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RESEARCH ARTICLE

Dietary restriction and life-history trade-offs: insights into mTOR pathway regulation and reproductive investment in Japanese quail

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ABSTRACT

Resources are needed for growth, reproduction and survival, and organisms must trade off limited resources among competing processes. Nutritional availability in organisms is sensed and monitored by nutrient-sensing pathways that can trigger physiological changes or alter gene expression. Previous studies have proposed that one such signalling pathway, the mechanistic target of rapamycin (mTOR), underpins a form of adaptive plasticity when individuals encounter constraints in their energy budget. Despite the fundamental importance of this process in evolutionary biology, how nutritional limitation is regulated through the expression of genes governing this pathway and its consequential effects on fitness remain understudied, particularly in birds. We used dietary restriction to simulate resource depletion and examined its effects on body mass, reproduction and gene expression in Japanese quails (*Coturnix japonica*). Quails were subjected to feeding at 20%, 30% and 40% restriction levels or *ad libitum* for 2 weeks. All restricted groups exhibited reduced body mass, whereas reductions in the number and mass of eggs were observed only under more severe restrictions. Additionally, dietary restriction led to decreased expression of *mTOR* and insulin-like growth factor 1 (*IGF1*), whereas the ribosomal protein S6 kinase 1 (*RPS6K1*) and autophagy-related genes (*ATG9A* and *ATG5*) were upregulated. The pattern in which *mTOR* responded to restriction was similar to that for body mass. Regardless of the treatment, proportionally higher reproductive investment was associated with individual variation in *mTOR* expression. These findings reveal the connection between dietary intake and the expression of *mTOR* and related genes in this pathway.

KEY WORDS: Dietary restriction, Gene expression, mTOR pathway, Reproduction, Resource allocation, *Coturnix japonica*

INTRODUCTION

Resource availability is a key driver of resource allocation decisions which define life-history trade-offs (Ng'oma et al., 2017; Zera and Harshman, 2001). When food is abundant, animals allocate resources towards current reproduction and away from somatic maintenance and further reproduction (English and Bonsall, 2019; Kooijman and Lika, 2014; Pontzer and McGrosky, 2022). The bias for reproductive investment may be accompanied by physiological costs, including oxidative stress and a reduced immune potential (Chang van Oordt et al., 2022; Metcalfe and Monaghan, 2013), which in turn affects future reproduction performance and the health span of the organism (Hassan et al., 2003; Mahrose et al., 2022; Pick et al., 2019). This phenomenon changes under limited resources when organisms must divert energy from reproduction to somatic maintenance (Carlsson et al., 2021; Flatt et al., 2013).

Dietary restriction (DR) is an intervention that mimics the depletion of resources, which regulates life-history traits almost uniformly in model organisms ranging from yeast to humans (Colman et al., 2014; Inness and Metcalfe, 2008; Simons et al., 2013). Reducing calorie intake affects the investment in self-maintenance, growth and reproduction antagonistically (McCracken et al., 2020; Regan et al., 2020). The classical resource allocation theory predicts that DR should lead to a linear decrease in reproduction in favour of self-maintenance (Shanley and Kirkwood, 2000). However, modest DR can maintain or even improve reproductive performance by activating cell-recycling mechanisms such as apoptosis and autophagy (Adler and Bonduriansky, 2014; Mahrose et al., 2022). When the level of DR becomes more severe, the organism must shift energy away to meet basal energetic requirements; thus, the reproduction rate will decline (Moatt et al., 2016; Ottinger et al., 2005; Shanley and Kirkwood, 2000).

Resource availability at the organismal level is monitored by the neuroendocrine system, which dynamically responds to internal signals via changes in physiology or gene expression (Maruska et al., 2018). The key mediators of this process are the nutrient-sensing pathways governed by insulin-like growth factor-1 (IGF-1) and mechanistic target of rapamycin (mTOR) (Johnson, 2018; Kapahi et al., 2017). The IGF-1/mTOR signalling pathway is activated by high nutrient availability and triggers growth and reproduction while downregulating cellular processes that maintain organismal and cellular homeostasis (e.g. apoptosis and autophagy) (Montoya et al., 2022; Papadopoli et al., 2019). In response to growth hormone and energy availability, IGF-1 is released into the bloodstream mainly from the liver and binds to its membrane receptor (IGF-1R) at the cellular membrane, which activates further intracellular molecular components (PI3K and Akt) that will, in turn, trigger mTOR activation (Feng and Levine, 2010).

mTOR serves as the central regulator of the nutrient-sensing pathway, integrating intracellular nutrient availability and extracellular signals to govern essential cellular processes, including metabolism, growth, proliferation and survival, thereby influencing tissue and organ growth (Papadopoli et al., 2019; Rabanal-Ruiz and Korolchuk, 2018). mTOR exists in two distinct

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complexes, known as mTORC1 and mTORC2, each with different functions and regulatory mechanisms. mTORC1 is primarily involved in regulating cell growth and protein synthesis in response to various environmental cues such as nutrient availability, energy status and growth factors (Panwar et al., 2023; Saxton and Sabatini, 2017; Takahara et al., 2020). In contrast, mTORC2 has diverse roles in cell survival, cytoskeletal organisation and metabolism (Sun et al., 2023; Szwed et al., 2021).

DR has been shown to downregulate the mTORC1 pathway through various mechanisms, primarily involving its upstream effectors, such as IGF-1 and intracellular amino acid deprivation (Sancak et al., 2010; Speakman and Mitchell, 2011). Furthermore, DR has a complex impact on mTORC2 activity. It has been observed that DR downregulates mTORC2 by inhibiting insulin/Akt signalling (Saxton and Sabatini, 2017; Yu et al., 2019). Conversely, there is evidence to suggest that DR can upregulate mTORC2 through the activation of the adenosine monophosphate-activated protein kinase (AMPK) pathway and inhibition of the mTORC1 pathway (Fu and Hall, 2020; Tulsian et al., 2018). Inhibition of mTORC1 downregulates the inhibitory effect of ribosomal protein S6 kinase 1 (RPS6K1) on mTORC2 (Ragupathi et al., 2024).

