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SAWADI FRANSISCO NDUNGURU

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Head of Doctoral School:
Prof. Dr. István Komlósi D.Sc.
professor

Supervisors:
Dr. Levente Czeglédi Ph.D.
professor
Dr. Ádám Zoltán Lendvai Ph.D.
associate professor

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

Author
SAWADI FRANSISCO NDUNGURU
PhD candidate

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Written by **Sawadi Fransisco Ndunguru**, certified MSc in Applied Zoology

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Dissertation supervisors:
Dr. Levente Czeglédi Ph.D.
Dr. Ádám Zoltán Lendvai Ph.D.

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Dr. ……………………….. ……………………………………

Dr. ……………………….. ……………………………………

The evaluation board:

Chairperson: Dr. ………………………….. …………………………………

Members: Dr. ………………………….. ………………………………….

Dr. ………………………….. …………………………………

Dr. ………………………….. …………………………………

Dr. ………………………….. …………………………………

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ACTB	Actin beta
4EBP1	4E-binding protein
Akt	protein kinase B
AMPK	adenosine monophosphate activated protein kinase
Deptor	DEP-domain-containing mTOR interacting protein
FOXO1	forkhead box O1
IGF1	Insulin-like growth factor 1 gene
IGF-1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 gene
IGF-1R	Insulin-like growth factor 1 receptor
IL-1β	interleukin-1 beta
mLST8	mammalian homolog of protein Lethal Sec 13 protein
mSin1	mammalian stress-activated protein kinase interacting protein 1
mTORC1	mechanistic target of rapamycin complex 1
mTORC2	mechanistic target of rapamycin complex 2
MyD88	myeloid differentiation primary response gene 88
NF-κβ	nuclear factor kappa beta
PRAS40	proline-rich AKT substrate of 40 KD
Protor	protein observed with Rictor-1
Raptor	regulatory-associated protein of mTOR
ROS	reactive oxygen species
Rictor	rapamycin-insensitive companion of mTOR
RPL19	ribosomal protein 19
RPS6K1	ribosomal protein serine 6 kinase 1 gene
STAT3	signal transducer and activator transcription 3
S6K1	ribosomal protein serine 6 kinase 1
TLR	Tall-like receptor

1. GENERAL INTRODUCTION

Environmental factors, particularly nutritional availability during growth and sexual maturation, have a profound influence on reproductive investment in birds. Adequate nutrient intake, both in quantity and quality, is essential to meet the physiological demands of development and reproduction (ALLEN & ULLREY, 2004; JARMAN *et al.*, 2024). During reproduction, birds experience increased nutritional demands to support the rapid development of reproductive organs (MFOUNDOU *et al.*, 2021; CHEN *et al.*, 2023). Rapid growth and development of reproductive structures may be driven by nutrient availability (WHELAN *et al.*, 2021; KORVER, 2023), ensuring offspring viability (KARLSSON & LILJA, 2008; VALCU *et al.*, 2019).

Maternal conditions during the prenatal period profoundly affect offspring development. Female birds allocate non-genetic factors such as hormones, nutrients and immunity into the egg components such as albumen, yolk, and shell (WILLIAMS & GROOTHUIS, 2015). The deposition of maternal resources is influenced by maternal health, the size of the egg, environmental factors such as habitat quality, and predation risk (REED *et al.*, 2009; DAROLOVÁ *et al.*, 2014; SÉCHAUD *et al.*, 2022). Preferred breeding sites and favourable male partners may also lead to greater resource deposition in the eggs (LAHAYE *et al.*, 2015; NANGSUAY *et al.*, 2015). In poultry, maternal social environments such as stocking density are crucial for properly depositing the egg resources necessary for embryo development (GENG *et al.*, 2020; WAN *et al.*, 2023). A fertilized egg is self-contained nutritional unit. The growth and development of avian embryos depend entirely on female-deposited resources (NASIR & PEEBLES, 2018; WAN *et al.*, 2023). At this stage, rapid metabolic activity depletes albumen early, leaving the yolk as the primary nutrient source before hatching (LIU *et al.*, 2021). The Japanese quail (*Coturnix japonica*) was used in the study because it is an ideal model for studying growth, development and sexual maturation due to its rapid development and established role in developmental biology (OTTINGER & BRINKLEY, 1978; OTTINGER, 2001). It has been widely used to investigate the influence of embryonic nutrition on postnatal traits

(AL-DARAJI *et al.*, 2012; LUQMAN *et al.*, 2020; SRITABTIM *et al.*, 2024a) and remains a cornerstone species in growth physiology research (ZHANG *et al.*, 2024).

Nutritional deficiency can arise due to environmental challenges or feed restriction, which can adversely affect embryonic development and delay sexual maturation (VAN DER KLEIN *et al.*, 2018; AFROUZIYEH *et al.*, 2021). For example, feed restriction in poultry while controlling for body weight can reduce energy reserves and impair sexual maturation (ZUIDHOF *et al.*, 2014; VAN DER KLEIN *et al.*, 2018; RADULOVIC *et al.*, 2021). It may also reduce hatchability due to increased embryonic mortality resulting from restricted crude protein (VAN EMOUS *et al.*, 2015). On the other hand, controlled feed restriction improves body weight uniformity and egg production (LU *et al.*, 2023), though it can delay the development of sexual organs such as ovaries and follicles, leading to reduced reproductive performance (CARNEIRO *et al.*, 2019; LU *et al.*, 2021; LU *et al.*, 2023).

Growth and reproduction are metabolically demanding and may induce oxidative stress, a condition arising when reactive oxygen species (ROS) overwhelm the body's antioxidant defences, leading to cellular damage (HOSSEINIAN & HASANZADEH, 2021; OKE *et al.*, 2024). Oxidative stress can impair immune system function, damage biomolecules such as proteins, lipids, DNA and contribute to ageing (RYAN *et al.*, 2010; ROMERO-HARO *et al.*, 2016). Such damage often occurs in fast-growing birds, such as broiler chickens in ad libitum feeding, where high-fat deposition and metabolic rates increase ROS production (RICKLEFS, 2006; DE BEER & COON, 2007). Alternatively, reduced food intake can limit dietary antioxidants, resulting in elevated levels of oxidative stress (GAVRILOV & GAVRILOVA, 2001; RAJMAN *et al.*, 2006). Such stress during development may reduce lifespan (BARJA, 2004). Nutritional interventions, such as specific nutrient supplements, can reduce oxidative stress and boost the total antioxidant capacity. Dietary methionine supplementation improved egg weight, one-day ducklings' body weight, and total antioxidant capacity (RUAN *et al.*, 2018; LUGATA *et al.*, 2024a). Little is known about whether specific nutrient supplementation in a restricted diet can improve plasma total antioxidant capacity and reproductive investment in sexually maturing Japanese quail.

In ovo nutrient supplementation provides an excellent opportunity to offset nutritional constraints during embryonic development. This strategy facilitates essential nutrient delivery to enhance embryonic growth and early postnatal growth and development, such as carbohydrates (RETES *et al.*, 2018; ÁCS *et al.*, 2022; ZANGERONIMO *et al.*, 2023), proteins (FOYE *et al.*, 2006; PEEBLES, 2018), amino acids (AJAYI *et al.*, 2022; LUGATA *et al.*, 2024a; NDUNGURU *et al.*, 2024a). Various delivery routes, such as air sac, yolk sac and amniotic fluid are effective (PEEBLES, 2018; AJAYI *et al.*, 2022). Still, the physiological and molecular mechanisms governing embryonic nutrient signalling and incorporation for postnatal growth remain elusive.

Two key nutrient-signalling pathways regulate embryonic development: the insulin/insulin-like growth factor-1 signalling (IIS) and the mechanistic target of rapamycin (mTOR), the intracellular nutrient-sensing pathway (XU & VELLEMAN, 2023; NDUNGURU *et al.*, 2024a). These pathways respond to nutrient availability, such as amino acids to regulate growth and reproduction. IGF-1, secreted mainly by the liver, mediates nutrient-induced growth through IGF-1 receptors that interact with mTOR complexes (SALTIEL & KAHN, 2001; YOON, 2017). Amino acids such as methionine and leucine enhance protein synthesis by activating these pathway. Embryonic methionine supplementation in Japanese quail improved body weight through day 21 (NDUNGURU *et al.*, 2024a), and leucine had similar effects, with growth beginning as early as day 3 post-hatch mediated by upregulation of hepatic *IGF1*, *mTOR*, and *RPS6K1* genes (NDUNGURU *et al.*, 2024b).

Rapid growth enhances reproductive success. (DINGEMANSE *et al.*, 2020), but can compromise immunity (WLAŹLAK *et al.*, 2023a). Chicken strains selected for fast growth are associated with adverse effects because they utilise more resources that cannot be spent on other vital organs, such as the immune system (VAN DER MOST *et al.*, 2011; ZERJAL *et al.*, 2021; DADFAR *et al.*, 2023). Fast-growing poultry lines often divert energy from immune organs to growth, weakening immune defences (Van Der Most *et al.*, 2011; Zerjal *et al.*, 2021). Cytokines, essential for immune system response and inflammation control may be reduced during early development (Song *et al.*, 2021). Balancing the I1S/mTOR signalling pathway is crucial because its overactivation promotes fast growth but suppresses autophagy and immune response (Gan *et al.*, 2023), while inhibition enhances immunity and longevity (Panwar *et al.*, 2023a). However, excessive mTOR inhibition increases the risk of delayed wound healing and dysregulates inflammation (Webber *et al.*, 2019; Raziyeva *et al.*, 2021). However, it is not well known whether mTOR-driven growth affecting birds' immune systems regulates the trade-off between growth and defence.

Rapamycin, a known mTOR inhibitor, has been used to study this balance. Originally developed as an immunosuppressant for transplant patients, rapamycin has been shown to extend lifespan and reduce inflammation in mammals (Blagosklonny, 2019; Phillips & Simons, 2023). Its effects on avian immunity are less understood due to unique lymphoid structures like the bursa of Fabricius and spleen (Kaiser *et al.*, 2005; Morgan, 2021). Understanding how rapamycin influences these cytokines can shed light on the immune response and the balance between growth and defence system.

1.1. Objective of the PhD Program

The overall objective of the PhD Program is to investigate the specific roles of different amino acid compositions in feed in modulating the biological pathways that control growth and development in Japanese quail. The specific objectives of the doctoral study are to investigate:

1. Effects of feed restriction and supplementation with leucine and methionine on the growth and development of ovaries and ovarian follicles during sexual maturation in Japanese quail
2. Impact of predictable and unpredictable feed access on the proportions of egg components in Japanese quail.

3. The influence of embryonic amino acids supplementation on growth and expression of mTOR signalling pathway genes in Japanese quail.
4. Effects of rapamycin treatment on growth and immune-related genes in the mTOR signalling pathway in Japanese quail.

2. LITERATURE REVIEW

2.1. The influence of feed restriction on growth and sexual maturation

Numerous studies have reported that insufficient nutrition in females has been attributed to stunted growth and delayed sexual maturation. This notion is true for rats (MATSUZAKI *et al.*, 2017; GUERGOLETTE *et al.*, 2025), pigs (PRUNIER & QUESNEL, 2000), cattle (DUITTOZ & KENNY, 2023), fish (CANOSA & BERTUCCI, 2023), also humans (CALCATERRA *et al.*, 2021). Some studies have indeed revealed that female animals have to grow to reach a minimum body weight for the initiation of sexual maturation (BOHLEN *et al.*, 2018; MONTENEGRO *et al.*, 2020). The pattern appears to correspond across the animal species, suggesting a potential biological mechanism regulating growth and sexual maturation. Corresponding studies have been demonstrated in poultry, supporting similar findings (BAHRY *et al.*, 2023; LU *et al.*, 2023). Allowing fast growth and body weight gain can rapidly increase early sexual maturation (SOLLER *et al.*, 1984). However, the rapid growth rate is accompanied by increased fat deposition, high motility and skeletal disorders due to *ad libitum* feed consumption in chickens (DE BEER & COON, 2007; SUZUKI *et al.*, 2019).

Feed restriction strategies have been used to regulate feed intake and address the challenges. Feed restriction involves limiting specific nutrients such as amino acids and reducing total calorie or protein intake without malnutrition (SAHRAEI, 2012). Feed restriction minimises poultry disorders caused by feed overconsumption, alleviates metabolic disorders, including ascites, mortality, and sudden death syndrome, and reduces feed costs (SAHRAEI, 2012). Feed restriction is used to control reproductive efficiency in poultry by delaying sexual maturity, allowing chickens to reach optimum body size before the beginning of egg production and reducing excessive body weight gain (SAHRAEI, 2012; CARNEIRO *et al.*, 2019). Additionally, feed restriction control excessive body weight gain, ultimately improving reproductive efficiency in poultry (DE BEER & COON, 2007). Despite its benefits, studies showed that feed restriction constrained the reproductive outputs by limiting the quality of the offspring and the number of reproductive attempts in birds due to insufficient energy inputs (HARGITAI *et al.*, 2022a; KUMAR *et al.*, 2022). Subsequently, reduced feed intake is proportional to the reduction of dietary antioxidants, which can lead to lower antioxidant capacity and increased risk of oxidative stress (HARGITAI *et al.*, 2022b).

The rapid development of ovaries occurs during the onset of the reproductive period when the small-sized ovarian follicles are recruited into the preovulatory hierarchy follicles (VÉZINA & WILLIAMS, 2003; GHANEM & JOHNSON, 2019; SUN *et al.*, 2021). The hierarchical follicles are rapidly deposited with yolk, which contains nutrients such as lipoprotein and increases their size (WANG *et al.*, 2022; NAVARA *et al.*, 2023; SONG *et al.*, 2023). At the beginning of follicular growth and development, the smallest follicles undergo complex developmental stages to the ovulating largest hierarchical follicles under the hormonal and nutrient signal influence (LI *et al.*, 2021). The smallest pre-hierarchical follicles are numerous small and large white follicles (SWF and LWF, respectively), usually with a size of about 2 to 5 mm diameter and the 5-6 smaller yellow follicles (SYF) about 5 to 10 mm diameter. The largest hierarchical follicle of the pre-ovulatory follicle is usually about five to six (F1-F6, with size from 10 mm and above, F1 being the largest follicle. Variation in the ovarian follicles in chickens depends on the progressive development stages of growth (HLOKOE *et al.*, 2022), despite environmental factors such as nutrient availability. The reproductive maturation in birds is indicated by an increased female gonadosomatic index (GSI, gonadal weight expressed as a percentage of total body weight), also known as the ovary index. The ovary index measures sexual maturity in relation to sexual development and investment in gonads for reproductive roles (COELHO *et al.*, 2021; JIANG *et al.*, 2022), which determines reproductive readiness.

2.2. Feed restriction and plasma antioxidant capacity

Early-age environmental constraints, such as food availability during fast growth at the onset of sexual maturation, can affect reproductive investment (BAHRY *et al.*, 2023). Environmental factors limit female investment in reproduction, affecting their physiological condition. Studies have reported that feed restriction decreases the level of dietary antioxidants, therefore diminishing the antioxidant capacity and elevating oxidative stress levels (BACOU *et al.*, 2021), and decreasing the reproductive investment (ALONSO-ALVAREZ *et al.*, 2017). Oxidative stress occurs when there is an imbalance between the production of ROS and the body's antioxidant defence system, detoxifying these reactive intermediates (HOSSEINIAN & HASANZADEH, 2021). ROS are highly reactive by-product molecules produced by the organism during cellular aerobic metabolism that cause oxidative stress due to oxidative damage (JUAN *et al.*, 2021). Oxidative stress resulting from ROS can negatively impact various cellular functions essential for growth and reproductive success (KARLSSON & LILJA, 2008; VALCU *et al.*, 2019). The body's antioxidant defence system includes enzymes including dismutase, glutathione peroxidase, protein and dietary products such as carotenoids and vitamins, which regulate ROS accumulation (ZHAO *et al.*, 2024). Despite the harmful effects of feed restriction, it has been commonly used to control chickens' reproduction and oxidative balance (PANG *et al.*, 2023; OKE *et al.*, 2024) (PAN *et al.*, 2005).

The plasma total antioxidant capacity (TAC) is a biomarker for the body's defence system against oxidative stress, helping to counteract the effects of ROS during growth and reproductive maturation (OKE *et al.*, 2024). The antioxidant defence system acting against ROS includes those that are synthesized by the body, such as antioxidant enzymes (e.g. Glutathione peroxidase, Superoxide dismutase) and proteins and those that are obtained through diet (e.g. vitamins, tocopherols, carotenoids, tocotrienols) (SURAI, 2020; DESBRUSLAIS & WEALLEANS, 2022). However, all classes of antioxidants are dependent on the overall antioxidant capacity and oxidative stress (MAVROMMATIS *et al.*, 2021), with a significant amount of antioxidants obtained from dietary sources and modified by dietary availability (ALAN & MCWILLIAMS, 2013). A low diet protein intake resulted in an imbalanced antioxidant activity by intercepting the energy balance that led to increased oxidative stress (TARRY-ADKINS *et al.*, 2010; MANIAM & MORRIS, 2012). Although feed-deprived individuals have reduced antioxidant defence systems, it is not clear whether feed restriction will affect plasma total antioxidant activity. Despite the fact that nutrient oxidative stress has decreased production and reproductive performance, resulting in economic losses (BACOU *et al.*, 2021).

Among the significant constituents of animal diet, more attention is given to protein as it constitutes the biologically active compounds in the body than other macronutrients such as carbohydrates and lipids (BESKI *et al.*, 2015; YUAN *et al.*, 2023). Proteins contain amino acids, essential building blocks for cell and tissue formation, growth, and reproduction and are important in antioxidant regulation (BESKI *et al.*, 2015; BU *et al.*, 2015; RUAN *et al.*, 2018). For example, a study on ducks showed that dietary methionine improved egg weight, one-day ducklings' body weight, and total antioxidant capacity (RUAN *et al.*, 2018). Similarly, the increased serum levels of amino acids, such as leucine and methionine, are accompanied by increased body weight, ovary weight, oviduct weight and egg white during the egg-laying cycles in pigeons (REN *et al.*, 2021).

2.3. Embryonic development

Avian embryonic development is dependent on the availability of the deposited egg resources. Materials such as nutrients, hormones, and enzymes are maternally deposited into the egg for embryo consumption during growth.

2.3.1. Major egg components and composition

As opposed to mammals, the embryonic development of avian species takes place in the egg, where all maternal resources are deposited. This is one of the disadvantages as it necessitates providing all essential maternal resources into the egg before egg laying (SOCKMAN, 2018). In poultry, chicken egg composition provides essential nutrients for human consumption (NABER, 1979; KUANG *et al.*, 2018; RÉHAULT-GODBERT *et al.*, 2019). The most significant part of the egg is albumen, taking up to 60-70% of the total egg weight, which consists of 88% water, 11% proteins, and 1% is taken by carbohydrates and minerals (CAMPBELL *et al.*, 2003; SUN *et al.*, 2019). The egg white (albumen) is a viscous fluid that surrounds the egg and contains a water reservoir and proteins; apart from being a nutrient region, it acts as a cushioning material to absorb shock and protect the developing embryo (DA SILVA *et al.*, 2019). However, this composition is not universal; it differs across the avian species (SUN *et al.*, 2017; SUN *et al.*, 2019). For example, an egg contains 56% of the albumen in chicken and turkey, while a goose and duck have a lower 53% of the albumen, while quails contain about 60% (TOLIK *et al.*, 2014). Next to albumen, the nutrient-rich part, the egg yolk, comprises lipid layers, essential proteins and vitamins. Yolk forms the primary source of nutrients for the developing embryo during the incubation period (PETIT *et al.*, 2022). The yolk in quail, chicken goose, duck, turkey and goose eggs comprises about one-third of the egg weight but about one-fifth of the ostrich (TOLIK *et al.*, 2014). From the outside, the egg is encased in a porous eggshell containing calcium carbonate minerals that allows gaseous exchange while protecting against bacteria and moisture exchange (TSAI *et al.*, 2006). The eggshell and shell take about 7.4% of quail eggs, 12% of other poultry species, and about 20% of ostrich eggs (SUN *et al.*, 2019). The Japanese quail egg size ranges from 11 to 13-gram; relatively large sizes eggs from other avian species, such as turkey and ostrich, result in an even more extensive package of nutrient composition compared to small-sized quail eggs (GENCHEV, 2012; JEKE *et al.*, 2018). Variations are determined by the composition and proportions of amino acids in the egg that form the nutrition value for the developed embryo (SUN *et al.*, 2019). Meanwhile, egg albumen is regarded as the most balanced source of amino acids for human consumption (SWENDSEID *et al.*, 1959).

2.3.2. Embryonic nutrient supplementation

The prenatal period is a critical phase for the growth and development of vital tissues and organs, establishing a solid connection between embryonic and postnatal growth and development (TONG *et al.*, 2013; TAHMASEBI & TOGHYANI, 2016; AZHAR *et al.*, 2022). In avian species, the environmental conditions are closely regulated by the maternal influence, where parent (s), have a programmatic effect on embryonic development (GROOTHUIS & SCHWABL, 2008; GROOTHUIS *et al.*, 2019; WIDOWSKI *et al.*, 2022). The deposition of maternal resources into the eggs depends on several factors, including genetic factors, nutrition, and behaviour resulting from management and rearing conditions (ALI *et al.*, 2016; PEIXOTO *et al.*, 2020; GONG *et al.*, 2023).

Embryonic nutrient supplementation has been invented to address sufficient nutritional demands not met by parental deposition. The technique involves injecting substances directly into the developing embryo. The method has been widely studied and proven to enhance performance in the poultry industry, therefore supporting embryonic growth and subsequent postnatal growth (GAO *et al.*, 2017; NAYAK *et al.*, 2017; GAO *et al.*, 2018; NAYAK *et al.*, 2018). Several substances, including health-beneficial compounds such as antibiotic feed additives that include herbs, probiotics, nutraceuticals, drugs, immunostimulants, and other nutrients such as carbohydrates, proteins, minerals, vitamins, and amino acids, have been delivered to the developing embryo using the *in ovo* technique to enhance growth in the poultry industry (ARAIN *et al.*, 2022). Supplementation of essential amino acids has achieved a significant milestone in poultry production (Table 1).

Table 1. Summary of studies investigating impacts hatchability, hatching body weight and postnatal body weight with exclusive amino acids feeding

Reference	Amino acid	Dosage	Injection site	Species/ Strain	Hatchability (%)	Hatching weight (g)	Final body weight (g)
NAYAK *et al.* (2017)	Arg; Trp	2.5 mg Arg; 2.5 mg Trp, 2.5 mg Arg+Trp	Amniotic fluid	Broiler chicken (Cobb 400)	NS	NS	NS
TAHMASEBI & TOGHYANI (2016)	Arg; Thr	35mg Arg; 25mg Thr; 35+25mg Arg and Thr	Amniotic fluid	Broiler chicken (Ross 308)	NS	NA	↑
NABI *et al.* (2022)	Arg; Thr	25 mg L-Arg; 25 mg L-Thr; 25mg L-Arg and L-Thr	Amniotic fluid	Broiler chicken	↑	NA	↑
GAO *et al. (*2017)	Arg	3; 6; or 12 mg L-Arg	Amniotic fluid	Broiler chicken (Arbor Acres)	↓	NS	↑
RUFINO *et al.* (2019)	Gln	2.5; 5; 7.5; 10; and 12.5 mg	Amniotic fluid	Chicken (Rhode Island Red)	NS	NS	NS
NAYAK *et al.* (2018)	Trp	2.5 mg Trp	Amniotic fluid	Broiler Chicken	↑	↑	↑
FARIAS *et al.* (2023)	Met	2.5; 5;7.5; 10; and 12.5 mg	Amniotic fluid	Layer chicken (Rhode Island Red)	↑	↑	↑
KADAM *et al.* (2008)	Thr	10; 20; 30; and 40 mg	Yolk sac	Broiler chicken	NS	NS	NS
AZHAR *et al.* (2022)	Arg	2.5; 5; and 7.5 mg	Air sac	Chicken	NS	↑	↑

Zhang *et al.* (2017)	Arg	1.14 mg	Amniotic fluid	Domestic pigeon (Columba livia)	↑	NS	NS
Gao *et al.* (2018)	Arg	6 mg	Amniotic fluid	Broiler chicken (Arbor Acres)	NA	NA	↑
Zhu *et al.* (2019)	L-Lys	2.1 mg	Amniotic fluid	American King pigeon (Columba livia)	↓	↑	NS
Xu *et al.* (2019)	Hist	0.55 mg	Amniotic fluid	Domestic pigeon (Columba livia)	↑	NS	NS
Coskun *et al.*, (2018)	L-Lys; Met	2.1 mg L- Lys; 2.1 mg L-Met; or 1.05 L-Lys +1.05 L-Met mg	Air sac	Broiler chicken Ross 308	NS	NS	NS
Kermanshahi *et al.* (2017)	Thr	0.25 or 0.5 mg	Air sac or Amniotic fluid	Japanese quail (Coturnix japonica)	↑	↑	NA
Sözcü & Ak, (2020)	Gln	20; 40; 60; 80 mg	Air sac	Broiler chicken Ross 308	↑	↑	NA
Han *et al.* (2020)	Leu	4.525 mg	Air sac	Broiler chicken (Chunky strain)	NA	NA	↑
Azhar *et al.* (2022)	Arg	2.5; 5; and 7.5 mg	Albumin	Kampung chicken	NA	↑	↑
Moreira *et al.* (2012)	Thr	10.5; 21; 31.5 and 42 mg	Air sac	Broiler chicken (Ross 708)	NS	NS	NS
Nazem *et al.* (2019)	Met	20; 30; 40 or 50 mg	Yolk sac	Broiler chicken (Ross 308)	NS	↑	NA
Gao *et al.* (2018)	Arg	6 mg	Amniotic fluid	Broiler chicken (Arbour Acres)	NS	NS	↑
Han *et al.* (2020b))	Leu	9.05 mg	Amniotic fluid	Broiler chicken (Ross 308)	NA	NA	NS
Li *et al. (*2016)	Arg	100; 25; and 6.25 mg	Amniotic fluid	Broiler chicken (Ross)	NA	NS	↓
Odutayo *et al.* (2020)	Arg	11 and 20 mg	Amniotic fluid	Breeder chicken (FUNAAB Alpha)	NA	NS	↑
Alabi *et al.* (2021)	Thr	15; 30; and 45 mg	Amniotic fluid	Broiler chicken	↓	NS	↑
(Moreira Filho *et al. (*2018)	Thr	0; and 3.5 mg	Amniotic fluid	Broiler Chicken (Cobb500)	NS	↑	NA
Reicher *et al.* (2022)	Gln	4 and 10 mg	Amniotic fluid	Broiler Chicken (Cobb500)	NS	NA	NA
Salmanzadeh *et al. (*2016)	Arg	7 mg	Amniotic fluid	Layer chickec (White leghorn)	NA	NA	NA
	Gln	10; 20; 30; 40; and 50 mg	Albumin	Broiler chicken (Cobb500)	↑	↑	↑
Omidi *et al.* (2020)	Arg	5; and 10 mg	Amniotic fluid	Broiler chicken	NS	NS	NS
Chen *et al. (*2021)	L-Met	5; 10; and 20 mg	NA	Broiler chicken (Yellow feathered)	NA	NS	NA
Lugata *et al.*, (2024a)	DL-Met; L-Met	5 mg	Amniotic fluid	Chicken (Fertile Hungarian Partridge-Colored hen and TETRA-SL)	NA	NS	NA

Abbreviations: NA- Not applicable; NS-Not significant; ↑-Increased; ↓-Decreased

Embryonic amino acid feeding primarily focuses on improving poultry breeding, such as embryonic growth, hatchability, and postnatal growth. For example, *in ovo* delivery of glutamine, lysine, methionine, and the combination of lysine and methionine showed an increased hatchability compared to the negative control group without affecting chick weight at hatching in chicken and guinea fowl (SALMANZADEH *et al.*, 2016; COSKUN *et al.*, 2018; IDOWU O.P.A *et al.*, 2024). Other studies observed that *in ovo*, amino acids such as lysine, arginine, and methionine had no significant negative effects on hatchability but improved postnatal growth performance in domesticated pigeons (ZHANG *et al.*, 2017; ZHU *et al.*, 2019). Considering that farms and laboratories designed for high productivity in poultry may not impose strict nutrient intake limits (DOUHARD *et al.*, 2021).

Despite the promising results on hatchability, hatching weight, and postnatal growth, inconsistencies persist, as shown in Table 1. Injection of the substance in the egg may cause physical trauma to the embryo, which may lead to increased stress due to handling and injection itself (EBRAHIMI *et al.*, 2012; KUCHARSKA-GACA *et al.*, 2017). Similarly, injection of the amino acids in the egg can interfere with respiratory activity, as the air sac, the major respiratory part, is obstructed, which may be the source of embryo mortality (ROTO *et al.*, 2016; DAS *et al.*, 2021). The alteration of the egg's internal environment due to increased concentrations of nutrients induces an osmotic imbalance that affects embryonic development and viability (ROTO *et al.*, 2016).

