Received: 19 September 2023 | Revised: 3 January 2024 | Accepted: 12 January 2024

DOI: 10.1111/mec.17296

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Paternal starvation affects metabolic gene expression during zebrafish offspring development and lifelong fitness

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Funding information
Human Frontier Science Program, Grant/Award Number: HFSP R0025/2015

Handling Editor: Philine G. D. Feulner

Abstract

Dietary restriction in the form of fasting is a putative key to a healthier and longer life, but these benefits may come at a trade-off with reproductive fitness and may affect the following generation(s). The potential inter- and transgenerational effects of long-term fasting and starvation are particularly poorly understood in vertebrates when they originate from the paternal line. We utilised the externally fertilising zebrafish amenable to a split-egg clutch design to explore the male-specific effects of fasting/starvation on fertility and fitness of offspring independently of maternal contribution. Eighteen days of fasting resulted in reduced fertility in exposed males. While average offspring survival was not affected, we detected increased larval growth rate in F1 offspring from starved males and more malformed embryos at 24 h post-fertilisation in F2 offspring produced by F1 offspring from starved males. Comparing the transcriptomes of F1 embryos sired by starved and fed fathers revealed robust and reproducible increased expression of muscle composition genes but lower expression of lipid metabolism and lysosome genes in embryos from starved fathers. A large proportion of these genes showed enrichment in the yolk syncytial layer suggesting gene regulatory responses associated with metabolism of nutrients through paternal effects on extra-embryonic tissues which are loaded with maternal factors. We compared the embryo transcriptomes to published adult transcriptome datasets and found comparable repressive effects of starvation on metabolism-associated genes. These similarities suggest a physiologically relevant, directed and potentially adaptive response transmitted by the father, independently from the offspring's nutritional state, which was defined by the mother.

KEYWORDS
development, non-genetic, paternal, starvation, transcriptome

1 | INTRODUCTION

Dietary restriction in the form of caloric restriction and intermittent fasting is a putative key to the extension of lifespan and health-span (Adler & Bonduriansky, 2014; Kapahi et al., 2017). The benefits of intermittent fasting for organismal health have been reported in a range of invertebrates and vertebrates, including humans (Honjoh et al., 2017; Mitchell et al., 2019; Pan et al., 2022). Despite its beneficial effects on organismal health, dietary restriction may have negative side effects on several life-history traits including reproductive fitness, although the direction of effects reported varies across species and studies. In females, dietary restriction appears to generally have positive effects on egg quality and extend reproductive fitness (Nehra et al., 2012; Sun et al., 2021). In contrast, the effects of diet on male reproduction are less clear. High-calorie diets generally have negative effects on male fertility (e.g. Rato et al. (2014)), whereas the effects of dietary restriction on male fertility and reproduction vary: Dietary restriction had a negative effect on sperm count, sperm motility and capacitation ability in young 2-month-old Sprague–Dawley rats (*Rattus norvegicus*) (Li et al., 2021), but had no effects on ejaculate quality in 2.5-month-old rats that were also exposed to heat stress (Aydilek et al., 2015) and led to a positive effect on ejaculate quality in aged 12-month-old Wistar rats (Jesús et al., 2022). A randomised trial of dietary restriction in obese men showed an increase in sperm count and sperm concentration in the trial group under caloric restriction (Andersen et al., 2022), whereas a systematic meta-analysis on the impact of dietary manipulation on sperm has reported that food reduction led to alterations in ejaculate traits in mammals, arthropods and fish, but the extension of the effects varied across taxa (Macartney et al., 2019). The conflicting results for the effects of dietary restriction on male reproductive fitness suggest species and context dependence and warrant further investigation.

Environmental effects on parental condition may be passed on to the offspring through the gametes in the form of epigenetic information that contributes to regulation of genome activity in the next generation (Immler, 2018; Mousseau & Fox 1998; Rando, 2016; Wolf & Wade, 2009). This phenomenon is generally referred to as intergenerational where the next generation offspring is directly affected (Perez & Lehner, 2019) and transgenerational inheritance, when the transmission of environment information occurs across multiple generations in the absence of the direct exposure (Skinner, 2008). Environmental effects on the mother are widely known to contribute to intergenerational inheritance and the underlying mechanisms have been extensively studied. For example, maternal obesity and hormonal imbalance can predispose the offspring to type 2 diabetes and obesity; or maternal zinc deficiency can impact offspring development reducing birth weight, altering the offspring's lipid metabolism or increasing their resistance to insulin; maternal gestational exposure to cadmium can lead to hormonal changes in the F2; and maternal stress can increase the levels of stress hormones in their grandchildren (Lawless et al., 2023; Lecoutre et al., 2021; Mbiydzenyuy et al., 2022; Şanlı & Kabaran, 2019; Santilli & Boskovic, 2023; Sanusi et al., 2021; Stäubli & Peters, 2021).

In contrast, the consequence and importance of environmental exposure of the father on the subsequent generations is less well understood. One contributing factor to the limited understanding on paternal transgenerational inheritance is the masking effect of variable maternal factors deposited in the egg (i.e. RNAs and proteins) (Rauwerda et al., 2016). Besides, in animals developing in utero, the maternal environment continues to exert direct influence on the growing embryo (Lacal & Ventura, 2018) and makes the quantification of paternal effects on offspring fitness challenging. One way to assess the potential effects of paternal condition on offspring fitness is the analysis of sperm and ejaculate parameters. In house mice (*Mus musculus*) for example, paternal prenatal deficiency of folate led to altered levels of DNA and histone methylation in sperm and the offspring of these males suffered from higher rates of birth defects (Lambrot et al., 2013). Similarly, paternal consumption of alcohol in mice led to impaired foetal and placental growth and altered metabolism of the offspring (Bedi et al., 2022). These alterations in the offspring were accompanied by changes in the levels of sperm H3K4me3, a histone post-translational modification found in promoters of active genes, which correlated with CCCTC-binding factor (CTCF), a transcription factor associated to the three-dimensional organisation of the chromatin. Furthermore, disruptions in CTCF-binding sites were observed in the placenta of offspring from the fathers exposed to alcohol, with associated changes in gene expression in this tissue.

Parental dietary restriction is also known to affect the following generations. In the nematode *Caenorhabditis elegans* for example, dietary restriction resulted in lifespan expansion in the individuals undergoing the treatment with effects carried over for up to three generations (Ivimey-Cook et al., 2021). Similarly, in human populations, famine experienced by one generation may lead to increased risk of hyperglycaemia for at least two subsequent generations (Li et al., 2017). These examples indicate that parental diet can dramatically influence offspring fitness. However, in these studies, neither the mechanism nor the parental origin of the effect of the dietary restriction was investigated. Nevertheless, these examples support the idea that male dietary interventions may have far-reaching consequences not only for the males themselves but also for the next generations. Dietary restriction has been advertised as a new way to extend health- and lifespan but its potential negative side effects are not known.

The physiological responses to dietary restriction are modulated key metabolic pathways and changes in gene expression including changes in well-conserved nutrient sensing pathways such as Peroxisome Proliferator-Activated Receptors (PPARs), the AMP-activated protein kinase (AMPK) pathway or the mechanistic target of rapamycin (mTOR) (Sung et al., 2023). PPAR is a key pathway related to the sensing of fatty acids and is involved in energy metabolism. In fasting conditions, PPARα triggers the production of ketone

bodies through fatty acid oxidation (Hashimoto et al., 2000), which is required by the cells to maintain the energy levels during the lack of sources of glucose. mTOR is inhibited by caloric restriction and may trigger autophagy, favouring the recycling of cellular components and impeding cell growth (Efeyan et al., 2013). The correct function of autophagic activity is also necessary during early embryo development due to the highly dynamic nature of the early cells and the need for quick recycling of cellular components (Wada et al., 2014). These changes are accompanied by alterations in several other pathways because of the metabolic switch experienced by the cells. In the rainbow trout *Oncorhynchus mykiss* for example, food deprivation induced changes in the expression of glucose, lipid metabolism, blood function and immune response-related genes in the liver (Salem et al., 2007). Lipid and insulin metabolism-related genes are also deregulated in a model of skeletal muscle aging in rats exposed to caloric restriction between 15 and 30 months of age (Ham et al., 2022). However, it is unclear whether and how these changes in nutrient sensing and metabolic rates in starved individuals may cause lasting effects that are transmitted to the next generation.

While the transmission mechanisms of parental condition to offspring are currently not fully understood, this transmission of information associated with parental life experiences across generations may have a wider ecological and evolutionary impact (Ashe et al., 2021; Lind & Spagopoulou, 2018; Skinner & Nilsson, 2021). Whether these responses are simple side effects and deleterious or anticipatory beneficial responses with a potential role in adaptation is currently debated. Offspring of zebrafish exposed to hypoxia were found to be more resistant to this condition (Ragsdale et al., 2022) and in *C. elegans*, parental infection with the pathogen *Pseudomonas vranovensis* promoted offspring resistance to subsequent infections (Burton et al., 2020). In contrast, dietary restriction in *C. elegans* negatively affected the next up to three generations, regardless of their environment (Ivimey-Cook et al., 2021). The effects of parental conditions on the next generations likely depend on the environmental factors in question, the species and the parental origin and disentangling maternal from paternal effects is key.

