

**EVOLUTIONARY INSIGHTS INTO SEX-SPECIFIC LIFE
HISTORY STRATEGIES, RESOURCE ALLOCATION,
AND PARENTAL INVESTMENT IN MALE-PREGNANT
SYNGNATHID FISH: A STUDY OF *HIPPOCAMPUS
ERECTUS* AND *SYNGNATHUS TYPHLE***

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The cure for boredom is curiosity.

There is no cure for curiosity.

- Dorothy Parker

SUMMARY

Sexual dimorphism extends far beyond physical characteristics and encompasses profound differences in reproductive strategies between males and females, shaping their entire life histories. These differences arise from anisogamy, where eggs are large and energetically costly to produce, while sperm are smaller and relatively inexpensive. As a result, females tend to invest more energy per reproductive unit and exhibit a positive correlation between fitness and longevity. Conversely, males invest less in each reproductive act and may prioritize mating frequency over longevity. In some species, females allocate resources to gestation, lactation, and other aspects of parental care, influencing their metabolic rates and immune responses. In contrast, males may engage in riskier behaviours associated with sexual competition, which can affect their physiological traits and hazard risk. These differences in reproductive strategies contribute to varying metabolic needs between sexes, influencing resource allocation for immune defence, metabolic rates, and overall lifespan. In fact, while the mechanisms of ageing are broadly conserved, emerging evidence suggests that there are sex-specific rates of senescence and age-related diseases.

For a considerable duration, research has largely overlooked the exploration of male and female biological and biomedical distinctions. While there are some signs of change, the fields of gerontology and reproductive research continue to concentrate primarily on a limited selection of model species, adhering to the conventional sex roles where females invest more in parental care while males compete for access to females. However, across the animal kingdom, organisms not only exhibit diverse rates of senescence but also encompass a wide array of sex determination mechanisms, sex roles, mating systems, sexual dimorphism, and numerous other facets. In particular, the Syngnathidae family challenges these norms with the evolution of male pregnancy. Seahorses and pipefish, members of the Syngnathidae family, present a unique opportunity to address the gaps in our understanding of sex-specific ageing and resource allocation in the realm of reproductive strategies. Their unique biological traits, ranging from male pregnancy to flexible sex roles and diverse mating systems, offer an unparalleled opportunity to explore resource allocation dynamics between males and females in different reproductive strategies. Additionally, the Syngnathidae family challenges conventional wisdom about fertility reduction with age, as some species exhibit increased reproductive outputs as they age due to indeterminate growth patterns.

The first chapter addresses how limited resources affects male and female lined seahorse (*Hippocampus erectus*) development, gene expression and gut microbial composition. The primary objectives were to investigate how caloric restriction (CR) influences this non-model species, which has not been previously studied in caloric restriction gerontology research. Additionally, we aimed to explore resource allocation for growth and somatic maintenance versus reproduction in a species with male pregnancy. To achieve this, we conducted a five-month experiment involving 3-month-old juvenile seahorses. These seahorses were subjected to either an every other day feeding schedule resulting in a caloric deficit or an ad libitum (AL) feeding regimen. At the study's conclusion, the fish were

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euthanized, and we collected head kidney, liver, gonads for gene expression analysis, and hind gut tissue for microbial composition assessment. Our findings revealed that CR had a more pronounced impact on the growth of male seahorses, leading also to a delayed formation of the brood pouch. This delay suggests that males require significant energy for pouch formation and may postpone reproduction until more food becomes available. In contrast, the morphological differences between CR and AL females were less noticeable, but females exhibited a greater number of differentially expressed genes in their ovaries and liver compared to males. Many of these genes were associated with energy and lipid metabolism, particularly in the ovaries, indicating that female seahorses likely invest more in rapid growth and adjust their reproductive output accordingly. These results emphasize the importance of considering a wider range of species when studying the connection between reproductive strategies and energy allocation. Such considerations contribute to the understanding of sex-specific life history trajectories, shedding light on the ultimate causes of immune dimorphism and lifespan dimorphism.

The second chapter delves into the study of sex-biased gene expression in the broadnosed pipefish (*Syngnathus typhle*). While previous research has primarily focused on identifying genes with biased expression in conventional model species and their implications for sex-specific health issues and senescence rates, our aim was to unravel how this pattern manifests in a species with male pregnancy and sex-role reversal, where females compete for access to males. To address this, we collected younger, older, females and males, including pregnant and non-pregnant males. After euthanizing the fish, we dissected their brain, head kidney, and gonads, and subjected the collected tissues to RNA sequencing. Our findings revealed a complex pattern of gene expression in the broadnosed pipefish. Some genes and pathways that exhibit sex-biased expression in species with conventional reproductive roles showed a reversed pattern in pipefish, such as those related to stress response and immune regulation. Conversely, certain aspects, like energy and lipid metabolism in females, appeared to be conserved based on biological sex. This study underscores the significance of exploring a diverse range of animal models with distinct sex roles, mating systems, and sexual selection. Such research enhances our understanding of whether sex-biased genes are truly biased by gender or by biological sex, ultimately bridging the gap between medical applications and fundamental research.