Several factors, including the injection site, can also influence the final results (Table 1, Figure 1). For example, egg yolk is mainly composed of lipids, the energy reserve, which may not be an ideal position for *in ovo* amino acid feeding (ANTON *et al.*, 2006; ALTUNTAŞ & AYDIN, 2014; ABEYRATHNE *et al.*, 2022). The injection concentration of 5 mg per egg of threonine into the air sac increased body weight gain in the Japanese quail; however, a similar amount of amino acid injected under the air sac (amniotic fluid) had no difference in body weight gain compared to the control (KERMANSHAHI *et al.*, 2017). Injection into the amniotic fluid provides the embryo direct access to amino acids (OHTA *et al.*, 2001; ZHANG *et al.*, 2018; KOP-BOZBAY & OCAK, 2019a). The injection timing differed among the studies; however, this variation is also based on the species type, the genetic response of embryonic development as shown to differentiate from chicken and pigeons (ZHANG *et al.*, 2017; XU *et al.*, 2019; ZHU *et al.*, 2019) and Japanese quail (KERMANSHAHI *et al.*, 2017). Most studies in chicken injected the amino acid between the 14 and 18 days of embryonic development. Late injections close to hatching, such as on the 18th day of incubation, showed increased hatchability (NAYAK *et al.*, 2018). At the end of embryonic development, the amniotic fluid is consumed by the developing embryo and sent into the gastrointestinal tract, a process initiated on the 13th day of the incubation (UNI & FERKET, 2004; BIESEK *et al.*, 2023). Thus, the observed increase in hatching weight can be attributed to the enhancement of the gastrointestinal tract and muscle growth during embryonic development, a crucial factor in addressing the early postnatal nutritional adaptation challenges (UNI & FERKET, 2004; GAWEŁ *et al.*, 2022).

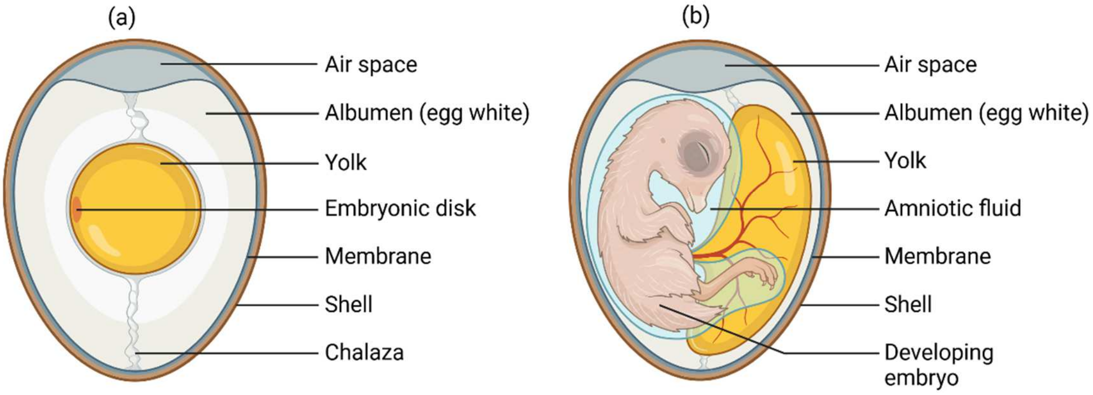

Figure 1. Structure of an avian egg (a) Before the incubation (b) Developing embryo showing potential embryonic amino acid feeding positions. Source (ROTO et al., 2016).

2.4. The mechanism controlling growth and development

Signalling molecules, such as hormones, are crucial during development for regulating growth in an organism and coordinating the development of different organisms to attain systemic growth (BLUMBERG *et al.*, 2009). The signalling molecules act as the internal cue, enabling the organisms to adjust their growth response to the current external environmental factors. Although the internal cues contribute to determining the overall body size, growth in birds generally remains flexible and is prone to adaptation to external (environmental) conditions, particularly nutrition (PESTI & CHOCT, 2023; RUUSKANEN, 2024). Meanwhile, in the target tissues, the signalling molecules interact with local cues such as nutrients to control physiological processes, including cell growth, proliferation, and autophagy, which determine the final functional body size. Two distinct pathways regulate critical aspects of development: the insulin/insulin growth-like factor-1 signalling (IIS) hormonal pathway and the mechanistic target of rapamycin (mTOR), the intracellular nutrient-sensing pathway controlling growth, ageing and reproduction. Similarly, the growth hormone (GH)/IGF-I system, insulin, and thyroid hormones are nutrient-sensitive regulators necessary for postnatal growth and development (AL-SAMERRIA & RADOVICK, 2021). Also, the sexual steroids such as estrogens are crucial for the development during sexual maturation in female birds. Sex hormones (estrogens, androgens) and growth hormone (e.g., thyroid hormone) play a significant role in regulating metabolism, growth, and gene expression in various tissues, including the liver and skeletal (KENESSEY & OJAMAA, 2006; FAULDS *et al.*, 2012). These hormones can directly modulate components of the IGF-1/mTOR pathway or indirectly influence its activity through upstream or downstream signalling molecules (KHAN *et al.*, 2025).

2.4.1. The role of Insulin/Insulin-like Growth Factor (IGF) Signalling pathway (IIS) in regulating growth

The insulin/IGF signalling pathway (IIS) is a highly conserved system that regulates various body physiological processes, including metabolism, growth and ageing. Under the influence of growth hormone (GH), IGF-1 is secreted from the liver hepatocyte and influences phenotypic expression (LIU *et al.*, 2016) . Beyond the storage organ for energy reserves such as glycogen, the liver integrates and transmits signals that regulate growth and metabolic activities (TREFTS *et al.*, 2017). In vertebrates, IGF-1 is the primary ligand of this pathway that controls embryonic and post-natal growth and development by stimulating energy metabolism, cell proliferation, migration, differentiation, and protein synthesis, affecting growth and survival in response to energy and nutrient status (LODJAK & VERHULST, 2020; REGAN *et al.*, 2020; BEATTY *et al.*, 2022). The IGF-1 hormone has structural homology with insulin, IGF-1 exerts its functional effects by binding with high affinity to specific cell receptors, the IGF-1 receptor (IGF-1R) triggering the downstream signalling cascades of both the mitogen-activated protein (MAP) kinase and phosphoinositide 3-kinases (PI3K) to protein kinase B (PKB)/Akt signalling pathway in target tissues (POREBA & DURZYNSKA, 2020; NAGAO *et al.*, 2021). The PI3K induces the recruitment of protein kinase B (PKB)/Akt, leading to stimulation of the activity of the mTOR pathway by phosphorylating the TSC1/TSC2 or PRAS40, the negative regulators of mTOR and thus activating it to regulate cell growth, proliferation and protein synthesis (SALTIEL & KAHN, 2001; YAKAR & ADAMO, 2012) through activating a conserved kinase cascade (Figure 2).

2.5. The mechanistic target of rapamycin (mTOR) pathway as a nutrient sensor

2.5.1. General overview of mTOR

The mechanistic target of rapamycin (mTOR) pathway is a nutrient-sensing pathway that is the least understood but most studied molecular checkpoint in molecular biology. It is a core protein complex pathway identified during the screening for resistance to the immunosuppressant drug rapamycin from the soil bacteria *Streptomyces hygroscopicus* in Easter Island, named in reference to the island's indigenous name, Rapa Nui (SABATINI, 2017). The mTOR protein complex is a highly conserved serine/threonine kinase from yeast to mammals that has a functional unit of two complexes referred to as the rapamycin-sensitive complex 1 (mTORC1) and rapamycin-insensitive complex 2 (mTORC2) (SAXTON & SABATINI, 2017) (Figure 2). The mTORC1 regulates ribosomal biogenesis, cell growth and proliferation, mRNA translation, autophagy, and mitochondria metabolism (PANWAR *et al.*, 2023a). It consists of three unique components: the mTOR, regulatory proteins associated with mTOR (RAPTOR) and mammalian lethal associated with sec 13 protein 8 (mLST8) (JACINTO *et al.*, 2004). Raptor is involved in mTOR phosphorylation, and dysregulation of mTOR inhibits the phosphorylation of downstream effectors of p70 ribosomal S6 kinase 1 (RPS6K1), eukaryotic Initiation factor 4E binding protein 1 (4EBP1) (DANN & THOMAS, 2006). mLST8 is a positive regulator that stabilises the association of the other components. Other mTORC1 components include proline-rich Akt substrate 40 kDa (PRAS40) (LAPLANTE &

SABATINI, 2009) and DEP-domain containing mTOR-interacting protein (DEPTOR) (AGRAWAL *et al.*, 2014). PRAS40 inhibits the direct binding of substrates through the interaction with Raptor, reducing mTOR activity (LAPLANTE & SABATINI, 2009). Phosphorylation of PRAS40 by mTOR relieves negative regulation and, therefore, increases mTOR activity. Upon activation of mTORC1, it is recruited to the lysosome, where it interacts with small GTPase Ras homologs enriched in the brain (Rheb) (DANN & THOMAS, 2006). The mTORC2 complex is important in controlling cytoskeletal dynamics and cell survival and is primarily composed of mTOR and mLST8, but mTORC2 contains a rapamycin-insensitive companion of mTOR (RICTOR) (LAMMING *et al.*, 2014). Furthermore, mTORC2 also contains a negative regulator called DEPTOR and mammalian stress-activated MAK-interacting protein 1 (mSin1), which assemble the mTORC2 on the plasma membrane (EBNER *et al.*, 2017). Although the identification of the function of each mTOR complex is generally accurate, studies indicate a significant cross-talk and co-regulation between the complexes, presenting a considerable challenge in studying and manipulating the pathway (XIE & PROUD, 2014; PANWAR *et al.*, 2023a). Among the two complexes, mTORC1 is the most characterised and plays a crucial role in mRNA translation, protein synthesis and growth, which are the key areas of investigation in the experimental chapters of this thesis.

2.5.2. The mTORC1 upstream

The mTORC1 pathway serves as a central hub for several key activating factors upstream of the signalling pathway; these include energy status, stress, hormones, hypoxia, insulin, growth factors, and amino acids, which together regulate the downstream processes such as protein synthesis, autophagy, cell differentiation and proliferation, cell growth ribosomal biogenesis (FERNANDES & DEMETRIADES, 2021). The activated mTOR is known for its vital activity in regulating gene expression at the transcription and translation level, consequently forming a central intracellular target for controlling growth and ageing in organisms (PANWAR *et al.*, 2023b). Growth factors such as insulin and insulin-like growth factor 1 (IGF-1) are some of the most studied hormones that influence growth in birds (LENDVAI *et al.*, 2021). At the same time, amino acids and glucose are crucial nutritional interventions that affect growth, and both are known for their ability to stimulate the activation of the mTORC1 signalling pathway (LEPRIVIER & ROTBLAT, 2020; TAKAHARA *et al.*, 2020).

2.5.3. The mTORC1 downstream

Previous studies indicate that mTORC1 is a key regulatory protein complex responsible for cell growth and acts as the indirect downstream effector of the AKT to promote growth by responding to growth factors such as IGF-1 and amino acids (LATRES *et al.*, 2005; FENG & LEVINE, 2010). The mTORC1 directly activates the ribosomal protein serine 6 kinase (S6K) family members, which is essential in protein synthesis and influences growth (DUFNER & THOMAS, 1999; BIGOT *et al.*, 2003). Protein synthesis depends on nutrient availability in rats and mice that activated the S6K1. For example, the activity of S6K1 in chicken muscles was activated by refeeding the food-deprived individuals (BIGOT *et al.*, 2003). Similarly, it has been shown that supplying amino acids in the rat adipocytes after the period of deprivation stimulated the activity of S6K1 that subsequently influences protein synthesis and growth (FOX *et al.*, 1998).

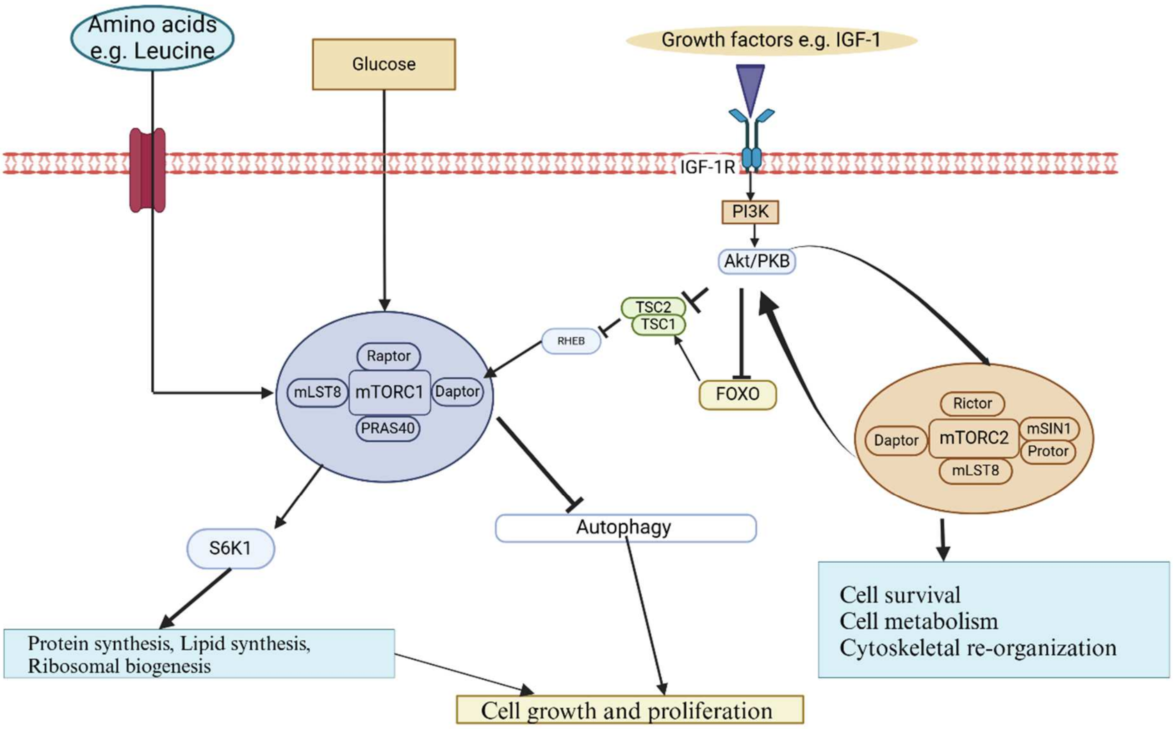

Figure 2. A schematic representation of the mTOR structure illustrates how key signalling pathways regulate mTORC1 and mTORC2, along with their component parts. Multiple signals, including growth factors and amino acids, activate mTORC1. Once activated, mTORC1 stimulates anabolic processes such as protein synthesis, nucleotide synthesis, and ribosomal biogenesis. It also inhibits catabolic processes like autophagy and promotes cell growth and proliferation. The mTORC2 regulates cell survival and proliferation through interaction with Akt and other downstream proteins once PI3K is activated. Source (SABATINI, 2017; TAKAHARA et al., 2020).

2.5.4. The role of rapamycin in regulating growth and immunity

Organisms' environments are complex and associated with interactions with pathogens, environmental stressors, malnutrition, and inappropriate management for domestic birds. For their growth and survival, birds have evolved an innate and adaptive immune system crucial to defend against challenges and maintain health and productivity. Increased white blood cell counts can refer to a stronger immune system, while a decrease in white blood cells such as leucocytes, macrophages, natural killer cells, and lymphocytes may indicate immunosuppression (WLAŹLAK *et al.*, 2023b). In birds, the effective activation of the signalling proteins and innate humoral inflammatory cytokines triggering the immune system also forms part of the immune defence system (WLAŹLAK *et al.*, 2023b). The activation of innate and humoral adaptive immune systems is associated with energy utilisation, demanding tight regulation. Immunosuppression can lead to detrimental effects by reducing growth and reproduction but can also increase susceptibility to diseases and higher mortality rates (WLAŹLAK *et al.*, 2023b; ALISPAHIC *et al.*, 2024). This concept describes the trade-off between growth and defence. Depending on the current environmental conditions, birds with limited resources will prioritise their demands towards either growth or the immune system. Birds must optimise the balance between growth and immune systems to survive and reproduce successfully, which is important in agricultural productivity. The avian immune system shares the major histocompatibility complex (MHC) and the toll-like receptors with mammals (EDWARDS *et al.*, 1995; SHULTZ & SACKTON, 2019). However, the molecular mechanisms regulating growth and defence trade-offs remain less understood in birds.

The mTOR pathway responds to diverse endogenous and exogenous cues and is important for growth, reproduction, lifespan, metabolism, and immune regulation (DOBRENEL *et al.*, 2016; GONZÁLEZ & HALL, 2017). Rapamycin is an immunosuppressive drug that can optimize the balance between growth and immune system defence through the mTOR pathway (WEICHHART *et al.*, 2015). The immunosuppressive impact of rapamycin is attributed to the inhibition of the mTOR pathway (WEICHHART *et al.*, 2015). The suppression of mTOR by rapamycin can produce varying effects on cytokine secretion, influenced by both types of activated dendritic cells and the length of treatment (HAIDINGER *et al.*, 2010; KATHOLNIG *et al.*, 2013). In humans, short-term administration of rapamycin leads to an increase in the production of proinflammatory cytokines, including interleukin (IL)-1β, IL-12, and the activation of the nuclear factor kappa beta (NF-κβ) pathway (HAIDINGER *et al.*, 2010). In contrast, long-term treatment with rapamycin may impair innate immunity by lowering the expression of these proinflammatory cytokines. The study on mouse models demonstrated that rapamycin treatment reduced the gene expression of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α (KANG *et al.*, 2024).

The mTOR signalling pathway is crucial for sustaining intestinal mucosal immunity. Key aspects of intestinal morphology, such as villus height and crypt depth, are vital for effective nutrient digestion and absorption in birds (LIU *et al.*, 2016). The alterations in these morphological structures can also indicate intestinal immunity status (KHAJEH BAMI *et al.*, 2022). Rapamycin-induced mTOR inhibition decreases the concentration of jejunal immunoglobulin A in jejunum and negatively impacts the intestinal structure in mice (SAMPSON *et al.*, 2016). Studies on mTOR inhibition also explored the importance of upstream signalling pathways, such as TLR4/MyD88/MAPK and downstream NF-κB signalling pathways (YU *et al.*, 2016; ZHOU *et al.*, 2018a)(Figure 3). Treatment with rapamycin led to decreased expression of inflammatory markers in models of inflammation, which is linked to the suppression of TLR4/MyD88/MAPK and NF-κβ signalling pathways (YU *et al.*, 2016; ZHOU *et al.*, 2018a). Within these pathways, the myeloid differentiation primary response 88 (MyD88) plays a crucial role in initiating downstream signalling cascades, including the activation of nuclear factor kappa beta (NF-κβ), a key transcription that regulates inflammatory and immune responses (ZHOU *et al.*, 2018a). Rapamycin modulates the inflammatory response through the mTOR/STAT3 pathway (Figure 3). The signal transducer and activator of transcription 3 (STAT3), an important downstream molecule of the mTOR pathway, plays a significant role in immune regulation (SÄEMANN *et al.*, 2009). In an autoimmune mouse study, response to rapamycin treatment led to the suppression of mTOR/STAT3 pathway (HOU *et al.*, 2017a).

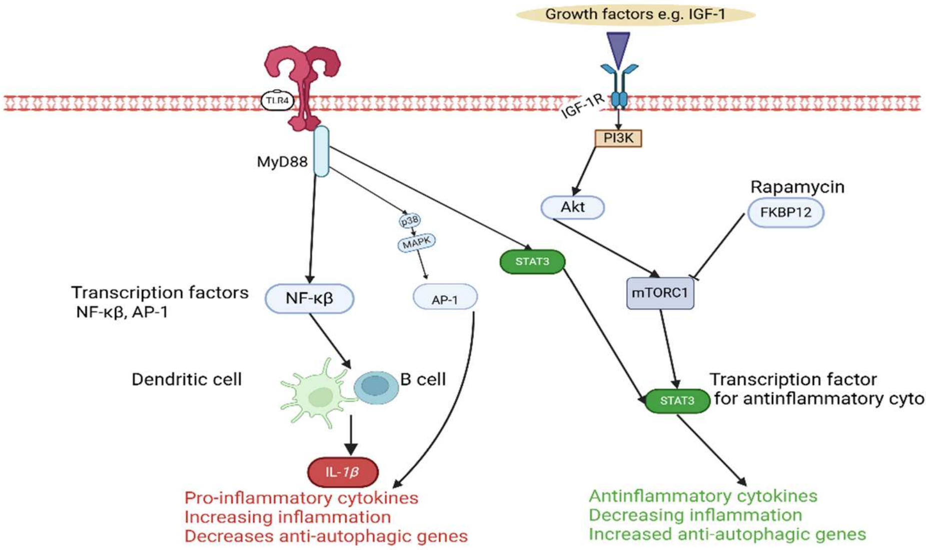

Figure 3. Schematic representation of the MyD88/MAP signalling pathway. Source (MAYO et al., 2012)

3. MATERIALS AND METHODS

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

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

3.1.1. Experimental animal, housing, experimental design and measurements

Japanese quails used in this experiment were reared in the animal house of the Institute of Animal Science, Biotechnology and Nature Conservation, Faculty of Agriculture and Food Sciences and Biotechnology and Environmental Management, University of Debrecen. The basal (control) grower feed was prepared according to the NRC (1994) recommendation for quails on a corn-wheat-soybean meal basis. The birds in the control group were full-feed (feed intake plus an extra 5% feed) and had free access to water. At the age of 5 weeks, we selected 40 female quails with similar body weight and kept them in individual cages in the same room with a controlled temperature of 23-25 °C and humidity of 60-65%. Birds were allowed free access to breeder feed and water for one week of the acclimation period. During this time, individual weekly body weight changes and daily feed intake to the nearest (0.01 g accuracy) were measured to determine the appropriate amount of feed for each bird during the experiment.

A two-week experimental period began when the quails reached six weeks old. Birds were divided into five experimental groups (8 birds per group): ad libitum as a control feed, a 20% restricted feed (DR20) (i.e. given 80% of their average individual feed intake), a restricted feeding supplemented with 20% of the recommended amount of methionine (DR20+Met), a restricted feeding supplemented with 20% of the recommended amount of leucine (DR20+Leu) and a restricted feeding supplemented with 20% leucine and 20% methionine of the recommended amount (DR20+Leu+Met) based on the individual feed intake (Table 2, Appendix 1).

Table 2. Composition and nutrient content of the experimental feeds

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

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

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

3.1.2. Measurements

At the end of the experimental period, body weight was measured using digital scale VWR software version 6.02 with 0.01 g accuracy (Avantor, Radnor, PA, USA). After measurements, birds were sacrificed by cervical dislocation and collecting ovaries and follicles, then measured ovary weight using a analytical balance (0.1 g accuracy), three biggest hierarchical follicle weights, the first (F1), second (F2) and third (F3) to nearest 0.01 g accuracy, and follicle diameter to nearest 0.1 mm accuracy using vernier calliper and counted number of the first three biggest hierarchical follicles (F1, F2, and F3) (Figure 4). To determine the reproductive investment, we computed the female ovary index of each bird in the experimental group. The ovary index is a proportional ovary weight expressed as a percentage of total body weight calculated using the formula described previously (JIANG *et al.*, 2022).

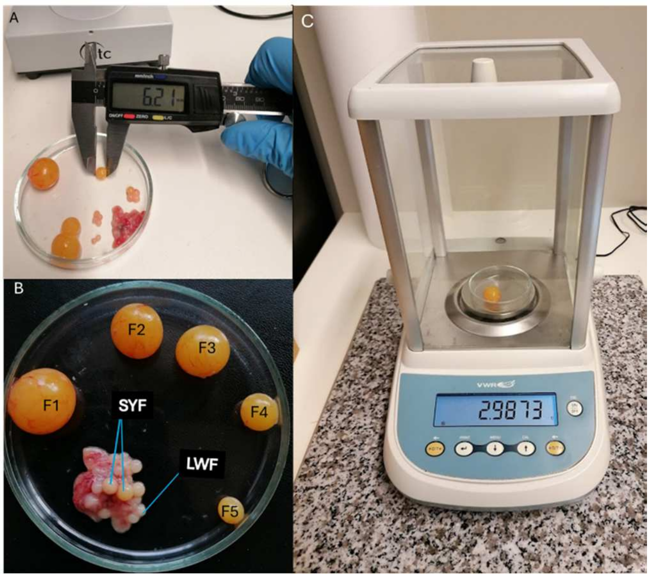

Figure 4. Ovarian follicle measurement. A: Diameter measurements, B: Isolated follicles from the ovary (F1-F5) hierarchical follicles, SYF-small yellow follicles, LWF-large white follicles, C: analytical scale weighing hierarchical follicles.

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

3.2.1. Animals and housing

The four-week-old Japanese quail chicks were purchased from a commercial quail breeder (Budai Fürjészet, Hungary). The quail was kept in the animal house until it reached 8 weeks of age. In this experiment, two trials were conducted. The first trial focused on quantitative feed restriction. In this trial, 40 8-week sexually matured female quails with similar body weights (≈275.3±3.64 g) were selected and placed in individual cages. The quails were acclimated for seven days under experimental conditions before the experiment began. During the acclimation period, quails were fed ad libitum every morning, and their feed intake was measured at 24-hour intervals. This allowed us to calculate the average daily feed intake for each bird (Appendix 1). Additionally, the live initial and final body weights were recorded during the acclimation time with a digital balance (± 0.1 g), allowing us to analyse any changes in live weight over time. However, no significant changes were observed in either body weight or feed throughout the acclimation period ($p > 0.05$). After the acclimation period, quails were randomly distributed into four treatment groups consisting of 20% restriction ad libitum as a control feed, a 20% restricted feed (DR20: i.e. given 80% of their average individual feed intake), 30% restriction (DR30: i.e. given 70% of their average individual feed intake), 40% restriction (DR40: .e. given 80% of their average individual feed intake) and a control group that received full feed.

In the second trial, 24 sexually matured birds were placed in individual cages. After acclimation, quails were randomly assigned to three experimental groups. The control group received ad libitum feed throughout the experimental period, equal to their daily individual feed intake. The unpredictable fed group (UNPR) received the same total amount as the control group throughout the experiment, but with daily variability ranging from the lowest amount, 30% per day, to 170% per day of their respective feed intake. The restricted group (DR40) received an average of 60% of their feed intake (Figure 5). The trial was applied for sixteen days (Figure 5). The experimental house in both trials was maintained at 24 ± 3 °C, with relative humidity levels between 60% and 75%. A standardised lighting regimen was implemented with a 12:12 light-to-dark cycle controlled using timers. The basal diet for the quails was formulated based on corn, wheat, and soybean meal to meet the specific nutrient requirements for the breeder quails, as outlined in Table 1 (NRC, 1994).

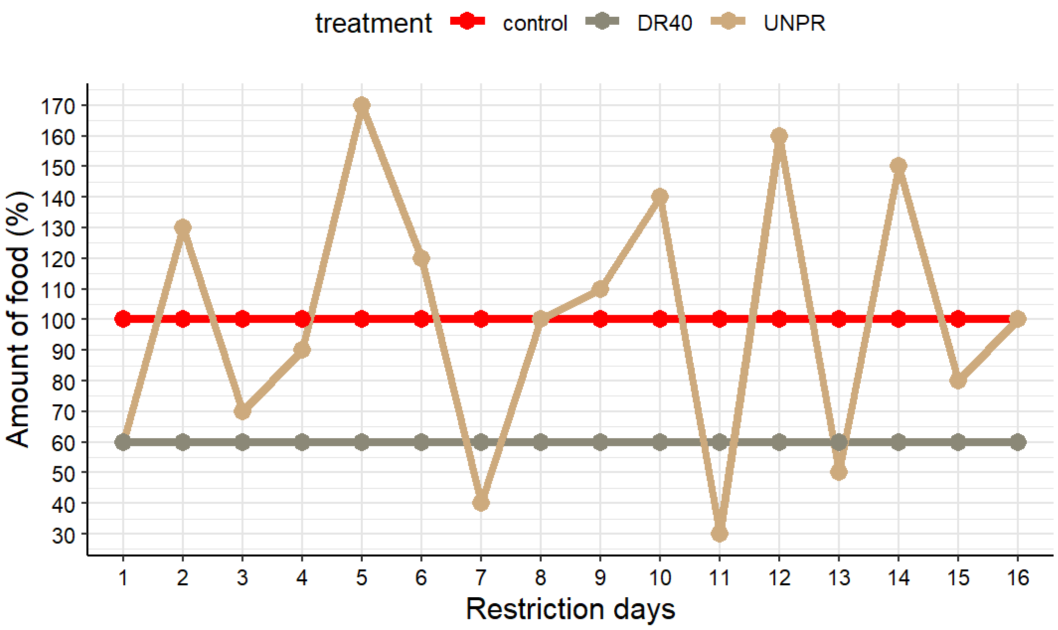

Figure 5. Dietary treatments over sixteen days of the experimental period. The red colour (control) indicates that the feed is fully available throughout the experiment. The grey (DR40) indicates a constant 40% dietary restriction throughout the experiment (60% food access). The tan colour (UNPR) indicates unpredictable feed access, with fluctuating feed amounts of an average of 100%. Each treatment is based on an individual average daily feed intake under ad libitum conditions measured during acclimation (i.e. 100%).

3.2.2. Egg trait measurements

Eggs were collected daily. Each egg was marked individually and immediately weighed using a digital laboratory analytical balance (± 0.01 g). Eggs were carefully broken with the blunt end of a knife, and the eggshells were washed, air-dried and weighed using an analytical balance (± 0.01 mg). The eggshell ratio was calculated as the percentage of eggshell weight to egg weight. The egg yolk and albumen were separated into a clean container, and the weight of both components was measured (± 0.01 g). Eggshell, yolk and albumen ratio, expressed in percentage in figures, were calculated as the ratios of the eggshell, yolk and albumen weights to the whole egg weight, respectively. The egg yolk to albumen ratio was calculated by dividing the yolk weight by that of albumen weight.