In our study, we assessed the importance of paternal starvation for male reproductive fitness and its potential effects on following generations using zebrafish as a model species. Zebrafish represent an excellent model for paternal effect studies because they are external fertilisers and embryos develop *ex utero*, which allows to perform in vitro fertilisation (IVF) experiments to control for maternal effects by using a split batch design, where each male fertilises eggs from more than one female and each female has her eggs fertilised by more than one male. We exposed male zebrafish to a period of starvation as a dietary restriction intervention and tested its effects on male fertility assessed by ejaculate quality and fertilisation success rates, offspring development and embryo metabolic gene expression profiles and offspring reproductive later in life.

2 | MATERIALS AND METHODS

2.1 | Animal model

We used wild-type Zebrafish *Danio rerio* from the AB strain obtained from ZIRC (Zebrafish International Resource Center, University of Oregon, Eugene, USA) and maintained at the SciLifeLab zebrafish platform at Uppsala University (https://www.scilifelab.se/facilities/zebrafish/) and the Controlled Ecology Facility (CEF) at the University of East Anglia (UEA, UK). The fish were kept in 3-L tanks in a recirculating rack system (Aquatic Habitats [Uppsala] and Techniplast [UEA]) at 26.4 ± 1.4°C and a 12:12 diurnal light cycle. Prior to the experiments, fish were fed three times a day with a mixture of dry pellets and live artemia.

2.2 | Feeding regime

For the experiments, males were randomly split into 3-L tanks assigned to the two experimental groups, referred to as fed and starved (Figure 1). Each male was assigned an ID number and weighed and imaged for posterior identification through pigmentation and fin shape features. Males were maintained with wild-type companion females at a total fish density of 10–16 fish per 3-L tank for the duration of the experiment. For each experiment, we replicated the number of tanks ranging from a minimum of three up to seven tanks per treatment. The numbers vary for the different traits as not all fish always laid or produced gametes for IVF. Upon splitting into experimental groups, fish in the starvation treatment were completely deprived of food while fish from the fed treatment were kept in the ad libitum feeding regime where they were fed three times a day under standard lab conditions (mix of dry pellets and live artemia three times a day). The starvation conditions were kept for 18 days (Figure 1). In zebrafish, a full cycle of spermatogenesis takes three weeks on average with the time between the last mitotic division and the sperm release into the spermatogenic tubule being six days (Leal et al., 2009). Here, the 18 days of starvation allowed exposure for one spermatogenic cycle.

2.3 | In vitro fertilisation

On day 18, fish were put into breeding tanks and separated from wild-type females with dividers, allowing visual and olfactory contact. Breeding tanks were then covered with black cloths to avoid any light-induced oviposition the following morning (Westerfield, 2007).

Females and males were prepared for in vitro fertilisation and anaesthetised using 1.0–3.0 mg/L metomidate hydrochloride (AquacalmTM). Males were weighed to control for the effects of feeding regime and imaged to match their ID with the data collected on day 0 (fed males $n = 49$, starved males $n = 49$). Males were then

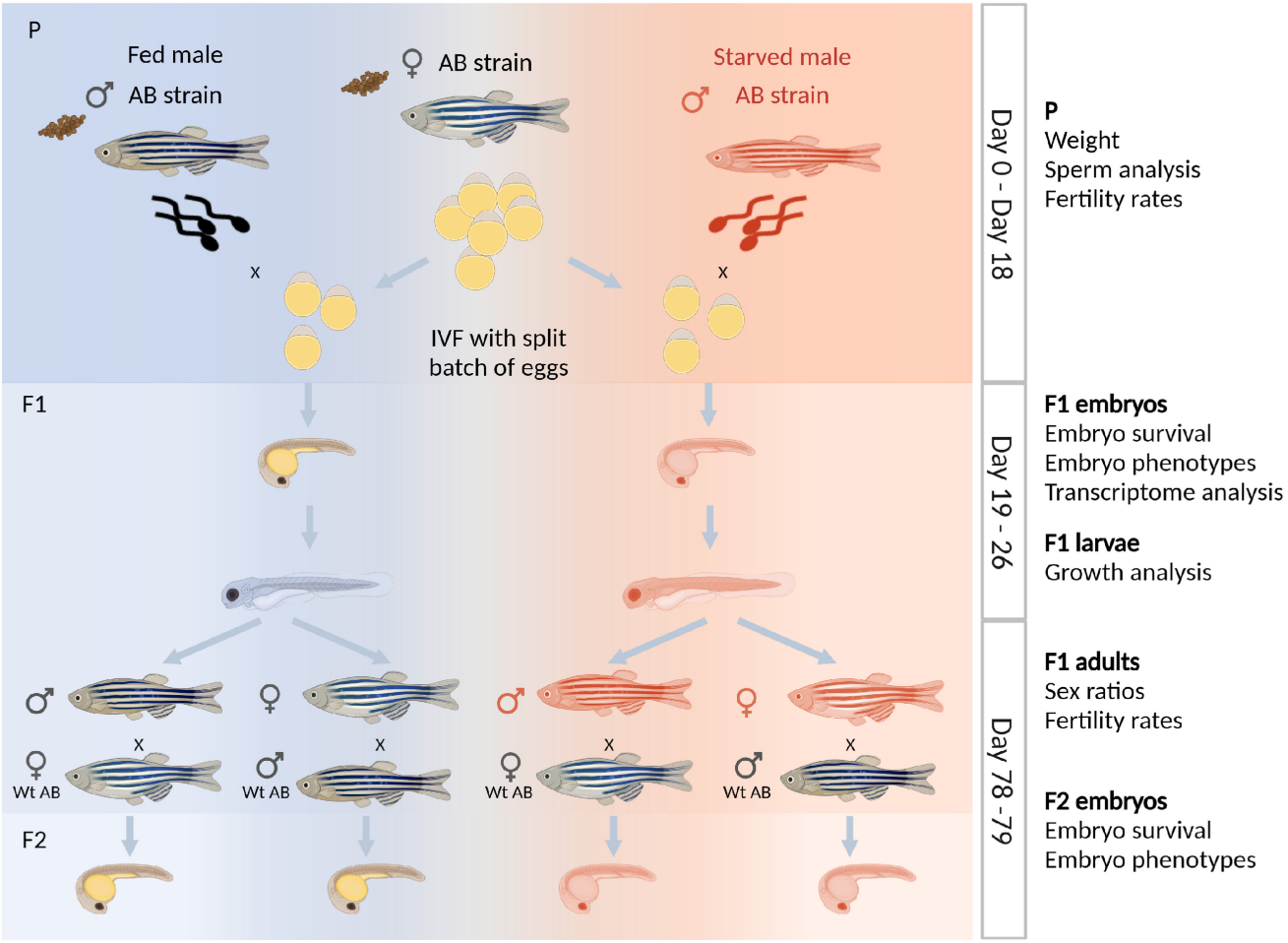


FIGURE 1 A zebrafish model to study the intergenerational effects of paternal starvation. Experimental design for IVF using a split-clutch design: All males from the AB strain were weighed at the beginning of the experiment (Day 0) and then randomly split into fed and starved groups. Starved males were fully deprived of food whereas fed males were fed a standard diet of a mix of dry and live (artemia) food three times a day. All males were kept with an equal number of females for the duration of the experiment. After 18 days, males were weighed again, and ejaculates were collected. Eggs were collected from healthy wild-type AB non-experimental females and split into two equal halves for IVF. Sperm from one fed and one starved male, respectively, were used to fertilise one half of the clutch of eggs. The sperm collected was used to fertilise eggs from two different females. On day 19, embryos were collected at prim-5 stage (24 hpf) for transcriptome analyses. Larval length was measured on days 5 and 8 postfertilisation. Part of the F1 larvae was grown to adulthood. F1 males and females were crossed to wildtype AB fish and their offspring was obtained by natural spawning. The phenotypes of the F2 embryos were studied at 2 and 24 hpf. The timeline of the experimental setup and data collected is shown on the right side of the design. Created with BioRender.com.

placed on a soft and wet sponge and squeezed gently in cranio-caudal direction to collect the ejaculate under a dissecting microscope (Nikon SMZ800). From each male, 0.2–0.8 μL of ejaculate were collected and transferred into a 0.2-mL Eppendorf tube containing 80 μL of Hank's buffer (HBSS) and kept on ice for 5–10 min until IVF. Females were placed on 15-cm Petri dishes and gently stripped to obtain eggs. Clutches used for IVFs contained 20–300 high quality eggs and they were used within 1 min after stripping. To create the first generation (F1), we used a split clutch design to perform IVFs (Figure 1). Sperm samples were mixed gently, and each ejaculate and egg clutch were divided into two parts. In vitro fertilisation was performed simultaneously for both experimental groups. The sperm from each experimental male was used to fertilise split clutches from two different females when sperm availability allowed this second fertilisation. The sperm of males from seven independent tanks was used in seven rounds of IVF.

The reproductive success for each fish was assessed by analysing the number of fertilised eggs at 2h post-fertilisation (hpf) (fed fathers: 36.265 ± 24.870, $n = 3874$; starved fathers: 41.592 ± 22.412, $n = 3947$; data shown as mean ± SD), embryo survival at 24 hpf (fed males: 31.673 ± 23.112, $n = 1777$; starved males: 35.3273 ± 21.697, $n = 2038$; data shown as mean ± SD) and embryo defects at 2 (embryos from fed males 6.020 ± 6.217; embryos from starved males 5.041 ± 4.247; data shown as mean ± SD) and 24 hpf (embryos from fed males 4.592 ± 5.697; embryos from starved males 6.265 ± 6.360; data shown as mean ± SD).

