Surviving on limited resources: effects of caloric restriction on growth, gene expression and gut microbiota in a species with male pregnancy (*Hippocampus erectus*).

Freya Adele Pappert[1,2], Vincent Alexander Wüst[1], Carmen Fontanes Eguiguren[3], Olivia Roth[1,2]

[1]Marine Evolutionary Biology, Zoological Institute, Christian-Albrechts-Universität Kiel, Am Botanischen Garten 1-9, 24118 Kiel, Germany

[2]Evolutionary Ecology of Marine Fishes, Helmholtz-Centre for Ocean Research Kiel (GEOMAR), 24105 Kiel, Germany

[3]University of Vienna, Djerassipl. 1, 1030 Vienna, Austria

*Corresponding author: Freya Adele Pappert, Marine Evolutionary Biology, Zoological Institute, Christian-Albrechts-Universität Kiel, Am Botanischen Garten 1-9, 24118 Kiel, Germany. Telephone: +49 4318804224; E-mail: fpappert@zoologie.uni-kiel.de

Abstract

The positive health effects of caloric restriction (CR) have been widely studied in a few conventional model species, displaying typically conventional reproductive roles, with females investing more in parental care. This creates a bias in the current literature about the impact of CR between males and females in species with unconventional reproductive strategies. In this study, we investigated the effects of CR on growth, gene expression, and intestinal microbiota in the lined seahorse *Hippocampus erectus.* Juvenile seahorses were subjected to ad libitum (AL) or every other day fasting for 5 months. Total mRNA sequencing of liver, head kidney and gonad tissue, along with 16SrRNA amplicon sequencing of hind intestine samples was done to investigate sex-adverse effects of fasting in a species with male pregnancy and thus distinct life-history strategies leading to sex-specific trade-offs in resource allocation. Our results indicate that fasting caused a general stunting effect and delayed brood pouch development in males, reflecting its energetic demands and possibly delaying reproduction till more resources are available. We found both organ and sex-specific gene expression responses to CR, with liver and ovaries displaying the strongest variation. In the liver of CR female seahorses, numerous genes linked to energy and lipid synthesis were upregulated, and in the ovaries of the same, we found downregulation of inflammatory markers. These results reflect the fasted female's need to allocate more resources to eggs to increase reproductive chances earlier in life compared to males. There was no sex-specific response to CR in the microbiota, rather both fasted males and females displayed similar higher alpha-diversity and increased abundance of Proteobacteria and Bacteroidetes compared to AL fed seahorses. Our research suggests that male and female reactions to nutrient scarcity may depend on their resource allocation to reproduction and parental investment, rather than directly on their biological sex. Our findings highlight the importance of using species displaying a broader range of reproductive strategies, sex role, mating system and life-history strategies when studying the sex-specific effects of CR.

Keywords: caloric restriction, fasting, seahorse, life-history, male pregnancy

1. Introduction

Caloric restriction (CR) exerts significant physiological effects, positively impacting various metabolic pathways, reducing oxidative damage, promoting DNA repair and autophagy, improving cardiovascular health, and mitigating gut dysbiosis, among other benefits [1,2]. Extensive research has demonstrated that lifelong CR increases lifespan in a range of species, including yeast [3], nematodes [4], fruit flies [5], and rodents [2,6]. Some claim it also has rejuvenating properties: mice, rhesus monkey and humans fed on calorie-restricted diets had DNA methylation patterns equal to their younger counterparts [7] (epigenetic clocks are based on methylation values that change with age), while the proinflammatory gut of aged mice was reversed to a younger healthier microbial composition after two months of CR [8]. The gut microbiome plays a crucial role in various aspects of our health, including the synthesis of vitamins, fine-tuning of the immune system, maintaining the integrity of the intestinal barrier, and influencing the modification of bile acids, neurotransmitters, and hormones. Fasting can lead to changes in mucus production, which in turn affects microbial diversity [9].

CR is believed to induce a state of hormetic stress, tricking the body into perceiving a scarcity of food and prompting it to conserve energy until more nutrients become available [10]. According to Kirkwood's disposable soma theory, longevity requires investing in somatic maintenance, which reduces the resources available for reproduction [11]. When animals lack sufficient energy for reproduction, they prioritize extending their lifespan until resources become abundant again, at which point they resume breeding [12]. This strategy aims to maximize their overall reproductive success over time [13]. Some species naturally have long periods of fasting during hibernation periods when food is scarce, including many reptiles and fish [14,15]. However, this theory is challenged when we observe species with long lifespans, high caloric diets and high reproductive outputs, such as queen bees, ants and termites [16,17]. Moreover, if CR is done excessively it can have negative health impact in humans (e.g. extreme weight loss, lethargy, diminished libido etc.) [2,13,18,19].

Studies from several mice strains and fruit flies suggest that females may respond better to CR with respects to increased health- and lifespan than males [20,21]. On the other hand, every other day (EOD) fasting extended maximum lifespan in laboratory line killifish but not in wild-derived lines [22], fasting further extended lifespan in males, but not in females, and the transcriptomes of killifish livers were impacted in a sex-specific manner [23]. Nonetheless, most biomedical research historically neglected sex differences, underlining that the ultimate and proximate drivers of this sexual dimorphism are still poorly understood. As proximate drivers, improved regulation of mitochondria in females [19], variations in sex steroids [24], adiponectin/leptin levels, immune function, and body fat distribution have been proposed [20,25]. These differences may be explained by distinct reproductive strategies and specific energy allocation as ultimate drivers. Females often invest more in the production of energetically costly and large eggs, while males produce inexpensive sperm (anisogamy) [26]. Moreover, in many taxa, females bear the burden of additional parental care, such as pregnancy and lactation in mammals [27,28]. As females are typically the limiting sex due to their time-dependent parental care responsibilities, males often compete for access to females and may allocate more energy to courting and mate guarding [26,29]. Despite the general consensus that different species showcase varying sexual dimorphism with respect to the effects of CR, most research to date has focused predominantly on species with conventional life history, with female showing increased parental care [30]. However, sex roles in the animal kingdom are not constrained and have evolved with a high degree of flexibility across species [26,31,32], leaving a gap in our understanding of how CR plays out in species with diverse reproductive strategies. Understanding the ultimate drivers of sexual dimorphism in the effects of

CR on health across different species is not merely an academic curiosity but holds significant implications for various fields and aspects of human and animal health.

In the realm of ray-finned fishes, we can find numerous forms of parental care, many of which are dominated by paternal care, such as egg guarding, oral brooding to full viviparity [32,33]. Male pregnancy in syngnathid (seahorses, pipefishes and seadragons) is a unique phenomenon that evolved once in vertebrates and has since diversified across approximately 300 species within the Syngnathidae family [34,35]. Syngnathids have evolved various methods of caring for their offspring, ranging from basic egg attachment to the male's tail to complex forms of male pregnancy involving structures analogous to mammalian placentas [36]. These structures supply embryos with nutrients, oxygen, and immunological components [33,37,38]. Their unique reproductive biology allows us to disentangle the cost of investment into the brooding structure vs. investment into egg production, which are two energetically costly traits typically found in females. We can leverage this feature, providing a valuable opportunity to uncover insights into how divergent life histories and reproductive trade-offs affect the sexual dimorphic responses to CR.

For this purpose, we designed an experiment focusing on how CR, implemented through intermittent fasting (IF), affects development, gut microbiota and aging pathways in the lined seahorse, *Hippocampus erectus*. Male seahorses have one of the highest forms of male pregnancy. The female lined seahorse will transfer her eggs to the male's pouch, which he then fertilizes with his sperm. The embryos are embedded within the walls of the fully enclosed pouch containing blood vessels that supply nutrients and oxygen, on top of providing a controlled environment [39,40]. Investment into the male brooding structure is considered to be energetically demanding, but to what degree this is comparable to the lipid rich female eggs is not well known. Thus, we also sought to understand how CR influences the cost of reproductive and brooding structure development, and how CR might restrict allocating energy into reproductive development.

In our study we took 52 three-month-old juvenile (not sexually mature) seahorses, which were divided into two feeding groups: Ad libitum (AL) and IF. IF encompasses various eating patterns, including CR, where animals consume their allotted calories in a short period and spend a prolonged time in a fasted state between meals. In addition to CR, other IF paradigms such as every-other-day (EOD) fasting and time-restricted feeding (TRF) have been shown to provide similar benefits to overall health, even without reducing overall calorie intake [20,41,42]. The AL group was fed twice daily, while the CR group was fed EOD, resulting in a CR of approximately 50%. This estimate is due to the fact that seahorses lack a stomach, and are thus limited with the amount of food they can consume in one go during feeding [43]. This avoids any negative effects of overeating in the AL and ensures us that the EOD feeding group is truly undergoing CR.

Throughout the study, we measured the body weight of the seahorses at four different time points over a period of 5 months. By using three-month-old juveniles, it allowed us to examine any potential physiological developmental differences in pouch formation, the critical reproductive structure in male seahorses. While in females we could compare, at the end of fasting period, the ovary masses to asses egg production. Using RNA sequencing we analyzed transcriptome-wide differential gene expression between AL and CR diets, and comparing male vs female in RNA-Seq from liver, head-kidney, and gonad tissue. Furthermore, we also sampled hind-gut and conducted microbiota analysis using 16S rRNA amplicon sequencing to investigate the composition

of the gut microbiota and assess potential changes induced by fasting. In parallel to the main experiment, we also sacrificed three-year-old and four-month-old seahorses (males and females), and assessed the same parameters (i.e., RNA-Seq and 16S rRNA-Seq). By comparing young and old seahorses to our AL and CR treatment groups, we hoped to additionally assess whether CR had a rejuvenating effect on tissue expression or microbial gut composition, providing insights into the potential long-term effects of CR on aging and overall health.