Last but not least, the third chapter explores the impact of parental reproductive age on pregnancy duration, offspring number, offspring morphology, and offspring differential gene expression in *Syngnathus typhle*. Our primary objective was to unravel whether reproductive age plays a significant role in a species that experiences increased fertility and reproductive output as individuals age, and to explore the potential influence of somatic and mutation accumulation in parents on offspring health. This work gravitates away from conventional research, which predominantly focuses on maternal effects on offspring due to the female's dual role in costly egg production and increased parental care, including pregnancy and lactation in mammals. By using the broadnosed pipefish, we aimed to disentangle these two costly female traits. Males in this species gestate embryos for a month but still serve as sperm producers. To address these questions, we mated younger and older male and female pipefish. We also conducted whole-body RNA sequencing on the resulting offspring. Our findings indicate that offspring older and larger parents tended to have also larger body sizes, potentially

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advantageous for foraging and enhanced swimming abilities to evade predators. However, when examining differential gene expression, we found that paternal lineage had a more substantial impact on offspring gene expression phenotype, while the maternal side exhibited negligible effects. Notably, offspring from older fathers were particularly affected, with enriched pathways related to DNA replication, translation, telomere extension, and neurogenesis, among others. These results underscore the significance of understanding which parent spends more time with their offspring (i.e., parental care and/or pregnancy) and thus has closer contact, allowing offspring to shape their future phenotype based on inherited environmental cues from that parent. While information transmitted through gametes remains important, the future of research in inheritance lies in transgenerational phenotypic plasticity. It is imperative to comprehend how male and female reproductive roles intertwine with this process, shedding light on the complex mechanisms of inheritance.

ZUSAMMENFASSUNG

Der Geschlechtsdimorphismus geht weit über die körperlichen Merkmale hinaus und umfasst tiefgreifende Unterschiede in den Fortpflanzungsstrategien von Männchen und Weibchen, die ihre gesamte Lebensgeschichte prägen. Diese Unterschiede ergeben sich aus der Anisogamie, bei der die Eier groß und energetisch kostspielig zu produzieren sind, während die Spermien kleiner und relativ preiswert sind. Infolgedessen investieren die Weibchen tendenziell mehr Energie pro Fortpflanzungseinheit und weisen eine positive Korrelation zwischen Fitness und Langlebigkeit auf. Umgekehrt investieren Männchen weniger in jeden Fortpflanzungsakt und geben der Paarungshäufigkeit möglicherweise Vorrang vor der Langlebigkeit. Bei einigen Arten wenden die Weibchen Ressourcen für die Trächtigkeit, die Laktation und andere Aspekte der elterlichen Fürsorge auf, was sich auf ihre Stoffwechselraten und Immunreaktionen auswirkt. Im Gegensatz dazu lassen sich Männchen auf risikoreichere Verhaltensweisen ein, die mit sexuellem Wettbewerb verbunden sind, was sich auf ihre physiologischen Eigenschaften und ihr Gefahrenrisiko auswirken kann. Diese Unterschiede in den Fortpflanzungsstrategien tragen zu unterschiedlichen Stoffwechselbedürfnissen zwischen den Geschlechtern bei und beeinflussen die Ressourcenzuweisung für die Immunabwehr, die Stoffwechselraten und die Gesamtlebensdauer. Während die Mechanismen des Alterns weitgehend konserviert sind, gibt es Hinweise darauf, dass es geschlechtsspezifische Raten der Alterung und altersbedingter Krankheiten gibt.

Lange Zeit hat die Forschung, die Erforschung der biologischen und biomedizinischen Unterschiede zwischen Männern und Frauen weitgehend vernachlässigt. Obwohl es einige Anzeichen für einen Wandel gibt, konzentrieren sich die Bereiche Gerontologie und Reproduktionsforschung nach wie vor in erster Linie auf eine begrenzte Auswahl von Modellarten und halten an den herkömmlichen Geschlechterrollen fest, bei denen die Weibchen mehr in die elterliche Fürsorge investieren, während die Männchen um den Zugang zu den Weibchen konkurrieren. Im gesamten Tierreich weisen die Organismen jedoch nicht nur unterschiedliche Alterungsraten auf, sondern auch ein breites Spektrum an Mechanismen zur Geschlechtsbestimmung, Geschlechterrollen, Paarungssystemen, Geschlechtsdimorphismus und zahlreichen anderen Aspekten. Insbesondere die Familie der Syngnathidae stellt diese Normen mit der Evolution der männlichen Schwangerschaft in Frage. Seepferdchen und Seenadeln, die zur Familie der Syngnathidae gehören, bieten eine einzigartige Gelegenheit, die Lücken in unserem Verständnis der geschlechtsspezifischen Alterung und Ressourcenverteilung im Bereich der Fortpflanzungsstrategien zu schließen. Ihre einzigartigen biologischen Merkmale, die von der männlichen Schwangerschaft bis hin zu flexiblen Geschlechterrollen und unterschiedlichen Paarungssystemen reichen, bieten eine einmalige Gelegenheit, die Dynamik der Ressourcenverteilung zwischen Männchen und Weibchen bei verschiedenen Fortpflanzungsstrategien zu untersuchen. Darüber hinaus stellt die Familie der Syngnathidae die konventionelle Annahme in Frage, dass die Fruchtbarkeit mit dem Alter abnimmt, da einige Arten aufgrund unbestimmter Wachstumsmuster mit zunehmendem Alter eine erhöhte Reproduktionsleistung aufweisen.