The activity of mTOR and its downstream effectors is further regulated through transcriptional regulation/mRNA expression and post-translational modifications (Deng et al., 2014; Mierziak et al., 2021; Rollins et al., 2019). While post-translational modifications and specific amino acid availability induce mTOR activation, general resource availability is responsible for adaptive changes in gene expression (Efeyan et al., 2015; Mierziak et al., 2021; Sandri et al., 2013). Although the final activity of mTOR is influenced by multiple factors, higher expression of the *mTOR* gene can potentially increase the pool of available mTOR protein for activation, while lower gene expression leads to reduced protein production and availability (Buccitelli and Selbach, 2020). Studies on model organisms have predominantly focused on the post-translational activation of mTOR and its downstream effectors (Laplante and Sabatini, 2012; Papadopoli et al., 2019). However, the impact of DR on the differential expression of mTOR signalling genes and their role in mediating fitness remain to be fully elucidated.

mTORC1 performs its effect on fitness traits through a number of downstream effectors, including RPS6K1 (Guo and Yu, 2019; Nojima et al., 2003) and autophagy-related genes (Ma et al., 2018). Under excess food availability, the mTORC1/RPS6K1 pathway facilitates protein synthesis and subsequent cell growth and proliferation (Fenton and Gout, 2011; Tulsian et al., 2018). Under DR, the mTORC1/autophagy pathway recycles cell contents for energy substitution and reduces oxidative stress (Chung and Chung, 2019). Hence, *RPS6K1* and autophagy genes (including *ATG9A* and *ATG5*) are the best representative candidate genes of the mTOR downstream pathways. Studying the expression of these genes and their relationship to fitness traits under different DR levels is critically important to understand their role beyond post-translational regulation.

Previous studies on DR have primarily focused on model organisms other than birds, and except for some production-related experiments on chickens (Deng et al., 2014; Hao et al., 2021; She et al., 2019), understanding the mechanism of mTOR signalling in avian biology remains largely unclear. A recent study investigating the ecological perspective of mTOR activation using a proxy gene *Telomere Maintenance 2* (*TELO2*) suggests that mTOR plays a key role in telomere maintenance in free-living great tits (*Parus major*) (Casagrande et al., 2023). Additionally, the study proposed that mTOR could be viewed as a regulator of trade-offs among life histories. Studies conducted in mammals have indicated that a higher nutritional intake and metabolic rate upregulate the mTOR pathway, leading to a subsequent downregulation of autophagy (Escobar et al., 2019). This, in turn, exposes organisms to oxidative damage and cellular senescence. However, DR without malnutrition has been found to mitigate these effects (Carroll and Korolchuk, 2018). Birds have higher metabolic rates, circulating glucose levels and body temperature than mammals, while they live twice as long as size-matched mammals (Barja, 1998). Despite the fact that a higher metabolic rate contributes to oxidative and glycoxidative damages, birds suffer less compared with mammals at a given body size (Jimenez et al., 2019). This metabolic paradox may be due to a different diet–fitness relationship in birds, which can be elucidated using dietary manipulation experiments.

In the present study, we aimed to observe the effects of DR gradient on liver *mTOR* mRNA expression, as well as its main upstream and downstream effectors, using adult female Japanese quails (*Coturnix japonica*) as an experimental avian model system. The liver serves as a primary site for the intricate nutrient metabolic pathway, which significantly influences the proper functioning of the entire body. Being a major organ responsible for regulating homeostasis, the liver plays a crucial role in nutrient regulation, protein synthesis and detoxification processes. Nearly all genes involved in the nutrient-sensing pathway exhibit differential expression in the liver and display a robust correlation with the overall functioning of the body, ultimately determining fitness traits (Baloni et al., 2019; Gokarn et al., 2018).

We hypothesised that under DR, the expression of target genes involved in the mTOR pathway would play a crucial role in mediating resource availability and influencing fitness traits. We predict reduced *mTOR*, *IGF1*, *IGF1R* and *RPS6K1* expression and upregulation of autophagy-related genes (*ATG9A* and *ATG5*). Furthermore, we anticipated that variations in *mTOR* expression will be associated with proportional differences in reproductive investment. These findings will provide insights into the relationship between gene expression, resource allocation and fitness traits.

MATERIALS AND METHODS

Experimental animals and housing

The experiment was approved by the Ethical Committee for animal use of the University of Debrecen, Hungary (Protocol No. 5/2021/DEMAB) and followed all institutional and national regulations.

We purchased 4 week old Japanese quail (*Coturnix japonica* Temminck & Schlegel 1848) chicks from a commercial quail breeder (Budai Fürjészet, Dunavecse, Hungary) and housed them in the animal house of the Institute of Agricultural Research and Educational Farm of the University of Debrecen. The birds were kept in cages in groups of 10 for an additional 4 weeks (until they reached maturity) before being subjected to the experimental treatment. At the age of 8 weeks, 32 female birds of similar body mass were selected and housed in individual cages (18.5 cm long×21 cm wide×18.5 cm high) for a 7 day acclimation period on *ad libitum* feed and water. This exclusive focus on female subjects was chosen to specifically include egg traits as representative reproductive parameters in the study. The experimental room was maintained at a temperature of 24±3°C and 60–75% relative humidity. Photoperiod was fixed at a 12 h:12 h light:dark daily cycle and regulated using an LED Lighting Dimming System. The basal feed for experimental quails was formulated as a breeder quail

ration (20% CP; 12.13 MJ kg^{-1} ME; National Research Council, 1994) based on corn, soybean and wheat (see Supplementary Materials and Methods and Table S1).

Experimental design

During the acclimation period, the daily feed intake of each individual was measured for seven consecutive days. Approximately 50 g feed was weighed on a digital scale (±0.1 g) and provided in a 200 g capacity plastic feeder each morning between 08:00 and 09:00 h. The following day (24 h later), the remaining feed was weighed again, and the feeders were replenished with fresh feed. Daily feed intake was measured as the difference between the mass of the offered food and the remaining food. Because of the design of the feeders, food spillage was negligible. The average daily feed intake was calculated as the mean of the seven measurements; the average feed intake for each day during the acclimation period was 29.67±3.73 g. We also measured the live body mass of each bird at the beginning and at the end of the acclimation period to analyse mass change. At the start of the experimental treatment, there was no significant temporal pattern of either body mass or feed intake. Birds were regularly laying eggs during the acclimation period.

After acclimation, 32 female birds were randomly assigned to four dietary treatments. The birds were fed with 80% (DR20), 70% (DR30) and 60% (DR40) of their average individual feed intake, while the control group was fed *ad libitum* (ADL). The experiment lasted for 14 days. The amount of feed left daily in the ADL group was measured and analysed to detect any significant changes in temporal intake. However, no significant changes were observed.

Measurements and sampling

Immediately after lights-on in the morning, we removed all the feeders to maintain similar feeding conditions (empty gut) between the ADL and food-restricted birds during the measurement and sampling points. We measured body mass at the beginning of the experiment (day 0) and on days 7 and 14 of the restriction period. We collected eggs daily, measured their mass and recorded the identity of the respective hen. Body mass and egg mass were measured using a digital scale (±0.1 g). On day 14, all birds were euthanised, and a liver sample was immediately collected, rapidly frozen on dry ice and stored at −80°C until further assays.