Studying fasting in seahorses offers us a captivating opportunity to explore the intricate connections between reproductive strategies and life history trade-offs in energy allocation from a novel perspective. By choosing this fish model with male pregnancy, we aimed to shed light on how the roles of sex (sperm and egg production) and investment into the brooding structure may shape the evolutionary responses to periods of food scarcity. Ultimately, we hope that unraveling the mysteries of sexual dimorphism in the effects of CR has far-reaching implications for human health, biology, and ecology. By adopting a multidisciplinary approach, researchers can make significant strides in addressing this intriguing question and translating the findings into practical applications for health and beyond.

2. Material and methods

2.1 Study organism and design

Fifty-two three-month-old juveniles *Hippocampus erectus* from an aquarium bread population kept at the GEOMAR Helmholtz centre for ocean research in Kiel were used for the experiment. Fish were separated into groups of two or three and housed in 20 l aquaria with a recirculating system, with conditions similar to their natural environment (temperature 23-25°C, salinity 30-35 PSU, nitrate 20-50 mg/l, phosphate below 1 ppm). To minimize contamination, an ozone generator was placed above the tanks, and a 20% water change was conducted weekly. The filters, plastic seagrass, and oxygen tubes were cleaned separately with hot water every two weeks, and any food or waste was suctioned and removed approximately thirty minutes after feeding to prevent the formation of bacterial films.

We allocated twenty-six seahorses to an AL feeding regime and another twenty-six to an EOD regime, which will be named CR in this manuscript for consistency. The tanks were divided into 9 AL and 9 CR, with alternating tanks placed next to each other to reduce tank location effects. In the AL group, there were three juvenile seahorses in each of the eight tanks, and one tank had only two seahorses, the same applied to CR. All fish were fed with defrosted mysids (the frozen food is thawed under cold running water). The feeding schedule was as follows: the AL group was fed twice a day, in the morning and in the evening; the CR group was fed every other day, i.e., one day they got no food, the next day they were fed like the AL group (in the morning and in the evening). This resulted in a calorie deficit for the CR group, as seahorses do not have stomachs meaning overeating during feeding time is unlikely. To ensure that any observed effects in the CR group were due to a reduction in calories and not nutrient deficiency, both groups were given vitamins. The supplements were provided equally on the days when both groups were fed and included 15 µl of Microbe-Lift Garlic feed supplement (contains pure garlic, which has been shown to have a positive effect on bacterial diseases in fish [44]), 15 µl of JBL Atvitol multivitamin drops for aquarium fish and half a capsule (730mg) of Omega-3 1000 (unsaturated omega-3 fatty acids and vitamin

E). These additives were left in the defrosted food for 2-3 minutes to ensure absorption. Seahorses were monitored daily during feeding for a health check and/or any abnormal swimming behaviours.

At the start of the experiment, all animals were weighed and measured for length before being placed in their respective tanks. For the first seven days, we gradually reduced the food in the IF group by feeding them only once in the morning on fasting days to minimize stress and facilitate habituation. Starting from day eight, the exact feeding regime was implemented, with the IF group receiving no mysids on alternate days. The study spanned a total of 157 days, which roughly equates to five months. During which, pouch development was closely monitored to determine when sexual maturation was taking place in the respective treatment groups, and to ensure that we could separate in a timely fashion males and females in same sex-tanks to prevent mating. Furthermore, to monitor growth they were weighed in a small beaker, with previously calibrated tank water, roughly every 50 days. Upon termination of the dietary experiment, the seahorses were euthanized with an overdose of MS-222 (Tricaine methane sulfonate; 500 mg/l; Sigma-Aldrich). They were weighed and measured for total body length (snout to tail), including weighing of female ovaries and measuring width and length of pouch in males. They then were dissected to extract liver, head kidney, and gonads for gene expression analysis, which were immediately preserved in RNA later, and hind-gut which was placed in a sterile Eppendorf tube and immediately stored at -80°C. The tissue samples in RNAlater were stored at 4°C for three days before being transferred to long-term storage at -20°C.

Additionally, to assess how CR influences aging compared to the AL treatment, we also took four-month-old juveniles (control young, CY) and roughly three-year-old adult (control old, CO) seahorses that were kept in the GEOMAR stocks. They were also euthanized with 500 mg/l of MS-222, then measured for weight and body length and dissected to retrieve organs, which were stored as described above.

2.2 RNA and DNA extraction, library preparation and sequencing

RNA extraction of liver, head kidney, and gonads was performed using the RNeasy Mini Kit from Qiagen (Venlo, Netherlands) according to the manufacturer's instructions. The concentration of the extracted RNA was measured using a Peqlab NanoDrop ND-1000 spectral photometer (Erlangen, Germany), and the samples were subsequently stored at -80°C. Samples were sent to BGI Tech Solutions in Hong Kong for library preparation and mRNA sequencing. For library preparation the DNBSEQ Eukaryotic Strand-specific mRNA library was utilized, and the sequencing of stranded mRNA was carried out on the DNBseq platform. The sequencing parameters included 150bp reads and 25M clean paired-end reads per sample. BGI Tech Solutions in Hong Kong conducted this process on a total of 40 randomly selected individuals to optimize costs. The samples added up to 120, which consisted of 5 males and 5 females from the AL, CR, CO, and CY groups, resulting in 40 individuals multiplied by the tree tissue types.

To extract the microbiota from the hindgut, DNA extraction was performed using the DNeasy Blood & Tissue Kit (QIAGEN, Germany) following the manufacturer's protocol. Subsequently, a 16S PCR was conducted to verify the success of the extraction. The library preparation for a total of 57 individuals was performed by the Institute for Experimental Medicine (UKSH, Campus Kiel). This group included 37 seahorses from the fasting experiment with additionally five young and old seahorses: 5 AL, 11 CR, 5 CY, and 5 CO females, as well as 10 AL, 11 CR, 5 CY, and 5 CO males. Sequencing of the amplicons of the V3-V4 hypervariable region (341f/806r)

was conducted using the Illumina MiSeq platform (Illumina, USA) with 2x 300-bp paired-end read settings at the IKMB.

2.3 Data analysis

2.3.1 Morphology

In our experiment we complied stringently to daily animal welfare screening processes and seahorses were euthanized when scores were not good, resulting in a total of 38 individuals who survived till the conclusion of the experiment. Among them, the survivors encompassed 16 individuals belonging to the AL group, while 22 individuals belonged to the CR group. For the purpose of the morphology analysis, we included only the 38 individuals which made it to the end of the experiment (and separately 10 individuals from old vs. young seahorses). To investigate the effect of treatment (AL or CR) and sex on weight (in grams [g]) and total body length (in centimetres [cm]), we compared the measurements. Specifically, we had 11 AL males, 5 AL females, 11 CR males, and 11 CR females. It is worth noting that we initially couldn't divide equally the seahorses by sex at the beginning of the experiment as they were not yet mature, resulting in a smaller number of AL females compared to the other groups. Statistical analysis was done in Rstudio (v.4.2.2) [45], and tests were considered significant when p-values were smaller than 0.05. Due to the unbalanced design, we performed a Kruskal-Wallis test for the different variables (treatment and sex), for both weight and length. Following the rejection of the Kruskal-Wallis test, we perform post hoc procedure with the Dunn's test for the interaction variables. The same analysis was done to compare weight difference of ovaries between fasted and non-fasted female seahorses and for pouch width in male seahorses. Additionally, to measure the effectiveness of the different treatments on the same individuals over time for the repeated weight measurements we did a Friedman test.

2.3.2 Differential gene expression analysis

The effects of CR were characterized by constructing differential expression signatures associated with each tissue. The integrity and quality of the RNA Illumina sequencing results from BGI Tech Solution were thoroughly assessed and controlled using FastQC (v.0.11.9) [46]. The adapters had already been trimmed by BGI Tech Solutions. Regrettably, during the run, six samples failed, all of which were ovarian tissue and belonged to female seahorses (two AL, one CR, two OC, and one YC). Moving on to the alignment process, we used STAR (v.2.7.9)a [47] to map the reads to a genome assembly of *Hippocampus erectus* (BioProject: PRJNA347499). Subsequently, read counts were obtained using TPMCalculator [48]. In order to investigate potential distinctions among dietary groups within specific tissues, we partitioned the dataset by tissue. On logarithmically transformed count data we employed the "adonis2" function and the "bray" method, and conducted a PERMANOVA analysis (Permutational Multivariate Analysis of Variance Using Distance Matrices). Our examination evaluated the influences of diet and sex in the liver and head kidney, while concentrating exclusively on the effects of diet in the ovaries and testes. We also did a principal component analysis (PCA) with multiple PC's to visualise group clustering and possible outliers (Figure 2 and S2)For differential gene expression (DGE) analysis we first used the edgeR package (v.3.40.2) 49, to scale the count data to counts per million (cpm) and filtered it based on a minimum threshold of 10 counts in at least 5 libraries (since each group had at least 5 replicates). We performed this process separately for each

organ, resulting in different gene sets for each tissue: 13,015 genes for the liver, 16,470 genes for the head kidney, 17,411 genes for the testes, and 16,897 genes for the ovaries. To address composition biases, we normalized the data using the trimmed mean of values (TMM) with the calcNormFactors function. This step calculated normalization factors for each sample, effectively eliminating composition biases between libraries. Next, we utilized the limma package (v.3.54.1) [50] for DGE analysis. limma is a linear model-based method that employs an empirical Bayes approach and the voom method to convert count data to a continuous scale. It is particularly useful in accounting for batch effects and reducing technical variation. The voom method, on the other hand, is well-suited for handling library sizes that vary significantly [51]. To compare several groups, we created a matrix of independent contrasts, enabling us to perform a one-way analysis of deviance (ANODEV) for each gene. Subsequently, we estimated coefficients of interest between groups to determine log fold changes using the contrasts.fit function. We then applied empirical Bayes moderation to shrink the estimated variance and performed a moderated t-test to identify differentially expressed genes (DEGs). For further downstream analysis, we focused only on adjusted P-values below 0.05, ensuring that the observed treatment effects were statistically significant.