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

3.3.1. Experimental design

Freshly laid Japanese quail (*Coturnix japonica*) eggs were collected and kept at room temperature between 16 °C and 18 °C for 1-5 days. On the day of laying, eggs were weighed on a digital scale (±0.01 g accuracy). To eliminate the effect of differences in egg weight during the experiment, only eggs with similar weights (11 ± 0.5 g) were included in the experiment. A total of 200 eggs were weighed and randomly selected; half were injected with 1 mg L-methionine dissolved in 50 µl saline solution, while the remaining eggs received physiological saline solution as a control group (Figure 6).

Figure 6. Injection of amino acid and saline solution into the Japanese quail eggs.

3.3.2. Preparation and in ovo injection of methionine

A batch of the amino acid solution was prepared by dissolving crystalline L-methionine (CAS No. 63-68-3, Sigma Aldrich, BioUltra, > 99.5%) in 0.9% physiological saline solution (Sigma Aldrich). Saline and amino acid solutions were sterilised by autoclaving. The broad end site of the eggs was disinfected with 70% ethanol before injecting the amino acids solution, then a hole was made using a sterile 26G needle. 50 µl of either the L-methionine or saline solution was injected into the egg yolk using a 50 µl ethanol sterilised Hamilton syringe (Figure 7). After solution injection, the eggs were sealed with candle wax at the injection point. The injection of the amino acid and saline solution was performed before the beginning of incubation on embryonic day zero. Immediately after the injections, the eggs were transferred to the automatic turning incubator (WQ-63 Model 2021 Version 2, AGROFORTEL, Budapest, Hungary). The set incubation temperature was at 37.8 ± 0.5 °C and relative humidity at 50-60% until 14 days. On day 8 of embryonic development, eggs were candled with a flashlight and removed from those where embryonic development had not started or had stopped (Figure 7). On day 14 of the incubation, eggs were transferred from the incubator tray to the hatching tray. The hatching tray was portioned based on the treatment groups to separate the eggs and avoid mixing of chicks during hatching. On day 14 of the incubation, eggs were transferred from the incubator trails to the hatching tray and the temperature was reduced to 35.5 °C while the relative humidity was increased to 65–70%.

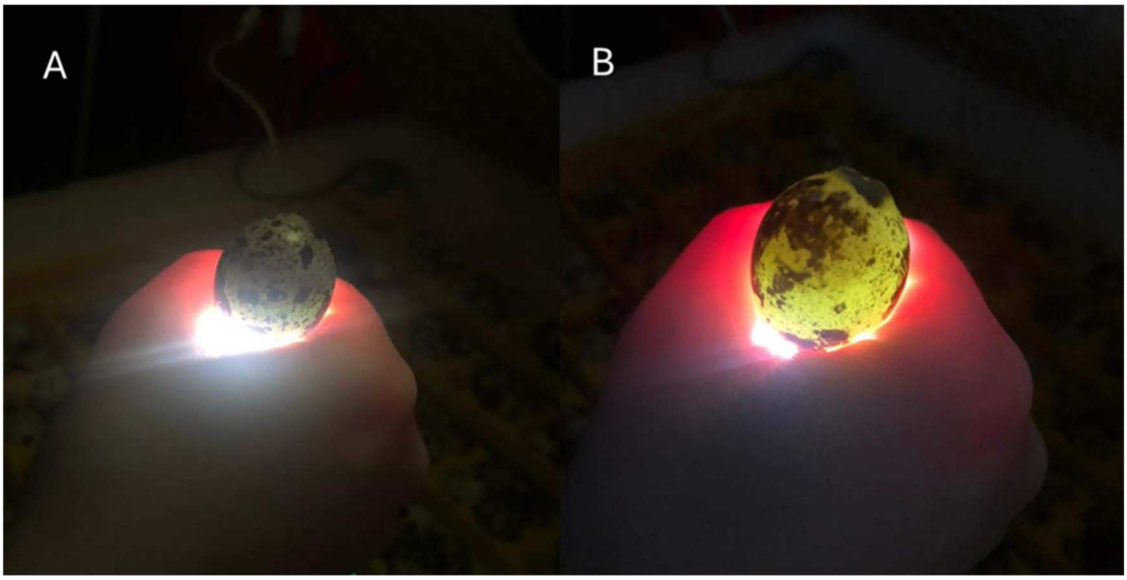

Figure 7. Candling of Japanese quail eggs. A: Opaque egg showing a developing embryo, B: Translucent showing dead embryo, embryonic development observed.

3.3.3. Rearing the experimental chicks

The hatched chicks were transferred from the incubator to the chick-rearing cages (40 cm long × 50 wide × 40 cm high) and reared for three weeks (21 days) (Figure 8). Chicks were given ad libitum feed and water as recommended (NRC, 1994) (Table 3). Quail chicks were kept under uniform standard management conditions throughout the experimental period. During the experimental chick-rearing period, body weight was measured using an electronic digital scale (± 0.01 g), and tarsal, head, and wing lengths were measured using a vernier calliper with ± 0.01 mm accuracy on days 1, 3, 5, 7, 14, and 21. All measurements were taken by the same person who was unaware of the treatment subjected to experimental groups.

Figure 8. Rearing cages for Japanese quail chicks.

Table 3. Composition and nutrient level of the basal diet of the quail chicks

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

*[a] 1 kg premix provided: 1*10^{6} NE vitamin A, 2*10^{5} NE vitamin D3, 4900 mg/kg vitamin E, 200 mg vitamin K3, 150 mg vitamin B1, 500 mg vitamin B2, 1200 mg Ca-d-Pantothetane, 400 mg vitamin B6, 2 mg vitamin B12, 11 mg biotin, 2502 mg niacin, 60 mg folic acid, 30^{5} mg choline chloride, 13200 mg Zn, 1920 mg Cu, 9612 mg Fe, 13200 mg Mn, 180 mg I, 42 mg Se, 12 mg Co.*

3.3.4. Sample collection

After recording body weight, a random sample of 8 quail chicks from each experimental group was selected for blood and tissue sampling on day 1 and 21 post-hatching (Figure 9). The blood samples were collected using the heparinised capillary tubes (~65 µl for day-old and ~80 µl for 21-day-old birds) by venipuncture using a 26G sterile needle. After sampling, the blood samples were immediately centrifuged in 1000g, separated, and then plasma from red blood cells using a Hamilton syringe, stored at -20 °C for further laboratory analyses. To avoid the potential effects of diel variation in circulating IGF-1, blood samples on day 21 were always collected at the same time as the first samples on day 1 (LENDVAI *et al.*, 2021). After blood sample collection, birds were sacrificed by cervical dislocation liver sample collection, snap-frozen in dry ice, and then stored at -80 °C for further hormonal and gene expression analyses (Figure 10).

Figure 9. Measurement and sample collection. A: Body weight measurement, B: Tissue sampling of Japanese quail chicks.

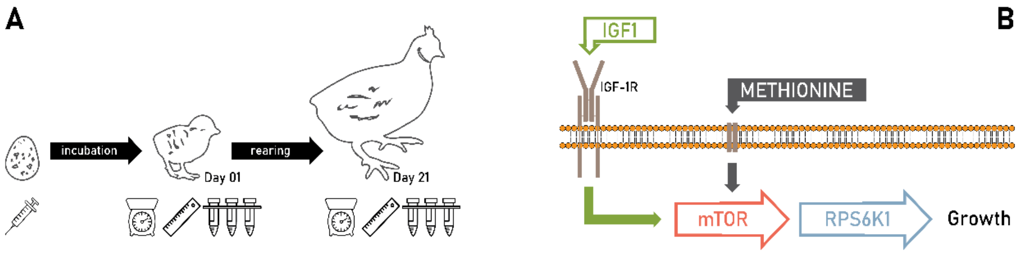

Figure 10. General schematic representation of the experimental protocol. A syringe icon indicates the L-methionine manipulation before incubation; the lab scale, the ruler and the centrifuge tubes represent body weight measurements, morphological measurements, and blood plus tissue sampling, respectively. B: Simplified diagram showing the investigated elements of the nutrient-sensing pathway (*IGF1*: insulin-like growth factor 1, *mTOR*: mechanistic target of rapamycin, *RPS6K1*: ribosomal protein serine 6 kinase 1).

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

3.4.1. Experimental design

A total of 336 freshly laid eggs were collected from 33-week-old, 70-laying Japanese quails (Coturnix japonica) over five days and kept at a temperature between 16 °C and 18 °C. On the day of the start of the incubation, eggs were weighed on the digital scale (0.01g accuracy). Eggs with similar weights (11.0 ± 0.5 g, 100 eggs) were selected for incubation to reduce the effects of egg weight differences. The eggs were divided into two experimental groups (50 eggs each) and incubated in an automatic turning incubator (WQ-63-WQ-98 Model 2021 Version 2, AGROFORTEL, S.R.O., Budapest, Hungary). On day 8 of the embryonic development, eggs were candled, and those containing dead embryos and unfertilised eggs were removed from the incubator. The incubation conditions were as in experiment 3.4 above.

3.4.2. Preparation and in ovo injection of leucine

The leucine solution (50 mg leucine/ml saline solution) was prepared by dissolving crystalline L-leucine (reagent grade; Sigma Aldrich, purity > 98%) in a 0.9% physiological saline solution (Braun Melsungen, Germany). Before injection, eggs were disinfected on the eggshell with 70% ethanol. Then, at the site of injection, the broad side of the egg was incised using a 26G sterile needle. We injected 50 µl into each egg, with 2.5 mg/egg of the leucine solution into the amniotic fluid using a Hamilton syringe on embryonic day ten (ED10). Then, the second group received 50 µl of the physiological saline solution (Braun Melsungen, Germany), each serving as a control group. After the injection, the hole was sealed with candle wax and transferred to the incubator to resume incubation. Based on the average leucine content of eggs and the average weight of the eggs, the injection represents an average 2.0% increase in leucine content.

3.4.3. Rearing experimental hatchlings

Hatched quail chicks from each experimental group were immediately transferred to cages (40 cm long × 50 wide × 40 cm height) and reared for an average of three weeks (21 days) in groups of their treatments. Chicks were given free water and feed access (Table 3). Quails were reared in standard conditions and measurement was done as described in experiment 3 above.

3.4.4. Sample collection

Eight chicks (One and 21 days old) from each experimental group were randomly selected for sample collection after body weight recording. Birds were sacrificed due to cervical dislocation. The whole gastrointestinal (GIT) length was measured post-scarification on one-day-old and 21-day-old chicks using a ruler (nearest 0.1cm). Changes in the size of the gastrointestinal tract reflect how nutrient supplements affect the physical growth of the digestive system, which is vital for efficient nutrient absorption and overall development of chicks. Then, liver samples were collected, snap-frozen in dry ice, and stored at −80 °C for further gene expression assay (Figure 11).

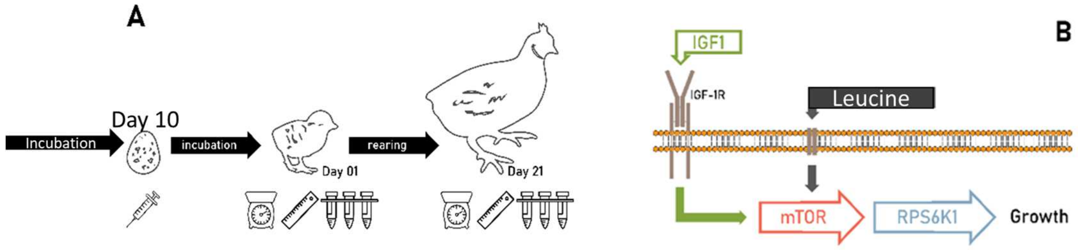

Figure 11. General schematic representation of the experimental protocol. A syringe icon indicates the L-leucine manipulation before incubation; the lab scale, the ruler and the centrifuge tubes represent body weight measurements, morphological measurements, and blood plus tissue sampling, respectively. B: Simplified diagram showing the investigated elements of the nutrient-sensing pathway (*IGF1*: insulin-like growth factor 1, *IGF1R*: insulin-like growth factor 1 receptor, *mTOR*: mechanistic target of rapamycin, *RPS6K1*: ribosomal protein serine 6 kinase 1, *FOXO1*: forkhead box O1 transcription factor).

3.5. Experiment 5: Effects of rapamycin treatment on body weight changes, relative gene expression and histomorphological parameters

3.5.1. Experimental design

In this experiment, 40 5-week-old quails with similar body weights (average weight of females: 154.6 ± 5.2 g; males: 180.43 ± 7 g) were assigned into two experimental groups: the control and the rapamycin-treated groups. Birds were provided with free access to feed and water ad libitum, and the basal diet for quails was formulated as a breeder quail ration (24% CP; 12.13 MJ/kg ME) based on corn, soybean, and wheat (NRC, 1994). Each experimental group consisted of 20 birds, 10 females and 10 males. Then, quails were injected subcutaneously with 1 mg/kg body weight rapamycin every second day for two weeks (7 times, once daily at 10:00 hours). The purity of rapamycin powder was 99% (J62473.MC, Thermo Fisher Scientific, Waltham, MA, USA). Before injection, the rapamycin powder was dissolved in 100% ethanol to yield a 25 mg/ml stock solution. The stock solution was diluted with isotonic saline solution (B. Braun, Budapest, Hungary) to a final concentration of 1 mg/ml and the injection volume for each bird's live weight (Liu et al., 2016). The control group received the rapamycin vehicle (ethanol with isotonic saline solution) in an equal volume to the rapamycin-injected group.

3.5.2. Growth measurement and relative spleen weight

The body weight was measured at the beginning of the experiment (day 1) and on days 3, 5, 7, 10, 12, and 14 before the injections and at the end of the experiment (day 15) using an electronic scale (± 0.01 g). The average daily weight gain was calculated. At the end of the experiment, the birds were sacrificed by cervical dislocation. The whole spleen was aseptically excised, collected, transferred to the liquid nitrogen and stored at -80 °C for later RNA isolation. The relative spleen weight was also measured as the percentage of the total live weight of each bird (SŁAWIŃSKA *et al.*, 2014).

3.5.3. Measurement of intestinal histomorphology

To evaluate the villus height (VH), crypt depth (CD), villus height to crypt depth ratio (VH: CD), and total mucosa thickness and define the absorptive function of the intestinal segment of the small intestine (terminal ileum), the paraformaldehyde-fixed intestinal segments of the terminal ileum were used. The intestinal tissues were stained using hematoxylin-eosin staining on 20 samples (n = 5 birds from each sex per treatment). The samples for morphological measurement were randomly selected from the sampled birds. The stained segments were examined using an Olympus BX61 light microscope paired with a DP71 camera (Olympus Corporation, Shinjuku, Tokyo, Japan) to capture images. The images were examined using cellSens Entry imaging software and the arbitrary line measurement tool. Villus height was measured from the tip of the villus to the crypt-villus junction. Crypt depth was defined from the base of the crypt up to the villus-crypt axis, and total mucosa thickness was determined from the top of the villus to the wall of the intestine involving villus height, crypt depth, and muscular mucosa (MUNYAKA *et al.*, 2012). The measurements were taken from 12 ileal villi, crypts, and mucosa sections, allowing the calculation of the average villus height (VH), crypt depth (CD), VH:CD ratio, and total mucosa thickness for each sex within each treatment group.

3.6. Laboratory analyses

3.6.1. Total antioxidant capacity analyses

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

3.6.2. Analysis of amino acid concentration in Japanese quail eggs

Before the amino acid solution was injected into the eggs, the amount of amino acids in the Japanese quail egg was quantified from freshly laid eggs using a pool of 12 eggs. Determining amino acid concentration in eggs was done in the accredited Central Laboratory of the Agriculture and Food Products, Faculty of Agricultural and Food Sciences and Environmental Management University of Debrecen, Hungary. The protein content of the samples was determined using the Kjeldahl method (LYNCH & BARBANO, 1999). Then, the nitrogen content of the sample was converted into ammonium salt by boiling it in concentrated sulphuric acid. 14 ml of concentrated sulphuric acid and two catalyst tablets containing selenium were added. The sample was destructed at 420 °C when placed on a destructive block (VELP DKL Kjeldahl). After cooling the sample, it was distilled on a VELP UDK-149 distiller. An automatic titrator (VELP TITROLINE 5000) was applied, and the nitrogen content was calculated. The protein content of the samples was calculated from the nitrogen content using a conversion factor (6.25). Measurements were repeated four times with CV% <10%.

For protein hydrolysis, the same amount of protein was measured into a hydrolysis tube with Teflon top with 6N HCl and was reacted at 110°C for 23 h in an oven (Memmert UN55, Buechenbach, Germany). The samples were applied to the amino acid analysis after cooling and filtering through a regenerated cellulose filter (0.2 µm, Whatman 10463040 Spartan syringe filter). For total amino acid analysis, AAA500 amino acid analyser (INGOS Ltd, Praha, Czech Republic) with low-pressure ion exchange chromatography with post-column derivatisation with ninhydrin (INGOS Ltd, Praha, Czech Republic) and photometric detection at 210 and 254 nm was used. Amino acid standard mixture (INGOS Ltd, Praha, Czech Republic) was applied as a reference. The recovery was higher than 95%.

3.6.3. Enzyme-Linked Immunosorbent Assay (ELISA)

The circulating plasma IGF-1 levels were measured by a competitive enzyme-linked immunosorbent assay (ELISA) as described previously (MAHR *et al.*, 2020). The samples were analysed in duplicate on a single plate. Non-sterile Nunc™ 96-Well Polypropylene MicroWell™ Plates (Thermo Scientific™) were coated using 100 µl antibody raised against IGF-1 in rabbits and incubated overnight at 4 °C. After coating, the plate was wrapped well with cling film and incubated overnight at 4 °C. The unbounded capture antibody was washed three times with 250 µl washing buffer in each well and forcefully hit against a sheet of clean absorbent to dry the coated wells. The capture antibody was incubated for 2 hours at room temperature (25 °C) with 20 µl standard concentrations (in two-fold serial dilutions in duplicate starting at 1000 ng/ml to 1.954 ng/ml of synthetic chicken IGF-1 sample or 20µl of sample diluent and 100 µl biotinylated IGF-1 as a tracer was pipetted into each well using a multichannel pipette. The diluent was prepared by adding 11 µl of the plasma samples and diluted with 9 µl phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO4 in 1000 ml ddH_2O, pH 7.4) to make a complete 20 µl of assay volume. The blank wells for non-specific binding and total binding were filled with 20 µl assay buffer. After all target samples were loaded into the wells, reference samples and standards were also loaded into the specific wells. Then, the microplate was briefly shaken to ensure all loaded content settled at the bottom of the well and incubated. After incubation, the microplate was washed thrice with 250 µl of PBS buffer containing 0.025% Tween 20.

After washing, 100 µl of streptavidin-horseradish peroxidase conjugate (HRP) was added to all wells using a multichannel pipette and incubated at room temperature for 30 minutes, followed by another washing cycle (3 times). Then, 100 µl of tetra-methyl-benzidine (TMB) was added to the wells and incubated at room temperature for 30 minutes. The enzymatic reaction was stopped by adding 100 µl of 1M H_2SO_4, and optical density was measured at 450 nm (reference at 620 nm) and immediately read absorbance (OD) by using Magellan^{TM} microplate reader for F50 software (TECAN Trading AG, Switzerland). The calibration curve was fitted using a 4-parametric log-logistic curve that follows the sigmoid-shaped curve, and concentrations of unknown samples were read off from this curve. In this assay, we were interested in measuring the concentration of free circulating plasma IGF-1.

3.6.4. Total RNA isolation

For the first experiment, the total RNA was isolated from frozen liver tissue using TRIzol reagent and Direct-zol™ RNA MiniPrep (Zymo Research; Orange, CA, USA) according to the manufacturer's protocol, including the DNA digestion step. About 25 mg of tissue samples were measured and placed in RNAase-free tubes. To each tube, 600 µl of TRIzol reagent was pipetted and homogenised using a D1000 rotor hand-held homogeniser 130W with an adjustable speed of 8,500-30,000 RPM (Sigma Aldrich) for 30 seconds for efficient lysis. The homogenised tissues were centrifuged at 16,000 RPM for 30 seconds and transferred to the supernatants into RNase-free tubes at low temperatures (4 °C). Subsequently, the same amount (600 µl) of 95-100% ethanol as the Tri-Reagent was added and mixed thoroughly with the homogenised samples. The mixture was transferred into Zymo-Spin™ IIC column tubes and placed on the collection tubes. Then, the mixture was centrifuged for 30 seconds in 16000g, and the flow-through was discarded. In the DNase treatment stage, the DNase 1 was used to remove genomic DNA, which consisted of the following steps: 400 µl RNA wash buffer was added to Zymo-Spin™ IIC column tubes followed by 30 seconds centrifugation in 16000g and discard of the flow-through from the collection tubes. 5 µl DNase I and 75 µl DNA digestion buffer were measured and mixed to make a volume of 80 µl. The resulting solution of 80 µl was added to each sample in a Zymo-Spin™ IIC column tube and incubated for 15 minutes at room temperature. After incubation, 400 µl Direct-zol™ RNA Pre-wash buffer was added, followed by 30 seconds of centrifugation and repeated the step. To completely remove the Pre-wash buffer, 700 µl RNA wash buffer was added and centrifuged for 2 minutes. Then, the column tubes were placed on Eppendorf tubes, and 60 µl of DNAase/RNAase-free water was pipetted directly onto each column tube and centrifuged to elute RNA samples. RNA integrity was checked by 1.5% agarose gel electrophoresis, and RNA concentration (ng/µl) and percentage purity (%) were determined spectrophotometrically by HTX SYNERGY multi-mode plate reader (Agilent BioTek, BioTek Instruments Inc, USA).

In experiments 2, 3 and 4 different RNA isolation and cDNA isolation Kits were used. The total RNA was isolated using the peqGOLD Total RNA Kit following the manufacturer's protocol, which also included the DNA digestion step using the peqGOLD DNase Digestion Kit (VWR Life Science, USA). About 25 mg of the frozen liver tissue samples were excised and homogenised in 700 µl TRK Lysis buffer using a D1000 rotor hand-held homogeniser 130W with an adjustable speed of 8,500-30,000 RPM (Benchmark Scientific, USA). The homogenate was centrifuged for 5 minutes in 13000 x g and transferred the lysate to the peqGOLD RNA Homogenizer Column tube inserted into a 2 mL Collection Tube and centrifuged again for 1 minute in 13000 x g. An equal volume of 70% ethanol was added to the filtrate, vortexed, and the sample was transferred to the peqGOLD RNA Mini Column tube and centrifuged for 1 minute at 10000 x g. 250 µl RNA Wash Buffer 1 was added to the Mini Column tube and centrifuged for 1 minute in 10000 x g, followed by a DNA digestion step. In the DNA digestion step, 75 µl of the DNase 1 stock solution was added to the Mini Column Tube and incubated for 15 minutes at room temperature. After incubation, 250 µl of the RNA Wash Buffer 1 was added and followed by 1 minute centrifugation in 10000 x g (this step was repeated). Finally, it was followed by maximum centrifugation of the RNA pellet for

2 to dry the sample. Then, 60 µl of Nuclease-free Water was added and centrifuged to elute the RNA sample. The RNA concentration (ng/µl) and percentage purity (%) were determined using an HTX Synergy multi-mode Microplate Reader Spectrophotometer (Agilent BioTek, BioTek Instruments Inc, USA). The RNA integrity was checked using Qubit^{TM} RNA IQ Assay according to the manufacturer's protocol (Life Technologies Corporation, Netherlands) using the Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, USA) (Figure 12).

Figure 12. Sample preparation and RNA integrity check: A: Centrifuge, B: Invitrogen Qubit 4 Fluorometer, and C: mini cooler box.

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

The reverse transcription was performed using the qScript cDNA synthesis kit (Quantabio Reagent Technologies; QIAGEN Beverly Inc., USA) in a 20 µl final volume containing 5x cDNA supermix, 200 ng RNA template and distilled water, using PCRmax Alpha thermal cycler (Cole-Parmer Instrument Co. Ltd., UK). The conditions consisted of reverse transcription at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. The intron-spanning forward and reverse primers for quail were designed by using the Oligo 7 software and checked for target identity using Primer-Blast software of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov, Table 4). The quantitative PCR was performed in Agilent AriaMx Real-time PCR System (Agilent Technologies, USA) and applied 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne; Tartu, Estonia), 2 ng cDNA template, 200 nM of each primer, and distilled water in a 10 µl final volume of each sample in duplicates. The relative gene expression changes were normalised against the *RPL19* gene as the most stable reference gene selected from 6 housekeeping genes such as *ACTB*, *GAPDH*, *RPL19*, *RPS8, 18S*, and *RPL13* by three algorithms delta Ct, Best Keeper, NormFinder (Simon *et al.*, 2018; Vitorino Carvalho *et al.*, 2019). Raw data were collected with Aria AgilentMx 1.8 software. The relative gene expression of the target genes (*mTOR*, *RPS6K*1, and *IGF1*) was reported in fold change calculated using the double-ΔCT method (Schmittgen & Livak, 2008). The sample with the highest delta Ct value was considered as a calibrator to calculate the double-ΔCT value (Pabinger *et al.*, 2014). The results of the rapamycin treatment experiment were determined as fold changes of the expression of target genes in the experimental group compared to the control group.

In experiments 4 and 5 cDNA synthesis was done using LunarScript® RT SuperMix Kit according to the manufacturer protocol (New England Biolabs, Inc. USA). For 20 µl of cDNA synthesis, a 4 LunaScript® RT SuperMix reaction mix containing hexamer and oligo-dT primers, dNTPs. Murine RNase Inhibitor and Luna® Reverse Transcriptase were used. 200 ng of total RNA was added, followed by RNase/DNase-free water to reach a 20 µl mix. The following thermos cycling conditions for cDNA synthesis were set: 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. The synthesised cDNA samples were diluted 10-fold and stored at -20 °C until further gene expression analysis (RT-PCR). The details of all experiments have be summarised in Table 5.