Zusammenfassung

Das erste Kapitel befasst sich damit, wie begrenzte Ressourcen die Entwicklung, die Genexpression und die Zusammensetzung des Darmmikrobioms von männlichen und weiblichen Seepferdchen (*Hippocampus erectus*) beeinflussen. Die primären Ziele waren zu untersuchen, wie kalorische Reduktion (CR) diese Nicht-Modellspezies beeinflusst, die bisher in der Gerontologie-Forschung zur kalorischen Restriktion nicht untersucht wurde. Darüber hinaus wollten wir die Ressourcenzuteilung für Wachstum und somatische Erhaltung gegenüber der Reproduktion bei einer Spezies mit männlicher Schwangerschaft untersuchen. Um dies zu erreichen, führten wir ein fünfmonatiges Experiment mit 3 Monate alten jungen Seepferdchen durch. Diese Seepferdchen wurden entweder alle zwei Tage gefüttert, was zu einem Kaloriendefizit führte, oder sie wurden ad libitum (AL) gefüttert. Am Ende der Studie wurden die Fische eingeschläfert, und wir sammelten Kopfnieren, Leber und Keimdrüsen für die Genexpressionsanalyse sowie Hinterdarmgewebe für die Bewertung der mikrobiellen Zusammensetzung. Unsere Ergebnisse zeigten, dass CR einen stärkeren Einfluss auf das Wachstum der männlichen Seepferdchen hatte, was auch zu einer verzögerten Bildung des Brutbeutels führte. Diese Verzögerung deutet darauf hin, dass die Männchen viel Energie für die Bildung des Brutbeutels benötigen und die Fortpflanzung möglicherweise aufschieben, bis mehr Nahrung zur Verfügung steht. Im Gegensatz dazu waren die morphologischen Unterschiede zwischen CR- und AL-Weibchen weniger auffällig, aber die Weibchen wiesen im Vergleich zu den Männchen eine größere Anzahl unterschiedlich exprimierter Gene in ihren Eierstöcken und ihrer Leber auf. Viele dieser Gene standen im Zusammenhang mit dem Energie- und Fettstoffwechsel. Diese Ergebnisse unterstreichen, wie wichtig es ist, bei der Untersuchung des Zusammenhangs zwischen Fortpflanzungsstrategien und Energieallokation ein breiteres Spektrum von Arten zu berücksichtigen. Solche Überlegungen tragen zum Verständnis geschlechtsspezifischer Lebensverläufe bei und werfen ein Licht auf die letztendlichen Ursachen von Immundimorphismus und Dimorphismus der Lebensspanne.

Das zweite Kapitel befasst sich mit der Untersuchung der geschlechtsspezifischen Genexpression in der Seenadel *Syngnathus typhle*. Während sich frühere Forschungsarbeiten in erster Linie auf die Identifizierung von Genen mit verzierter Expression bei konventionellen Modellarten und deren Auswirkungen auf geschlechtsspezifische Gesundheitsprobleme und Alterungsraten konzentrierten, war es unser Ziel, herauszufinden, wie sich dieses Muster bei einer Art mit männlicher Schwangerschaft und Geschlechtsrollenumkehr manifestiert, bei der die Weibchen um den Zugang zu den Männchen konkurrieren. Um dies zu untersuchen, sammelten wir jüngere und ältere Weibchen und Männchen, einschließlich trächtiger und nicht trächtiger Männchen. Nach der Tötung der Fische seziierten wir ihr Gehirn, die Kopfnieren und die Keimdrüsen und unterzogen die gesammelten Gewebe einer RNA-Sequenzierung. Unsere Ergebnisse zeigten ein komplexes Muster der Genexpression beim Breitnasenpfeifenfisch. Einige Gene und Signalwege, die bei Arten mit konventioneller Fortpflanzungsfunktion eine geschlechtsspezifische Expression aufweisen, zeigten bei den Pfeifenfischen ein umgekehrtes Muster, wie z. B. solche, die mit der Stressreaktion und der Immunregulation zusammenhängen. Umgekehrt schienen bestimmte Aspekte, wie der Energie- und Fettstoffwechsel bei Weibchen, je nach biologischem Geschlecht konserviert zu sein. Diese Studie unterstreicht die Bedeutung der Erforschung einer Vielzahl von Tiermodellen mit unterschiedlichen

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Geschlechterrollen, Paarungssystemen und sexueller Selektion. Eine solche Forschung verbessert unser Verständnis dafür, ob geschlechtsspezifische Gene tatsächlich durch das Geschlecht oder durch das biologische Geschlecht beeinflusst werden, und schließt letztlich die Lücke zwischen medizinischen Anwendungen und Grundlagenforschung.