2.3.3 Gene Ontology analysis

Gene ontology (GO) analysis was performed using the g:Profiler web tool (accessed on July 6, 2023). This tool enabled us to conduct gene enrichment analysis by comparing the significantly DEGs between AL and CR seahorses across the different organs. To annotate the genes of *Hippocampus erectus*, we employed OrthoFinder and conducted a homology-based search using *Danio rerio* (GRCz11) as a reference species. Adjusted statistical significance was set for "g:SCS threshold" value at 0.05, focusing exclusively on annotated genes. We treated numeric IDs as "ENTREZGENE_ACC" and limited the data sources to GO biological processes (BP), as well as biological pathways from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Reactome (REAC) databases. To enhance the specificity of our analysis, we applied a maximum term size of 1000 to exclude overly broad categories and prioritize more informative results.

2.3.4 Microbiome data analysis

The 16S rRNA amplicon sequencing analysis was performed via QIIME2 platform (v.2022.8.3) [52]. With dada2 the raw paired-end illumine reads were initially demultiplexed and then amplicon primers for 16S V3-V4 region were trimmed. It was then quality controlled by “denoising” sequences (in order to better discriminate between true sequence diversity and sequencing errors). Further filtering of low-quality reads followed (manual trimming at forward read at 250 bp and reverse at 210 bp), then forward and reverse samples were merged together and possible chimeras removed. A phylogenetic tree was constructed using Fasttree (v.2.1.11) that infers approximately-maximum-likelihood based on the longest root [53]. Interactive α rarefaction curves were computed for max_depth of 20’000 to determine whether the samples were sequenced deeply enough to capture all the bacterial community members. Then samples were classified for the V3/V4 hypervariable region based on taxonomic groups using the SILVA v138 database and a naïve Bayes based classifier [54]. After which the amplicon sequence variants (ASV) were filtered to remove sequences from chloroplasts and mitochondria and exported at genus level as an operational taxonomic unit (OTU) for further analysis in RStudio (v.2022.07.2).

To reduce noise, we applied a filtering threshold, including only OTU's that were present in at least three samples across the entire dataset. Additionally, two control male samples (CO and CY) were excluded from the analysis as they did not contain any OTUs. After these steps, the remaining samples consisted of 5 AL, 11 CR, 5 CY, and 5 CO for females, and 10 AL, 11 CR, 4 CY, and 4 CO for males. OTU counts were normalized across samples to ensure data comparability.

α-diversity was measured using the Shannon and Simpson index with the vegan package (v.2.6.4) 55. β-diversity was assessed using the Bray Curtis dissimilarity matrix and Jaccard distance (vegdist function), with the former considering abundance and the latter based on presence-absence (Fig. 4B and Fig. S3B-C). Hypothesis testing for both α- and β-diversity, and their respective metrics, was performed using PERMANOVA with both diet and sex as factors (x ~ diet*sex, data=data, permutations=999), preceded by testing for normality with a Shapiro-Wilk normality test. To compare groups, pairwise.adonis2 v0.4 56 was used as a post-hoc test, with Bonferroni correction for p-value adjustment to account for multiple testing. To visualise the results, we used boxplots α-diversity and for β-diversity we used a non-metric multidimensional scaling (NMDS) with 95% confidence ellipses to aid in interpretation. To gain insights into the relationship between taxa composition and diet and age variables, we further examined the OTUs using the *envfit* function from the vegan package (v.2.6.4). This allowed us to fit the OTUs to the NMDS ordination space and calculate the correlation between the ordination scores of OTUs and the treatment variables. By employing permutation tests with 999 iterations, we obtained p-values to assess the significance of these correlations. We then plotted the OTUs that significantly explained the NMDS Bray-Curtis ordination (p.val < 0.001, Fig. S3E).

Relative abundance comparisons were made for the most frequent OTU (Figure S3D and also Figure 4C-F) and Wilcoxon signed-rank test for significant differences between groups using FDR correction (CR, AL, YC, CO and AL male, CR male, AL female, CR female). It was observed that the genus *Vibrio* from the family *Vibrionaceae* exhibited the highest frequency and variation across samples, ranging from 6% to 99% (Figure S3D). Despite *Vibrio* prevalence not showing significant differences across treatment (Fig. S3E) we realised, upon further analysis, that this high variation introduced noise and reduced the statistical potency of downstream analyses of α- and β-diversity (Figure S3A-C). Consequently, we performed two analysis one including the genus *Vibrio* with results in supplementary material (Fig. S3A-E) and another analysis excluding this OTU (Fig. 4A-C). Following the removal, the data was re-normalized, and analysis and hypothesis testing repeated.

3. Results

3.1 Calorie restriction reduces growth

EOD feeding had a significant impact on growth in the CR group (Fig. 1). By the end of the experiment, the AL group had a larger overall size and was heavier compared to the CR group (Fig. 1A and S1A-C, $p < 0.001$). Prior to beginning the diet treatment, the seahorses had a mean weight of 0.55 ± 0.29 g and a mean length of 4.58 ± 0.95 cm (Tables S1 and S6, and Figs. S1A and C, Kruskal-Wallis p = NS). After 157 days, the AL group had a mean weight of 8.09 ± 3.12 g, while the CR group had a mean weight of 3.51 ± 1.17 g (Fig. 1A, Kruskal-Wallis $p < 0.001$). The Friedman test for the repeated measures of weight were also strongly significantly different from

one another (Fig. S1B, $p < 0.001$). Closer one-way analysis revealed that weight measurements on day 55 already showed stark contrasts (AL 3.12 ± 1.29 vs. CR 1.17 ± 0.44 g, Kruskal-Wallis $p < 0.001$) and even more on day 111 (AL 7.66 ± 2.84 vs. CR 2.88 ± 0.91 g, Kruskal-Wallis $p < 0.001$) (Fig. S1B).

In terms of size, on day 157 the AL group had a mean length of 12.93 ± 1.68 cm, compared to 10.28 ± 1.08 cm for the CR group (Fig. S1C, Kruskal-Wallis $p < 0.001$). The post-hoc analysis examining the treatment effects within each sex revealed noticeable differences between males and females. Males showed a significant difference in weight (AL 9.46 ± 2.55 g vs. CR 4.18 ± 0.79 g, adj.p.val < 0.003 with $\chi2 = 26.625$, Fig. 1B) and length (AL 13.75 ± 1.27 cm vs. CR 10.76 ± 0.37 cm, adj.p.val < 0.002 with $\chi2 = 22.524$, Fig. S1D). In contrast, females showed no significant difference in weight (AL 5.1 ± 1.97 g vs. CR 2.84 ± 1.13 g, NS, Fig. 1B) or length (AL 11.11 ± 0.78 cm vs. CR 9.81 ± 1.35 cm, NS, Fig. S1D). We did find a significant interaction between treatment and sex for both weight and lenght (Kruskal-Wallis < 0.001, Table S6), but these interactions were non-significant upon correction for multiple testing (e.g., AL male vs AL female and/or CR male vs CR female, see Table S6).

Throughout the fasting period, we closely monitored the fish and documented the emergence of pouch formation in male seahorses, as they were not sexually mature at the beginning of the experiment. On day 19, we observed the first signs of pouch formation in an AL seahorse, and by day 28, we were able to distinguish between males and females in all AL seahorses. As a result, we made minor adjustments within the AL tanks to ensure that males and females would not mate. On day 39, pouch formation was documented in the CR group, and by day 47, we could confidently determine the sex of all CR individuals. The pouch measurements taken at the end of the experiment (day 157), showed significant differences in width (AL 0.77 ± 0.12 vs. CR 0.5 ± 0.1 cm, Kruskal-Wallis < 0.004, Fig. 1D) and length (AL 2.6 ± 0.47 vs. CR 1.82 ± 0.17 cm, Kruskal-Wallis < 0.005, Fig. S1H) between treatment groups.

With regard to the ovarian weight taken at the end of the fasting experiment, we could not find significant differences between AL and CR (AL 0.23 ± 0.15 vs. CR 0.15 ± 0.08 g, NS, Fig. 1C). This could be confounded by the low number of biological replicates in AL (only 5 compared to 11 in CR). In the supplementary materials we also compare morphological parameters of AL and CR treated seahorses with the young and old control groups (Fig. S1E-G).