RNA extraction and cDNA synthesis

Total RNA from liver tissue was isolated using the TRIzol reagent (Direct-zol™ RNA MiniPrep, Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's protocol, including the DNA digestion step. RNA concentration and purity were measured using an HTX Synergy Multi-Mode Microplate Reader spectrophotometer (Agilent BioTek, BioTek Instruments Inc., Santa Clara, CA, USA). RNA integrity was checked by 1% agarose gel electrophoresis with ethidium bromide staining (see Supplementary Materials and Methods for a more detailed protocol). Reverse transcription was performed using the qScript cDNA synthesis kit, following the manufacturer's protocol (Quantabio Reagent Technologies, Qiagen Beverly Inc., Beverly, MA, USA) in a PCRmax Alpha Thermal Cycler (Cole-Parmer Ltd, Vernon Hills, IL, USA) (see Supplementary Materials and Methods for a more detailed protocol).

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed using EvaGreen qPCR Mix (Solis BioDyne, Teaduspargi, Estonia) according to the manufacturer's protocol. Intron-spanning gene-specific primer pairs for quails were designed using Oligo7 software and obtained from Integrated DNA Technologies (BVBA-Leuven, Belgium) (Table S2). We checked for target identity using Primer-Blast software of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) (see Supplementary Materials and Methods for a more detailed protocol).

Among the most frequently used reference genes in birds, β-actin (*ACTB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and 18S ribosomal RNA (RN18S), we selected the best reference gene, *ACTB*, using NormFinder, BestKeeper and deltaCt algorithms. The $2^{-\Delta\Delta Ct}$ method was employed to analyse the relative changes in mRNA expression of the target genes (*mTOR*, *RPS6K1*, *IGF1*, *IGF1R*, *ATG9A* and *ATG5*) (Livak and Schmittgen, 2001).

Statistical analysis

All analyses were performed using R v.4.1.2 'Bird Hippie' (http://www.R-project.org/). We fitted four models to analyse our data depending on the data source and relationship of variables. We used a linear model to analyse single-time data such as relative mRNA expression, hereafter gene expression, and the regression of one-to-one parameters. To analyse body mass across restriction time and restriction levels, we used linear mixed-effects models using 'lme4' (Bates et al., 2015) and 'lmerTest' packages v.3.1-3 (Kuznetsova et al., 2017), considering individuals and experimental blocks as a random intercept. To analyse egg mass across days and restriction levels, we used generalised linear-quadratic mixed-effect models using the 'mgcv' package v.1.8-40 (Wood, 2017) to incorporate non-linear forms of the predictor restriction days. We used generalised linear mixed-effects models of the family logit using the 'aod' package v.1.3.2 (https://cran.r-project.org/package=aod) to analyse the binary response variable (daily egg laying). In mixed models, individual bird ID was included as a random intercept to control for repeated measures. Akaike's information criterion (AICc) was used to choose the best-supported models (Burnham and Anderson, 2010). The log of fold-change was used to analyse relative gene expression. The Tukey test was used as a *post hoc* test with $P<0.05$ significance level and bars set as means±s.e.m. To see the multivariate regression of gene expression against the fitness traits and resource allocation strategy, we used principal component analysis (PCA) using the 'prcomp' function from the 'stats' package to avoid multicollinearity between the predictor variables (http://www.R-project.org/). We used the 'ggbiplot' package to visualise the clustered data against treatments (http://github.com/vqv/ggbiplot). We used Kaiser's rule to retain PCs for further analysis (Kaiser, 1960). We analysed the impact of individual genes on each fitness trait using linear regression. We analysed the resource allocation strategy using the proportional change in egg mass as a function of change in body mass compared with the pre-treatment status. We used trigonometric calculation to determine the direction of the resulting vector in radians ($-\pi/2$ to $\pi/2$) that reflects resource allocation strategy. A value of zero corresponds to no re-allocation; positive and negative values indicate re-allocation towards reproduction and self-maintenance, respectively. This method allowed us to analyse the relationship between individual gene expression and resource allocation strategy across treatment groups.

RESULTS

Dietary restriction affects body mass

Experimental groups did not differ in their initial body mass, but DR reduced it significantly (treatment: $F_{3,28}=13.832$, $P<0.0001$; time: $F_{2,56}=61.826$, $P<0.0001$; treatment×time interaction: $F_{6,56}=12.262$, $P<0.0001$). Birds grouped under all restriction levels showed a

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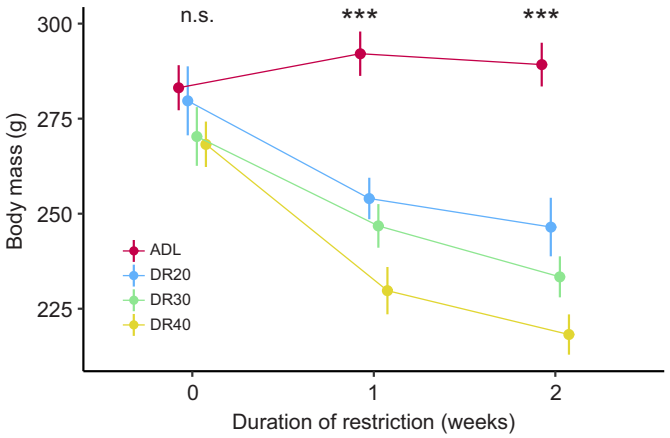

Fig. 1. The effect of different dietary restriction levels on the body mass of female quails at different time points. All food-restricted groups (DR20, DR30, DR40) showed significantly lower body mass compared with the *ad libitum*-fed group (ADL) at both week 1 and week 2 time points. Data are means±s.e.m. from 8 birds per group and were statistically analysed by two-way ANOVA. n.s., not significant; ***P<0.001.

significant reduction in body mass compared with the ADL birds at both week 1 and week 2 (Fig. 1; Table S3). The DR40 treatment also resulted in significantly lower body mass than the DR20 treatment at both time points (week 1: P=0.039, week 2: P=0.012; Table S3), while the other food-restricted groups did not differ significantly. All food-restricted groups showed significantly reduced body mass at both weeks compared with their respective initial body mass (Table S3). The DR30 and DR40 groups showed a significant (P=0.035) and marginally non-significant (P=0.080) body mass reduction from week 1 to week 2, respectively, while the DR20 group showed no further significant variation from week 1 to week 2 (P=0.332; Table S3).

Severe dietary restriction reduces reproductive traits

The level of DR and the restriction period significantly explained daily egg-laying probability. Food-restricted birds decreased daily egg-laying probability in comparison to the ADL group (Fig. 2A, Table 1). Additionally, overall probability of daily egg laying was significantly reduced across the restriction period (P=0.004). Concerning the total number of eggs laid in the 14 days, treatment showed a significant effect ($F_{3,24}$=5.448, P=0.045). The DR40 group laid significantly fewer eggs than the ADL group (t=286, P=0.039), while the DR20 and DR30 did not show a significant variation from the ADL (Fig. 2B).