Im dritten Kapitel schließlich werden die Auswirkungen des Reproduktionsalters der Eltern auf die Dauer der Schwangerschaft, die Anzahl der Nachkommen, die Morphologie der Nachkommen und die unterschiedliche Genexpression der Nachkommen bei *Syngnathus typhle* untersucht. Unser primäres Ziel war es, herauszufinden, ob das Fortpflanzungsalter bei einer Spezies, deren Fruchtbarkeit und Fortpflanzungsleistung mit zunehmendem Alter der Individuen zunimmt, eine bedeutende Rolle spielt, und den potenziellen Einfluss der somatischen und Mutationsakkumulation bei den Eltern auf die Gesundheit der Nachkommen zu untersuchen. Diese Arbeit weicht von der konventionellen Forschung ab, die sich vorwiegend auf die Auswirkungen der Mutter auf die Nachkommen konzentriert, da das Weibchen eine Doppelrolle bei der kostspieligen Eiproduktion und der verstärkten elterlichen Fürsorge, einschließlich Schwangerschaft und Laktation bei Säugetieren, spielt. Mit Hilfe der Seenadeln wollten wir diese beiden kostspieligen weiblichen Eigenschaften entflechten. Die Männchen dieser Art tragen die Embryonen einen Monat lang aus, dienen aber weiterhin als Spermienproduzenten. Um diese Fragen zu klären, haben wir Fische unterschiedlicher Größe miteinander verpaart, was als Anhaltspunkt für das Fortpflanzungsalter dient, und anschließend verschiedene Parameter gemessen. Außerdem führten wir an den Nachkommen eine Ganzkörper-RNA-Sequenzierung durch. Unsere Ergebnisse deuten darauf hin, dass die Nachkommen älter und größere Eltern tendenziell eine größere Körpergröße haben, was für die Nahrungssuche von Vorteil sein könnte, und dass sie besser schwimmen können, um Fressfeinden zu entgehen. Bei der Untersuchung der unterschiedlichen Genexpression stellten wir jedoch fest, dass die väterliche Abstammung einen größeren Einfluss auf den Phänotyp der Genexpression der Nachkommen hatte, während die mütterliche Seite vernachlässigbare Auswirkungen zeigte. Besonders betroffen waren die Nachkommen älterer Väter, bei denen unter anderem die Wege der DNA-Replikation, der Translation, der Telomer-Verlängerung und der Neurogenese angereichert waren. Diese Ergebnisse unterstreichen, wie wichtig es ist, zu verstehen, welcher Elternteil mehr Zeit mit seinen Nachkommen verbringt (d.h. elterliche Betreuung und/oder Schwangerschaft) und somit einen engeren Kontakt hat, der es den Nachkommen ermöglicht, ihren künftigen Phänotyp auf der Grundlage der von diesem Elternteil ererbten Umweltfaktoren zu gestalten. Während die über die Keimzellen übertragenen Informationen weiterhin wichtig sind, liegt die Zukunft der Vererbungsforschung in der transgenerationalen phänotypischen Plastizität. Es muss unbedingt verstanden werden, wie die männlichen und weiblichen Fortpflanzungsrollen mit diesem Prozess verflochten sind, um die komplexen Mechanismen der Vererbung zu erhellen.

INTRODUCTION

The evolution of ageing

Senescence, often termed ageing, is described as a gradual decline in physiological function and reproductive capacity over time and is a nearly universal phenomenon of multicellular organisms (Hughes and Reynolds 2005; Viña, Borrás, and Miquel 2007). There are numerous theories that attempt to explain the evolutionary origins of ageing and they are not necessarily mutually exclusive. The first hypothesis of why ageing occurs came from Weismann, that stated that only organisms that reproduce asymmetrically should age, as they replace worn out individuals with younger ones (Weismann 1891). If sexual division is perfectly symmetrical, it is impossible for selection to distinguish between mother and daughter cells, for both are either equally damaged or intact and reproductive payoff from improving the maintenance is equal (Partridge and Barton 1996). Many following ageing theories are based on the fact that we have extrinsic hazard risks that lead to our deaths (e.g. accidents, predation illnesses), also coined extrinsic mortality (Hughes and Reynolds 2005). This would equate to the rate of ageing in each organism being correlated to your environmental hazard risk. We do see this in birds that senesce at a slower rate compared to mammals, given that they can fly and avoid predation (Holmes, Flückiger, and Austad 2001). And yes, this is also true for bats living on average longer than mammals constrained to the ground (S. N. Austad and Fischer 1991).

But what is happening at an intrinsic biological level? Hamilton proposed that the force of natural selection would be stable until first reproduction, after which it would decline with age in organisms with overlapping generations (W. D. Hamilton 1966). In other words, a gene that influences an individual's survival or reproductive abilities during their early years exerts a more significant impact on their Darwinian fitness compared to a gene with an equivalent effect size that becomes evident later in life as extrinsic mortality is unavoidable (W. D. Hamilton 1966; Hughes and Reynolds 2005). Linked to this population genetic theory are many other senescence hypotheses from mutation accumulation (MA), to antagonistic pleiotropy (AP) and the disposable soma theory (DST). The physiological decline that comes with MA as we age happens precisely because the force of natural selection becomes weaker over time (Viña, Borrás, and Miquel 2007). In the case of AP, alleles with beneficial early life effects on fitness but deleterious in later years (highly polymorphic genes) will be selected for, and increase in frequency over evolutionary time within a population (Hughes and Reynolds 2005). The DST is a concept that falls under the larger umbrella of AP (Kirkwood 1977). It suggests that the body's maintenance and repair processes are metabolically costly, and any energy used for reproduction is then not available for somatic maintenance. For example, if an allele increases the amount of energy allocated to reproduction, it will have the side effect of decreasing the amount of energy allocated to repair (Van Den Heuvel, English, and Uller 2016a).