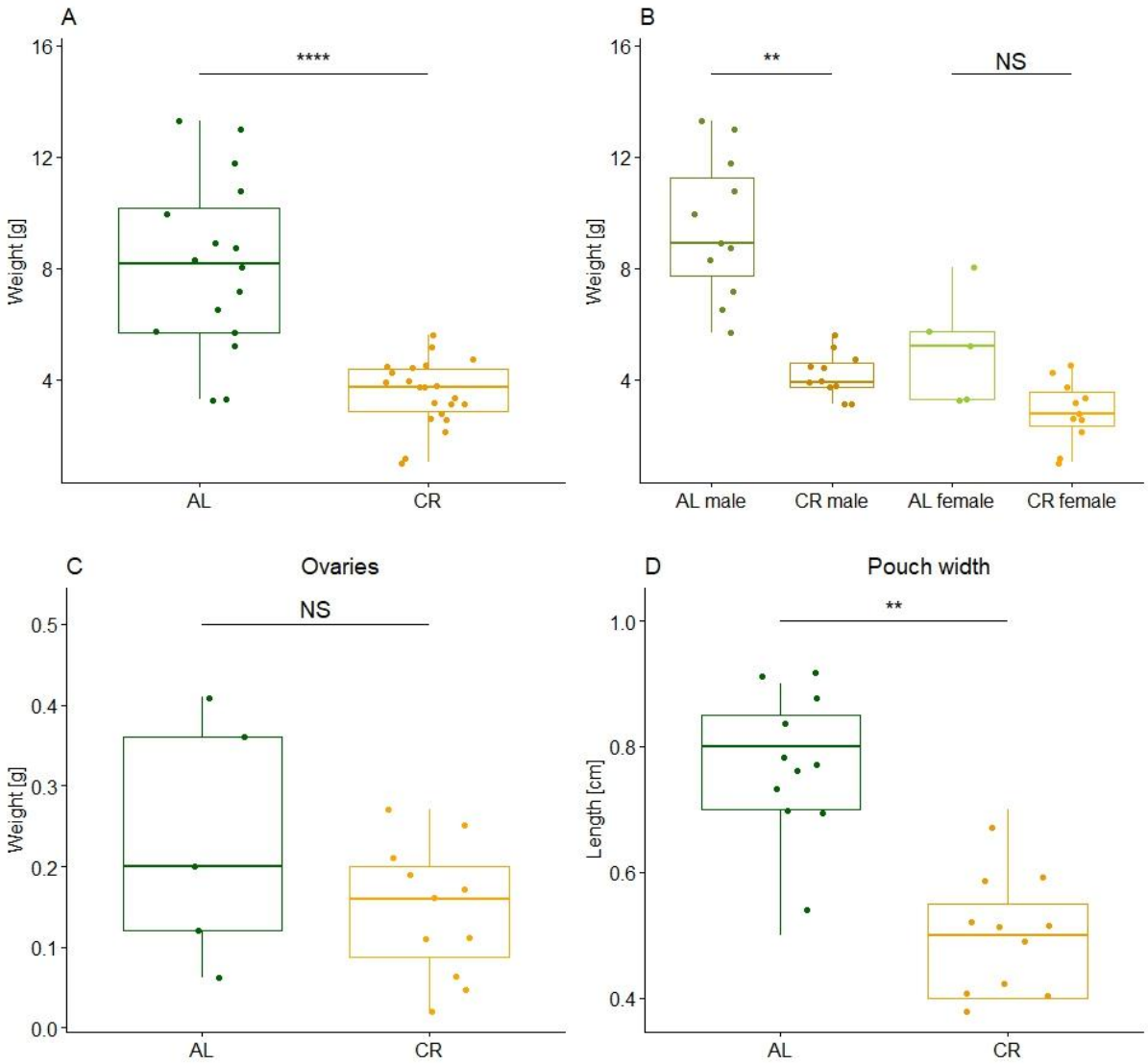


Figure 1: Boxplots depict morphological measurements of weight [g] on the y-axis, obtained at the end of a five-month diet treatment. The x-axis is divided into treatment groups: (A) showcasing weight differences between AL (16 individuals) and intermittent fasting (CR) (22 individuals); (B) represents weight differences with 11 AL males, 11 CR females, 5 AL females, and 11 CR females; and (C) depicts weight of ovaries between AL and CR females; D) side difference in pouch width. Significance thresholds are indicated with three asterisks (Kruskal-Wallis; ** < 0.001) to emphasize differences between treatment effects and within sex groups.

3.2 Liver and ovarian tissue show stronger response to the effects of fasting

The results of the DGE revealed the strongest effects of the fasting period in the liver tissue (PERMANOVA; $p < 0.001$), no significant interactive effects of sex and diet could be identified. This was also true after correcting for multiple testing with AL vs. CR showing an adj.p.val of 0.012. However, in head kidney, we found a very strong effect of sex (PERMANOVA; $p < 0.001$), and a smaller effect of diet (PERMANOVA; $p = 0.035$), and no significant effect for the interaction. Similarly, after correction, we found no significant difference between AL

and CR groups. In gonadal tissue, we found no significant effects between the dietary groups in testes, while we did find a very significant difference in ovaries (PERMANOVA; $p < 0.001$) (full stats Table S6).

A total of 940 significantly DEGs were observed between AL and CR (limma; adj. p-value < 0.05), whereas only 67 DEGs were identified in the head kidney (Table S1). When dividing the contrast comparisons for sex, we found no significantly DEGs between CR or AL in females and only 4 in males in head kidney (Table S1). While dietary sex-specific treatment was vastly different for females and males in liver, with the first having 375 DEGs and 83 in males (adj.p.val < 0.05) (Table S1). In the gonadal tissues we found no DEGs between AL and CR in testes, but 366 in ovaries (Table S1). We were further interested in identifying potential similar tissue senescence patterns between the dietary treatment groups (AL and CR) and younger (CY) or older (CO) seahorses. Overall, there was a significant difference for both dietary treatment groups and control groups in the liver irrespective of pairwise comparison (adj.p.val < 0.006). In the head kidney we found less of a significant difference between AL vs. CO (pairwise.adonis; adj.p.val < 0.05) compared to CR vs. CO (adj.p.val < 0.03), but overall significant difference between AL vs. CY and CR vs. CY (adj.p.val < 0.006). The interactions in ovaries and testes did initially reveal some marginally significant differences, but upon post-hoc correction they were no longer significant. A more in-depth description of the results from the limma DGE analysis comparing the young and old control groups to the dietary treatment groups can be found in the supplementary material in Table S1.

These results are reflected also in the PCA revealing distinct clustering of the different tissue types, which can be seen clearly from PC1, PC2 and PC3 (Fig. S2). However, despite tissue specific differences, in PC4 and PC5 the data cluster based on the different groups, with clear distinction between AL and CR, and also between the juvenile and adult controls (Fig. 2). The results of the PCA did not reveal significant overlaps between the CR treated group and the juvenile control group, as there was a lot of variation between individual age control samples, particularly in liver as seen in the PCA plot (Fig. 2).

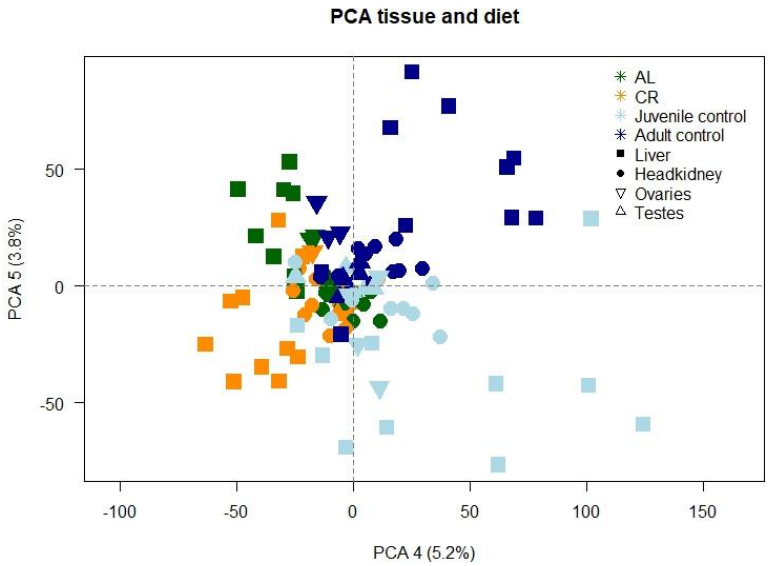


Figure 2: Principal Component Analysis (PCA) plots depicting organ separation and grouping of treatment groups. This particular PCA 4 and 5 depicts grouping of samples based on diet treatment and age groups (PCA 1 and 2 are in supplementary

material Figure S2). The groups are color-coded as follows: CR (orange), AL (green), juvenile control (light blue), and adult control (dark blue).

Gene enrichment analysis revealed that in liver of females the genes most significantly affected by CR were associated with lipid, steroid, and cholesterol metabolic processes, ATP biosynthetic process, cellular respiration, and oxidative phosphorylation (Table S2, sheet A). Conversely, in the liver of male seahorses, CR led to enrichment of nucleotide and ribonucleoside metabolic processes, as well as oxidative phosphorylation and ATP biosynthetic process (Table S2, sheet B). In the ovaries, the genes affected by CR showed a disproportionate downregulation (Fig. 3). These downregulated genes were associated with various catabolic processes, vascular development, haemostasis, and the immune system (Table S2, sheet C).

