Daphnia magna modifies its gene expression extensively in response to caloric restriction revealing a novel effect on haemoglobin isoform preference

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Daphnia magna modifies its gene expression extensively in response to caloric restriction revealing a novel effect on haemoglobin isoform preference

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Abstract

Caloric restriction (CR) produces clear phenotypic effects within and between generations of the model crustacean *Daphnia magna*. We have previously established that micro-RNAs and cytosine methylation change in response to CR in this organism, and we demonstrate here that CR has a dramatic effect on gene expression. Over 6,000 genes were differentially expressed between CR and well-fed *D. magna*, with a bias towards up-regulation of genes under caloric restriction. We identified a highly expressed haemoglobin gene that responds to CR by changing isoform proportions. Specifically, a transcript containing three haem-binding erythrocruorin domains was strongly down-regulated under CR in favour of transcripts containing fewer or no such domains. This change in the haemoglobin mix is similar to the response to hypoxia in *Daphnia*, which is mediated through the transcription factor hypoxia-inducible factor 1, and ultimately the mTOR signalling pathway. This is the first report of a role for haemoglobin in the response to CR. We also observed high absolute expression of superoxide dismutase (SOD) in normally fed individuals, which contrasts with observations of high SOD levels under CR in other taxa. However, key differentially expressed genes, like SOD, were not targeted by differentially expressed micro-RNAs. Whether the link between haemoglobin and CR occurs in other organisms, or is related to the aquatic lifestyle, remains to be tested. It suggests that one response to CR may be to simply transport less oxygen and lower respiration.

KEYWORDS

caloric restriction, *Daphnia*, ecological genomics, gene expression, methylation, micro-RNA

1 | INTRODUCTION

Caloric restriction (CR) is the reduction in dietary intake of calories without undernutrition (Koubova & Guarente, 2003). CR induces marked phenotypic changes in many organisms. Most notably an increase in longevity has been observed in various arthropods, rodents, yeast and possibly in humans (Heilbronn & Ravussin, 2003; Kapahi, Kaeberlein, & Hansen, 2017; Lakowski & Hekimi, 1998; Redman & Ravussin, 2011; Sohal & Weindruch, 1996; Walford, Harris, & Weindruch, 1987). This occurs through CR-mediated

delays in the onset of processes and diseases associated with ageing (Koubova & Guarente, 2003; Most, Tosti, Redman, & Fontana, 2017). As a result, moderate CR could well be beneficial to human health, although this remains to be confirmed by sufficiently long-running clinical trials (Most et al., 2017).

The transcriptomic response to CR has been investigated in a variety of model organisms spanning mammals, invertebrates and yeasts (Choi et al., 2018; Ding et al., 2014; Dobson et al., 2018; Heintz et al., 2017; Kapahi et al., 2017; Kim et al., 2016; Matthews et al., 2017; Regan et al., 2016; Wood et al., 2015). Neuroprotective qualities of CR have been attributed to an altered gene expression profile of CR rats due to an altered response to oxidative stress and histone deacetylase activity, in addition to changes to insulin-signalling pathway and longevity-associated gene levels (Wood et al., 2015). In *Drosophila*, CR responses have been characterized at the whole organism and tissue level (Ding et al., 2014; Dobson et al., 2018; Kapahi et al., 2017; Regan et al., 2016), and there is an overall down-regulation of genes under CR (Ding et al., 2014). However, despite the popularity of *Daphnia magna* for studying life history traits and the effects of environmental stressors (Boersma, Spaak, & De Meester, 1998; Garbutt & Little, 2014, 2017; Lampert, 1987; Latta IV et al., 2011; Mitchell & Lampert, 2000; Orsini et al., 2016), the gene-level response to CR has not previously been studied in this species.

1.1 | Environmental stressors and caloric restriction in *Daphnia magna*

There is a wealth of existing phenotypic data in *Daphnia*, including demonstrations of CR-mediated lifespan increase (Latta IV et al., 2011), which we confirm and expand upon here. There are also clear maternal effects due to CR where *D. magna* mothers exposed to CR produce offspring that are (a) larger at birth, (b) feed at a slower rate than offspring of abundantly fed mothers and are (c) more resistant to parasitism (Garbutt & Little, 2014, 2017). Furthermore, as *Daphnia* are aquatic invertebrates it is possible that they respond to CR in a manner that is partially distinct from terrestrial invertebrates. We explore this novel perspective in the context of haemoglobins here.

1.2 | *Daphnia* haemoglobins and the link to CR

Haemoglobin (henceforward "Hb") production was observed to increase in response to food levels in *D. magna* (Fox et al., 1951; Zeis, 2020). However, no further studies have considered the relationship between nutrition and Hb in *Daphnia*. By contrast, it is well established that the response to hypoxia and hyperthermal stress in *D. magna* and *D. pulex* causes changes in Hb concentration and subunit components to occur (Cuenca Cambronero, Zeis, & Orsini, 2018; Gerke, Börding, Zeis, & Paul, 2011; Lai et al., 2016; Lyu et al., 2015; Zeis et al., 2003). This suggests that there is phenotypic plasticity in the *Daphnia* Hb response to stress. Interestingly, acclimation to hypoxic conditions in *D. magna* results in smaller adults without impacting on clutch size during the first five broods (Seidl, Paul, & Pirow, 2005), a phenotype which is similar to that for the offspring of CR individuals (Garbutt & Little, 2014). Links between nutrition and hypoxia have not been explored widely, although in *Drosophila* a low-protein CR regime partially reverses the reduction in lifespan caused by hypoxia (Vigne & Frelin, 2006). Indeed, hypoxia increases haemoglobin levels across vertebrates and aquatic crustaceans, which suggests a Hb gene response to oxygen stress occurs across taxa (Harrison, Greenlee, & Verberk, 2018).

Daphnia magna encodes 12 di-domain Hb genes [as annotated in the gene set: (Orsini et al., 2016)] that result from a complex series of duplications (Colbourne et al., 2011; Zeis, 2020). Sixteen Hb di-domains are aggregated to form the free-circulating *D. magna* Hb molecule (Zeis, 2020). Different combinations of Hb isoforms produce structurally distinct Hb molecules, which in turn have different oxygen-binding characteristics (Zeis, 2020). The induction of Hb genes in *Daphnia* is mediated by the transcription factor hypoxia-inducible factor (HIF) (Gorr, Cahn, Yamagata, & Bunn, 2004; Zeis, 2020), a heterodimer formed from HIF-1α and HIF-1β proteins. The HIF-1 complex is an important factor in extending longevity of *Caenorhabditis elegans* under CR (Di Chen & Kapahi, 2009; Lee, Hwang, & Kenyon, 2010; Y. Zhang, Shao, Zhai, Shen, & Powell-Coffman, 2009). Increased expression of HIF-1 alone was responsible for increased lifespan and was triggered by elevated concentrations of mitochondrial reactive oxygen species (Lee et al., 2010).

1.3 | Control mechanisms underpinning CR responses

The free radical theory of ageing has been linked to CR (Gladyshev, 2014; Liochev, 2013; Vina, Borras, Abdelaziz, Garcia-Valles, & Gomez-Cabrera, 2013). The theory hypothesizes that ageing results from accumulated oxidative damage due to reactive oxygen species. Superoxide dismutase (SOD), a key component of the theory (Gladyshev, 2014), defends against reactive oxygen species (ROS) by converting abundant superoxide into hydrogen peroxide and oxygen. The link between SOD and longevity is supported by experiments showing that the absence of SOD genes corresponds to decreased lifespans across a range of taxa (Muid, Karakaya, & Koc, 2014; Muller et al., 2006; Oka, Hirai, Yasukawa, Nakahara, & Inoue, 2015).

Four nutrient-sensing signalling pathways are often implicated in lifespan extension as a result of CR (Kenyon, 2010). They are (a) the insulin/insulin-like growth factor (IGF-1), (b) the mechanistic target of rapamycin (mTOR) signalling pathway, (c) 5' adenosine monophosphate-activated protein kinase (AMP Kinase) and (d) sirtuin signalling protein-modulated pathways. These pathways are complex and interlinking, with mTOR signalling potentially acting as the ultimate modulator of the other three (Johnson, Rabinovitch, & Kaeberlein, 2013).

