DR. JACK HEARN (Orcid ID : 0000-0003-3358-4949)
PROF. T.J. LITTLE (Orcid ID : 0000-0002-8945-0416)

Article type : Original Article

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

Jack Hearn*[1], Jessica Clark[2], Philip J. Wilson[3], and Tom J. Little[4]

[1]Department of Vector Biology, Liverpool School of Tropical Medicine, Liverpool, United Kingdom
[2]Institute of Biodiversity, Animal Health & Comparative Medicine, University of Glasgow, United Kingdom
[3]School of Geosciences, University of Edinburgh, Edinburgh, United Kingdom
[4]Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, Edinburgh, United Kingdom
*corresponding author: Jack.Hearn@lstmed.ac.uk

Email addresses:

JH: Jack.Hearn@lstmed.ac.uk
JC: jessica.clark@glasgow.ac.uk

PJW: phil.wilson@ed.ac.uk
TJL: Tom.Little@ed.ac.uk

Keywords

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

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 6000 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 super-oxide 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.

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 deacetlyase activity, in addition to changes to insulin-signalling pathway and longevity-associated gene levels (Wood et al., 2015). In *Drosophila* CR responses have been characterised 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, Frederick, & Pfrender, 2011; Mitchell & Lampert, 2000; Orsini et al., 2016), the gene-level response to CR has not previously been studied in this species.

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 (1) larger at birth, (2) feed at a slower rate than offspring of abundantly fed mothers, and are (3) 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.

Daphnia Haemoglobins and the link to CR

Haemoglobin (henceforward "Hb") production was observed to increase in response to food levels in *D. magna* (Fox, Gilchrist, & Phear, 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 hyper-thermal 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).

Control mechanisms underpinning CR responses

The free radical theory of ageing has been linked to CR (Gladyshev, 2014; Liochev, 2013; Viña, Borras, Abdelaziz, Garcia-Valles, & Gomez-Cabrera, 2013). The theory hypothesises that ageing results from accumulated oxidative damage due to reactive oxygen species. Super-oxide 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 1) the insulin/insulin-like growth factor (IGF-1), 2) the mechanistic target of rapamycin (mTOR) signalling pathway, 3) 5' adenosine monophosphate-activated protein kinase (AMP Kinase), and 4) 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).

Characterizing transcriptomic responses to CR

To characterise 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.

Materials and Methods

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), RUYAK1-6 (Russia). Prior to the experiments, replicates of all genotypes were put through three generations of acclimation to harmonise environmental effects arising from variation in stock conditions. During this period, each individual was maintained in a 60ml glass jar filled with artificial pond medium (Kluttgen et al., 1994). This was changed twice weekly and when offspring were produced. Each individual was fed ~6.25 x 10^6 *Chlorella vulgaris* cells daily and was maintained on a 12:12 L:D cycle at 20ºC. We estimate cell numbers by measuring the daily optical absorbance of 650nm 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 x 10^6 cells as per acclimation) and one was assigned to a caloric restriction (CR: ~1.4 x 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.

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: Supplementary File S2, clone longevity input data: Supplementary 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.

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 x 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 x 10^6 algal cells/day or a caloric restricted (CR) diet of 1 x 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 hours, then the five G_0 *D. magna* individuals were homogenised in 700ul Qiazol Qiagen reagent ID: 79306), and stored at -70°C until further processed. RNA was extracted using miRNeasy mini kits (Qiagen Cat No./ID: 74106) 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 4000 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 was deposited in the European Nucleotide Archive under Bioproject PRJEB25137.

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 Trimmommatic (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 (1) an excellent gene-set is available for *D. magna* (Orsini et al., 2016, 2018), (2) 'alignment-free' mapping approaches to transcripts are at least the equal of genome-based alignments for RNASeq (C. Zhang, Zhang, Lin, & Zhao, 2017), and (3) the *D. magna* 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 (Supplementary 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 dataset. 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 non-zero $\log_2$-fold change analyses (i.e $\log_2$-fold change 1 and 2) which 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 penalised 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, & 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 Supplementary File S2.

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) sub-categories 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, Supplementary 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: Supplementary File S2). We ran 1000 iterations of GSEA as the results were variable between runs, and selected only those KEGG terms found significant in >95% of runs.

Interaction between expression level and differential methylation

Average TPM gene expressions calculated in tximport and normalised 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 datasets in this work is given in Supplementary 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 normalised 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).

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 normalised 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.

Results

Effect of CR on longevity

Cox's proportional hazards ANOVA (see Supplementary File S2 for R code and model output) of the survivorship data (Supplementary File S3) showed that CR *D. magna* lived longer on average than NF *D. magna* ($\chi 2 = 13.26$, $p = 0.0003$; 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 ($\chi 2 = 155.4$, $p < 0.0001$), and there was a significant interaction between genotype and food level for differences in longevity (Figure 1; $\chi 2 = 29.4$, $p = 0.0001$).

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 (Supplementary Table S1: read filtering and alignment rates, Supplementary 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% (Supplementary 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, Supplementary 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 super-oxide dismutases which are involved in protection from oxidative damage, including the most expressed gene (Dapma7bEVm009708) at a mean expression of 847468.627 TPM. This was over 6-fold greater than the next gene, a Vitellogenin-1 precursor (Dapma7bEVm014991) (Supplementary 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 hemoglobin 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 uncharacterised genes (Supplementary Table S5).

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 (Supplementary File S5: DRIMSeq transcript expression levels for DTU significant genes). Of these, 32 genes have a mean gene expression normalised count level greater than 1000 TPM and five greater than 10000 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.

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 (Supplementary 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 Supplementary 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 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).

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 (Supplementary 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 CR with a $\log_2$-fold change of 1 or greater intersected with differentially methylated regions. One was an uncharacterised 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 hyper-methylated under CR.

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: Supplementary 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 toward 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 = 10277 TPM), which is important for DNA repair functions, was up-regulated under CR and was negatively correlated with nine miRNAs (Supplementary 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.

Discussion

Firstly, 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 global, as over 6000 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 organised 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 super-oxide 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.

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 to 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 hypothesise 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 underly 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* knock-down 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 suggests 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).

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 (Supplementary 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, 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 triacyglycerol 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).

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 super-oxide dismutase (SOD) was down-regulated under CR in *D. magna,* this gene has also shown varying responses to CR (or links to longevity) in other systems such as yeast (Mesquita et al., 2010). Over-expression 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 hypothesise 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. 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.

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 form 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 lies 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 et al., 2015, 2017; Asselman, De Coninck, Pfrender, & De Schamphelaere, 2016; 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 (for example, 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*.

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 15063 versus 5278 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.

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, this is a potential down-stream mechanism through which lifespan increases are mediated by CR. We also find that highly-expressed copper-zinc super-oxide 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 if respiration is suppressed under CR through changes to the haemoglobin mix in *D. magna*, and if this response occurs in other organisms.

Acknowledgements

This research was funded by the Wellcome Trust Institutional Strategic Support Fund (Round 2, University of Edinburgh). The funder was not involved in study design, and collection, analysis, or interpretation of data and in writing the manuscript.

References

[dataset] Hearn, J., Clark, J., Wilson, P.J., & Little, T.J.; 2019; Differential expression in response to caloric restriction in *Daphnia magna*; European Nucleotide Archive; PRJEB25137.

Alexa, A., & Rahnenfuhrer, J. (2016). topGO: Enrichment Analysis for Gene Ontology. Available at: https://bioconductor.org/packages/release/bioc/html/topGO.html.

Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data.

Genome Biol, *11*. doi:10.1186/gb-2010-11-10-r106

Anders, S., Reyes, A., & Huber, W. (2012). Detecting differential usage of exons from RNA-seq data. *Genome Res*, *22*. doi:10.1101/gr.133744.111

Andrews, S. (2010). FastQC: A quality control application for high throughput sequence data. Available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc.

Asselman, J. (2019). Bioinformatic Analysis of Methylation Patterns Using Bisulfite Sequencing Data. In S. J. Brown & M. E. Pfrender (Eds.), *Insect Genomics: Methods and Protocols* (pp. 157–175). New York, NY: Springer New York. doi:10.1007/978-1-4939-8775-7_12

Asselman, J., Coninck, D. I. M. De, Beert, E., Janssen, C. R., Orsini, L., Pfrender, M. E., … Schamphelaere, K. A. C. De. (2017). Bisulfite Sequencing with *Daphnia* Highlights a Role for Epigenetics in Regulating Stress Response to Microcystis through Preferential Differential Methylation of Serine and Threonine Amino Acids. *Environmental Science & Technology*, *51*(2), 924–931. doi:10.1021/acs.est.6b03870

Asselman, J., De Coninck, D. I. M., Pfrender, M. E., & De Schamphelaere, K. A. C. (2016). Gene Body Methylation Patterns in *Daphnia* Are Associated with Gene Family Size. *Genome Biology and Evolution*, *8*(4), 1185–1196. doi:10.1093/gbe/evw069

Asselman, J., De Coninck, D. I. M., Vandegehuchte, M. B., Jansen, M., Decaestecker, E., De Meester, L., … De Schamphelaere, K. A. C. (2015). Global cytosine methylation in *Daphnia magna* depends on genotype, environment, and their interaction. *Environmental Toxicology and Chemistry*, *34*(5), 1056–1061.