Table 4. Primer sequence of the target genes (*IGF1, IGFR, mTOR, RPS6K1, FOXO1, IL-1β, myD88, NF-κB, STAT3*) and Reference gene (*RPL19*)

Gene	Gene name	Primer sequences (5' → 3') (forward/reverse)	NCBI GenBank	Fragment size (bp)
RPL19	ribosomal protein L19	F: CATCGGTAAGAGGAAGGGT	XM_015885843.1	163
		R: ACGTTGCCCTTGACCTTCAG		
mTOR	mechanistic target of rapamycin	F: CCGAAGCATTGAATTGGCCCT	XM_015882433.2	116
		R: CATCTCTCAAAGGCAGCGGACC		
RPS6K1	ribosomal protein S6 kinase 1	F: AGGCAGGAACCCTCCGTGCAA	XM_015883670.2	106
		R: AGCTCAAACTGCGAAGGGTCGG		
IGF-1	insulin-like growth factor-1	F: CACTATGCGGTGCTGAGCTGGTT	XM_015867574.2	118

		R: TCCCCTTGTGGTGTAAGCGTCT		
IGF1R	Insulin-like growth factor 1 receptor	F: TACAACTACCGCTGCTGGACCAC R: AGGCACTCAGGATGGCAACAC	XM_015873184.2	107
FOXO1	forkhead box O1	F: TGAGCGAGATCTGCGAGTTCAT R: AGGAAGCTCCCGTTGTCGAACA	XM_015851898.1	102
IL-1β	Interleukin-1 beta	F: CGTGCTGGAGTCACCCACACA R: CACGGGGACGGTACAGAGCGAT	XM_015882931.2	98
myD88	Myeloid differentiation primary response 88	F: CTGGGCCGTCACGATGTGCT R: CTGTCTACCGCCGGGACCTG	XM_015852905.2	119
NF-κB	Nuclear factor-kappa B	F: TGCATCGTTTGAGAGCTCCGGTT R: CTTTACGCAGACGCACAGCTT	XM_015860720.2	77
STAT3	Signal transducer and activator of transcription 3	F: GGTCATCAAAACCGGTGTGCAGT R: GCTGCAACATCACCAGAGTCCT	XM_015885854.1	120

Table 5. Summary table showing the main features of the experiments

Feature	Description
Experiment 1: Effects of dietary restriction and amino acid supplementation	
Animal Model	Female Japanese quail
Age at Start	5 weeks (selection and acclimation), the experiment began at 6 weeks
Experimental Duration	2 weeks
Housing Conditions	Birds kept in individual cages, 23–25 °C, 60–65% humidity
Acclimation Period	1 week, with free access to breeder feed and water
Number of Birds	40 total (8 birds per group, 5 groups)
Experimental Groups	**Control**: Full feed, **DR20**: 20% feed restriction, **DR20+Met**: DR20 + 20% methionine, **DR20+Leu**: DR20 + 20% leucine 5. **DR20+Met+Leu**: DR20 + 20% methionine + 20% leucine
Diet Composition	Based on NRC (1994) recommendations; corn–wheat–soybean meal base
Feed Intake Measurement	Daily feed intake and weekly body weight during acclimation to set restricted feed levels
Nutrient Constants	All feeds had the same ME (12.13 MJ/kg), crude protein (24%), lysine (1.3%), etc.
Amino Acid Adjustments	Met and/or Leu increased only in the respective supplemented groups
Key Measurements Taken	Final body weight (to 0.01 g), Ovary weight (to 0.1 g), Follicle weight (F1–F3) (to 0.01 g), Follicle diameter (to 0.1 mm), Follicle count (F1–F3), Ovary index (ovary weight as % of body weight)
Method of Sacrifice	Cervical dislocation
Experiment 2: Effects of dietary restriction	
Trial 1	

Animal Model	Female Japanese quail
Age at Start	5 weeks (selection and acclimation), the experiment began at 6 weeks
Experimental Duration	14 days
Housing Conditions	Kept in Individual cages, 24 ± 3 °C, 60–6 5% humidity, 12:12 light–dark cycle.
Acclimation Period	1 week, with free access to breeder feed and water
Number of Birds	40 female quail total (8 birds per group)
Experimental Groups	Control (100%), DR20 (80%), DR30 (70%), DR40 (60%)
Diet Composition	Based on NRC (1994) recommendations corn–wheat–soybean meal base
Egg collection frequency	Daily egg collection
Feed Intake Measurement	Daily feed intake and weekly body weight during acclimation to set restricted feed levels
Diet Composition	Based on NRC (1994) recommendations corn–wheat–soybean meal base
Egg collection frequency	Daily egg collection
Parameters measured	Egg weight, Eggshell weight & ratio, Yolk and albumen weights & ratios, Yolk-to-albumen ratio
Experiment 2: Effects of unpredictable feed access	
Trail 2	
Number of birds	24 female quails (8 birds per group)
Age at Start	5 weeks (selection and acclimation), the experiment began at 6 weeks
Experimental duration	16 days
Housing Conditions	Kept in individual cages, 24 ± 3 °C, 60–6 5% humidity, 12:12 light dark cycle
Acclimation Period	1 week, with free access to breeder feed and water
Experimental Groups	Control (100% ad libitum), UNPR (30–170% feed daily; avg 100%), DR40 (60% constant restriction)
Feed Intake Measurement	Daily feed intake and weekly body weight during acclimation to set restricted feed levels
Diet Composition	Based on NRC (1994) recommendations corn–wheat–soybean meal base
Egg collection frequency	Daily egg collection
Parameters Measured	Egg weight, Eggshell weight & ratio, Yolk and albumen weights & ratios, Yolk-to-albumen ratio
Experiment 3: Effects of in Ovo injection of L-methionine	
Sample Size	200 eggs (approx. 100 control, 100 treated)
Treatment	In ovo injection of 1 mg L-methionine in 50 μl 0.9% saline (control = 50 μl saline only)
Injection Timing	Embryonic day 0 (before incubation starts)

Injection Method	Injection into yolk at the broad end using a sterile Hamilton syringe; sealed with candle wax
Incubation and hatching conditions	Temperature: 37.8 ± 0.5 °C; Humidity: 50–60%; Duration: 14 days and transferred to hatching tray on day 14; Temperature: 35.5 °C; Humidity: 65–70%
Post-Hatching Rearing	Chicks reared for 21 days in cages (40×50×40 cm); standard diet (Table 3); feed and water ad libitum
Measurements	Body weight, tarsal, head, and wing length on days 1, 3, 5, 7, 14, and 21 post-hatch
Measurement Tools	Digital scale (±0.01 g), Vernier calliper (±0.01 mm)
Sampling for Analysis	8 chicks/group sampled on days 1 and 21 for blood and liver tissue
Blood Collection	Venipuncture with 26G needle; plasma separated and stored at -20 °C
Tissue Collection	Liver collected post-mortem, snap-frozen, stored at -80 °C
Analysis Focus	Hormonal (IGF-1) and gene expression related to nutrient-sensing (IGF1, mTOR, RPS6K1 pathways)
Experiment 4: Effects of in Ovo injection of L-leucine	
Sample Size	100 eggs selected (50 leucine-treated, 50 control) from 336 collected eggs
Treatment	In ovo injection of 1 mg L-leucine in 50 µl 0.9% saline (control = 50 µl saline only)
Injection Timing	Embryonic day 10 (after incubation starts)
Injection Method	Into the amniotic fluid at the broad end using a sterile Hamilton syringe; sealed with candle wax
Incubation and hatching conditions	Temperature: 37.8 ± 0.5 °C; Humidity: 50–60%; Duration: 14 days and transferred to hatching tray on day 14; Temperature: 35.5 °C; Humidity: 65–70%
Post-Hatching Rearing	Chicks reared for 21 days in cages (40×50×40 cm); standard diet; feed and water ad libitum
Measurements	Body weight, tarsal, head, and wing length on days 1, 3, 5, 7, 14, and 21 post-hatch, gastrointestinal tract (GIT) length on days 1 and 21
Measurement Tools	Digital scale (±0.01 g), Vernier calliper (±0.01 mm)
Sampling for Analysis	8 chicks/group sampled on days 1 and 21 for liver tissue and GIT length measurement
Blood Collection	Venipuncture with 26G needle; plasma separated and stored at -20 °C
Tissue Collection	Liver samples snap-frozen and stored at −80 °C for gene expression assays
Analysis Focus	Nutrient-sensing gene expression (IGF1, IGF1R, mTOR, RPS6K1, FOXO1)
Experiment 5: Effects of rapamycin treatment	
Animal Model	5-week-old Japanese quail (*Coturnix japonica*)
Sample Size	40 birds total: 20 rapamycin-treated (10 males, 10 females) and 20 control (10 males, 10 females)
Treatment	Subcutaneous injection of **1 mg/kg body weight rapamycin**, every **second day** for 2 weeks (7 injections

	total). Injected with vehicle (ethanol + saline solution), equal volume as treatment for the control.
Rapamycin Preparation	Dissolved in ethanol (25 mg/ml stock), diluted to 1 mg/ml in isotonic saline
Breeder diet composition	Based on NRC (1994) recommendations corn–wheat–soybean meal base
Body Weight Measurements	Days 1, 3, 5, 7, 10, 12, 14, and 15 (pre-injection each time); average daily gain calculated
Spleen Analysis	Spleen collected post-mortem, weighed (relative to live weight), snap-frozen in liquid nitrogen and stored at -80 °C for RNA isolation
Histomorphology Analysis	**Terminal ileum** tissues fixed, stained (H&E), and analyzed for: Villus Height (VH), Crypt Depth (CD) and VH:CD Ratio, Total Mucosa Thickness
Tissue Sample Size	20 birds (5 males and 5 females per group)
Microscopy Tools	Olympus BX61 light microscope, DP71 camera, and cellSens Entry software
Measurement Technique	Arbitrary line tool used for 12 sections per sample (villi, crypts, mucosa)
Analysis Focus	Impact of rapamycin on growth, spleen gene expression (IGF1, IL-1β, MyD88, NF-κβ, STAT3), and small intestinal absorptive capacity (Crypt depth, Villus height and Mucosa thickness)

3.7. Statistical analyses

All statistical analyses were performed using R version 4.3.3 ‘Angel Food Cake’ (http://www.r-project.org/) using the RStudio user interface. Graphs (images) were visualized using the *ggplot* function provided by the ‘ggplot2’ package version 3.4.3 (VAIDA & BLANCHARD, 2005). Akaike’s information criterion corrected for small sample sizes (AICc) was used for model selection (GUTHERY *et al.*, 2003). The residuals versus fitted values were plotted to check for homogeneity of variance before the statistical analysis. Where the residuals appear not to have a uniform vertical spread across the range of fitted values, a log transformation was used to normalise the data skewness. To determine hatchability, a chi-squared test (χ^2) was used to test whether there is a statistically significant difference between observed and expected hatching percentages between the treatment groups.

The effects of amino acid supplementation on top of dietary restriction on body weight change, final body weight, growth rate, number of hierarchical follicles and TAC with the function *lm* using a linear mode (SEARLE *et al.*, 1980; KUZNETSOVA *et al.*, 2017). To account for this variability and allow the direct effects of treatments to be observed, we included the final body weight in the model when analysing ovary weight, ovary index, follicle weight and follicle diameter. To analyse the effects of dietary restriction and amino acid supplementation on ovary weight, ovary index, hierarchical follicle weight and diameter as response variables, a linear additive model while controlling for the effect of body weight by including the final body weight was used (SEARLE *et al.*, 1980; KUZNETSOVA *et al.*, 2017).

Yolk weight, albumen weight, and shell weight, a polynomial mixed-effect model of the Gaussian family (DEMIDENKO, 2013), was used to incorporate the polynomial nonlinear pattern of the variables across days as a random effect. A function lmer from the lme4 package v. 1.1.31 (BATES *et al.*, 2015) was used to define fixed and random effects and estimate model parameters. To analyse the yolk-to-albumen ratio, a Generalised Additive Mixed-Effects Model (GAMM) from the Gaussian family, using the gamma function from mgcv package v. 1.8.42 (WOOD, 2017), was used to capture nonlinear patterns across treatment days with a smooth function included as part of the fixed effects, while the fluctuating components of the smooth function were treated as random effects. A Generalised Linear Mixed-Effect Model (GLMM) from the beta family distribution was used to analyse shell, albumen, and yolk ratios and presented them as percentages in graphs to clearly present the proportions. The GLMM using glmmTBM from the glmmTBM package v. 1.1.8 (BROOKS *et al.*, 2017), was fitted because these variables were constrained between 0 and 1 (ratio). In the saturated models of all variables, the treatment and treatment period were considered fixed effects, while individual bird identity and nonlinear components of the polynomial and additive models were treated as random effects.

For IGF-1, a linear model was used to analyse the effects of IGF-1 levels as a response variable during treatment and days as independent variables. Growth in terms of increasing body weight and development of morphological traits (wing, head, tarsal, and feather lengths) across the days (1, 3, 5, 7, 14, and 21) were analysed using a linear mixed model with body weight and morphological traits as dependent variables, treatment, and days as fixed factors, while individual bird identity as random factor. Where the interaction effect of treatment and day was significant, estimated marginal means were used to compare body weight/morphological traits between the treatment groups within each day using the 'emmeans' package (SEARLE *et al.*, 1980). The p-values were calculated using the Tukey HSD test (NANDA *et al.*, 2021).

The effects of rapamycin on body weight were analysed by a linear mixed model with bird identity as a fixed factor across the days, while treatment and sex were fixed factors. Histomorphology parameters were analysed using a linear model. For gene expression, the quantification of cycle values was obtained from the RT-PCR amplification using the Agilent AriaMx 2.0 software. Among the most frequently used reference genes in bird gene expression studies were analyzed for their stability. The selected reference genes are β-cytoskeletal actin (*ACTB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein L19 (*RPL19*) using ΔCt, NormFinder, and Best Keeper algorithms. The *RPL19* was finally considered the most stable reference gene for normalization. We calculated the relative gene expression levels using the $2^{-\Delta\Delta Ct}$ methods described previously (SCHMITTGEN & LIVAK, 2008). The linear model was also fitted to analyse the effects of treatment on the relative gene expression of *IGF1R, mTOR*, *IGF1*, *RPS6K1* and *FOXO1, IL-1β, NF-κβ, STAT3, MyD88.*

A one-way Analysis of Variance (ANOVA) was used to assess the statistical significance among the treatment groups in all analysed parameters where only one factor was involved. A two-way ANOVA was used to determine the statistical difference among the treatments involving two factors. Estimated marginal means were calculated using the *emmeans* package.(SEARLE *et al.*, 1980). Pairwise comparisons were adjusted for multiple testing using the Tukey method, with statistical significance set at $p < 0.05$.

4. RESULTS AND DISCUSSION

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

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

The initial body weight was similar among the treatment groups ($p = 0.884$, Table 5). Dietary restriction treatment significantly reduced the final body weight ($p = 0.009$, Table 5). However, supplementation of leucine, methionine and their interaction on top of restricted feeding restored the final body weight similar to the control group ($p > 0,05$ for all, Table 5).

Body weight changes were affected by dietary treatments (Table 5). The dietary restriction showed a significant negative change in body weight compared to the control group ($p < 0.001$; Table 5). The multiple comparison showed a significant positive change in the body weight compared to the control group by supplementing methionine or leucine individually or in combination within a restricted diet (DR20+Met: $t = 4.394$, $p = 0.001$; DR20+Leu: $t = 4.501$, $p < 0.001$; DR20+Leu+Met: $t = 3.143$, $p = 0.027$, Table 5). All other pairwise comparisons were not significant ($p > 0.05$, Table 5). The dietary restriction and amino acid supplementation significantly affected growth rate; the results were similar to body weight changes ($p < 0.001$, Table 5).

These results indicate that nutrient status can positively influence the body mass necessary for attaining sexual maturity. We previously showed in our recent study in Japanese quails that 20% of an 8-week-old feed-restricted group experienced moderate weight loss, with severe weight loss observed in 30% and severe 40% feed restriction, which was accompanied by reduced egg mass, number and laying probability (REDA *et al.*, 2024a). Other studies also reported individuals under 20% feed and energy between 15 to 21 weeks of age during sexual maturity (VAN DER KLEIN *et al.*, 2018; LU *et al.*, 2021) or 10% feed restriction (URDANETA-RINCON & LEESON, 2002) in chickens resulted in reduced body mass compared to *ad libitum* feeding. In contrast, a 20% dietary restriction in broiler chickens at the age of 8 to 16 days showed no significant difference in body weight compared to the ad libitum-fed group at 42 days (BUTZEN *et al.*, 2013). While alternating skip-a-day for the period of a week, feed restriction increased body weight in chickens than the continuous feed restriction and ad libitum or three-day restriction feeding (TESFAYE *et al.*, 2009; CARNEIRO *et al.*, 2019). These contradictory results may be due to differences in bird age, developmental stage and severity of restriction.

Table 6. Comparative effects of amino acid supplementation on initial body weight, final body weight, change in body weight and growth rate in Japanese quails reared on restricted feeding

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

Values are in mean values ± standard error of the mean (SE) from sample size (n) = 8 for each treatment group.

Supplementing dietary-restricted individuals with methionine and leucine individually or in their combination mitigated the effects of dietary restriction and increased body mass, growth rate and positive change in body mass compared to the restricted diet. Essential amino acids are well-known for their role in protein synthesis and are essential for supporting growth development (JOHNSON, 2014). Our study in Japanese quail showed that embryonic supplementation of methionine or leucine increased postnatal growth and development (NDUNGURU *et al.*, 2024b; NDUNGURU *et al.*, 2024a). Based on the diet regulation hypotheses, animals can optimise their nutritional balance by selecting the types and amount of food to be consumed to meet their requirements (RAUBENHEIMER *et al.*, 2022). Specific nutrient intake provides a nutrient balance and can evaluate how organisms respond when confined to a diet that differs in specific nutrient composition to optimize growth.

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

Dietary restriction significantly reduced ovary weight compared to the control ($p < 0.001$; Figure 13A). The supplementation of Leu or combination of leucine and methionine with the dietary restriction significantly restored ovary weight similar the control group ($p > 0.05$). Multiple comparison showed ovary weight significantly increased compared to the restricted group with amino acid supplementation (DR20+Leu: $t = -2.913$, $p = 0.047$, DR20+Leu+Met: $t = -2.939$, $p = 0.045$, Figure 13A). All other compared groups were like a control group ($p > 0.05$, Figure 13A). Dietary restriction marginally reduced the ovary index compared to the control group ($p < 0.001$, Figure 13B). The supplementation of Leu or combination of leucine and methionine with the dietary restriction marginally restored ovary index similar to the control group ($p = 0.655$).

Dietary restriction during the sexual maturation stage decreased ovary mass and ovary index. Nutritional status regulates the critical, highly demanding sexual maturation stage by influencing the development of reproductive organs and improving their function (Rizzoto et al., 2019; Yin et al., 2023). The growth and development of sexual organs occur rapidly closer to the sexual maturation stage Lu et al., 2023; Yin et al., 2023). Adequate nutrient and energy intake is essential for gonadal development, which is important for synthesis of the hormone prior to sexual maturation. Studies show that restricted diets reduce ovary development and reproductive performance in birds (Pan et al., 2014; Lu et al., 2023; Yin et al., 2023). Another study reported that despite the increasing body weight, increased feed allocation due to ad libitum feeding did not stimulate ovarian development in 54-week-old chickens compared to the restricted diet (McGovern et al., 1997).

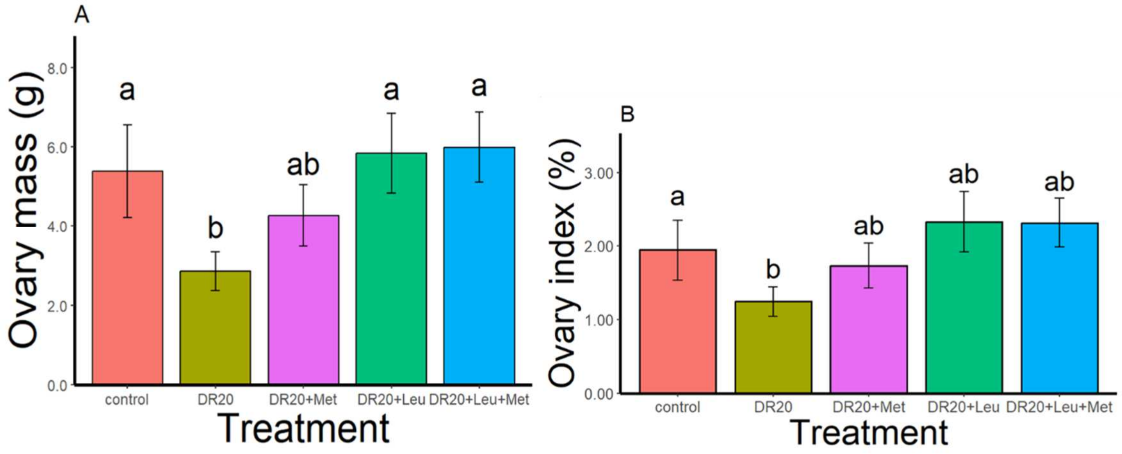

Figure 13. Effects of feed restriction and amino acid supplementation on A: Ovary weight. B: Ovary index of Japanese quails at the age of 8 weeks. Control: full-fed; DR20: 20% feed restriction; DR20+Met: 20% feed restriction + 20% methionine; DR20+Leu: 20% feed restriction+20% leucine; DR20+Met+Leu: 20% feed restriction + 20% methionine + 20% leucine. Bars represent the mean ± standard error mean (SE) from 8 birds per group. Means without a common superscript differ significantly ($p < 0.05$).

However, our study revealed that supplementing leucine and methionine or their combination within a restricted diet improved ovary growth, which increased ovary mass and index, matching the levels comparable to the full-fed group but above the dietary-restricted group. It has been observed that the ovary index typically increases during the sexual maturation stage and peaks of nutrient availability during the breeding season (KIMARIO et al., 2020).. Previous studies have shown that amino acid supplementation, such as methionine and valine, improved oviducts and follicle development in quails and chickens (Bunchasak & Silapasorn, 2005; Hanafy & Attia, 2018). Increasing ovary mass is often proportional to ovary index, a key indicator of reproductive organ development and maturation (Jiang *et al.*, 2022; Li *et al.*, 2022). The observed increase in ovary mass and index following amino acid supplementation suggests a compensatory reproductive investment that mitigates the effects of dietary restriction.

4.1.3. Effects of feed restriction and amino acid supplementation on hierarchical follicle weight and diameter

Dietary treatment had no significant effect on the weight of hierarchical follicles compared to the control group ($p > 0.05$ for all treatments; Table 6). Supplementation with leucine, methionine, or their combination did not have a significant effect on follicle weight compared to the restricted group ($p > 0.05$ for all treatments, Table 6). Dietary treatment had no significant effect on the diameter of hierarchical follicles compared to the control group ($p > 0.05$ for all treatments; Table 6). Supplementation with leucine, methionine, or their combination did not have a significant effect on follicle diameter compared to the restricted group ($p > 0.05$ for all treatments, Table 6).

Table 7. Comparative effects of amino acid supplementation on follicle weight and follicle diameter of Japanese quails at the age of 8 weeks during dietary restriction

Variables	Follicles	Treatments				
		Control	DR20	DR20+Met	DR20+Leu	DR20+Leu+Met
Follicle weight (g)	F1	2.690±0.10 (n=4)	1.996±0.07 (n=8)	2.157±0.25 (n=5)	2.462±0.18 (n=5)	2.548±0.26 (n=7)
	F2	1.610±0.09 (n=5)	1.122±0.11 (n=5)	1.033±0.24 (n=7)	1.464±0.19 (n=8)	1.590±0.24 (n=8)
	F3	0.575±0.9 (n=5)	0.356±0.07 (n=5)	0.351±0.11 (n=8)	0.640±0.04 (n=4)	0.589±0.18 (n=8)
Follicle diameter (mm)	F1	18.240±0.34 (n=4)	14.874±0.23 (n=8)	16.181±0.92 (n=5)	17.420±0.67[a] (n=5)	17.696±0.74 (n=7)
	F2	14.895±0.42 (n=5)	12.996±0.28 (n=5)	12.190±1.07 (n=7)	14.170±0.71 (n=8)	14.622±0.83 (n=8)
	F3	9.920±0.55 (n=5)	8.858±0.13 (n=5)	8.644±0.99 (n=8)	10.853±0.30 (n=4)	10.660±0.72 (n=8)

Values are in mean values ± standard error of the mean (SE) from (sample size=n) each treatment group. Control: Control feed; DR20: 20% dietary restriction (Control-20%); DR20+Met: 20% feed restriction + 20% methionine; DR20+Leu: 20% dietary restriction+20% leucine; DR20+Met+Leu: 20% dietary restriction + 20% methionine + 20% leucine.

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

The average number of hierarchical follicles (F1, F2, and F3) exhibited a significant response to dietary treatment (Figure 14). Interestingly, dietary restriction and leucine supplementation did not show a significant difference in follicle counts compared to the control group ($p = 0.654$). In contrast, supplementation with methionine, whether administered alone or in combination with leucine within a restricted diet, resulted in a significant increase in follicle numbers ($p < 0{,}001$). Multiple comparison showed a significant difference compared to the restricted group in methionine and combination with leucine (DR20+Met: $t = 8.216$, $p < 0.001$; DR20+Leu+Met: $t = 6.390$, $p < 0.001$, Figure 14), but not leucine supplementation alone (DR20+Leu: $t = 0.913$, $p = 0.887$, Figure 14).

Dietary restriction did not affect the hierarchical follicle's counts or size (weight and diameter). In poultry studies, selection for rapid growth has been attributed to the increase in the number of follicles (MFOUNDOU *et al.*, 2021). The generation of multiple follicle sets is likely to occur with a significant increase in the number follicles (ASSERSOHN *et al.*, 2021). Nutritional status has a significant influence on the follicle growth and development in birds (EITAN & SOLLER, 2009). Overfeeding during sexual maturation results in rapid growth and excessive follicular growth and the occurrence of multiple follicles leading to disruption of the ovulation process and low egg production (EITAN & SOLLER, 2009; ASSERSOHN *et al.*, 2021). Reduced feed intake in immature broiler birds decreased excessive follicular development and multiple ovulation, resulting in sufficient egg production (RENEMA *et al.*, 1995; HOCKING, 2004; DIAZ & ANTHONY, 2013). Contrasting findings showed that reduced energy intake and feed intake resulted in reduced body weight and a lower number of yellow follicles in chickens and reduced size (LU *et al.*, 2023; GHOLAMI-SOLTANMORADI *et al.*, 2024).

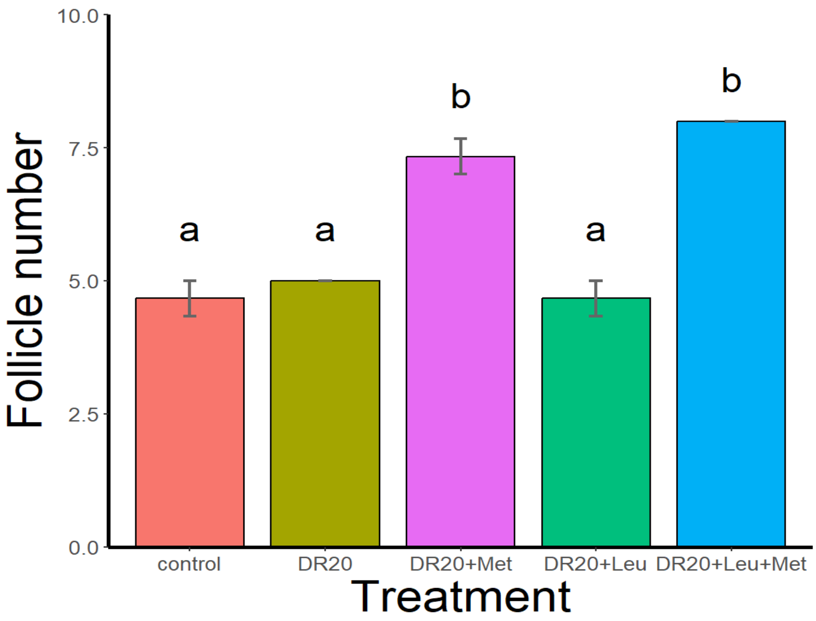

Figure 14. The average number of hierarchical follicles in each treatment of Japanese quails at 8-week-old. Control: Full-fed; DR20: 20% feed restriction (Control-20%); DR20+Met: 20% feed restriction+20% methionine; DR20+Leu: 20% feed restriction+20% leucine; DR20+Met+Leu: 20% feed restriction+20% methionine + 20% leucine. Bars represent the mean ± standard error (SE) from 8 birds in each group. Means without a common superscript differ significantly ($p < 0.05$).

Our study revealed that diet-restricted quail supplemented with methionine or a combination of leucine and methionine increased the number of follicles without altering the follicle size, whereas leucine alone did not. These variations in follicle numbers suggest that different amino acids may differentially influence ovarian follicular differentiation through distinct metabolic pathways. During growth and sexual development, primordial germ cells differentiate into smaller follicles (LENGYEL *et al.*, 2024; SRITABTIM *et al.*, 2024b) in which specific essential amino acids could play a role (LANSFORD & CHENG, 2024). Methionine is important in committing specific cell lineages by influencing unique pathways, such as the methionine cycle, and promoting epigenetic modification crucial for cellular differentiation (Ju et al., 2023; Zhang et al., 2020). However, the potential role of leucine is cell proliferation and metabolism, supporting protein synthesis and metabolic activities through the mechanistic target of the rapamycin (mTOR) pathway, but less in differentiation or lineage commitment compared to methionine (CORREIA *et al.*, 2022). These results suggest that nutrient-dependent growth and development of hierarchical follicles may vary based on size and developmental stage. Such nutrient variation requirements may be crucial during critical follicle development, such as the shift to high-level follicles.

4.1.5. Effects of feed restriction on plasma total antioxidant capacity

Dietary treatments affected total antioxidant capacity levels in a similar way to body weight (Figure 15). Dietary restriction significantly reduced antioxidant capacity compared to the control group ($p = 0.021$; Figure 15). However, supplementing methionine or leucine individually or in combination show no significant difference ($p = 0.442$). Multiple comparison showed all amino acid supplemented groups within a restricted diet were not significantly different compared to the dietary-restricted group ($p > 0.05$ for all, Figure 15).

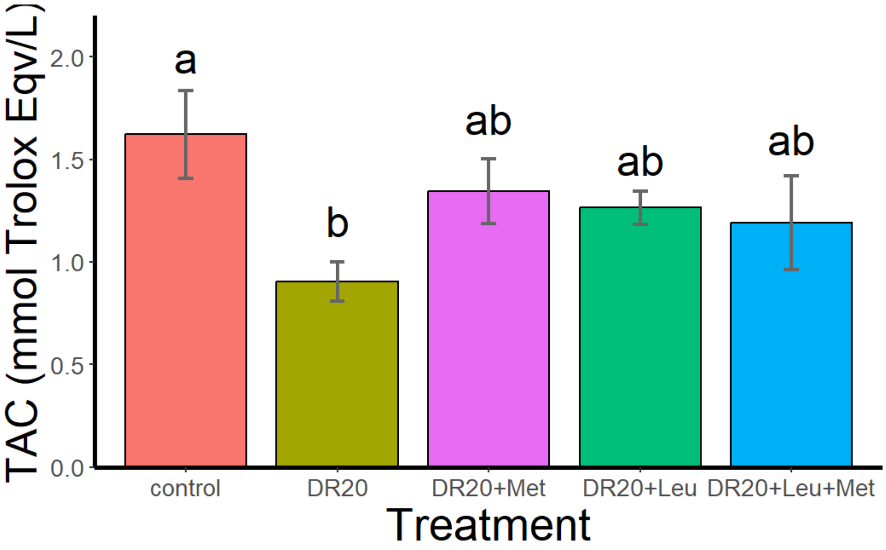

Figure 15. Effects of amino acid supplementation on top of restricted feeding on total antioxidant capacity (TAC) in Japanese quails at the age of 8 weeks. control: Full-fed; DR20: 20% feed restriction; DR20+Met: 20% feed restriction + 20% methionine; DR20+Leu: 20% feed restriction+20% leucine; DR20+Met+Leu: 20% feed restriction + 20% methionine + 20% leucine. Bars represent the mean ± standard error of the mean (SE) from sample size (n =7 for each group). Means without a common superscript differ significantly ($p < 0.05$). Abbreviations: TAC (mmol Trolox Eqv/L) = millimole Trolox equivalency per litre.

Feed restriction reduced the plasma total antioxidant capacity. However, supplementation with leucine or methionine or their combination increased the plasma total antioxidant activity. Both amino leucine and methionine acids have a more substantial oxidative capacity with a significant function in providing oxidative energy through the regulation of protein metabolism (Chang *et al.*, 2015; Zhang *et al.*, 2024; Lugata *et al.*, 2024a). Oxidative stress negatively impacts ovarian and follicular development in poultry (Wang *et al.*, 2021). The increased total antioxidant activity is proposed as a potential signal for combating and monitoring stress during growth and sexual maturation (Oke *et al.*, 2024).

4.1.6. Association between the variable and plasma total antioxidant capacity

Regardless of the treatment, all parameters significantly correlated with the plasma total antioxidant capacity (final body weight: t = 2.859, df = 30, R^2 = 0.214, p-value = 0.008; growth rate: t = 2.817, df = 30, R^2 = 0.209; p-value = 0.008; ovary weight: t = 2.285, df = 30, R^2 = 0.148, p-value = 0.029, and ovary index: t = 1.997, df = 30, R^2 = 0.148, p-value = 0.055; Figure 16). There was no significant correlation between dietary treatment and follicle measurements, weight and diameter (p > 0.05 for all).

