crucial genes of interest in a nutrient-sensing pathway (BUTLER and LEROITH, 2001; FORBES et al., 2020; LODJAK et al., 2018).

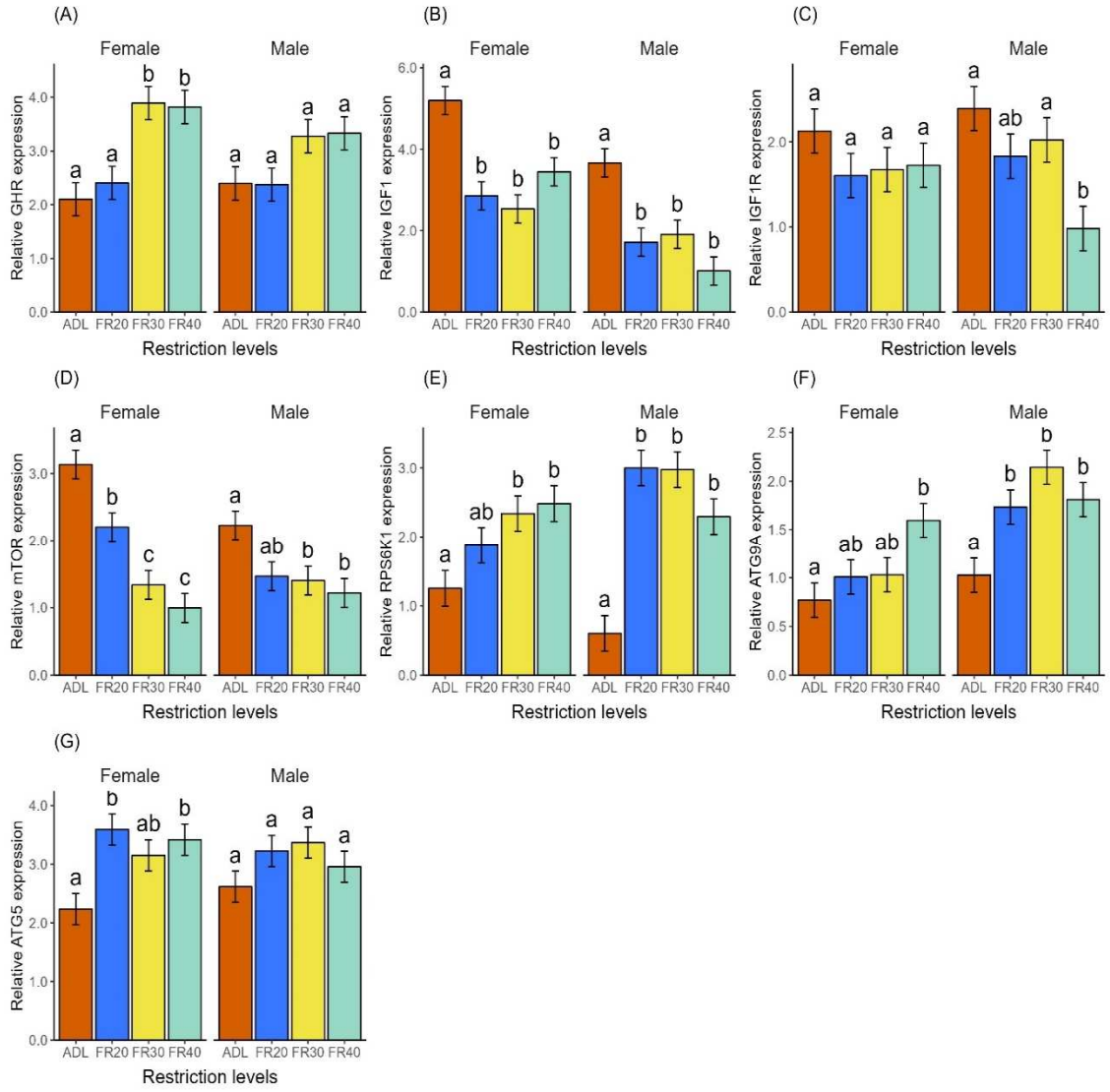

Figure 7. Effect of feed restriction on expression of certain mTOR pathways genes in female and male Japanese quails. (A) growth hormone receptor (GHR), (B) insulin-like growth factor 1 (IGF1), (C) insulin-like growth factor 1 receptor (IGF1R), (D) mechanistic target of rapamycin (mTOR), (E) ribosomal 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.

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. IGF-1 and its receptor (IGF-1R) play pivotal roles in growth and development across various organisms. IGF-1, mainly secreted in the liver, acts as a critical mediator

of cell growth, differentiation, and survival. It exerts its effects by binding to its specific receptor, which triggers a cascade of signalling, mainly the mTOR pathway, responsible for cellular proliferation (IPSA et al., 2019; NEIRIJNCK et al., 2019). The directional contradiction of *GHR* and *IGF1* gene expression in response to FR was against the previously established conclusion and needs further study from transcription through posttranscriptional modification to translation and posttranslational modification.

The *mTOR* gene expression showed a significant and gradual decrease across the FR levels (Figure 7D, Table 7). 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). The expression of the *mTOR* gene is crucial for the cellular production of the mTOR protein, which is then assembled into mTORC1 or mTORC2 complexes along with other component proteins (SZWED et al., 2021). The reduced *mTOR* gene expression could contribute to a lower mTORC1 abundance for activation (REDA et al., 2024b). Concurrently, FR can downregulate the expression of potential mTORC1 upstream activators. On the other hand, in normal nutritional conditions, activated mTORC1 suppresses mTORC2 activation by phosphorylating S6K1 (LIU et al., 2013; OH and JACINTO, 2011; SZWED et al., 2021; WU et al., 2022). The mTORC1-activated S6K1 phosphorylates mTORC2 at Rictor and Sin1 members of the complex and impairs mTORC2 as a negative feedback loop for mTORC1 activation (JULIEN et al., 2010; RAGUPATHI et al., 2024; WU et al., 2022). Consequently, reduced *mTOR* gene expression during FR may have a positive impact on the activation of mTORC2 and subsequent cell survival under dietary stress. mTORC2 is also activated by the energy stress sensor AMPK under restriction conditions (SZWED et al., 2021).

Interestingly, the expression of *RPS6K1* was increased in response to the treatments in both sexes (Table 7), showing an increasing trend with the severity of the restrictions in females (Figure 7E). We expected that downregulation of *mTOR* expression would reduce the expression of *RPS6K1* and would subsequently reduce body weight (BAE et al., 2012). However, the result contradicted our assumptions (REDA et al., 2024a; REDA et al., 2024b). While a consistent response is expected (BUCCITELLI and SELBACH, 2020), the expression of *RPS6K1* gene and the phosphorylated S6K1 protein might respond differently to upstream effectors. Protein expression of S6K1 kinase alone is not sufficient to initiate ribosomal protein translation; instead, it needs to be

phosphorylated by the activated mTORC1 kinase (HOLZ et al., 2005). Hence, the mechanism of action of *mTOR* and *RPS6K1* gene expression, their total protein expression, their phosphorylated expression, and their impact on shaping production are of future research interests. Although there is growing evidence for the correlation between gene expression and protein abundance (BUCCITELLI and SELBACH, 2020; KOUSSOUNADIS et al., 2015; NIE et al., 2006), post-transcriptional modification may alter the biological function of these genes. Previous studies have reported reduced *RPS6K1* expression in the liver of overfed geese (HAN et al., 2015). Currently, the paucity of research reporting the effect of FR on *RPS6K1* gene expression hinders the generalisation of the observed patterns.

Table 7. Output of two-way ANOVA from a linear model, indicating the effect of feed restriction on expression of liver mTOR pathway genes

Response variables	Explanatory Variables	Sum Sq	Mean Sq	df	F-value	p-value
GHR	treatment	25.56	8.52	3	11.09	<0.001
	sex	0.71	0.71	1	0.93	0.340
	treatment × sex	2.12	0.77	3	0.92	0.437
IGF1	treatment	57.22	19.07	3	19.80	<0.001
	sex	32.79	32.79	1	34.05	<0.001
	treatment × sex	7.01	2.34	3	2.42	0.075
IGF1R	treatment	6.71	2.24	1	4.13	0.010
	sex	0.01	0.01	3	0.02	0.895
	treatment × week	3.17	1.06	1	1.95	0.132
mTOR	treatment	22.92	7.64	3	20.83	<0.001
	sex	1.84	1.84	1	5.02	0.029
	treatment × sex	3.84	1.28	3	3.49	0.021
RPS6K1	treatment	25.62	10.05	3	18.94	<0.001
	sex	0.75	0.75	1	1.59	0.212
	treatment × sex	6.48	2.16	3	479	0.005
ATG9A	treatment	6.06	2.02	3	8.17	<0.001
	sex	5.34	5.34	1	21.59	<0.001
	treatment × sex	2.18	0.73	3	2.93	0.041
ATG5	treatment	9.32	3.11	3	5.49	0.002
	sex	0.05	0.05	1	0.09	0.760
	treatment × sex	2.11	0.70	3	1.24	0.303

Furthermore, the restriction treatment significantly increased autophagy genes (Table 7). 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 7F). All restricted groups

showed higher *ATG5* gene expression compared to the ADL groups, though treatments showed no statistical variation in the male groups (Figure 7G, Table 7). The effect of FR is consistent with previous studies on other organisms (GREEN et al., 2022; YANG et al., 2022). Autophagy genes are responsible for the initiation, elongation and integrity of autophagosomes, which in return are crucial for the degradation and recycling of cellular contents in response to nutritional deficiency (JUDITH et al., 2019; KARANASIOS et al., 2016; PIEKARSKI et al., 2014). Systemic and cellular nutritional limitations are reported to induce the initiation of the autophagy process (BAGHERNIYA et al., 2018; CHUNG and CHUNG, 2019). The effect is governed by upstream effectors, including *mTOR* expression and activation levels its respective kinase (REDA et al., 2024b), which is discussed in detail in Section 4.1.3, about the relationship of genes.

4.1.5. Sex difference in hepatic gene expression

Sex significantly affects the expression of most of the measured mTOR pathway genes (Table 7). 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 8A). 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 8B). 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 8C). Although little is known about the sex differences on 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; MCMURTRY et al., 1997; TÓTH et al., 2022; TÓTH et al., 2018). For instance, a study in chickens suggested a strong correlation between the plasma levels of IGF-1 and the expression of the *IGF1* gene in the liver (BURNSIDE and COGBURN, 1992; GIACHETTO et al., 2004; KITA, 1998; KITA et al., 1996). Therefore, the evidence indicating sex-specific levels of plasma IGF-1 could align with the hepatic gene expression patterns we observed. Reduced *IGF1* gene expression due to FR also suggests a corresponding effect on circulating IGF-1 levels (KITA, 1998; KITA et al., 1996). Therefore, the current study suggests sex-specific *IGF1* gene expression but not treatment-specific differences between females and males (REDA et al., 2024a). This may also highlight that the IGF-1/IGF1R axis plays a pivotal role in sexual dimorphism, influencing the distinct

physiological and morphological differences between males and females upon interaction with sex steroids (ASHPOLE et al., 2017; METER et al., 2022; REDA et al., 2024a; TÓTH et al., 2022).

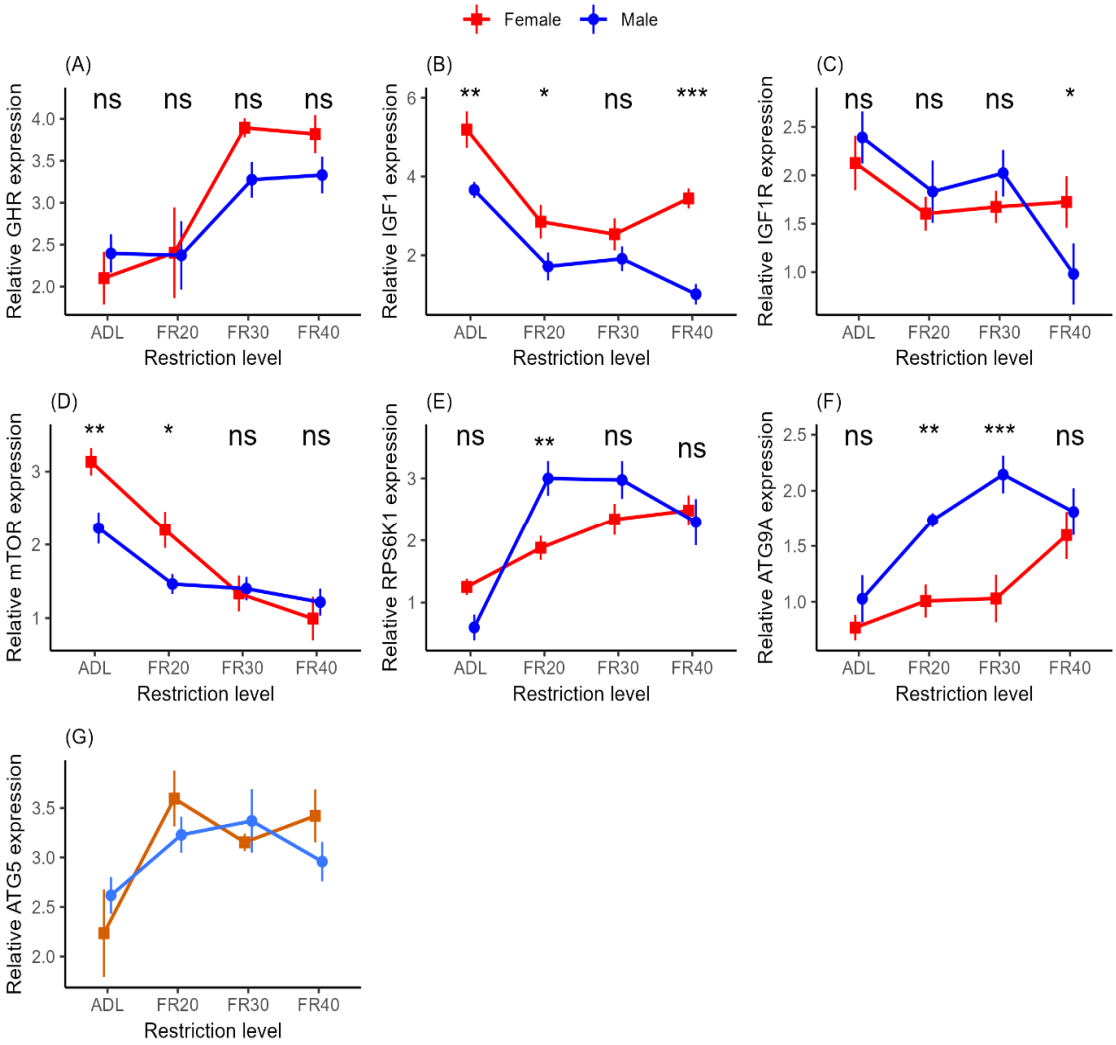

Figure 8. Sex-specific effects of dietary restriction in Japanese quails. (A) growth hormone receptor (*GHR*), (B) insulin-like growth factor 1 (*IGF1*), (C) insulin-like growth factor 1 receptor (*IGF1R*), (D) mechanistic target of rapamycin (*mTOR*), (E) ribosomal protein S6 kinase 1 (*RPS6K1*), (F) autophagy-related 9A (*ATG9A*), (G) autophagy-related 5 (*ATG5*). Dots and vertical bars represent the mean ± SEM from 8 birds per group. Abbreviations: ADL, *ad libitum*; FR20, 20% restriction; FR30, 30% restriction; FR40, 40% restriction. 'ns', not significant at $p < 0.05$; '*' significantly different at $p < 0.05$; '**' significantly different at $p < 0.01$; '***' significantly different at $p < 0.001$.

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 8D). 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 4), 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; YU and HENSKE, 2006). Females need more feed than males to maintain their larger body weight and egg production may force their *mTOR* expression to respond strongly to restriction in females than in males (REDA et al., 2024b).

Females exhibited lower *RPS6K1* expression in the FR20 group with no difference in the ADL, FR30, and FR40 groups (Figure 8E). The pattern of increase was more pronounced in males in the FR20 and FR30 groups. Since S6K1 plays a critical role in ribosomal translation (MA et al., 2015a; UM et al., 2006), its gene expression would contribute to maintaining the level of the respective kinase for phosphorylation. This, in turn, would help reduce the loss of body weight that we observed in females but less in males. The result was contrary to our assumptions. While there was no sexual difference in *ATG5* gene expression (Figure 8G), *ATG9A* showed lower expression in females compared to males (Figure 8F). 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 8F).

At the expense of anabolic progressions and stress, FR has a critical role in maintaining pathways required to retain cellular function. In conditions of scarce resources, autophagy serves as a cytoprotective mechanism through recycling of damaged organelles and malformed proteins, a process in which cells break down their components to provide energy and nutrients(JIA and LEVINE, 2007; MORSELLI et al., 2010). Restriction treatment upregulates autophagy, one way, through inhibition of mTOR activity that facilitates the nuclear localisation of Transcription Factor EB (TFEB), the ATG transcription factors in the nucleus(MARTINA et al., 2012; SCIARRETTA et al., 2022). Accordingly, 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 keep 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. In

females, there was a moderate increase in *ATG9A* expression and a rapid reduction in *mTOR*.

4.1.6. Association among gene expressions

Most of the analysed genes were significantly associated (Figure 9). Unexpectedly, *GHR* expression had no significant correlation with expression of *IGF1* and *IGF1R* genes, while significantly and negatively correlated with *mTOR* expression and positively with *RPS6K1*, *ATG9A* and *ATG5* expressions. The *IGF1* gene expression showed a significantly positive correlation with *mTOR* expression, whereas a negative correlation with expression of *RPS6K1* and *ATG9A* genes. The direction of association of *IGF1* to others genes was in line with our assumption except the non-significant association with *GHR* and negative association with RPS6K1. The *mTOR* also showed a positive correlation with *IGF1R*, while negatively correlated with *RPS6K1* and *ATG9A* gene expression. The negative association of *mTOR* expression with *GHR* and *RPS6K1* expression was out of our assumption. The expression of *RPS6K1* significantly and positively correlated with both autophagy genes, which was contrary our assumption.