Auld, S. K. J. R., Hall, S. R., Housley Ochs, J., Sebastian, M., & Duffy, M. A. (2014). Predators and patterns of within-host growth can mediate both among-host competition and evolution of transmission potential of parasites. *The American Naturalist*, *184*(S1), S77–S90.

Bewick, A. J., Vogel, K. J., Moore, A. J., & Schmitz, R. J. (2017). Evolution of DNA methylation across insects. *Molecular Biology and Evolution*, *34*(3), 654–665. doi:10.1093/molbev/msw264

Boersma, M., Spaak, P., & De Meester, L. (1998). Predator-mediated plasticity in morphology, life history, and behavior of *Daphnia*: the uncoupling of responses. *The American Naturalist*, *152*(2), 237–248.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. doi:10.1093/bioinformatics/btu170

Brown, M. K., Chan, M. T., Zimmerman, J. E., Pack, A. I., Jackson, N. E., & Naidoo, N. (2014). Aging induced endoplasmic reticulum stress alters sleep and sleep homeostasis. *Neurobiology of Aging*, *35*(6), 1431–1441. doi:10.1016/j.neurobiolaging.2013.12.005

Calfon, M., Zeng, H., Urano, F., Till, J. H., Hubbard, S. R., Harding, H. P., … Ron, D. (2002). IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature*, *415*(6867), 92–96.

Chadwick, S. R., & Lajoie, P. (2019). Endoplasmic Reticulum Stress Coping Mechanisms and Lifespan Regulation in Health and Diseases. *Frontiers in Cell and Developmental Biology*, *7*, 84. doi:10.3389/fcell.2019.00084

Choi, K.-M., Hong, S.-J., van Deursen, J. M., Kim, S., Kim, K. H., & Lee, C.-K. (2018). Caloric restriction and rapamycin differentially alter energy metabolism in yeast. *The Journals of Gerontology: Series A*, *73*(1), 29–38.

Cohen, E., Bieschke, J., Perciavalle, R. M., Kelly, J. W., & Dillin, A. (2006). Opposing activities protect against age-onset proteotoxicity. *Science*, *313*(5793), 1604–1610.

Colbourne, J. K., Pfrender, M. E., Gilbert, D., Thomas, W. K., Tucker, A., Oakley, T. H., … others. (2011). The ecoresponsive genome of *Daphnia pulex*. *Science*, *331*(6017), 555–561.

Cuenca Cambronero, M., Zeis, B., & Orsini, L. (2018). Haemoglobin-mediated response to hyper-thermal stress in the keystone species *Daphnia magna*. *Evolutionary Applications*, *11*(1), 112–120.

Di Chen, E. L. T., & Kapahi, P. (2009). HIF-1 modulates dietary restriction-mediated lifespan extension via IRE-1 in *Caenorhabditis elegans*. *PLoS Genetics*, *5*(5).

Ding, F., Gil, M. P., Franklin, M., Ferreira, J., Tatar, M., Helfand, S. L., & Neretti, N. (2014). Transcriptional response to dietary restriction in *Drosophila melanogaster*. *Journal of Insect Physiology*, *69*(C), 101–106. doi:10.1016/j.jinsphys.2014.05.002

Dobson, A. J., He, X., Blanc, E., Bolukbasi, E., Feseha, Y., Yang, M., & Piper, M. D. W. (2018). Tissue-specific transcriptome profiling of *Drosophila* reveals roles for GATA transcription factors in longevity by dietary restriction. *Npj Aging and Mechanisms of*

Disease, *4*(1), 5. doi:10.1038/s41514-018-0024-4

Enright, A. J., John, B., Gaul, U., Tuschl, T., Sander, C., & Marks, D. S. (2003). MicroRNA targets in *Drosophila*. *Genome Biology*, *5*(1), R1. doi:10.1186/gb-2003-5-1-r1

Fox, H. M., Gilchrist, B. M., & Phear, E. A. (1951). Functions of haemoglobin in *Daphnia*. *Proceedings of the Royal Society of London. Series B-Biological Sciences*, *138*(893), 514–528.

Fridrich, A., Hazan, Y., & Moran, Y. (2019). Too Many False Targets for MicroRNAs: Challenges and Pitfalls in Prediction of miRNA Targets and Their Gene Ontology in Model and Non-model Organisms. *Bioessays*, *41*(4), 1800169.

Garbutt, J. S., & Little, T. J. (2014). Maternal food quantity affects offspring feeding rate in *Daphnia magna*. *Biology Letters*, *10*(7), 20140356.

Garbutt, J. S., & Little, T. J. (2017). Bigger is better: changes in body size explain a maternal effect of food on offspring disease resistance. *Ecology and Evolution*, *7*(5), 1403–1409.

Gerke, P., Börding, C., Zeis, B., & Paul, R. J. (2011). Adaptive haemoglobin gene control in *Daphnia pulex* at different oxygen and temperature conditions. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *159*(1), 56–65.

Gladyshev, V. N. (2014). The free radical theory of aging is dead. Long live the damage theory! *Antioxidants & Redox Signaling*, *20*(4), 727–731. doi:10.1089/ars.2013.5228

Gleixner, E., Ripp, F., Gorr, T. A., Schuh, R., Wolf, C., Burmester, T., & Hankeln, T. (2016). Knockdown of Drosophila hemoglobin suggests a role in O_2 homeostasis. *Insect Biochemistry and Molecular Biology*, *72*, 20–30.

Gorr, T. A., Cahn, J. D., Yamagata, H., & Bunn, H. F. (2004). Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna* is hypoxia-inducible factor-dependent. *Journal of Biological Chemistry*, *279*(34), 36038–36047.

Graham, A. M., & Barreto, F. S. (2019). Novel microRNAs are associated with population divergence in transcriptional response to thermal stress in an intertidal copepod. *Molecular Ecology*, *28*(3), 584–599.

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., … Regev, A. (2013). *De novo* transcript sequence reconstruction from RNA-seq using

the Trinity platform for reference generation and analysis. *Nat. Protocols*, *8*(8), 1494–1512. Retrieved from http://dx.doi.org/10.1038/nprot.2013.084

Hansen, K. D., Langmead, B., & Irizarry, R. A. (2012). BSmooth: from whole genome bisulfite sequencing reads to differentially methylated regions. *Genome Biology*, *13*(10), R83.

Hansen, M., Chandra, A., Mitic, L. L., Onken, B., Driscoll, M., & Kenyon, C. (2008). A role for autophagy in the extension of lifespan by dietary restriction in *C. elegans*. *PLoS Genetics*, *4*(2), e24.

Harrison, J. F., Greenlee, K. J., & Verberk, W. C. E. P. (2018). Functional hypoxia in insects: definition, assessment, and consequences for physiology, ecology, and evolution. *Annual Review of Entomology*, *63*, 303–325.

Hearn, J., Blaxter, M., Schönrogge, K., Nieves-Aldrey, J.-L., Pujade-Villar, J., Huguet, E., … Stone, G. N. (2019). Genomic dissection of an extended phenotype: Oak galling by a cynipid gall wasp. *PLoS Genetics*, *15*(11), e1008398–e1008398. doi:10.1371/journal.pgen.1008398

Hearn, J., Chow, F. W.-N., Barton, H., Tung, M., Wilson, P., Blaxter, M., … Little, T. J. (2018). *Daphnia magna* microRNAs respond to nutritional stress and ageing but are not transgenerational. *Molecular Ecology*, *27*(6), 1402–1412.

Hearn, J., Pearson, M., Blaxter, M., Wilson, P. J., & Little, T. J. (2019). Genome-wide methylation is modified by caloric restriction in *Daphnia magna*. *BMC Genomics*, *20*(1), 197. doi:10.1186/s12864-019-5578-4

Heilbronn, L. K., & Ravussin, E. (2003). Calorie restriction and aging: review of the literature and implications for studies in humans. *The American Journal of Clinical Nutrition*, *78*(3), 361–369.

Heintz, C., Doktor, T. K., Lanjuin, A., Escoubas, C., Zhang, Y., Weir, H. J., … Mair, W. B. (2017). Splicing factor 1 modulates dietary restriction and TORC1 pathway longevity in *C. elegans*. *Nature*, *541*(7635), 102–106. doi:10.1038/nature20789

Helwak, A., Kudla, G., Dudnakova, T., & Tollervey, D. (2013). Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. *Cell*, *153*(3), 654–665.