Organisms have to balance the cost and benefits of either a) investing in high fertility at an early age ensuring to pass on as quickly as possible their genetic information, or b) they invest in maintaining the soma and living long enough to be able to have ample opportunity to mate (Thomas B.L. Kirkwood and Austad 2000). These two opposing strategies are based on a continuum across many species from

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those with short lives investing more intensely in early reproduction to those with longer lives and longer reproductive periods (Jones et al. 2014). However, the long-held view that senescence was expected to begin at the age of first reproduction has been overturned after the overmounting evidence of the diversity of senescence patterns across the tree of life (Cayuela et al. 2019; Trifonova et al. 2018). Some species display accelerated or rapid ageing, for instance, mayflies are insects with extremely short adult lifespans (few hours or days) (Brittain 1982), or killifish with roughly 16 weeks lifespan (Kim, Nam, and Valenzano 2016) or the pacific salmon (semelparous) senescing rapidly after reproducing (B. J. Crespi and Teo 2002). Other species show slow or negligible senescence and have an extended lifespan such as rougheye rockfish (up to 200 years) and the greenland shark (roughly 400 years) (Stenvinkel and Shiels 2019).

Although organisms may show variable forms of senescence, the most studied model organism in gerontology (e.g. *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*) have revealed conserved mechanistic pathways for ageing (Holtze et al. 2021), also referred to as the “Hallmarks of ageing”, encompassing genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication (López-Otín et al. 2013) and in a more updated review also disabled macroautophagy, chronic inflammation and dysbiosis (López-Otín et al. 2023) (Figure 1). There is currently much research underway trying to target mechanisms related to these cellular pathways, in the hope to either slow down or even revert the senescence rate and extend lifespan (Meron et al. 2022). For example, it has been observed across countless studies that decreasing food intake actually activates our survival circuit genes, promoting a range of beneficial health effects and prolonging the maximum lifespan (Fontana, Partridge, and Longo 2010; López-Otín et al. 2013). Given that organisms have evolved in constantly changing environments with periods of nutrient instability, it makes sense that nutrient-sensitive genes are highly conserved across the animal kingdom, as predicting future nutritional needs is crucial to survival and reproduction (Murphy 2023). Indeed, it is rational to consider the majority of “longevity” pathways as pathways primarily aimed at optimizing reproduction, and deciphering the regulation of lifespan requires a comprehensive grasp of its connection to reproductive processes. Exploring how these systems interact and the timing of their communication will have a profound impact on our strategies for developing interventions related to both reproduction and ageing (Murphy 2023).

Since ageing is a complex process with various facets, employing numerous model species can provide us with valuable insights into distinct aspects of the numerous ageing mechanisms. So far, ageing research has been predominantly biased to a few species (e.g., yeast, nematode worms, fruit flies or mice) due to well-known advantages, such as availability, short generation times, pre-existing information (e.g. well-annotated genomic and transcriptomic sequencing data), and well-regulated husbandry practices (Holtze et al. 2021; Mitchell et al. 2015, 20). However, narrowing our perspective to a few model species hampers our ability to fully understand the mechanisms governing ageing and their relationship with reproduction. For example, fish make up approximately half of all existing vertebrate species (over 20,000) and not only have evolved various forms of mating systems, sex roles,

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parental investments, but also have various forms of ageing from rapid, gradual, and negligible senescence (Holtze et al. 2021; Trifonova et al. 2018). They also make good comparative models with mammals as they have similar organs, have an adaptive immune system and may suffer similar old age pathologies as senescing mammals (Froehlich et al. 2013; D. N. Reznick, Ghalambor, and Nunney 2002). Nonetheless, while mammals and birds tend to have fairly constant fecundity across age groups, fish fecundity tends to increase with age as numerous species display indeterminate growth and thus can increase the number of eggs and offspring over time (Barneche, White, and Marshall 2018). Such factors might cause different life-history choices and thus some fish species may respond differently to typically targeted ageing pathways. So far, many fish species have not been investigated at all, and only a few have been used in gerontology studies primarily zebrafish, guppies, and killifish (Harel et al. 2015; Henriques et al. 2013; D. Reznick, Bryant, and Holmes 2006), leaving ample room for further research endeavours.