The mTOR pathway not only affects translation but is also a key regulator of gene transcription by regulating the activity of specific transcription factors, epigenetic mechanisms or by affecting RNA stability (LAPLANTE and SABATINI, 2013). The modification of transcription factors is important for their activation, translocation, interaction, stability, and binding affinity (FILTZ et al., 2014; SUKUMARAN et al., 2020). mTORC1 phosphorylates transcription factors in response to resource availability, which in turn regulates several essential genes. Evidence shows that mTORC1 itself can function as a transcription factor when it is localised in the nucleus (JIANG, 2010; TSANG et al., 2010). Therefore, activated mTORC1 can upregulate the transcription of *IGF1*, *IGF1R,* and the *mTOR* gene itself. In the case of FR, the inhibition of mTOR can lead to a decrease in the expression of genes involved in growth and reproduction, including *IGF1*. Contrarily, 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; NAPOLITANO and BALLABIO, 2016). These transcriptional factors are mainly related to maintenance of cellular homeostasis by regulating autophagy and lysosomal genes at the transcriptional level during nutritional deficiency (INOKI et al., 2012; MARTINA et al., 2012). The

correlation analysis in the present study revealed that the expression of *ATG9A* and *ATG5* is negatively related to *mTOR* expression (Figure 9), indicating that the downregulation of mTOR mediates the upregulatory effect of FR on autophagy genes.

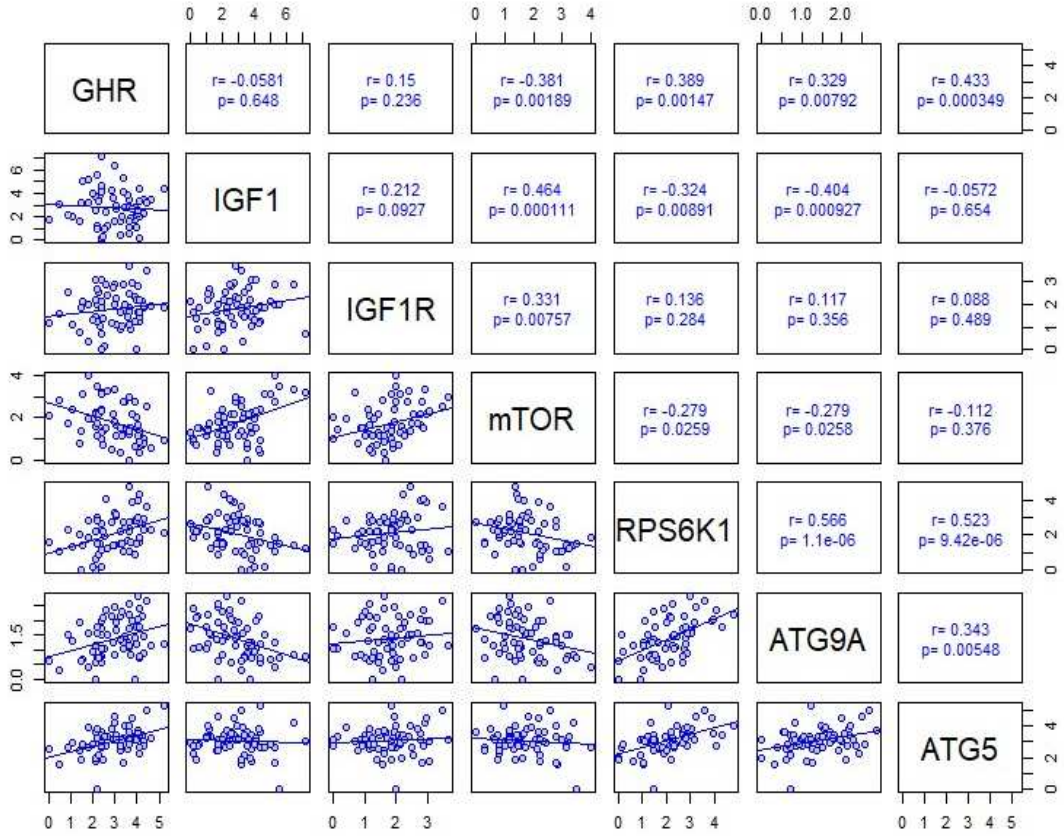

Figure 9. Pearson correlation among expression of mTOR pathway genes.

4.1.7. Association of expression individual mTOR pathway genes with production traits

To observe how individual genes are associated with body weight and main egg production traits, we employed linear. The linear association indicated that genes have a significant association with body weight and egg production traits. The *GHR* expression significantly and negatively affected female body weight (Figure 10A) and positively affected egg weight (Figure 10C). The expression of *IGF1* (Figure 10D-F) and *mTOR* (Figure 10J-L) significantly and positively explains all traits. *IGF1R* expression showed significant association only with male body weight (Figure 14G). The *RPS6K1* expression showed a negative relationship with female body weight (Figure 10M) and egg weight (Figure 10O), while the *ATG9A* expression showed a negative association with all the three variables (Figure 10P-R). The *ATG5* showed no significant association with any of the response variables and were presented in the association panel plot.

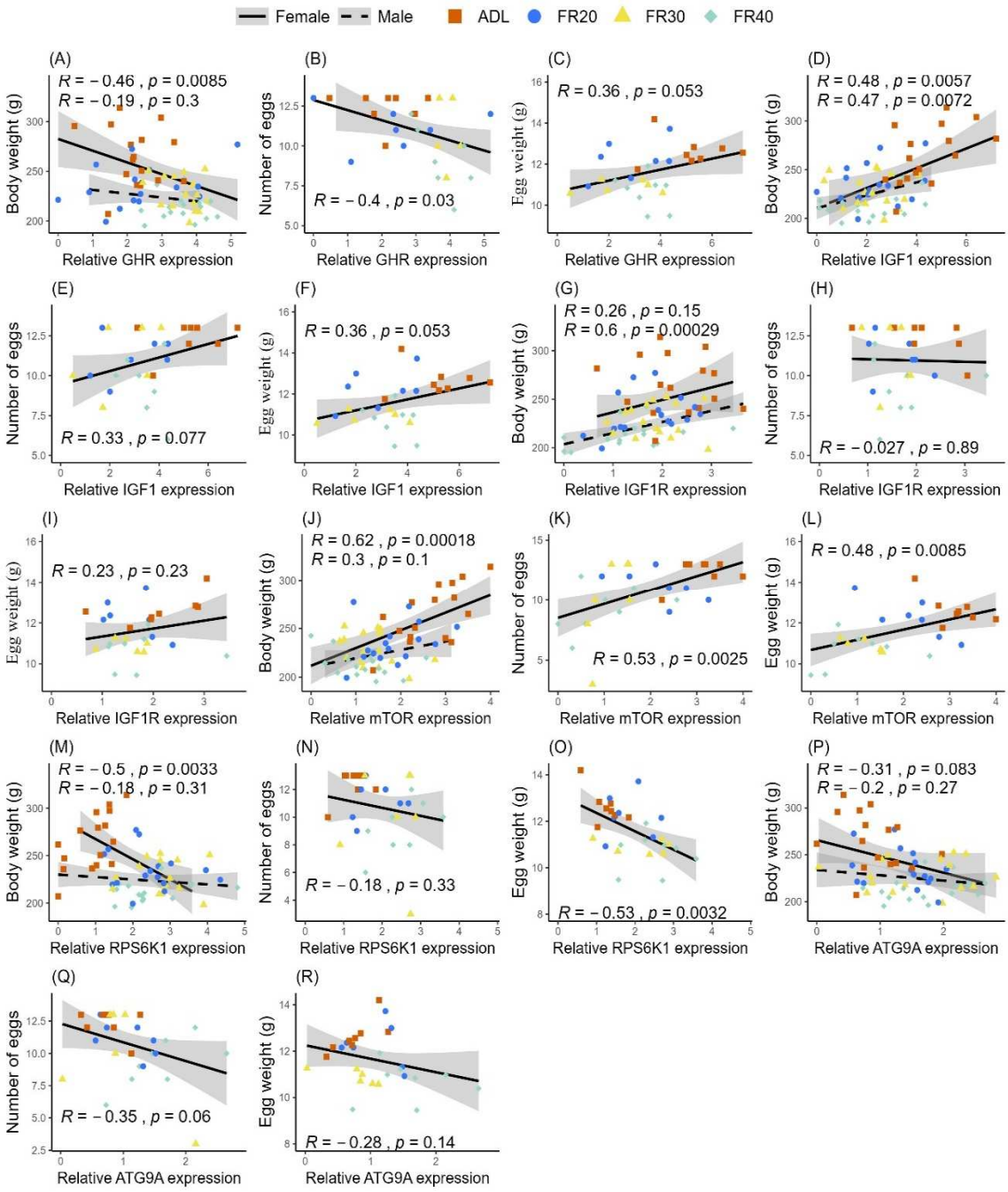

Figure 10. Linear association analysis of individual liver relative gen expressions with body weight, egg number and egg weight. Note: In association of body weight with genes, the first R and P values are for females and the scond R and P values are for males. (A) GHR with body weight, (B) GHR with egg number, (C) GHR with egg weight, (D) IGF1 with body weight, (E) IGF1 with egg number, (F) IGF1 with egg weight, (G) IGF1R with body weight, (H) IGF1R with egg number, (I) IGF1R with egg weight, (J) mTOR with body weight, (K) mTOR with egg number, (L) mTOR with egg weight, (M) RPS6K1 with body weight, (N) RPS6K1 with egg number, (O) RPS6K1 with egg weight, (P) ATG9A with body weight, (Q) ATG9A with egg number, (R) ATG9A with egg weight. Egg weight was the mean egg weight of week 2 of the experiment.

4.1.8. 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. 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 11). The elliptical biplot indicated a clustering of liver *mTOR* and *IGF1* expression around the control treatment and have positive influences on the PC1, while *RPS6K1, GHR* and autophagy genes expression clustered around the groups received restricted treatments and showed negative influences on PC1 (Figure 11). Finally, PC1 significantly and positively explained body weight, egg number, and egg weight, whereas PC2 significantly and negatively explained body weight while having a significant influence on egg number and weight (Table 8).

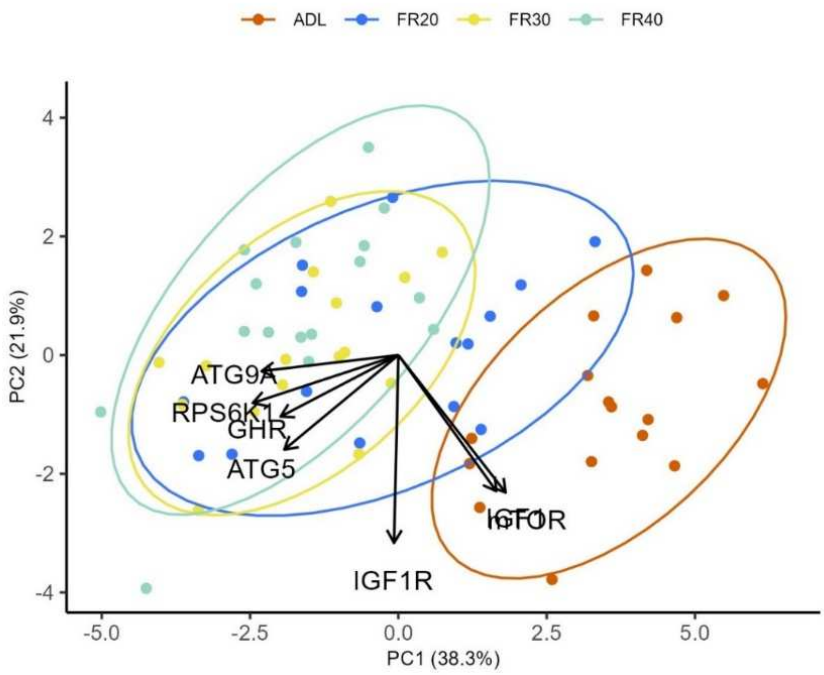

Figure 11. A biplot of PCA for the liver gene expression and body weight in Japanese quails treated with different dietary restriction levels for two weeks. Clustering is based on dietary restriction levels and a dimensional indication of genes in line with the restriction levels. The ellipsoids are defined by the treatment groups. IGF1 and mTOR are overlapped on the ellipse.

Table 8. Output of the multiple linear regression of the conserved PCs (from gene expression) predicting body weight, egg number and egg weight

Response variable	Predictor	Estimate	SE	*t-value*	*p-value*	R^2	*RSE*
Body weight	Intercept	23.41	2.52	93.60	<0.001	0.49	20.12
	PC1	9.42	1.55	6.09	<0.001		
	PC2	-9.81	2.05	-479	<0.001		
Egg number	Intercept	10.28	0.41	25.04	<0.001	0.28	2.12
	PC1	0.70	0.23	-3.00	0.006		
	PC2	-0.41	0.34	-1.21	0.237		
Egg weight	Intercept	11.36	0.19	60.74	<0.001	0.37	0.94
	PC1	0.37	0.11	3.343	0.002		
	PC2	-0.28	0.15	-1.84	0.077		

4.1.9. Resource allocation strategy

Birds effectively allocate resources toward body weight, reproduction, or self-maintenance based on resource availability (Figure 12). In week 1, the FR20 and FR30 groups increased relative reproductive investment (Figure 12C), 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 12D), where resources are re-allocated towards reproduction. At the end of the experiment, individual variation in allocation strategy was only related to *mTOR* expression ($t = -3.118$, $p = 0.008$). 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 12B).

We found that variation in the gene expression pattern was coordinated and related to production parameters and resource allocation. At a severe FR, the reduced egg number and egg weight are aligned with low *IGF1* (Figure 10E,F) and *mTOR* (Figure 10K,L) expression, suggesting that these genes are associated with the effect of FR on reproduction. However, individual variation in resource allocation strategy was only related to *mTOR* expression. Stronger restrictions induced an increasing reduction of *mTOR* expression, but irrespective of the treatment, individuals with relatively lower *mTOR* expression had a proportionally larger reproductive investment. This may seem surprising because mTORC1 is required for and thought to promote reproduction (GUO et al., 2018; MCLAUGHLIN et al., 2011). 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.

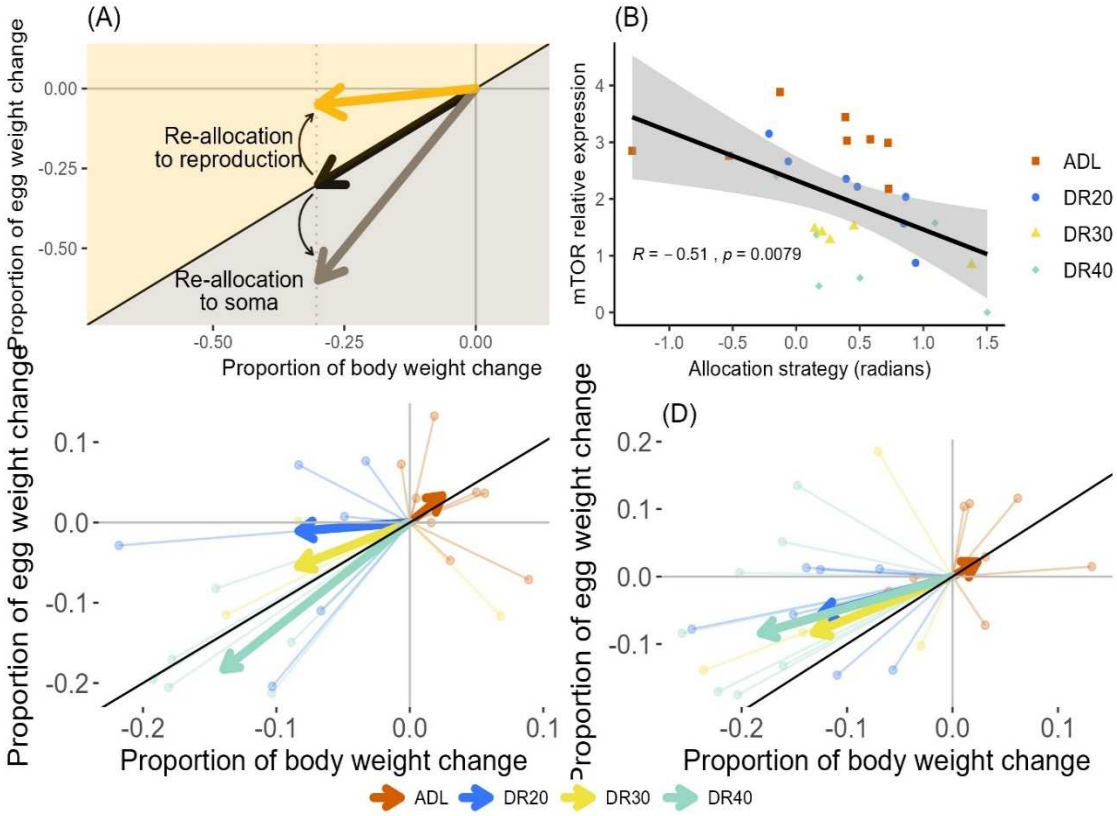

Figure 12. Effect of feed restriction on resource allocation decision. (A) A conceptual figure illustrating resource allocation decisions. The x- and y-axis show the proportional change in body weight and egg weight, respectively, during the experimental period (compared to the pre-treatment body weight 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.

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 12B) 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). The upregulated cellular maintenance helps to preserve the follicle pool and maintain reproductive potential (ENGLISH and BONSALL, 2019), while activation of autophagy-related genes promotes oocyte maturation (ZHOU et al., 2019). Rapamycin treatment, which downregulates mTORC1 was also found to stimulate oocyte maturation by increasing the expression of autophagy-related genes (LEE et al., 2015). In our study, un-inhibition (i.e. upregulation) of recycling mechanisms may have channelled resources towards reproduction.

4.1.10. 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: $F_{3,49} = 0.41$, $p = 0.743$; treatment × week: $F_{6,111.19} = 0.88$, $p = 0.511$; treatment × sex: $F_{3,49} = 0.13$, $p = 0.944$; treatment × week × sex: $F_{6,111.19} = 1.53$, $p = 0.174$). Additionally, IGF-1 levels remained unchanged throughout the restriction periods in both males and females (week: $F_{2,111.19} = 1.76$, $p = 0.177$; week × sex: $F_{2,111.19} = 2.02$, $p = 0.137$,). 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 13). Our data also further revealed repeatable individual variation in IGF-1 levels across the restriction period with high repeatability (repeatability: 0.81). Within-treatment variation in body weight had no significant association with IGF-1 levels ($p = 0.539$). IGF-1 levels were also not associated with individual-centred body weight ($p = 0.421$). Additionally, the egg weight and the number of eggs showed no significant relationship with IGF-1 levels (egg weight: $p = 0.579$; egg number: $p = 0.523$).