Eggs were considered unfertilised if there was no cell division within 2 h after collection or were classified as dead if they were green or black in colour. Embryos were compared with diagrams describing the embryonic zebrafish development to study any abnormalities (Kimmel et al., 1995). Those embryos deviating from the diagrams were classified as abnormal. Embryos at 24 hpf were classified as dead if they had no yolk or showed abnormal black colour.

2.4 | Natural spawning and reproductive fitness

The reproductive fitness of all experimental males (P) was studied by crossing them with non-experimental wild-type female fish from an independent AB population by natural spawning (fed males $n=27$, starved males $=32$). One day before natural spawning, fed and starved males were individually paired up with AB females in individual breeding tanks and were kept separated by a divisor, allowing for visual and olfactory contact. The next morning, the divisor was removed to let each pair spawn. Breeding tanks were checked every half hour and eggs were collected and transferred into Petri dishes containing a E3 and 0.1% methylene blue solution to avoid fungal growth. Plates were kept in an incubator set at 28°C.

Reproductive success for each experimental male in P was assessed by analysing the number of fertilised eggs (fed males: 112 ± 53.778, $n=2784$. starved males: 88.688 ± 42.815, $n=1608$; data shown as mean ± SD). The data were collected from three independent rounds of breeding with males from four separate tanks per treatment.

A standardised number of F1 offspring sired upon IVF with fed or starved were reared into adulthood for assessment of their reproductive fitness males (13.957 ± 8.544, from 23 fed or starved fathers). F1 larvae were kept in groups of 50 and, at the age of 2 months, fish were re-distributed into 3-L tanks in mixed sex groups of 14–16 fish per tank (survival at 2 months in fish from fed fathers 10.22 ± 8.18, survival in fish from starved fathers 10.57 ± 7.70). F1 males and females were crossed by natural spawning with wild-type females or males, respectively, following the procedure described above.

Similarly to the P males, the reproductive success of F1 males was assessed analysing fertilised eggs (Fertilised eggs of females from fed fathers: 109 ± 59.556, $n=944$; fertilised eggs of females from starved fathers: 94.905 ± 64.10, $n=2327$; eggs fertilised by males from fed fathers: 114.167 ± 88.21, $n=1398$; eggs fertilised by males from starved fathers: 174.67 ± 103.65, $n=535$; shown as mean ± SD), embryo defects at 2 hpf (64-cells) (number of abnormal eggs from offspring of fed fathers: 2.1 ± 2.73, $n=2342$. Number of abnormal eggs from offspring of starved fathers: 1.52 ± 2.54, $n=2862$; shown as mean ± SD), embryo survival at 24 hpf (live embryos from offspring of fed fathers: 71.75 ± 64.142, $n=1435$; live embryos from offspring of starved fathers: 80.29 ± 55.64, $n=1927$; shown as mean ± SD), and embryo defects at 24 hpf (abnormal embryos from offspring of fed fathers: 0.3 ± 0.57; abnormal embryos from offspring of starved fathers: 10.0 ± 29.33; shown as mean ± SD).

2.5 | Embryo/larvae phenotype

Upon confirmation of fertilisation (2 hpf), we placed individual fertilised eggs onto 12-well plates for monitoring of embryos phenotypes which included:

1. Hatching rate every 2 h from 48 to 58 hpf (healthy embryos from fed fathers at 48 hpf: 1.25 ± 2.81, embryos from starved fathers at 48 hpf: 1.64 ± 2.79, embryos from fed fathers at 50 hpf: 6.36 ± 6.993, embryos from starved fathers at 50 hpf: 5.21 ± 5.62, embryos from fed fathers at 52 hpf: 11.93 ± 8.37, embryos from starved fathers at 52 hpf: 11.214 ± 7.089, embryos from fed fathers at 54 hpf: 16.18 ± 8.78, embryos from starved fathers at 54 hpf: 14.964 ± 7.1, embryos from fed fathers at 56 hpf: 18.11 ± 8.46, embryos from starved fathers at 56 hpf: 17.25 ± 6.995, embryos from fed fathers at 58 hpf: 22.26 ± 5.95, embryos from starved fathers at 58 hpf: 21.652174 ± 4.386; shown as mean ± SD). Hatching rate was defined as the number of larvae that hatched at each time point analysed out of the total number of live embryos.
2. Yolk utilisation at 24 hpf and 5 days post-fertilisation (dpf) (yolk diameter in embryos from fed fathers at 24 hpf: 0.6387 ± 0.0277 mm, yolk diameter in embryos from starved fathers at 24 hpf: 0.639 ± 0.026 mm, yolk area in embryos from fed fathers at 5 dpf: 0.00476 ± 0.00168 cm^2, yolk area in embryos from starved fathers at 5 dpf: 0.0044 ± 0.0016 cm^2, yolk length in embryos from fed fathers at 5 dpf: 3.83 ± 0.12 mm, yolk length in embryos from starved fathers at 5 dpf: 3.802 ± 0.14 mm)
3. Growth rate between 5 and 8 dpf (length of larvae from fed fathers at 5 dpf 3.82 ± 0.12 mm, length of larvae from starved fathers at 5 dpf 3.808 ± 0.139 mm, length of larvae from fed fathers at 8 dpf 4.28 ± 0.20 mm, length of larvae from starved fathers at 8 dpf 4.32 ± 0.17 mm).

Embryos from fed fathers included in the hatching rate analysis $n=610$, embryos from starved fathers $n=611$. For the yolk utilisation and the growth rate analyses, a picture of each embryo was taken under a dissection microscope at 24 hpf, 5 dpf and 8 dpf. The measurements were taken for yolk diameter at 24 hpf (embryos from fed fathers $n=252$, embryos from starved fathers $n=258$), larval length (larvae from fed fathers $n=175$, larvae from starved fathers $n=180$), yolk length and lipid droplet at 5 dpf (larvae from fed fathers $n=70$, larvae from starved fathers $n=80$) and larval length at 8 dpf (larvae from fed fathers $n=287$, larvae from starved fathers $n=278$).

2.6 | Computer-assisted sperm analysis

To assess sperm motility, we used computer-assisted sperm analysis (CASA; ISAS; Proiser, R+D, S.L.). We used 2 µL of ejaculate, which was activated with 3 µL of tank water at 28°C on a Cytonix 4 Chamber slide (MicroTool B4 Slide, 20 µm depth). A recording taken every 10 s starting 10 s post-activation until 60 seconds post-activation. We

recorded sperm movement using a brightfield microscope (UOP UB203i trinocular microscope; Proiser) at 100× magnification and a black and white video camera (782 M monochrome CCD progressive camera; Proiser). The recordings were analysed using ISAS v1 software (Proiser) with the following settings: frame rate: 50 frames/s; frames used: 50; particle area: 5–50 μm2; threshold measurements for VCL: slow, 10–45 μm/s; medium, 45–100 μm/s; rapid, >100 μm/s (Alavioon et al., 2017).

2.7 | RNA extraction

For transcriptome analysis, we used one to three 24-hpf embryos from each clutch that were manually dechorionated and flash frozen in 1.5 mL Eppendorf tubes. RNA extraction was performed with the RNeasy micro kit (Qiagen) following the manufacturer's instructions. Briefly, samples were lysed in RLT buffer passing the embryos through a needle and syringe. Lysates were passed through a MinElute spin column and centrifuged for 15 s at 13000*g*. Samples were washed with buffer RW1 and treated in-column with DNase I diluted in buffer RDD for 15 minutes at room temperature (RT). The digestion reaction was stopped with RW1 buffer and centrifuge step. Columns were washed with RPE buffer and 80% ethanol followed by a 5-minute centrifuge step to remove any ethanol residues. Finally, we eluted the RNA in 14 μL of elution buffer and proceeded with quality assessment by TapeStation HS RNA tapes (Agilent) (Table S1).

2.8 | Library preparation and RNA-seq

The library preparation was performed with the Lexogen QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen). RNA was normalised to 13.5 ng/μL and the RNA input for prep = 67.5 ng per sample. The protocol for high-quality samples was used. SIRV set 3 were spiked into the samples at a very low level to allow technical evaluation of library prep and sequencing performance.

Eighteen cycles of PCR were performed. Libraries were quantified with Picogreen assay (Fisher Scientific) and sizes were determined with a HS D1000 tape (Agilent) (Table S2). Libraries were pooled to 4 nM final concentration and spiked with 1% phiX and processed on a NextSeq flow cell (150 cycles mid output) for sequencing on a NextSeq 500.

2.9 | RNA-seq analysis

Following Lexogen guidelines, sequencing adapters, polyA read through and low-quality tails were removed by using the *bbduk* tool of the *bbmap* package (parameters: *k* = 13, ktrim = *r*, useshortkmers = *t*, mink = 5, qtrim = *r*, trimq = 10, minlength = 20) (Bushnell, 2014). RNA-seq reads were then mapped to the zebrafish reference genome (GRCz11.96) using STAR with default parameters (version: 2.6.0c) (Dobin et al., 2023). Gene expression levels were estimated by the read counting module embedded within the STAR tool (–quantMode GeneCounts). Differentially expressed genes were identified by using edgeR (Robinson et al., 2010) using the TMM method to normalise raw read counts, whereas common, trended and tagwise dispersions were estimated by maximising the negative binomial likelihood (default). Lowly expressed genes were removed (only genes expressed >1 TMM in at least 50% of the samples were retained) and differentially expressed genes were tested performing quasi-likelihood *F*-tests (glmQLFit and glmQLTest), including the maternal genetic background of each embryo pair in the design of the linear model. Only genes showing FDR < 0.1 were considered as differentially expressed (115 downregulated genes and 30 upregulated). GO enrichment analysis was performed by using topGO (Alexa & Rahnenfuhrer, 2022) on the GO terms associated with the up- and down-regulated genes separately. In both analyses, GO terms associated with genes expressed >1 TMM in at least 50% of the samples were used as background. The statistical significance of the enrichments was tested with a Fisher's Exact Test (algorithm = 'weight'), then, GO terms associated with less than two significant genes were discarded prior to FDR calculation (Benjamini & Hochberg, 2023). Significant threshold was imposed to FDR < 0.1.