Hurlbert, S. H. (1984). Pseudoreplication and the design of ecological field experiments. *Ecological Monographs*, *54*(2), 187–211.

Ilan, L., Osman, F., Namer, L. S., Eliahu, E., Cohen-Chalamish, S., Ben-Asouli, Y., … Kaempfer, R. (2017). PKR activation and eIF2α phosphorylation mediate human globin mRNA splicing at spliceosome assembly. *Cell Research*, *27*(5), 688–704.

Johnson, S. C., Rabinovitch, P. S., & Kaeberlein, M. (2013). mTOR is a key modulator of ageing and age-related disease. *Nature*, *493*(7432), 338–345. doi:10.1038/nature11861

Jørgensen, S., Schotta, G., & Sørensen, C. S. (2013). Histone H4 lysine 20 methylation: key player in epigenetic regulation of genomic integrity. *Nucleic Acids Research*, *41*(5), 2797–2806.

Kanehisa, M., & Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*, *28*(1), 27–30.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M. (2015). KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Research*, *44*(D1), D457--D462.

Kapahi, P., Kaeberlein, M., & Hansen, M. (2017). Dietary restriction and lifespan: Lessons from invertebrate models. *Ageing Research Reviews*, *39*, 3–14. doi:10.1016/j.arr.2016.12.005

Kenyon, C. J. (2010). The genetics of ageing. *Nature*, *464*(7288), 504–512. doi:10.1038/nature08980

Kertesz, M., Iovino, N., Unnerstall, U., Gaul, U., & Segal, E. (2007). The role of site accessibility in microRNA target recognition. *Nature Genetics*, *39*(10), 1278–1284.

Kim, S.-S., Choi, K.-M., Kim, S., Park, T., Cho, I.-C., Lee, J.-W., & Lee, C.-K. (2016). Whole-transcriptome analysis of mouse adipose tissue in response to short-term caloric restriction. *Molecular Genetics and Genomics*, *291*(2), 831–847. doi:10.1007/s00438-015-1150-3

Klumpen, E., Hoffschröer, N., Zeis, B., Gigengack, U., Dohmen, E., & Paul, R. J. (2017). Reactive oxygen species (ROS) and the heat stress response of *Daphnia pulex*: ROS-mediated activation of hypoxia-inducible factor 1 (HIF-1) and heat shock factor 1 (HSF-1) and the clustered expression of stress genes. *Biology of the Cell*, *109*(1), 39–64.

Koubova, J., & Guarente, L. (2003). How does calorie restriction work? *Genes & Development*, *17*(3), 313–321.

Krueger, F., & Andrews, S. R. (2011). Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics*, *27*(11), 1571–1572.

Krüger, J., & Rehmsmeier, M. (2006). RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Research*, *34*(suppl_2), W451--W454.

Kvist, J., Gonçalves Athanàsio, C., Shams Solari, O., Brown, J. B., Colbourne, J. K., Pfrender, M. E., & Mirbahai, L. (2018). Pattern of DNA Methylation in *Daphnia*: Evolutionary Perspective. *Genome Biology and Evolution*, *10*(8), 1988–2007. doi:10.1093/gbe/evy155

Lai, K.-P., Li, J.-W., Chan, C. Y.-S., Chan, T.-F., Yuen, K. W.-Y., & Chiu, J. M.-Y. (2016). Transcriptomic alterations in *Daphnia magna* embryos from mothers exposed to hypoxia. *Aquatic Toxicology*, *177*, 454–463.

Lakowski, B., & Hekimi, S. (1998). The genetics of caloric restriction in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(22), 13091–13096. doi:10.1073/pnas.95.22.13091

Lampert, W. (1987). Feeding and nutrition in *Daphnia*. In R. H. Peters & R. De Bernardi (Eds.), *Daphnia* (p. 502). Men. Ist. Ital. Idrobiol.

Land, S. C., & Tee, A. R. (2007). Hypoxia-inducible factor 1α is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. *Journal of Biological Chemistry*, *282*(28), 20534–20543.

Latta IV, L. C., Frederick, S., & Pfrender, M. E. (2011). Diet restriction and life history trade-offs in short-and long-lived species of *Daphnia*. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *315*(10), 610–617.

Le, T. D., Zhang, J., Liu, L., Liu, H., & Li, J. (2015). MiRLAB: An R based dry lab for exploring miRNA-mRNA regulatory relationships. *PLoS ONE*, *10*(12), 1–15. doi:10.1371/journal.pone.0145386

Lee, S.-J., Hwang, A. B., & Kenyon, C. (2010). Inhibition of respiration extends *C. elegans* life span via reactive oxygen species that increase HIF-1 activity. *Current Biology*, *20*(23), 2131–2136.

Leiser, S. F., & Kaeberlein, M. (2010). The hypoxia-inducible factor HIF-1 functions as both a positive and negative modulator of aging. *Biological Chemistry*, *391*(10), 1131–1137.

Lewis, S. H., Ross, L., Bain, S. A., Pahita, E., Smith, S. A., Cordaux, R., … Sarkies, P.

(2020). Widespread conservation and lineage-specific diversification of genome-wide DNA methylation patterns across arthropods. *PLOS Genetics*, *16*(6), 1–24. doi:10.1371/journal.pgen.1008864

Li, E., & Zhang, Y. (2014). DNA methylation in mammals. *Cold Spring Harbor Perspectives in Biology*, *6*(5), a019133.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. doi:10.1186/s13059-014-0550-8

Love, M. I., Soneson, C., & Patro, R. (2018). Swimming downstream: statistical analysis of differential transcript usage following Salmon quantification. *F1000Research*, *7*.

Luis, N. M., Wang, L., Ortega, M., Deng, H., Katewa, S. D., Li, P. W.-L., … Kapahi, P. (2016). Intestinal IRE1 is required for increased triglyceride metabolism and longer lifespan under dietary restriction. *Cell Reports*, *17*(5), 1207–1216.

Lukasik, A., Wójcikowski, M., & Zielenkiewicz, P. (2016). Tools4miRs – one place to gather all the tools for miRNA analysis. *Bioinformatics*, *32*(17), 2722–2724. doi:10.1093/bioinformatics/btw189

Lyu, K., Wang, Q., Li, Z., Chen, R., Zhu, C., Liu, J., & Yang, Z. (2015). Age-dependent survival and selected gene expression in *Daphnia magna* after short-term exposure to low dissolved oxygen. *Journal of Plankton Research*, *37*(1), 66–74.

Lyu, K., Zhu, X., Wang, Q., Chen, Y., & Yang, Z. (2013). Copper/Zinc Superoxide Dismutase from the Cladoceran *Daphnia magna*: Molecular Cloning and Expression in Response to Different Acute Environmental Stressors. *Environmental Science & Technology*, *47*(15), 8887–8893. doi:10.1021/es4015212

Mao, X. R., & Crowder, C. M. (2010). Protein misfolding induces hypoxic preconditioning via a subset of the unfolded protein response machinery. *Molecular and Cellular Biology*, *30*(21), 5033–5042.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1). Retrieved from http://journal.embnet.org/index.php/embnetjournal/article/view/200

Matai, L., Sarkar, G. C., Chamoli, M., Malik, Y., Kumar, S. S., Rautela, U., … Mukhopadhyay, A. (2019). Dietary restriction improves proteostasis and increases life span through endoplasmic reticulum hormesis. *Proceedings of the National*

Academy of Sciences, *116*(35), 17383 LP – 17392. doi:10.1073/pnas.1900055116

Matthews, D., Diskin, M. G., Kenny, D. A., Creevey, C. J., Keogh, K., & Waters, S. M. (2017). Effect of short term diet restriction on gene expression in the bovine hypothalamus using next generation RNA sequencing technology. *BMC Genomics*, *18*(1), 1–14. doi:10.1186/s12864-017-4265-6

Mesquita, A., Weinberger, M., Silva, A., Sampaio-Marques, B., Almeida, B., Leão, C., … Ludovico, P. (2010). Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H_2O_2 and superoxide dismutase activity. *Proceedings of the National Academy of Sciences*, *107*(34), 15123 LP – 15128. doi:10.1073/pnas.1004432107

Miranda, K. C., Huynh, T., Tay, Y., Ang, Y.-S., Tam, W.-L., Thomson, A. M., … Rigoutsos, I. (2006). A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. *Cell*, *126*(6), 1203–1217.

Mitchell, S. E., & Lampert, W. (2000). Temperature adaptation in a geographically widespread zooplankter, *Daphnia magna*. *Journal of Evolutionary Biology*, *13*(3), 371–382.

Most, J., Tosti, V., Redman, L. M., & Fontana, L. (2017). Calorie restriction in humans: An update. *Ageing Research Reviews*, *39*, 36–45. doi:10.1016/j.arr.2016.08.005

Muid, K. A., Karakaya, H. Ç., & Koc, A. (2014). Absence of superoxide dismutase activity causes nuclear DNA fragmentation during the aging process. *Biochemical and Biophysical Research Communications*, *444*(2), 260–263.