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; KAZEMI et al., 2020; 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). IGF-1 is considered to play a crucial role in governing life-history trade-offs in growing organisms (LEWIN et al., 2017; REGAN et al., 2020). Disparities in IGF-1 concentrations

among individuals may result in differences in growth rates and sizes (LODJAK et al., 2018). However, the effect of IGF-1 could be variable across species, sexes, and ages of organisms. For instance, a study on bearded reedlings (*Panurus biarmicus*) reported that heavier individuals, who have higher initial circulating IGF-1, were more likely to decrease IGF-1 in response to FR than lighter ones (TÓTH et al., 2022). Previous reports showed that the levels of circulating IGF-1 increased with age during early postnatal development, whereas they gradually decreased after maturity and reached their stable minimum level (LODJAK and VERHULST, 2020; ROBERTS et al., 1990).

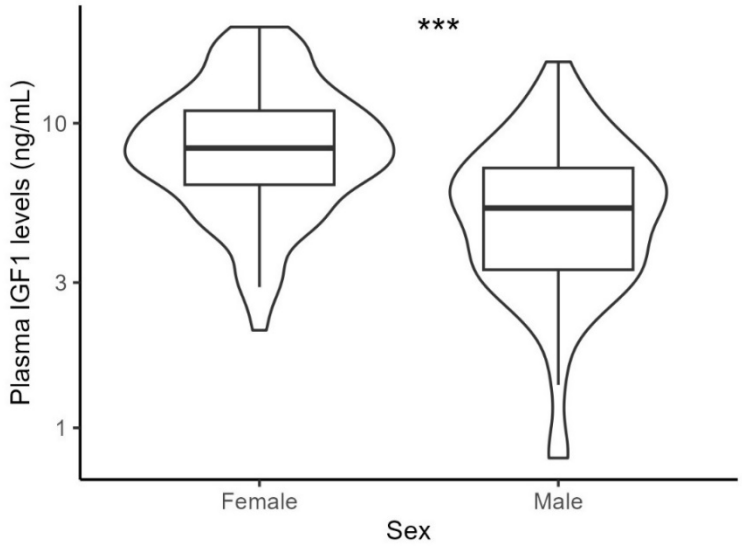

Figure 13. Sex difference on plasma IGF-1 levels. Data were analysed from 32 birds per sex group in three time points. Data were collected on day 0 (initial), week 1 and week 2 periods of the experiment.

Birds develop early-life insulin resistance, leading to high circulating glucose levels (SATOH, 2021). This, in turn, inhibits growth hormone secretion, a crucial event for the activation of IGF-1 secretion. Conversely, the high insulin levels due to insulin resistance stimulate consistent hepatic IGF-1 secretion at the adult stage (HOUSTON and O'NEILL, 1991; SCANES and BRAUN, 2013). In line with this notion, a recent study at different development stages of chickens revealed that the expression of the IGF system components sharply dropped after hatching and showed no variation between fast and slow growers (GUO et al., 2023). 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). Therefore, the reaction to nutritional cues in mature birds might also be diminished, which could explain the less IGF-1 response observed across all levels

of FR in our study. We also observed large and highly repeatable individual differences in plasma IGF-1 across time points, consistent with earlier studies in birds and mammals (LENDVAI et al., 2020; OBESE et al., 2008; ROBERTS et al., 1990; TÓTH et al., 2022), suggesting that differences among individuals could potentially influence how adaptive responses to varying nutritional availability are formed.

Despite the weak response to FR, a distinct difference between females and males was observed (REDA et al., 2024c): females exhibited 64.7% higher IGF-1 levels than males (Figure 13). Our findings are consistent with previous reports across different species, suggesting that IGF-1 exhibits sex-specific expression (ASHPOLE et al., 2017; BACON et al., 1993; BAÉZA et al., 2001; JOHNSON et al., 1990; METER et al., 2022; TÓTH et al., 2022; YUAN et al., 2009). Notably, the larger sex exhibits higher IGF-1 levels, particularly during growth and maturation. Though the exact mechanisms underlying the sex-related differences in IGF-1 levels are complex and multifactorial, reproductive investment, sex hormones and sex-based genetic variations are suggested as major contributors to the difference (JØRGENSEN et al., 2005; KERRIGAN and ROGOL, 1992; LIU et al., 2016).

A study on the Eastern fence lizard (*Sceloporus undulates*), a species with female-biased sexual size dimorphism, also revealed that expression of hepatic *IGF1* is reduced in males with increasing testosterone levels, leading to the smaller body size in males than females (DUNCAN et al., 2020). Contrastingly, in species where males are larger, such as brown anole lizards (*Anolis sagrei*) (COX et al., 2017), yellow catfish (*Pelteobagrus fulvidraco*) (MA et al., 2015b) and chicken (YADAV et al., 2023), testosterone induced the expression of *IGF1* gene, which also showed a male-biased pattern. In Madagascar ground geckos (*Paroedura picta*), ovarian hormones suppress *IGF1* expression in females, resulting in smaller females (METER et al., 2022). In the latter species, the overexpression of *IGF1* in males occurs well after the rise in circulating testosterone levels during sexual maturation. This implies the stimulatory effect of testosterone on IGF-1, thereby contributing to the larger body size observed in males. In Japanese quails, males and females grow at a similar rate up until the age of four to five weeks, but males reach sexual maturity one to two weeks earlier than females (OTTINGER et al., 2005). Testosterone levels significantly begin to rise at the age of four weeks and reach their peak stage at six weeks (OTTINGER and BRINKLEY, 1978). During this period, males exhibit a slower growth rate than females, who gain weight by increasing adipose tissue and mature slowly over one or two weeks (LEE et al., 2020; YANG et al., 2013).

This indicates that in species characterised by larger females in size dimorphism, elevated testosterone levels after sexual maturation in males may inhibit the expression of the *IGF1* gene, thereby reducing the secretion of IGF-1 and consequently slowing growth.

4.1.11. Effect of feed restriction on plasm triglyceride levels

The linear mixed-effect model revealed a significant treatment and treatment-by-restriction-period interaction effect (Table 9). 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 14; Appendix Table 4). However, in the first treatment week, FR20 and FR40 treatments imposed a reduction in the levels of plasma triglycerides, while the FR30 treatment showed a significant reduction compared to the ADL-fed group (Appendix Table 4). In males, all restricted groups showed significantly lower plasma triglyceride levels than the control group in weeks 1 and 2 (Figure 14, Appendix Table 4).

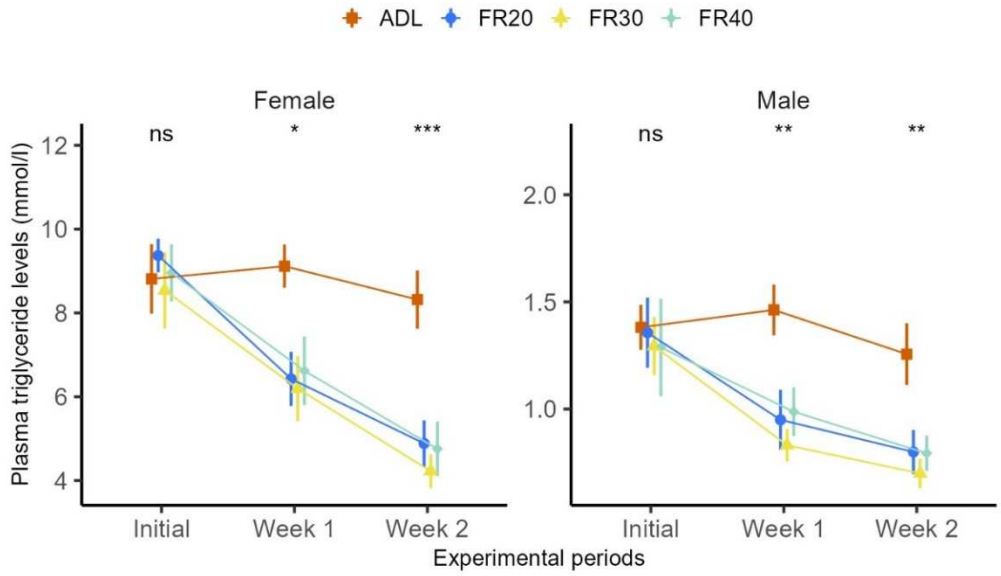

Figure 14. Effect of feed restriction on plasma levels of triglycerides across the two weeks restriction period in females and males Japanese quail. Data are represented by the mean ± SEM and were statistically analysed from the linear mixed-effect model. Note the different scales for the sexes, reflecting the significant disparity in female and male triglyceride values.

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 (Figure 14, Appendix Table 5). In case of the trend from week 1 to week 2, FR30 and FR40 of female groups showed a significant reduction in week 2, while all other groups in both sexes showed no significant

reduction (Appendix Table 5). Furthermore, sex significantly affects plasma triglyceride levels at all-time points, with females having more than six-fold higher triglyceride levels than males (Figure 14; Appendix Table 6). This disparity in plasma triglyceride levels between males and females also remained significant after controlling for body size. Body weight had a significant association with plasma triglyceride levels (Figure 15). In females, triglyceride levels showed a significant positive association with body weight at all-time points, while no significant relationship was observed in males (Figure 4; Figure A6). We did not observe significant associations between triglyceride levels and egg traits.

Table 9. Output of the linear mixed-effects model showing the effect of feed restriction treatment on plasma triglyceride levels across the time points (week) in female and male Japanese quails

Fixed factors	NumDF	DenDF	F-value	P-value
treatment	3	52	11.45	<0.001
week	2	120	46.48	<0.001
sex	1	52	1305.07	<0.001
treatment × week	6	120	4.10	0.001

Abbreviations: NumDF, numerator degree of freedom; DenDF, Denominator degree of freedom

The circulating triglyceride levels have been reduced at all restriction gradients compared to the *ad libitum* group (Figure 14). Previous evidences from different species suggest that dietary restriction can reduce triglyceride levels (MAHONEY et al., 2006; TEOFILOVIĆ et al., 2022; ZHAN et al., 2007). Intriguingly, the effect did not exhibit a proportional increase with the severity of restriction (REDA et al., 2024c). Although there is a lack of previous evidence, this absence of a dose-response relationship could suggest the presence of a threshold effect beyond which further reductions in triglycerides are impeded. This may indicate a potential resource re-allocation strategy, favouring the maintenance of circulating triglyceride levels at the expense of other traits, which warrants further investigation. While the observed effect trend in males aligned with the trend in body weight (REDA et al., 2024a), females exhibited a different pattern, wherein the effect on body weight intensified with the severity of restriction (REDA et al., 2024b). 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). We also observed a threshold-like uniform decrease in *IGF1* gene expression in males and females and *mTOR* gene expression in males (REDA et al., 2024a; REDA et al., 2024b).

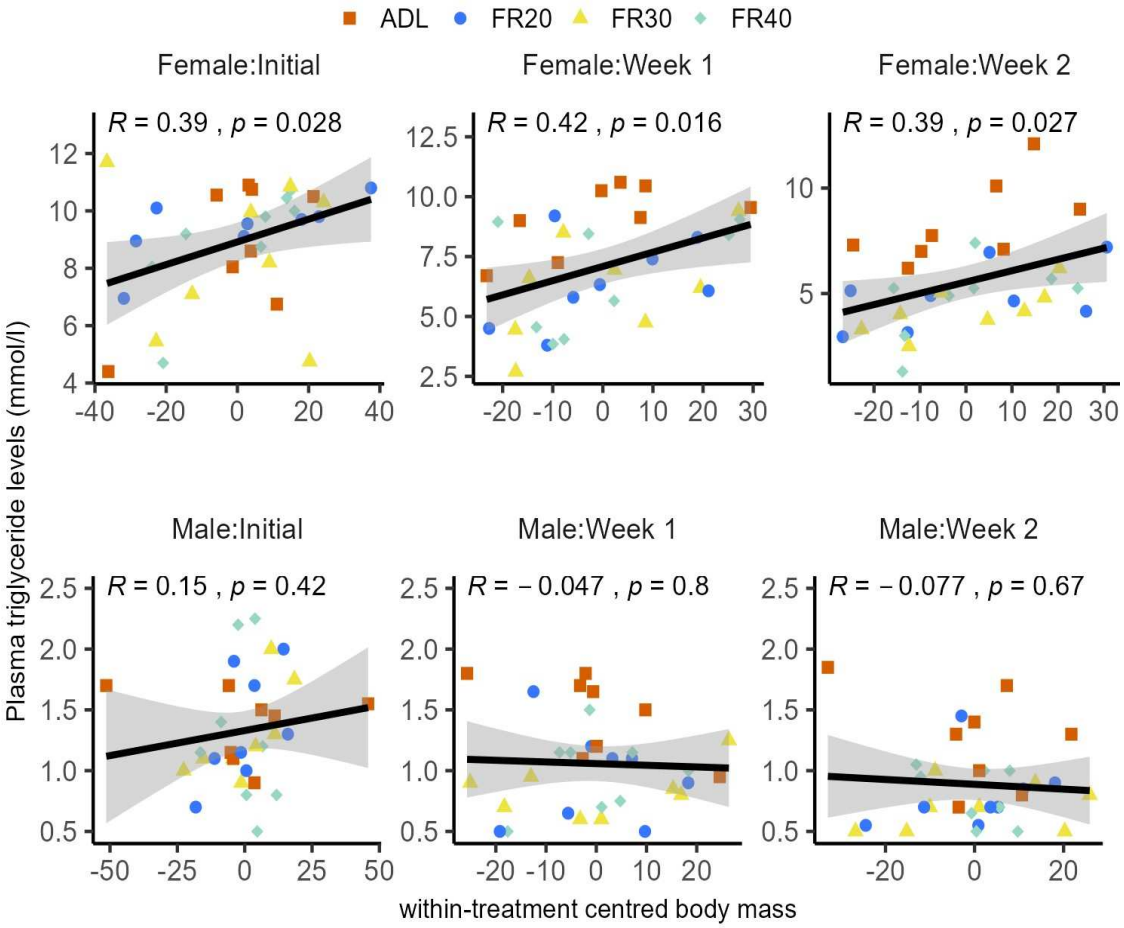

Figure 15. Relationship between body weight and plasma triglyceride levels in females (top panels) and males (bottom panels) at three-time points of the experiment. Note: Body weight data was subjected to within-treatment centring to remove the treatment interference.

Triglycerides are mainly derived from dietary sources, particularly from dietary fats and carbohydrates via the liver (HAVEL et al., 1962). They are prime indicators of fat metabolism (ARAÚJO et al., 2019). In birds, triglycerides serve as energy sources for essential activities such as flight, reproduction, and parental care (FOWLER and WILLIAMS, 2017; KERN et al., 2007). Hence, 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 in humans (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).

Female quails exhibited over six-fold higher circulating triglycerides than males (Figure 14). These values fall within the reference ranges reported for Japanese quails (SCHOLTZ et al., 2009) and indicate the high hepatic synthesis of fatty acids, cholesterol, and phospholipids, which are stimulated by oestrogen and play a critical role in lipid deposition in the yolk of laying hens (LEVEILLE et al., 1957; SALVANTE et al., 2007; WALZEM et al., 1999). Interestingly, in mammals, oestrogen is reported to reduce circulating triglyceride levels via inhibiting feeding behaviour and lipogenesis and promoting triglyceride uptake by adipocytes (ITO et al., 2021). This difference could be attributed to the tendency of females to have higher fat reserves, which is crucial for various fitness components, particularly reproduction, body weight, and immunity. We found a positive correlation between plasma triglyceride levels and body weight in females, while no such association existed in males (Figure 15). Within females, heavier individuals had higher triglyceride levels than lighter ones. This may be due to the necessity of triglycerides for body fat reserves and their importance in egg deposition for reproductive success. Body fat reserve can have a strong correlation with increasing body weight (BARNETT et al., 2015). Interestingly, triglyceride levels had no associations with either egg number or egg weight, although triglycerides are used as precursors of egg yolk formation and subsequent egg production (CUI et al., 2020).

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

4.2.1. Effect of unpredictable feeding on body weight

Treatment, sex, and time had a significant interaction effect on body weight (Table 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 16). In males, body weight of the unpredictably fed group did not differ in body weight compared to the control group at all-time points. The 40% restricted group (FR40) exhibited lower body mass 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 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, Appendix Table 7).

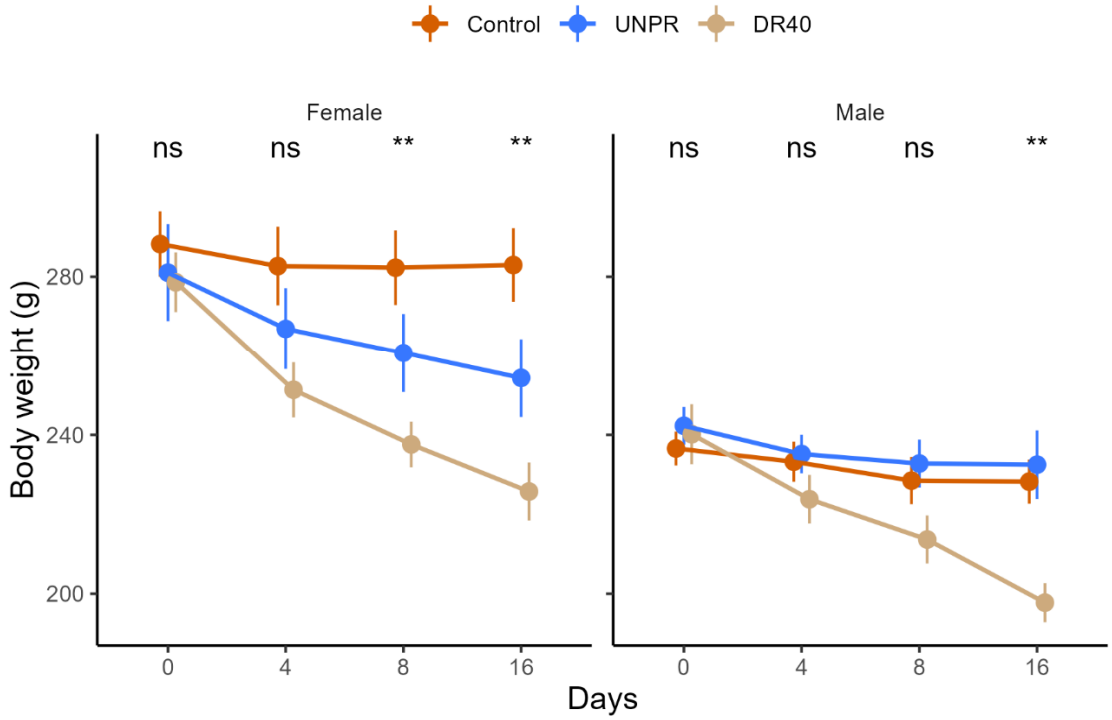

Figure 16. Effect of feed treatment on body weight of female and male quails across 16 days treatment period. Data are represented by the mean ± SEM from 8 birds per group. Abbreviations: 'ns', not significant at p < 0.05; '*' significantly different at p < 0.05; '**' significantly different at p < 0.01;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

We compared the body weight of each group at all time points and found that in the females, the UNPR and FR40 groups showed continuous reduction at every time point while the control group did not show that. In the males, the UNPR group showed significant reduction at the day 4 and kept with change until the next time points. The FR40 group in the males showed continuous reduction throughout the time points. Interestingly, males in the control group showed significant reduction at days 8 and 16 (Appendix Table 8). We also further compare males and females and found that females have significantly higher body weight than the males at all treatment levels and time points (Appendix Table 9).