To investigate the impact of random effects in our model, we used the R package glmmTMB2 to fit a negative binomial distribution to the gene expression levels, including condition (starved/fed) and maternal contribution as fixed effects and fish id as random intercepts. We applied the same thresholds to discard lowly expressed genes and to define a given gene as up-regulated, down-regulated or non-differentially expressed than the ones used in the previous edgeR analysis. In this second analysis, we identified 733 up-regulated genes and 802 down-regulated genes in starved versus fed fish. Of note, 19 of the 30 (63%) edgeR up-regulated genes and 82 of the 115 (71%) edgeR down-regulated genes were significantly up- or down-regulated according to the new glmmTMB analysis. When performing a gene ontology enrichment analysis on the up- and down-regulated genes highlighted by the glmmTMB analysis, we recapitulated the main results previously obtained by running the GO analysis on edgeR up- and down-regulated genes. While 1 of the 7 (14%) GO terms significantly enriched in up-regulated genes as defined by glmmTMB was also enriched in up-regulated genes identified with edgeR, virtually all the GO terms enriched in glmmTMB down-regulated genes were also enriched in edgeR down-regulated genes (Figure S4).

The gene co-expression network helps to identify genes with similar expression patterns forming clusters of expression in multiple samples. The network in this study was built with BioLayout V. 3.4 using expression values for the differentially expressed genes as input. The Pearson correlation cut-off was set to 0.94 and Markov clustering (MCL) approach was used to determine clusters of co-expression. The protein–protein interaction (PPI) network was built on STRING (V. 11.5) using the ensemble gene IDs of the DEGs and calling clusters of interaction through MCL with inflation parameter set to 2. STRING provides information on predicted

interactions and experimentally proved interactions give higher priority to those in zebrafish but it also includes proved interaction in other organisms. KEGG pathway and anatomical term enrichments of the PPI network clusters and DEG, respectively, were studied with ShinyGO. The chromosomal ideogram was made with Phenogram (Wolfe et al., 2013).

The intersection between DEGs in intestines of starved adults (Jawahar et al., 2022) and the offspring of starved males (this study) was done using the merge function in R. The odds ratio of the common expressed genes in the two datasets was calculated by using medcalc (https://www.medcalc.org/calc/odds_ratio.php).

Single-cell RNA-seq data from 24 hpf embryos (Lange et al., 2023) were used as reference to deconvolute anatomical information from our DEGs detected upon bulk RNA-seq data analysis. We intersected the list of differentially expressed genes in the 24 hpf embryos from our study with the reference list of genes expressed at this same stage across the various cell types of the embryo. We followed the detailed protocol in Marquez-Galera et al. (2022). Briefly, the anndata object was converted to an h5Seurat file and read into a seurat object. Single-cell data counts were normalised and PCA, and TSNE dimensionality reductions were applied. DEGs from bulk RNA-seq were then read and intersected with the single-cell data and shifts in the cell populations in the dimensionality reductions were used as indicators of cell populations enriched on these gene sets.

2.10 | Statistical analysis

All analyses were conducted using the statistical software R v. 4.1.2 (R Core Team, 2023). Linear models (LM) were fitted using the *lm* function, linear mixed-effects models were applied using *lmer* function and generalised linear mixed-effects models (GLM) were applied using the *glmer* function from *lme4* package on phenotypic data following a binomial response (e.g. embryo survival and fertilisation success). We included traits of interest as response variables, and treatment, time and sex where applicable as fixed effects with tank ID, Block and IVF round as random factor. We excluded random factors that explained no variation to avoid overparametrisation of the models. All full models used for each trait are presented in Data S1.

ANOVA type III was used to assess significance for individual fixed effects in each model. Figures for the phenotypic data, KEGG pathways, tissue specificity and transcription factor predicted motifs were produced with the *ggplot2* package.

3 | RESULTS

We exposed adult zebrafish males to one of two treatments: a fed treatment (control) where males were fed a standard *ad libitum* diet of dry and live (artemia) food three times a day (hereafter referred to as *fed* males) and a short-term starvation treatment where males were deprived of food for 18 days (hereafter referred to as *starved* males) (Figure 1). Starved males lost 21.83% of their weight by the end of the experimental period (day 18), an effect that was confirmed in three separate experiments (Figure 2a; Figure S1, Table S3) while fed males showed no significant change in weight. We assessed the effects of starvation on ejaculate parameters as a first indicator of possible effects on the germ cells and reproductive fitness which could lead to intergenerational effects (Tables S4–S6). We found no significant difference in sperm density between starved and fed males (Figure 2b) but sperm from starved males were overall slower (Figure 2c,d). We found a significant association between post-experimental male body weight and both total sperm number and sperm density. This association was positive for total sperm number (Table S2, Figure S1b), whereas sperm density showed a positive association with male body weight in fed males but a negative association in starved males (Table S8, Figure S1c). Fertilisation success did not differ between males from the two treatments in IVF assays (Figure 2e; Table S9) but it was significantly lower in starved males in natural spawning with wild-type non-experimental females compared to fed males (Figure 2f; Table S10). Similarly, the total number of eggs was reduced upon natural spawning in the starved males (Figure 2g).

3.1 | Paternal starvation effects on offspring survival, growth and reproductive success

When studying offspring survival and embryo malformations, we found no significant effect during the first 24 hpf or the first 2 months post-fertilisation (Figure 3a, Tables S11, S12). However, larvae from starved males were slightly but not significantly smaller at 5 dpf and grew faster compared to their half-siblings sired by fed males ending up significantly larger at 8 dpf showing no other morphological differences (Figure 3b; Table S13).

We further assessed hatching rate, which is known to be affected by a range of variables during embryo development (Pype et al., 2015; Scopel et al., 2021; Zajitschek et al., 2014). We checked for hatched larvae every 2 h between 48 and 58 hpf and found that the total hatching rate was slower in the clutches sired by starved males compared to the clutches sired by fed males (Figure 3c; Table S14).

During their first days of life, zebrafish embryos rely entirely on the nutrient supply from the yolk which is gradually absorbed until the embryo becomes a free feeding larva at 5 dpf (Anderson et al., 2011; Huang & Linsen, 2015). Therefore, as an indicator of nutrient consumption and metabolic activity, we measured the size of the yolk (length, diameter, and area) at 24 hpf and at 5 dpf. We found no significant effect of paternal starvation on yolk size at either time point (Figure S2a–c, Table S15–S17).

The sex ratio in the offspring upon reaching sexual maturity did not differ between starved and fed males (Figure S2d). When setting up male and female F1 offspring from starved and fed males with wild-type non-experimental fish for natural spawning, we