Table 1. Output of the generalised linear mixed model of the family logit to predict the probability of daily egg laying

	Estimate	s.e.	*z*-value	*P*-value	Variance
Predictors					
Intercept	2.93	0.54	5.37	<0.0001	
DR20	−1.38	0.67	−2.06	0.0391	
DR30	−1.71	0.67	−2.55	0.0108	
DR40	−1.60	0.66	−2.41	0.0158	
Day	−0.08	0.03	−2.82	0.0048	
Random effects					
Bird ID					1.186

The model is fitted with restriction treatment and restriction days as fixed effect and individual bird identity as random effect. Bird ID, individual bird. N=480 observations.

DR treatment significantly affected egg mass (treatment: $F_{3,25.68}$=5.18, P=0.006; day: $F_{2,309.91}$=24.89, P<0.0001; treatment×day: $F_{6,309.83}$=14.85; $P\leq$0.0001). The time-dependent trend indicated that egg mass was significantly reduced in the DR30 and DR40 groups starting from day 5 (Fig. 2C, Table 2). As for the ADL group, egg mass from the DR20 group showed no change throughout the restriction period. On the last days of the experiment, egg mass from the DR30 and DR40 groups showed improvement. Average egg mass in the 2 week restriction period was significantly lower in the DR30 and DR40 groups compared with that in the ADL group, while the DR40 group still showed significantly lower average egg mass than the DR20 group (Fig. 2D).

Dietary restriction affects gene expression

mTOR expression showed a significant and gradual decrease across the DR levels ($F_{3,28}$=15.424, P<0.0001; Fig. 3A; Table S4). However, the expression of *RPS6K1* showed an increased trend in response to the increasing severity of the treatments ($F_{3,28}$=7.522, P=0.001; Fig. 3B, Table S4). The DR treatment also significantly decreased *IGF1* expression ($F_{3,28}$=8.998, P=0.0002). All the food-restricted groups had significantly lower *IGF1* expression than the ADL controls (Fig. 3E; Table S4). Contrary to *mTOR* expression, the downregulation of *IGF1* did not intensify with the severity of the treatment. Despite a similar trend to *IGF1* expression, *IGF1R* expression remained statistically indistinguishable among the four groups ($F_{3,28}$=1.108, P=0.362; Fig. 3F; Table S4). Furthermore, the restriction treatment significantly increased *ATG9A* and *ATG5* expression (*ATG9A*: $F_{3,28}$=5.726, P=0.01, *ATG5*: $F_{3,28}$=4.117, P=0.05; Fig. 3C,D; Table S4).

Gene expression is related to fitness

The PC analysis indicated that cumulatively 63.7% of the variation was explained by PC1 and PC2 with an eigenvalue of 2.46 and 1.37, respectively. PC1 reflects the expression of *ATG9A*, *RPS6K1*, *ATG5*, *mTOR* and *IGF1*, whereas PC2 reflects mainly *IGF1R* and *mTOR* expression. *mTOR* and *IGF1* expression contributed positively, while *RPS6K1*, *ATG9A* and *ATG5* expression contributed negatively to variation in PC1 (Fig. 4). While *mTOR* and *IGF1* expression were positively correlated, both were negatively correlated with *RPS6K1* and *ATG9A* (Fig. S2).

Variation in both PC1 and PC2 significantly explained body mass. However, reproductive parameters (egg number and egg mass) were related to only PC1 (Table 3). Therefore, a significant increase in the positive contributor variables of PC1, *mTOR* and *IGF1*, and a decrease in the negative contributor variables, *RPS6K1*, *ATG9A* and *ATG5*, led to an increase in body mass, egg number and egg mass. A significant decrease in the major contributors to PC2, *IGF1R* and *mTOR*, significantly decreased the body mass. Individually, the expression of *mTOR* and *IGF1* significantly explained all fitness variables positively. *RPS6K1* expression showed a negative relationship with body mass and egg mass, while *ATG9A* expression was negatively related to body mass and egg number (Fig. S1). The Pearson correlation analysis of gene expression also indicated that *mTOR* expression showed an association with the expression of all other genes (Fig. S2).

mTOR expression is associated with resource re-allocation

Birds effectively allocate resources toward body mass, reproduction or self-maintenance based on resource availability (Fig. 5). On week 1, only the DR20 group increased relative reproductive investment (P=0.02; Fig. 5C), while the ADL controls and the two other treatment groups did not deviate

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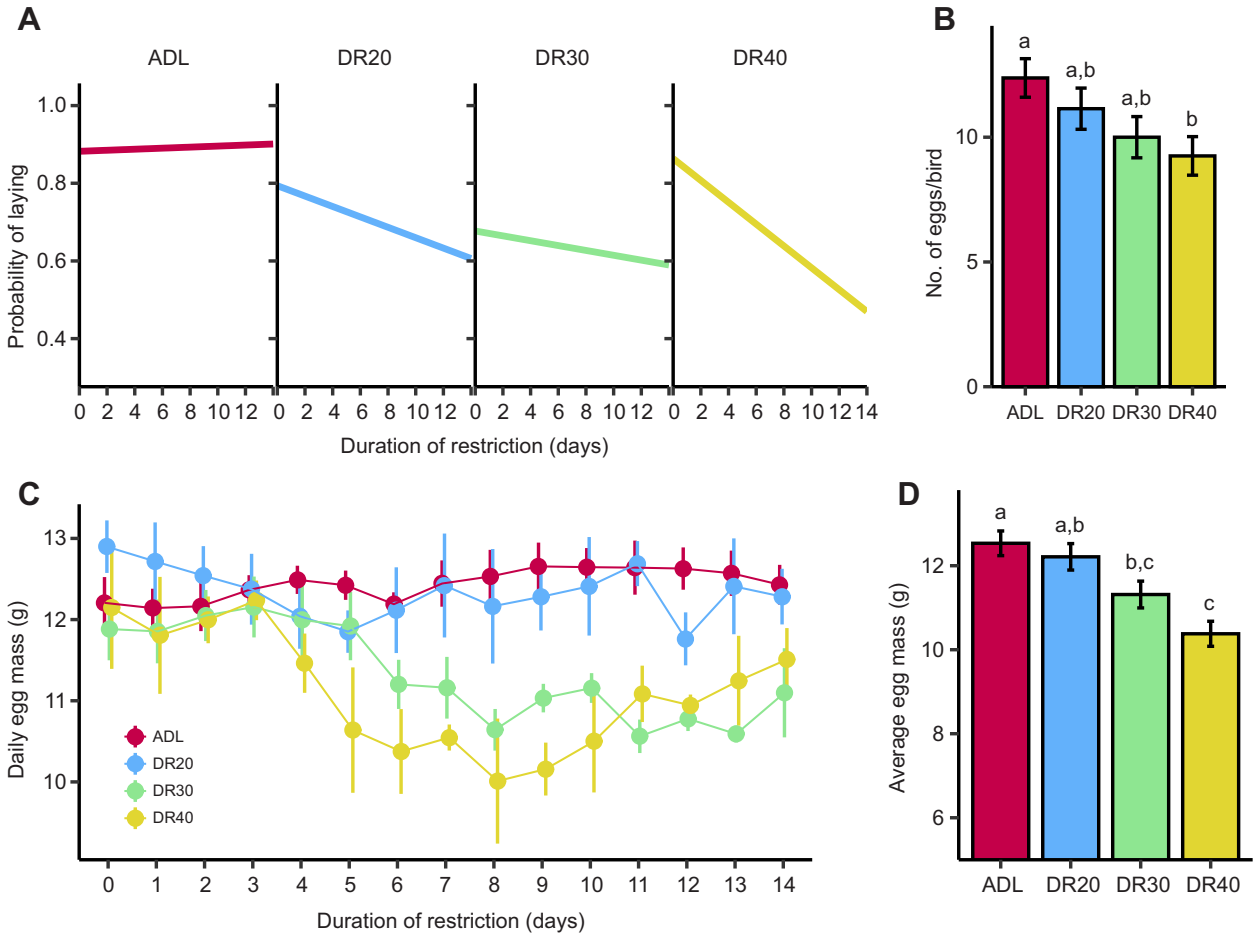