1.4 | Characterizing transcriptomic responses to CR

To characterize phenotypic and genotypic ageing in a single organism, we performed a whole organism longevity experiment on a set of eight *D. magna* genotypes each subjected to a calorically restricted and normal diet. To contrast gene expression between food levels, we selected one genotype that demonstrated increased longevity here. We have previously established that life history traits (Garbutt & Little, 2014, 2017), DNA methylation (Hearn, Pearson, Blaxter, Wilson, & Little, 2019) and miRNA expression in the selected strain (Hearn et al., 2018) respond to CR; indeed, the miRNA was sequenced from the same set of RNA extractions. Using gene- and transcript-level expression change of this CR-responsive genotype of *D. magna*, we now identify known and novel gene families and metabolic pathways implicated in CR.

2 | MATERIALS AND METHODS

2.1 | Longevity experiment

This experiment used eight genotypes from geographically dispersed populations. These were obtained from the *D. magna* diversity panel held in Basel, Switzerland (http://evolution.unibas.ch/ebert/research/referencepanel/). They are (clone ID, Country of Origin): BEK22 (Belgium), Clone 32 (UK), FIFAV1 (Finland), GBEL75 (UK), GG8 (Germany), ILPS1 (Italy), MNDM1 (Mongolia) and RUYAK1-6 (Russia). Prior to the experiments, replicates of all genotypes were put through three generations of acclimation to harmonize environmental effects arising from variation in stock conditions. During this period, each individual was maintained in a 60-ml glass jar filled with artificial pond medium (Klüttgen, Kuntz, & Ratte, 1994). This was changed twice weekly and when offspring were produced. Each individual was fed ~6.25×10^6 *Chlorella vulgaris* cells daily and was maintained on a 12:12 L:D cycle at 20°C. We estimate cell numbers by measuring the daily optical absorbance of 650 nm white light by the *Chlorella* culture, with 1.0 absorbance being equivalent to approximately 5×10^6 algal cells. Offspring from the second clutch initiated each generation, including the experimental generation.

From the acclimated females of all eight genotypes, two offspring from clutch two were taken. One was assigned to normal food (NF: ~6.25×10^6 cells as per acclimation) and one was assigned to a caloric restriction (CR: ~1.4×10^6 cells) treatment, which is approximately 20% of the amount of food available to NF replicates. Each food treatment and genotype combination were replicated 24 times. Date of birth and date of death were recorded. They were otherwise maintained identically to the acclimation period.

2.2 | Survival analysis

A Cox's proportional hazards model was used to test for differences in longevity between the two food treatments for all eight genotypes. This was done using the survival package in R (code used and model outputs: File S2, clone longevity input data: File S3). The response variable was days alive, with genotype, food treatment and their interaction (days alive = clone +food + clone*food) as fixed effects. There was no censoring as all individuals were followed from their day of birth to the day of death. We present the results of the analysis of deviance (ANOVA) for the Cox's model which performs χ2-tests of likelihood ratios of each model factor sequentially, which in this case was food, followed by clone, followed by their interaction.

2.3 | Material for RNA harvesting

Clone 32 (UK; Auld, Hall, Housley Ochs, Sebastian, & Duffy, 2014) was selected for RNA sequencing because it showed a longevity response in the above experiment, and it shows clear maternal effects under variation in maternal food—larger offspring are produced under CR (Garbutt & Little, 2014, 2017). This clone was also the focus of more detailed analysis of food and longevity (Clark, Wilson, McNally and Little, *In revision*) where it again showed lifespan extension in response to food restriction. To generate RNA, maternal lines of Clone 32 were first acclimatized for three generations in artificial pond medium at 20°C and on a 12h:12h light:dark cycle and fed 2.5×10^6 cells of the single-celled green algae *Chlorella vulgaris* daily. The treatment generation [G_0 in (Hearn et al., 2018)] was then split into two groups of eight replicates paired by mother and fed either a normal diet (NF) of 5×10^6 algal cells/day or a caloric-restricted (CR) diet of 1×10^6 algal cells/day per individual. Each replicate was formed of five *D. magna* reared in the same jar from birth.

After the birth of the first clutch of offspring in a replicate jar, the offspring were removed and the jar was treated for microbial contamination with tetracycline and ampicillin (as described in Hearn et al., 2018) over 24 hr, and then, the five G_0 *D. magna* individuals were homogenized in 700ul Qiazol Qiagen reagent ID: 79,306, and stored at −70°C until further processed. RNA was extracted using miRNeasy mini kits (Qiagen Cat No./ID: 74,106) and quality and integrity checked by Qubit (Thermo Fisher) fluorometer, nanodrop (Thermo Fisher) and Bioanalyzer (Agilent). No degradation was observed on Bioanalyzer total RNA traces. Extractions were subsequently halved, and one half was used to create small RNA libraries for miRNA expression analysed in Hearn et al. (2018). TruSeq-stranded mRNA-seq (Illumina, San Diego, USA) libraries were prepared by Edinburgh Genomics from the remaining RNA for eight normal food and eight caloric-restricted replicates as for the previous study. All libraries were multiplexed and sequenced on one lane of HiSeq 4,000 to at a targeted depth of 15 million read-pairs per sample yielding a total of at least 290 million read-pairs. Raw sequencing data generated by this project were deposited in the European Nucleotide Archive under Bioproject PRJEB25137.

2.4 | Differential gene expression and transcript usage analysis

Reads were adapter and quality trimmed with Cutadapt (version 1.16, options: -q 15 --trim-n -m 36) (Martin, 2011), and then Trimmomatic (version 0.36, default options) (Bolger, Lohse, & Usadel, 2014) to remove remaining residual adapter sequence. Fastq files were inspected using FastQC (Andrews, 2010) before and after quality filtering to confirm the removal of adapter-derived and low-quality sequences and reports combined using multiQC (version 1.8). We used a transcript-driven approach to quantify gene expression as (a) an excellent gene set is available for *D. magna* (Orsini et al., 2016, 2018), (b) "alignment-free" mapping approaches to transcripts are at least the equal of genome-based alignments for RNASeq (C. Zhang, Zhang, Lin, & Zhao, 2017), and (c) the *D. magn*a genome is still in draft form with many genes overlapping and/or spread across multiple scaffolds. Gene expression per replicate was quantified using Salmon v0.13.1 (Patro, Duggal, Love, Irizarry, & Kingsford, 2017) with parameters "salmon quant --dumpEq --validateMappings --rangeFactorizationBins 4 -l A --seqBias –gcBias" against the *D. magna* reference transcriptome (Orsini et al., 2016, 2018). The reference transcriptome was created by combining principal and alternative transcripts (downloaded from http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Genes/earlyaccess) and indexed with a k-mer size of 25.