Previous studies reported that while both the control and unpredictably fed groups consumed, on average, the same amount of feed, the unpredictably fed group significantly

reduced body weight (FOKIDIS et al., 2012). In broiler breeders, continued feed restriction and skip-a-day feeding are issues of controlling excessive body weight and animal welfare (DE BEER and COON, 2007; LINDHOLM et al., 2018; SWEENEY et al., 2022). However, continuous dietary unpredictability has not yet been studied in poultry. In rats, variably fed groups showed reduced body weight compared to continuously restricted feeding (ESCOBAR et al., 2007). There was no effect of long-term unpredictable feeding on the body weight of adult and juvenile zebra finches of both sexes (*Taeniopygia guttata*) (LYNN et al., 2023).

Table 10. Output of the linear mixed-effect model showing the effect of unpredictable treatment, sex and treatment days on body weight in Japanese quails

Factors	**NumDF**	**DenDF**	**F-value**	**P-value**
Treatment	2	42	5.64	0.007
Sex	3	126	149.31	<0.001
day	1	42	37.94	<0.001
Treatment × sex	6	126	36.12	<0.001
Treatment × day	2	42	1.55	0.224
Sex × day	3	126	4.99	0.003
Treatment × sex × day	6	126	2.95	0.010

Abbreviations: NumDF, numerator degree of freedom; DenDF, Denominator degree of freedom

4.2.2. 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 17A2) or the probability of daily egg laying ($p = 0.856$, 22A1). However, the 40% restriction (FR40) treatment significantly reduced both the total number of eggs ($p < 0.001$, Figure 17A2) and the probability of daily egg laying ($p < 0.001$, Figure 17A1). 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 17B2) and demonstrated a significant interaction with both the linear ($p < 0.001$) and quadratic ($p < 0.001$) terms of restriction time (Figure 17B1). This indicates that the impact of the 40% restriction on egg weight varies on the linear and quadratic components of the effect of treatment days. We could not find previous studies to corroborate our results. We predicted that birds might become stressed due to unpredictable dietary fluctuations, resulting in a negative impact on egg production and quality. Even though

female birds in the unpredictable feeding showed significant body weight reduction, all egg parameters were kept similar to the control group. Therefore, this outcome was contrary to our hypothesis. Similar to the previous result, FR40 significantly affected egg number and egg weight (REDA et al., 2024b).

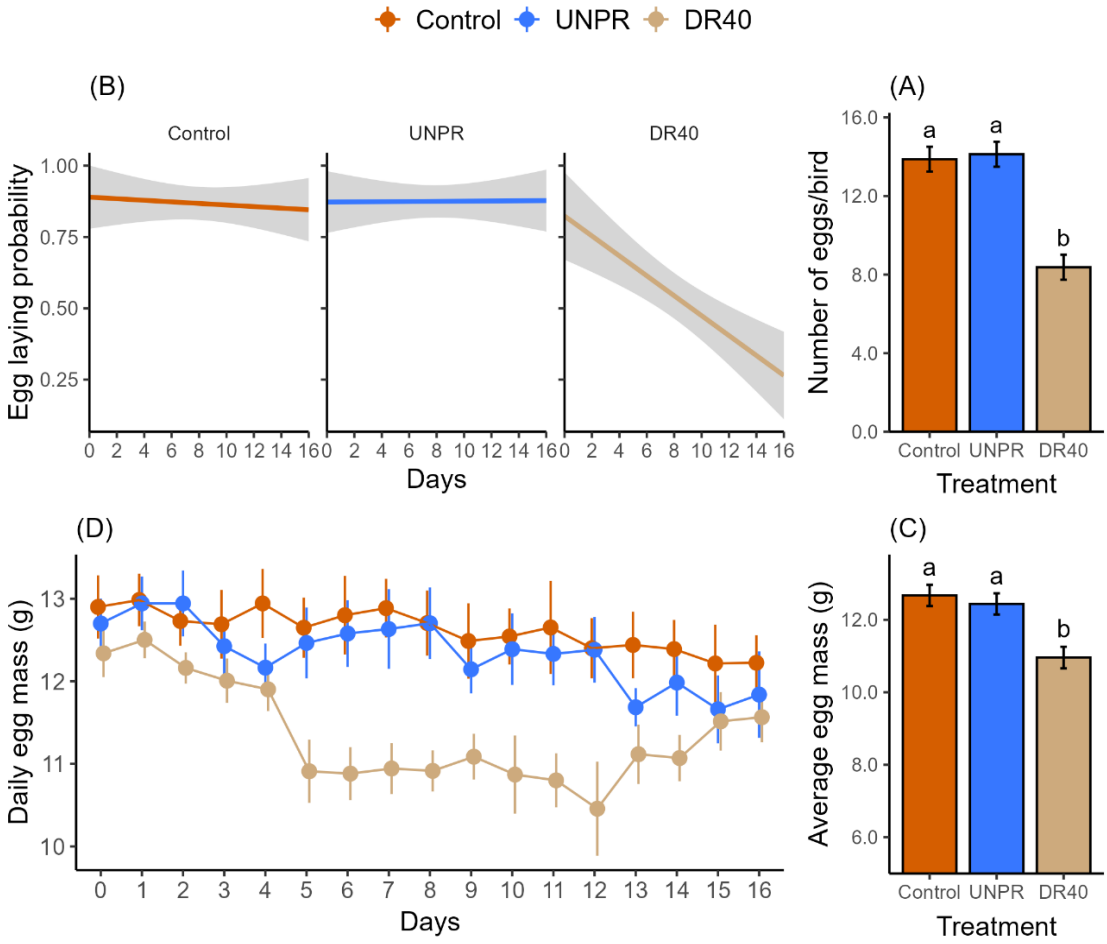

Figure 17. Effect of dietary treatment on egg number and egg weight. Abbreviations: Control, birds received equals to their daily feed intake; UNPR, unpredictable feeding; FR40: 40% restriction.

4.2.3. 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 (Table 11, Figure 18). 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 18A). 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 19A and 20A). In the liver, neither of the treatments showed a significant effect on *IGF1R* in both male and female groups (Figure 18B). 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 19B). 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 18C). In the muscle, FR40 in the female groups showed higher *FOXO1* gene expression compared to both control (0.058) and UNPR (0.014) (Figure 19C).

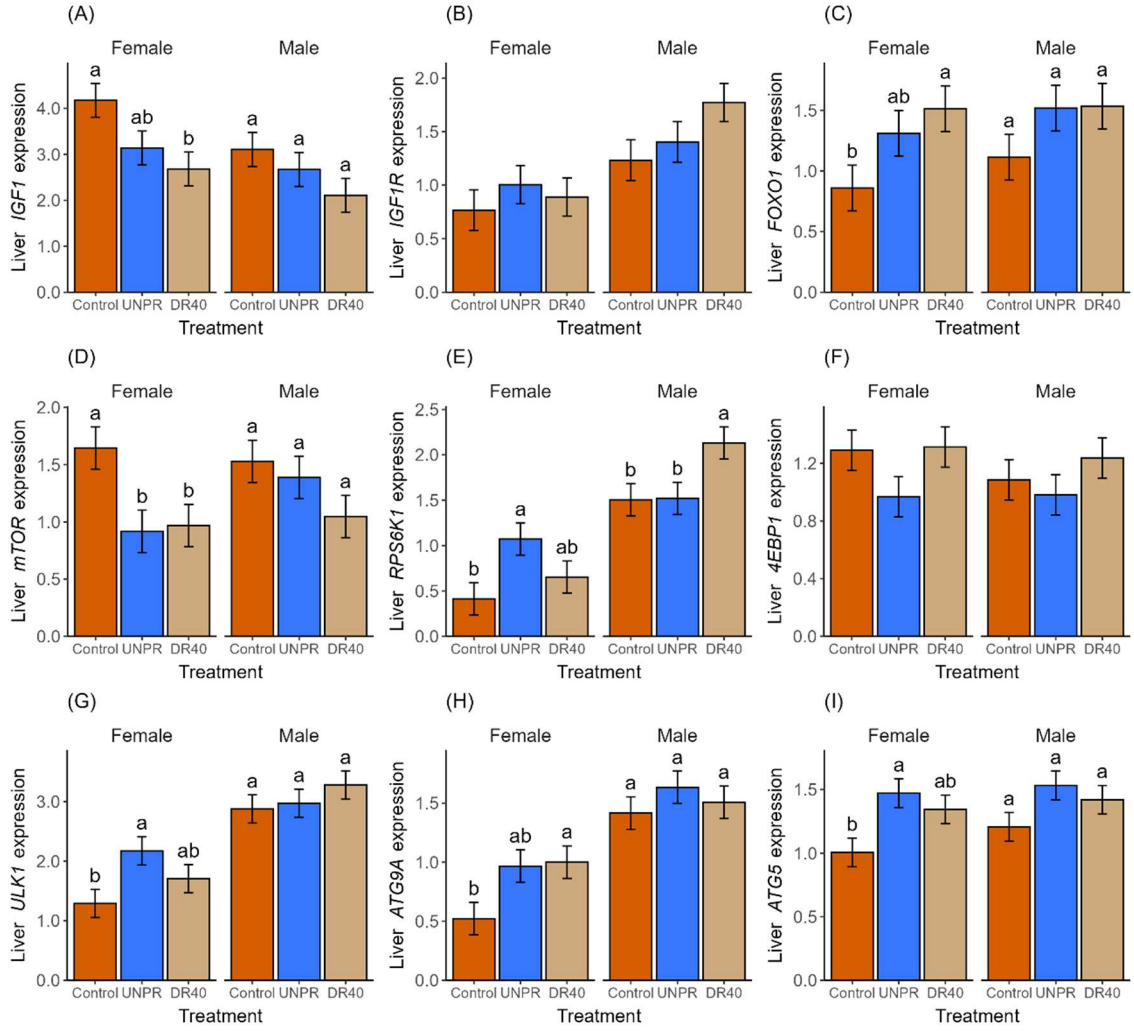

Figure 18. Effect of variable dietary treatment on expression of liver nutrient-sensing genes. (A) IGF1, insulin-like growth factor 1; (B) IGF1R, insulin-like growth factor 1 receptor; (C) FOXO1, forkhead box O1; (D) mTOR, mechanistic target of rapamycin; (E) RPS6K1, ribosomal protein S6 kinase 1; (F) 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 within sex are not significantly different at $p < 0.05$. Abbreviations: Control, birds received equal to their daily feed intake; UNPR, unpredictable feeding; FR40: 40% restriction.

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 18D) and muscle (UNPR: $p = 0.081$, FR40: $p = 0.003$, Figure 19D). 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 18E). In the muscle tissue, the trend due to dietary manipulation has changed, showing a reducing trend, although it was not statistically significant (Figure 19E). In the liver, *4EBP1* showed no significant effect of treatments in both females and males (Figure 18F). In the muscle, FR40 treatment increased *4EBP1* gene expression compared to both control and UNPR groups in both females and males (Figure 19F). Moreover, the control group exhibited lower expression values of liver *ULK1*, *ATG9A* and *ATG5* genes expression in females, while showing a similar trend but no significant variation in males (Figure 18G,H,I). In the muscle, while *ULK1* gene expression showed no change across all treatments in both sexes (Figure 19G), *ATG9A* showed an increasing trend in UNPR and FR40 groups with significant differences in females (Figure 19H).

One of the important biological mechanisms for regulating environmental/dietary conditions involves altering gene expression (LÓPEZ-MAURY et al., 2008). Organisms may respond to unpredictability through altering patterns of expression of certain genes (FEIGE-DILLER et al., 2022). Cells have the ability to acclimate to typical alterations within their native habitat by sensing environmental factors (EFEYAN et al., 2015; SUNG et al., 2023). This adaptation is always moderated through initiating specialized regulatory networks that have evolved to establish adaptive gene expression profiles (DWORKIN and LOSICK, 2001; HAMANN et al., 2021; MARTÍNEZ SOSA and PILOT, 2023). Genes in the nutrient-sensing pathway, including *IGF1*, *mTOR*, *FOXOs,* and their downstream effectors *RPS6K1*, *4EBP1* and autophagy genes, are therefore responsible for sensing nutritional availability and stability, and undergo adaptive changes in expression and link genotypes and molecular functions to the fitness outcomes (BENNETT-KEKI et al., 2023; EFEYAN et al., 2015; NOURMOHAMMAD et al., 2017). However, previously reported studies used constant *ad libitum* and restricted feeding, which did not consider the effect of variable feeding.

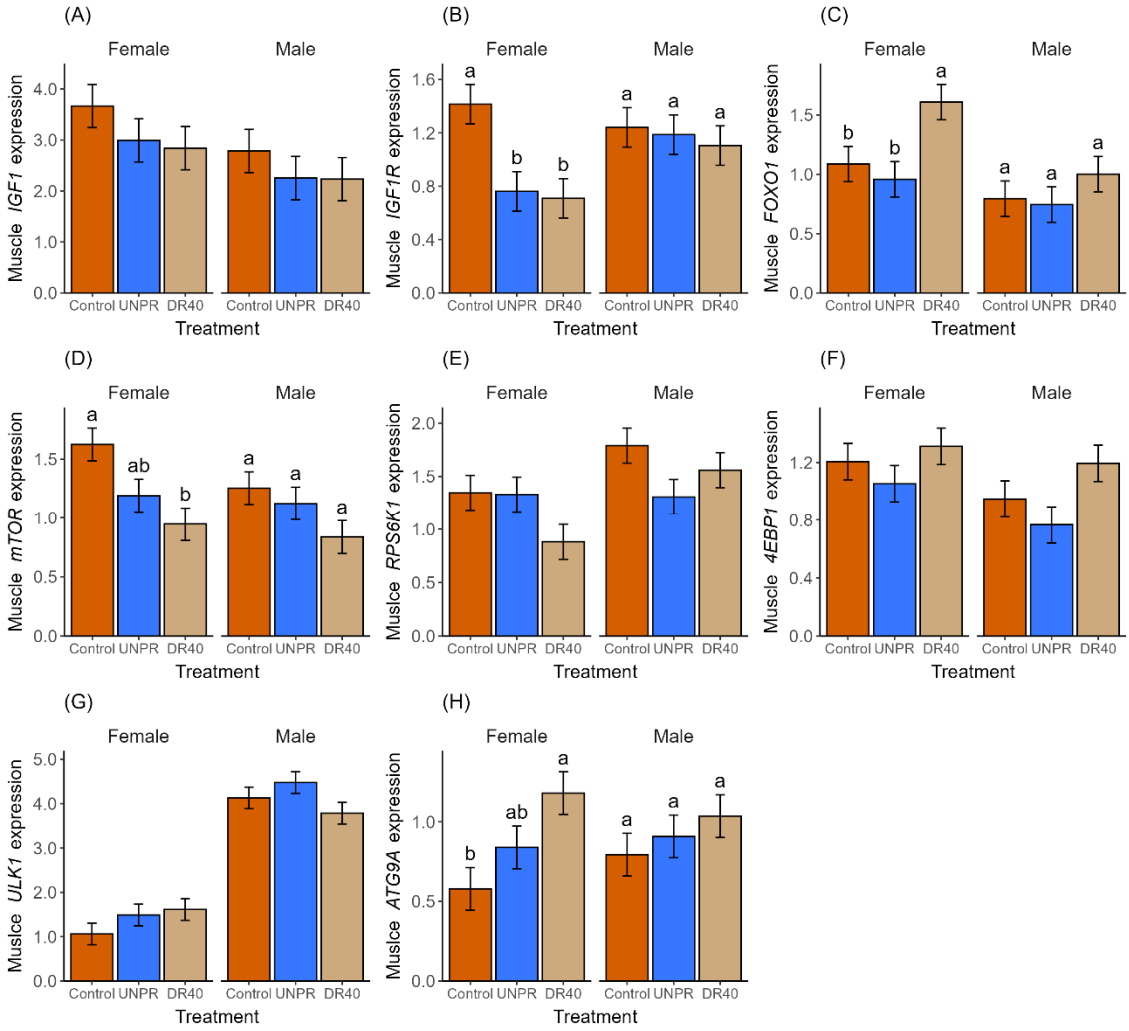

Figure 19. Effect of unpredictable dietary availability on expression of mTOR pathway genes in the muscle. Descriptions and Abbreviations from Figure 18.

Unlike constantly high or low feeding, a condition 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. The current study proved that unpredictable feeding affected the key mTOR pathway genes in both liver and muscle tissues. Interestingly, the reducing trend in the key anabolic genes, *IGF1* expression in the liver, *IGF1R* in muscle, and *mTOR* expression in both liver and muscle in response to UNPR and FR40 in females coupled with the body weight loss. The reduction in expression of these genes was also coupled with increasing expression of *FOXO1*, *ULK1,* and autophagy genes in the liver and *FOXO1* and *ATG9A* in the muscle, which was in line with our expectations. Upregulated mTOR is expected to inhibit the

action of transcription factors responsible for the initiation of transcription of energy homeostasis-regulating genes (MARTINA et al., 2012). The weak response to the dietary treatment in male body weight was coupled with the weak response in gene expression.