Fig. 2. Effect of dietary restriction on egg production and egg mass. (A) While all the food-restricted groups showed a slightly reduced probability of daily egg laying, the DR40 group showed the greatest reduction. (B) Only the DR40 group showed a significantly reduced total number of eggs. (C) Egg mass was reduced in the DR30 and DR40 groups starting on the fifth and sixth day, respectively. (D) Average egg mass was significantly lower in the DR30 and DR40 groups. Data are means±s.e.m from 8 birds per group. The Tukey test was used as a *post hoc* test with $P<0.05$ significance level. Different letters indicate a significant difference between means ($P<0.05$).

significantly from zero re-allocation, and all treatment groups had a similar strategy by the end of the second week ($P>0.4$; Fig. 5D). At the end of the experiment, individual variation in allocation strategy was only related to *mTOR* expression ($t=-3.118$, $P=0.004$). Irrespective of the treatment, individuals with lower *mTOR* values were more likely to invest proportionally more in reproduction than individuals with higher *mTOR* expression (Fig. 5B).

DISCUSSION

Understanding the evolution of resource allocation and its underlying mechanisms remains a major challenge in biology (Moatt et al., 2020; Ng'oma et al., 2017; Vedder et al., 2023). In this study, we decreased resource availability along a gradient of varying severity and investigated the body mass change, reproductive performance and expression of key genes in the nutrient-sensing IGF-1/mTOR pathway. Our study provided four key results.

First, we found that the severity of the feed restriction affected body mass differently (Fig. 1), indicating that our manipulation of resource availability was successful. Even a mild (20%) feed restriction decreased body mass in the first week, but birds could stabilise their body mass in the second week. The 30% and 40% restrictions resulted in stronger initial body mass losses and birds continued to lose mass in the second week. Birds with higher metabolic rate could be more sensitive to variation in food availability (Brzęk et al., 2012; Zhang et al., 2018).

Second, despite the effective treatments, reproductive performance dropped considerably only when food restriction was more severe. Under 20% food restriction, only the probability of daily egg laying was reduced, indicating that birds were more likely to skip some days in laying, whereas controls reliably laid an egg every day. However, the total number of eggs and the egg mass throughout the study remained similar in the DR20 group to that in ADL birds. In DR30 birds, while the number of eggs remained similar to that for ADL birds, the probability of egg laying and the mass of the eggs were reduced, especially in the second half of the experiment. In the most severely restricted hens (DR40), all three reproductive parameters were affected: the probability of egg laying decreased during the study, resulting in fewer and smaller eggs than for the controls. However, even under the highest restriction level, the birds continued reproduction, indicating that despite an overall reduction in their resource pool, they managed to maintain their reproductive performance, in some cases (DR20) even at the level of the ADL controls. However, depending on the magnitude of reduction in the available energy, birds had to face different trade-offs. At a low restriction level (DR20), individuals had to allocate more resources from a limited budget to reproduction, but they

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Table 2. Output of the linear quadratic mixed-effect model for testing the effect of dietary restriction on egg mass across the 14 day restriction period

	Estimate	s.e.	d.f.	*t*-value	*P*-value	Variance	R^2
Fixed effects							
Intercept	12.47	0.29	25.17	42.90	<0.0001		
DR20	−0.16	0.42	25.31	−0.38	0.7049		
DR30	−1.04	0.43	25.64	−2.43	0.0225		
DR40	−1.40	0.41	25.62	−3.39	0.0022		
poly(day, 2)1	2.96	1.24	308.73	2.38	0.0177		
poly(day, 2)2	−0.92	1.28	308.74	−0.72	0.4720		
DR20×poly(day, 2)1[1]	−5.16	1.89	308.92	−2.72	0.0069		
DR30×poly(day, 2)1	−10.39	1.98	311.63	−5.24	<0.0001		
DR40×poly(day, 2)1	−10.9402	1.95	309.26	−5.61	<0.0001		
DR20×poly(day, 2)2[2]	2.90	1.92	309.25	1.51	0.1307		
DR30×poly(day, 2)2	1.81	1.97	309.11	0.92	0.3580		
DR40×poly(day, 2)2	12.28	1.94	309.17	6.33	<0.0001		
Random effects							
Bird ID or group						0.649	
Residual						0.36	
Model							0.74
Fixed							0.35
Random							0.38

N=347 observations. [1]Linear term of a quadratic effect. [2]Quadratic term of the quadratic effect of day. We employed a linear quadratic mixed-effect model: *lmer*(*egg_mass* ∼ *treatment* * *poly*(*day, 2*)+(*1* | *birdID*)) to capture non-linear trends of egg mass across days.

could do it without compromising egg size. When resources became more limiting (DR30), birds had to trade off quality for quantity of reproduction. Under even more challenging conditions (DR40), egg number and egg mass plus body mass were compromised. Our analysis of resource allocation strategy supports the idea that birds invest in reproduction at moderate restriction (DR20), whereas they favour self-maintenance at more severe restriction levels (DR40) (Fig. 5). These results corroborate previous findings (Li et al., 2011; Mahrose et al., 2022), indicating that moderate restriction improves egg production at the expense of egg mass and body mass. Moderate DR has also been shown to have a positive effect on preserving reproductive capacity in mammals (Sun et al., 2021). Mild DR has