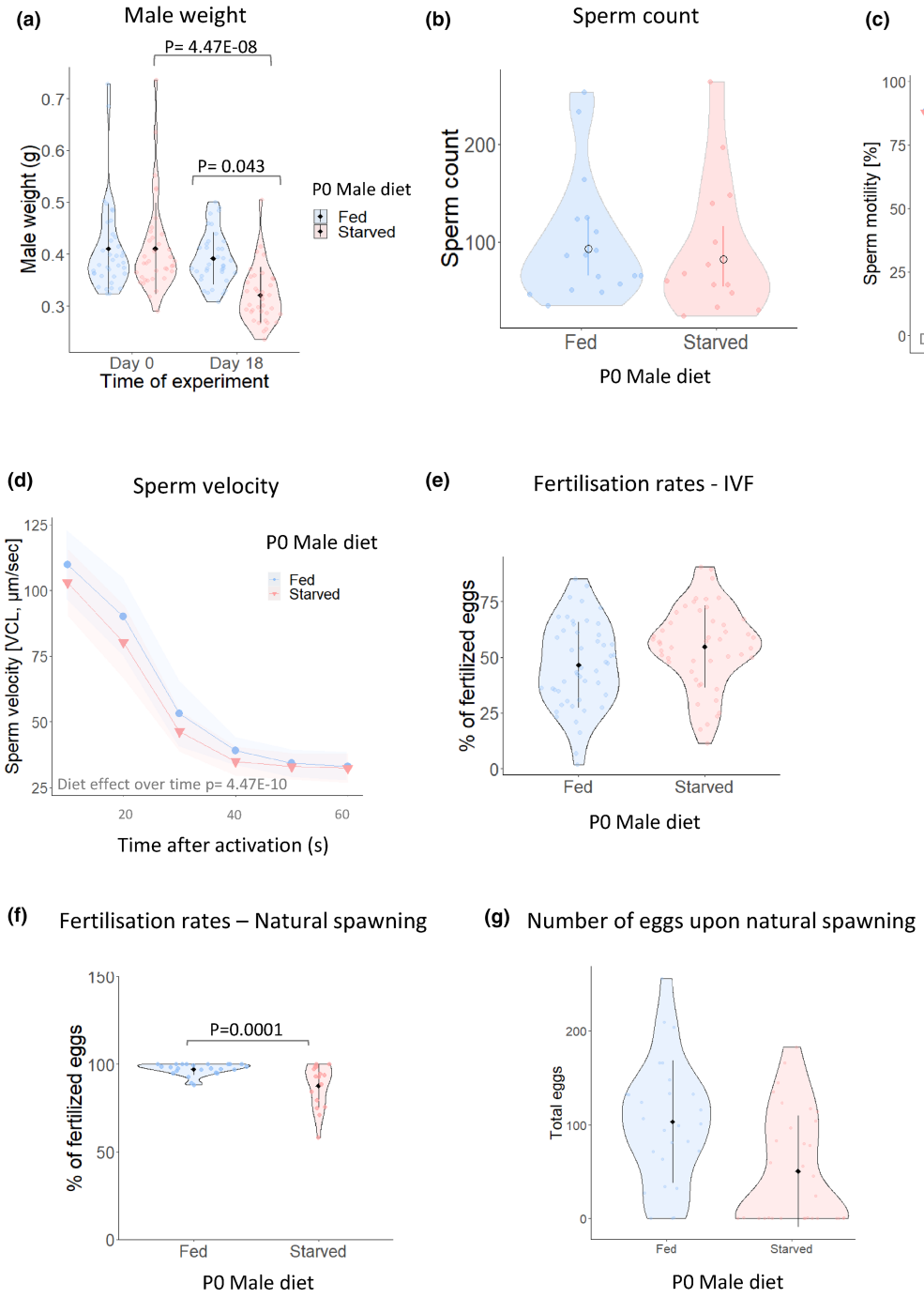


FIGURE 2 Starvation leads to altered male fertility traits. (a) Weight of fed and starved males on days 0 and 18 of the experiment. On day 18, the weight of starved males was significantly reduced by 21.83% in 3 independent experiments (starved group Day 0: 0.41 ± 0.08, Day 18: 0.321 ± 0.05, mean ± SD). Individual data points are represented as dots within the violin plots. $N = 37$. (b) Sperm count in fed and starved males. Bars represent the mean and 95% confidence intervals. (c) Percentage of sperm motility in fed and starved males across 6 time points after sperm activation. (d) Sperm velocity in fed and starved males at 6 time points after sperm activation. (e) Percentage of fertilised eggs at 64-cell stage (2 hpf) and produced by IVF. Individual data points within the violin plot represent the average egg survival rate per male and across 7 independent tanks. $N = 49$. (f) Percentage of fertilised eggs at 64-cell stage (2 hpf) and produced by natural spawning, Fed males $N = 27$, Starved males $N = 32$. Individual data points within the violin plots represent the average egg survival rate per male and across 3 independent rounds of breeding (g). Number of eggs produced per non-experimental females during natural spawning with fed or starved males. Black bars represent the mean ± SD. Shadowing in panels c and d represents 95% confidence intervals.

found no overall difference in fertilisation success, abnormal eggs or offspring survival during the first 24 hpf (Figure S2e; Figure 3d, Figure 4a, Tables S18–S20). However, we found a significantly higher percentage of abnormally developing F2 embryos at 24 hpf produced by the F1 offspring of starved males compared to the clutches produced by the F1 offspring of fed males (Figure 4b; Table S21).

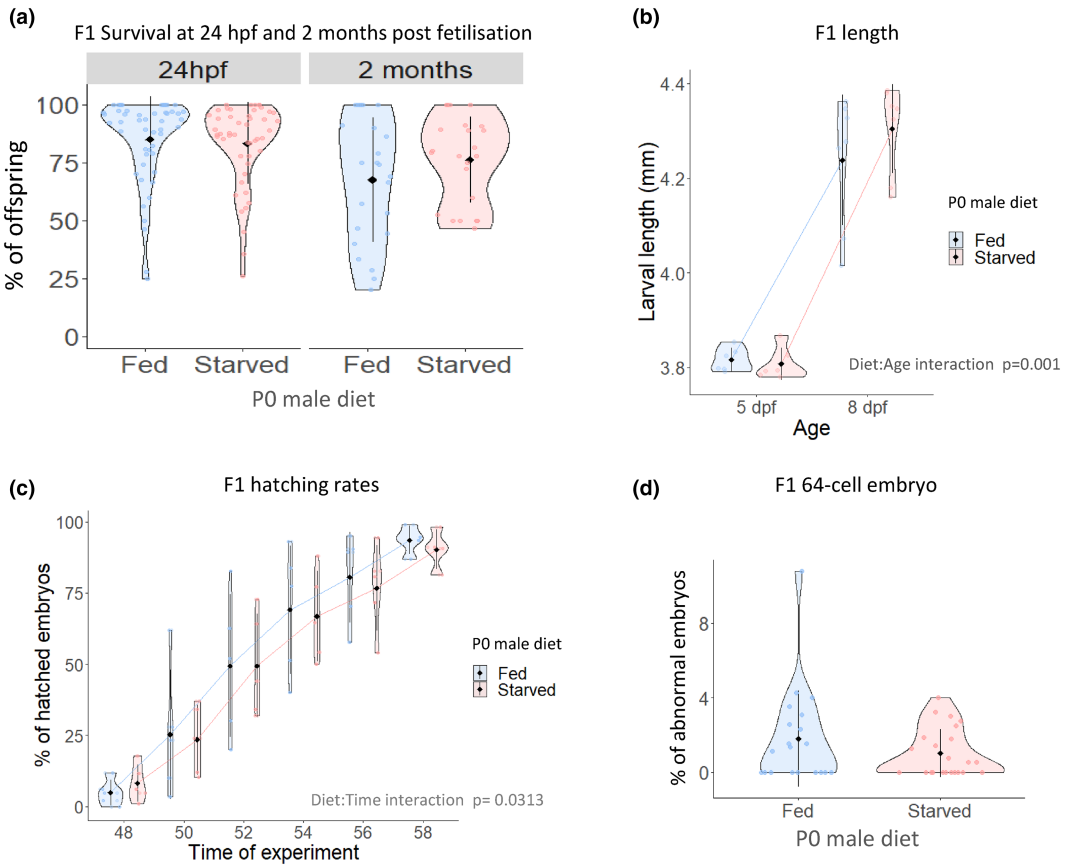


FIGURE 3 Paternal starvation significantly enhances larval growth but delays hatching. (a) F1 survival at 24 hpf (Left): The analysis of several embryo clutches revealed no statistically significant changes in survival between embryos from fed and starved fathers. Individual data points within the violin plot represent the average egg survival rate per male and across 7 independent tanks. F1 survival at 2 months post fertilization (Right). No significant differences were found at this stage. Individual data points within the violin plots represent average offspring survival (b). Larvae length: Larval growth was measured at 5 and 8 dpf in larvae from several clutches. Individual data points in the violin plot represent the average of larvae length per tank. (c) Hatching rates: The percentage of hatched embryos was analysed every 2 h between 48 and 58 hpf. (d) F1 abnormal eggs as determined at 64-cell stage (2 hpf). Eggs from males and females from fed and starved fathers were collected and analysed upon natural mating. No statistical differences were observed in the egg quality between groups or between groups and either sex.

3.1.1 | Impact of paternal diet on the F1 transcriptome

To trace the potential molecular changes underlying the observed phenotypic effects of paternal diet on early embryo development and adult reproductive fitness in the offspring, we explored the transcriptome of prim-5 embryos (24 hpf), a stage of development characterised by fast growth and dynamic expansion of transcriptome repertoire (White et al., 2017), which represents the most conserved transcriptomic stage among anamniotes fitting with the developmental hourglass model (Marlétaz et al., 2018). Prim-5 embryos from starved and fed males were generated in split-clutch IVF assays as described above.

Embryo transcriptome data showed significant variation as a result of maternal effects (Figure S3). Despite this variation in embryo transcriptomes, our analyses revealed significant effects of paternal diet on the transcriptome of F1 embryos. Differential gene expression analysis of 3′ end transcript identified 145 differentially expressed genes (DEGs) between embryos from starved and fed males (Figure 5a–c). The majority (115 genes) of the DEGs were downregulated in offspring from starved males while a smaller subset (30 genes) was upregulated (details in Table S22).

Next, we sought to understand the biological relevance of the observed gene expression changes. A GO term analysis of the 115 downregulated genes revealed significant enrichment for genes associated with lipid metabolism and lysosome (GO terms such as 'lipoprotein metabolic process', 'regulation of triglyceride catabolic process', 'positive regulation of fatty acid biosynthetic process', 'cholesterol homeostasis', 'cholesterol binding' and 'lysosome') (Figure 5d). In contrast, the GO term analysis of the 30 upregulated genes showed an enrichment for genes related to muscle development and contraction (GO terms such as 'skeletal muscle tissue development', 'sarcomere organisation' and 'myofibril assembly') (Figure 5e). Then we asked whether the genes showing similar trends of regulation are potentially co-regulated for example by chromosomal topological constraints. Gene location analysis indicated that DEGs were distributed across the entire genome, but we found small clusters of genes located on chromosomes 5 (*myhz* and *c9*), 12 (*ctsl*)

and 16 (*apoa* and *apoc*) (FDR <0.01) (Figure S5). These genes are related to muscle development, cellular response to oestrogen stimulus, proteolysis and lipid transport respectively. The deregulation of these clusters suggests a potential co-regulation of the expression of these genes. We explored this further by building a gene co-expression network to which we applied a Markov cluster algorithm (MCL clustering). Gene co-expression networks help to determine whether certain genes expressed similarly form clusters of gene expression and can highlight potential regulatory mechanisms (Gaiteri et al., 2014; Shi et al., 2010). In our analyses, the gene co-expression network showed that several of the genes clustering together on a chromosome were also found within the same co-expression network clusters (Figure S6a–c; Clusters 1, 4 and 5. A full list of the genes in each cluster is in Table S23).