4.2.4. Sexual difference in gene expression

Expression of nutrient-sensing genes showed sex-specific trends in both tissues (Table 11; Figure 20). In the control group, *IGF1* expression was significantly higher in females than in males. However, the statistical significance between sexes disappeared in the UNPR and FR40 groups (Figure 20A). Males scored higher *IGF1R* expression in all groups except the muscle of the control group (Figure 20B). Only muscle in the FR40 groups showed a sex difference in *FOXO1* expression, with females exhibiting significantly higher expression (Figure 20C). *mTOR* expression showed no significant variation between sexes in both tissues (Figure 20D).

Table 11. A simplified statistical summary of the effect of treatment, sex, tissue and their interaction on the expression of mTOR pathways sensing genes in Japanese quails

Gene	Treatment		sex		Tissue		Treat × sex		Treat × tissue		Sex × tissue		Treat × sex × tissue	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P
IGF1	6.23	0.003	9.93	0.002	0.65	0.422	0.77	0.738	0.54	0.587	0.007	0.933	0.08	0.921
IGF1R	0.38	0.681	16.9	<.001	0.73	0.394	1.83	0.167	5.46	0.006	3.46	0.066	1.96	0.147
FOXO1	7.19	0.001	1.15	0.287	7.91	0.006	0.92	0.400	2.39	0.097	733	0.008	0.09	0.909
mTOR	12.2	<.001	0.04	0.839	0.82	0.365	1.85	0.163	0.24	0.785	2.99	0.087	0.32	0.727
RPS6K1	0.06	0942	49.6	<.001	2.91	0.091	7.09	0.001	5.19	0.007	11.4	0.001	0.37	0.693
4EBP1	5.92	0.004	4.07	0.047	0.70	0.404	0.27	0.762	0.11	0.894	0.74	0.391	0.30	0.739
ULK1	3.37	0.039	215	<.001	7.48	0.007	1.15	0.320	0.42	0.658	26.4	<.001	2.74	0.702
ATG9A	7.27	0.001	22.0	<.001	13.2	<.001	1.89	0.157	1.07	0.348	16.8	<.001	0.02	0.978
ATG5	6.48	0.003	1.50	0.227			0.23	0.794						

RPS6K1 gene expression responds to treatment differently. In the liver, males exhibit significantly higher *RPS6K1* expression than females in the control and FR40 groups; expression in females increased due to UNPR treatment while not responding to FR40. Expression of liver *RPS6K1* in males increased in the FR40 while not responding to UNPR treatment. In the muscle, FR40 males exhibited significantly higher *RSP6K1* expression than females. Males tended to reduce muscle *RPS6K1* expression in the UNPR, while females tend to decrease in FR40 with no variation in the UNPR group (Figure 20E). Females tended to show higher *4EBP1* expression than males, although the response to treatment was similar (Figure 20F). Regarding the genes responsible for autophagy, males consistently showed significantly higher *ULK1* expression than females in all treatment groups and tissues (Figure 20G). Males showed significantly higher

ATG9A expression than females in all treatment groups in the liver, while there was no variation in the muscle tissue, and the trend of response to treatments was similar (Figure 20H). Females and males showed similar *ATG5* expression levels and responses to treatment (Figure 20I).

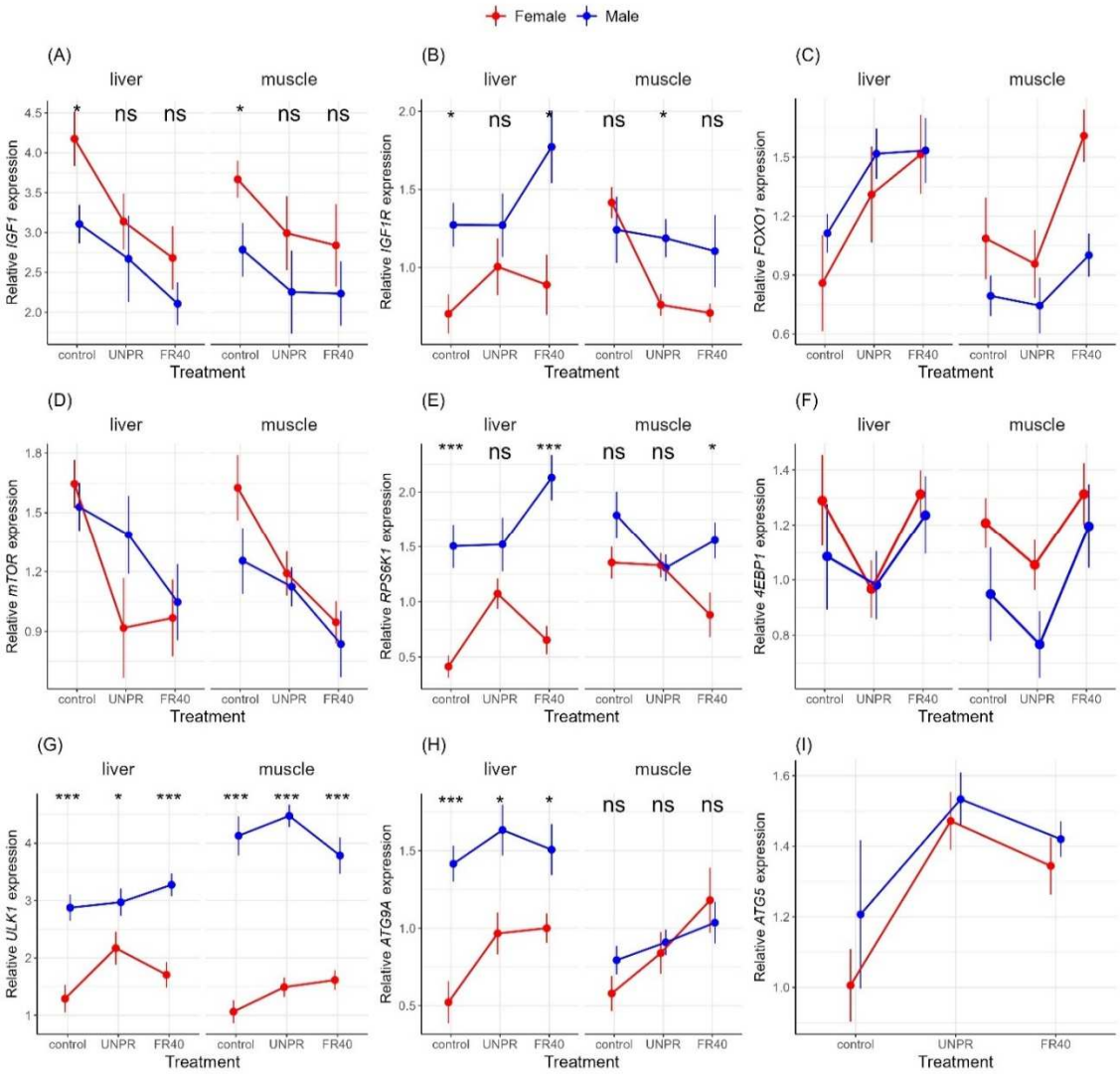

Figure 20. Sex-specific gene expression responses to treatment in liver and muscle tissues. Abbreviations: 'ns', not significant at $p < 0.05$; '*' significantly different at $p < 0.05$; '**' significantly different at $p < 0.01$; '***' significantly different at $p < 0.001$; Control, birds received equals to their daily feed intake; UNPR, unpredictable feeding; FR40: 40% restriction.

In sexually dimorphic organisms like Japanese quails, genes could be differentially expressed between males and females and could respond differently to treatments. Japanese quails are sexually size-dimorphic, with larger females having a more intensive reproductive investment than males (BALTHAZART et al., 1983; REDA et al., 2024a). The current sexual difference in gene expression of most of the genes is in

agreement with our findings in experiment I (REDA et al., 2024a). Studies in flies demonstrated that the impact of dietary restriction on fitness varies between sexes and is linked to sex-specific effects on the expression of genes involved in the mTOR pathway (BENNETT-KEKI et al., 2023; MCDONALD et al., 2021). The suggested rationale for these disparities is based on sex difference in nutritional demands and energy distribution. The disparity in gene expression between males and females can be attributed to divergent reproductive strategies, the regulatory role of sex hormones, and the requirement for particular optimal diets for reproduction (CAMUS et al., 2019; GEGENHUBER et al., 2022). Developing reports also indicated that the IGF-1/mTOR pathway plays a crucial role in sexual dimorphism (ASHPOLE et al., 2017; GÜRGEN et al., 2013).

4.2.5. Correlation and differential expression of genes in liver and muscle tissues

Genes showed a significant correlation in expression between liver and muscle tissues (Figure 21). All genes except *IGF1R* ($p = 0.347$, Figure 21B) showed a significant correlation in expression between both tissues in females. In female *IGF1R*, although there is no overall correlation, there is a strong correlation in expression between the tissues within each treatment group (control: $p = 0.01$, UNPR: $p < 0.001$, FR40: $p = 0.045$). Similarly, in males, except for *RPS6K1* expression ($p = 0.206$, Figure 21E), all genes showed a significant correlation between tissues. Furthermore, some genes showed tissue-specific expression (Table 11). *FOXO1* expressed significantly higher in the liver than in the muscle ($p = 0.006$). Additionally, *ULK1* showed significantly higher expression in muscle than in the liver ($p = 0.007$). Expression of *ATG9A* was significantly higher in the liver than in muscle ($p < 0.001$). *RPS6K1* showed tissue-specific trends in response to treatments. While UNPR in females ($p = 0.021$) and FR40 in males (0.029) showed significantly higher expression in the liver, the difference disappeared in muscle (Figures 19E and 20E). The *IGF1R* expression also showed tissue-specific response to treatments in female quails: while in the liver it did not respond to treatments, in the muscle both the UNPR and the FR40 showed significantly lower *IGF1R* expression than the control group.

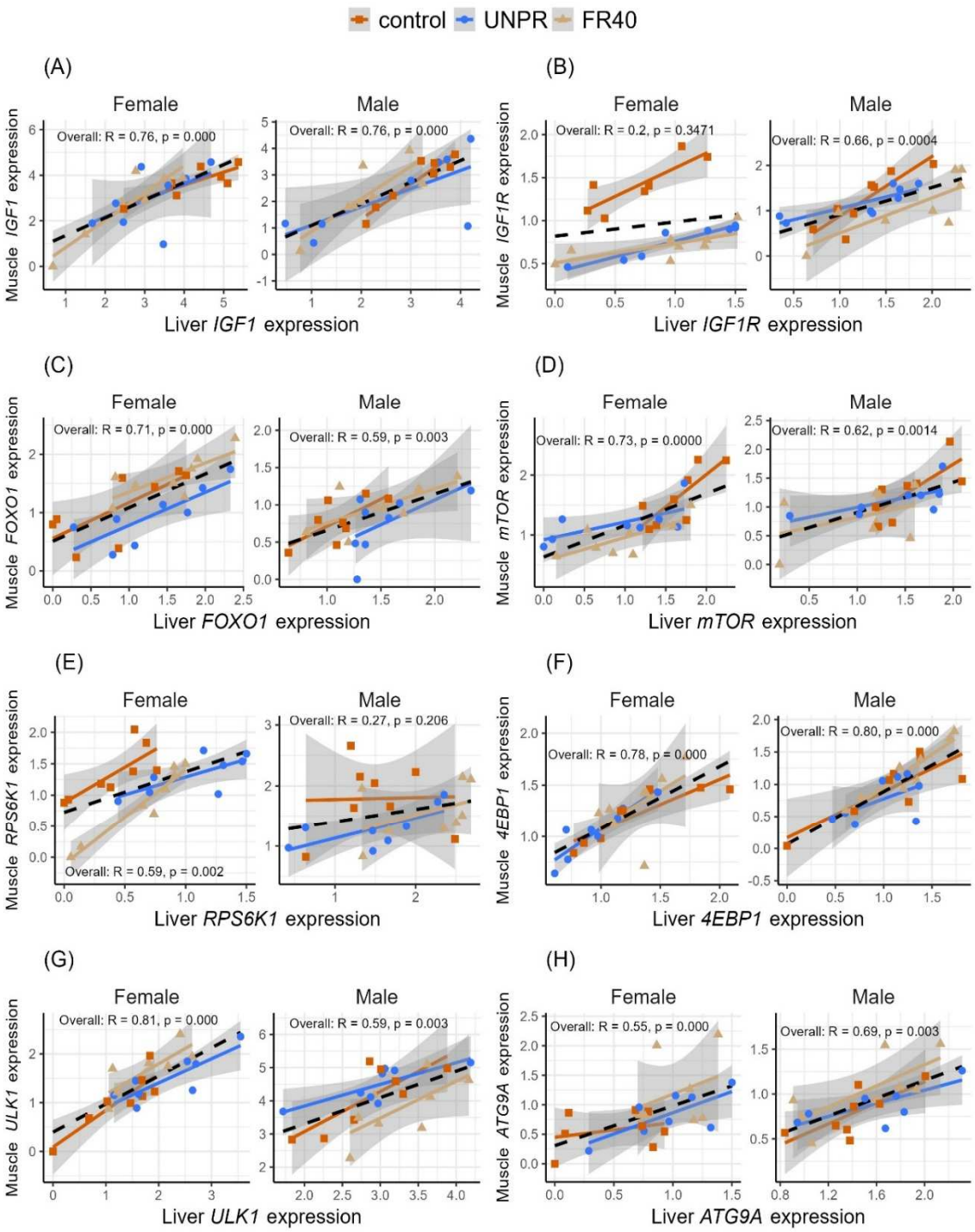

Figure 21. Expression of liver and muscle genes showed significantly positive correlation. We used Pearson correlation to evaluate the association of expression of a gene between liver and muscle tissues. Abbreviations: control, birds received equals to their daily feed intake; UNPR, unpredictable feeding; FR40: 40% restriction.

The liver and breast muscles are the crucial tissues in the metabolism and growth of birds. The liver is an active tissue involved in multiple metabolic functions (ZAEFARIAN et al., 2019). Genes involved in nutrient-sensing pathways show distinct expression

patterns in the liver and are strongly associated with the body's overall functioning, which in turn influences production-related traits (BALONI et al., 2019). Breast muscle is also the fast-growing skeletal muscle important for meat production in chickens and flight in wild birds (BIEWENER, 2011; TICKLE et al., 2018). Numerous genes are differentially expressed in muscles as a function of environmental cues (LI et al., 2020; MALILA et al., 2022). As different tissues carry out different roles, genes can be expressed differentially among the tissues of an organism in response to external or internal factors (GONZALEZ et al., 2005; ZHAO et al., 2024a). In our study, most of the genes showed a significantly positive correlation in the expression of the same gene between liver and breast muscle tissues. However, some genes, for example, *IGF1R* and *RPS6K1*, respond differently to dietary unpredictability. Additionally, irrespective of the effect of treatments, some genes showed higher expression in one tissue than the other. For example, *FOXO1* and *ATG9A* were more expressed in the liver than in the muscles of male quails, whereas, *ULK1* showed higher relative expression in the muscle than in the liver of males.

4.3. 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

In this experiment, we had seven treatments: control feeding, 20 restriction feeding (FR), methionine supplementation (FR+M), leucine supplementation (FR+L), methionine plus leucine supplementation (FR+ML), energy restriction (ER), and protein restriction (PR). We divide the dietary manipulation data into two categories: amino acid supplementation and energy and protein restriction; we also make statistical analyses accordingly. Within each manipulation category, we compared the treatments to each other and with control and FR, which served as positive and negative controls, respectively.

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

In this category, the control, FR, FR+M, FR+L, and FR+ML are compared to see the effect of methionine and leucine supplementation on top of restricted feeding.

4.3.1.1. Effect on body weight

The two-way ANOVA from the linear mixed-effects model showed a significant treatment and time interaction effect ($F_{8,70} = 6.37$, $p < 0.001$, Table 12). Initially, birds

had similar body weights, and during the experiment, they showed treatment-dependent divergent growth rates (Figure 22). At the end of the first week (day 7), treatments showed no significant difference (Figure 22A). 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.

Table 12. Output of the linear mixed-effects model showing the effect of amino acid supplementation on body weight across the time points (day)

Fixed factor	NumDF	DenDF	F value	Pr(>F)
Treatment	4	35	1.096	0.374
Day	2	70	8.448	0.001
Treatment × day	8	70	6.371	<0.001

Abbreviations: NumDF, numerator degree of freedom; DenDF, Denominator degree of freedom

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 ($p < 0.001$) and the second 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, FR+L, and FR+M groups did not show significant change across all time points. 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 on 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 (Table 13). 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; $p = 0.018$, Figure 22B). The body weight gain in 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).

The effect of 20% feed restriction on body weight was similar to our previous results (REDA et al., 2024b), and we found that FR significantly reduced body weight gain compared to the control feeding. The combined supplementation of leucine and methionine (FR+ML) led to an increase in body weight: 5.55 g from the initial weight to

day 7, 6.65 g from day 7 to day 14, and a total gain of 12.01 g from the initial weight to day 14. In contrast, the FR+L group showed an increase of 3.8 g by day 7 but no further weight gain between days 7 and 14 (0.74 g). The results suggest that combined supplementation of leucine and methionine may help mitigate the effects of restricted feeding.