Muller, F. L., Song, W., Liu, Y., Chaudhuri, A., Pieke-Dahl, S., Strong, R., … others. (2006). Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. *Free Radical Biology and Medicine*, *40*(11), 1993–2004.

Nowicka, M., & Robinson, M. D. (2016). DRIMSeq: a Dirichlet-multinomial framework for multivariate count outcomes in genomics. *F1000Research*, *5*.

Oka, S., Hirai, J., Yasukawa, T., Nakahara, Y., & Inoue, Y. H. (2015). A correlation of reactive oxygen species accumulation by depletion of superoxide dismutases with age-dependent impairment in the nervous system and muscles of *Drosophila* adults. *Biogerontology*, *16*(4), 485–501.

Orr, W. C., Mockett, R. J., Benes, J. J., & Sohal, R. S. (2003). Effects of Overexpression

of Copper-Zinc and Manganese Superoxide Dismutases, Catalase, and Thioredoxin Reductase Genes on Longevity in *Drosophila melanogaster*. *Journal of Biological Chemistry*, *278*(29), 26418–26422. doi:10.1074/jbc.M303095200

Orsini, L., Brown, J. B., Shams Solari, O., Li, D., He, S., Podicheti, R., … others. (2018). Early transcriptional response pathways in *Daphnia magna* are coordinated in networks of crustacean-specific genes. *Molecular Ecology*, *27*(4), 886–897.

Orsini, L., Gilbert, D., Podicheti, R., Jansen, M., Brown, J. B., Solari, O. S., … Frilander, M. J. (2016). *Daphnia magna* transcriptome by RNA-Seq across 12 environmental stressors. *Scientific Data*, *3*, 160030. Retrieved from http://dx.doi.org/10.1038/sdata.2016.30

Park, Y., & Wu, H. (2016). Differential methylation analysis for BS-seq data under general experimental design. *Bioinformatics*, *32*(10), 1446–1453.

Patro, R., Duggal, G., Love, M. I., Irizarry, R. A., & Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nature Methods*, *14*(4), 417–419. doi:10.1038/nmeth.4197

Pinzón, N., Li, B., Martinez, L., Sergeeva, A., Presumey, J., Apparailly, F., & Seitz, H. (2017). microRNA target prediction programs predict many false positives. *Genome Research*, *27*(2), 234–245.

Quinlan, A. R., & Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics*, *26*(6), 841–842. doi:10.1093/bioinformatics/btq033

Redman, L. M., & Ravussin, E. (2011). Caloric restriction in humans: impact on physiological, psychological, and behavioral outcomes. *Antioxidants & Redox Signaling*, *14*(2), 275–287.

Regan, J. C., Khericha, M., Dobson, A. J., Bolukbasi, E., Rattanavirotkul, N., & Partridge, L. (2016). Sex difference in pathology of the ageing gut mediates the greater response of female lifespan to dietary restriction. *Elife*, *5*, e10956.

Rubio-Patiño, C., Bossowski, J. P., De Donatis, G. M., Mondragón, L., Villa, E., Aira, L. E., … others. (2018). Low-protein diet induces IRE1α-dependent anticancer immunosurveillance. *Cell Metabolism*, *27*(4), 828–842.

Sanchez, D., López-Arias, B., Torroja, L., Canal, I., Wang, X., Bastiani, M. J., & Ganfornina, M. D. (2006). Loss of glial lazarillo, a homolog of apolipoprotein D, reduces lifespan and stress resistance in *Drosophila*. *Current Biology*, *16*(7), 680–

686.

Seidl, M. D., Paul, R. J., & Pirow, R. (2005). Effects of hypoxia acclimation on morpho-physiological traits over three generations of *Daphnia magna*. *Journal of Experimental Biology*, *208*(11), 2165–2175.

Sohal, R. S., & Weindruch, R. (1996). Oxidative Stress, Caloric Restriction, and Aging. *Science*, *273*(5271), 59–63. Retrieved from http://science.sciencemag.org/content/273/5271/59.abstract

Soneson, C., Love, M. I., & Robinson, M. D. (2015). Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Research*, *4*.

Steinkraus, K. A., Smith, E. D., Davis, C., Carr, D., Pendergrass, W. R., Sutphin, G. L., … Kaeberlein, M. (2008). Dietary restriction suppresses proteotoxicity and enhances longevity by an hsf-1-dependent mechanism in *Caenorhabditis elegans*. *Aging Cell*, *7*(3), 394–404.

Stephens, M. (2016). False discovery rates: a new deal. *Biostatistics*, *18*(2), 275–294.

Supek, F., Bošnjak, M., Škunca, N., & Šmuc, T. (2011). REVIGO summarizes and visualizes long lists of gene ontology terms. *PloS One*, *6*(7), e21800.

Tasaki, E., Kobayashi, K., Matsuura, K., & Iuchi, Y. (2018). Long-lived termite queens exhibit high Cu/Zn-superoxide dismutase activity. *Oxidative Medicine and Cellular Longevity*, *2018*.

Thadani, R., & Tammi, M. T. (2006). MicroTar: predicting microRNA targets from RNA duplexes. In *BMC bioinformatics* (Vol. 7, p. S20).

Vigne, P., & Frelin, C. (2006). A low protein diet increases the hypoxic tolerance in Drosophila. *PLoS One*, *1*(1).

Walford, R. L., Harris, S. B., & Weindruch, R. (1987). Dietary restriction and aging: historical phases, mechanisms and current directions. *The Journal of Nutrition*, *117*(10), 1650–1654.

Walker, D. W., Muffat, J., Rundel, C., & Benzer, S. (2006). Overexpression of a *Drosophila* homolog of apolipoprotein D leads to increased stress resistance and extended lifespan. *Current Biology*, *16*(7), 674–679.

Wang, Y., Branicky, R., Noë, A., & Hekimi, S. (2018). Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. *The Journal of Cell Biology*, *217*(6), 1915 LP – 1928. doi:10.1083/jcb.201708007

Wood, S. H., Dam, S., Craig, T., Tacutu, R., O'Toole, A., Merry, B. J., & de Magalhães, J. P. (2015). Transcriptome analysis in calorie-restricted rats implicates epigenetic and post-translational mechanisms in neuroprotection and aging. *Genome Biology*, *16*(1), 285.

Wouters, B. G., & Koritzinsky, M. (2008). Hypoxia signalling through mTOR and the unfolded protein response in cancer. *Nature Reviews Cancer*, *8*(11), 851–864.

Yu, G., Wang, L.-G., Han, Y., & He, Q.-Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. *Omics: A Journal of Integrative Biology*, *16*(5), 284–287.

Zeis, B. (2020). Hemoglobin in Arthropods-*Daphnia* as a Model. In U. Hoeger & J. R. Harris (Eds.), *Vertebrate and Invertebrate Respiratory Proteins, Lipoproteins and other Body Fluid Proteins* (pp. 163–194). Cham: Springer International Publishing. doi:10.1007/978-3-030-41769-7_6

Zeis, B., Becher, B., Goldmann, T., Clark, R., Vollmer, E., Bölke, B., … others. (2003). Differential haemoglobin gene expression in the crustacean *Daphnia magna* exposed to different oxygen partial pressures. *Biological Chemistry*, *384*(8), 1133–1145.

Zhang, C., Zhang, B., Lin, L.-L., & Zhao, S. (2017). Evaluation and comparison of computational tools for RNA-seq isoform quantification. *BMC Genomics*, *18*(1), 583.

Zhang, Y., Shao, Z., Zhai, Z., Shen, C., & Powell-Coffman, J. A. (2009). The HIF-1 hypoxia-inducible factor modulates lifespan in *C. elegans*. *PloS One*, *4*(7).

Zhu, A., Ibrahim, J. G., & Love, M. I. (2019). Heavy-tailed prior distributions for sequence count data: removing the noise and preserving large differences. *Bioinformatics*, *35*(12), 2084–2092.

Data Accessibility

Raw read data generated for this study has been deposited in the European Nucleotide Archive, Bioproject PRJEB25137. All scripts used for analyses are available in Supplementary File S2.

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.

Tables

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	3345	280	62
Down-regulated in CR	3063	52	11

Table 2. Overlap between significantly expressed genes at log_2-fold change 0 and hypo- or hyper-methylated genes. Percentages were calculated from total hypo- and hyper-methylated genes.

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

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 Supplementary 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)

Figures

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.

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.