To further understand the relationship between the DEGs and their gene products, we created a PPI network and determined clusters of interaction by applying the MCL clustering approach. Based on known, experimentally proved interactions in zebrafish and other organisms, and predicted interactions, this analysis revealed nine clusters of interacting proteins for the downregulated genes (clusters 1–5 showed enriched GO terms for lipoprotein metabolic process, proteolysis, monosaccharide metabolic process, ribosomal large subunit assembly and deoxyribonucleotide metabolic process respectively) and two interaction clusters for the upregulated genes (cluster 1 enriched for GO terms associated to muscle contraction and cardiac muscle contraction, among others) (Figure S7a,b). The analysis of the KEGG pathways enriched within these clusters revealed terms such as PPAR signalling and autophagy (Figure 6a) whose activity is related to the nutritional status of the cell including lipid metabolism. We found no KEGG pathways significantly enriched for the interacting genes in clusters 1 or 2 of the upregulated genes PPI network.

When interrogating the tissue localisation of the pool of downregulated genes based on the anatomy information in the generif database compared to a background list of the total genes expressed at 24 hpf, we found significant enrichment in the yolk syncytial layer (YSL), yolk, the enveloping layer (EVL), pancreatic bud and liver primordium (Figure 6b). We sought to further explore the tissue specificity of expression of DEGs at 24 hpf. To this end, we used existing single-cell RNA-seq dataset produced by the Zebrahub partners (Lange et al., 2023) to which we applied a dimensionality reduction approach (Marquez-Galera et al., 2022) to differentiate between cell-type-specific signals in bulk RNA-seq DEGs according to the single-cell RNA-seq data at 24 hpf. This approach allows us to increase the accuracy of the spatial distribution of the DEGs as well as the specificity to developmental stages

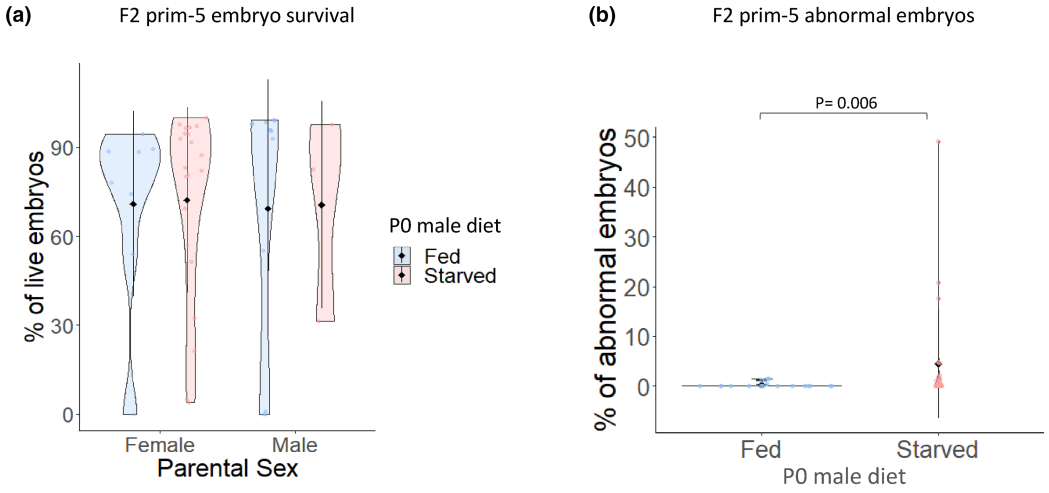
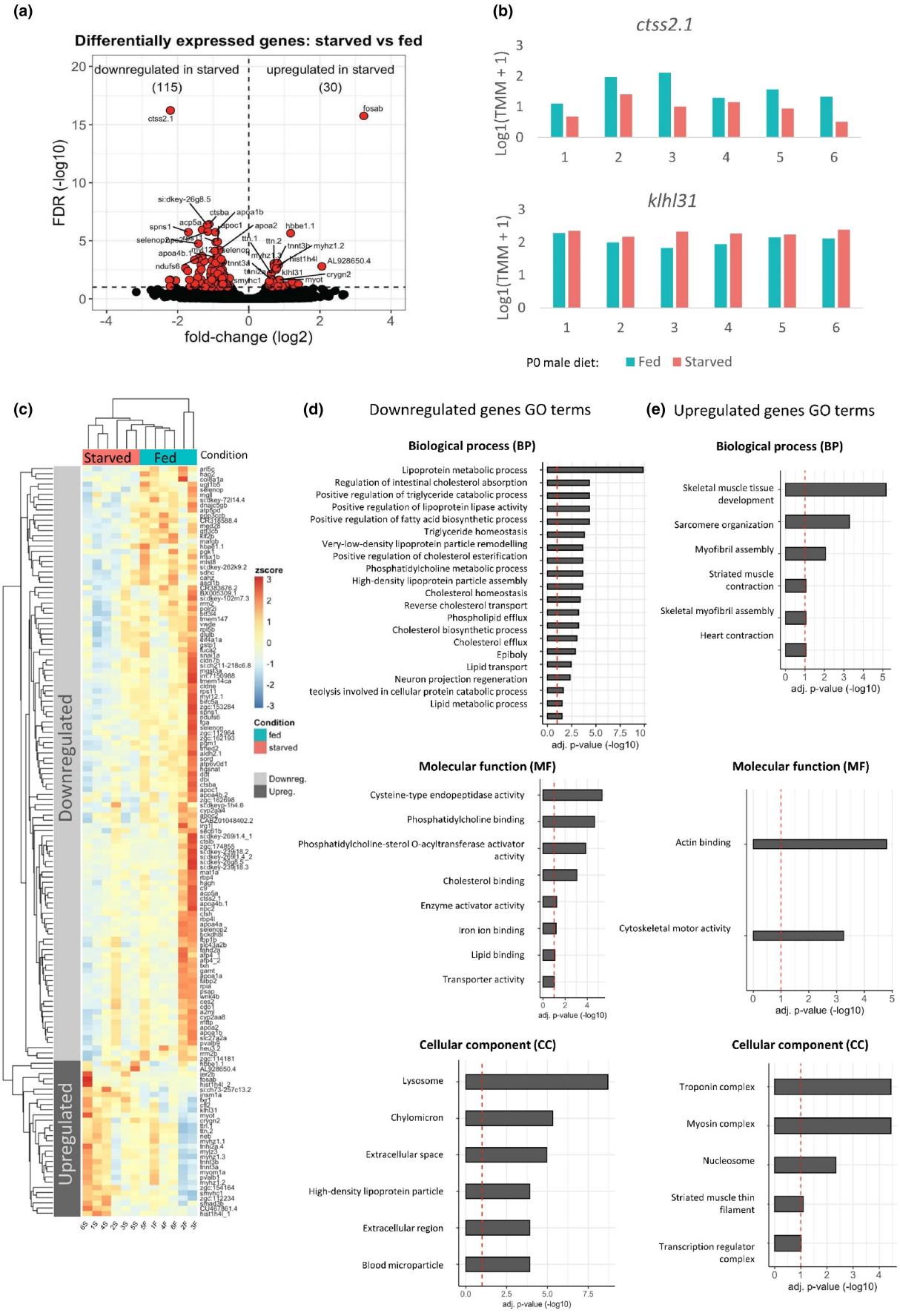


FIGURE 4 Paternal starvation does not alter F2 survival but is associated with F2 malformations at 24 hpf. (a) F2 survival at prim-5 (24 hpf): The analysis of several embryo clutches revealed no statistically significant changes on survival between embryos produced by the F1 (either females or males) of fed and starved fathers. Individual data points within the violin plot represent the average embryo survival rate per individual and produced by natural spawning. (b) F2 abnormal embryos at prim-5 stage (24 hpf). Increased number of abnormal embryos was observed in the group laid by F1 fish coming from starved fathers. No statistical differences were found when sex was considered as interaction factor.

FIGURE 5 Paternal starvation leads to changes in embryo transcriptome in prim-5 embryos. (a) Differentially expressed genes in offspring of starved and fed fathers: Volcano plot depicting the differentially expressed genes between embryos from starved and fed fathers. We found 130 genes downregulated and 15 genes upregulated (only genes expressed >1TMM in 50% of the samples, FDR <0.1). A total of 6 samples for each group were included in the analysis. (b) Example of expression levels in downregulated (top: *ctss2.1*) and upregulated (bottom: *klhl31*) genes in offspring of starved fathers. Examples are given for all the embryos included in the transcriptomic analysis. (c) Differential expression within experimental groups and deregulated genes: Heatmap showing the variation in downregulated genes (top) and upregulated (bottom) between embryos from starved and fed fathers. (d) GO terms for upregulated genes in embryos from starved fathers. (e) GO terms for downregulated genes in embryos from starved fathers.