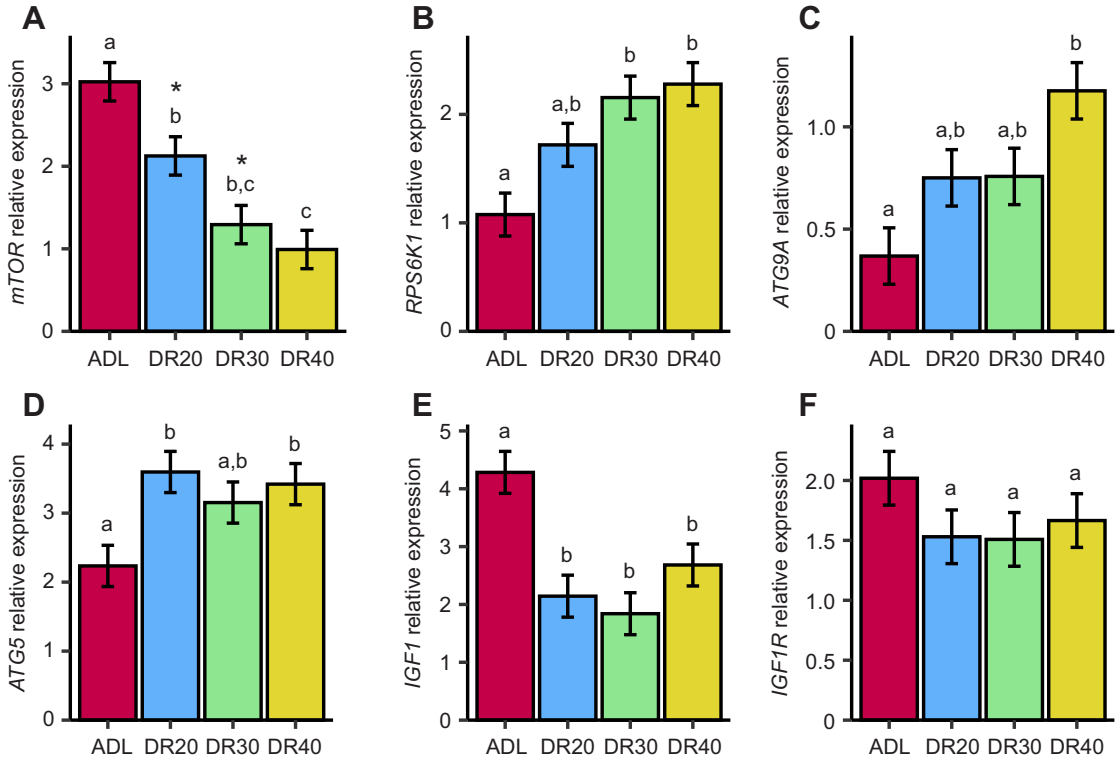

Fig. 3. Relative mRNA expression over the 14 day dietary restriction period. Dietary restriction had a significant effect on relative mRNA expression (log fold-change) of (A) mechanistic target of rapamycin (*mTOR*), (B) ribosomal protein S6 kinase 1 (*RPS6K1*), (C) autophagy-related 9A (*ATG9A*), (D) autophagy-related 5 (*ATG5*) and (E) insulin-like growth factor 1 (*IGF1*), whereas it had no significant effect on (F) insulin-like growth factor 1 receptor (*IGF1R*). Data are means±s.e.m. from 8 birds per group. The Tukey test was used as a *post hoc* test with $P<0.05$ significance level. Different letters indicate a significant difference between means ($P<0.05$). Dots above the letters indicate a marginally significant difference ($P<0.1$).

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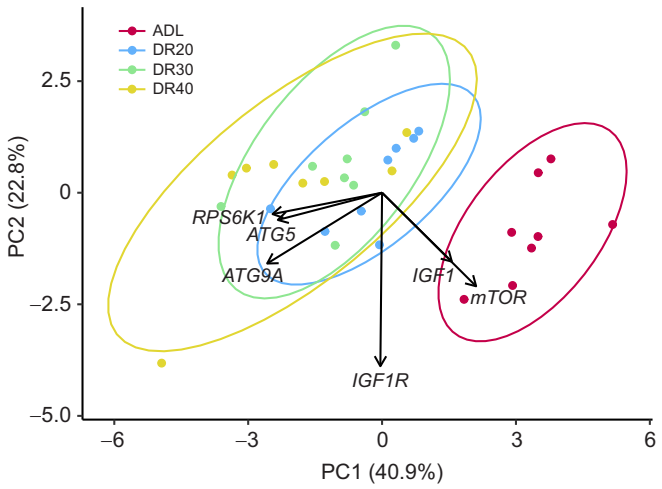

Fig. 4. Dimensional indication of principal components (PCs) of gene expression as independent variables clustered along with dietary restriction levels. The expression values of *IGF1* and *mTOR* are clustered around the ADL group, while catabolic autophagy genes and *RPS6K1* are clustered around the food-restricted groups.

for years been used in the poultry sector to avoid rapid growth and maintain reproductive life span and health span (Holmes and Ottinger, 2003). A study on rainbow trout indicated that a 20% restriction led to the production of bigger eggs than in fish fed *ad libitum* (Cardona et al., 2019) and suggested that organisms fed *ad libitum* seem to invest much of their energy for growth, while the moderately food-restricted organisms favour investing in their reproductive success.

Third, in response to our treatment, we found characteristic signatures in gene expression patterns. DR downregulated both *mTOR* and *IGF1* expression, albeit in different ways. The pattern of change in *mTOR* gene expression across treatments groups mirrored the variation in body mass loss and showed a dose-dependent reaction, where the downregulation of *mTOR* gene expression was proportional to body mass loss (Figs 1 and 3A; Tables S3 and S4). In contrast, *IGF1* expression was affected equally in all food-restricted groups. Although gene expression and circulating hormone levels may be temporally dissociated (Ndunguru et al., 2024), our results for *IGF1* expression suggest that relying solely on hormonal regulation might provide a limited picture of the physio-phenotypical adjustments in response to nutritional availability. Nutritional stress has been found to downregulate the expression of the *IGF1* gene, resulting in a decrease in circulating levels of IGF-1. The deficiency of IGF-1 has been observed to have pleiotropic effects (Lodjak and Verhulst, 2020). The result also indicates that *mTOR* expression is more sensitive to a gradient of nutritional deficiency. Although the specific mechanisms underlying the effect of DR on *mTOR* expression have not been thoroughly investigated, our findings shed light on the similarity between the effect of DR on gene expression and the previously studied effect on the abundance of activated mTORC1 (Velingkaar et al., 2020).

The expression of the *mTOR* gene is crucial for the cellular production of the mTOR protein, which is then assembled into mTORC1 or mTORC2 complexes along with other component proteins (Szwed et al., 2021). The reduced *mTOR* gene expression could contribute to a lower mTORC1 abundance for activation. Concurrently, DR can downregulate the expression of potential mTORC1 upstream activators. In normal nutritional conditions, activated mTORC1 suppresses mTORC2 activation by phosphorylating RPS6K1 (Liu et al., 2013; Oh and Jacinto, 2011; Szwed et al., 2021; Wu et al., 2022). mTORC1-activated RPS6K1 phosphorylates mTORC2 at Rictor and Sin1 members of the complex and impairs mTORC2 as a negative feedback loop for mTORC1 activation (Julien et al., 2010; Ragupathi et al., 2024; Wu et al., 2022). Consequently, reduced *mTOR* gene expression during DR may have a positive impact on the activation of mTORC2 and subsequent cell survival under dietary stress. mTORC2 is also activated by the energy stress sensor AMPK under dietary restriction conditions (Szwed et al., 2021).