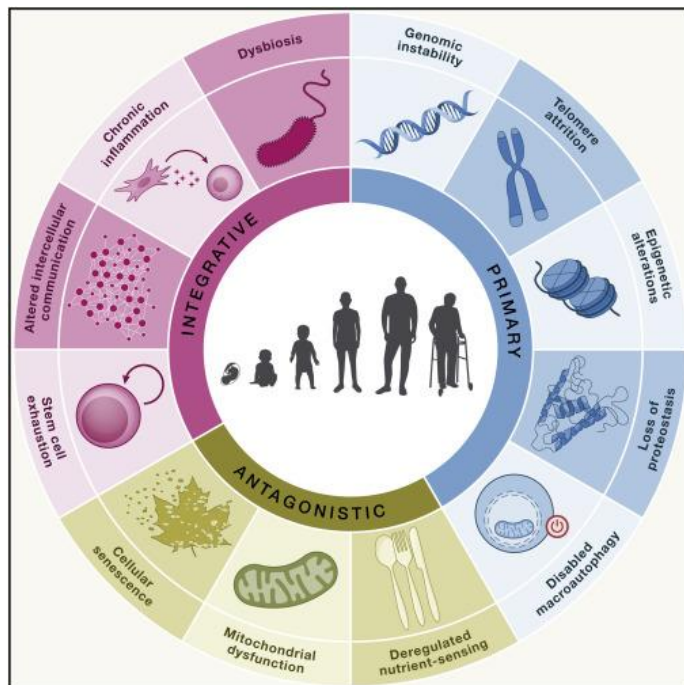


Figure 1: An illustration of the revisited Hallmarks of Ageing by López-Otín et al. 2023. Image used with copyright agreement (Elsevier 5650790181632).

Sexual dimorphism

Differences in senescence rates and lifespan vary not only across species but also between sex (Bronikowski et al. 2022; Jones et al. 2014). Although the mechanistic pathways for ageing (Hallmarks of Ageing), are thought to be conserved (López-Otín et al. 2013), there is accumulating evidence that ageing leads to sex-specific rates of senescence and age-related diseases (Vom Steeg and Klein 2016; Zuk 2009). Furthermore, rates of senescence in reproductive age varies between the sexes, with the ability to reproduce declining at different rates between males and females (Comizzoli and Ottinger

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2021). There are a number of species that exhibit female biased lifespans, including humans, a study looking at 101 mammal species, revealed that female's median lifespan is on average 18.6% longer than that of males, and in human females on average 7.8% longer (Lemaître et al. 2020). However, there are examples of some exhibiting male-biased lifespans or others lacking any sex-specific lifespan dimorphism. For instance, Brandt's bat and acorn woodpecker males are the longer lived sex (Clutton-Brock and Isvaran 2007; Podlutsky et al. 2005), and in the American beavers or the common dwarf mongoose lifespan is equivalent for both sexes (Clutton-Brock and Isvaran 2007).

Males and females frequently vary in a) genome composition and/or sex determination (e.g., sex chromosomes); b) physiological phenotype and life-history (e.g., hormonal milieu, adult sexual size dimorphism, reproductive investment); and c) ecological niches (e.g., breeding grounds, food availability) and environmental risk hazards (e.g., parasites, predators). There are numerous theories that have been put forward to explain the underlying mechanism that led to sex differences in ageing. Some examples include the asymmetric inheritance of mitochondria, imposing selection on mitochondrial genomes favouring female-specific needs (Gemmell, Metcalf, and Allendorf 2004), or the "unguarded X" hypothesis (Xirocostas, Everingham, and Moles 2020), which suggests that the sex with only one full sex chromosome (XY or ZW) might express more harmful genes being unable to compensate for deleterious alleles. Additionally, there is the "toxic Y" (or W) hypothesis (Bronikowski et al. 2022; Brown, Nguyen, and Bachtrog 2020), where the Y chromosome, rich in repetitive DNA, may accelerate ageing through misexpression with age. There is also the chromosome X (or Z) dosage compensation, where incomplete inactivation of the X chromosome leads to a biased expression of X-linked genes (Lucchesi and Kuroda 2015).

From an evolutionary standpoint, since males and females acquire fitness in different ways, divergent reproductive strategies may lead to different optima in lifetime. Maximum lifespan is affected by multiple loci, so alleles that are in the same loci can be selected in opposing directions in the two sexes, culminating in sexually antagonistic selection (SAC) and inter-locus sexual conflict (IRSC) (Bonduriansky et al. 2008; Maklakov and Lummaa 2013). This may have driven the evolution of genetically encoded differences in how energy is allocated between somatic maintenance and reproduction between males and females (T. B.L. Kirkwood and Rose 1991; Lemaître et al. 2020). In gonochoristic species, males (defined as sperm producers) and females (egg providers) invest in different traits, behaviours and reproductive demands which lead to life history trade-offs and distinct mortality risks (R. C. Brooks and Garratt 2017).

In general, females invest more energy per reproductive unit due to the size difference between eggs and sperm (anisogamy) (Clutton-Brock and Isvaran 2007). Consequently, female egg production is limited compared to males, who produce sperm in large quantities, meaning the longer a female lives, the more eggs she can produce, leading to a positive correlation between female fitness and longevity (G. C. Williams 1966). Conversely, male fitness is enhanced by increasing mating rates because sperm production is energetically cheaper (Bateman 1948; Pierotti and Clutton-Brock 1989). Additionally, in some species reproductive requirements of the female also include resource allocations for gestation,

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lactation and an overall higher parental investment (Lemaître et al. 2020; Shaw and Shaw 2009). In such light, females may be more reproductively constrained by the time they devote to their offspring and will favour investing in longevity and health span, compared to males with a lower burden of parental care (Clutton-Brock and Isvaran 2007).