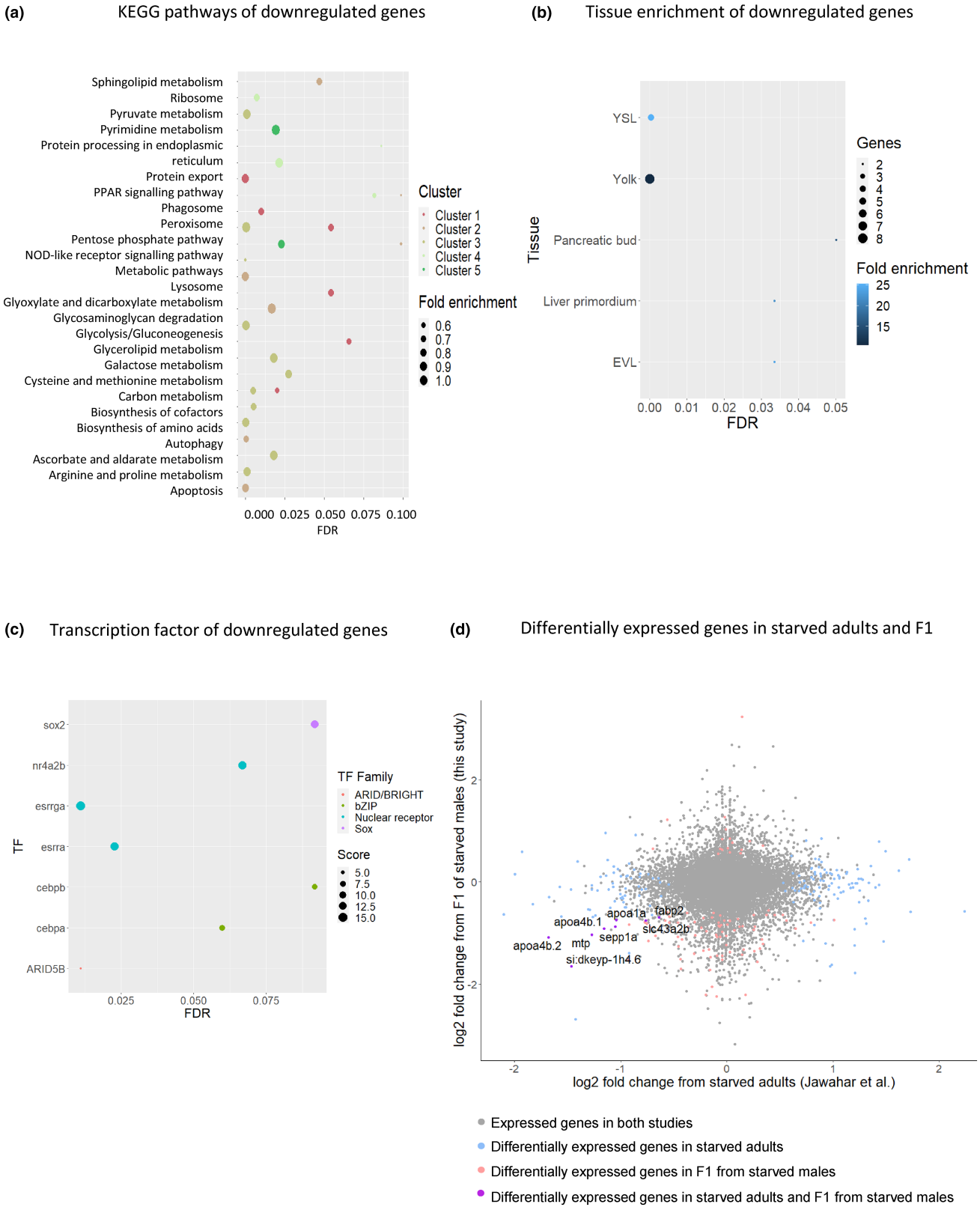


FIGURE 6 The downregulated genes in the offspring of starved males are associated to metabolic processes, show preference for yolk and YSL expression and correlate with altered genes in adults exposed to starvation. (a) KEGG pathways enriched in downregulated genes. (b) Tissue enrichment of downregulated genes. (c) Transcription factor enrichment in the subset of downregulated genes. (d) Intersection analysis between the DEGs in the datasets from Jawahar et al. (2022) and this study (Odds ratio = 4.9617, $p < .0001$).

(Figure S8). This analysis revealed an enrichment of the downregulated genes in cells associated with the haematopoietic system, blood island, hatching gland and periderm (tissue derived from the EVL) (Figure S8 mid panel). Note that the clusters defined in the single-cell data lack information for pancreatic bud and liver primordium. These are small cell populations which might be more difficult to pool in this type of approach. The absence of yolk and YSL information is given by the nature of the sample preparation for single-cell RNA-seq where embryos are dissociated, and samples are washed and spun leading to loss of the lipid-rich yolk in the supernatants; or yolks are directly removed with deyolking buffer (Farnsworth et al., 2020; Jiang et al., 2021).

Because of the lack of reference transcriptomic information for the yolk in the single-cell RNA-seq, we then sought to further confirm the findings provided by generif by searching the in situ hybridisation data in the zebrafish information network database (zfin) and associated available literature. This manual curation of the data allowed us to confirm the results reported by the generif database for all the YSL expressed reported genes (i.e. *fabp2*, *mttp*, *apoa4b.1*, *cldne*, *gamt*, *apoc2*, *apoc1* and *rbp4*) and added information on YSL expression on 29 additional genes (Table S22). The yolk and YSL are essential extra-embryonic structures involved in the metabolic activity of the early embryo. The downregulation of these genes in the yolk and YSL in embryos from starved fathers points to the impact of paternal nutrition on these tissues with possible impact on the metabolic activities of the offspring. The generif approach revealed no significant enriched tissues for the upregulated genes. However, the deconvolution of single-cell data with the upregulated genes showed that those genes are expressed in cardiac muscle cells, myotome, notochord, and muscle pioneers at 24 hpf (Figure S8 bottom panel). These results were further validated by the data available in zfin (Bradford et al., 2022).

Furthermore, when we explored the transcription factors (TFs) predicted to bind to the downregulated DEGs, we found enrichment for *cebpa* and *cebpb*, transcription factors among others. These factors, members of the bZIP family, are related to the digestive system development (Yuan et al., 2015) and have enriched expression in the YSL, liver and gut (Figure 6c) again pointing at the misregulation of genes involved in nutrient utilisation.

Finally, we queried the relationship between transcript abundance profiles upon direct exposure to starvation and offspring response. To this end, we compared the transcriptome data from the embryos from starved males in our study with existing transcriptome data from intestines of starved adult zebrafish (Jawahar et al., 2022) (Figure 6d). This dataset offers a good opportunity to focus on the impact of the dietary stressor which in our unexposed embryos seems to target a nutritionally relevant tissue in the early embryo as observed by the pool of deregulated YSL-associated genes. This transcriptome intersection analysis revealed a significant enrichment for a subset of genes that were downregulated in both studies (Odds ratio = 4.9617, $p < .0001$). These genes (*apoa1a*, *apoa4b.1*, *apoa4b.2*, *fabp2*, *mtp*, *sepp1a*, *slc43a2b* and *si:dkey p1h4.6*) belong to cluster 1 in our PPI network and are mostly involved in PPAR signalling. This finding indicates that the impact of starvation can affect the same metabolism pathway genes similarly in starved adults and in unexposed offspring.

4 | DISCUSSION

Our results provide clear evidence that paternal starvation not only affects male fertility but also directly affects offspring performance early and later in life by impacting the development of the F2 generation. We observed that paternal starvation led to reduced sperm velocity and impacted the F1, which showed enhanced growth, delayed hatching rates and altered transcriptome with changes in genes associated with metabolism and growth. The impact of paternal starvation was passed on to the unexposed F2 inducing higher malformation rates.

Sperm swimming velocity is known to affect fertilisation success in externally fertilising fish and any changes may directly affect male reproductive success (Gage et al., 2004). In our study, reduced sperm swimming velocity in starved males only negatively affected fertilisation success in natural spawnings, not in IVF assays. Nevertheless, our finding of reduced velocity in sperm suggests that starvation has a negative impact on spermatogenesis, potentially by affecting metabolic pathways that also determine sperm velocity. Fertility traits showed great variation among starved males suggesting that the overall condition of the males may interfere with the impacts of starvation, and that males that are generally in better condition may be more resistant. This idea is further supported by our finding of a significant association between total sperm number and density and male body weight and the significant interaction between male body weight and treatment. Genetic variation among males is the most likely explanation for the observed variation in our experiment, as a such variation has been reported in various zebrafish strains (Brown et al., 2012; Lange et al., 2013) and in recombinant inbred mice exhibiting marked differences in their response to dietary restriction (Liao et al., 2013). The potentially condition-dependent response to dietary restriction needs further investigation.

The changes triggered by paternal starvation leading to reduced fertility and sperm quality can be linked to non-genetic alterations transmitted to the offspring. When looking at the impact of this treatment on early-life performance in the resulting offspring, we found that while survival during early development and juvenile life stages were not significantly affected, hatching rate was slower and growth rate faster in larvae sired by starved males compared to their half-siblings sired by fed males. Our finding of faster growth was highly reproducible across our experiments and is comparable to effects of maternal starvation on offspring growth rate in *C. elegans* (Hibshman et al., 2016), and the observation of overgrown pups following reduced paternal protein diet and caloric restriction in mice (Morgan et al., 2021). Nevertheless, our results contrast with the reduced growth in larvae as a result of maternal starvation in zebrafish (Fan et al., 2019). This inconsistency between paternal and maternal effects of dietary restriction suggests that the impact of parental

diet is highly influenced by the conditions of the model, including species, age, sex and the nature of the dietary manipulation. Fish can undergo long periods of starvation that can exceed weeks and are more resistant than other species like mammals (Drew et al., 2008; Furné et al., 2012; Furne & Sanz, 2023; Wang et al., 2006). These differences among species are most likely directly linked to overall metabolic rates and the substantial differences between endotherm and ectotherm organisms.