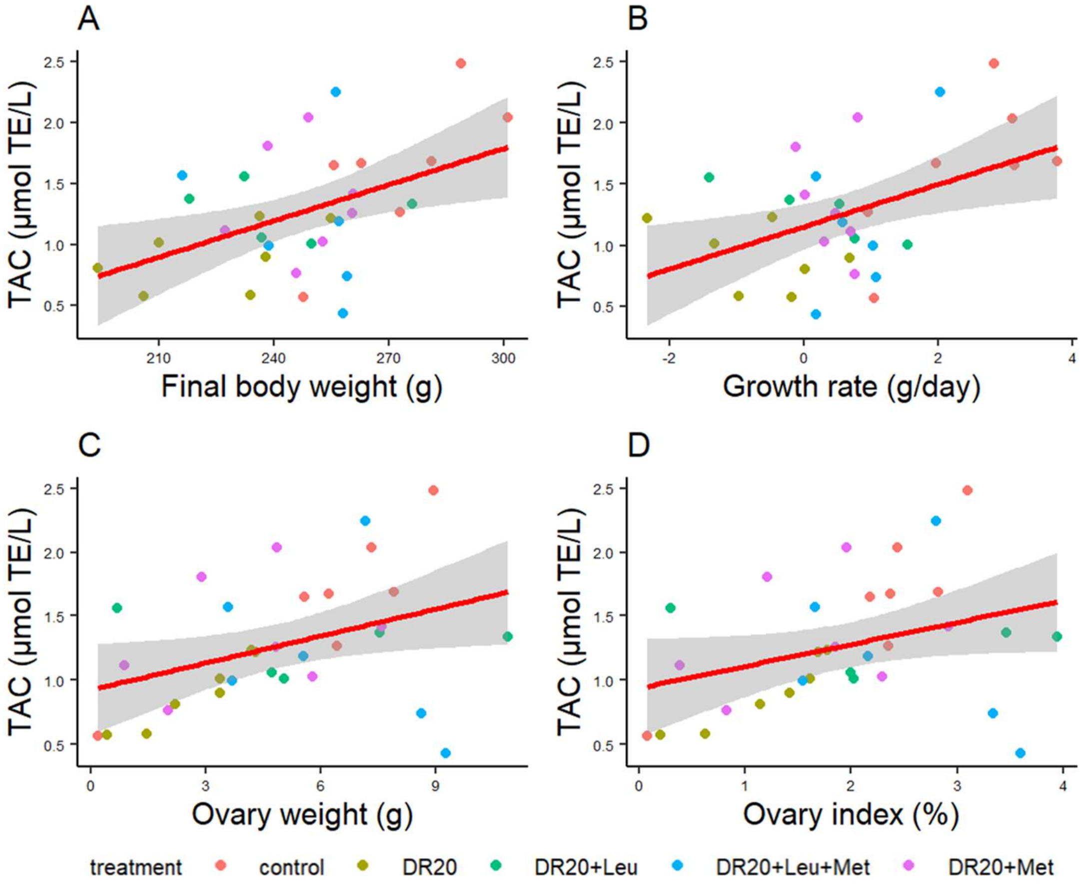

Figure 16. The association between plasma total antioxidant capacity (TAC) and final body weight, growth rate, ovary weight and ovary index. A: Final body weight (g), B: Growth rate (g/day), C: Ovary weight (g) and D: Ovary index (%). Treatments:- control: Full-fed; DR20: 20% feed restriction; DR20+Met: 20% feed restriction + 20% methionine; DR20+Leu: 20% feed restriction+20% leucine; DR20+Met+Leu: 20% feed restriction + 20% methionine + 20% leucine.

Regardless of the dietary treatment, the plasma total antioxidant capacity levels correlated to most of the parameters except follicles. These results suggest that birds could neutralize free radicals and other reactive species molecules despite dietary restriction. Nevertheless, the supplementation of methionine and leucine, in combination, boosted the plasma total antioxidant activity. These results provide insight into the importance of dietary composition optimization in managing stress, which could improve growth and sexual maturation in poultry; further research could explore the effects of specific amino acid composition in a range of avian species.

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

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

Egg yolk weight significantly varied across the duration of the treatment period ($p < 0.001$, Figure 17A, Table 7). However, the overall egg yolk weight showed no significant difference among the treatment groups (Figure 17B, Table 7). Treatment and its interaction with the treatment period significantly affected albumen weight throughout the restriction period ($p < 0.001$, Table 7). The DR40 showed the lowest overall albumen weight compared to the control ($p = 0.002$), DR20 ($p = 0.011$), and UNPR ($p = 0.016$), while the other groups showed no significant response to the dietary treatment (Figure 17C).

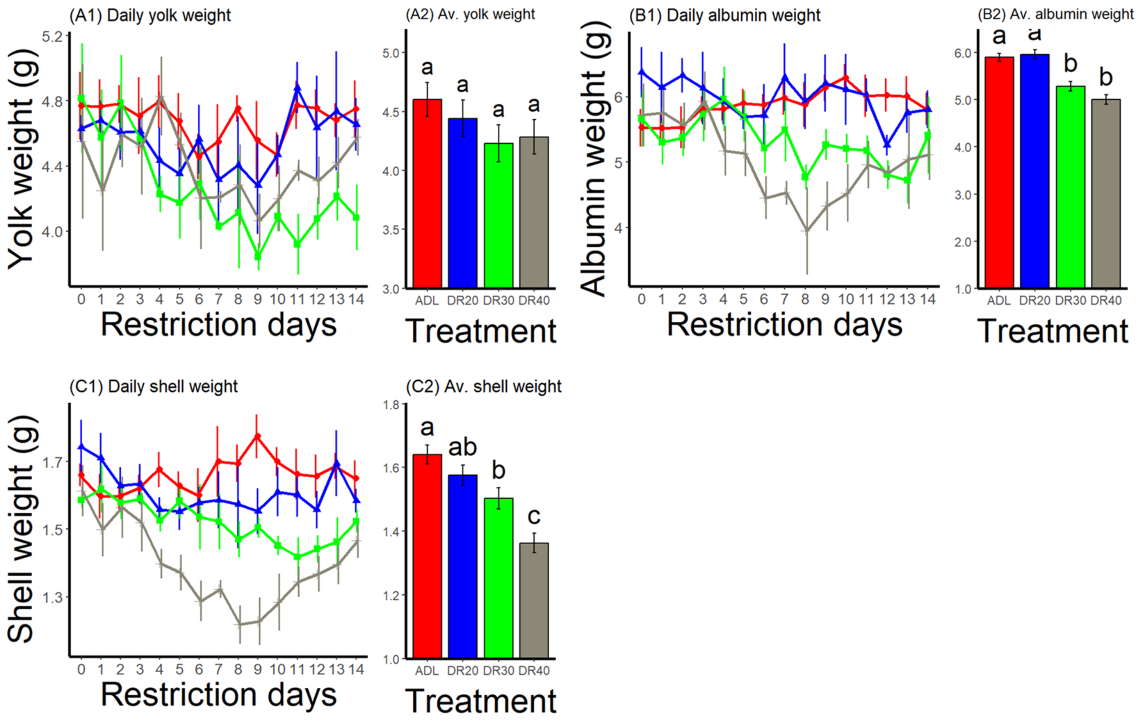

Figure 17. Feed restriction treatments on egg components of Japanese quail at 10-week age across the 14 treatment days. A1: daily yolk weight, A2: average yolk weight, B1: daily albumen weight, B2: average albumen weight, C1: daily shell weight, C2: average shell weight. Bars indicate the average value of the 14-day records of each variable. Tukey's test was applied to compare adjusted marginal means at $p < 0.05$ significance level. Means followed by a common letter are not significantly different at $p < 0.05$. Abbreviations: control: full feeding; DR20: 20% restriction; DR30: 30% restriction; DR40: 40% restriction.

Table 8. ANOVA output derived from models indicating the effect of predictable feed restriction treatment, treatment days, and their interaction on the weight of egg components in Japanese quail at 10-week age

Dependent variable*	Term	*NumDF*	*DenDF*	*F-value*	*P-value*
Yolk weight	treatment	3	23.636	0.940	0.437
	poly(day, 14)	2	300.547	9.617	<0.001
	treatment × poly(day, 14)	6	300.444	1.280	0.266
Albumen weight	treatment	3	25.789	4.677	0.010
	poly(day, 14)	2	303.28	7.122	<0.001
	treatment × poly(day, 14)	6	303.164	9.457	<0.001
Shell weight	treatment	3	25.557	15.546	<0.001
	poly(day, 2)	2	305.310	17.842	<0.001
	treatment × poly(day, 2)	6	305.119	8.202	<0.001

*Fitted models: For yolk weight and albumen weight: lmer(~treatment*poly(day,14) +(1|birdID), family = Gaussian()); For shell weight: lmer(~treatment * poly(day, 2)+(1|birdID), family=Gaussian()). Abbreviations: NumDF-Numerator degree of freedom; DenDF-Denominator degree of freedom.

Proportions of egg components relative to the egg weight showed varied trends with levels and duration of dietary restriction treatments (Figure 18, Table 9). Dietary restriction treatments and their interaction with the treatment period significantly affected the yolk ratio (Figure 18A, Table 9). The yolk ratio of the DR40 group was comparable to the control group ($p = 0.316$) but significantly different from the DR20 group ($p = 0.045$). There were no significant differences in yolk ratio among the other treatment groups ($p > 0.05$ for all groups). In contrast, the albumen ratio was significantly reduced in the severely restricted groups (Figure 18B). DR40 scored the lowest albumen ratio compared to the control ($p < 0.039$) and DR20 ($p < 0.011$), while no difference was observed with other treatment groups compared to the control ($p > 0.05$ for all groups). The shell ratio was significantly reduced in DR40 compared to the control group ($p < 0.007$) and DR30 ($p < 0.039$, Figure 18C). Treatment significantly affected the yolk-to-albumen ratio ($p < 0.001$, Table 9). The DR40 group exhibited a significantly high yolk-to-albumen ratio value compared to the control groups ($p < 0.001$), DR20 ($p = 0.002$), and DR30 ($p = 0.005$), while there was no significant difference observed among the other groups ($p > 0.05$; Figure 18D).

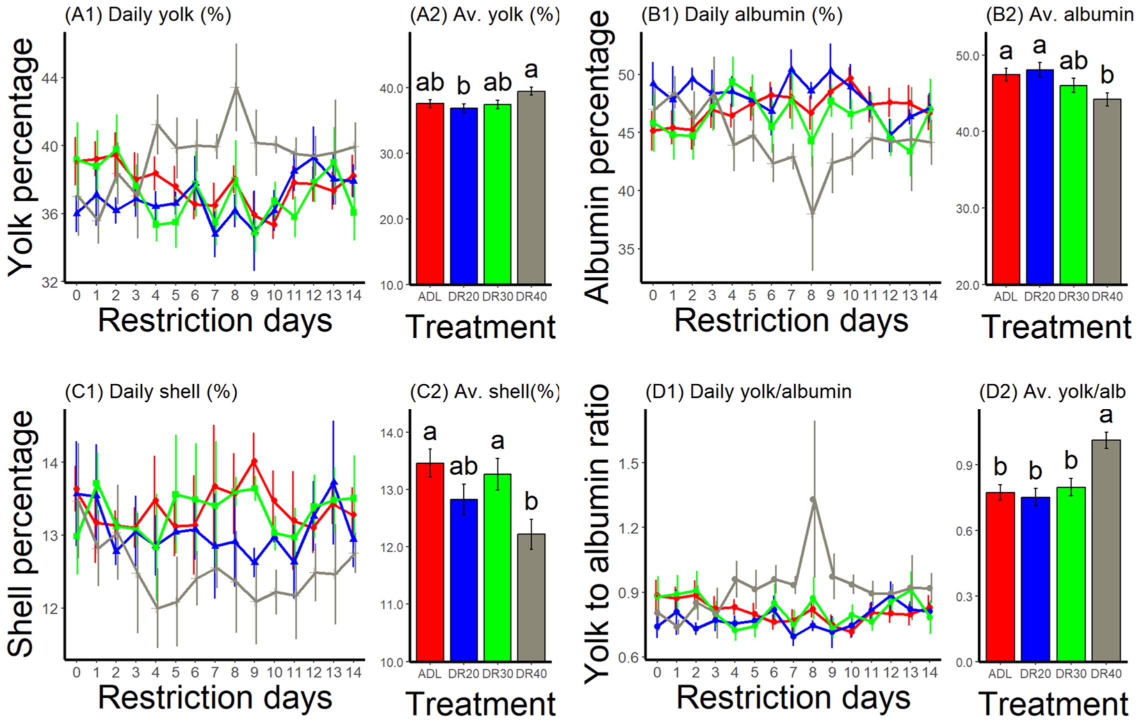

Figure 18. Variations of Japanese quail egg component proportions at 10-week age as affected by predictable feed restriction treatments across 14 treatment days. A1: daily yolk ratio, A2: average yolk ratio, B1: daily albumen ratio, B2: average albumen ratio, C1: daily shell ratio, C2: average shell ratio D1: daily yolk-albumen-ratio, D2: average yolk-albumen-ratio. Ratios other than the yolk-albumen ratio are calculated against the total egg weight and are presented as percentages for clarity in the presentation of proportions. Bars indicate the average value of the 14-day records of each variable. Tukey's test was applied to compare adjusted marginal means at $p < 0.05$ significance level. Means followed by a common letter are not significantly different at $p < 0.05$. Abbreviations: control: full feeding; DR20: 20% restriction; DR30: 30% restriction; DR40: 40% restriction.

Table 9. ANOVA output derived from models indicating the effect of predictable feed restriction treatment, treatment days, and their interaction on the proportions of egg components in Japanese quail at 10-week age

Dependent variable*	Term	NumDF	DenDF	F-value	p-value
Yolk ratio	treatment	3	20.296	2.165	0.124
	poly(day, 2)	2	299.980	0.916	0.401
	treatment × poly(day, 2)	6	299.737	5.227	<0.001
Albumen ratio	treatment	3	25.789	4.677	0.010
	poly(day, 2)	2	303.280	7.122	<0.001
	treatment × poly(day, 2)	6	303.160	9.457	<0.001
Shell ratio	treatment	3	27.302	2.950	0.050
	poly(day, 2)	2	281.159	0.856	0.426
	treatment × poly(day, 2)	6	828.937	0.901	0.495
Yolk to albumen ratio	treatment	3	20.563	3.822	0.0253
	s(day)	2	301.828	0.414	0.662
	treatment × poly(day, 2)	6	301.546	5.160	<0.001

*Fitted models: Yolk and shell ratio, lmer(~treatment*poly(day,2)*(1|birdID), family = beta_family()); Albumen ratio <-gamm(albumen_weight~s(day)+treatment, data=egg_quality_FR, random = list(birdID=~1)); Yolk to albumen ratio, <-lmer(yolkalbumen_ratio~treatment*poly(day,2)+(1|birdID), data=egg_quality_FR)). Abbreviations: NumDF-Numerator degree of freedom; DenDF-Denominator degree of freedom

4.2.2. Effects of unpredictable feed access on weights and proportions of egg components

Egg yolk weight showed a significant variation across the duration of the treatment period ($p < 0.001$, Figure 19A, Table 8). However, the overall egg yolk weight showed no significant difference among the treatment groups (Table 8). Treatment and duration significantly affected albumen weight with no interaction effects (treatment; $p < 0.001$, day; $p < 0.001$, Figure 19B, Table 8). The DR40 showed the lowest overall albumen weight compared to the control ($p = 0.017$, Figure 19B), but no significant difference was observed between the UNPR and the control group ($p = 0.728$). Dietary treatment and restriction period significantly affected eggshell weight ($p < 0.001$, Figure 19C, Table 8). Furthermore, treatment and its interaction with the treatment period significantly affected eggshell weight ($p < 0.001$, Table 8). The restriction group (DR40) scored significantly lower shell weight compared to the control ($p < 0.017$, Figure 19C). Similarly, the DR40 group significantly reduced shell weight compared to the UNPR groups ($p < 0.015$). However, there was no significant difference in eggshell weight between UNPR and the control group ($p = 0.998$).

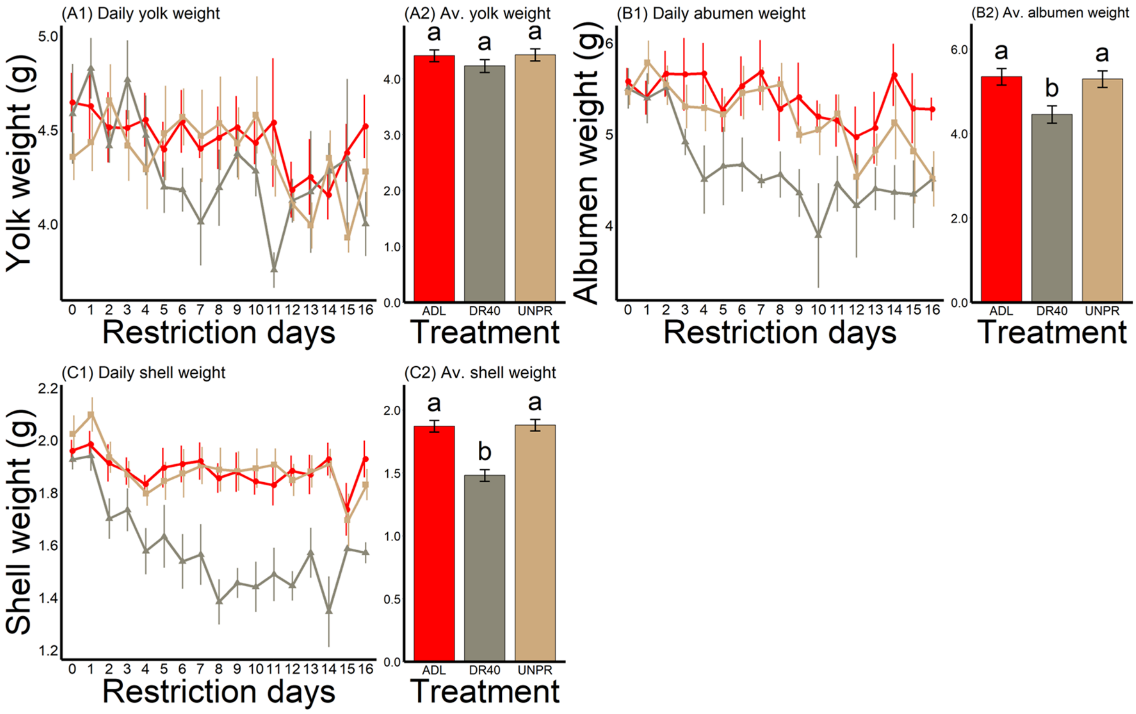

Figure 19. Variations of Japanese quail egg component weights at 10 weeks of age as affected by unpredictable feed access treatments across 16 treatment days. A1: daily yolk weight, A2: average yolk weight, B1: daily albumen weight, B2: average albumen weight, C1: daily shell weight, C2: average shell weight. Bars indicate the average value of the 16-day records of each variable. Tukey's test was applied to compare adjusted marginal means at $p < 0.05$ significance level. Means followed by a common letter are not significantly different at $p < 0.05$. Abbreviations: control: full feeding; DR40: 40% restriction; UNPR: Unpredictable feed access.

Table 10. ANOVA output derived from models indicating the effect of predictable feed restriction treatment, treatment days, and their interaction on the weight of egg components in Japanese quail at 10-week age

Dependent variable*	Term	NumDF	DenDF	F-value	P-value
Yolk weight	treatment	2	21.079	0.408	0.669
	poly(day, 2)	2	288.894	13.729	<0.001
	treatment × poly(day, 2)	4	287.13	1.996	0.095
Albumen weight	treatment	2	21.253	4.772	0.019
	poly(day, 16)	16	236.746	5.931	<0.001
	treatment × poly(day, 16)	32	239.478	1.356	0.105
Shell weight	treatment	2	21.415	4.432	0.0245
	poly(day, 2)	2	287.209	7.929	<0.001
	treatment × poly(day, 2)	4	285.738	2.711	0.03

*Fitted models: For yolk weight and shell weight: lmer(~treatment*poly(day,2) +s(day)+(1|birdID), family = Gaussian()); For Albumen weight: lmer(~treatment * poly(day, 16)+s(day)+((1|birdID), family=Gaussian()). Abbreviations: NumDF-Numerator degree of freedom; DenDF-Denominator degree of freedom

Egg production places a significant nutritional demand on female birds. The number, size, and characteristics of external and internal egg traits determine the requirement for investment in egg production and serve as key indicators of reproductive success (ANENE *et al.* 2023; ZHANG *et al.* 2023). Severe DR40 and DR30 feed restrictions but not moderate DR20 dietary restrictions affected albumen and shell weight, but the egg yolk weight remained unchanged in all treatment groups in both trials. In the second trial, unpredictable feed access had no significant effect on the egg weight components compared to the control group.

The albumen and yolk, together, make about 85% of the total egg composition, comprise nearly all the egg's interior content and are the major indicators of egg quality (SUN *et al.*, 2019; MALFATTI *et al.*, 2021). Both albumen and yolk determine the quality of table eggs and the quality of hatchlings (FINKLER *et al.*, 1998; NELSON *et al.*, 2010). We found that the restricted feeding treatment significantly reduced albumen weight (Figure 19) but did not affect yolk weight (Figure 19). The distribution of nutritionally and physiologically costly components between yolk and albumen is thought to be influenced by nutrient availability and reproductive strategy, which reflects an evolutionary balance in resource allocation (JAVŮRKOVÁ & MIKŠÍK, 2023; CHEN *et al.*, 2024). Under limited nutrient availability, yolk and albumen may have competing demands for essential nutrients. Birds may prefer to allocate more nutrients into the yolk rather than the albumen in a challenging nutritional condition for an appropriate supply of energy and nutrients, but at the same time, it may affect egg integrity and hydration. Studies reported that maternal investment in yolk determines the hatchling phenotype (JAVŮRKOVÁ & MIKŠÍK, 2023; CHEN *et al.*, 2024), which suggests that birds could prioritize reallocating resources to the yolk for proper embryo development. Differences in yolk and albumen variation due to dietary variability may also be associated with competition for egg size. Studies have reported reduced egg size under nutritional deficiency (Olawuni *et al.*, 1992; Reda *et al.*, 2024a). Therefore, the allocation of resources to yolk and albumen may be adjusted at different rates, which requires further study. A study on chickens reported in support of our result that albumen and yolk weight did not show change up to a 20% restriction level (Moreira *et al.*, 2012). Another study on chickens showed that albumen weight reacted quickly to decreased nutrient density, while yolk weight responded later to severely low nutrient density (Wu *et al.*, 2007). The variation was also reflected in the solid content of both components: albumen solid content significantly decreased with reduced nutrient density, whereas the solid content of the yolk showed no variation. Contrary to our results, another study reported an increasing albumen ratio and a decreasing yolk ratio with the severity of restriction on Japanese quail (Mahrose *et al.*, 2022). The insignificant effect of unpredictable feed access on egg yolk, albumen and shell weight egg components may suggest that these traits are affected only by constant deficiency. Though we cannot corroborate with other findings, our unpublished result showed that daily unpredictable feed access does not affect the number and weight of eggs, while it has a significant effect on the body weight of laying birds (Reda et al., unpublished). However, there is a possibility that a long period and more severe unpredictable feed access may change the current result.

Eggshell is one of the critical egg components affected by dietary conditions. Egg quality measures were reported to have a high correlation between shell weight and most egg components (Sooncharenying & Edwards, 1989; Ketta & Tůmová, 2018). Increased shell weight implies a well-packed shell mineral nutrient that increases their thickness to protect the embryo from infection, dehydration, and mechanical damage and encourage efficient embryo nutrient utilization (Hincke, 2012; Ketta & Tůmová, 2018; Narushin *et al.*, 2022). Therefore, birds invest a significant portion of available resources, including minerals and organic nutrients, on eggshells (Nakano *et al.*, 2003). In the current study, shell weight decreased with the severity of restrictions, while the unpredictable feed access group remained comparable to the control group. The study on Japanese quail found a decrease in shell traits under severely restricted levels, 35g/day/bird, as compared to ad libitum, 30, 20 g/day/bird, similar to our finding (MAHROSE *et al.*, 2022). However, this contradicts the finding of a different study on Japanese quail, which suggested that eggshell traits are not affected up to a 40% feed restriction level (ALI, *et al.*, 2007). Surprisingly, a linear increase in shell weight with increasing levels of DR (up to 30%) was reported in chickens, which contradicts our findings (OLAWUNI *et al.*, 1992). Under severe nutritional deficiency, birds can also reduce egg weight by shrinking the eggshell, negatively affecting shell weight. This could be at the expense of the egg production and embryo protection strategy. However, further study is needed on short and long-term dietary manipulations.

Dietary restriction treatments significantly affected the yolk ratio but not the restriction period (Figure 20A, Table 10). The yolk ratio of the DR40 group significantly differed from the control ($p = 005$) and UNPR ($p = 0.025$). There were no significant differences in yolk ratio between the control and UNPR treatment groups ($p = 0.799$, Figure 20A, Table 10). The albumin ratio was affected by the duration of the dietary restriction (Figure 20B, Table 10). In contrast, there was no significant difference in albumin ratio between the dietary treatment groups ($p > 0.05$, Figure 20B, Table 10). Dietary treatment and treatment period significantly affected the eggshell ratio (Figure 20C, Table 10). The eggshell ratio was significantly reduced in DR40 compared to the control group ($p < 0.005$) and UNPR ($p = 0.020$, Figure 20C, Table 10). There was no significant difference in eggshell ratio between the UNPR and the control group ($p = 0.886$). Both dietary treatment and duration of treatment significantly affected the yolk-to-albumen ratio, but not their interactions (Figure 20D, Table 10). The DR40 group exhibited a significantly high yolk-to-albumen ratio value compared to the control ($p < 0.045$) and UNPR ($p = 0.020$) groups. However, there was no significant difference in the yolk-to-albumen ratio between the UNPR and the control group ($p = 0.886$, Figure 20D, Table 10).

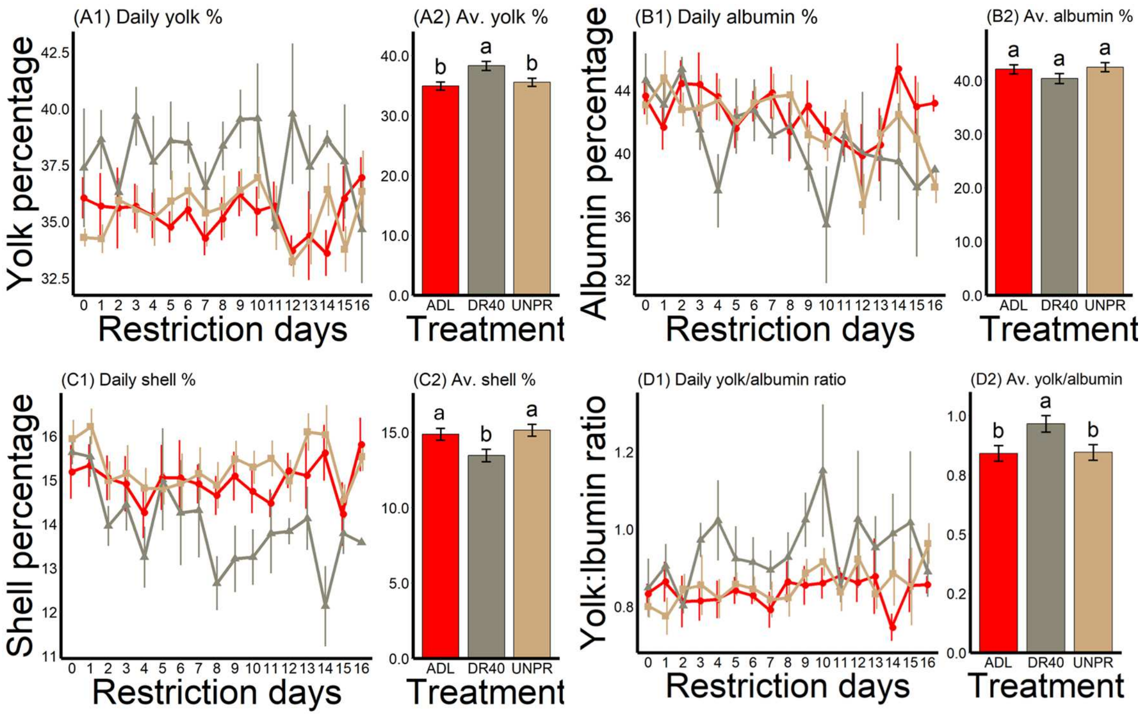

Figure 20. Variations of Japanese quail egg component proportions at 10-week age as affected by unpredictable feed access treatments across 16 treatment days. A1: daily yolk ratio, A2: average yolk ratio, B1: daily albumen ratio, B2: average albumen ratio, C1: daily shell ratio, C2: average shell ratio D1: daily yolk-albumen-ratio, D2: average yolk-albumen-ratio. Ratios other than the yolk-albumen ratio are calculated against the total egg weight and are presented as percentages for clarity in the presentation of proportions. Bars indicate the average value of the 16-day records of each variable. Tukey's test was applied to compare adjusted marginal means at $p < 0.05$ significance level. Means followed by a common letter are not significantly different at $p < 0.05$. Abbreviations: control: full feeding; DR40: 40% restriction; UNPR: Unpredictable feed access.