Contrary to our assumption, we also found that *RPS6K1* expression increased with the severity of dietary restriction (Fig. 3B). In response to phosphorylation by mTORC1, RPS6K1 initiates ribosomal translation, consequently promoting cell growth and differentiation (Saxton and Sabatini, 2017). Previous studies have reported reduced *RPS6K1* expression in the liver of overfed geese (Han et al., 2015) and in the brain of food-restricted mice (Ma et al., 2015). Therefore, we expected that downregulation of *mTOR* expression would reduce the expression of *RPS6K1* and subsequently reduce body mass (Bae et al., 2012). Despite the predicted decrease in *mTOR* expression, body mass and *RPS6K1* expression were negatively correlated (Fig. S1). While a consistent response is expected (Buccitelli and Selbach, 2020), expression of the *RPS6K1* gene and the phosphorylated RPS6K1 protein might respond differently to upstream factors. Protein expression of RPS6K1 kinase alone is not sufficient to initiate ribosomal protein translation; rather, it needs to be phosphorylated by the activated mTORC1 kinase (Holz et al., 2005). Hence, the mechanism of action of *mTOR* and *RPS6K1* gene expression, their total protein expression, their phosphorylated protein expression and their impact on shaping fitness traits are future research interests. Although there is growing evidence for the correlation between gene expression and protein abundance (Koussounadis et al., 2015; Nie et al., 2006), post-transcriptional modification may alter the

Table 3. Output of the multiple linear regression of principal components (PCs) from gene expression predicting body mass, egg number and egg mass

Response variable	Predictor	Coefficient	s.e.	*t*-value	*P*-value	R^2	RSE
Body mass	Intercept	246.83	4.13	59.70		0.48	23.39
	PC1	12.22	2.68	4.56	<0.0001		
	PC2	−9.12	3.59	−2.54	0.02		
Egg number	Intercept	10.72	0.41	26.34		0.22	2.23
	PC1	0.70	0.26	2.75	0.011		
	PC2	−0.082	0.35	−0.24	0.82		
Egg mass	Intercept	11.64	0.18	64.89		0.32	0.96
	PC1	0.36	0.12	3.13	0.004		
	PC2	−0.23	0.15	−1.53	0.14		

RSE, residual standard error.

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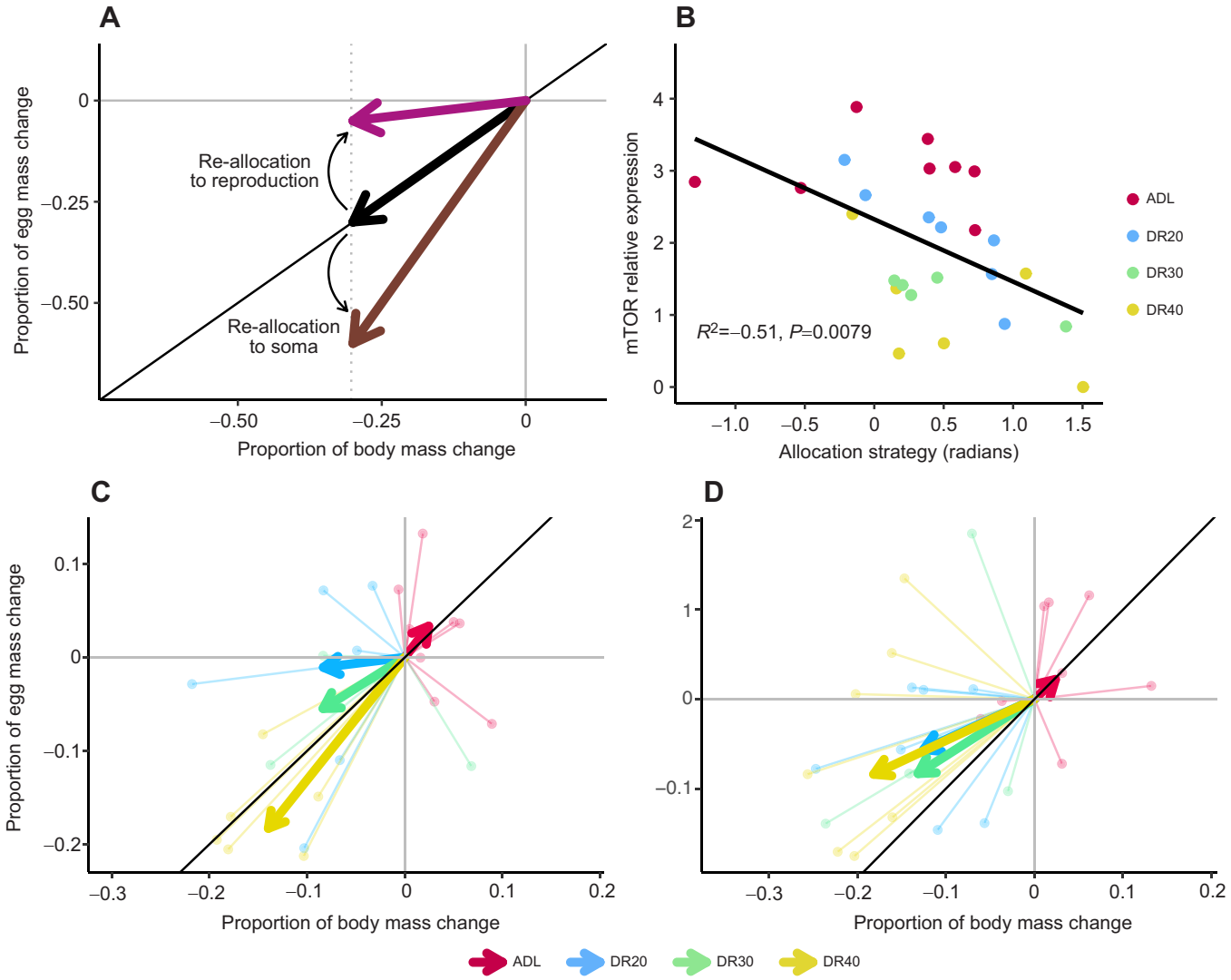