The integrity of germ cells is important, and their maintenance may come at the expense of somatic maintenance (Chen et al., 2020; Ivimey-Cook et al., 2023). This link between somatic health and reproductive fitness may help explain our findings of faster growth but reduced reproductive fitness in F1 offspring from starved males. The faster growth rate during larval stages suggests more efficient utilisation of the available resources towards muscle formation and may translate into potentially negative effects later in life in reproductive fitness, as indicated by the overall higher rate of abnormally developing embryos produced by the offspring of starved fathers. This means that the decay in reproductive fitness with enhanced growth observed in offspring from starved fathers could be either a cause or a consequence of a trade-off between soma and germline as influenced by the paternal environment and manifested early in development. These findings are interesting as they suggest that while short-term starvation in males may be beneficial for individual lifespan and health, they may have detrimental effects both on individual reproductive fitness and on adult condition and reproduction in the resulting offspring.

In our study, yolk size did not differ between embryos of starved and fed males. The difference in growth rate between offspring of starved and fed males therefore, appears not to be explained by changes in mobilisation of maternal provisioning during these early stages as measured by yolk resorption and may reflect metabolic differences in the growing cells of the embryos and larvae (Anderson et al., 2011; Huang & Linsen, 2015). However, the finding of a total of 145 DEGs, several of which robustly associated with major functions in body growth and fatty acid metabolism further supports the idea that the paternal condition directly influences gene expression in the offspring. Several metabolic pathways were enriched in the PPI clusters formed by the downregulated genes in this analysis and included PPAR signalling, autophagy and glycolysis/gluconeogenesis. These pathways are often found deregulated in conditions of direct exposure to starvation. For example, autophagy is triggered in conditions of nutrient deficiency upon mTOR pathway inhibition (Mizushima et al., 2004; Wong et al., 2015) and *ppar*α is overexpressed under fasting conditions (Kersten et al., 1999).

The misregulation of starvation-associated pathways and the pool of genes affected suggest a remarkable similarity of response in the offspring to that in the parent. This idea is further supported by the significant correlation with data from intestine of starved zebrafish adults (both males and females) (Jawahar et al., 2022) and the transcriptome of our unexposed embryos with a high proportion of misregulated genes being expressed in a nutrition-related tissue like the yolk. The deregulation of similar genes and pathways between parents and offspring has been described in studies in mammals but the response in offspring is often opposite to that observed in the parents (Carone et al., 2010; Fan et al., 2019). In our study, we report both: genes related to PPAR signalling mimic the direct response to a similar starvation paradigm, while autophagy and other metabolic-related genes show the opposite response to the expected upon starvation. This contrasting behaviour of gene expression could be underlying the desensitisation to the stressor exhibited by the offspring of parents exposed to similar conditions, as shown by increased resistance to starvation in the offspring of *C. elegans* upon maternal starvation (Hibshman et al., 2016). Further experiments are required to investigate whether this is the case in zebrafish but this comparable response to paternal diet between directly exposed adults and non-free feeding and unexposed offspring suggests a functional link to the paternal nutritional status and a directed transmission of response. This directed transmission of paternal information into the offspring could be instructive for the embryo on responses to nutrient availability in the environment despite the actual nutritional status, which in our experiments was controlled by the mother through the egg content.

Interestingly, we observed that a number of DEGs were located in proximity forming clusters in different chromosomes (5, 12 and 16). These genes respond similarly to paternal starvation. These two facts (clustering and unified response) could point to a co-regulation phenomenon behind the expression of these genes which seem to go hand-by-hand. This co-expression of neighbouring genes has been widely studied and confirmed across several models (Fukuoka et al., 2004; Purmann et al., 2007; Williams & Bowles, 2004) and could point to a conserved response to caloric imbalances.

We also applied different approaches to study the tissue specificity of the DEGs. The upregulated genes showed clear enrichment for GO terms and pathways related to muscle formation and contraction. These genes are mostly expressed in tissues related to these activities, namely muscle pioneers, notochord and cardiac muscle cells. We speculate that the significantly enhanced growth observed at 8 dpf could be a product of the transcriptional deregulation initiated at earlier stages and detected by our transcriptomic analyses at 24 hpf.

The downregulated genes are mostly expressed in the yolk and YSL, periderm, haematopoietic system, blood island and hatching gland at 24 hpf. The yolk is an extraembryonic tissue loaded with maternally supplied factors that operates as the metabolic hub of the early zebrafish embryo and provides nutritional support during the first days of life (Anderson et al., 2011; Huang & Linsen, 2015). Many of these yolk-associated genes are also expressed in the yolk sac of other vertebrates including humans, mouse and chicken (Cindrova-Davies et al., 2017) and their physiological roles in the yolk sac are highly conserved during the early stages of life, meaning that the metabolic response to paternal starvation could also be conserved in higher vertebrates. The altered gene expression caused by paternal starvation in this tissue could translate into metabolic defects which ultimately affect larval growth and fitness. However, metabolomics studies would be required to test this hypothesis.

The potential mechanisms that could lead to altered gene expression in extraembryonic and embryonic tissues are various and may trigger one or more changes in DNA methylation, histone modifications and small RNAs. For example, paternal high fat diet altered H3K4me3, a histone mark associated with increased gene expression. The changes in the deposition of H3K4me3 in sperm occurred in genes associated with placental formation, pointing to a potentially targeted impact of the paternal epigenome on the development of this tissue through direct regulation of gene expression (Pepin et al., 2022). On the other hand, tRNAs and miRNAs carried in the ejaculate can change upon protein restriction leading to transcriptomic changes in the offspring (Sharma et al., 2016). However, in externally fertilising fish, the impact of the ejaculate might be reduced by the dilution of the contents of the seminal fluid in water (Fitzpatrick, 2020). As the majority of the mis-regulated genes in our study appear downregulated, it is possible that the mechanisms that mediate paternal starvation effects on offspring transcriptome at the studied stage include a combination of these factors since, for example, the direct deposition of H3K4me3 in sperm in the metabolic pool of genes would not explain the decreased expression observed here.

Overall, our study confirms the effect of paternal starvation on offspring fitness from early development into late life and across at least two generations in zebrafish. The non-genetic alterations caused by paternal diet in the offspring can have adaptive or maladaptive consequences, which are determined by shifts in the environment and time of generation, among other factors (Stajic & Jansen, 2021). The similarity of the gene expression patterns in offspring of starved males and starved adult males (Jawahar et al., 2022) suggests anticipatory response by the offspring. This may potentially indicate adaptive or maladaptive response which could be assessed by analysis of F1 and F2 responses exposed to starvation.

4.1 | Limitations of the study

This study sought to measure the impact of paternal nutritional state in the offspring and to test the applicability of zebrafish as a model for this phenomenon. The limitations of this work include the lack of association between molecular and anatomical phenotypes due to the lack of stage-matched sampling. For example, transcriptomic data at 8 dpf would provide better insights into the molecular changes associated with the growth differences in the offspring of starved fathers. Future studies will require increased sample sizes which is of high relevance when analysing changes in the transcriptome of individual embryos triggered by the paternal environment. Furthermore, our work did not explore the epigenetic (or potentially genetic, including chromosomal aberrations) mechanisms underlying the observed morphological and molecular changes observed in the offspring. Nevertheless, we believe that our results will provide foundation for future studies aiming to explore the epigenetic mechanisms underlying starvation-associated phenotypic changes in the offspring.

AUTHOR CONTRIBUTIONS

AJG, CN, GA, DM and WR performed the experimental work; AJG did the molecular lab work; CN developed the experimental protocol; FA performed the RNA-seq bioinformatic analyses; RS supervised the bioinformatic analyses; AJG and SI performed the data analyses; AJG, FM and SI wrote the paper; FM and SI conceived the study design and supervised the project.

ACKNOWLEDGEMENTS

This work was funded by a Human Frontier Science Program research grant (HFSP R0025/2015). AJG was also supported by the PrecisionTox of EU Horizon2020 programme (Grant agreement: 965406) and SI was further supported by an ERC Starting Grant (HapSelA-336633). We thank Genomics Birmingham for sequencing; the BMSU facility at the University of Birmingham, SciLifeLab zebrafish platform at Uppsala University and the Controlled Ecology Facility (CEF) at the University of East Anglia (UEA, UK) for the animal care; the Zebrahub partners for allowing us to use their single-cell data for our transcriptomic comparisons; Bradley Cairns for his ideas on developing the zebrafish model for intergenerational studies and Rui Monteiro for his feedback on the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVIALIABLITY STATEMENT

Phenotypic data can be downloaded from the data repository Dryad (https://doi.org/10.5061/dryad.ncjsxkt2j) and the sequencing data can be downloaded from NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE253445 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE253445).

ETHICS STATEMENT

All animal works were performed under the Project Licence # P0C37E901, in accordance with the UK Home Office regulations and UK Animals (Scientific Procedures) Act 1986, and under Licence C200315/16 of the Swedish Board of Agriculture.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Jimenez-Gonzalez, A., Ansaloni, F., Nebendahl, C., Alavioon, G., Murray, D., Robak, W., Sanges, R., Müller, F., & Immler, S. (2024). Paternal starvation affects metabolic gene expression during zebrafish offspring development and lifelong fitness. *Molecular Ecology*, *33*, e17296. https://doi.org/10.1111/mec.17296