Table 11. ANOVA output derived from models indicating the effect of predictable feed restriction treatment, treatment days, and their interaction on the proportions of egg components in Japanese quail at 10-week age

Dependent variable*	Term	NumDF	DenDF	F-value	p-value
Yolk ratio	treatment	2	20.001	6.161	0.008
	poly (day, 2)	2	290.853	0.034	0.966
	treatment × poly (day, 2)	4	288.867	0.607	0.658
Albumen ratio	treatment	2	20.428	1.549	0.236
	poly (day, 2)	2	292.068	13.448	<0.001
	treatment × poly (day, 2)	4	290.200	2.0788	0.084
Shell ratio	treatment	2	20.563	2.950	0.074
	poly (day, 2)	2	301.828	7.799	<0.001
	treatment × poly (day, 2)	4	301.546	4.377	0.002
Yolk to albumen ratio	treatment	2	19.935	3.979	0.035
	poly (day, 2)	2	289.440	6.766	0.001
	treatment × poly (day, 2)	4	287.474	1.095	0.359

*Fitted models: Yolk and shell ratio, lmer(~treatment*poly(day,2)*(1|birdID), family = beta_family()); Albumen ratio <-gamm(albumen_weight~s(day)+treatment, data=egg_quality_FR, random =

list(birdID=~1)); Yolk to albumen ratio, <-lmer(yolkalbumen_ratio~treatment*poly(day,2)+(1|birdID), data=egg_quality_FR)). Abbreviations: NumDF-Numerator degree of freedom; DenDF-Denominator degree of freedom

The study demonstrated that the yolk ratio was notably increased in the severely restricted group (DR40) in both trials, with UNPR feed restriction comparable to the control group (Figure 20A). This was the opposite of albumen, which showed that the albumen ratio was lower in the severely restricted group (Figure 20B), except under UNPR feed restriction, where the albumen ratio remains comparable to that of the control group. The lack of differences may be due to a two-week age difference in Japanese quail birds between the two trials. The study in chicken demonstrated an increase in a flock age was proportional to the increase in yolk content and decrease in albumin content (AHN *et al.* 1997; NANGSUAY *et al.* 2011). Although, the inclusion of the age factor in the analysis model did not provide a difference in any of the traits. These results imply that in the DR40 group, the magnitude of reduction is lower in the yolk than in the total egg weight. Similarly, the shell ratio was significantly reduced in the severely restricted (DR40) group in both trials, and the difference among treatments indicated that the proportion of shell weight loss due to feed restriction was less compared to the loss of egg weight. While the UNPR feed restriction, the shell ratio remained similar to the control group (Figure 20C).

To assess the degree of change of yolk and albumen, we calculated the yolk-to-albumen ratio and found that DR40 has the highest value compared to other groups in both trials (Figure 20D). These results further confirmed that the severity of the effect of DR is higher on albumen than on yolk. Yolk is mainly made of water, fats, proteins, and other essential nutrients, while albumen is mainly made of water and proteins. Corresponding to our results, the study in chickens indicated a higher yolk-to-albumin ratio in chickens with low feed efficiency than with high feed efficiency (ANENE *et al.*, 2021). Feed efficiency is the measured feed conversion ratio in the poultry industry, showing how much feed the laying chicken consumes is converted into egg weight (ANENE *et al.*, 2021). Yolk contains lipids, which are absorbed at the end of the incubation period to provide energy for efficient hatching (LIU *et al.*, 2021). The observed higher yolk-to-albumen ratio in severely restricted feed indicates the bird deposited more nutrients to the egg yolk, providing the embryo with sufficient energy during development (NANGSUAY *et al.*, 2011; HADINIA *et al.*, 2019).

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

4.3.1. Amino acid concentration in Japanese quail eggs

The amino acid composition in the Japanese quail eggs is presented in Table 11. The amino acid composition in Japanese quail has been studied (GENCHEV, 2012). However, previous studies have demonstrated that amino acid composition in quail eggs may differ in response to their nutritional state (TOLIK *et al.*, 2014). These disparities in amino acid levels reflect variations in food composition in different geographical locations. It has also been shown that females can modulate the deposition of yolk nutrients in the growing and developing follicles (NAVARA *et al.*, 2023).

Table 12. Amino acid (m/m%) content of Japanese quail eggs. Concentrations were calculated from a pool of 12 freshly laid eggs

Amino acid	Whole egg	Egg yolk	Egg white
ASP	1.26	1.43	1.04
THR	0.73	0.82	0.60
SER	0.98	1.23	0.69
GLU	1.82	1.96	1.50
PRO	0.47	0.69	0.38
GLY	0.40	0.44	0.36
ALA	0.59	0.66	0.55
CYS	0.20	0.21	0.28
VAL	0.80	0.90	0.66
MET	0.42	0.38	0.36
ILE	0.65	0.77	0.50
LEU	1.11	1.20	0.85
TYR	0.54	0.65	0.42
PHE	0.68	0.68	0.64
HIS	0.36	0.44	0.27
LYS	1.18	1.20	0.87
ARG	0.58	0.80	0.37

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

Hatchability tended to be lower in the methionine-injected group compared to the control group. Only 31.15% of quail chicks hatched in the methionine-injected group and

51.47% in the control group. The Chi-square analysis indicated no significant difference in hatchability ($\chi^2 = 1.80$, df =1, p = 0.176). The similarity in hatchability indicates that embryonic methionine supplementation did not cause significant detrimental effects on the developing embryos regarding liveability. These results corroborate the findings of the other study that the injection of methionine in the amniotic fluid showed no significant impact on hatchability (COSKUN *et al.*, 2018). Previous findings showed that several factors, such as injection volume, injection site, and amino acid concentration, could affect hatchability (OHTA *et al.*, 2001; ZHAI *et al.*, 2011). For example, *in ovo*, the injection of 50 mg/mL methionine into the amniotic fluid of the chicken eggs reduced the hatchability by about 5% compared to the control group (COŞKUN *et al.*, 2014). Similarly, injection of the 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL methionine in chicken eggs decreased hatchability with increasing concentration compared to the control group, and it caused detrimental effects resulting in toxic effects that induced embryonic mortality (FARIAS *et al.*, 2023).

In contrast, the other study showed that injection of 7.5 mg/mL methionine in geese eggs did not significantly affect hatchability compared to the control group (DANG *et al.*, 2022). It has also been hatchability decreased when the injection volume increased as injecting 0.5 mL of amino acids into the albumen did not affect hatchability in ducks and broiler eggs compared to the control groups (BHANJA *et al.*, 2012; GAAFARBR *et al.*, 2013). Decreasing hatchability may be related to an unproportional increase in osmotic balance between the embryonic natural medium and injected solution due to increased amino acid concentration that increases osmolarity (FARIAS *et al.*, 2023). Under such circumstances, the ability of the embryo to absorb the injected amino acids may be limited due to changes in the physiological environment.

4.3.2 Effects of embryonic methionine body weight changes and morphological traits

The body weight at hatching through day 5 was not different between treatment groups. However, chicks in the L-methionine-injected group grew faster (treatment × age interaction, $p < 0.001$), starting from day 7; their body weight was consistently higher than controls till day 21 (Table 12). Wing, head, and tarsus length did not differ between treatments (p = 0.1148, p = 0.234, p = 0.372, respectively) and increased significantly with the age of chicks (p < 0.001, Table 12).

Table 13. Injecting methionine into the eggs enhanced postnatal body weight gains in Japanese quail chicks

Parameter	Age (day)	Treatment		Estimate	SE	df	t.ratio	p.value
		Control (emmean ±se)	Methionine (emmean ±se)					
Body weight (g)	1	9.124 ± 0.79 (16)	9.406 ± 1.17 (35)	-0.283	1.418	96.672	-0.200	0.842
	3	12.192 ± 1.61 (8)	11.571 ± 1.62 (8)	0.621	2.292	125.690	0.271	0.787
	5	19.729 ± 1.61 (8)	21.484 ± 1.62 (8)	-1.754	2.292	125.690	-0.765	0.446
	7	25.804 ± 1.61 (8)	31.396 ± 1.62 (8)	-5.592	2.292	125.690	-2.439	0.016
	10	39.229 ± 1.61 (8)	45.346 ± 1.62 (8)	-6.117	2.292	125.690	-2.668	0.008
	14	60.042 ± 1.61 (8)	69.484 ± 1.62 (8)	-9.442	2.292	125.690	-4.119	<0.001
	21	104.242 ±1.61 (8)	110.084 ± 1.62 (8)	-5.842	2.292	125.690	-2.548	0.012
Head length (mm)	1	21.151 ± 0.22 (16)	20.612 ± 0.33 (35)	0.539	0.399	80.909	1.350	0.181
	3	22.771 ± 0.44 (8)	22.323 ± 0.45 (8)	0.448	0.625	124.124	0.716	0.475
	5	24.521 ± 0.44 (8)	24.086 ± 0.45 (8)	0.435	0.625	124.124	0.696	0.488
	7	26.596 ± 0.44 (8)	26.573 ± 0.45 (8)	0.023	0.625	124.124	0.037	0.971
	10	29.421 ± 0.43 (8)	29.986 ± 0.45 (8)	-0.565	0.625	124.124	-0.903	0.368
	14	33.734 ± 0.43 (8)	33.823 ± 0.45 (8)	-0.090	0.625	124.124	-0.143	0.886
	21	39.196 ± 0.44 (8)	39.886 ± 0.45 (8)	-0.690	0.625	124.124	-1.103	0.272
Tarsus length (mm)	1	15.668 ± 0.69 (16)	15.919 ± 0.72 (35)	-0.250	0.362	122.525	-0.691	0.491
	3	17.158 ± 0.78 (8)	17.257 ± 0.78 (8)	-0.099	0.599	129.790	-0.165	0.869
	5	21.270 ± 0.78 (8)	21.194 ± 0.78 (8)	0.076	0.599	129.790	0.127	0.899
	7	24.020 ± 0.78 (8)	24.543 ± 0.78 (8)	-0.523	0.599	129.790	-0.872	0.385
	10	27.470 ± 0.78 (8)	28.519 ± 0.78 (8)	-1.049	0.599	129.790	-1.751	0.082
	14	31.258 ± 0.78 (8)	32.069 ± 0.78 (8)	-0.811	0.599	129.790	-1.354	0.178
	21	36.408 ± 0.78 (8)	37.694 ± 0.78 (8)	-1.286	0.599	129.790	-2.147	0.034
Wing length (mm)	1	13.703 ± 0.67 (16)	14.538 ± 0.99 (35)	-0.835	1.202	98.458	-0.694	0.489
	3	16.183 ± 1.37 (8)	15.434 ± 1.38 (8)	0.750	1.948	125.887	0.385	0.701
	5	24.671 ± 1.37 (8)	25.296 ± 1.38 (8)	-0.625	1.948	125.887	-0.321	0.749
	7	35.021 ± 1.37 (8)	32.771 ± 1.38 (8)	2.250	1.948	125.887	1.155	0.250
	10	48.221 ± 1.37 (8)	44.434 ± 1.38 (8)	3.787	1.948	125.887	1.944	0.054
	14	65.346 ± 1.37 (8)	63.684 ± 1.38 (8)	1.662	1.948	125.887	0.853	0.395
	21	86.358 ± 1.37 (8)	84.184 ± 1.38 (8)	2.175	1.948	125.887	1.116	0.266

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

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

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

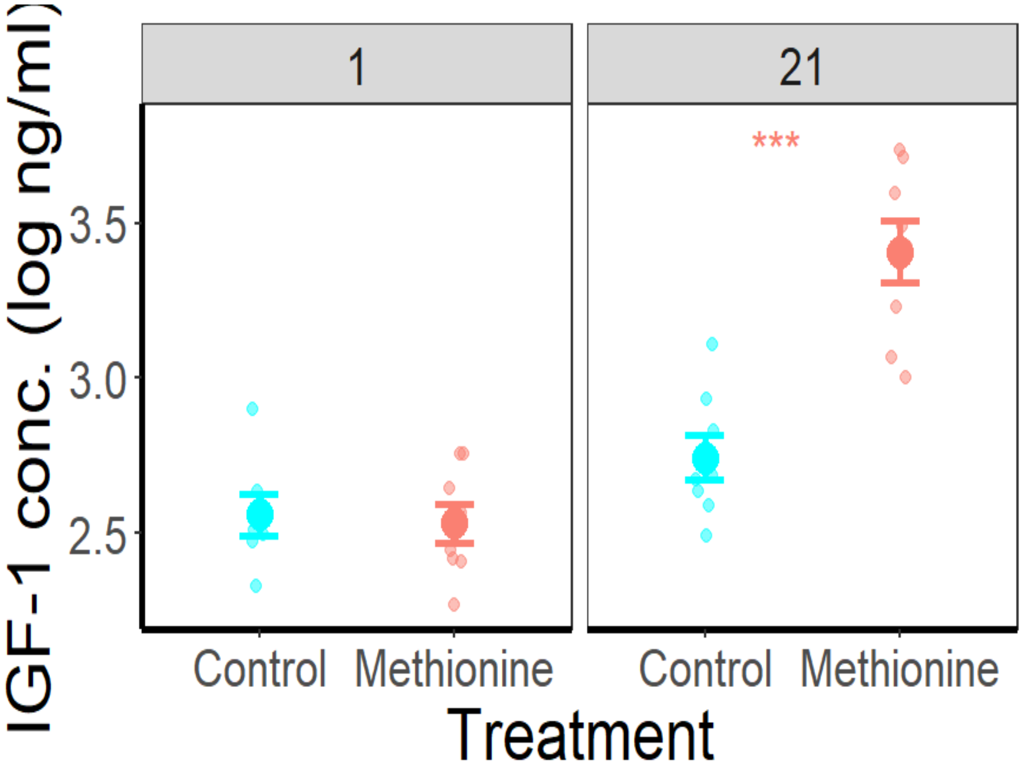

Figure 21. Injecting methionine in eggs increased the postnatal IGF-circulating levels in Japanese quail chicks. The asterisks denote the significant difference between the treatment groups at $p < 0.001$. Numbers at the top of the panels indicate the chick's age in days (1-day-old and 21-day-old chicks).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

Embryonic methionine programming did not increase the body weight of the offspring from day 1 through day 6; some birds had a slight drop in body weight from day 3. The increased body weight at the higher level from the methionine-injected group than the control reflects its role in modulating the offspring's phenotype, observed 7 days post-hatch through 21 days of the experiment. The findings of this study agree that nutrient developmental programming during critical development windows can have short-term and longer-term consequences in the offspring (ANDRIEUX *et al.*, 2022). Maternally deposited nutrients into the egg are vital during embryonic development and are essential in understanding the growth and developmental trajectories of the postnatal offspring. For example, mothers allocate non-genetic resources, including nutrients in the eggs, to control the growth and development of the embryos and subsequent offspring (REED & CLARK, 2011). Maternal egg depositions can impact the offspring's development and phenotypes connected to the offspring’s fitness (PRICE, 1998). In our study, the result of the body weight corroborates the findings of some studies that demonstrated that *in ovo,* nutritional administration of different 50 mg/mL or 2 mg/mL of methionine solutions improved the hatching body weight and postnatal growth in broiler chicken compared to the control group (COŞKUN *et al.*, 2014; COSKUN *et al.*, 2018).

Biometric measurements showed that methionine programming increased only the wing on day-old chicks but did not affect the head and tarsi length from day 3 through day (Table 11). Our result suggests that maternal methionine programming improved post-hatch tarsal length growth and increased body weight; this is attributed to the increased protein synthesis, which might also be necessary during growth (WEN *et al.*, 2014; WILLEMS *et al.*, 2015). Although the Japanese quail in a methionine-treated group showed a gain in body weight, the measured body condition using the scaled weight index remained unchanged and non-significant compared to the control group throughout the experimental period. These results indicate that quail chicks originating from methionine treatment did not achieve a significantly high proportion of body weight gain compared to the control group. Despite low body condition, methionine treatment facilitated the enhancement of skeletal growth and body weight increase with body weight became more quickly evident, revealing the effectiveness of the treatment in promoting growth and development in birds. Although our study found that the treatment did not accelerate growth at hatching, recent studies showed that L-methionine supplementation in one day old broiler chicks promoted faster growth and development (SHEN *et al.*, 2015; AKTER *et al.*, 2020). In contrast, early-age (3-6 days) dietary methionine supplementation in blue tits and magpie nestlings hindered growth (SOLER *et al.*, 2003; BROMMER, 2004). In contrast, supplementation in great tits nestlings from day 9 did not affect their growth rate

(WEGMANN *et al.*, 2015). The small impact of body size increase in nestlings may indicate a causal relationship between IGF-1 and early postnatal development, as IGF-1 levels were significantly higher in pied flycatchers 7 days old compared to those 13 days old (LODJAK *et al.*, 2018).

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

The relative mRNA expression of *IGF1, mTOR, and RPS6K1* was influenced by methionine treatment in quail chicks. On day-old chicks, the relative mRNA expression of *IGF1* significantly increased in a methionine-treated group than in the control group ($p = 0.024$); on reaching day 21, the relative mRNA expression of *IGF1* was equal among the groups ($p = 0.835$, Figure 22A). Further, on day-old chicks, the relative mRNA expression of *mTOR* significantly increased in the methionine-treated group than in the control group ($p = 0.001$), while on reaching day 21, the relative mRNA expression of mTOR disappeared ($p = 0.763$, Figure 22B). However, the relative mRNA expression of *RPS6K1* on day-old chicks did not differ significantly between the methionine-treated and control group ($p = 0.083$), whereas on 21 days old chicks, significantly increased in methionine-treated than in the control group ($p < 0.001$, Figure 22C).

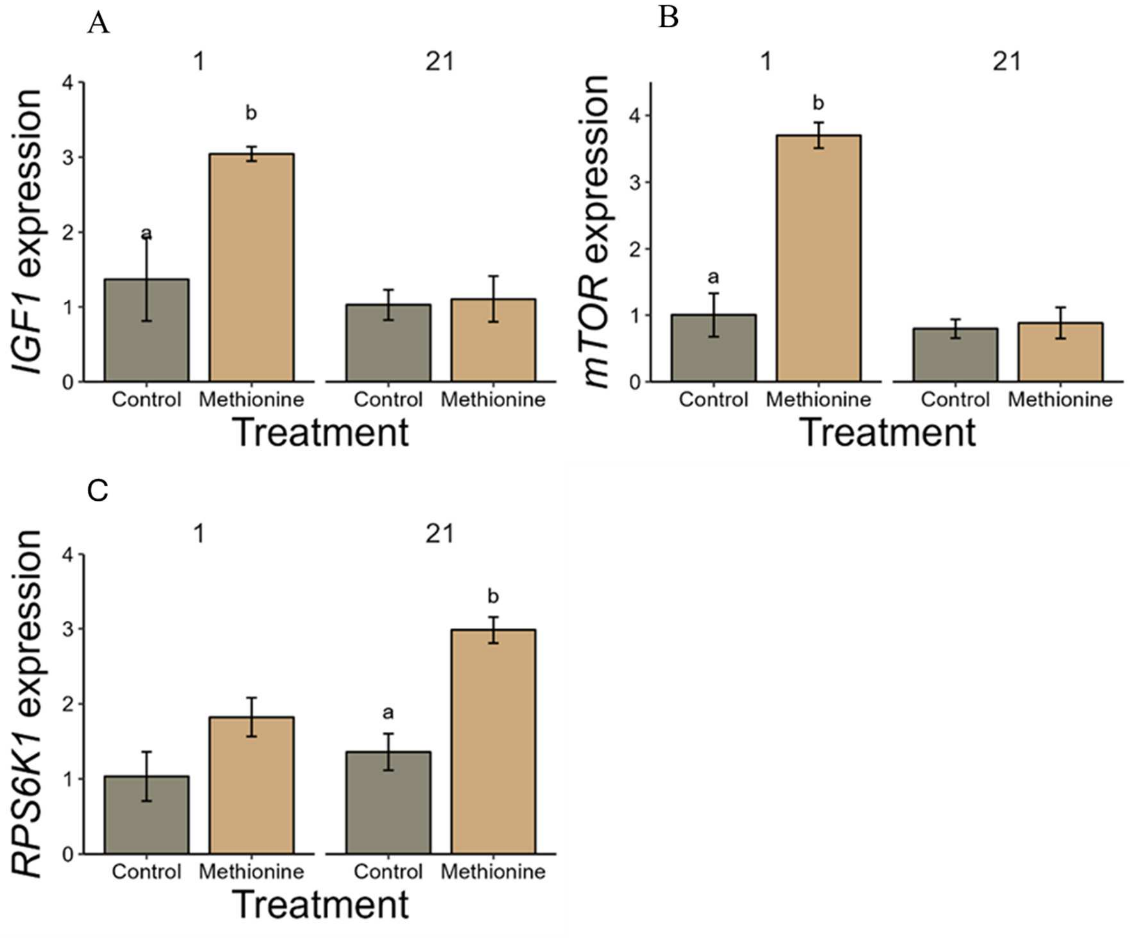

Figure 22. Gene expression patterns in fold change affected methionine in the nutrient pathway. A: relative expression of *Insulin-like growth factor 1 (IGF1),* B: *mechanistic target of rapamycin (mTOR),* C: *ribosomal protein serine 6 kinase 1 (RPS6K1*). The numbers on top of the bars indicate the age of the chicks (1-day-old and 21-day-old chicks). The bars and the error bars indicate the mean and standard error of the mean (mean+SE). Tukey's test was applied to compare adjusted marginal means at $p < 0.05$ significance level. Means followed by distinct letters are significantly different at $p < 0.05$.

After hatching, one-day-old chicks' body weight and morphological measurements did not differ among the treatment groups. However, at this chick age, embryonic methionine administration significantly increased *IGF1* and *mTOR* relative gene expression compared to the control group. Although initially there was an increase in the relative gene expression of *mTOR* and *IGF1*, the circulating levels of IGF-1 hormones were comparable among the treatment groups after hatching. Moreover, body weight and morphological measurements stayed the same up to the age of one week (seven days). Starting from day 7 onwards, Japanese quail chicks injected with embryonic methionine consistently outperformed the control group in terms of body weight increase, a trend that persisted through day 21. However, despite the ability of the embryonic methionine to significantly increase body weight, its effect on the skeletal measurements, including wing and head and lengths, was not manifested, except for tarsus length, which became significantly larger in the methionine injected group 21 days postnatal.

Three weeks later, after hatching, there was a significant restructuring of the physiological and relative gene expression patterns. During this time, quails treated with methionine exhibited a notable increase in the circulating levels of IGF-1 and enhanced the relative gene pattern of *RPS6K1* compared to the control group. Meanwhile, the relative gene expression levels of *mTOR* and *IGF1* no longer show any differences between the treatment groups. The difference between protein and relative gene expression levels suggests that proteins may undergo the post-translational modification regulation. The post-translational modification could be associated with instability of messenger RNA (mRNA) or enhanced protein half-life which often influenced by the post-translational modification that can alter the protein levels (IDEKER *et al.*, 2001; CSERNUS *et al.*, 2023). The increased production of circulating IGF-1 protein after hatching may be facilitated by the upregulated hepatic relative gene expression. However, delays may occur during the translation process from the mRNA to the protein as well as post-translational modification that could affect both the secretion and stability of circulating IGF-1 protein levels (GEDEON & BOKES, 2012). These delays may account for the observed lag in increasing circulating levels of IGF-1 hormone and manifested until 21 days. On the other hand, even if the hepatic *IGF1* relative gene expression has returned to the baseline control levels, other tissues or organs such as the brain, muscles and kidney may still respond to the treatment by synthesizing additional circulating IGF-1 levels to perform the specific function (SJÖGREN *et al.*, 1999; POREBA & DURZYNSKA, 2020). This mechanism could also contribute to the delayed increased in the circulating IGF-1 levels. Notably, chicks of different ages, such as 1-day old and 21-day old, may experience distinct physiological states at different developmental stages. The difference physiological responses can influence the response of the chicks to the treatments and regulation of circulating IGF-1 levels.

Nutritional programming during the critical developmental stages is important and has short and long-term effects on the offspring (JHA *et al.*, 2019; ABDEL-MONEIM *et al.*, 2023). The effect modulated by methionine treatment in this study showed an increased relative gene expression of *mTOR* and *IGF1* on one-day old Japanese quail chicks indicating that these genes are crucial during both embryonic and early postnatal growth and development in birds. The circulating IGF-1 levels and *RPS6K1* gene relative expression showed its greater importance during the late postnatal developmental stage with the noticeable increase observed during the juvenile stage. The results indicate that mTOR is crucial during both embryonic and early postnatal growth and development, while RPS6K1 is essentially important and reorganised during postnatal growth. Additionally, it was observed that an increase in body weight was accompanied by high levels of circulating IGF-1 measured later during the postnatal period, indicating that methionine programming enhances growth and development by promoting IGF-1 synthesis (WEN *et al.*, 2017). The activity of circulating levels of IGF-1 has been observed in birds during developmental stages (LODJAK *et al.*, 2018; LODJAK & VERHULST, 2020). This may elucidate the delayed effects of our methionine treatment on body weight, which only became pronounced one week postnatal. This observation is supported by a lack of significant differences in body weight between methionine and the control group of on-day-one chicks and first-week chicks despite their different developmental stages, which became significantly different after one week. The weak relationship observed in our study among the quail chicks of growth in body weight shows the mere association between the early postnatal growth and circulating IGF-1 levels, which significantly increased in day 7 days older flycatchers than the 13 days old chicks (LODJAK *et al.*, 2018).

The results of this study demonstrate that early maternal investment influences the phenotypic expression of offspring. Mothers regulate embryonic growth and development and their resulting offspring by providing non-genetic resources such as nutrients allocated into eggs (WIDOWSKI *et al.*, 2022). Evidence from studies showed that early maternal investment in eggs can affect the performance of offspring (BENTON *et al.*, 2005; WIDOWSKI *et al.*, 2022). Previous studies demonstrated that the major mechanisms of maternal programming involved the direct transfer of hormones into the eggs (DARRAS, 2019; GROOTHUIS *et al.*, 2019). In this study, it was revealed that methionine nutritional signals can promote postnatal growth and development through specific nutrient-sensing pathways.

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

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

Similar to methionine, treatment with leucine did not significantly affect hatching success compared to the control group ($\chi2 = 0$, df = 1, $p = 0.59$). However, a noteworthy trend indicated an increase in hatching success in the embryonic leucine-supplemented group (65.1%) compared to the control placebo group (50%). Similar results were reported when the blend of branched-chain (BCAA) leucine, valine and isoleucine was injected into the broiler chicken eggs on day 7 or 14, showing no significant difference in hatchability compared to the control group (BHANJA & MANDAL, 2005). Similarly, the study also reported that the injection of BCAA into the amnion, yolk sac, or albumen of broiler chicken eggs did not significantly affect hatchability compared to the control (KOP-BOZBAY & OCAK, 2019a). In contrast, the study focusing on turkey eggs indicated that administration of embryonic blend BCAA in the ratio of 3 leucine: 1 valine: 2 isoleucine on day 24 of incubation adversely affected hatchability (KOP-BOZBAY & OCAK, 2019a). These differences in hatchability outcomes suggest that other factors, including species type, timing, and amino acid concentration, may also be critical in influencing hatchability.

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

We found that one-day-old chicks had no difference in body weight after hatching between the treatment groups. However, we observed a significant interaction between the treatment and the age of the individuals on body weight ($p < 0.001$). On reaching three days old, body weight increased consistently in the leucine-injected compared to the control group on all days until 21 days old (Table 13). We also found the interaction between treatment and age on the head ($p < 0.001$), wing ($p < 001$) and tarsus ($p < 0.001$, Table 13) lengths. While tarsus length did not differ in 1-day old individuals, it increased consistently high starting from 3 days old chicks in leucine injected group (Table 13), with no difference in head length on day and two days old chicks but differed starting five days (Table 13), and wing length differed from 7 days old chicks (Table 13) until 21 days (Table 13). We observed a significant interaction between treatment and age on GIT length ($p < 0.001$). Still, there was no difference between the injected and the control group in the one-day-old ($p = 0.173$); however, the GIT length increased significantly in the leucine-injected than the control in the 21-day-old chicks ($p = 0.021$, Figure 23).

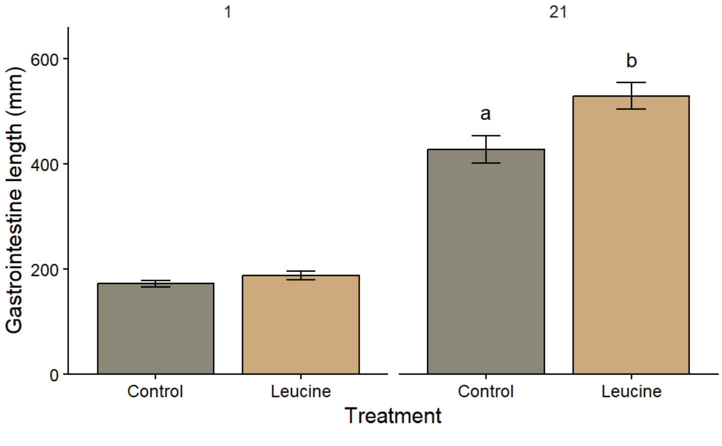

Figure 23. Gastrointestinal length as affected by leucine treatment. The numbers on top of the bars indicate the age of the chicks (1-day-old and 21-day-old chicks). The bars and the error bars indicate the mean and standard error of the mean (mean+SE). Tukey's test was applied to compare adjusted marginal means at $p < 0.05$ significance level. Means followed by distinct letters are significantly different at $p < 0.05$.