The Salmon results were converted into a gene-by-replicate expression matrix for input to DESeq2 (Love, Huber, & Anders, 2014) with the R package tximport (Soneson, Love, & Robinson, 2015). Read mapping rates per replicate were taken from the resulting Salmon log files (Table S1). Differential gene expression between caloric restriction and normal food replicates was tested using DESeq2, with "mother" fit as a blocking factor (~ mother + condition). Relationships between replicates, treatment and mother were assessed through a principal component analysis (PCA) plot generated in DESeq2, which was based on the top 500 most-variable genes in the data set. We incorporated $\log_2$-fold changes into our significance test and report genes significant at $\log_2$-fold change thresholds of 1, and 2 as this is more robust than post hoc filtering of genes by $\log_2$-fold change alone. This method results in s-values for the nonzero $\log_2$-fold change analyses (i.e. $\log_2$-fold change 1 and 2) that are analogous to q-values (Stephens, 2016; Zhu, Ibrahim, & Love, 2019). S-values are a measure of the chance that the sign (+ or -) of the $\log_2$-fold change for the gene is question is incorrect. We applied a significance threshold of 0.005 to the s-values as recommended by DESeq2 and apeglm (Zhu et al., 2019) package authors, as s-values are less conservative than q-values. We also performed significance testing without imposing a $\log_2$-fold change threshold (referred to as 0 $\log_2$-fold change) in DESeq2, with a q-value threshold of 0.05. We advise caution on the use of $\log_2$-fold change thresholds, however. Although they helped us to identify a general trend in the data, the method penalized high expression genes. This is because the variance in gene expression increases with the level of gene expression (Anders & Huber, 2010), meaning that high expression genes have wider confidence intervals on their predicted $\log_2$-fold changes; although DESeq2 does model for this "overdispersion." We also considered genes differentially expressed at $\log_2$-fold change 0 with overall expression of greater than 10,000 length-scaled transcripts per million (TPM) when interpreting the results. Differential transcript usage (DTU) was tested in DRIMSeq (Nowicka & Robinson, 2016) and DEXSeq (Anders, Reyes, & Huber, 2012), and the overall false discovery rate (OFDR) for a two-step procedure (here referring to gene and transcript level) was calculated in stageR following Love, Soneson, and Patro (2018), with "mother" fit as a blocking factor as for DESeq2. Only those genes that exhibited significant DTU in both DRIMSeq and DEXSeq after OFDR were considered further. Differential gene and transcript analysis scripts are given in File S2.

2.5 | Topgo and gene set enrichment analysis

Gene ontology term enrichment was tested in topGO (Alexa & Rahnenfuhrer, 2016) for each category of significant gene from gene- and transcript-level analyses using *D. magna* GO term annotations following Hearn et al. (2018) and Hearn, Pearson, et al. (2019). A conservative p-value threshold of 0.01 was applied with no multiple testing correction in line with topGO author recommendation (Section 6.2. The adjustment of *p*-values, topGO manual: https://bioconductor.org/packages/release/bioc/html/topGO.html). We considered enriched GO terms of the biological process (BP) and molecular function (MF) subcategories for discussion. We clustered the significantly enriched GO terms in two-dimensional semantic space and treemaps of higher-order processes using REVIGO (Supek, Bošnjak, Škunca, & Šmuc, 2011) following Hearn, Blaxter, et al. (2019) to identify patterns in enrichment and reduce redundancy in GO terms. We adapted the REVIGO webserver-produced R script for each category of enriched GO terms to combine groups of terms by colour according to the REVIGO treemap categories (R script, File S2).

Gene set enrichment analysis (GSEA) of *D. magna* KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs (Kanehisa & Goto, 2000; Kanehisa, Sato, Kawashima, Furumichi, & Tanabe, 2015) was applied to identify KEGG gene pathways up- or down-regulated under CR. GSEA was run for all genes in the expression experiment using their $\log_2$-fold changes from the $\log_2$-fold change zero differential gene expression experiment. To obtain KEGG annotations for each gene, the *D. magna* gene set was annotated with trinotate (Haas et al., 2013) and gene-to-KEGG annotations were input to the universal enrichment protocol of clusterProfiler (Yu, Wang, Han, & He, 2012); a q-value cut-off of 0.05 was used to determine significance (R scripts: File S2). We ran 1,000 iterations of GSEA as the results were variable between runs, and selected only those KEGG terms found significant in > 95% of runs.

2.6 | Interaction between expression level and differential methylation

Average TPM gene expressions calculated in tximport and normalized in DESeq2 for CR and NF were correlated with the proportion of

methylation within genes for the corresponding treatment using data taken from Hearn, Pearson, et al. (2019). A schematic of the relationships between data sets in this work is given in File S1. Proportion methylated was defined as the count of methylated reads over the total reads at CpG sites within a genic region (exons + introns) aligned against the *D. magna* genome assembly (version 2.4) using Bismark (Krueger & Andrews, 2011), see Hearn, Pearson, et al. (2019) for further detail. Genic regions were defined from the *D. magna* reference annotation (downloaded from http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Genes/earlyaccess/dmagset7finloc9c.puban.gff.gz). CpG counts for CR or NF were extracted by combining CpG counts of the six replicates per treatment from Hearn, Pearson, et al. (2019) from Bismark bam files. Average methylation rates within genic regions per treatment were calculated using bedtools (Quinlan & Hall, 2010). We then combined CpG averages with corresponding average TPM expression for each gene, calculated from the normalized Salmon count matrix. Genes that had expression greater than 10 TPM, at least 5% CpG methylation, and did not overlap another gene in *D. magna* annotation were retained for the correlation analysis. We applied Spearman's rho and Kendall's tau calculated in base R to the results, because of the lack of normality in the proportions of methylation and mean expression data. Furthermore, we intersected the list of differentially expressed genes at each $\log_2$-fold threshold and differential transcript usage results with the differentially methylated regions identified in response to CR from Hearn, Pearson, et al. (2019).

2.7 | MiRNA target prediction and mRNA-MiRNA expression correlation

MiRNA targets of differentially expressed miRNAs identified in Hearn et al. (2018) were predicted in the 3' untranslated regions (3' UTRs, following Graham & Barreto, 2019) of differentially expressed genes using PITA (Kertesz, Iovino, Unnerstall, Gaul, & Segal, 2007), RNAhybrid (Krüger & Rehmsmeier, 2006), miRanda (Enright et al., 2003), MicroTar (Thadani & Tammi, 2006) and rna22 (Miranda et al., 2006) implemented on the tools4miRs webserver (Lukasik, Wójcikowski, & Zielenkiewicz, 2016). Only those 3' UTRs predicted as targets by at least four programs were considered further. The DESeq2 normalized count matrices for differentially expressed miRNAs (taken from Hearn et al., 2018), and mRNAs at $\log_2$-fold change 0 were supplied as input to miRLAB for Pearson's correlation of expression levels (Le, Zhang, Liu, Liu, & Li, 2015). The correlation results were intersected with the list of predicted miRNA to mRNA targets. MiRNA-mRNA pairs with greater than 0.5 or less than −0.5 correlation in expression level were considered further.

3 | RESULTS

3.1 | Effect of CR on longevity

Cox's proportional hazards ANOVA (see File S2 for R code and model output) of the survivorship data (File S3) showed that CR *D. magna* lived longer on average than NF *D. magna* ($\chi^2 = 13.26$, $p = .0003$; Figure 1, part A). Clone 32 was one of the clones that demonstrated increased longevity under CR (Figure 1, Part B). Genotypes also differed in their lifespans ($\chi^2 = 155.4$, $p < .0001$), and there was a significant interaction between genotype and food level for differences in longevity (Figure 1; $\chi 2 = 29.4$, $p = .0001$).

3.2 | Expression experiment

A median of 23,365,302 read-pairs were sequenced per sample with a range of 21,452,900–26,752,873, and FastQC revealed no issues with read quality after trimming (Table S1: read filtering and alignment rates, File S4: multiQC report). Read mapping rates of greater than 80% were recorded for all replicates, except one (3H2, NF) that had a mapping rate of 74% (Table S1).

The number of genes differentially up- or down-regulated in CR at the 0 $\log_2$-fold change level was similar, at 3,345 an 3,064, respectively, CR (Table 1, Tables S2–S4). This global response was reflected in the PCA plot of replicates (Figure 2), in which the first component explains 49% of the variance and separates all sample pairs by treatment. Variance between mothers in the PCA (different colours in Figure 2) can be explained by "jar effects," such as differences in the micro-environment of our incubators (Hurlbert, 1984). This was also true for miRNAs derived from the same RNA extractions (Hearn et al., 2018). However, with increasing $\log_2$-fold change thresholds there was a bias towards genes being up-regulated in CR (Table 1).