On the other hand, males typically face higher costs associated with increased sexual competition, which involve maintaining conspicuous sexual traits (e.g., larger size, weapons, brighter colours) or riskier behaviours (e.g., territoriality and courtship), often resulting in a shorter adult lifespan (Clutton-Brock and Isvaran 2007; Lemaître et al. 2020). This concept aligns with one of the leading theories of ageing, suggesting that increased external mortality leads to accelerated senescence (P. D. Williams et al. 2006). Nonetheless, the strong selection imposed by sexual competition on male condition may favour alleles with positive effects on somatic maintenance and lifespan (Bonduriansky et al. 2008), with also mating success in males increasing with age and size, potentially favouring slower senescence (Graves 2007).

Furthermore, it is important to consider how sex-hormones (e.g., testosterone and oestrogen) play a pivotal role in regulating these distinct female and male traits. Hormones are pleiotropic, meaning they control multiple traits simultaneously (Flatt, Tu, and Tatar 2005). Since many fundamental aspects of the endocrine system are evolutionarily conserved (Cox, McGlothlin, and Bonier 2016), it is possible for sexual conflict to emerge regarding how sex-hormone production should govern the same genes in individuals of different sexes. For instance, oestrogens can act as antioxidants (Borrás et al. 2021) and promote telomere repair (Bayne et al. 2008), while testosterone can boost aggression, providing a competitive edge but also elevating extrinsic hazard risk (R. C. Brooks and Garratt 2017), and both can modulate differently the immune system (Taneja 2018).

A dimorphic immune response also contributes to sex-specific health and life-spans. Females mount a stronger immune response than males, but are also more susceptible to autoimmune diseases, potentially due to excessively reactive immune responses (Pennell, Galligan, and Fish 2012). The intricate balance of life-history trade-offs associated with parental care investment between sexes is likely to influence the heightened immune sensitivity required for improved survival chances for both parent and offspring (Figure 2). In species with pregnancies, heightened immune sensitivity could potentially result in miscarriages when the developing foetus is perceived as "foreign." This may favour a temporary reduction in female sensitivity during pregnancy rather than a permanent decrease over lifetime, potentially driving the development of adaptable immune responses (C. Jessica E. Metcalf and Graham 2018). In general, sex-dependent selection optimizes energy allocation for either somatic maintenance or reproduction, resulting in varying resource distribution for immune defence, metabolic needs, and overall mortality risk.

Although studies investigating the proximate and ultimate drivers of sex-lifespan dimorphism is growing, so far research has neglected to test how differences in sex-specific senescence could look in organisms with diverging or plastic biological sex and genders. In the vast and diverse realm of the animal kingdom, biological sex and sex roles are far from a rigid, exhibiting remarkable flexibility and

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adaptability in response to various environmental factors. One fascinating phenomenon that highlights this fluidity is sequential hermaphroditism, a strategy seen in many fish species (e.g., protogynous for female-to-male, protandrous for male-to-female, and serial bidirectional) (De Mitcheson and Liu 2008; Edgecombe et al. 2021). Furthermore, traditional sex roles in parenting are not always observed. For instance, in many bird, insect, amphibia and fish lineages, parental care responsibilities are shared more equitably between males and females, and in some cases, they exhibit complete sex-role reversals (Anders Berglund, Widemo, and Rosenqvist 2005; Houde 2001; Liker, Freckleton, and Székely 2013). In teleost fishes, male parental care is highly prevalent, taking on various forms, such as nest guarding, oral brooding, egg cleaning, and even male pregnancy, highlighting the adaptability and diversity of reproductive strategies in the animal kingdom (Blumer 1982; Smith and Wootton 1995). Investigating how resource allocation for somatic maintenance, reproductive strategies, and immune defence varies in organisms that diverge from conventional sex-roles could shed light on the trade-offs that influence sex-specific diseases and ageing patterns.