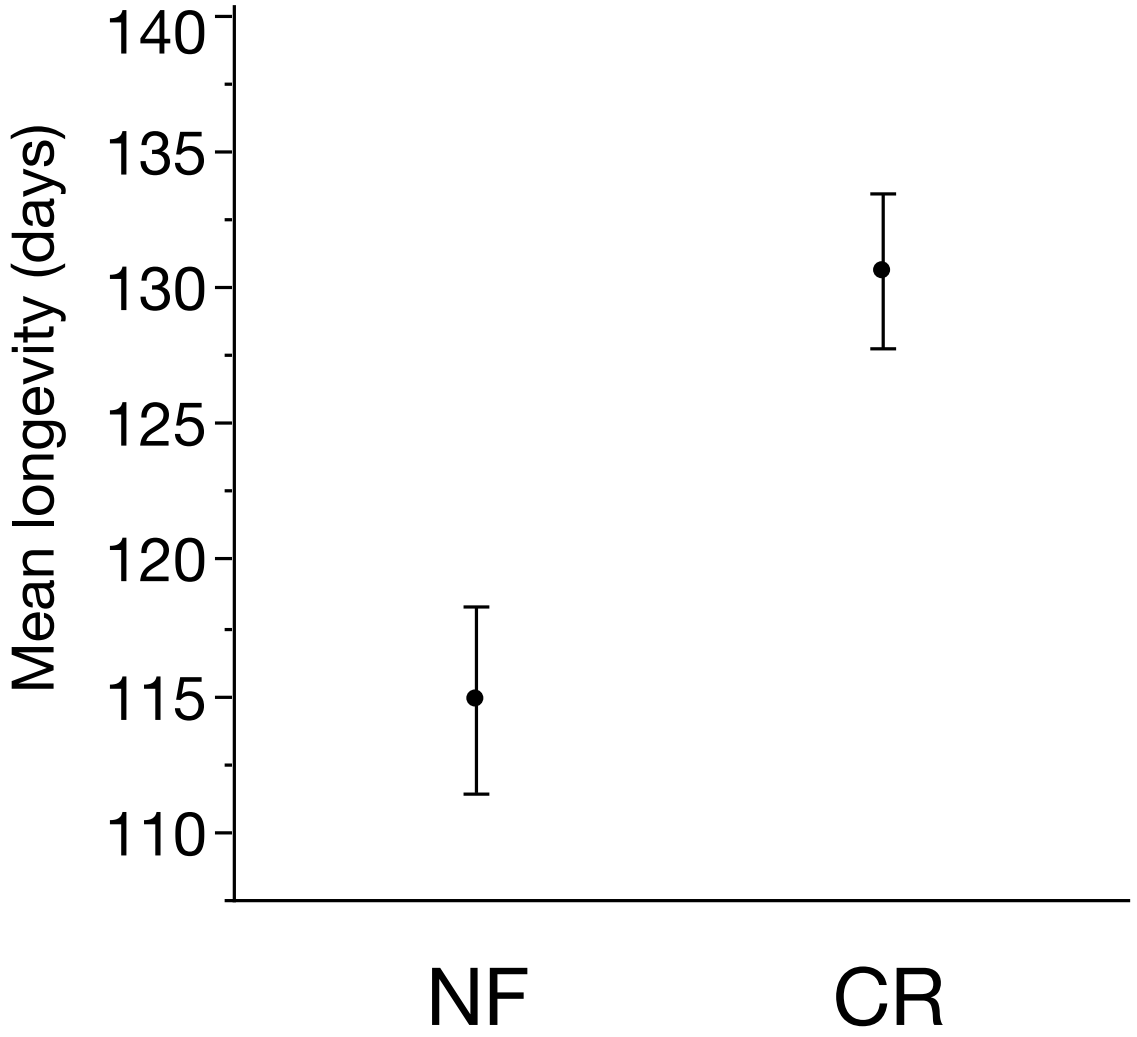
Figure 3. Transcript proportion plots and protein domains for differentially used transcripts of an up-regulated di-domain haemoglobin. Transcripts 11, 12, and 21 of gene Dapma7bEVm014981 show DTU across conditions in DEXSeq as does transcript 12 in DRIMSeq adjusted results. Part A) Bars represent proportions 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.

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

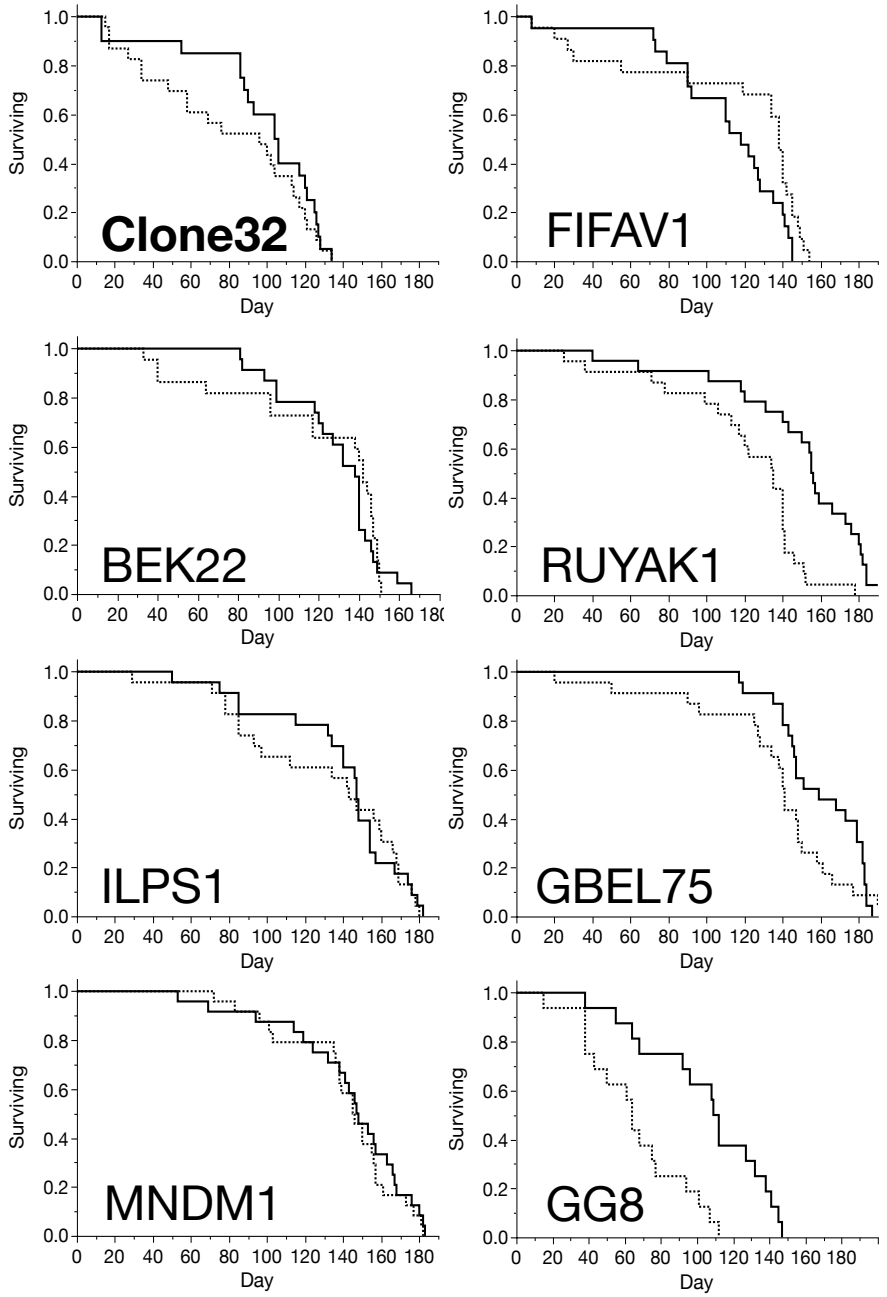
p-value with scales inset next to each plot. A) Down-regulated in CR Biological Process GO terms, B) Up-regulated in CR Biological Process GO terms C) Down-regulated in CR molecular function GO terms, D) Up-regulated in CR molecular function GO terms. GO terms associated with numbers can be found in Supplementary Table S11.