There were 75 genes with a mean expression greater than 10,000 TPM that were significant at the $\log_2$-fold change 0 threshold and up-regulated in CR, versus 15 that were down-regulated. Four of the 15 genes down-regulated under CR were superoxide dismutases that are involved in protection from oxidative damage, including the most expressed gene (Dapma7bEVm009708) at a mean expression of 847,468.627 TPM. This was over 6-fold greater than the next gene, a Vitellogenin-1 precursor (Dapma7bEVm014991) (Table S5: gene descriptions and expression levels). Two further vitellogenin-1 precursor genes were in the top five CR up-regulated genes (Dapma7bEVm018415 and Dapma7bEVm029595), as was a di-domain haemoglobin precursor (Dapma7bEVm029622). By contrast, the gene lists for the $\log_2$-fold changes 1 and 2 were composed of genes at relatively modest expression levels and contained a high proportion of uncharacterized genes (Table S5).

3.3 | Differential transcript usage and HB-related genes

For differential transcript usage, 498 transcripts corresponding to 294 genes were significant in the DEXSeq analysis and 327 transcripts from 187 genes for the DRIMSeq analysis. There was an overlap of 181 transcripts and 112 genes between the two methods (File S5: DRIMSeq transcript expression levels for DTU significant genes). Of these, 32 genes have a mean gene expression normalized count

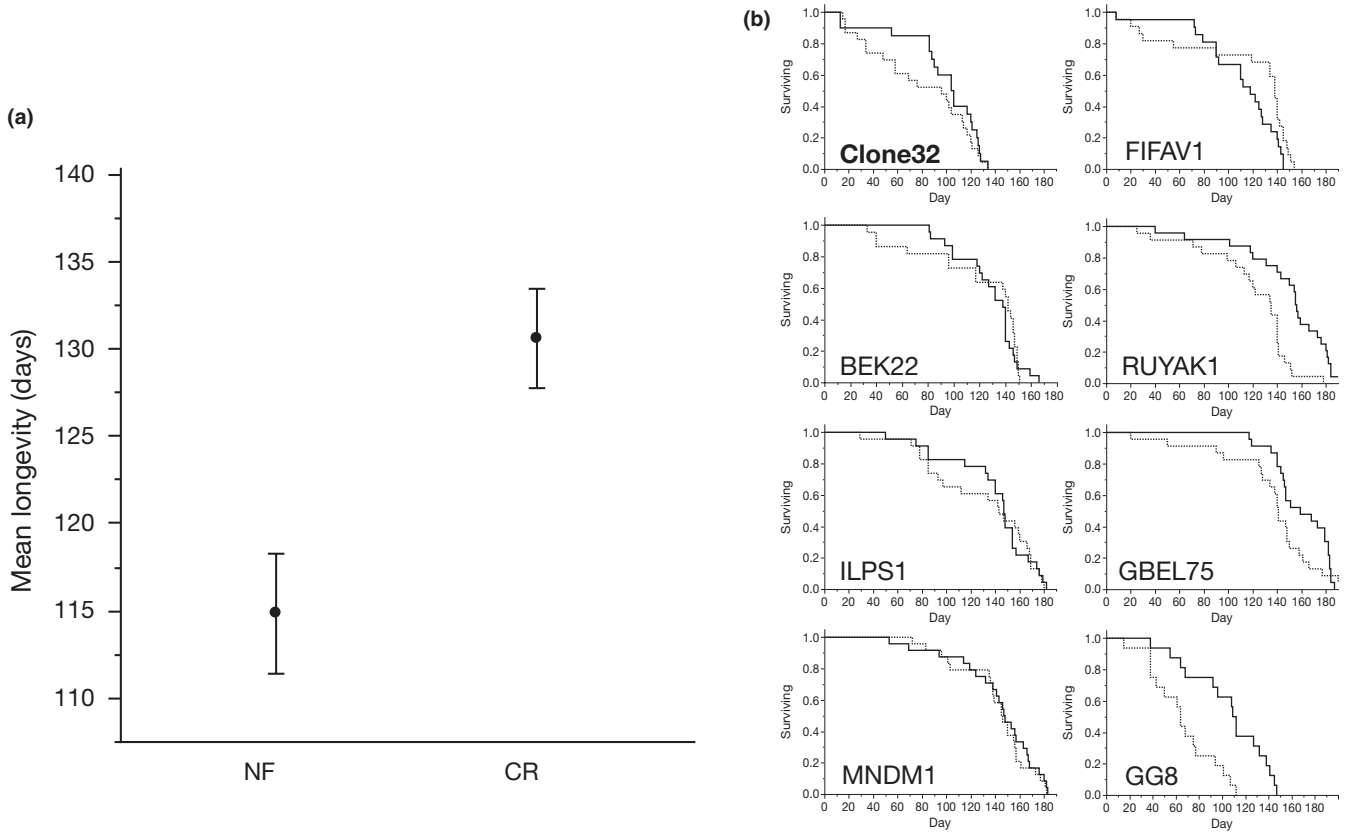

FIGURE 1 **CR *Daphnia magna* lived approximately 15 days longer than NF *D. magna* on average.** A) Point estimates are mean longevity across clones for each treatment, and whiskers are standard errors. B) Survivorship between treatments varied among the eight different *D. magna* genotypes (dashed line = NF, solid line = CR); Clone 32 is labelled in bold in the upper-left plot

TABLE 1 Significantly differentially expressed genes at each $\log_2$-fold change threshold. More genes were up-regulated in CR replicates

	$\log_2$-fold change 0	$\log_2$-fold change > 1	$\log_2$-old change > 2
Up-regulated in CR	3,345	280	62
Down-regulated in CR	3,063	52	11

level greater than 1,000 TPM and five greater than 10,000 TPM. The most highly expressed DTU exhibiting gene was a di-domain haemoglobin precursor (Figure 3, Dapma7bEVm014981) with a TPM of 65,675. The isoform most abundant in CR replicates ("Transcript 21," Figure 3) does not contain a haem-binding erythrocruorin domain, whereas an isoform containing three such domains ("Transcript 12," Figure 3) is down-regulated in CR. A further di-domain haemoglobin was also in this high expression group (Dapma7bEVm015367, TPM 13,532).

Following from this result, we found that one Hb trans-inducer HIF-1α (Dapma7bEVm009543) was up-regulated significantly in CR ($\log_2$-fold change 0) at a mean expression of 6,903 TPM versus 4,558 in NF. Three of the HIF-1 co-dimer HIF-1β genes were DE at this threshold, the highest expressed gene was down-regulated in CR at a mean 2,316 TPM versus 2,667 in NF. The two other HIF-1β genes were up-regulated in CR, but had lower average overall expression at 464 and 391 TPM, respectively. An mTOR protein kinase (Dapma7bEVm000341) was also up-regulated under CR at $\log_2$-fold change 0 albeit with a modest $\log_2$-fold change overall (0.14) and mean expression of 2,434 TPM.

3.4 | Gene ontology and gene set enrichment analyses

For molecular function GO term enrichment, 20 and 21 terms were significantly up- and down-regulated at $\log_2$-fold change 0, respectively (Table S6). Biological process GO terms differ in that 25 terms are enriched for genes down-regulated in CR at $\log_2$-fold change 0, versus 8 that were up-regulated (REVIGO clustering of GO terms, Figure 4 and Table S7).