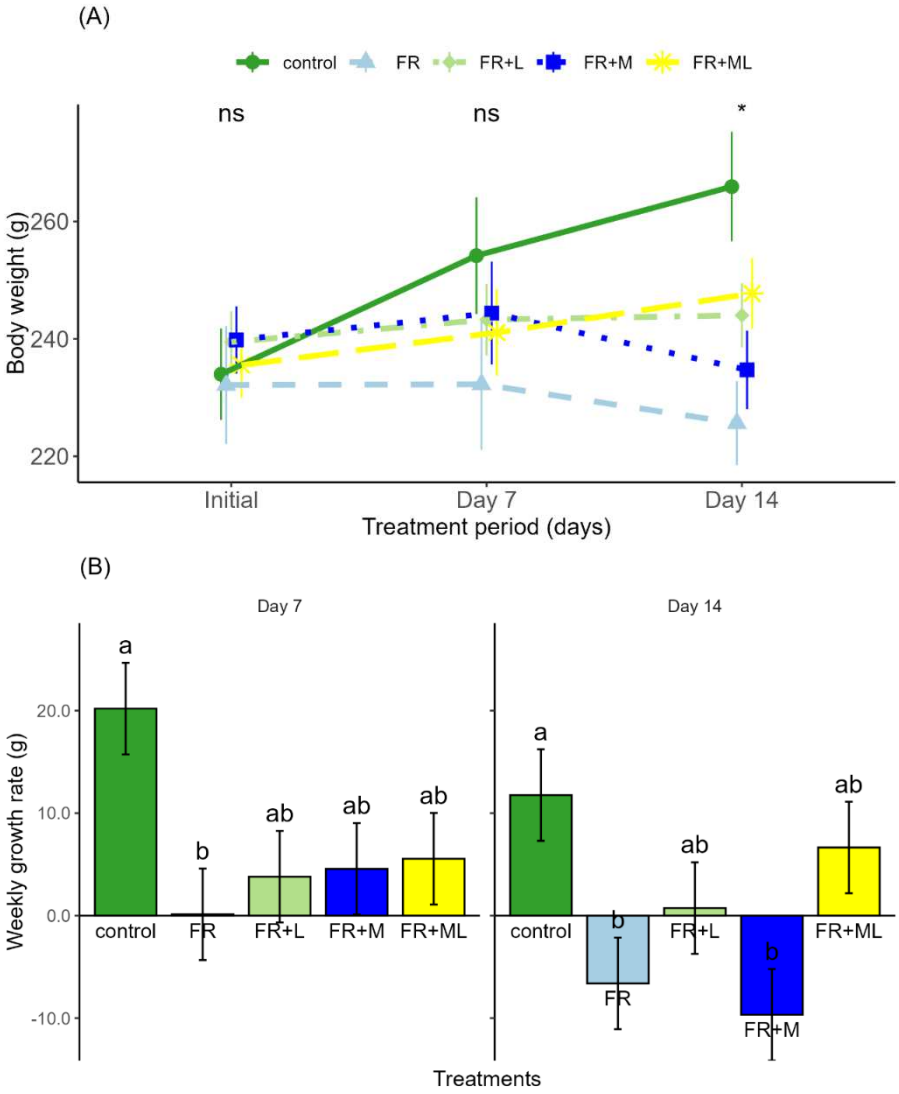

Figure 22. Effect of amino acid supplementation on top of restricted feeding on (A) body weight (B) body weight gain of Japanese quails. (A) Data are represented by the mean ± SEM from 8 birds per group across three time points. (B) Weekly body weight gain was determined by subtracting Day 1 weight from initial weight and Day 14 weight from Day 7 weight. 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: 'ns', not significant at $p < 0.05$; '*' significantly different at $p < 0.05$, 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, combination of 20% methionine and leucine supplemented on top of 20% restriction.

Our goal with the supplementation was to ensure that the leucine, methionine, or combined leucine and methionine levels were quantitatively balanced with those of the control groups, while other nutrients are restricted. Consequently, the supplemented amounts of these amino acids matched what the control groups received, based on their feed consumption. Supplementing these amino acids on top of restricted feeding could lead to varying fitness outcomes, potentially causing an increase, maintain, or decrease. The increase or maintenance could be due to improving metabolic efficiency or the decrease could be due to an amino acid imbalance (PARK, 2006). Previous studies reported that up to 30% leucine supplementation on the starter broiler chicken increased final body weight and body weight gain, while those above that had no effect compared to the recommended level (KRATEI and SHAHIR, 2021). Another study reported that about 30% methionine supplementation at the top of the recommended level did not change the body weight gain of fast-growing and slow-growing broilers (WEN et al., 2017). Other studies also reported that above 30% - 90% methionine supplementation did not have significant body weight change. These results slightly corroborate our findings, though the base on which amino acid were supplemented was different.

Table 13. Output of the linear mixed-effects model showing the effect of amino acid supplementation on weekly body weight gain across the time points (day)

Fixed factors	NumDF	DenDF	F value	Pr(>F)
Treatment	4	70	6.166	<0.001
Day	1	70	4.935	0.030
Treatment × day	4	70	0.834	0.508

Abbreviations: NumDF, numerator degree of freedom; DenDF, Denominator degree of freedom

4.3.1.2. Effect of amino acid supplementation on hepatic gene expression

Treatments did not show a significant effect on liver *GHR* gene expression ($p = 0.127$), while there is a reducing trend in response to FR (Figure 23A). 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 FR-treated groups (Figure 23B). All the supplemented groups showed a clearly increasing trend compared to the unsupplemented restricted group. Treatments had no significant effect on liver *IGF1R* gene expression (p = 0.665; Figure 23C).

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 the FR (Figure 23D). 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 23D). Treatments did not show a significant effect on liver *FASN* gene expression (p = 0.447; Figure 23E).

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 23F). 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 23G). Treatments had no significant difference in liver *ATG5* gene expression (Figure 23H). The effect of FR on the expression of liver *mTOR* pathway genes was similar to our previous reported result (REDA et al., 2024a; REDA et al., 2024b). Feed restriction downregulates the anabolic genes, including *IGF1* and *mTOR*, whereas, downregulates the catabolic genes, including the autophagy genes, and was discussed in Section 4.1.2.

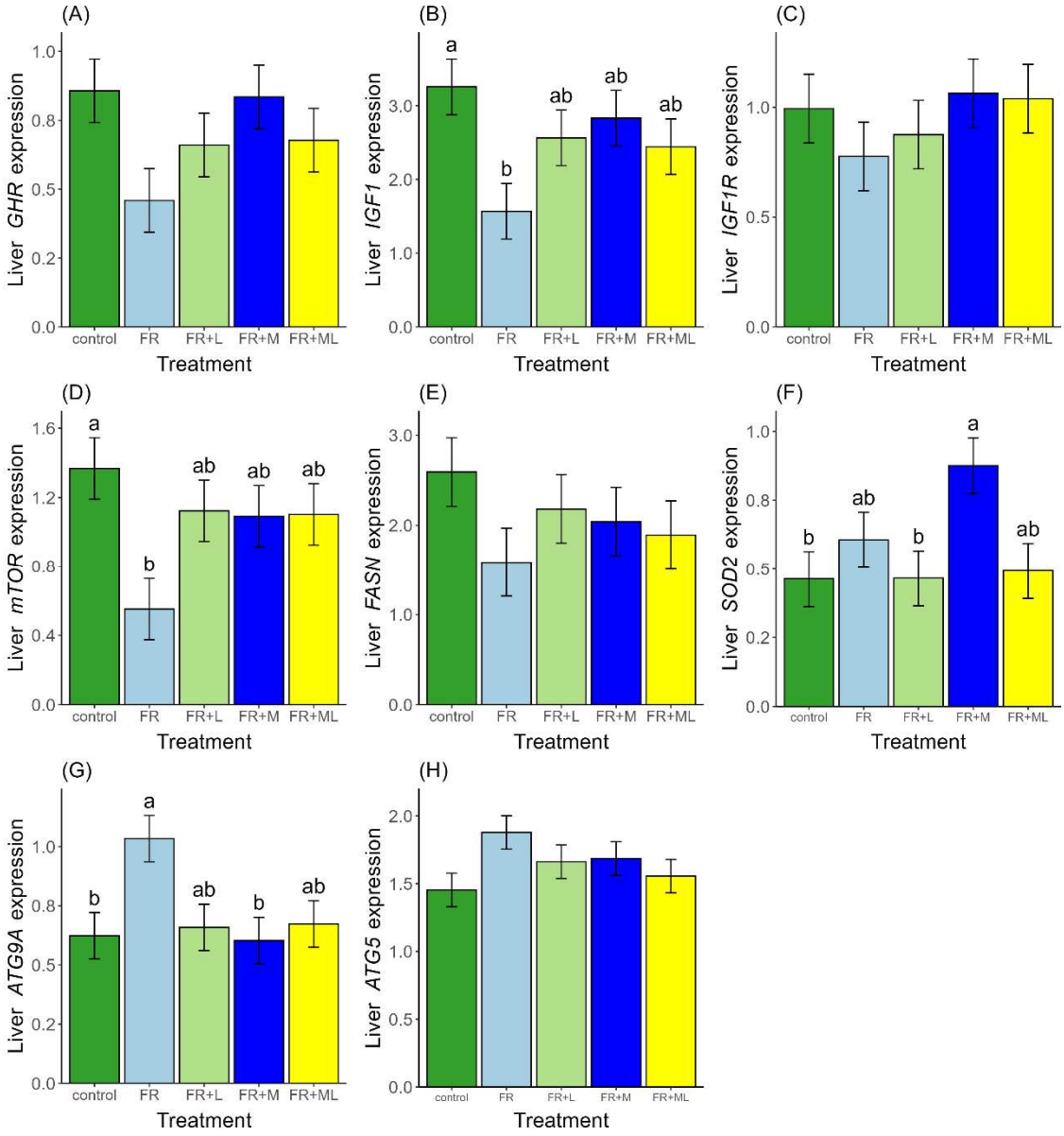

Figure 23. Effect of amino acid supplementation on the top of feed restriction on expression of mTOR pathway genes. (A) growth hormone receptor (GHR), (B) insulin-like growth factor 1 (IGF1), (C) insulin-like growth factor 1 receptor (IGF1R), (D) mechanistic target of rapamycin (mTOR), (E) Fatty acid 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

In addition to being used as building blocks of protein synthesis, amino acids regulate multiple pathways, from transcription to translation processes (KIMBALL and JEFFERSON, 2006). One of the pathways regulated by amino acid signalling is the mTOR pathway (DEMETRIADES et al., 2014; SHIMOBAYASHI and HALL, 2016), a pathway that governs physiological decisions of organisms to coordinate their reproduction, growth, and lifespan (GUO and YU, 2019; PAPADOPOLI et al., 2019). The mTOR largely maintains a cellular balance between anabolism and catabolism in response to the other signals such as growth factors, amino acids, and stress (KIM et al., 2008; MAO and ZHANG, 2018). The impact of growth factors in the absence of amino acids is much less in the process of regulating mTOR signalling (HARA et al., 1998; PROUD et al., 2001; TAKAHARA et al., 2020). Previous reports suggested that the variation in the availability of some essential amino acids, particularly leucine (KENNEDY and LAMMING, 2016; SURYAWAN et al., 2020) and methionine (ATHERTON et al., 2010; WEN et al., 2014), plays a crucial role in the regulation of the pathway.

In line with our findings, DENG et al. (2014) reported that 20% and 40% of dietary leucine supplementation increases the expression of mTOR pathways genes in the pectoral muscles of neonatal chicks. Another study reported that 30%, 60%, and 90% leucine supplementation on top of control feeding gives divergent expression of mTOR pathway anabolic genes at different portions of the intestine of neonatal broiler chicks (CHANG et al., 2015). The study reported that leucine supplementation reduces the expression of duodenum mTOR pathway anabolic genes. Conversely, expression of jejunum and ileum mTOR pathway anabolic genes increased at the 30% and 60% supplementation, whereas it tends to reduce at 90% supplementation. The tissue-specific variation across the studies was interesting. Furthermore, methionine supplementation was reported to increase liver *IGF1* and *GHR* gene expression in the starter and growers of broilers (DEL VESCO et al., 2015). *In-ovo* leucine and methionine feeding also showed a significant increase in mTOR pathway anabolic genes (NDUNGURU et al., 2024, Under review).

Our study differed in that supplementation was applied on top of restricted feeding to investigate whether the supplementation of these amino acids restored expression of mTOR pathway genes. We did not find previous studies to directly corroborate our findings. Accordingly, supplementation with leucine, methionine, or both showed an observable effect on gene expression. The GHR, IGF1, and mTOR genes clearly showed an increasing trend compared to the unsupplemented restricted group. In addition to

increasing gene expression, supplementation with these amino acids could facilitate activation of mTORC1 (TAKAHARA et al., 2020). The role in increasing mTOR expression and activation of mTORC1 kinase could affect the expression of genes downstream of mTOR, including autophagy genes, which are important for intracellular nutrient recycling from damaged cellular components. This process may allow the body to use available and recycled amino acids more efficiently.

In our study, the amino acid-supplemented groups showed a similar *ATG9A* expression as the control group. Interestingly, the specific effect of methionine supplementation on *SOD2* expression could suggest the role of methionine in increasing antioxidants gene expression (CATANESI et al., 2021). These all support our hypothesis that dietary supplementation of these essential amino acids restore the expression of mTOR pathway genes that can be downregulated due to feed restriction. Interestingly, supplementing combination of leucine and methionine in the same group did not have an additive effect. It was not different from individual leucine or methionine supplementations. Therefore, supplementation with a single amino acid (leucine or methionine) could sufficiently fill the gap of dietary limitations on the expression of the mTOR pathway gene.

4.3.2. Effect of energy and protein restriction

4.3.2.1. Effect on body weight

Body weight was significantly lower on the energy restriction (ER) feeding compared to the control and protein restriction (PR) feedings (Figure 24, Table 14). In the first week, the trend suggested observable variation among treatment groups (Figure 24A). However, Tukey's HSD adjustment for family-wise comparisons revealed no significant differences among the treatments. 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 24A). 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 ($p = 0.038$). While the PR fed groups showed increasing body weight with time, the ER fed group did not show any increment (Figure 24).

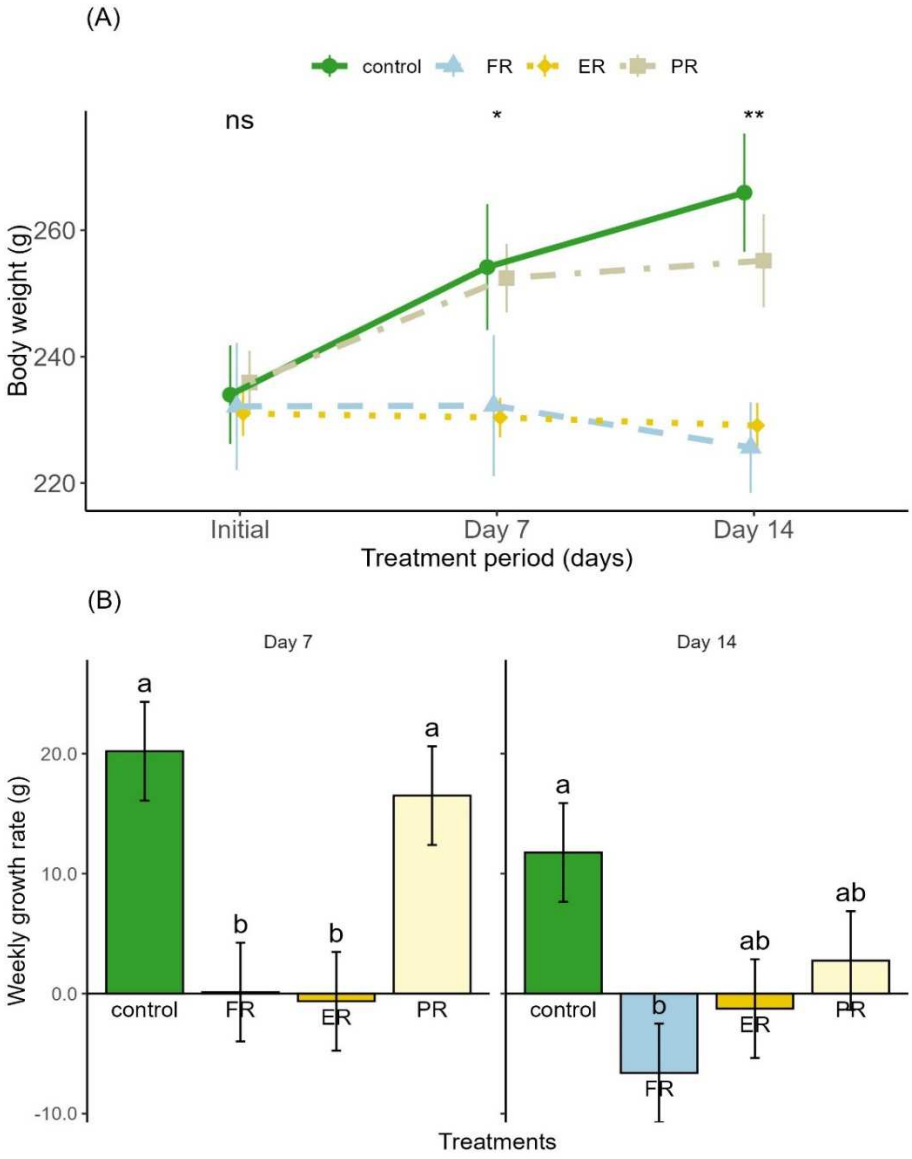

Figure 24. Effect of energy and protein restriction on (A) body weight and (B) weekly body weight gain. Abbreviations: Control, birds received full of their daily feed intake; FR, 20% restricted feeding; PR, 20% protein restriction; ER, 20% metabolisable energy restriction. Other explanations from Figure 22.

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 24B, Table 15). This finding revealed that a 20% metabolisable energy reduction from standard feeding (NRC, 1994) is enough to attenuate the growth of maturing birds. Throughout the treatment period, the energy-restricted group did not show any body weight gain. While feed consumption was higher in the 20% energy restricted group, the effect on body weight was similar to that of the 20% feed restricted group. Therefore, metabolisable energy deficiency could substitute

feed restriction (total feed reduction). During energy deficiency, the animal body increases the breakdown of body reserves to use as an alternative energy source and starts to reduce body weight (ARAFA et al., 1985; MASORO, 2009). 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. The IGF-1/AMPK/mTOR pathway is the key pathway mediating the metabolic effects of energy restriction.