Table 14. The effects of embryonic leucine on the growth and development of morphological traits in Japanese quail

Parameter	Age (days)	Treatment		Estimate	SE	df	t.ratio	p.value
		Control (emmean±se)	Leucine (emmean±se)					
Body weight (g)	1	9.212 ± 1.90 (16)	9.885 ± 1.71 (20)	-0.673	2.558	61.563	-0.263	0.794
	3	11.628 ± 2.72 (8)	17.324 ± 2.19 (11)	-5.696	3.498	91.795	-1.628	0.107
	5	16.384 ± 2.89 (8)	25.483 ± 2.38 (9)	-9.099	3.752	98.442	-2.425	0.017
	7	25.800 ± 2.99 (8)	39.532 ± 2.49 (8)	-13.731	3.824	100.189	-3.591	<0.001
	10	30.867 ± 2.89 (8)	50.219 ± 2.49 (8)	-19.352	3.824	100.189	-5.061	<0.001
	14	40.250 ± 2.89 (8)	68.819 ±2.49 (8)	-28.569	3.824	100.189	-7.471	<0.001
	21	63.122 ± 3.10 (8)	93.407 ± 2.49 (8)	-30.285	3.977	103.713	-7.616	<0.001
Head length (mm)	1	20.075 ± 0.42 (16)	19.730 ± 0.38 (20)	0.345	0.573	57.603	0.602	0.549
	3	22.133 ± 0.62 (8)	22.934 ± 0.49 (11)	-0.802	0.774	89.645	-1.036	0.303
	5	23.497 ± 0.64 (8)	25.128 ± 0.53 (9)	-1.631	0.827	96.805	-1.971	0.052
	7	24.197 ± 0.64 (8)	27.387 ± 0.54 (8)	-3.191	0.843	98.676	-3.787	<0.001
	10	27.830 ± 0.64 (8)	30.899 ± 0.55 (8)	-3.070	0.843	98.676	-3.643	<0.001
	14	28.797 ± 0.64 (8)	32.575 ± 0.55 (8)	-3.778	0.843	98.676	-4.484	<0.001
	21	29.847 ± 0.68 (8)	33.725 ± 0.55 (8)	-3.878	0.875	102.425	-4.434	<0.001
Tarsus length (mm)	1	16.175 ± 0.44 (16)	16.015 ± 0.40 (20)	0.160	0.594	58.856	0.269	0.789
	3	17.801 ± 0.63 (8)	19.901 ± 0.51 (11)	-2.100	0.805	90.373	-2.608	0.011
	5	20.737 ± 0.67 (8)	22.398 ± 0.55 (9)	-1.662	0.862	97.372	-1.929	0.057
	7	21.170 ± 0.67 (8)	23.664 ± 0.57 (8)	-2.494	0.878	99.204	-2.841	0.005
	10	24.870 ± 0.67 (8)	28.176 ± 0.57 (8)	-3.306	0.878	99.204	-3.766	<0.001
	14	26.703 ± 0.67 (8)	31.001 ± 0.57 (8)	-4.298	0.878	99.204	-4.896	<0.001
	21	28.863 ± 0.71 (8)	32.976 ± 0.57 (8)	-4.113	0.912	102.880	-4.511	<0.001
Wing length (mm)	1	15.250 ± 1.35 (16)	15.320 ± 1.21 (20)	-0.070	1.811	86.453	-0.039	0.969
	3	17.128 ± 2.02 (8)	17.795 ± 1.61 (11)	-0.667	2.584	101.051	-0.258	0.797
	5	22.546 ± 2.17 (8)	25.702 ± 1.77 (9)	-3.156	2.803	104.398	-1.126	0.263
	7	25.346 ± 2.17 (8)	31.806 ± 1.87 (8)	-6.460	2.867	105.365	-2.253	0.026
	10	38.879 ± 2.17 (8)	50.531 ± 1.87 (8)	-11.652	2.867	105.365	-4.064	<0.001
	14	46.229 ± 2.17 (8)	61.256 ± 1.87 (8)	-15.027	2.867	105.365	-5.241	<0.001
	21	57.485 ± 2.35 (8)	70.244 ± 1.87 (8)	-12.759	3.007	107.542	-4.244	<0.001

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

The results of this study indicated that one day after hatching, body weight, gastrointestinal tract length, and length of wings, tarsi and head were not significantly different among the treatment groups in Japanese quail chicks. During the postnatal period, feed intake can significantly influence the growth and development of birds (WANG *et al.*, 2018; WANG *et al.*, 2024). However, the study did not measure the postnatal feed intake. Japanese quail chicks in a leucine-injected group showed significant fast growth; reaching day 7, they were heavier than the control group. Similarly, some morphological features in the leucine injected group showed the positive effects of leucine injection early, whereby tarsal length increased by day 3 and body weight increased by day 5, but the head length and tarsal length only reached a significant difference from the control group on day 7. This study used a 2.0% increase in leucine in Japanese quail eggs. Similarly, the study revealed that embryonic feeding of 0.1% of BCAA blend, which includes leucine prior to the incubation, improved only the embryonic growth, showing no difference in the body weight of the newly hatched broiler chicken (KITA *et al.* 2015). On the contrary, the embryonic feeding with a 0.2% BCAA blend in turkey eggs on day 22 of the incubation period resulted in a decrease in embryo weight (KOP-BOZBAY & OCAK, 2019b). Nevertheless, the body weight increase of the freshly hatched turkey poults resulted from a 0.2% BCAA bled embryonic feeding accompanied by the growth and development of the skeletal muscles (KOP-BOZBAY & OCAK, 2019b). Correspondingly, the study by Bhanja and others indicated that embryonic supplementation of BCAA in broiler chicken eggs on day 7 or 14 did not have a significant effect on body weight at hatching but improved postnatal body weight gain (BHANJA & MANDAL, 2005). These differences in the growth of the offspring following embryonic amino acid feed result from the difference in species, as well as the time or amount of amino acids supplemented into the egg. Despite the difference in other studies, the overall findings of this study are consistent with our recent study (NDUNGURU *et al.*, 2024a), which showed that embryonic feeding of amino acids improved the overall postnatal growth performance in Japanese quail.

Gastrointestinal growth and development are crucial for effective nutrient absorption to ensure that chicks meet their nutritional demands. In chickens, it has been observed that a significant increase in intestinal length occurs between the ages of four to eight days postnatal (RAVINDRAN & ABDOLLAHI, 2021). At this time, the chicken body weight increased exponentially more than six times compared to the hatching body weight (RAVINDRAN & ABDOLLAHI, 2021). This study clearly showed that embryonic leucine positively enhanced gastrointestinal length measured on day 21. It plays a vital role in maintaining the intestinal health. Embryonic leucine influences the innate and adaptive immune system and maintains the integrity of the intestinal mucosa for optimal nutrient absorption critical for growth and development in chicks (CHANG *et al.*, 2015; KIM *et al.*, 2022). A previous study showed that dietary leucine enhanced intestinal development by activating the mTOR signalling pathways in broiler chickens (CHANG *et al.*, 2015).

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

We found no significant interaction between treatment and age of chicks on *IGF1* gene expression (p = 0.702). However, we observed a significant difference in *IGF1* gene expression between the groups of 1-day-old chicks (p = 0.023) and 21-day-old chicks (p = 0.042). The *IGF1* gene expression increased significantly in the leucine-injected group compared to the control group (Figure 24A). We found a marginally significant interaction between treatment and the age of chicks in *IGF1R* gene expression (p = 0.071). We also found a significant difference in *IGF1R* gene expression in day-one-old chicks between the two groups (p = 0.026). *IGF1R* gene expression was significantly higher in the leucine-injected group than in the control group (Figure 24B). There was no significant difference in *IGF1R* gene expression between the groups in 21-day-old chicks (p = 0.435). We found a significant interaction between treatment and age on *mTOR* gene expression (p = 0.021). In 1-day-old chicks, we observed a significant difference in *mTOR* gene expression between the two groups (p = 0.009; control: n = 8, leucine: n = 8). The mTOR gene expression increased significantly in leucine injected group than in the control group (Figure 24C). We observed no difference in *mTOR* gene expression between the two groups in 21-day-old chicks (p = 0.579). We found no interaction effects between treatment and age on *RPS6K1* gene expression (p = 0.251). We observed the difference in *RPS6K1* gene expression in day-old chicks between the two groups (p = 0.022). The *RPS6K1* increased markedly in one-day-old chicks in the leucine injected group compared to the control group (Figure 24D). The maximum *RPS6K1* gene expression was not different between the two groups in 21-day-old chicks (p = 0.977).

We detected no significant interaction between the treatment and age on *FOXO1* gene expression (p = 0.665). Similarly, neither 1-day-old chicks (p = 0.702) nor 21-day-old chicks (p = 0.432) had a significant difference in *FOXO1* gene expression (Figure 24E).

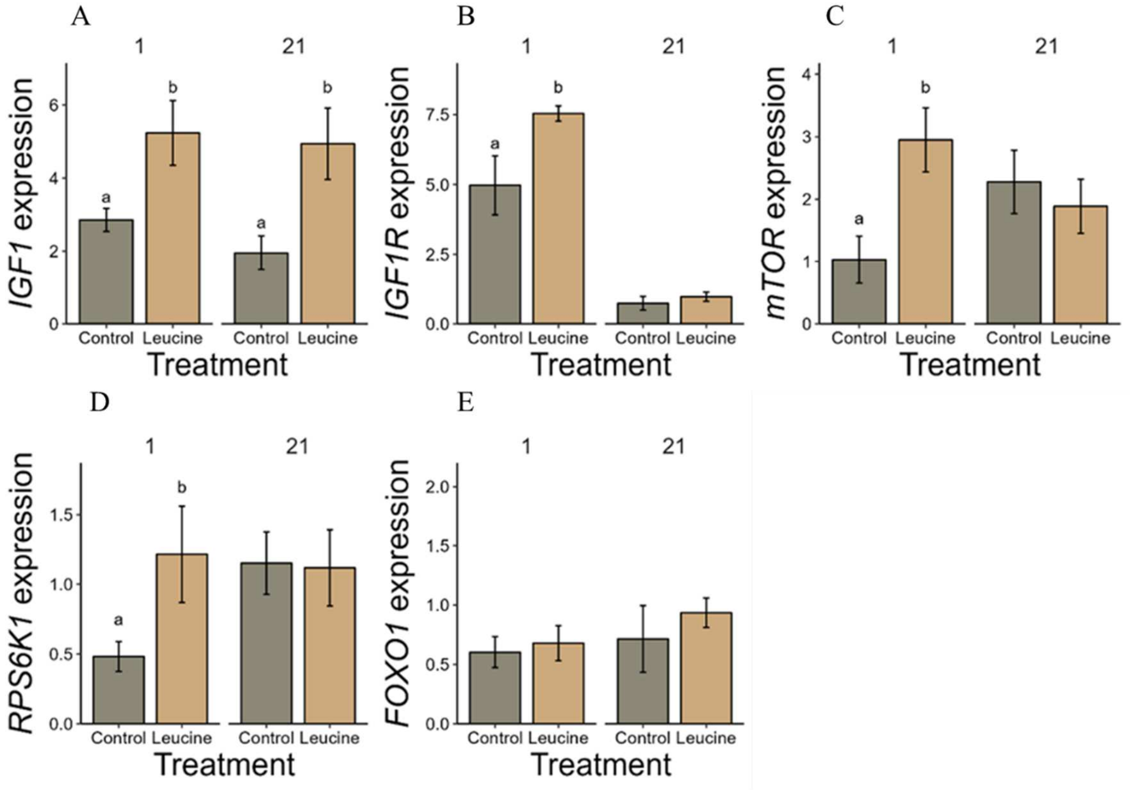

Figure 24. Gene expression patterns in fold change affected leucine treatment in the nutrient-sensing pathway.A: Insulin-like growth factor 1 (*IGF1*), B: Insulin-like growth factor 1 receptor (*IGF1R*), C: mechanistic target of rapamycin (*mTOR*), D: ribosomal protein serine 6 kinase 1 (*RPS6K1*) and E: Forkhead box protein O1 (*FOXO1*). The numbers on top of the bars indicate the age of the chicks (1-day-old and 21-day-old chicks). The bars and the error bars indicate the mean and standard error of the mean (Mean+SE). Tukey's test was applied to compare adjusted marginal means at $p < 0.05$ significance level. Means followed by distinct letters are significantly different at $p < 0.05$.

This study showed that leucine treatment altered the hepatic gene expression patterns related to the nutrient-sensing IGF-1/mTOR pathway. The gene expression changes in this pathway were detected at the hatching stage. It is exciting because the phenotypic differences in growth were not evident until days 3, 5, or 7 for tarsus length, body weight, and wing and head length, respectively. Consequently, the upregulation of relevant genes transpired several days prior to the manifestation of the growth. The findings of this study align with our recent study, where we demonstrated that a 2%

increase in embryonic methionine also had a significant effect on the developmental route of the Japanese quails (NDUNGURU *et al.*, 2024a). Notably, it was observed that the impact of embryonic leucine appears to be more pronounced than those of methionine, with phenotypic differences noticed earlier and affecting most of the measured skeletal traits. Additionally, the *IGF1* relative gene expression persisted until day 21, whereas in the methionine treatment experiment, the *IGF1* relative gene expression had already become similar to that of the control group by day 21 (NDUNGURU *et al.*, 2024a). Despite the differences in the type of amino acid used in the two experiments, it is essential to note that leucine was supplemented at a later embryonic developmental stage (day 10) compared to methionine, which was supplemented before beginning the incubation (day 0). In this case, future studies are necessary to determine whether the later stage of embryonic development contributes to the more substantial growth-promoting effect that was observed with leucine or whether leucine itself is more effective in activating the IGF-1/mTOR pathway, leading to greater phenotypic expression.

The relative expression of *mTOR* and the key downstream effector, ribosomal protein S6 kinase 1 (*RPS6K1*), significantly increased at hatching due in the leucine treatment group compared to the control coinciding with *IGF1* and *IGF1R* expression. In animals, mTOR is crucial in regulating protein synthesis, cell differentiation, proliferation, and overall growth in response to nutrients such as leucine and several growth factors (WANG & PROUD, 2006; SAXTON & SABATINI, 2017; REHMAN *et al.*, 2023). Under nutrient availability such as leucine, mTOR complex 1 (mTORC1) is activated, which subsequently activates phosphorylates of the vital downstream effector, including the RPS6K1 proteins (CRUZ *et al.*, 2019; REHMAN *et al.*, 2023). Contrary to *IGF1,* the relative expression of *IGF1R*, mTOR, and RPS6K1 in a leucine-injected group returned to the baseline control levels on day 21. To some extent, this pattern mirrors the results observed from embryonic methionine, where an initial increase was observed in relative gene expression of mTOR but faded by the study (21 days) in Japanese quail (NDUNGURU *et al.*, 2024a). However, this study reveals a striking difference, where

RPS6K1 relative gene expression was solely upregulated on day 21 following embryonic methionine supplementation (NDUNGURU *et al.*, 2024a); in contrast, it is only significantly expressed at hatching in the case of embryonic leucine supplementation. *RPS6K1* serves as an essential downstream effector of the mTOR pathway, and its activation requires the mTOR-mediated phosphorylation crucial for promoting protein synthesis, cell size enhancement, and growth facilitation (UM *et al.*, 2006; MA & BLENIS, 2009; MAGNUSON *et al.*, 2012). The contrasting pattern of *RPS6K1* relative expression between methionine and leucine indicates that specific amino acids have unique and significant roles in shaping postnatal growth and development in Japanese quail. The results of this study corroborate the previous research that demonstrated the profound effect of dietary leucine supplementation in influencing post-hatch growth in chicken broilers (DENG *et al.*, 2014) and zebrafish (*Danio rerio*) (ZHU *et al.*, 2020), which boosted *mTOR* and *RPS6K1* relative expression in breast muscles with increasing age. However, a contrary result was observed where dietary leucine supplementation in broilers across the developmental stages (1 to 10 days, 11 to 21 days, and 22 to 35 days) failed to influence the relative expression of *mTO*R and *RPS6K1* nor did it enhance the postnatal growth performance (ZEITZ *et al.*, 2019). These results suggest that embryonic leucine may offer more substantial and lasting growth benefits than postnatal supplementation, highlighting the potential nutritional strategies for optimizing growth and development in birds. Additionally, the results suggest the regulatory mechanisms that reinstate homeostasis following the IGF-1/mTOR signalling pathway alteration.

The balance between protein synthesis and degradation is essential for animal growth and development. The protein synthesis is regulated by the protein kinase B (Akt)/mTOR pathway, while protein degradation is influenced by the Akt/FOXO1 pathway (DENG *et al.*, 2014; ZEITZ *et al.*, 2019). FOXO1, a transcription factor downstream of Akt, is essential in regulating cell survival, growth and development (ZHOU *et al.*, 2012; FURTADO *et al.*, 2021). During nutrient scarcity, FOXO1 initiates autophagy and proteasomal gene transcription, leading to the breakdown of muscle protein for glucose production (gluconeogenesis) (HAEUSLER *et al.*, 2010; LANGLET *et al.*, 2017; TESSERAUD *et al.*, 2021). However, excessive autophagy and protein degradation can be detrimental, causing cell death and reduced stunted growth (FURTADO *et al.*, 2021; TESSERAUD *et al.*, 2021). This study found no significant difference in the relative gene expression of the *FOXO1* between the embryonic leucine-supplemented and control quail chicks at the age of 1 day and 21 days. It has been demonstrated in mammals that increasing the relative expression of *FOXO1* can reduce the accumulation of toxic proteins (FURTADO *et al.*, 2021), suggesting the positive effects of protein metabolism and may influence growth in poultry. A study in broiler chickens revealed that thermal manipulation during embryonic development increased the *FOXO1* relative expression and promoted growth (AMAZ *et al.*, 2024). In the other study, dietary supplementation of leucine in broiler chicken at various growth stages (1 to 10 days, 11 to 21 days, and 22 to 35 days) did not influence *FOXO1* relative gene expression (ZEITZ *et al.*, 2019). Similarly, high *FOXO1* gene transcript levels were reported in broilers compared to layers, indicating the crucial role of FOXO genes, including *FOXO1* gene expression, in regulating growth and metabolism in poultry (WILLSON *et al.*, 2018). Despite the conflicting findings from different studies, the evidence suggests that the *FOXO1* gene plays an essential role in modulating growth in poultry.

Supplementing embryos with a subtle dose of leucine promotes the activation of the crucial components of the nutrient-sensing insulin-insulin signalling (IIS) and the mammalian target of the rapamycin (mTOR) pathway, resulting in marked improvement of the postnatal growth in Japanese quail. Conducting comparative studies across the avian species and developmental stages can help gain insights into how specific nutrients fundamentally influence growth and development. Additionally, exploring the synergistic effects of leucine during the embryonic stage in conjunction with essential amino acids may help uncover the significant interaction that influences growth and development postnatal. The findings of this study underscore the importance of nutritional cues in forming early maternal investment and opening new avenues for further enhancing poultry productivity on a large scale.

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

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

The rapamycin treatment did not significantly affect body weight throughout all days nor at the end of the experiment (Table 14). There was no significant effect on body weight gain (Table 14). However, we observed the sex differences in spleen weight where female quails grew faster and increased body weight compared to males. Relative spleen weight was significantly higher in females than in male birds, and the rapamycin treatment resulted in significantly lower spleen values than the control group (Table 15).

Table 15. Body weights of the quails on injection days

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

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

Table 16. Effect of rapamycin on growth and relative spleen in Japanese quail

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

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

Rapamycin is a potent specific mTOR pathway inhibitor clinically approved in numerous studies (TSANG *et al.*, 2007; CHEN *et al.*, 2016). Studies have suggested that rapamycin treatment in mammals has induced a negative effect on inhibiting appetite, which in turn reduces feed intake (SCARPACE *et al.*, 2016). Reducing food intake permits the utilisation of stored body reserves, such as fats, to produce energy for other metabolic activities (ROSS *et al.*, 2015). Reduced feed intake and extra burning of the energy stored due to rapamycin treatment have reduced animal body weight in mammals (ROSS *et al.*, 2015; SCARPACE *et al.*, 2016). In this experiment, quails treated with rapamycin were expected to decrease their body weight and weight gain. However, despite females being heavier and growing faster than male quails, rapamycin treatment did not influence body weight and body weight gain compared to the control group.

Contrary to the result of this experiment, rapamycin treatment in the same concentration used in this study (1 mg/kg of body weight) for 6 days decreased the body weight of broiler chickens (LIU *et al.*, 2016). Furthermore, rapamycin treatment at a concentration of 2 mg/kg for 3 weeks or 5 weeks also resulted in lower body weights in rats (DEBLON *et al.*, 2012; SCARPACE *et al.*, 2016). Additionally, rapamycin treatment significantly increased body weight loss in the mice aged 25 months more than 3 months compared to their control groups (SCARPACE *et al.*, 2016). In humans, significant body weight loss was observed in females treated with rapamycin compared to females (MANNAA *et al.*, 2023). These reductions are generally due to the loss of appetite and alternative energy utilisation from the deposited fat weight. These contradictory results may be related to individual differences such as age, sex, time of exposure, and genetic variability.

Among the immune effector organs is the spleen, which is involved in adaptive immune responses such as the production of cytokines, cell differentiation, pathogen clearance, lymphocyte production and balancing the cellular inflammatory responses (MOTA & MADDEN, 2022). Accordingly, the reduction of spleen weight is used as an indirect measure of immunosuppression (VINCZE *et al.*, 2022). In this experiment, a smaller spleen weight was observed in rapamycin-treated quails than in control birds. The spleen weight was reduced when a rapamycin analogue was applied at a concentration of

2.5 mg/kg/day for 14 days, indicating the immunosuppression effect likely to reduce immune activity and immune signalling pathway (SIMLER *et al.*, 2002). However, because body weight remained unaltered, this suggests that the energy resources saved from the reduced immune investment were not further invested or redirected toward increasing body size. At this stage, it is possible that birds were already at their physiological weight limit under the experimental conditions, where the energy balance remained stable. In addition, some studies have indicated proportionally smaller spleens in male quails compared to females, which is consistent with previous findings in birds, as a more pronounced decrease in male bird species compared to females, suggesting sex-specific effects in immune regulation (MØLLER *et al.*, 1998; VINCZE *et al.*, 2022).

4.5.2. Effects of rapamycin treatment on relative gene expression

The rapamycin treatment did not significantly affect *mTOR* expression compared to the control birds ($p = 0.401$); none of the sex effects ($p = 0.129$) or interaction ($p = 0.903$, Table 16). Rapamycin treatment significantly decreased *IGF1* in male quails compared to the expression of male control birds ($p = 0.038$; Table 16), with an interaction effect between treatment and sex ($p = 0.036$). Interleukin-1β (IL-*1β*) expression did not significantly differ between the treatment groups ($F_{1,26} = 3.128$; $p = 0.088$), and neither sex effect was observed ($p = 0.922$) nor interaction ($p = 0.752$, Table 16). *MyD88* expression was significantly higher in rapamycin-injected males compared to the control male group ($p < 0.011$) with an interaction effect ($p = 0.0005$; Table 16). However, *NF-κβ* expression did not significantly differ between rapamycin-treated and control group ($p = 0.851$), and neither sex effect was observed ($p = 0.149$) nor interaction (Table 16). Rapamycin did not significantly affect *STAT3* expression ($p = 0.377$), and neither sex effects ($p = 0.239$) nor interaction effects (Table 16).

Table 17. The relative gene expression in Japanese quails treated with 1 mg/kg body weight rapamycin.

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

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

Rapamycin is recognised for its ability to reduce the levels of inflammatory markers by inhibiting the mTOR pathway as well as the upstream TLR-4/MyD88/MAPK pathways and the downstream NF-κβ pathways (ZHOU *et al.*, 2018b). MyD88 primarily functions as an adaptor protein that relays signals from TLR-4, which then activates other complexes, leading to mTOR phosphorylation and activation of transcription factors like NF-κB (ZHOU *et al.*, 2018a). Unexpectedly, the *MyD88* expression was higher in male quails treated with rapamycin than in the control males; a notable difference was observed between male and female birds. Additionally, the gene expression level of the *NF-κβ* did not change in the rapamycin-treated birds compared to the control. Supporting these findings, the increased gene expression of *MyD88* was also noted in rapamycin-treated rats in comparison to the control rats (LI *et al.*, 2019).

This study found that rapamycin treatment did not alter the expression of *STAT3* as a downstream mTOR effector, and phosphorylation of both molecules indicates the activation of the mTOR-STAT3 pathway (HOU *et al.*, 2017b). It has been reported that rapamycin treatment decreases the p-mTOR/mTOR and p-STAT3/STAT3 proteins in comparison to the experimental autoimmune encephalomyelitis-immunised mice treated with 1 mg/kg for 21 to 30 days following immunological challenge (HOU *et al.*, 2017b). The results imply that rapamycin suppresses the mTOR/STAT3 pathway.

In some cases, rapamycin can promote the activation of the immune system through the expression of MyD88, which can potentially increase energy expenditure for immune investment and reduce energy resources for body size increase (HU *et al.*, 2024). However, the lack of expression changes in *NF-κβ* and *STAT3* may reflect species-specific immune regulation as it differs across different taxa (DIMITROV & WHITE, 2016) and context-dependent effects of rapamycin depending on the external or environmental stimuli (POWELL *et al.*, 2012). Similarly, under normal conditions, immunomodulatory responses can induce an immune response while suppressing excessive inflammatory stress, resulting in trade-offs between body size and immune defence due to dysregulation of the mTOR pathway (LI *et al.*, 2024). Therefore, rapamycin's action on the immune signalling pathway could also depend on a particular species' baseline immune status.

The study also examined the effects of rapamycin treatment in Japanese quail on expression of *mTOR* and *IGF1* genes. The results showed that rapamycin treatment did not significantly affect the expression or the *mTO*R gene. Collaborating to our finding, in immature mice (23-25-day-old), a single intraperitoneal dose (10 mg/g of body weight) rapamycin administration failed to alter the expression of mTOR transcript (SIDDAPPA *et al.*, 2014). Similarly, in chicken the proportion of mTOR phosphorylation in jejunal mucosa was inhibited by rapamycin treatment at the concentrations of 0.4 and 1 mg/kg/day for 6 days), highlighting its regulatory role in promoting intestinal mucosa (LIU *et al.*, 2016). mTOR if the downstream of IGF-1 in the nutrient pathway plays a crucial role in energy metabolism, protein synthesis and cellular growth (LODJAK & VERHULST, 2020; NDUNGURU *et al.*, 2024b). In contrast to mTOR, the study found that lower *IGF1* expression in male quails in response to rapamycin treatment compared to the control males. Similar to the findings of this study, rapamycin administration of 1.5 mg/kg of body weight for 2 weeks daily resulted into reduced muscle *IGF1* gene expression in fine flounders (*Paralichthys adspersus*) during the refeeding (FUENTES *et* *al.*, 2015). Furthermore, the *IGF1* expression revealed sex specific effects with lower expression levels observed in males than in females. Our recent study in Japanese quail also demonstrated the sex specific effect whereby *IGF1* gene expression (REDA *et al.*, 2024b) and circulating IGF-1 levels were consistently lower in males across different dietary treatment conditions (REDA *et al.*, 2024b). Genetic variation and sex hormones can majorly contribute to the sex differences in IGF-1 expression (JØRGENSEN *et al.*, 2005; LIU *et al.*, 2016).

4.5.3. Effects of rapamycin treatment on intestinal morphology

The crypt depth was higher in rapamycin-injected male quails than in control male birds (p = 0.001), but females showed overall higher crypt depths than male birds (p = 0.004, Figure 26A). Neither the rapamycin treatment (p =0.298), sex ($F_{1,181}$ = 1.471; p = 0.226), nor their interaction (p = 0.248) affected ileal villus height (Figure 26B). The rapamycin injection decreased the VH: CD ratio (p = 0.002) in treated males compared to the control male birds, and we also observed a higher VH: CD ratio in male birds compared to females (p = 0.026, Figure 26C). Total mucosa was thicker in females (p = 0.016, Figure 26D), but the rapamycin treatment did not alter the mucosa thickness compared to the control group.

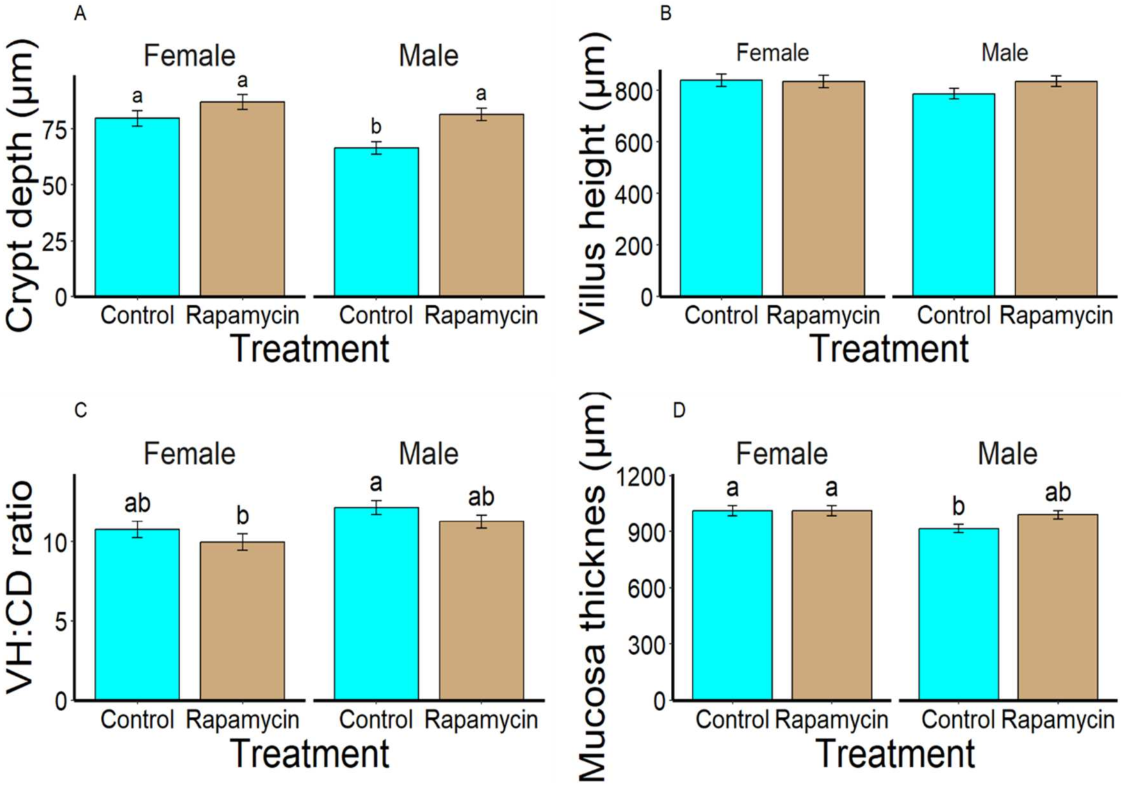

Figure 25. Intestinal histomorphology traits in the Japanese quail (Coturnix japonica) treated with 1 mg/kg body weight rapamycin. Error bars represent means ± standard errors of the mean (n = 5 birds of each sex per treatment). A: crypt depth (µm), B: villus height (µm), D: VH: CD ratio and D: mucosa thickness (µm). Groups with distinct letters are significantly different from each other at $p > 0.05$.