By contrast to the Biological process GO terms, for the GSEA analysis of KEGG terms five of the six enriched terms were groups of genes up-regulated under CR (representative GSEA result, Figure 5). The five KEGG terms significantly enriched in CR were for serine/threonine-protein kinase/endoribonuclease IRE1 (K08852), preprotein translocase subunit SecA (K03070), serine/threonine-protein kinase/endoribonuclease IRE2 (K11715), chitinase (K01183) and cytochrome P450 family 4 (K15001). The single term enriched in CR down-regulated genes was for apolipoprotein D and lipocalin

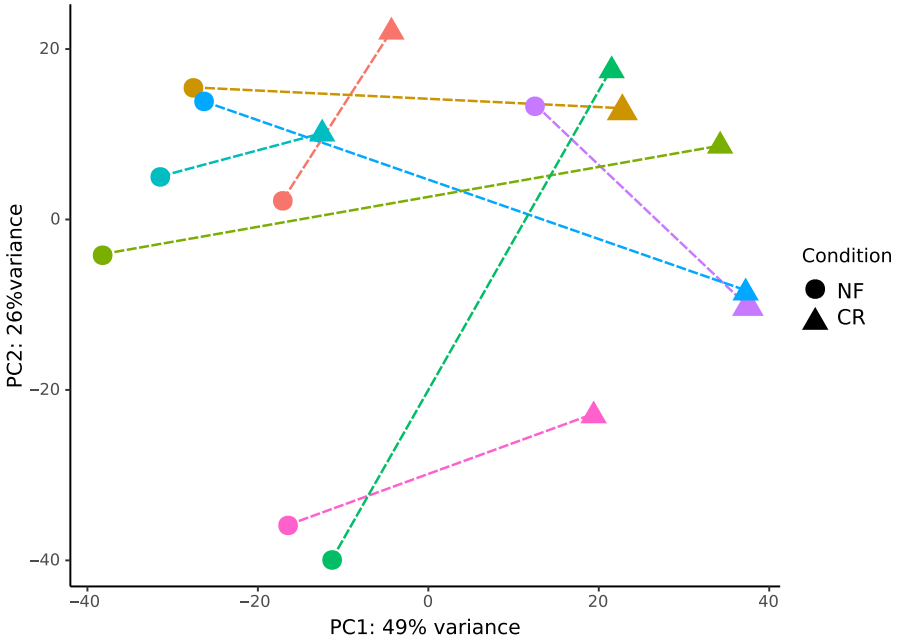

FIGURE 2 DESeq2 generated PCA plots of regularized logarithm transformation of data of replicates in the nutrition experiment. Shapes define treatment, and colour defines mother of each pair of replicates. Figure legend: NF = normal food, CR = caloric restriction. Dashed lines indicate relationship between replicates paired by mother; these are always divided along the x-axis in the same direction

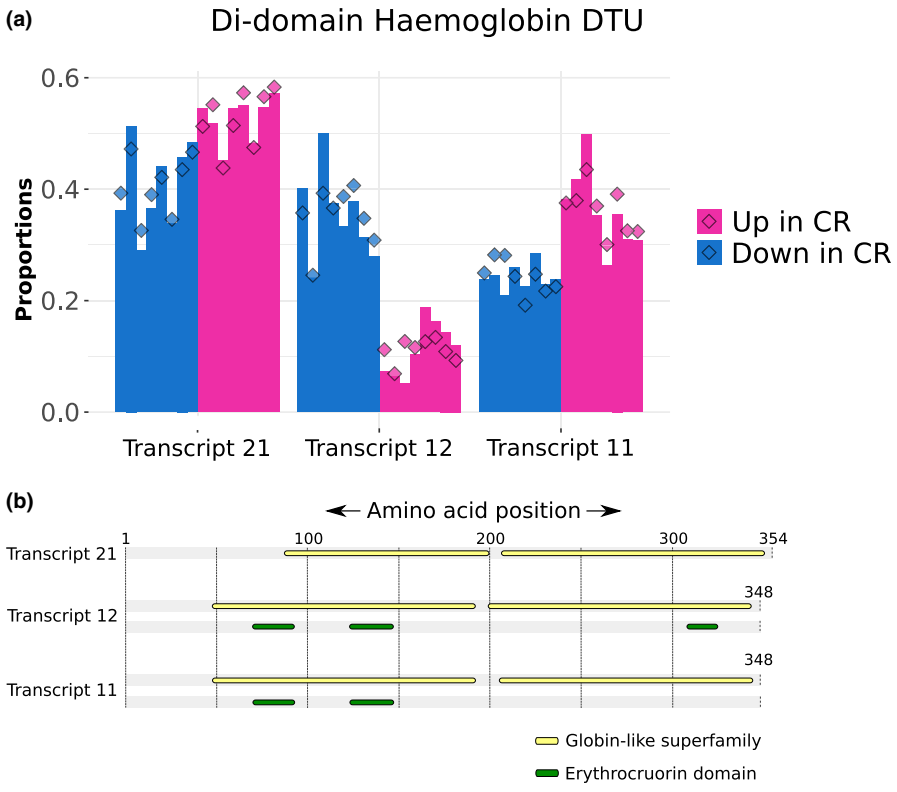

FIGURE 3 Transcript proportion plots and protein domains for differentially used transcripts of an up-regulated di-domain haemoglobin. Transcripts 11, 12 and 21 of gene Dapma7bEVm014981 show DTU across conditions in DEXSeq as does transcript 12 in DRIMSeq-adjusted results. Part A) Bars represent proportions of total gene expression for that transcript per replicate, and diamonds DRIMSeq fitted values: Up in CR = transcript up-regulated in CR; Down in CR = transcript down-regulated in CR. B) InterProScan predicted globin-like super-family (IPR009050) and erythrocruorin (IPR002336) protein domains for transcripts 11, 12 and 21, which shows the varying number of erythrocruorin domains. Transcript 21 does not encode an erythrocruorin domain at all

family protein (K03098). GSEA chitinase (K01183) up-regulation in CR overlaps with GO enrichment for chitin metabolism and chitin binding (Figure 4, B and D).

3.5 | Overlap with differentially methylated regions

Over 2,000 genes that met our criteria (i.e. expression greater than 10 and at least 5% methylation) were included in the correlations with CpG methylation rate for each of the NF and CR comparisons. Weak, significant and highly concordant negative correlations were found for both comparisons for Kendall's tau and Spearman's rho (File S6). Previously, we identified 115 and 192 genes containing regions of hypo- or hypermethylated at CpG sites under CR, respectively (Hearn, Pearson, et al., 2019). For both categories of methylation, there was little signal of co-regulation with gene expression. The majority of overlap occurs at the $\log_2$-fold 0 level for which thousands of genes were significantly up- and down-regulated under CR implying a high degree of overlap by chance (Table 2). Only two genes up-regulated in