Table 14. Output of the linear mixed-effects model showing the effect of energy or protein restriction treatment on body weight across the time points (days)

Fixed factors	NumDF	DenDF	F value	Pr(>F)
Treatment	3	28	2.682	0.066
Day	2	56	11.919	<0.001
Treatment x day	6	56	7.538	<0.001

Abbreviations: NumDF, numerator degree of freedom; DenDF, Denominator degree of freedom

In contrast with previous studies in chickens (ABDEL-HAFEEZ et al., 2016; KESHAVARZ, 1984; URBAN et al., 2018), our finding revealed no effect of a 20% protein restriction on the body weight of maturing quails. The similar body weight gain of the PR group in the first week compared to the control group, despite having similar feed intake, was remarkable. However, in the second week, the PR group showed a reduced trend in body weight gain compared to the control group. This suggests that with increasing time, the PR group may experience a faster decline in body weight gain than the control group. Protein restriction induces fibroblast growth factor 21 (FGF21) expression to regulate body weight through the mTOR pathway (HILL et al., 2022; HILL et al., 2017).

Table 15. Output of the linear mixed-effects model showing the effect of energy or protein restriction treatment on body weight across the time points (days)

Fixed factors	NumDF	DenDF	F value	Pr(>F)
treatment	3	28	2.682	0.066
Day	2	56	11.919	<0.001
treatment x day	6	56	7.538	<0.001

Abbreviations: NumDF, numerator degree of freedom; DenDF, Denominator degree of freedom

4.3.2.2. Effect on hepatic gene expression

Neither the energy restriction nor the protein restriction showed significant difference in the expression of all the anabolic genes compared to the control group (Figure 25A-E), except that the ER showed a noticeable reducing trend in mTOR gene expression (Separate comparison: $p = 0.069$; Figure 25D). Unlike the effect on body weight (Figure 24), energy restriction did not show a significant effect on mTOR pathway anabolic genes. 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 fed groups ($p = 0.05$), which was also similar effect with the FR treated group (Figure 25G). In response to systemic energy reduction, the intracellular energy deficiency activates the adenosine monophosphate-activated protein kinase (AMPK) and suppresses mTOR pathway, as a result, facilitates autophagy genes expression for cellular homeostasis (XU et al., 2012). 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 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 25), whereas, showed significantly higher *mTOR* gene expression compared to the FR group (Figure 25D). Protein restriction is expected to down regulate mTOR pathway through inducing FGF21 expression (LI et al., 2024; WELLES et al., 2020). As proteins are sources of amino acids, their scarcity could contribute to cellular amino acid deficiency and affect mTOR pathways genes (BENSALEM et al., 2023; WANG et al., 2017). However, our study did not confirm this narrative.

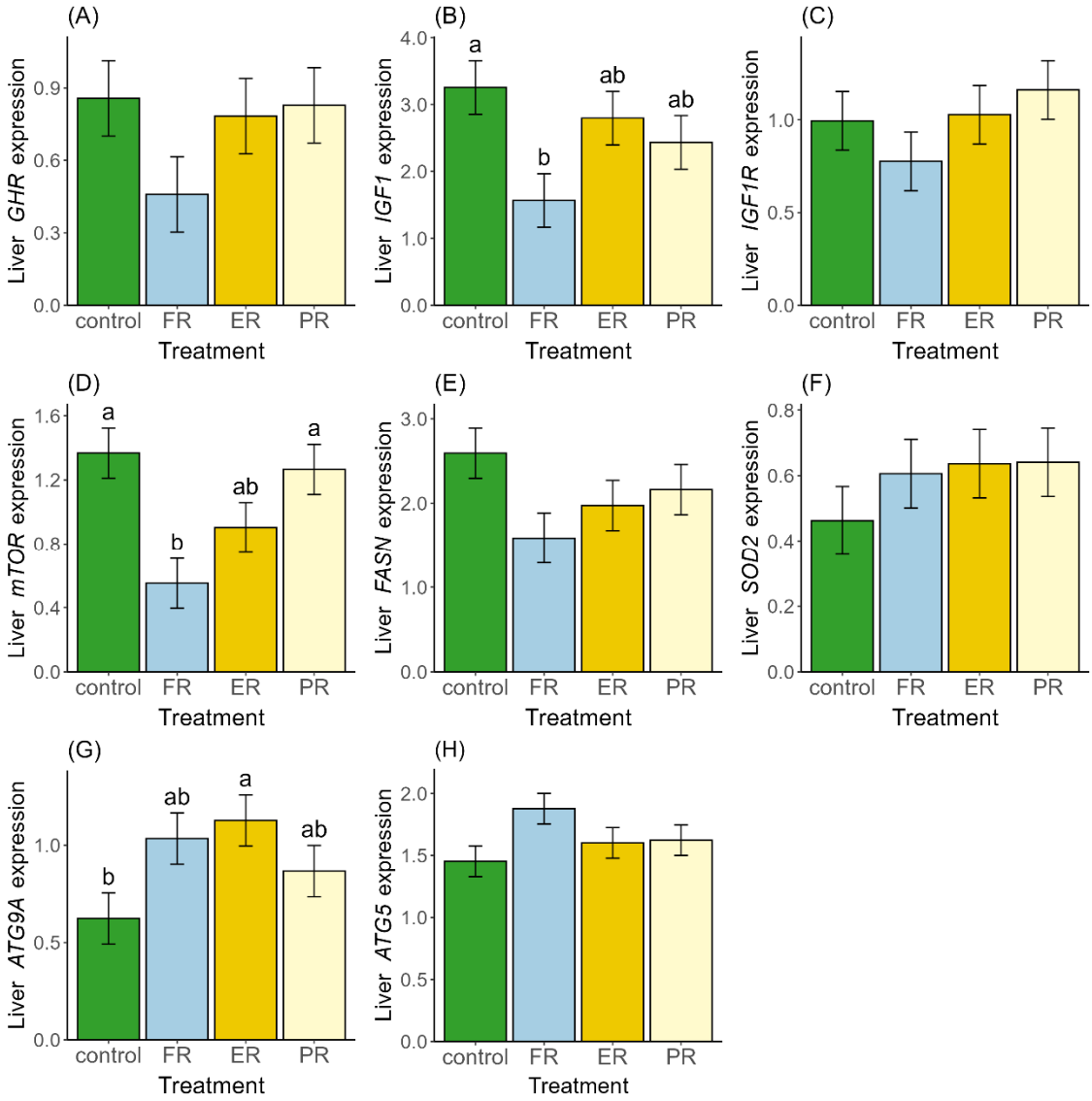

Figure 25. Effect of energy and protein restrictions on expression of mTOR pathway genes. Abbreviations: Control, birds received full of their daily feed intake; FR, 20% restricted feeding; PR, 20% protein restriction; ER, 20% metabolisable energy restriction. Other explanations from Figure 22.

4.3.2. Association of variables

Expression of genes in the mTOR pathway has a significant correlation with each other. Therefore, we should have to use principal component analysis (PCA) to avoid multicollinearity of variables and reduce the number of variables that should be considered in the association analysis. Accordingly, the first two components with eigenvalues greater than 1 have been retained for further association. Expression of *GHR*, *IGF1*, *mTOR* and *FASN* gene are the major contributor for PC1, while expression of *IGF1R*, *ATG9A* and *SOD2* are the major contributor for PC2 (Appendix Table 7). We further performed a linear regression using the PCs as dependent variables (Figure 26),

and found that PC1 had a significant positive relationship ($p < 0.001$), whereas PC2 had a significant negative relationship with body weight ($p < 0.001$). The result corroborated our previous finding; that expression of *IGF1* and *mTOR* positively, whereas ATG genes negatively correlated with body weight (REDA et al., 2024a; REDA et al., 2024b).

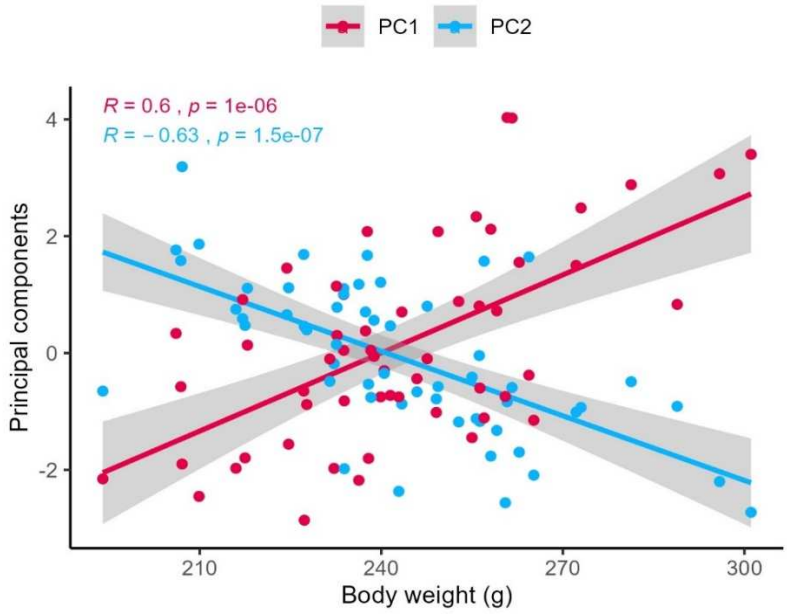

Figure 26. Overall association of body weight with principal components derived from genes expression. Note: Principal components are derived from the eight genes. PCs having Eigenvalue of above one are retained for relationship analysis.

5. CONCLUSIONS

Overall, dietary limitation, dietary unpredictability, and specific nutrient manipulation had remarkable effects on the mTOR pathway in Japanese quails. FR induced 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 reallocation 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 all.

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. The anabolic genes of *RPS6K1* and *GHR* showed sex-specifically intensified expression in the restricted groups, contrary to our assumption. It is also evident from the study that there is a notable disparity in IGF-1 levels between females and males, with females exhibiting 64.7% higher compared to males, supporting the notion of sex-specific IGF-1 secretion patterns. However, there was no directional response in circulating IGF-1 levels due to FR, which could be attributed to the maturity status of the birds. Maturation likely promotes local tissue IGF-1 production for paracrine and autocrine utilization. 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 triglycerides to maintain a circulating energy source and support egg production. Triglyceride levels also exhibited a sex-specific association with body weight, demonstrating a positive relationship in females but no significant relationship in males. 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. Egg production and egg weight did not respond to unpredictable feeding. The unpredictable feeding affected 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. Additionally, gene expression in the liver and the muscle showed a strong correlation, which indicates that mTOR pathway genes are coherently expressed in the liver and muscle tissues. Overall, we can conclude that the two weeks of daily unpredictable feeding have an observable impact on female birds, while the effects on males are weak. The difference between females and males may be attributed to reproduction and physiological mechanisms. Birds kept laying consistent numbers and egg weight throughout the dietary fluctuation, which may be at the expense of their body weight. Expression of genes could have mediated the effect of unpredictability, as most genes in females are either downregulated or upregulated.

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 has shown an increasing trend in expression of liver *GHR*, *IGF1,* and *mTOR* genes, whereas it showed a reduced trend in expression of *ATG9A*. Interestingly, methionine supplementation remarkably increased *SOD2* expression, a gene encoding 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 fills 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 birds, similar to the effect of restricted feeding. However, the effect was not coupled with an effect on the expression of anabolic 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. This also indicated the effect of low energy on alternative pathways that can promote autophagy without significantly affecting mTOR activation. The non-significant effect of a 20% protein restriction was remarkable. Neither body weight nor expression of the mTOR pathways were affected. The result was contrary to our assumption, which we 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.

6. 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.

7. 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.

8. SUMMARY

The mechanistic target of rapamycin (mTOR) pathway is a conserved pathway involved in a multitude of cellular processes. It maintains cellular balance between anabolism and catabolism in response to cues such as growth factors and nutrients (KENNEDY and LAMMING, 2016). It regulates several growth-related processes, including nuclear transcription, ribosome biogenesis, translation, metabolism, and autophagy. As a result, the pathway is proposed to affect life-history traits, including growth, reproduction, immunity, and ageing (PAPADOPOLI et al., 2019; SAXTON and SABATINI, 2017). nutrient-sensing As a central cellular nutrient-sensing unit, mTOR receives upstream signalling from GH-promoted IGF-1 and systemic energy sensing AMPK and senses intracellular amino acids (CONDON and SABATINI, 2019; LAPLANTE and SABATINI, 2009). In effect, mTOR regulates downstream effectors of transcription and translation factors to facilitate mRNA transcription and translation (MA and BLENIS, 2009; ROCZNIAK-FERGUSON et al., 2012; ZHAO et al., 2024b).

Despite the fundamental importance of this process, how different nutritional states are regulated through the expression of genes governing this pathway and its consequential effects on fitness remains understudied. Additionally, the vast majority of our current knowledge about the mTOR pathway originates from model organisms, such as yeast, nematodes, fruit flies, and mice, however, little is known about its regulation and functioning in birds. Some of the effects of mTOR signalling seem to be specific to different species of organisms. As birds have some special physiological characteristics, they are important model organisms for studying the upstream effects on the evolutionarily highly conserved mTOR signalling pathway and resulting lower stream effects. Therefore, we used Japanese quail (*Coturnix japonica*) as an avian model to investigate the effect of nutritional cues on mTOR signalling pathway and its association with life-history traits. Accordingly, we conducted three experiments: applying 1) gradients of feed restriction levels; 2) unpredictable feeding treatment; 3) various nutrient restrictions (restricting whole feed, calories, proteins, or methionine) and supplementation of methionine and leucine on top of restricted feeding.

In the Experiment 1, we used feed restriction to mimic nutritional deficiency to study the mechanistic link between gene expression and life-history traits. Nutritional limitations affect an organism's physiology through conserved metabolic pathways, one of which is proposed to be the mTOR pathway. Feed restriction is a robust laboratory

method for studying molecular and physiological mechanisms of life history. However, the impact of varying levels of feed restriction and its sex-specific responses is understudied, and in birds, it remains unknown. Quails were exposed to 20%, 30%, and 40% feed restriction (FR) or *ad libitum* feeding to investigate the expression of key mTOR pathway genes, some physiological traits, and body weight and reproduction traits. The experiment was conducted on 32 female and 32 male quails and lasted two weeks. The study was applied in an individual-bird based cage system with controlled light, temperature, and humidity conditions. We measured initial, week 1, and week 2 body weights, and blood samples were collected on these days. We recorded egg parameters daily. Gene expression was measured using real-time qPCR from the liver tissue sample at the end of week 2. Plasma IGF-1 was measured using a competitive ELISA assay, while the level of triglycerides was measured using a photometric method with a half-automatic analyser. We found that body weight was significantly reduced in all restriction levels at both week 1 and week 2 in females. In males, only the severely restricted level (40%) showed a significant reduction. Egg number was significantly reduced only in the 40% restricted feeding. Egg weight was reduced in the 30% and 40% restricted feeding.

We found differential gene expression signatures in the key mTOR pathway genes in both sexes. Expression of the *mTOR* gene showed feed restriction-dose-dependent reduction and was proportional to body weight losses in females, whereas in males, it decreased similarly across all restriction levels. *IGF1* gene expression was equally decreased at all restriction levels in both sexes. Surprisingly, *RPS6K1* expression increased at all restriction levels, and *GHR* increased in the 30% and 40% restriction levels in both sexes, which is also different from studies reported from mammalian model species (CAO et al., 2001; MA et al., 2015a). *ATG9A* expression was increased at all restriction levels in males while only at 40% restriction in females. The expression of *ATG5* was higher at all restriction levels in females, while there was no effect of feed restriction in males. The result indicated a sex difference in the expression of mTOR pathway genes. Furthermore, we found observable sexual differences in plasma IGF-1 and triglyceride levels. IGF-1 levels exhibited a marked sexual difference, with females having 64.7% higher IGF-1 levels than males. Females showed more than six-fold higher triglyceride levels than males. The difference is attributed to the sexually size-dimorphic nature and differential energy allocation strategies (BARNETT et al., 2015; COX et al., 2022; MA et al., 2015b).

Moreover, our study showed a resource allocation trade-off between body maintenance and reproduction, depending on the level of resource availability. Birds received 70% and 80% of their feed intake, re-allocating resources to reproduction, with a proportional loss in body weight. However, birds received only 60% of their intake, re-allocating feed to body maintenance, with a proportional egg weight loss. The study also revealed that regardless of the treatment, proportionally higher reproductive investment was associated with individual variation in *mTOR* expression.

In the second experiment, we used unpredictable feeding to study the effect of nutritional fluctuation on the mTOR pathway genes and their corresponding effects on body weight and reproduction. The predictability of feed availability is important for organisms to adjust their genetic, physiological, and morphological appearances. If dietary conditions are unforecastable, organisms face a stressful challenge to cope with the condition; as a result, the biological mechanisms could be changed (FOKIDIS et al., 2012; LYNN et al., 2023; MUNN et al., 2010). However, we have limited knowledge of whether the mTOR pathway mediates unpredictability. Therefore, we feed quails either daily unpredictably, predictably with a 40% restriction, or with full feeding (control). The unpredictably fed birds received the same amount of total feed 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. The experiment lasted for 16 days and was conducted on 24 female and 24 male quails of six months of age, selected based on sex-based uniform body weight. The experiment was applied based on individual cage systems. Body weight was recorded on days 0, 4, 8, and 16 of the experiment, while egg traits were measured daily. The expression of mTOR pathway genes was measured from liver and muscle tissues and collected at the end of the experiment. During the measuring and sampling days, the unpredictably fed birds received full of their individual intake to ensure consistent feeding conditions with the control group and prevent any short-term effects of underfeeding or overfeeding.

The study revealed that unpredictable feeding reduced body weight and affected gene expression only in females. In the liver, expression of *IGF1* and *mTOR* was reduced due to dietary unpredictability. *RPS6K1, FOXO1, ULK1, ATG9A*, and *ATG5* showed an increasing trend in the unpredictably fed group. In the muscle, only *mTOR* and *IGF1R* decreased due to the variable feeding. Unpredictable feeding did not affect any of the egg traits. Although liver gene expression was strongly correlated with the expression of the corresponding gene in the muscle, the response to treatment was tissue- and sex-specific.

The third experiment was applied based on amino acid and macronutrient manipulation. Amino acids are reported as potent activators of mTOR (CONDON and SABATINI, 2019; SHIMOBAYASHI and HALL, 2016). Low levels of amino acids downregulate mTOR signalling in a wide range of organisms (JIN et al., 2021; TRAUTMAN et al., 2022). The mechanisms through which specific amino acids activate mTOR are not fully understood, although withdrawal of some essential amino acids efficiently downregulates the signalling (TRAUTMAN et al., 2022). Additionally, dietary protein regulates the mTOR pathway through upstream signals (FGF21) and as a source of essential amino acids (LI et al., 2024). Systemic energy content also has a crucial regulatory role in the mTOR pathway via regulation of the AMPK pathway (GONZÁLEZ et al., 2020). Here, we tested how supplementation with 20% leucine, 20% methionine, or both on top of restricted feeding affected the mTOR pathway. We also examine the effect of a 20% protein or 20% energy restriction under full feeding conditions. We compared all these treatments with full-balanced feeding or a 20% feed restriction.