3.3 Sex-dimorphic effects of fasting on gene expression in liver and ovaries

A more in-depth comparison of particular transcripts from these pathways that were differentially expressed in the respective organs is represented in Figure 3 (and TableS3). The downregulation of several genes in the female liver in response to CR could have implications for various cellular processes (Table S3 sheet A). Notably, genes associated with oxidation mechanisms, such as retinol dehydrogenase 14b (*rdh14b*) (logFC: 1.042, adj. p.val: 0.015), which is involved in the metabolism of vitamin A [57], and glutaredoxin (*glrx*) (logFC: 0.794, adj. p.val: 0.031), which could impact antioxidant defence by maintaining the balance of cellular thiol/disulphide redox state cellular redox regulation [58]. The downregulation of STEAP family member 3 (*steap3*) (logFC: 0.945, adj. p.val: 0.031) and erythropoietin receptor (*epor*) (logFC: 2.005828, adj. p.val: 0.0279595) could be synergistic, as the first has ferrireductase activity meaning it can reduce Fe3+ (ferric iron) to Fe2+ (ferrous iron) [59], and the latter regulates blood cell production [60]. Furthermore, several genes involved in gene expression regulation were downregulated by CR in female liver. These included histone deacetylase 1 (*hdac1*) (logFC: 0.917, adj. p.val: 0.031), which removes acetyl groups from lysine residues on histone proteins, leading to transcriptional repression [61]. The downregulation of macroH2A.1 histone (*macroh2a1*) (logFC: 0.688, adj. p.val: 0.034) suggests a potential role in gene silencing, and in regulating glucose metabolism and the leptin receptor gene (*lepr*) in mice liver [62]. Additionally, the downregulation of the spen family transcriptional repressor (*spen*) (logFC: 1.111, adj. p.val: 0.034) is known to be involved in regulating Notch/RBP-Jκ signaling and Ras/MAP kinase signaling pathways [63]. Hormone metabolism was also affected by CR in female liver, particularly progesterone, as indicated by the downregulation of cytochrome P450 family 17 subfamily A (*cyp17A2*) (logFC: 1.290, adj. p.val: 0.041) and progesterone receptor (*pgr*) (logFC: 1.206, adj. p.val: 0.032). The downregulation of *cyp17A2* implies a decrease in the metabolism of progesterone to androstenedione [64]. CR also resulted in the upregulation of genes involved in various metabolic processes in the female liver, several were associated with cholesterol synthesis and regulation. These included cytochrome P450, family 51 (*cyp51*) (logFC: -5.1978, adj.p.val: 0.020362) [65], mevalonate kinase (*mvk*) (logFC: -3.146, adj.p.val: 0.024) [66], squalene epoxidase (*sqle*) (logFC: -4.812, adj.p.val: 0.029) [67], hormone-sensitive lipase (*lipe*) (logFC: -1.699; adj.p.val: 0.0005), and lipase hepatic A (*lipca*) (logFC: -1.939, adj.p.val: 0.013) [68]. Furthermore, other genes involved in energy metabolism and ATP production were upregulated by CR in the female liver. These genes play a crucial role in combating oxidative stress, such as isocitrate dehydrogenase (NADP+) 2 (*idh2*) (logFC: -1.795, adj.p.val: 0.0005), known for its importance in oxidative stress response and glucose sensing [69]. Additionally, several subunits of the mitochondrial electron

transport chain, including NADH:ubiquinone oxidoreductase subunit B9 (*ndufb9*) (logFC: -0.631, adj.p.val: 0.015) and NADH:ubiquinone oxidoreductase subunit A2 (*ndufa2*) (logFC: -0.875, adj.p.val: 0.035), showed upregulation in CR [70]. Moreover, CR led to the upregulation of genes involved in antioxidant defence mechanisms, such as peroxiredoxin 4 (*prdx4*) (logFC: -1.157, adj.p.val: 0.035) and peroxiredoxin 6 (*prdx6*) (logFC: -0.858, adj.p.val: 0.049) [71]. Additionally, retinol dehydrogenase 12 (*rdh12*) (logFC: -2.916, adj.p.val: 0.019) was found to be upregulated in CR-fed seahorses. Interestingly, an autophagy-related 13 homolog (*atg13*) (logFC: -0.639, adj.p.val: 0.028) and acetoacetyl-CoA synthetase (*aacs*) (logFC: -3.382, adj.p.val: 0.02), an enzyme involved in degradation of ketone bodies (particularly acetoacetate) for energy production were also upregulated in CR.

In the liver of male seahorses, CR resulted in the downregulation of a limited number of genes (Fig. 3, TableS3 sheet B). Among these, Coenzyme Q8A genome duplicate b (*coq8ab*) (logFC: 1.872, adj.p.val: 0.005) was downregulated in CR, involved in coenzyme Q biosynthesis, which is essential for mitochondrial electron transport chain and energy production. Additionally, beta-carotene oxygenase 1 (*bco1l*) (logFC: 2.271, adj.p.val: 0.023) was downregulated. This gene plays a role in the cleavage of beta-carotene into retinal, a vital precursor of vitamin A [72]. Conversely, CR led to the upregulation of several genes associated with energy metabolism in the male seahorse liver. Notably, Isocitrate dehydrogenase (NADP(+)) 2 (*idh2*) (logFC: -1.811, adj.p.val: 0.0008) and NADH:ubiquinone oxidoreductase subunit B9 (*ndufb9*) (logFC: -0.906, adj.pval: 0.0008) were found to be upregulated. These genes were also upregulated in the liver of fasted female seahorses, mentioned above. Similarly, lipase genes such as lipase gastric (*lipf*) (logFC: -1.381, adj.p.val: 0.005) and lipase hepatic A (*lipca*) (logFC: -2.19, adj.p.val: 0.007) showed significant upregulation, also consistent with the results in female seahorses. Furthermore, CR modulated here the expression of genes involved in nutrient sensing. For example, lactate dehydrogenase D (*ldhd*) (logFC: -1.149, adj.p.val: 0.013), known for its role in converting pyruvate to lactate and oxidizing NADH to NAD+ at the end of glycolysis. Fetuin B (*fetub*) (logFC: -4.464, adj.p.val: 0.008), a glycoprotein known to impair glucose metabolism and induce a form of insulin resistance [73], was also downregulated in AL compared to CR. Additionally, CR influenced the expression of genes involved in metabolism. Thimet oligopeptidase, upregulated under CR, (*thop1*) (logFC: -1.544, adj.p.val: 0.005) has been extensively studied and shown to regulate energy metabolism, with knockout thop1 mice displaying improvements in lipolysis and increased oxidative metabolism [74]. Selenoprotein P (*selenop2*) (logFC: -1.431, adj.p.val: 0.018), one of numerous selenoproteins predominantly involved in oxidoreductase reactions, is likely involved in the transport of the essential nutrient Selenium (Se) [75]. Interestingly, genes involved in regulating the circadian rhythm were upregulated in the liver of CR male seahorses. This included clock circadian regulator b (*clockb*) (logFC: -1.2, adj.p.val: 0.013) and cryptochrome circadian regulator 3 (*cry3a*) (logFC: -1.472, adj.p.val: 0.005). The circadian rhythm is tightly linked to the daily cycle of nutrient intake and metabolic control. CLOCK, a key transcription factor of the circadian clock machinery, modulates the chromatin environment by directly modifying histone acetaltransferase (HAT) activity (HAT function of CLOCK may be controlled by changing cell energy levels [76]. *Cry3a*, orthologous to human *cry1*, is one of the major molecular clock drivers involved in autoregulating a transcriptional loop with roughly 24-hour periodicity, and CLOCK binds to the promoter region of *cry1* [77].

No significantly differentially expressed genes were found in the testes, while a significant number were identified in the ovaries, with 356 being upregulated in AL and 10 downregulated compared to CR (Fig. 3). Among the

upregulated genes in the ovaries of CR compared to AL (Table S3 sheet D), MID1 interacting protein 1a (*mid1ip1a*) stood out (logFC: -1.799, adj.p.val: 0.019). In humans, this gene encodes an E3 ubiquitin ligase of the Tripartite Motif (TRIM) family and is located near the pseudoautosomal boundary on the X chromosome [78]. Another interesting gene was Claudin 7 (*cldn7b*) (logFC: -0.983, adj.p.val: 0.038), which is involved in maintaining epithelial compartmentalization and controlling solute transfer across cell monolayers. Cell culture studies have speculated that *cldn7* may play a role in cancer cell survival, growth, and invasion [79]. Rhophilin Rho GTPase binding protein 2 (*rhpn2*) (logFC: -1.115, adj.p.val: 0.035) was also upregulated in CR seahorses. Rhophilin proteins, including Rhpn2, act as scaffold proteins with PDZ and Bro1 domains, participating in protein complex assembly, intracellular signalling, proteolysis, and endosome targeting [80]. On the other hand, among the many hundreds of upregulated genes in AL, and therefore downregulated in CR, several metabolic enzymes and genes involved in ATPase H+ transporting V1 and V0 were identified (*atp6v1f, atp6v1e1b, atp6v0cb*, logFC > 0.8; adj.p.val < 0.04). Additionally, two acyl-CoA synthetase (ACS) enzymes related to lipid metabolic regulation, ACS bubble gum (*acsbg*) and long-chain ACS (*acsl*), were downregulated in CR. Both enzymes play significant roles in reproduction, and *acsl* is linked to immune response in the rainbow trout, being upregulated under hypoxia to enhance tolerance [81,82]. Several nutrient-sensing-related genes were also found, which are crucial for regulating longevity pathways. Forkhead box O1 a (*foxo1a*) (logFC 0.976; adj.p.val: 0.022) is a FOXO transcription factor that integrates insulin action with nutrient and energy homeostasis. It interacts with the insulin receptor substrate and regulates metabolism, cellular proliferation, stress resistance, apoptosis, and longevity [83]. Another gene, sestrin 2 (*sesn2*) (logFC 1.283; adj.p.val 0.023), inhibits the mTORC1 pathway. It acts as a sensor for leucine, a specific amino acid. When leucine binds to sestrin2, it disrupts the interaction between sestrin2 and GATOR2, ultimately leading to mTORC1 activation [84]. Similarly, TELO 2 interacting protein 2 (*tti2*) (logFC 1.035, adj.p.val: 0.036) was identified, which binds to mTORC1, stimulating the downstream cascade for cell growth [85].