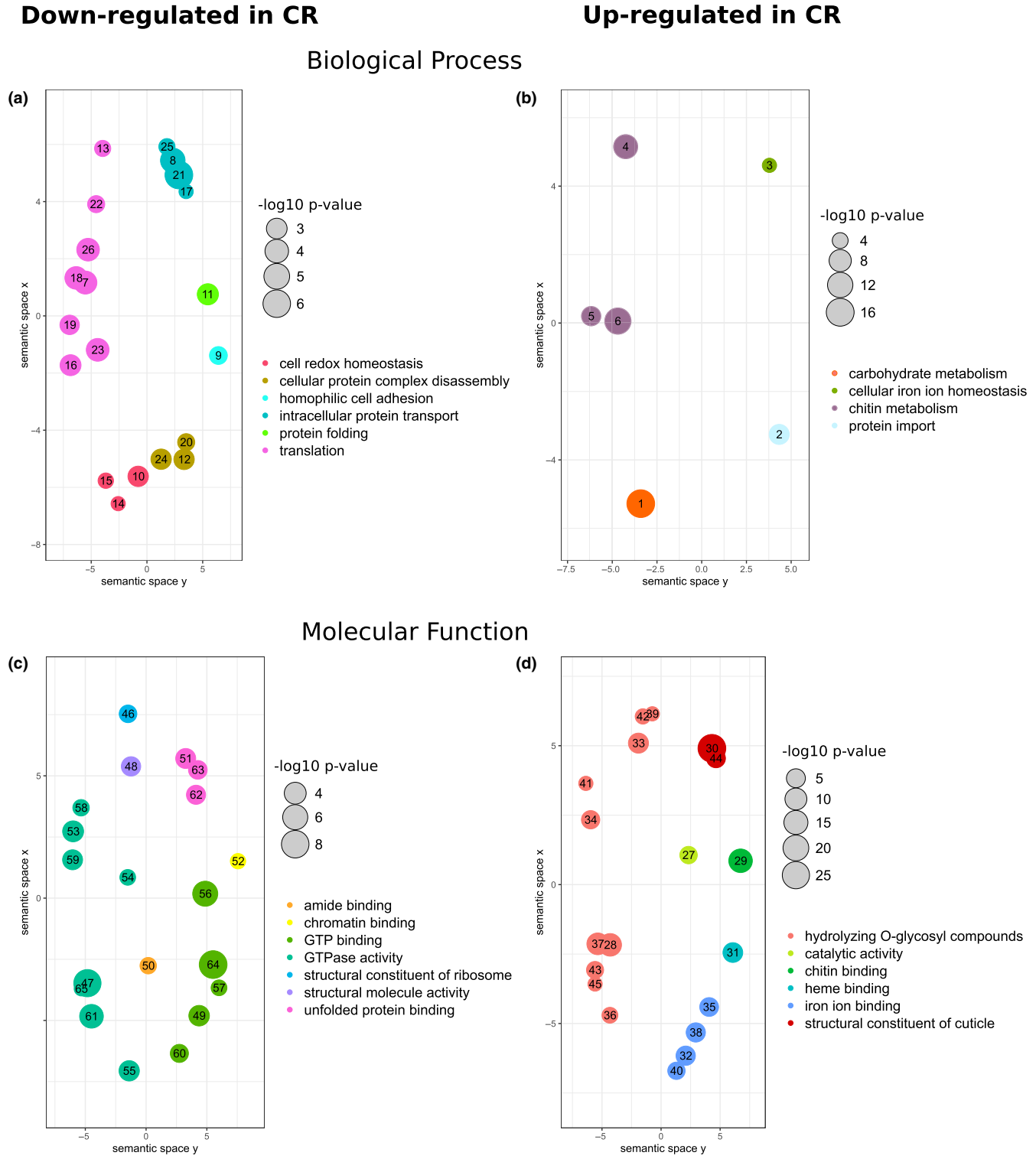

FIGURE 4 REVIGO scatter plots of enriched biological process GO terms for Caloric Restriction versus Normal Food. GO terms are grouped by REVIGO into broader categories indicated by colour and legend, Circle size is $-\log_{10}$ of the topGO enrichment p-value with scales inset next to each plot. A) Down-regulated in CR Biological Process GO terms, B) up-regulated in CR Biological Process GO terms C) down-regulated in CR molecular function GO terms and D) up-regulated in CR molecular function GO terms. GO terms associated with numbers can be found in Table S11

CR with a $\log_2$-fold change of 1 or greater intersected with differentially methylated regions. One was an uncharacterized gene (Dapma7bEVm028334), which was identified as hypo-methylated under CR in Hearn, Pearson, et al. (2019), and the other an integral membrane protein (Dapma7bEVm027395), which was hypermethylated under CR.

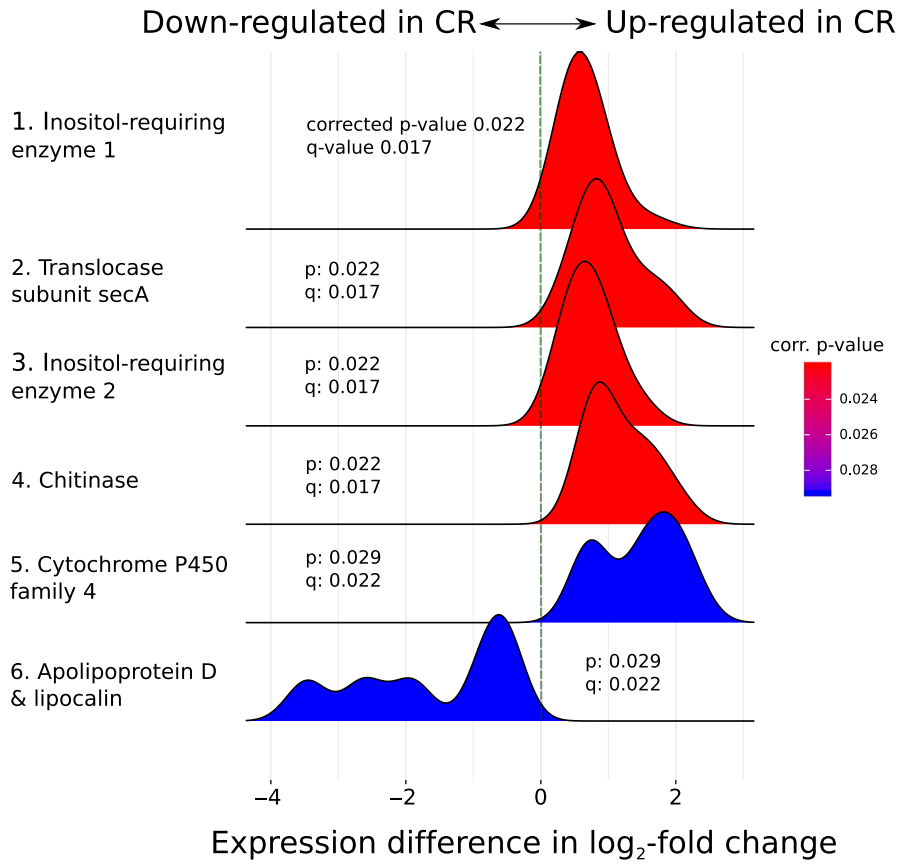

FIGURE 5 Gene Set Enrichment Analysis ridge plot for significant KEGG terms. Ridge plots are density plots of the frequency of $\log_2$-fold change values per gene within each enriched KEGG group, which helps to interpret the up- or down-regulation of that KEGG category. The plot was created in clusterProfiler using KEGG orthologue annotations and $\log_2$-fold changes per gene calculated by DESeq2 during differential expression analysis. X-axis is $\log_2$-fold change in expression for genes present in each KEGG category plotted, with positive values indicating up-regulated expression in CR replicates and negative values down-regulated expression in CR replicates. Peaks are coloured by corrected p-value as shown by the legend, and corrected p-value and q-values are shown per KEGG category. KEGG term complete definitions: 1 = K08852: serine/threonine-protein kinase/endoribonuclease IRE1; 2 = K03070: preprotein translocase subunit secA; 3 = K11715: serine/threonine-protein kinase/endoribonuclease IRE2; 4 = K01183: chitinase; 5 = K15001: cytochrome P450 family 4; 6 = K03098: apolipoprotein D and lipocalin family protein

TABLE 2 Overlap between significantly expressed genes at $\log_2$-fold change 0 and hypo- or hypermethylated genes. Percentages were calculated from total hypo- and hypermethylated genes

	Up-regulated in CR	Down-regulated in CR
Hypo-methylated	20 (10%)	48 (25%)
Hypermethylated	16 (14%)	28 (24%)

3.6 | MiRNA interaction

In total, 240 miRNA-mRNA pairs were predicted by the combined target-site method, which was composed of 117 genes and 34 miRNAs (miRNA-mRNA pairs and miRbase homologs: Table S8). Many miRNAs were predicted by our combined approach to target multiple genes, hence the discrepancy between number of genes and miRNAs. As for the expression analyses, these miRNA-mRNA pairs showed a bias towards genes expressed more highly under CR (Table 3). The strongest negative correlation for genes up-regulated in CR was −0.71, and the strongest positive correlation was 0.87. There was little overlap between these genes and other genes of interest, but a Histone-lysine N-methyltransferase Suv4-20 (Dapma7bEVm018601, mean expression = 10,277 TPM), which is important for DNA repair functions, was up-regulated under CR and was negatively correlated with nine miRNAs (Table S8: gene description for gene numbers included in Table 3). Four of the genes exhibiting DTU were predicted to have miRNA targets, of these only, a Para-nitrobenzyl esterase (Dapma7bEVm000405), was annotated.

4 | DISCUSSION

First, we established that CR has an effect on average lifespan across eight different genotypes of *D. magna*, in line with previous results (Garbutt & Little, 2014, 2017; Latta IV et al., 2011). Focussing on Clone 32, we explored the molecular basis of CR through difference in gene expression with normal food levels. This difference was

TABLE 3 Overlap between significantly expressed genes at $\log_2$-fold change 0 (and $\log_2$-fold change 2) and predicted correlation in expression between miRNA-mRNA pairs. A positive correlation means miRNA-mRNA pairs were both highly or lowly expressed, and a negative correlation that they exhibit reciprocal expression. Annotations for the genes in each category are given in Table S10

	Up-regulated in CR	Down-regulated in CR
Pearson's r > 0.5	33 (6)	5 (1)
Pearson's r < −0.5	33 (7)	4 (0)

global, as over 6,000 genes respond to treatment (Table 1. Log-fold change 0), and more genes were significantly up-regulated under CR with increasing $\log_2$-fold change thresholds (Table 1).