We found that combined supplementation of leucine and methionine supports partial increase in body weight gain compared to the feed-restricted group, reducing body weight gain during the two-week treatment period. Supplementing leucine, methionine, or both partially increased the expression of liver *GHR*, *IGF1*, and *mTOR* genes and decreased the expression of the *ATG9A* gene. Methionine supplementation showed a significant increase in *SOD2* gene expression compared to all groups. Overall, leucine and methionine supplementation showed the expected result of partially filling the gap due to feed restriction but were not equal to the full feeding group. Energy restriction significantly affected body weight, similar to the balanced feed restriction group. However, the effect was not coupled with an effect on the expression of the anabolic genes. Energy restriction increased the expression of *ATG9A*, which may be necessary for recycling cellular waste and fuelling other functions. Protein restriction did not affect body weight or the expression of mTOR pathway genes. Finally, the expression of mTOR pathway genes showed a strong correlation with the body weight of birds.

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10. PUBLICATIONS IN THE FIELD OF RESEARCH

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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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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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List of other publications

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12. STATEMENTS

Statement

I wrote this thesis in the framework of the University of Debrecen Doctoral School of Animal Science for the purpose of obtaining a doctoral degree (Ph.D.) at the University of Debrecen.

Debrecen, 2024. …….

.....

Gebrehaweria Kidane Reda

PhD candidate

Statement

We hereby certify that the doctoral candidate **Gebrehaweria Kidane Reda** has carried out his work under our supervision within the framework of the above-mentioned Doctoral School between 2020-2024. The candidate has made a decisive contribution to the results of the thesis through his independent creative work, and the thesis is the candidate's independent work. We recommend that the thesis be accepted.

Debrecen, 2024. ……..

.....	.....
Dr. Levente Czeglédi (Prof.)	Dr. Ádám Z. Lendvai
Supervisor	Supervisor

13. ANNEXES

Appendix Table 1. All pairwise body weight comparisons of treatment groups across all time points in females and males based on emmeans, in the feed restriction experiment

Sex	Time point[1]	*Contrast*	*Estimate*	*t-ratio*	*p-value*
Female	Initial (day 0)	ADL – FR20	3.45	0.40	0.978
		ADL – FR30	12.82	1.48	0.459
		ADL – FR40	14.88	1.72	0.329
		FR20 – FR30	9.38	1.08	0.702
		FR20 – FR40	11.43	1.32	0.557
		FR30 – FR40	2.05	0.24	0.995
	Week 1 (day 7)	ADL – FR20	38.09	4.40	0.001
		ADL – FR30	45.3	5.23	<0.001
		ADL – FR40	62.34	7.20	<0.001
		FR20 – FR30	7.21	0.833	0.838
		FR20 – FR40	24.25	2.80	0.039
		FR30 – FR40	17.04	1.96	0.219
	Week 2 (day 14)	ADL – FR20	42.74	4.93	<0.001
		ADL – FR30	55.84	5.23	<0.001
		ADL – FR40	71.01	8.20	<0.001
		FR20 – FR30	13.1	1.51	0.440
		FR20 – FR40	28.27	3.26	0.012
		FR30 – FR40	15.18	1.75	0.312
Male	Initial (day 0)	ADL – FR20	4.21	0.55	0.945
		ADL – FR30	-5.45	-0.72	0.889
		ADL – FR40	5.64	0.74	0.879
		FR20 – FR30	-9.66	-1.27	0.585
		FR20 – FR40	1.42	0.19	0.997
		FR30 – FR40	11.09	1.46	0.469
	Week 1 (day 7)	ADL – FR20	15.53	2.05	0.187
		ADL – FR30	14.12	1.86	0.259
		ADL – FR40	21.9	2.89	0.030
		FR20 – FR30	-1.40	-0.18	0.997
		FR20 – FR40	6.38	0.84	0.835
		FR30 – FR40	7.78	1.02	0.736
	Week 2 (day 14)	ADL – FR20	16.31	2.15	0.154
		ADL – FR30	15.01	1.98	0.212
		ADL – FR40	31.66	4.17	<0.001
		FR20 – FR30	-1.30	-0.17	998
		FR20 – FR40	15.35	2.02	0.196
		FR30 – FR40	16.65	2.19	0.141

[1]Pooled Std.error for females = 8.66, df = 35.5 Pooled Std.error for males = 7.59, df = 42.9; Emmeans adjusted for Tukey-test was used as *post hoc* test with $p < 0.05$ significance level for all mean comparisons, including in the following tables.

Appendix Table 2. All pairwise body weight comparisons of time points across all treatment groups in females males based on emmeans, in the feed restriction experiment.

Sex	Treatments	Contrast	Estimate	*t-ratio*	*p-value*
Female	ADL	Initial – week 1	-8.95	-1.704	0.213
		Initial – week 2	-6.09	-1.159	0.482
		Week 1 – week 2	2.86	0.545	0.849
	FR20	Initial – week 1	25.69	4.891	<0.001
		Initial – week 2	33.2	6.321	<0.001
		Week 1 – week 2	7.51	1.43	0.332
	FR30	Initial – week 1	23.52	4.479	0.001
		Initial – week 2	36.92	7.03	<0.001
		Week 1 – week 2	13.4	2.551	0.035
	FR40	Initial – week 1	38.51	7.332	<0.001
		Initial – week 2	50.05	9.529	<0.001
		Week 1 – week 2	11.54	2.197	0.081
Male	ADL	Initial – week 1	-0.54	-0.128	0.991
		Initial – week 2	-0.16	-0.039	0.999
		Week 1 – week 2	0.37	0.089	0.996
	FR20	Initial – week 1	10.77	2.56	0.035
		Initial – week 2	11.94	2.83	0.017
		Week 1 – week 2	1.16	0.28	0.959
	FR30	Initial – week 1	19.04	4.52	<0.001
		Initial – week 2	20.30	4.82	<0.001
		Week 1 – week 2	1.26	0.30	0.952
	FR40	Initial – week 1	19.04	3.73	0.001
		Initial – week 2	20.30	6.14	<0.001
		Week 1 – week 2	1.26	2.41	0.050

[1]Female Std.Error, 5.25; Female df, 56; Male Std.Error, 4.21; Male df, 56.

Appendix Table 3. Pairwise comparison of body weight of females and males at all the feed restriction gradients and time points based on emmeans.

Treatment	Time point	Contrast	Estimate	*t-ratio*	*p-value*
ADL	Initial weight	Female – male	43.25	5.14	<0.001
	Week 1 weight	Female – male	51.66	6.14	<0.001
	Week 2 weight	Female – male	49.17	5.84	<0.001
FR20	Initial weight	Female – male	44.01	5.23	<0.001
	Week 1 weight	Female – male	29.10	3.46	<0.001
	Week 2 weight	Female – male	22.75	2.70	0.008
FR30	Initial weight	Female – male	24.98	2.97	0.004
	Week 1 weight	Female – male	17.99	2.14	0.035
	Week 2 weight	Female – male	7.06	0.84	0.404
FR40	Initial weight	Female – male	34.01	4.04	0.001
	Week 1 weight	Female – male	11.22	1.33	0.186
	Week 2 weight	Female – male	9.82	1.18	0.246

Std.Error, 8.42; df, 85

Appendix Table 4. Pairwise comparisons of plasma triglyceride levels of all feed restriction levels at all-time points in female and male groups based on emmeans.

Sex	Time point	Contrast	Estimate[1]	*t-ratio*	*p-value*
Female	Initial (day 0)	ADL - FR20	-0.09	-0.63	0.921
		ADL - FR30	0.04	0.26	0.994
		ADL - FR40	-0.03	-0.19	0.997
		FR20 - FR30	0.13	0.89	0.808
		FR20 - FR40	0.06	0.44	0.971
		FR30 - FR40	-0.07	-0.45	0.969
	Week 1 (day 7)	ADL - FR20	0.37	2.55	0.061
		ADL - FR30	0.44	2.98	0.020
		ADL - FR40	0.37	2.49	0.071
		FR20 - FR30	0.06	0.43	0.973
		FR20 - FR40	-0.01	-0.06	0.999
		FR30 - FR40	-0.07	-0.49	0.961
	Week 2 (day 14)	ADL - FR20	0.55	3.77	0.002
		ADL - FR30	0.69	4.68	<0.001
		ADL - FR40	0.64	4.34	<0.001
		FR20 - FR30	0.13	0.91	0.802
		FR20 - FR40	0.08	0.56	0.942
		FR30 - FR40	-0.05	-0.34	0.986
Male	Initial (day 0)	ADL – FR20	0.05	0.30	0.991
		ADL – FR30	0.08	0.46	0.966
		ADL – FR40	0.16	0.96	0.773
		FR20 – FR30	0.03	0.17	0.998
		FR20 – FR40	0.11	0.66	0.911
		FR30 – FR40	0.08	0.49	0.960
		ADL – FR20	0.49	2.67	0.023

	ADL – FR30	0.57	3.37	0.006
	ADL – FR40	0.42	2.49	0.068
Week 1 (day 7)	FR20 – FR30	0.08	0.48	0.962
	FR20 – FR40	-0.07	-0.40	0.978
	FR30 – FR40	-0.15	-0.88	0.814
	ADL – FR20	0.45	2.67	0.044
	ADL – FR30	0.57	3.39	0.006
	ADL – FR40	0.45	2.68	0.043
Week 1 (day 14)	FR20 – FR30	0.11	0.71	0.891
	FR20 – FR40	0.001	0.01	1.000
	FR30 – FR40	-0.12	-0.70	0.895

[1]Female Std.Error, 0.15; Female df, 65; Male Std.Error, 0.17; Male df, 78.2.

Appendix Table 5. Pairwise comparisons of all restriction time points of log triglyceride levels at each restriction levels based on emmeans.

Sex	Treatment	Contrast	Estimate[1]	*t-ratio*	*p-value*
Females	ADL	Initial - Week 1	-0.06	-0.44	0.890
		Initial - Week 2	0.041	0.31	0.947
		Week 1 - Week 2	0.10	0.77	0.721
	FR20	Initial - Week 1	0.41	3.09	0.009
		Initial - Week 2	0.69	5.23	<0.001
		Week 1 - Week 2	0.28	2.14	0.092
	FR30	Initial - Week 1	0.34	2.58	0.033
		Initial - Week 2	0.69	5.24	<0.001
		Week 1 - Week 2	0.35	2.66	0.027
	FR40	Initial - Week 1	0.33	2.53	0.037
		Initial - Week 2	0.71	5.36	<0.001
		Week 1 - Week 2	0.37	2.83	0.017
Males	ADL	Initial - Week 1	-0.05	-0.36	0.932
		Initial - Week 2	0.12	0.81	0.698
		Week 1 - Week 2	0.18	1.17	0.476
	FR20	Initial - Week 1	0.38	2.53	0.037
		Initial - Week 2	0.52	3.45	0.003
		Week 1 - Week 2	0.14	0.92	0.629
	FR30	Initial - Week 1	0.43	2.88	0.015
		Initial - Week 2	0.61	4.06	<0.001
		Week 1 - Week 2	0.18	1.18	0.470
	FR40	Initial - Week 1	0.20	1.35	0.373
		Initial - Week 2	0.41	2.73	0.023
		Week 1 - Week 2	0.21	1.38	0.359

[1]Std.Error, 0.13; df, 56.

Appendix Table 6. Pairwise comparisons of females and males for log plasma triglyceride levels at all the treatment levels and time points (week) based on emmeans.

Treatment	Week	Contrast	Estimate[1]	*t-ratio*	*p-value*
ADL	Initial	Female - Male	1.84	11.63	<0.001
	Week 1	Female - Male	1.98	12.54	<0.001
	Week 2	Female - Male	1.88	11.88	<0.001
FR20	Initial	Female - Male	2.03	12.83	<0.001
	Week 1	Female - Male	1.84	11.67	<0.001
	Week 2	Female - Male	1.95	12.37	<0.001
FR30	Initial	Female - Male	1.97	12.48	<0.001
	Week 1	Female - Male	1.90	12.00	<0.001
	Week 2	Female - Male	1.92	12.14	<0.001
FR40	Initial	Female - Male	1.81	11.47	<0.001
	Week 1	Female - Male	1.80	11.39	<0.001
	Week 2	Female - Male	1.73	10.96	<0.001

[1]Std.Error, 0.16; df, 144.

Appendix Table 7. Pairwise comparisons of body weight of all treatments levels at all-time points in female and male groups based on emmeans of the unpredictable feeding experiment

Sex	Day	Contrast	Estimate	*t-ratio*	*p-value*
Female	Day 0	control - UNPR	7.20	0.67	0.78
		control - FR40	9.60	0.89	0.65
		UNPR - FR40	2.40	0.22	0.97
	Day 4	control - UNPR	15.81	1.46	0.317
		control - FR40	31.31	2.90	0.015
		UNPR - FR40	15.50	1.44	0.331
	Day 8	control - UNPR	21.54	1.99	0.125
		control - FR40	44.67	4.14	<0.001
		UNPR - FR40	23.14	2.14	0.092
	Day 16	control - UNPR	28.57	2.65	0.029
		control - FR40	57.21	5.30	<0.001
		UNPR - FR40	28.64	2.65	0.029
Male	Day 0	control - UNPR	-5.71	-0.53	0.858
		control - FR40	-3.59	-0.33	0.941
		UNPR - FR40	2.12	0.20	0.979
	Day 4	control - UNPR	-1.94	-0.18	0.982
		control - FR40	9.44	0.87	0.659
		UNPR - FR40	11.37	1.05	0.547
	Day 8	control - UNPR	-4.30	-0.40	0.916
		control - FR40	14.82	1.37	0.363
		UNPR - FR40	19.12	1.77	0.190
	Day 16	control - UNPR	-4.28	-0.40	0.917
		control - FR40	30.46	2.82	0.019
		UNPR - FR40	34.74	3.22	0.007

[1]Pooled Std.error = 10.8, df = 47.06;

Appendix Table 8. Pairwise comparisons of body weight of all time points at treatment levels in female and male groups based on emmeans of the unpredictable feeding experiment

Sex	Treatment	Contrast	Estimate[1]	*t-ratio*	*p-value*
Female	Control	day0 - day4	5.54	1.88	0.242
		day0 - day8	5.95	2.02	0.186
		day0 - day16	5.28	1.79	0.283
		day4 - day8	0.41	0.14	0.999
		day4 - day16	-0.26	-0.09	1.000
		day8 - day16	-0.68	-0.23	0.996
	UNPR	day0 - day4	14.15	4.80	<0.001
		day0 - day8	20.29	6.89	<0.001
		day0 - day16	26.65	9.05	<0.001
		day4 - day8	6.14	2.08	0.164
		day4 - day16	12.50	4.24	<0.001
		day8 - day16	6.36	2.16	0.140
	FR40	day0 - day4	27.25	9.25	<0.001
		day0 - day8	41.02	13.92	<0.001
		day0 - day16	52.89	17.95	<0.001
		day4 - day8	13.78	4.68	<0.001
		day4 - day16	25.64	8.70	<0.001
		day8 - day16	11.86	4.03	0.001
Male	Control	day0 - day4	3.34	1.13	0.670
		day0 - day8	8.09	2.74	0.035
		day0 - day16	8.35	2.83	0.027
		day4 - day8	4.75	1.61	0.376
		day4 - day16	5.01	1.70	0.327
		day8 - day16	0.26	0.09	1.000
	UNPR	day0 - day4	7.11	2.41	0.080
		day0 - day8	9.50	3.22	0.009
		day0 - day16	9.79	3.32	0.006
		day4 - day8	2.39	0.81	0.849
		day4 - day16	2.68	0.91	0.801
		day8 - day16	0.29	0.10	1.000
	FR40	day0 - day4	16.36	5.55	<0.001
		day0 - day8	26.50	8.99	<0.001
		day0 - day16	42.40	14.39	<0.001
		day4 - day8	10.14	3.44	0.004
		day4 - day16	26.04	8.84	<0.001
		day8 - day16	15.90	5.40	<0.001

[1]Pooled Std.error = 2.95, df = 126

Appendix Table 9. Pairwise comparisons of females and males for body weight at all the treatment levels and time points (days) based on emmeans of the unpredictable feeding experiment

Treatment	Day	Contrast	Estimate	*t-ratio*	*p-value*
Control	Day 0	female - male	51.65	4.78	<0.001
	Day 4	female - male	49.45	4.58	<0.001
	Day 8	female - male	53.79	4.98	<0.001
	Day 16	female - male	54.73	5.07	<0.001
UNPR	Day 0	female - male	38.74	3.59	0.001
	Day 4	female - male	31.70	2.94	0.005
	Day 8	female - male	27.95	2.59	0.013
	Day 16	female - male	21.88	2.03	0.048
FR40	Day 0	female - male	38.46	3.56	0.001
	Day 4	female - male	27.57	2.55	0.014
	Day 8	female - male	23.94	2.22	0.032
	Day 16	female - male	27.97	2.59	0.013

[1]Pooled Std.error = 10.80, df = 47.06;

Appendix Table 7. Contribution of original variables to the principal components in the amino acid and macronutrients manipulation experiment

Variables	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**	**PC8**
GHR	0.442	0.273	0.163	-0.201	0.409	0.512	-0.078	0.477
IGF1	0.445	-0.256	0.160	0.115	0.400	-0.477	-0.524	-0.190
IGF1R	0.324	0.445	-0.280	0.096	-0.478	0.235	-0.461	-0.337
mTOR	0.431	-0.042	-0.569	0.198	-0.156	-0.354	0.291	0.464
FASN	0.517	-0.181	0.090	-0.213	-0.008	0.143	0.571	-0.548
ATG9A	-0.022	0.530	0.080	-0.658	-0.020	-0.524	0.063	0.002
ATG5	0.152	0.325	0.666	0.514	-0.263	-0.186	0.212	0.121
SOD2	-0.157	0.491	-0.297	0.393	0.593	-0.034	0.216	-0.304