The expression of several immune-related genes was downregulated in seahorses under CR compared to AL feeding conditions in the ovaries of female seahorses (Fig. 3 and Table S3 sheet D). Notably, toll-like receptors (TLRs) 21 (logFC: 1.674; adj.p.val: 0.043) and 22 (logFC: 2.665; adj.p.val: 0.039) were among the downregulated genes. TLRs are pattern-recognition receptors that play a role in detecting pathogen-associated molecular patterns (PAMPs). In fish, TLR22 specifically is involved in recognizing aquatic double-stranded RNA viruses and influencing downstream signalling through the NF-κB and MAPK inflammatory pathways [86,87]. Furthermore, the downregulation of cytokine-like interleukin 12 receptor beta 2a (*ilrb2a*) (logFC: 2.555, adj.p.val: 0.021) is noteworthy. IL-12 plays a crucial role in controlling cell-mediated immunity by stimulating the production of interferon (IFN)-γ from T cells and natural killer cells [88]. Additionally, the downregulation of M17, an IL-6 subfamily cytokine with similarities to mammalian IL-6, is known to induce the proliferation and activation of macrophages in goldfish [89]. Similarly, the downregulation of macrophage expressed 1 tandem duplicate 2 (*mpeg1.2*) (logFC: 2.838; adj.p.val: 0.03) is worth noting. This gene is induced by infection and has anti-bacterial functions [90]. Moreover, interferon regulatory factor 5 (*irf5*) (logFC 1.3, adj.p.val: 0.042) was downregulated, playing a role in both antiviral and inflammatory responses [91]. Additionally, two chemokine receptors and one ligand (logFC > 2.5, adj.p.val < 0.02) as well as tumor necrosis factor (*tnf*) (logFC: 1.66; adj.p.val: 0.038) were downregulated, all of which are important in the inflammatory response [92].

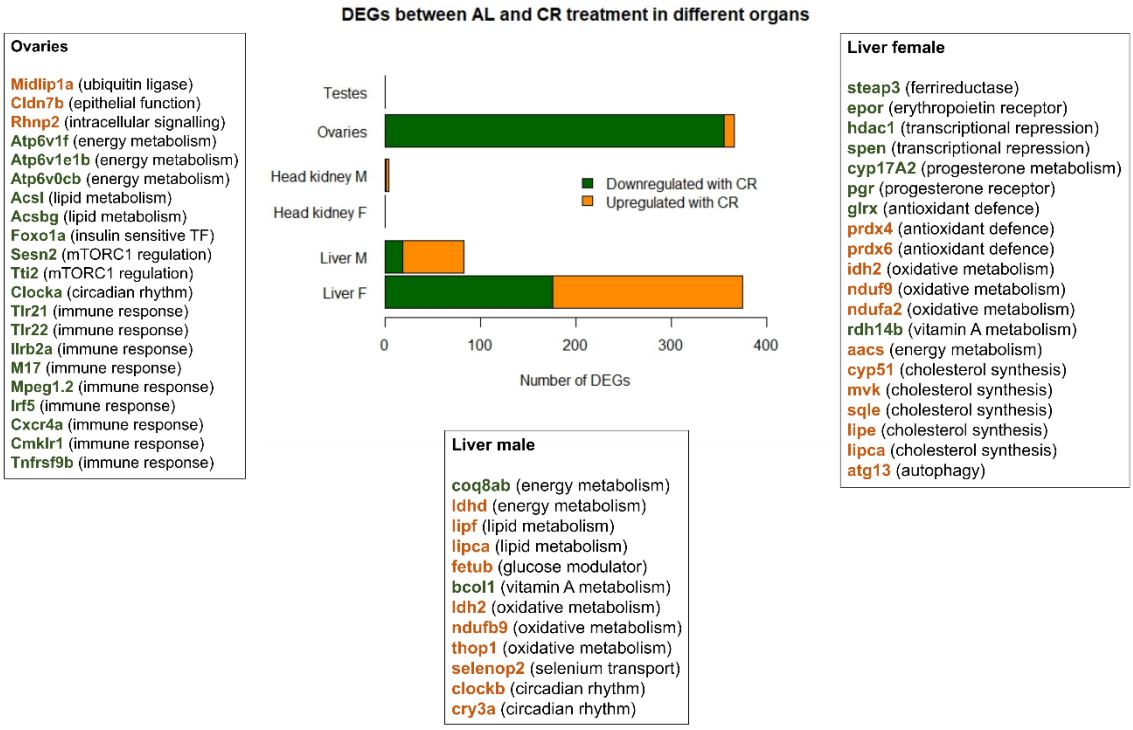


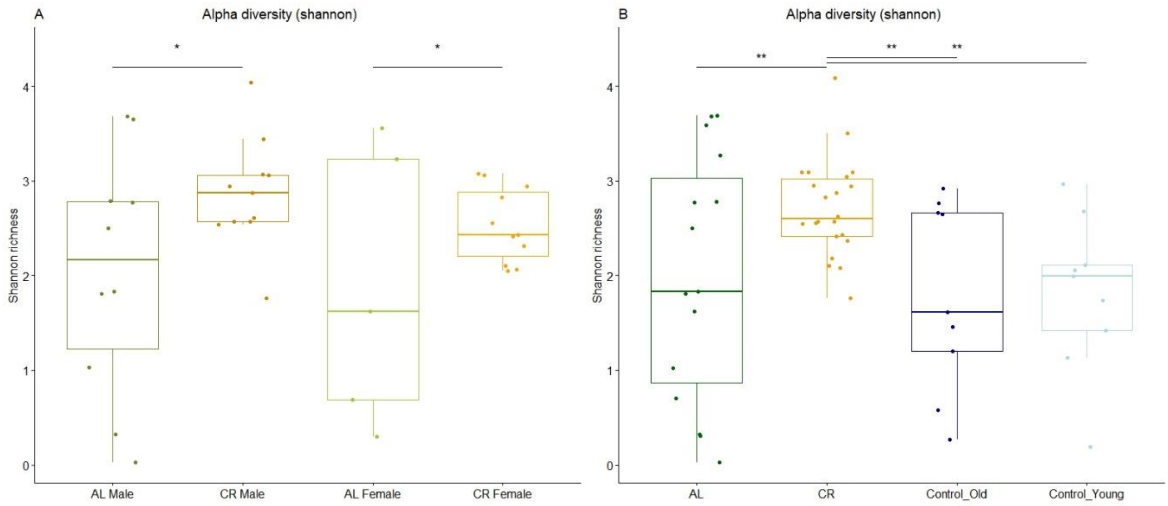
Figure 3: Barplot depicting DEGs between AL and CR-fed seahorses in different organs, categorized by sex (M = male and F = female). Upregulated genes in AL (downregulated in CR) are shown in green, while downregulated genes in AL (upregulated in CR) are shown in orange. Relevant DEGs discussed in the text for liver, male, female, and ovaries are indicated in surrounding boxes. Supplementary material TableS3 provides an overview of the DEGs (with *Danio rerio* orthologues) with additional details.

3.3 Calorie restriction promotes greater gut microbial diversity

After removing singletons and filtering, the dataset consisted of 200 unique OTUs. At the phylum level, CR seahorses had overall the highest abundance compared to AL and the young and old age groups, including Proteobacteria 11.7%, then Bacteroidetes 7.8% and then Myxococcota 1.95%. Conversely, AL had overall the lowest with Proteobacteria 1.95% and Bacteroidetes 0.97%. Whilst the age groups had CY with Proteobacteria at 7.8% and Bacteroidetes 2.93%, for CO 4.88% and 2.44% respectively. Hypothesis testing of α-diversity for Shannon index indicated a significant effect between treatment and age groups ($p.val < 0.02$) but not sex. Howbeit, post-hoc showed that these significant differences were only between CR and all other groups (Fig. 4B). A closer pairwise comparison between the two dietary groups and sex revealed significant differences between fasted males and AL fed ($p.val = 0.033$), similar for females ($p.val = 0.025$), but no differences between AL males and female or CR (Fig. 4A). Overall, fasted seahorses displayed a moderate to relatively high diversity compared to other groups (AL, CO, CY), with the bacterial taxa more evenly distributed across CR individuals (Figs. 4A-B), indicative of a more complex and diverse microbial community. In comparison, AL exhibited greater variation among individuals, with Shannon index values ranging from near 0, indicating few dominant taxa, to values near 4, indicating higher diversity with several coexisting species (Fig. 4A).