By bringing together several distinct analyses incorporating differential transcript usage, gene-level differential expression, gene ontology enrichment, gene set enrichment analysis, micro-RNA and methylation changes, we were able to make distinct biological inferences. The following discussion is organized by these inferences, each of which roughly corresponds to a result from a distinct analysis. In part 1, we focus on the haemoglobin response that emerged from the DTU analysis with support from gene expression results; part 2, discusses the endoplasmic reticulum stress–response results from GSEA; part 3, down-regulation of superoxide dismutase is a gene-level response identified in DESeq2; part 4, shows overlap in GO and GSEA down-regulated in CR processes; part 5, asks why differential methylation identified in response to CR in the same clone (Hearn, Pearson, et al., 2019) did not impact upon gene expression; finally, part 6 examines miRNA correlations that were biased towards genes up-regulated in CR.

4.1 | A haemoglobin response to CR in *D. magna*

Fewer genes had differential transcript usage detected than differential gene expression. This may reflect biological reality as the eight replicates per condition give reasonable power in DTU analyses (by comparison with simulated data in Love et al., 2018). However, our average sequencing depth per replicate of 17.2 mega-bases is lower than the 30–40 mega-bases simulated by Love et al., 2018, which could have resulted in under-sampling of lowly expressed isoforms. Alternatively, this could be because we were conservative in our approach to filtering DTUs by only taking the union of DEXSeq and DRIMSeq. Gene isoforms may also be under-annotated, although the gene set used as a reference was from a comprehensive bioinformatic construction of *D. magna* gene expression after exposure to twelve environmental stressors (Orsini et al., 2016).

DTU analysis identified a highly expressed Hb gene that responded to CR by changing isoform proportions (Figure 3). We believe it is the first time haemoglobin genes have been implicated in CR in *D. magna*. This is concordant with the observed changing Hb protein levels in response to food in *Daphnia* (Fox et al., 1951). The gene involved, Dapma7bEVm014981, has many different isoforms with the three most highly expressed in this experiment exhibiting varied proportions of expression between CR and NF. Most strikingly, transcript 12, which encodes three erythrocruorin (IPR002336) domains, is down-regulated strongly in CR (Figure 3). These erythrocruorin domain provide the scaffold for a Haem-binding pocket (http://www.ebi.ac.uk/interpro/entry/InterPro/IPR002336/) and hence oxygen affinity of the haemoglobin gene product. Based on these results, we hypothesize that *D. magna* moderates its haemoglobin mix in response to CR by reducing respiration, leading to a lower proportion of haemoglobin containing erythrocruorin domains under CR. This is evidenced by a change in the proportions of expressed isoforms of gene Dapma7bEVm014981 under CR in favour of isoforms without (transcript 21) an erythrocruorin domain or two domains only (transcript 11). Thus, variation in erythrocruorin domains could underlie the observed correlation between oxygen-binding capacity and structurally distinct Hb gene isoforms (Zeis, 2020).

As far as we are aware the effect of stress, here in the form of CR, on haemoglobin isoform expression has not been established prior to this work. We believe will be a fruitful area of future research in *Daphnia* and other organisms. For example, mammalian RNA-dependent protein kinase R (PKR) is an intracellular sensor of stress that has recently been shown to also be essential for globin gene expression generally (Ilan et al., 2017). It could potentially also act in conditions of cellular stress to modulate haemoglobin expression, while in *Drosophila* knockdown of a globin gene expressed in the trachea reduces survival under hypoxia (Gleixner et al., 2016; Harrison et al., 2018), presumably due to a role in providing adequate oxygen levels. More immediately, studies of Hb response to environmental stressors in *Daphnia* should consider differential isoform usage following our insights here, in addition to Hb gene-copy usage.

The Hb mix of *D. magna* is known to change in response to hypoxia due to the action of the dimeric hypoxia-inducible factor 1 (Cuenca Cambronero et al., 2018; Zeis, 2020). We identified that the HIF-1α component of the Hb gene transcription factor HIF-1 is up-regulated in CR, as are two HIF-1β genes. However, the most highly expressed HIF-1β is down-regulated in CR. The discordant patterns of HIF-1 component expression suggest HIF-1 regulation is not straightforward. Indeed, in *C. elegans* HIF-1 can promote or limit longevity (Leiser & Kaeberlein, 2010). The involvement of HIF-1 does suggest a previously unidentified overlap between hypoxia and CR response in *Daphnia*. This may explain the similar phenotypic effects on body size in offspring under hypoxia and CR, with little associated impact on reproduction of CR and hypoxia (Garbutt & Little, 2017; Hearn et al., 2018; Seidl et al., 2005). It also indicates that the mTOR pathway is active in the *D. magna* response to CR (Land & Tee, 2007), which was supported here by up-regulation under CR of an mTOR protein kinase (Dapma7bEVm000341).

4.2 | The endoplasmic reticulum stress response under CR

The serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme 1 KEGG category (IRE1, K08852) was up-regulated under CR in clusterProfiler GSEA analysis (Figure 5). This category of genes consisted of 103 genes, of which 97 were up-regulated in CR. Approximately two-thirds (61) of these genes are annotated as serine protein kinases, two of which are specifically IRE1 endoribonuclease (Table S9). When compared to the DESeq2 gene expression results, 56 of these genes were also up-regulated in CR at log-fold change 0. IRE1 is a sensor protein in the unfolded protein response that lowers stress in the endoplasmic reticulum. When activated, it initiates a transcription factor (X-box binding protein 1) that up-regulates endoplasmic reticulum-associated degradation genes (Calfon et al., 2002). HIF-1-mediated lifespan increase in *C. elegans* under CR was suppressed in both an IRE1 deletion mutant, and when the X-box binding protein 1 was knocked down with RNAi (Di Chen & Kapahi, 2009). Transient CR-derived stress in *C. elegans* larvae causes a robust IRE1-dependent unfolded protein response to be maintained into adulthood, which is an example of hormesis (Matai et al., 2019). The up-regulation of IRE1 reported here links the CR response in *D. magna* to protein homeostasis in the ER, and the dysregulation of which is strongly linked to ageing in general (Brown et al., 2014; Chadwick & Lajoie, 2019; Cohen, Bieschke, Perciavalle, Kelly, & Dillin, 2006; Steinkraus et al., 2008). This gene has further roles in CR in other organisms. It regulates the increased usage of intestinal triacylglycerol in *Drosophila*, which mediates the metabolic response of midgut epithelium to CR (Luis et al., 2016). While in mice, the up-regulation of IRE1 in response to a reduced protein diet protects against cancer (Rubio-Patiño et al., 2018).

4.3 | Superoxide dismutase was down-regulated under CR

Several of the well-known candidate response genes to CR were significantly up-regulated under CR, including sirtuins, IGF and the mTOR protein kinase. None of these genes showed large differences in their mean expression and $\log_2$-fold changes were modest.