Fig. 5. Effect of dietary restriction on resource allocation decision. (A) A conceptual figure illustrating the resource allocation decision. The *x*- and *y*-axes show the proportional change in body mass and egg mass, respectively, during the experimental period (compared with the pre-treatment body mass and egg mass, respectively; thus, all vectors start from the origin). The angle of the vectors (radians) illustrates the allocation strategy. The solid black line shows where $y=x$, i.e. when there is no re-allocation: a unit change in body mass is accompanied by the same amount of correction in egg mass (0 radian). The space above the identity line (positive radians) indicates reproductive re-allocation: in response to a change of available resources, the individual allocates more to reproduction at the cost of self-maintenance. In contrast, the space under the identity line (negative radians) indicates re-allocation towards self-maintenance: the same change in body mass is associated with a proportionally larger reduction in reproductive investment. (B) Individual allocation strategy related to *mTOR* expression. (C,D) Resource allocation of individuals (thin lines) and the median response of the respective treatment group (thick vectors). In the first week (C), the DR20 and DR30 groups invest more in reproduction in response to a decrease in body mass, while the DR40 group tends to re-allocate resources to self-maintenance. (D) Over two weeks, all food-restricted groups show reproductive re-allocation, albeit to different degrees. The longer vectors for the DR40 group also illustrate that this treatment imposed a higher cost than in the other two DR treatments (where the lengths of the vectors are similar). The ADL control group remained unchanged over time.

biological function of these genes. Currently, the paucity of research reporting the effect of DR on *RPS6K1* gene expression hinders the generalisation of the observed patterns, although two recent experiments from our laboratory corroborate this pattern (G.K.R., S.F.N., B.C., R.K., C.S., L.C. and Á.Z.L., unpublished; Reda et al., 2024). In a parallel study, we found that the same food restriction treatments affected gene expression patterns similarly in males, including an upregulation of *RPS6K1* expression (Reda et al., 2024), and another independent study also confirmed this result (G.K.R., S.F.N., B.C., R.K., C.S., L.C. and Á.Z.L., unpublished). Therefore, our current results suggest that *RPS6K1* may be critical in resource allocation decisions. The other genes of interest are the autophagy-related genes, *ATG9A* and *ATG5*, the genes involved in autophagosome formation, elongation and closure. Both genes showed a tendency to be upregulated in all dietary restriction groups (Fig. 3C).

mTOR expression was positively related to *IGF1* expression and expression of its signalling receptor *IGF1R* (Fig. S2). The mTOR pathway not only affects translation but also is a key regulator of gene transcription by regulating the activity of specific transcription factors and epigenetic mechanisms or by affecting RNA stability (Laplante and Sabatini, 2013). The modification of transcription factors is important for their activation, translocation, interaction, stability and binding affinity (Filtz et al., 2014; Sukumaran et al.,

2020). mTORC1 phosphorylates transcription factors in response to resource availability, which in turn regulate several essential genes. Evidence shows that mTORC1 itself can function as a transcription factor when it is localised in the nucleus (Jiang, 2010; Tsang et al., 2010). Therefore, activated mTORC1 can upregulate the transcription of *IGF1*, *IGF1R* and *mTOR* itself. In the case of DR, the inhibition of mTOR can lead to a decrease in the expression of genes involved in growth and reproduction, including *IGF1*. Contrarily, under scarce resources (DR), the downregulation of mTORC1 allows the nuclear localisation and activity of transcription factor EB (TFEB) and upregulates autophagosome formation through coordinating the expression of genes involved in autophagy such as *ATG9A* and *ATG5* (Martina et al., 2012; Napolitano and Ballabio, 2016). These transcriptional factors are mainly related to the maintenance of cellular homeostasis by regulating autophagy and lysosomal genes at the transcriptional level during nutritional deficiency (Inoki et al., 2012; Martina et al., 2012). The correlation analysis in the present study revealed that the expression of *ATG9A* and *ATG5* is negatively related to *mTOR* expression (Fig. S2), indicating that the downregulation of mTOR mediates the upregulatory effect of DR on autophagy genes.

Finally, we found that variation in the gene expression pattern was coordinated and related to fitness parameters and resource allocation. At a severe DR, the reduced egg number and egg mass aligned with low *IGF1* and *mTOR* expression, suggesting that these genes are associated with the effect of DR on reproduction (Fig. S1). However, individual variation in resource allocation strategy was only related to *mTOR* expression. Stronger restrictions induced an increasing reduction of *mTOR* expression, but irrespective of the treatment, individuals with relatively lower *mTOR* expression had a proportionally larger reproductive investment. This may seem surprising because mTORC1 is required for and thought to promote reproduction (Guo et al., 2018; McLaughlin et al., 2011). The resource re-allocation hypothesis suggests that organisms shift resources between reproduction and somatic maintenance when faced with limited resources (Moatt et al., 2020; Regan et al., 2020), a process mediated by the mTOR pathway. When nutrition is limited, mTORC1 activity is downregulated, triggering alternative pathways (Johnson et al., 2013; Li et al., 2015). In our study, the higher resource re-allocation to reproduction at a lower individual *mTOR* expression may have triggered upregulation of the autophagy pathway and recycling of damaged cell contents as an energy substitution for the nutrient deficit (Adler and Bonduriansky, 2014; Chung and Chung, 2019). The upregulated cellular maintenance helps to preserve the follicle pool and maintain reproductive potential (English and Bonsall, 2019), while activation of autophagy-related genes promotes oocyte maturation (Zhou et al., 2019). Rapamycin treatment, which downregulates mTORC1, was also found to stimulate oocyte maturation by increasing the expression of autophagy-related genes (Lee et al., 2015). In our study, removal of inhibition (i.e. upregulation) of recycling mechanisms may have channelled resources towards reproduction.

In conclusion, this study revealed that resource limitation-induced allocation trade-offs are associated with a differential expression of nutrient-sensing genes. A limited energy budget induces a lower expression of *mTOR* and *IGF1* and a higher expression of *RPS6K1*, *ATG9A* and *ATG5* differently at different restriction levels, and leads to overall lower fitness values. However, individuals showing relatively lower *mTOR* expression invest proportionally more in reproduction, which contradicts the established premise that mTOR mediates resource allocation towards reproduction. This apparent paradox may be resolved by a deeper understanding of mTOR's stimulatory, suppressive and permissive functions.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: G.K.R., S.F.N., L.C., Á.Z.L.; Methodology: G.K.R., S.F.N., B.C., G.G., R.K., C.S., L.C., Á.Z.L.; Software: G.K.R., Á.Z.L.; Validation: G.K.R., L.C., Á.Z.L.; Formal analysis: G.K.R.; Investigation: G.K.R., S.F.N.; Resources: G.K.R., L.C., Á.Z.L.; Data curation: G.K.R.; Writing - original draft: G.K.R.; Writing - review & editing: G.K.R., S.F.N., B.C., G.G., R.K., C.S., L.C., Á.Z.L.; Visualization: G.K.R., Á.Z.L.; Supervision: L.C., Á.Z.L.; Project administration: L.C., Á.Z.L.; Funding acquisition: L.C., Á.Z.L.

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Data availability
All data and R software code are available from figshare: https://doi.org/10.6084/m9.figshare.25558716.v1.

ECR Spotlight
This article has an associated ECR Spotlight interview with Gebrehaweria Reda.

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