Results from the β-diversity (for both Bray Curtis and Jaccard) showed no significant difference between sexes (Fig. 4C), but a highly significant difference between AL and CR (Figs. 4C-D, p.val < 0.001) (for results including Vibrio genus see Figs. S3B-D). Similarly, as with the results from the α-diversity, CR individuals clustered more closely together compared, while there was more of an overlap between all other groups (Fig. 4D).

Within the β-diversity NMDS plots, comparing just dietary groups in Fig. 4C and also treatment groups and age groups (Fig. 4D), showed that the *Brevinema* genus (family *Brevinemataceae*) correlated the strongest with AL seahorses (p.val < 0.001, Fig. 4C). Comparing overall abundance of *Brevinema* between groups revealed indeed the highest abundance of this taxa within these individuals compared to all others (Fig. S3F). On the other hand, taxa such as, *Thalassotalea* (family *Colwelliaceae*), *Pseudoalteromonas* (family *Pseudoalteromonadaceae*), *Marinobacterium* (family *Nitrincolaceae*) and *Arcobacteraceae* (no genus identified) strongly correlated with seahorses on a fasting diet (Fig. 4C, see supplementary material for relative abundance). These trends were the same for both male and female fasted seahorses. We found no significant overlap between the bacterial communities driving the differences between AL and CR, and CO and CY (Fig. 4D). Furthermore, there were strong similarities between the age groups and their gut microbial composition, indicating that age did not have an effect.



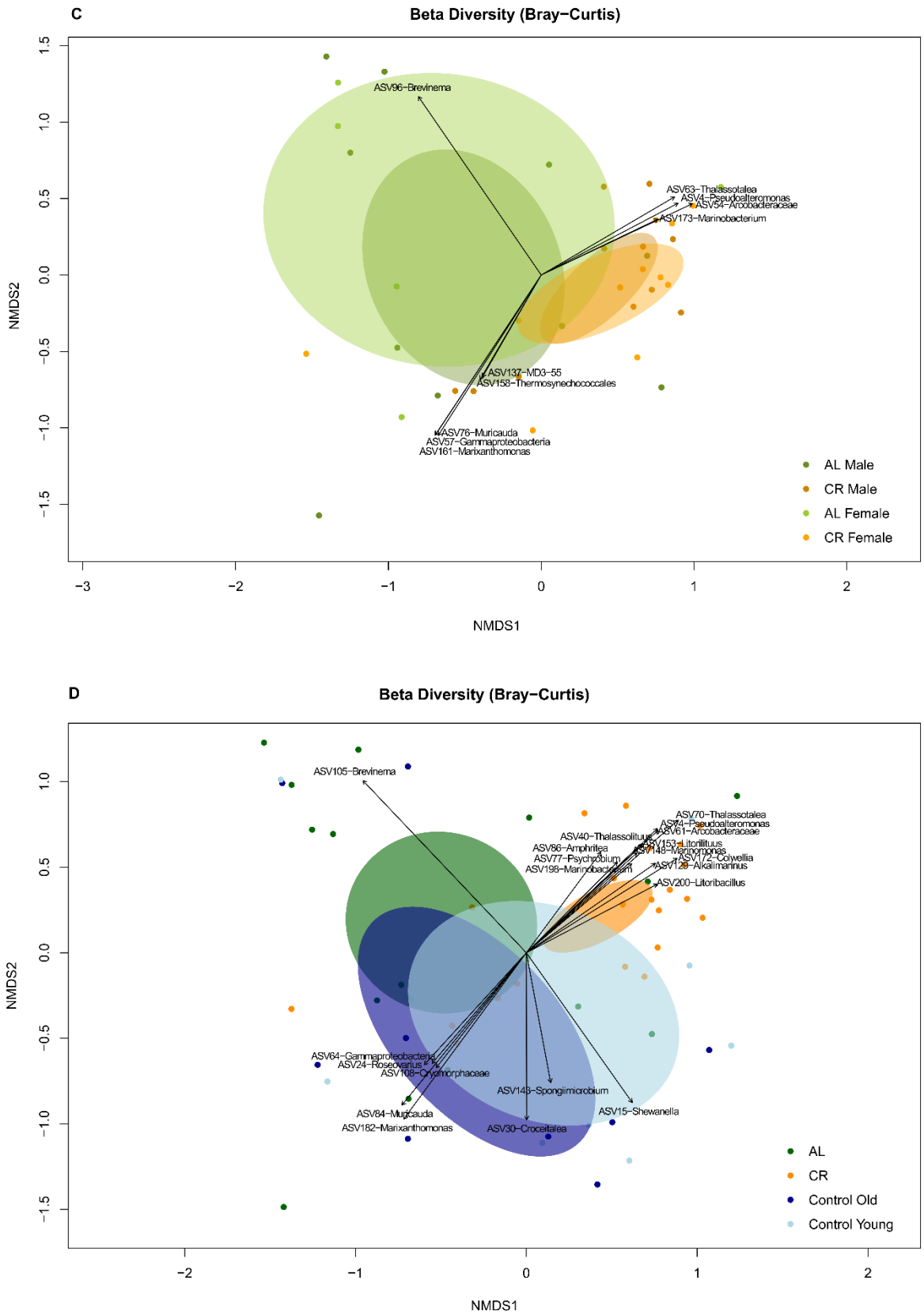


Figure 4: Microbial biodiversity analysis from hind gut tissue. A) The first panel displays α-diversity boxplots for treatment groups AL and CR along for males and females. The x-axis represents the groups, while the y-axis shows the Shannon richness.

P values represent adonis pairwise comparisons with * < 0.05. B) Shannon richness comparing dietary treatment groups with age groups. P values represent adonis pairwise comparisons with ** < 0.005. C) NMDS diversity plot for Bray-Curtis metric with AL and CR samples of males and females. D) NMDS diversity plot for Bray-Curtis metric comparing dietary treatment groups with age groups.

4. Discussion

The beneficial health effects of CR are well documented across various model species (yeast, nematode worms, fruit flies and rodents), with positive effects encompassing improved metabolism, reduction in inflammation to improvement of the gut microbiome [93,94]. This has led to believe that the effects of CR have evolutionary conserved mechanisms across all organisms. However, a meta-analysis revealed that such effects were twice as strong in the five model species compared to non-models [95]. This could be because model organisms have been subjected over many generations to laboratory conditions and are genetically more responsive to CR compared to non-model species displaying more genetic diversity. It is, however, probably a simple case of a bias in the literature, where studies on model organisms that show significant positive effects of CR are more likely to be published, while similar studies on non-model organisms may be less well-documented or less accessible. This bias highlights our limited understanding of how fasting affects non-model systems with different evolutionary adaptations to food availability and also diverse reproductive systems with female and male-specific trade-offs for energy allocation [20,93].

Our study sought to understand the effects of CR, through an alternating fasting regimen, in *Hippocampus erectus*. In this non-model species, males take on the role of the pregnant sex, incurring costly investment into development of brooding structure, while still producing sperm [96,97]. Conversely, females produce energy-rich eggs. *Hippocampus erectus* unique reproductive biology allowed us to disentangle the cost of investment into the brooding structure vs. investment into egg production, which are two costly traits typically found in females. Furthermore, seahorses have simple digestive systems and lack a stomach leading to potentially interesting responses to food deprivation as they typically eat at frequent intervals [43].

As discussed in the result section, one of the first expected effects of CR was the reduction of body mass. Fasting led to a slower growth trajectory (Fig. S1B), which is a typical effect observed in most other studies, in particular when fasting starts at a juvenile age [2]. Body weight was significantly different at the end of the fasting period between CR and AL (Fig. 1A), but there was a more pronounced effect between CR and AL males compared to females (Fig. 1B). This could be indicative of differences in growth investment between the sexes and their respective vulnerability to food shortages. Furthermore, we also recorded a delayed pouch formation of roughly twenty days in the CR male seahorses compared to their AL counterparts, with pouch width and length being on average smaller in CR individuals on day 157 compared to AL (Figs. 1D and S1H). Such differences, are potentially partly due to the slower developmental trajectory of CR seahorses, but likely reflect the energy investment that is necessary to develop the pouch tissue.

In female seahorses, ovarian weight between AL and CR did not reach significance (Fig. 1C), although the exact content of fat or nutrient deposits in the eggs was not measured as the ovaries were used for RNA sequencing. However, seeing as there were stark contrasts between the number of DEGs in this tissue between AL and CR

(Fig. 3), we believe that differences in nutrient allocation to the eggs could explain differences in gene expression. Overall, studies, primarily in mammals, have shown that differences in early-life history strategies in face of adversity, may lead males to prioritize somatic growth and females to allocate resources to other physiological adjustments [98]. This may also be reflected to some extent in *H. erectus*, as not only are they, in spite of male pregnancy, monogamous (per breeding season) and females will typically pick their preferred male [99], but sex-steroid hormones are not reversed with males predominantly producing androgens and females oestrogens [100].

There was heterogeneity between the number of significantly DEGs amongst the different organs (Fig. 3 and Table S1). Liver and ovaries showed strong sex-dependent effects of fasting, as for head kidney where there was generally sex-specific DGE but no differences between fasted and non-fasted individuals (Fig. 3). The latter could imply that either the head kidney is not an energy-demanding organ and/or that the pathways affected by CR do not severely impact its regular functioning, compared to liver tissue where energy storage and mobilization are critical during period of reduced calorie intake.