The mucosal morphology is crucial in maintaining intestinal homeostasis but less is known about this relation with mTOR complexes. As we hypothesized, rapamycin can induce changes in the gut histomorphology by mTOR inhibition. Intestinal alterations can refer to digestive functions, as higher villi heights and lower crypt depths can provide a more excellent surface for digestion and absorption of nutrients (MUNYAKA *et al.*, 2012). A VH: CD ratio increase further refers to improved intestinal development (SONG *et al.*, 2019). In this study, rapamycin did not alter the villus height. Rapamycin-treated male birds showed higher crypt depth than the control males, but the males had lower crypt depth than female birds. In addition, rapamycin decreased the ileal VH: CD ratio in male quails compared to the control male birds, but VH: CD ratio was lower in the female groups. Total mucosa thickness did not change between the treatments, but female birds provided thicker mucosa than male quails. Rapamycin resulted in a decreased villus height and VH: CD ratio in the duodenum and jejunum in young male broiler chickens when rapamycin was applied at 0.4 and 1 mg/kg BW through 6 days (LIU *et al.*, 2016). These results indicate the critical role of the mTOR (especially TORC1 of the mTOR multicomplex) pathway in protection against intestinal atrophy (LIU *et al.*, 2016).

5. CONCLUSIONS

The results showed that amino acid supplementation of 20% leucine (14.2 g/kg) or 20% methionine (4.5 g/kg) of the basal diet individually or in combination with feed restriction alleviated the effects of 20% feed quantitative feed restriction and increased body weight, ovary weight, ovary index, hierarchical follicle number and plasma total antioxidant capacity. The study highlights the importance of specific nutrient composition, particularly essential amino acids, in reversing the adverse effects of feed restriction on growth, reproduction, and oxidative stress in birds. Improvement of these parameters is essential for reproductive performance and increased productivity. Investigating the role of these specific amino acids across the avian species is essential to reveal their potential benefits.

Duration, amount, and timing of feed restriction can account for the quality of the laid eggs. Unpredictable feed access did not affect the yolk, albumin or eggshell weights. It maintained all the egg properties of egg components, highlighting its importance in maintaining the egg quality and improved reproductive capability compared to 20%, 30%, and 40% feed restrictions. The yolk-to-albumin ratio was high in 40% of feed restrictions. However, unpredictable feed access did maintain all the egg component proportions, and the yolk-to-albumin ratio remained unaffected. The unpredictable feed access preserved the proportions of the egg components, indicating that this approach may effectively improve egg quality traits and overall productivity in the poultry industry.

The embryonic feeding of 1 mg of methionine stimulated the nutritional transfer, which activated the nutrient-sensing pathway (ISS/mTOR) and influenced past-natal growth. Embryonic methionine supplementation increased the expression of hepatic IGF1 and mTOR genes at hatching. The expression of the mTOR downstream effect RPS6K1 and circulating IGF-1 hormone was observed later during postnatal growth, which was also accompanied by increased body weight. These results show that specific nutritional cues may have phenotypic programming effects by sequentially activating specific nutrient-sensing pathways and achieving transgenerational phenotypic plasticity.

Similarly, the embryonic feeding of 2.5 mg of leucine increased the expression of hepatic *IGF1*, *IGF1R, mTOR*, and *RPS6K1* genes. The increased expression of these genes was accompanied by increasing post-natal growth. How embryonic leucine feeding did not influence the expression of the hepatic *FOXO1* gene. The results outline the role of specific nutrients, such as essential amino acids, in enhancing postnatal growth by stimulating the nutrient-sensing pathway. Specific nutrient intervention during the embryonic period can improve postnatal growth performance in the poultry industry. Further investigations should focus on the balance of the essential and non-essential amino acids on early embryonic development in birds.

The rapamycin treatment did not significantly affect body weight. However, female quails grew faster and increased body and relative spleen weight more than males, and the rapamycin treatment resulted in significantly lower spleen weight values. Low spleen weight implies more stable immune cells to produce cytokines. Rapamycin treatment also exerted sex-specific effects: it decreased the hepatic expression of *IGF1* while increasing *MyD88* genes in male compared to female birds. The results suggest that rapamycin has the potential for immune system activation through the MyD88/MAP pathway and is responsible for pro-inflammatory cytokines and increasing inflammation. Reducing the spleen weight suppresses the activation of T-cells and B-cells, which enhances the memory for strong and long-lasting immune response. Further, studies should investigate age-dependent rapamycin response on growth and immune regulation across avian species.

6. NEW SCIENTIFIC RESULTS

1. Two-week-long (from 6 to 8 weeks of age) restricted feeding (80%) of diets supplemented with 20% (4.2 g/kg) leucine or with 20% (0.9 g/kg) methionine above the recommended level (21.2 g/kg leucine and 4.5 g/kg methionine) or the combined treatment significantly increased final body weight of female Japanese quails compared to the restricted feeding (80%) of diets supplemented with the required amount of leucine and methionine. Furthermore, the +20% methionine treatment increased the number of hierarchical follicles, while the +20% leucine treatment increased ovary mass, and the combined treatment of the two amino acids increased both parameters compared to the restricted feeding (80%) of diets supplemented with the required amount of leucine and methionine.
2. The 20% continuous feed restriction of quails for 14 days did not significantly affect egg yolk, albumen, and eggshell weights compared to the ad libitum-fed control group. In contrast, the 30% and 40% continuous feed restrictions resulted in significant reductions of albumen and eggshell weight compared to the control group. The unpredictable daily feed supply ranging from 30% to 170% of the daily feed intake of quails for 16 days did not significantly affect yolk weight, albumen weight, eggshell weight or their proportions.
3. In comparison with the control group, the *in ovo* feeding of 1 mg (≈ 2%) methionine above the average methionine concentration of eggs (48.72 mg/egg) on E0 into the egg yolk significantly increased postnatal body weight of Japanese quails from day 7 to day 21 as well as the gene expression of hepatic IGF1 and mTOR on day 0, RPS6K1 on day 21, and plasma IGF1 concentration on day 21.
4. In comparison with the control group, the *in ovo* feeding of 2.5 mg (≈ 2%) leucine above the average leucine concentration of eggs (127.6 mg/egg) on E10 into the allantoic fluid significantly increased postnatal body weight of Japanese quails from day 5 to day 21 as well as the gene expression of hepatic IGF1 on day 1 and 21, mTOR and RPS6K1 on day 1.
5. Subcutaneous treatment of 1 mg/kg body weight of rapamycin every second day for two weeks (7 times, once daily at 10:00 hours) resulted in significantly lower relative spleen weight of male and female, and in higher crypt depth of male Japanese quails but did not influence the body weight of the experimental animals.
6. Subcutaneous treatment of 1 mg/kg body weight of rapamycin every second day for two weeks (7 times, once daily at 10:00 hours) significantly decreased the splenic gene expression of IGF1 while increased the expression of MyD88 in male Japanese quails, and did not affect the gene expression of mTOR, NF-κβ, IL-1β and STAT3 compared to the control birds.

7. PRACTICAL RESULTS

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

8. SUMMARY

Nutrition is crucial in avian growth and development, particularly during embryogenesis and sexual maturation. Birds require nutrient resources both in quantity and quality to suit their physiological needs (ALLEN & ULLREY, 2004; JARMAN *et al.*, 2024). Adequate nutrition supports overall growth, enabling birds to attain adult size and sustain their reproductive roles (RUFFINO *et al.*, 2014; MILENKAYA *et al.*, 2015). Females allocate non-genetic nutrients into the egg components, such as albumen, yolk, and shell, that are necessary for the development of the embryos (WILLIAMS & GROOTHUIS, 2015). However, the deposition of maternal nutrients into the egg depends on various mothers' quality, mothers' breeding sites (MOUSSEAU, 1998; DAROLOVÁ *et al.*, 2014), and social environments such as stocking density in poultry (GENG *et al.*, 2020). Proper maternal resources deposition into the egg enhances proper embryonic and post-natal growth and development (GROOTHUIS *et al.*, 2005; GROOTHUIS *et al.*, 2019). The growth and development of avian embryos are dependent on female-deposited resources. Albumen and yolk are the only sources of nutrients for the developing embryo. Most part of albumen is largely depleted three or four days before hatching, making yolk materials the primary nutrient source during the final stages of development (LIU *et al.*, 2021).

Nutritional deficiency can arise, particularly during intentional feed restriction or environmental challenges, adversely affecting embryonic development and later sexual maturation (AFROUZIYEH *et al.*, 2021). Feed restriction has been used in poultry production to control body weight gain and increase productivity (SAHRAEI, 2012; AFROUZIYEH *et al.*, 2021; RADULOVIC *et al.*, 2021). Feed restriction during the growing phase of chicken and quails improved uniformity of body weight at first laying and increased egg number and average egg weight and development of reproductive organs (LU *et al.*, 2023).

Early nutrient supplementation provides an excellent opportunity during embryonic development to resolve the nutritional constraints resulting from insufficient maternal nutrient deposition in the egg (JHA *et al.*, 2019). This strategy facilitates essential nutrient delivery to enhance embryonic growth and early postnatal growth and development, such as carbohydrates (RETES *et al.*, 2018; ÁCS *et al.*, 2022; ZANGERONIMO *et al.*, 2023), proteins (FOYE *et al.*, 2006; PEEBLES, 2018), amino acids (AJAYI *et al.*, 2022; LUGATA *et al.*, 2024a; NDUNGURU *et al.*, 2024b). However, the mechanism through which the embryo signals nutrient availability, absorption, and incorporation for post-natal growth is less understood. Additionally, the embryonic nutrient programming mechanisms, including the physiological and molecular mechanisms, remain less determined.

Two distinct nutrient-signalling pathways regulate critical aspects of development: the insulin/insulin growth-like factor-1 signalling (ISS) hormonal pathway and the mechanistic target of rapamycin (mTOR), the intracellular nutrient-sensing pathway. Specific nutrients, such as amino acids, are essential in shaping the growth and developmental processes through the ISS and mTOR pathways (XU & VELLEMAN, 2023). Therefore, balancing the ISS/mTOR regulation is crucial for optimising both growth and the immune system in birds. Rapamycin is a mTOR inhibitor crucial for balancing growth and immunity. Rapamycin is an immunosuppressant suggested to extend lifespan, enhance immunity and reduce inflammation (BLAGOSKLONNY, 2019; ZHANG *et al.*, 2021; PHILLIPS & SIMONS, 2023). Exploring the immunomodulatory mechanism of rapamycin in birds is important because of the unique architecture of the avian immune system, the spleen, and the immune regulatory organ (SHINI *et al.*, 2010). The Japanese quail (*Coturnix japonica*) has long been a model animal for studying growth, ageing and sexual maturation and are known for their rapid growth and attainment of early sexual maturation just six to eight weeks after hatching (OTTINGER & BRINKLEY, 1978; OTTINGER, 2001). They have been used to study the relationship between nutrients, growth and the endocrine system (HOSSEINIAN & HASANZADEH, 2021), controlling cell differentiation, growth and homeostasis (ZHANG *et al.*, 2014). Also, this species has been a catalyst in exploring areas including the influence of early-life conditions such as embryonic development and the influence of embryonic nutrients on post-natal growth and development (AL-DARAJI *et al.*, 2012; LUQMAN *et al.*, 2020).

Feed restriction was used to imitate the low intake during intentional feed restriction or adverse environmental conditions. Poultry feed restriction has been used to control excessive weight gain from ad libitum intake to improve growth and reproductive performance (SAHRAEI 2012). Essential amino acids are important in protein synthesis, in turn promoting growth and sexual maturation and have been used as a supplement in poultry production (ALAGAWANY *et al.*, 2021). The results showed that 20% feed restriction at the stage of sexual maturation in birds significantly decreased final body weight, ovary weight, hierarchical numbers and follicle diameter. Amino acid supplementation of 20% leucine or 20% methionine individually or in combination with feed restriction alleviated the effects and increased body weight, ovary weight, ovary index, and hierarchical follicle number and diameter. However, a 20% feed restriction did not significantly affect plasma total antioxidant capacity. Nevertheless, supplementing 20% leucine, 20% methionine, or their combination increased the plasma antioxidant capacity. The study highlights the importance of specific nutrient composition, particularly essential amino acids, in reversing the adverse effects of feed restriction on growth, reproduction, and oxidative stress in birds at the onset of reproductive maturation. Feed restriction by 20% and 20% methionine or leucine supplementation to the restricted feed can be used to control excessive body weight gain in poultry to improve productivity.

The amount of intake during feed restriction and daily variability in feed intake can account for the quality of the laid eggs. Four feed restriction regimes were used for this objective: constant feed restriction of 20%, 30%, and 40%, and unpredictable feed access that varied between 30% and 170% of their daily feed intake. Constant feed restriction did not affect the egg yolk weight. 20% feed restriction had no effect on eggshell and albumen weight, but these parameters were reduced with 30% and 40% feed restrictions. The lowest albumen weight was observed in 40% feed restriction. However, unpredictable feed access did not have any effect on the yolk, albumin or eggshell weights. The proportion of egg yolk was highly maintained at 30% and 40% feed restrictions, while the albumen and shell proportions were lower at 30% and 40%. The yolk-to-albumin ratio was high in 40% of feed restrictions. However, unpredictable feed access did maintain all the egg component proportions, and the yolk-to-albumin ratio remained unaffected. The unpredictable feed access preserved the proportions of the egg components, indicating that this approach can effectively improve egg quality traits and overall productivity in the poultry industry.

The third objective aimed to determine the mechanism through which embryonic feeding of methionine and leucine can influence post-natal growth and development in Japanese quail. Before incubation, 1 mg (approx.. 2% increase in the egg) of methionine was injected into the embryonic egg. Embryonic feeding of methionine increased the expression of hepatic *IGF1* and *mTOR* genes at hatching. The expression of the mTOR downstream effect RPS6K1 and circulating IGF-1 hormone was observed later during post-natal growth, which was also accompanied by increased body weight. These results show that specific nutritional cues may have phenotypic programming effects by sequentially activating specific nutrient-sensing pathways and achieving transgenerational phenotypic plasticity. In the second trial, 2.5 mg (approx. 2% increase in the egg) of leucine was injected into the embryonic egg. The results showed that 2.5 mg leucine upregulated the expression of hepatic *IGF1*, *IGF1R, mTOR*, and *RPS6K1* genes. The increased expression of these genes was accompanied by increasing post-natal growth. How embryonic leucine feeding did not influence the expression of the hepatic *FOXO1* gene. The results outline the role of specific nutrients, such as essential amino acids, in enhancing postnatal growth by stimulating the nutrient-sensing pathway. Nutritional intervention during the embryonic period can improve the post-natal growth performance in the poultry industry.

Growth and defence are important for birds to survive and reproduce. Therefore, birds should maintain and optimise the balance between growth and immune response. Despite being involved in nutrient sensitivity, little is known about how the mTOR pathway regulates growth and defence. To answer this question, the 1 mg/kg body weight of rapamycin, an immune suppressive drug, was injected in Japanese quail birds after a day (for 7 days) in 14 days of the experimental period. The results showed that treatment did not affect body weight changes. However, reduced *IGF1* gene expression, which is involved in regulating growth. However, female quails grew faster and increased body weight. Relative spleen weight increased in females than in male birds, and the rapamycin treatment resulted in significantly lower spleen weight values. Low spleen weight implies more stable immune cells to produce cytokines. Rapamycin treatment decreased the hepatic expression of *IGF1* and *MyD88* genes in male compared to female birds. However, the relative expression of hepatic *mTOR*, Interleukin-1β *(IL-1β)*, *NF-κβ* and *STAT3* remained unaffected. *IGF-1* and m*TOR* are key regulators of protein synthesis and cellular proliferation, impacting growth, but rapamycin had no effect on growth, reflecting the gene expression in the mTOR pathway. In a dose-dependent manner, rapamycin could simultaneously balance growth performance and activate the body's immune system (O'SHEA *et al.* 2022).

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10. LIST OF PUBLICATIONS

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List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (1)

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

Foreign language scientific articles in international journals (5)

2. **Ndunguru, S. F.**, Reda, G. K., Csernus, B., Gulyás, G., Knop, R., Szabó, C., Lendvai, Á. Z., Czeglédi, L.: Amino acid supplementation supports growth and reproductive development under dietary restriction.
   Front. Anim. Sci. 6, 1-13, 2025. EISSN: 2673-6225.
   DOI: http://dx.doi.org/10.3389/fanim.2025.1622877
   IF: 2.4 (2024)
3. Reda, G. K*., **Ndunguru, S. F*.**, Knop, R., Lugata, J. K., Csernus, B., Gulyás, G., Szabó, C., Lendvai, Á. Z., Czeglédi, L.: Reproductive resilience and trade-offs: egg component allocation under nutritional constraints in Japanese Quails.
   Avian Res. 16 (3), 1-12, 2025. ISSN: 2053-7166.
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4. 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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5. **Ndunguru, S. F.**, Reda, G. K., Csernus, B., Knop, R., Lugata, J. K., Szabó, C., Lendvai, Á. Z., Czeglédi, L.: Embryonic Leucine Promotes Early Postnatal Growth via mTOR Signalling in Japanese Quails.
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6. **Ndunguru, S. F.**, Reda, G. K., Csernus, B., Knop, R., Gulyás, G., Szabó, C., Czeglédi, L., Lendvai, Á. Z.: Embryonic methionine triggers post-natal developmental programming in Japanese quail.
   J. Comp. Physiol. B-Biochem. Syst. Environ. Physiol. 194, 179-189, 2024. ISSN: 0174-1578.
   DOI: http://dx.doi.org/10.1007/s00360-024-01542-8
   IF: 1.6

Hungarian abstracts (1)

7. **Ndunguru, S. F.**, Czeglédi, L.: Az aminosav-összetétel és a nyersfehérje-tartalom hatása a Japán fürj petefészek fejlődésére = Amino acid composition and crude protein influence on ovarian follicular traits in quails.
   In: 39. Óvári Tudományos Nap : Konferencia : "Az agrár-, élelmiszer- és vidékgazdaság kihívásai" : Absztrakt kötet. Szerk.: Molnár Zoltán, Némethné Wurm Katalin, Széchenyi István Egyetem Albert Kázmér Mosonmagyaróvári Kar, Mosonmagyaróvár, 120, 2023. ISBN: 9786156443243

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8. **Ndunguru, S. F.**, Reda, G. K., Csernus, B., Lendvai, Á. Z., Czeglédi, L.: In ovo amino acids: Insights from domesticated and laboratory model species.
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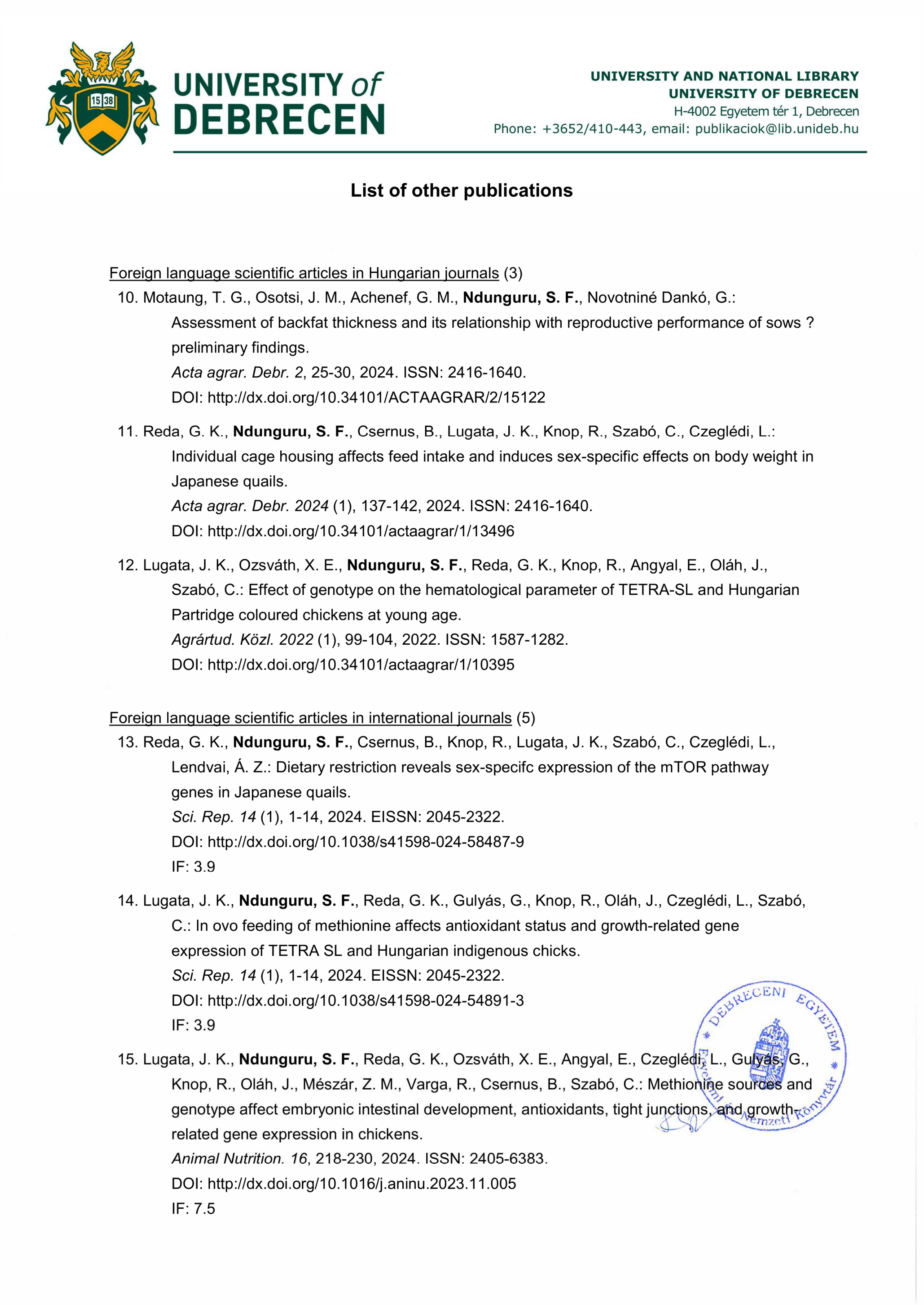

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List of other publications

Foreign language scientific articles in Hungarian journals (3)

10. Motaung, T. G., Osotsi, J. M., Achenef, G. M., **Ndunguru, S. F.**, Novotniné Dankó, G.: Assessment of backfat thickness and its relationship with reproductive performance of sows ? preliminary findings.
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12. Lugata, J. K., Ozsváth, X. E., **Ndunguru, S. F.**, Reda, G. K., Knop, R., Angyal, E., Oláh, J., Szabó, C.: Effect of genotype on the hematological parameter of TETRA-SL and Hungarian Partridge coloured chickens at young age.
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Foreign language scientific articles in international journals (5)

13. Reda, G. K., **Ndunguru, S. F.**, Csernus, B., Knop, R., Lugata, J. K., Szabó, C., Czeglédi, L., Lendvai, Á. Z.: Dietary restriction reveals sex-specifc expression of the mTOR pathway genes in Japanese quails.
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14. Lugata, J. K., **Ndunguru, S. F.**, Reda, G. K., Gulyás, G., Knop, R., Oláh, J., Czeglédi, L., Szabó, C.: In ovo feeding of methionine affects antioxidant status and growth-related gene expression of TETRA SL and Hungarian indigenous chicks.
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    Ecol. Evol. 14 (5), 1-28, 2024. ISSN: 2045-7758.
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<u>Foreign language abstracts</u> (4)

18. Achenef, G. M., Lendvai, Á. Z., Czeglédi, L., Knop, R., Szabó, C., Reda, G. K., **Ndunguru, S. F.**, Almira, F. N.: Effect of arginine on Japanese quail performance: Preliminary findings.
    In: IX. Gödöllői Állattenyésztési Tudományos Nap: Előadások és poszterek összefoglaló kötete = 9th Scientific Day of Animal Breeding in Gödöllő : Book of Abstracts of Presentations and Posters /szerk. Bényi Erzsébet, Bodnár Ákos, Pajor Ferenc, Póti Péter, Magyar Agrár- és Élettudományi Egyetem Szent István Campus, Gödöllő, 54, 2024. ISBN: 9789636231064

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21. Almira, F. N., Reda, G. K., Csernus, B., **Ndunguru, S. F.**, Knop, R., Szabó, C., Lendvai, Á. Z., Czeglédi, L.: Sex-specific effects of feed restriction on adiponectin and its receptors in Japanese quail.
In: 32nd International Symposium Animal Science Days : Book of Abstracts. Szerk.: Birgit Fürst-Waltl, Sarah-Joe Burn, Johann Sölkner, Werner Zollitsch, Unversität für Bodenkultur Wien, Wien, 26, 2024. ISBN: 9783900397159

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12. STATEMENTS

STATEMENT

I wrote this thesis within the framework of the University of Debrecen Doctoral School of Animal Science to obtain a doctoral degree (Ph.D.).

Debrecen, 09. August 2025.

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Sawadi Fransisco Ndunguru

PhD candidate

STATEMENT

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

Debrecen, 09. August 2025.

Dr. Ádám Zoltán Lendvai
Co-supervisor

Dr. Levente Czeglédi
Supervisor

Appendix 1. Average daily food intakes (ADFI (g)) of female Japanese quail during week 1 and week 2 of the treatment period.

Trial 1

Day	**Control**		**DR20**		**DR30**		**DR40**	
	ADFI	SEM	ADFI	SEM	ADFI	SEM	ADFI	SEM
0	30.41	1.20	28.93	1.23	29.91	0.98	29.82	2.21
1	32.46	0.51	22.46	2.00	21.32	2.64	17.86	1.20
2	32.96	2.10	22.93	2.10	21.43	0.22	17.86	0.88
3	32.85	1.20	22.36	1.06	21.43	0.47	17.86	0.89
4	31.03	1.80	23.50	1.23	21.43	1.00	17.86	1.23
5	33.50	0.89	23.63	0.59	21.43	1.54	17.86	2.12
6	35.24	0.23	23.64	2.10.	21.43	2.30	17.86	0.88
7	33.79	1.32	23.64	0.89	21.43	2.88	17.86	2.33
8	33.94	0.50	22.43	0.99	21.43	1.02	17.41	0.88
9	34.63	0.89	23.54	1.58	21.43	0.00	17.86	1.85
10	32.89	1.29	23.64	2.00	21.43	0.89	17.51	2.02
11	32.61	1.28	23.64	1.00	21.43	1.99	16.74	1.96
12	31.56	2.30	23.64	2.70	21.43	1.18	17.54	0.86
13	30.69	1.88	23.64	0.75	21.43	2.10	17.86	1.23
14	33.18	1.05	23.64	1.22	21.43	0.95	17.86	0.56

Trial 2

Day	**Control**		**DR40**		**UNPR**	
	ADFI	SEM	ADFI	SEM	ADFI	SEM
0	25.02	1.90	23.31	1.70	24.49	2.10
1	23.54	0.69	13.14	2.39	13.30	0.56
2	23.46	2.80	12.88	1.23	26.05	1.99
3	23.34	0.96	13.30	0.88	16.40	1.25
4	23.66	1.71	14.09	0.88	21.21	0.88
5	23.30	1.50	13.90	0.65	29.84	0.56
6	23.53	0.99	14.83	2.02	26.55	2.33
7	24.66	0.65	15.13	3.10	10.07	1.23
8	25.37	1.32	15.35	0.86	25.85	1.85
9	24.58	2.15	15.98	1.81	28.48	0.89
10	24.50	0.87	15.92	2.60	33.38	0.99
11	26.18	0.94	15.99	0.98	9.30	1.98
12	26.39	1.25	15.84	1.23	36.03	2.02
13	26.14	1.21	15.64	2.17	14.15	1.58
14	25.83	0.89	15.99	0.91	36.11	2.5
15	25.03	1.98	15.99	2.53	21.46	1.96
16	25.63	0.59	15.89	1.78	25.73	1.56

ADFI of DR20, DR30 and DR40 were calculated from individual birds. Abbreviations: ADFI, average daily feed intake; SEM, standard error of mean; ADL, *ad libitum*; DR20, 20% restriction; DR30, 30% restriction; DR40, 40% restriction and UNPR, Unpredictable feed access