Copper–zinc superoxide dismutase (SOD) was down-regulated under CR in *D. magna*, and this gene has also shown varying responses to CR (or links to longevity) in other systems such as yeast (Mesquita et al., 2010). Overexpression of copper-zinc and manganese SOD in *Drosophila* did not increase lifespan (Orr, Mockett, Benes, & Sohal, 2003), and in termites, increased longevity of queens was associated with enzyme activity and not expression level (Tasaki, Kobayashi, Matsuura, & Iuchi, 2018). The effect of SOD disruption on lifespan varies by experimental context in *Drosophila* (Wang, Branicky, Noë, & Hekimi, 2018). In *D. magna*, copper-zinc SOD is known to increase in expression in response to copper, ammonia and hypoxia levels as they are considered to be general stress response factors (Lyu, Zhu, Wang, Chen, & Yang, 2013). We hypothesize that up-regulated SOD production under NF was due to greater ROS production from higher respiration levels than CR, which was compensated for by greater SOD expression. An alternative explanation is that dissolved oxygen content was lowered in NF rearing jars by increased respiration levels versus CR jars, leading to a hypoxia-induced stress response known to occur in *D. magna* and *D. pulex* in low oxygen conditions (Klumpen et al., 2017; Lyu et al., 2013). This could also explain the Dapma7bEVm014981 Hb gene expressing differential transcripts in response to hypoxia through oxygen depletion in NF versus CR. Arguing against this interpretation was the up-regulation of the stress response IRE-1 genes in CR and not in NF. This is a gene that was switched on under hypoxia in human tumour cells (Wouters & Koritzinsky, 2008) and protected against hypoxia *in C. elegans* (Mao & Crowder, 2010). As a result, we might have expected to see this class of genes more highly expressed (at levels equal to or greater than CR) if NF replicates were also hypoxic. Nevertheless, if reduced respiration is shown to be a hallmark of CR in *Daphnia*, oxygen levels between treatments should be checked to avoid a confounding effect on experimental outcomes.

4.4 | Several processes were repressed under CR

Guanosine triphosphate (GTP)-related molecular function GO terms dominated enrichment in genes down-regulated under CR. The down-regulation of a GTPase has a key role in life extension due to CR in *C. elegans* (M. Hansen et al., 2008). This is hypothesized to stimulate recycling organelles and cytoplasmic proteins (autophagy), which promotes increased lifespans through down-stream mechanisms (M. Hansen et al., 2008).

Apolipoprotein D and other lipocalin genes (K03098) were down-regulated under CR. In *Drosophila*, overexpression of Apolipoprotein D increased lifespan by 18% and tolerance to starvation and could act as a scavenger of toxic products in defence against oxidative stress (Sanchez et al., 2006; Walker, Muffat, Rundel, & Benzer, 2006). Our hypothesis that cellular respiration is reduced in CR predicts that oxidative stress and damage will be correspondingly higher under NF conditions. This would explain the decrease in demand for defensive molecules like Apolipoprotein D under CR in our experiment.

4.5 | A weak correlation between gene methylation and expression

We observed a negative correlation between genes with a methylated CpG portion greater than 5% and gene expression. The highly concordant results for CR and NF comparisons indicate there is no effect of CR on this relationship. In *D. magna*, links between methylation and alternative splicing have been observed previously (Asselman et al., 2017; Kvist et al., 2018), and DNA methylation is enriched in gene bodies. We saw no overlap between the DTU analysis

and previously identified genes overlapping differentially methylated regions (DMRs), which is perhaps not surprising given the expression–methylation correlations between CR and NF expression and CpG methylation are almost identical. These results contrast with (Kvist et al., 2018) in which methylation at exons two to four exhibits significant positive correlations with gene expression in *D. magna*. Our focus on the response to a treatment differs from Kvist et al., (2018), which defined the evolutionary conservation of methylation across gene bodies in *D. magna*.

We suspect that the lack of concordance between the differentially methylated regions identified in Hearn, Pearson, et al. (2019) and gene expression data lie with the methylation analysis. That study used a "smoothing" approach which combines methylation effects across sites in close proximity (K. D. Hansen, Langmead, & Irizarry, 2012). This approach, although tailored to the sparsely CpG methylated *D. magna* genome, was developed on mammalian genomes with somatic CpG methylation rates of 70%-80% (Li & Zhang, 2014). By contrast *D. magna* has a much lower methylation rate of 0.5%–1% (Asselman, 2019; Asselman, De Coninck, Pfrender, & De Schamphelaere, 2016; Asselman et al., 2015, 2017; Hearn, Pearson, et al., 2019; Kvist et al., 2018) as do most arthropods sampled, with exceptions (Bewick, Vogel, Moore, & Schmitz, 2017; Lewis et al., 2020). We recommend that other researchers explore per site-based methods of assessing differential methylation in arthropods (e.g. Park & Wu, 2016), which do not make assumptions about the underlying pattern of methylation. We are currently applying such approaches to forthcoming research in order to definitively answer if CR influences gene expression though methylation in *D. magna*.

4.6 | miRNA correlations are biased towards CR

A robust miRNA response was observed to CR in Hearn et al. (2018), originating from the same total RNA samples included in this study. Here, we observed greater miRNA targeting of genes up-regulated in CR at $\log_2$-fold change 0 for both positive and negative correlations (33 in each case, Table 3). This is in keeping with the general bias towards up-regulation in CR across this experiment, but we do not see overlap in the miRNA correlated gene lists with Hb-related genes or SOD discussed above. Of note, there is a negative correlation between nine miRNAs and Histone-lysine N-methyltransferase Suv4-20 (Dapma7bEVm018601). This gene is significantly up-regulated at $\log_2$-fold change 1 under CR (average TPM 15,063 versus 5,278 in NF). Suv4-20 trimethylates histone H4 lysine 20 and has an important role in DNA repair and genomic stability (Jørgensen, Schotta, & Sørensen, 2013). However, we must interpret the results with caution as computational miRNA-mRNA target inference is prone to false positives (Fridrich, Hazan, & Moran, 2019; Pinzón et al., 2017). This is because animal miRNA seed binding is "wobbly" and does not require perfect complementarity between miRNA and mRNA. Because of this, Fridrich et al. (2019) recommend biological interpretation only when further experimental support is available, even when taking the overlap of multiple prediction programs as we have done.

Demonstrating that an mRNA is regulated by specific miRNAs will require the development of a crosslinking, ligation and sequencing of hybrids (CLASH) protocol for the *D. magna* Argonaute (AGO) proteins (Helwak, Kudla, Dudnakova, & Tollervey, 2013). The CLASH method isolates the AGO-miRNA-mRNA complexes that form during mRNA silencing by miRNA. Sequencing of the interacting RNA in the AGO protein can then be used to identify which miRNAs are bound to what mRNAs under the experimental condition surveyed. Even if the link between miRNA targeting and Suv4-20 is not borne out in future such experiments, the differential expression result indicates a potential link between CR, the histone epigenome and DNA repair.

5 | CONCLUSIONS

We first showed that caloric restriction increases the lifespan of *D. magna* across multiple genotypes. We then chose a CR-responsive genotype to survey the transcriptome of young adults after their first clutch and detected a number of canonical stress and CR responses. By contrasting differential transcript usage between CR and NF, we also showed that the haemoglobin isoform mix of a highly expressed *D. magna* Hb gene is reduced for isoforms containing erythrocruorin domains. We speculate that this may reduce the overall respiration levels of CR individuals and partially explain the observed increased lifespan under CR. Components of the transcription factor that controls Hb gene transcription, HIF-1, are also differentially expressed linking the response to hypoxia with that for CR. An mTOR protein kinase is also differentially expressed and is known to modulate HIF-1. The mTOR pathway is implicated in CR responses of diverse organisms, which is also the case in *D. magna*. The endoplasmic reticulum is implicated in CR through up-regulation of IRE1 as identified by GSEA, and this is a potential down-stream mechanism through which lifespan increases are mediated by CR. We also find that highly expressed copper–zinc superoxide dismutase genes are down-regulated under CR by orthodox gene expression analysis. This gene is associated with longevity in several taxa, but has a variable response to CR. Here, we propose that SOD is down-regulated due to lower levels of cellular respiration in CR, resulting in lowered oxygen stress. Differential miRNA expression is biased towards CR, but functional inference is difficult. DNA methylation shows no crossover with gene expression here. Future work should test whether respiration is suppressed under CR through changes to the haemoglobin mix in *D. magna*, and whether this response occurs in other organisms.

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AUTHOR CONTRIBUTIONS

JH and TJL designed this research and wrote the paper. JH, TJL, PJW and JC performed research. PJW and JC maintained *D. magna* genotypes used in this study.

DATA AVAILABILITY STATEMENT

Raw read data generated for this study have been deposited in the European Nucleotide Archive, Bioproject PRJEB25137 (Hearn, Clark, Wilson, & Little, 2019). All scripts used for analyses are available in File S2.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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