Females were not only more strongly impacted by CR in the liver compared to males, but their reproductive organs appeared to have variation in gene expression compared to no significant effects in testes (Fig. 3). These results align with studies in conventional model organism [20]. However, sperm is cheaper to produce and thus it would make sense that pre-copulatory energy allocation in gonads is still higher in female seahorses with fasting likely affecting more severely the lipid energy stored in the eggs. Yet, from a morphological perspective on body reshaping, male seahorses showed stronger response to CR for latency in pouch development, meaning that pre-copulatory resource allocation to grow this tissue is likely costly.

In the ovaries, for instance, genes linked to lipid synthesis, such as *acsbg* and *acsl*, were downregulated in CR, reflecting a different regulation of fat stores [101]. Reproductive functions in females, typically depend on the energy reserves of fat in adipose tissue and from these fat cells the hormone leptin is produced, which then is reduced during fasting due to the loss of body fat [2]. In the liver of CR females, the regulatory expression of the leptin receptor (*macroh2a1*) was downregulated (Fig.3). Interestingly, recent studies in mice and humans have revealed that leptins have numerous roles in regulating among other things immune functions and oocyte maturation, and as leptin correlates with progesterone concentrations [102], we also found a downregulation of *pgr* and *cyp17A2* in the liver of fasting females (Fig. 3). On a different note, CR females had further upregulated genes involved in antioxidative stress, such as *idh2*, *prdx4* and *prdx6* (Fig.3), increased autophagy (*atg13*) and increased ketosis (*aacs*), with such pathways being typically also upregulated in other studies [2].

In the ovaries of AL females, we found a strong inflammatory upregulation (Fig. 3). TLRs 21 and 22 lead to upregulation of inflammatory pathways [86,87], same as several chemokines, *tnf*, *irf5*, and *M17* (analogous to mammalian IL-6) which are all strong pro-inflammatory cytokines in fish [89,91,92]. This might be attributed to how females prime their eggs for transgenerational plasticity. Previous research conducted in *Syngnathus typhle*, a member of the *Syngnathidae* family, has shown that when parents are exposed to environmental stressors, such as elevated temperatures, the effects of transgenerational immune priming disappear [103]. This phenomenon is likely a result of parents allocating more resources to their own survival or preparing their offspring for different types of environmental stressors that are not necessarily pathogen-related.

On a different note, anti-inflammatory effects of CR have been well documented in other animal models and reducing inflammation can both hinder the onset of aging, reduce the occurrence of chronic autoimmune conditions and improve gut health [1,2,8]. Further confirmation for the beneficial effects of fasting can be found by the upregulation of both *sesn2* and *tti2* in AL seahorses compared to CR (still in ovarian tissue), which in turn leads to the activation of mTORC1 [84 85]. Pathways such as SIRTs, AMPK, IGF-1 tend to induce cell repair and autophagy, while mTOR leads to cell growth [2,10]. Additionally, *foxO1a* was upregulated in AL ovaries (Fig. 3), and FoxO is found typically elevated in response to high levels of reactive oxygen species (ROS) having a dual role in combating oxidative stress, being both pro-survival and pro-apoptotic [104]. In some studies *FoxO1a* has been shown to have positive effects by promoting, among other things, DNA repair, but it was also upregulated in hyperglycemic mice and its removal led to improvement of insulin levels [104]. Furthermore, we did observe a potential rejuvenation effect in the ovaries of fasting female seahorses, as pairwise comparisons between CR and CY found no differences, while similar pathways were enriched when comparing CR vs. AL or vs. CO (Tables S1 and S2, sheet D). In light of these findings, it would appear that the ovaries of AL females may have higher stores of lipids and increased lipid synthesis whilst also promoting cell growth, compared to fasted females having pathways upregulated for cell repair and regeneration. This suggests that female seahorses, despite not being the pregnant sex, when fasted are reshaping their phenotype from a state of high reproductive output to increased somatic maintenance.

In the liver of CR male seahorses we found upregulation of genes controlling circadian rhythm (*cklockb* and *cry3a*), which are known to be impacted by daily nutrient level fluctuations [42,105]. While female seahorses actually had the *clocka* gene downregulated in CR in ovaries, rather than liver (Fig. 3). Studies in mice found sex-specific signaling of circadian clock genes, with *cry1* being significantly higher in the liver of CR males [106]. A good regulation of the circadian clock has benefits for overall health, and particularly they are known to be tightly impacting the lining of the gut mucosa [105]. On this note, we did not observe a significant sex-specific effect of CR on the microbial composition, so it would not appear that the sex-specific regulation of circadian clock genes is strongly controlling the gut microbiome in males. Yet, overall, we did observe a meaningful effect of the fasting treatment for both α- (Fig. 4A) and β-diversity (Fig. 4B) to all other groups (AL, CO, CY).

Studies have shown that the gut microbiota play a pivotal role in influencing fish health by fostering the development of the gut epithelium, enhancing the immune system's functioning and acting as a protective barrier, impeding the colonization of harmful pathogens within the gut [107,108]. Taxa richness was high in all CR seahorses, compared to the strong individual fluctuations of AL individuals and the control young and old groups (Figs. 4B). Research has shown that the more diverse a community of bacteria is, the more stable and healthy environment [109]. A recent systematic review of the impact of different fasting regimes in various animal models and humans, showed that most types of fasting lead to an increase in α-diversity, although the abundance or absence of certain bacterial communities does change with the duration or intensity of fasting [9]. The clear effects of fasting on the microbial composition in seahorses are evident from the NMDS plots (Figs. 4C and D), with CR seahorses being very distinct to all other groups (AL, CO and CY). However, we did not find sex-specific effects of fasting on the microbial community (Figs. 4A and C). Major differences were seen in the higher abundance of Proteobacteria (11.7%) and Bacteroidetes (7.8%) in CR compared to AL fed groups (1.95% and 0.97% respectively). These results are consistent with other studies showing a higher relative abundance of Bacteroidetes and Proteobacteria

in fasting vertebrates (including fish) [108,110]. Bacteroidetes are known for their involvement in breaking down complex carbohydrates and producing short-chain fatty acids [111] and some members are also known to possess the capability to utilize mucosal glycans secreted by the host, thereby providing the host with extra energy [112]. While Proteobacteria are believed to have a significant role in priming the gut for colonization by strict anaerobic bacteria, which are essential for maintaining a healthy gut [113]. Among the Proteobacteria, the genera with highest relative abundance in CR were *Pseudoalteromonas*, *Thalassotalea* and *Marinobacterium* (Figs. 4C and S3G-H). In marine eukaryotes, *Pseudoalteromas* consist of marine species with wide activities such as anti-bacterial, algicidal, anti-viral and certain *Pseudoalteromonas* isolates specifically hinder the settlement of common fouling organisms [114]. *Thalassotalea* occur in marine environments and are an aerobic and chemo-organotrophic genus of bacteria, thus playing an essential role in nutrient cycling and decomposition of organic matter [115]. The ability to efficiently utilize various forms of energy from the environment may make these microbes more abundant and provide additional energy for the host during fasting.

Taken together, our findings suggest, that fasting in *Hippocampus erectus* shows sex-specific tissue gene expression, but an equal shift in bacterial diversity and abundance in the hind gut. The sex-specific effects appear more biased towards females for gene expression and likely reflect pre-copulatory energy investment in egg production, compared to cheaper sperm production in males [26], and the intricate relationship between female sex-steroid hormones being regulated by lipid stores. Contrary, CR had a stronger effect on body mass and pouch development in males, reflecting the developmental time and energy necessary to create the pouch tissue in preparation of pregnancy. In future research we would like to investigate the importance of sex hormones in responding to fluctuations in nutrient levels, and measure reproductive output of fasted pregnant males.

Our results in *H. erectus*, may only reflect part of the picture as teleost species exhibit many forms of mating behaviours and sex-roles, with differences in sex-specific parental investment [32] to switches in biological sexes over their lifetime [116]. Nonetheless, our work underscores the importance of considering a diverse range of model species with diverse reproductive strategies, sex-roles and mating system when exploring the effects of fasting, as reproduction and resource availability are tightly linked. Further research will hopefully lead to a more comprehensive and nuanced understanding of its impacts on sex-specific health and lifespan dimorphism, life history trade-offs in resource allocation, and the evolutionary adaptations that may occur in the face of nutrient scarcity due to climate change.

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Data availability statement

Additional information is available in the supplementary materials. Refer to Tables S1 to S6 for comprehensive details: Table S1 presents a summarized overview of numerical outcomes derived from the *limma* DGE analysis, encompassing various groups and organs. For the results of the GO analysis, consult Table S2. Visual insights from the DGE analysis concerning the specified organs and treatment groups are presented in Figure S3. Table S4 offers the results of the 16S rRNA amplicon sequencing analysis conducted in QIIME2, detailing all OTUs at the genus level. Lastly, Table S5 encompasses morphological findings, specifically the length and weight measurements of CR, AL, CY, and CO seahorses. Table S6 displays the full statistical analysis for morphology, RNA-Seq and 16S rRNA-Seq. The raw sequencing RNA-Seq data, 16S rRNA-Seq and metadata used in this study are available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject ID PRJNA1004169 (RNA-Seq: SUB13725814; 16S rRNA-Seq: SUB13759493)

Ethics statement

The work was carried out in accordance with the German animal welfare law and with the ethical approval given by the Schleswig-Holstein Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Ditgitalisierung (MELUND) (permit no. 1315/2021). No wild endangered species were used in this investigation.

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