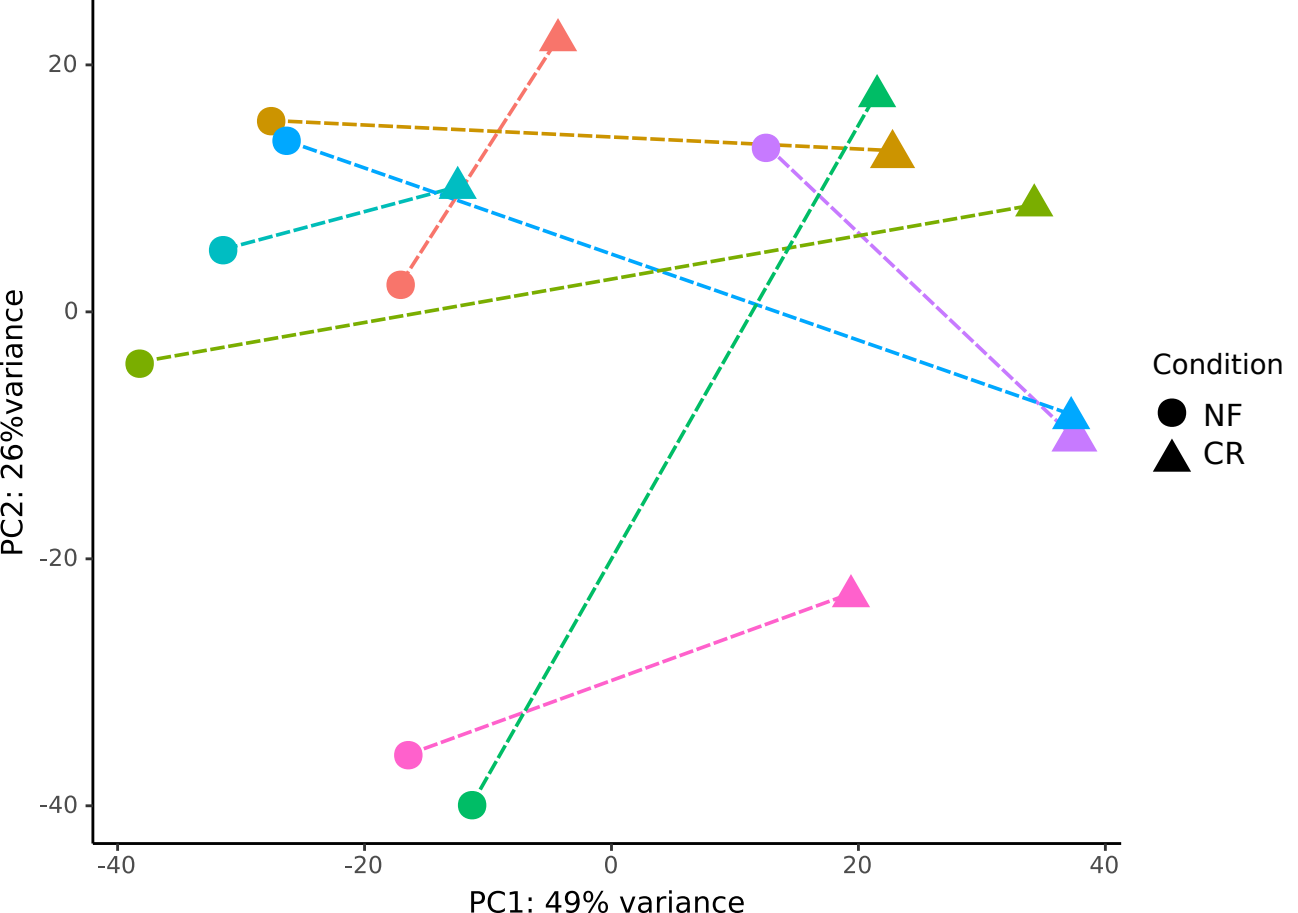
Figure 5. Gene Set Enrichment Analysis ridge plot for significant KEGG terms. Ridge plots are density plots of the frequency of log_2 fold-change values per gene within each enriched KEGG group, which helps to interpret the up- or down-regulation of that KEGG category. The plot was created in clusterProfiler 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.

A)

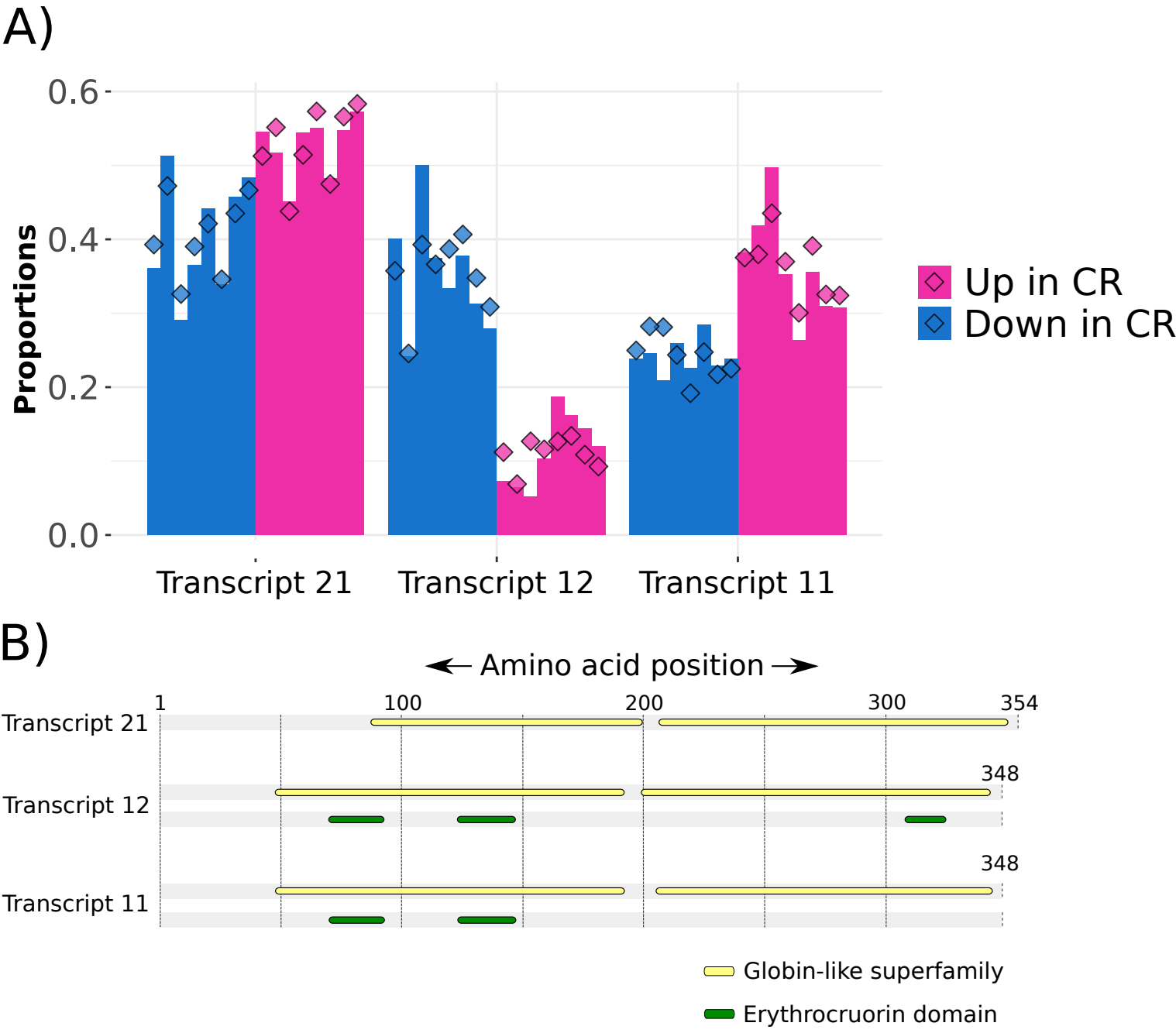

Mean longevity (days)
140
135
130
125
120
115
110
NF
CR

B)


Surviving
Day
Clone32
FIFAV1
BEK22
RUYAK1
ILPS1
GBEL75
MNDM1
GG8


20
0
-20
-40
PC2: 26%variance
-40
-20
0
20
40
PC1: 49% variance
Condition
NF
CR

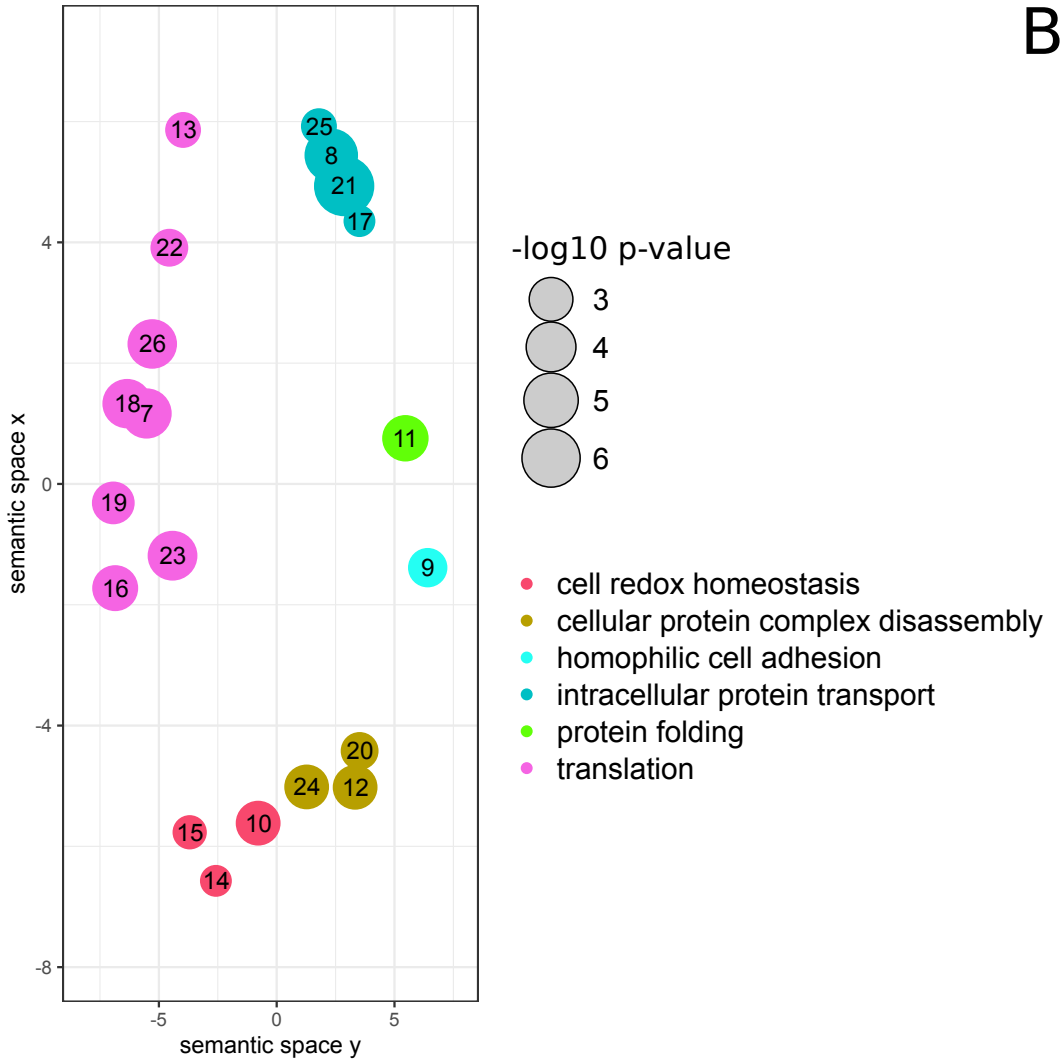
Di-domain Haemoglobin DTU



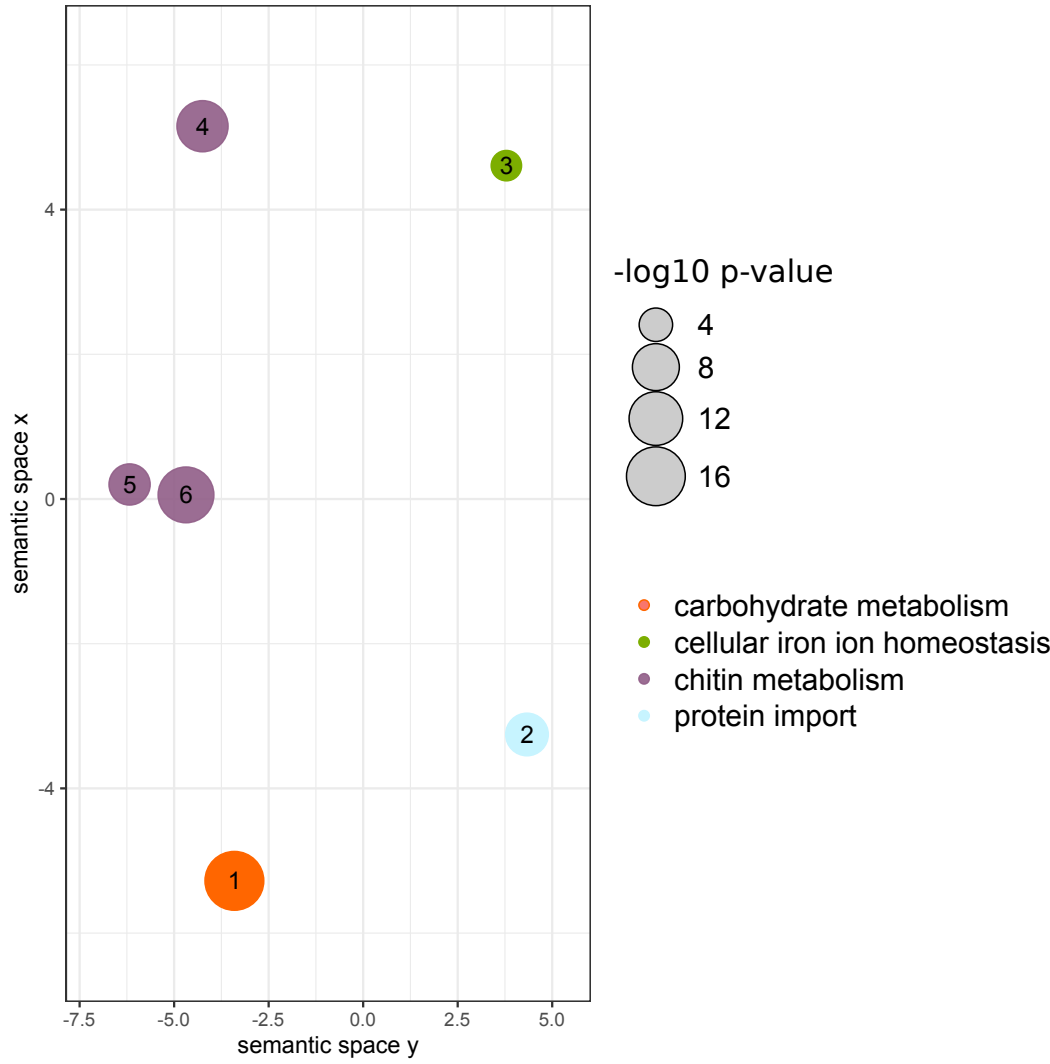
Down-regulated in CR **Up-regulated in CR**

Biological Process

A)

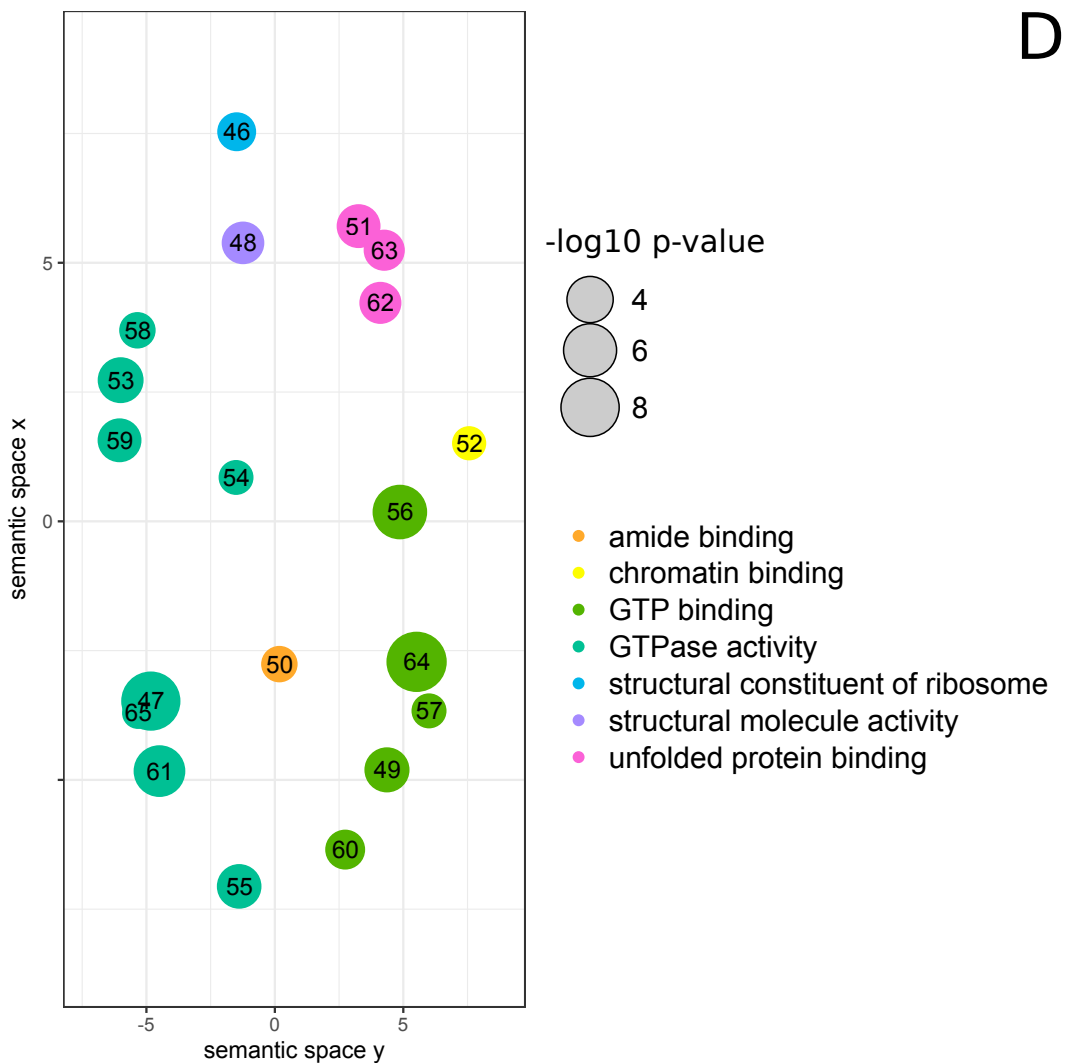


B)

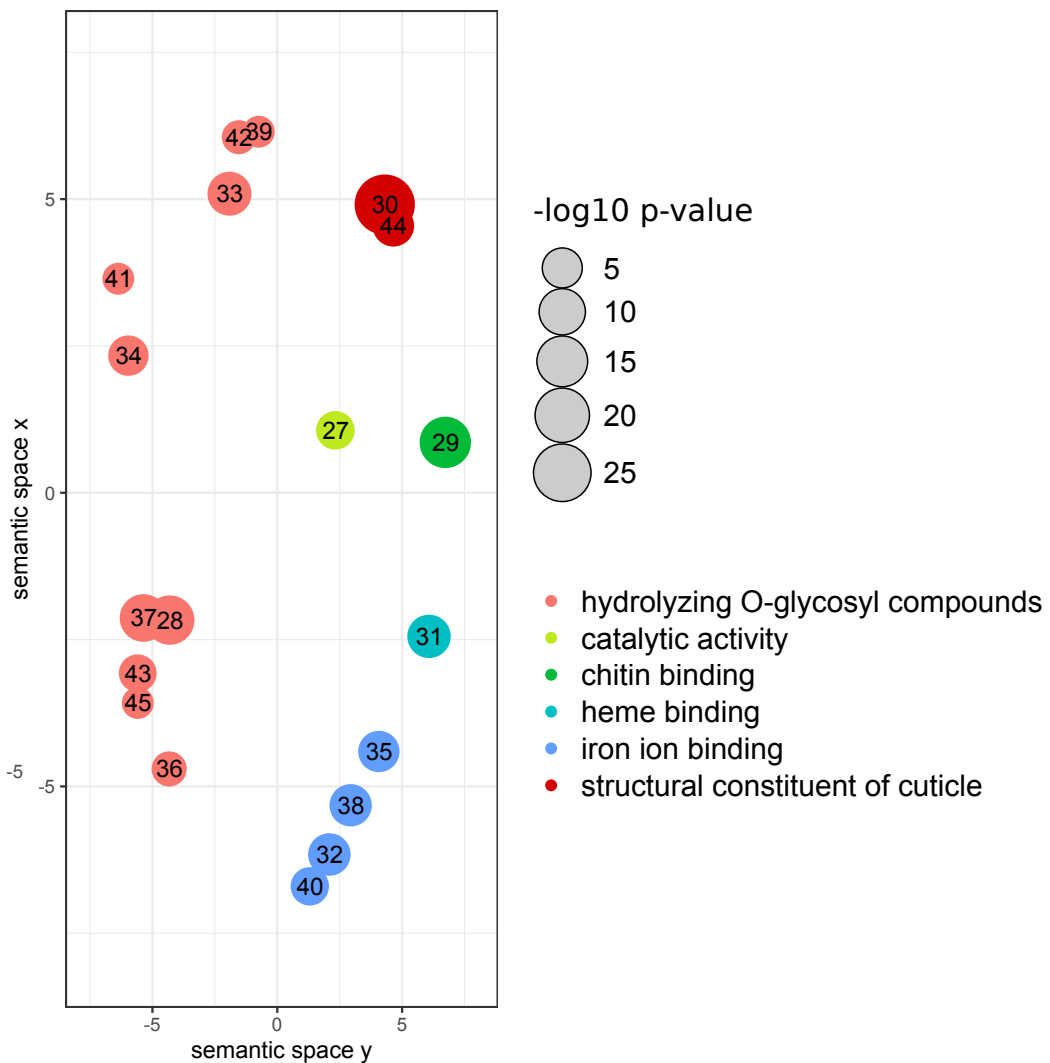


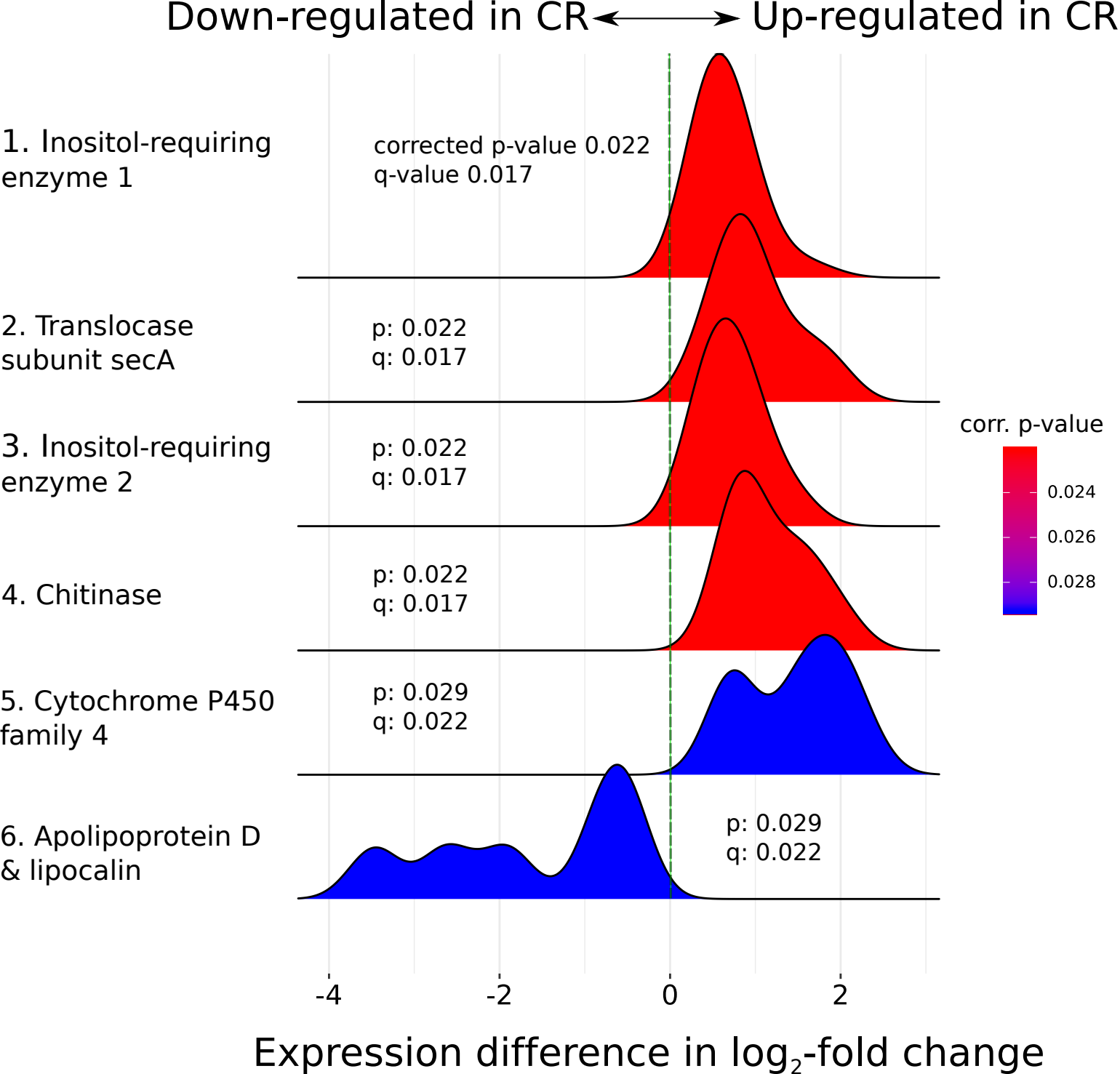
Molecular Function

C)



D)




Down-regulated in CR
Up-regulated in CR
1. Inositol-requiring enzyme 1
corrected p-value 0.022
q-value 0.017
2. Translocase subunit secA
p: 0.022
q: 0.017
3. Inositol-requiring enzyme 2
p: 0.022
q: 0.017
4. Chitinase
p: 0.022
q: 0.017
5. Cytochrome P450 family 4
p: 0.029
q: 0.022
6. Apolipoprotein D & lipocalin
p: 0.029
q: 0.022
corr. p-value
0.024
0.026
0.028
-4
-2
0
2
Expression difference in log2-fold change