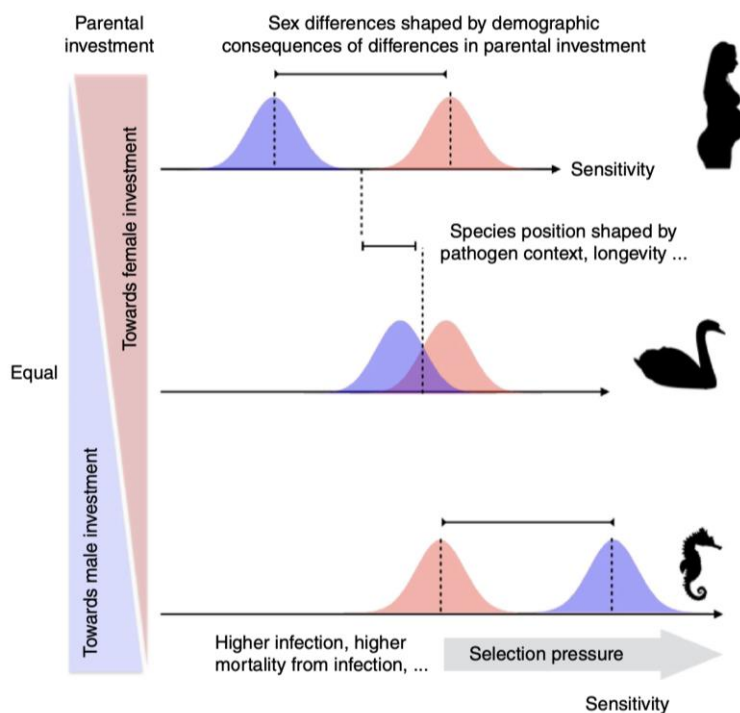


Figure 2: Projected associations between parental investment and sensitivity evolution of the immune system. Illustration taken from Metcalf and Graham 2018 (CC-BY). Enhanced parental investment by females (moving upward from the centre) or males (moving downward from the centre). In species where both sexes contribute equally to parenting (e.g., swans, middle), immune sensitivity is expected to be similar, with overlapping distributions near the centre. However, in species where females invest more in parenting (e.g., mammals like humans, top), females are likely to exhibit greater immune sensitivity, whereas the opposite pattern holds true for species where males invest more (e.g., seahorses, bottom).

Parental investment and trans-generational plasticity

Parent-offspring conflict emerges from disagreements regarding the allocation of resources, both in terms of energy and time (Trivers 1974). This parental investment varies based on parent sex, age, and genetic conflicts (Werren and Beukeboom 1998). Viviparity, or live-bearing, represents a high form of parental investment. It allows embryos to develop within a parent, offering protection from predators and harsh environments. Notably, viviparity has independently evolved on more than 150 occasions in vertebrates, spanning the taxonomic spectrum from fishes and amphibians to reptiles, and even occurring once in mammals (Blackburn 2015).

While examining the complex strategies of parental investment, including resource allocation and the costs associated with viviparity, it becomes evident that nature has devised a range of mechanisms to enhance the survival prospects of offspring. One such case is the capacity for trans-generational plasticity (TGP), a mechanism which happens whenever environmental signals encountered by one or both parents before fertilization lead to alterations in how offspring respond to their surroundings (Jennifer M. Donelson et al. 2018). This is not to be confused with phenotypic plasticity, which refers to the ability of an organism within its lifetime to exhibit varying physical traits in response to its specific environmental conditions (Price, Qvarnström, and Irwin 2003). This adaptability is significant because it allows the same genetic makeup (genotype) to produce advantageous physical traits when faced with rapid changes in the environment, enabling the organism to effectively deal with such fluctuations (Price, Qvarnström, and Irwin 2003). However, phenotypic plasticity gains particular significance when it extends over multiple generations, a phenomenon referred to as TGP. As an illustration, in the case of fish, when parents are exposed to elevated temperatures, it elevates the thermal resilience of their offspring (J. M. Donelson et al. 2012; Salinas and Munch 2012).

Given the heightened susceptibility of newly born offspring to environmental challenges, it is anticipated that parents across diverse taxa will adjust their offspring's traits based on the conditions in their local environment (Munday et al. 2013). These adjustments can be beneficial when the parental environment reliably predicts the offspring's conditions but can become disadvantageous in the event of abrupt environmental changes (i.e., environmental mismatch) (Burgess & Marshall 2014). An extreme example for this is illustrated by comparing individuals exposed to the Dutch Hunger Winter 1944-1945 with those born after, where parental and early-prenatal food scarcity followed by ample nutrition in adulthood possibly served as a trigger for chronic diseases in adults (Schulz 2010; Stein et al. 1975).

As we delve deeper into the intricate web of reproductive strategies, from the dynamics of parental investment, the physiological complexities of viviparity, to the adaptive mechanisms like TGP, we uncover a rich tapestry of factors shaping the traits and survival of offspring. These evolutionary strategies not only underscore the critical role parents play in preparing their offspring for the environment but also raise intriguing questions about how parental age might intersect with these mechanisms. As parents invest in the future through their reproductive choices, both maternal and paternal age effects can come into play, potentially influencing the fitness and adaptability of the next generation.

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As mentioned earlier, there are differences in reproductive investment between males and females of different species and there are trade-offs to consider when either parents decides to invest energy in their own offspring compared to themselves (B. Crespi and Semeniuk 2004). How much energy a father or a mother decided to put into rearing their offspring is often in a fine balance with what the environment can provide for them in nourishment and how physiologically healthy they are (Rödel et al. 2016). Advancing age is frequently correlated with a decrease in fertility and thus the quantity and quality of offspring (American Society for Reproductive Medicine 2014; Beamonte-Barrientos et al. 2010). Older mothers have been shown to induce more frequent chromosomal abnormalities offspring, resulting in spontaneous abortions and/or birth defects, also in humans (Descamps et al. 2008; Grande et al. 2012; Robinson, Mar, and Lummaa 2012). Markers measured in the mother such as oxidative stress or telomere length at birth, can affect the biological age of the offspring and such effects are strongest when resource availability is scarce (Van Den Heuvel, English, and Uller 2016b). Nevertheless, particularly in species where the young are not born precocial and are thus very vulnerable, having a young mother may lead to a higher offspring death rate (Kroeger et al. 2020). As she is physiologically and/or intellectually not mature enough to sustain a pregnancy or care adequately for her progeny, and may rather trade off her own somatic maintenance and survival against investing too much in her young, leading to higher mortality in her offspring (Kroeger et al. 2020).

Paternal age can also negatively affect the lifespan and health of offspring. Sperm quantity and quality can decline with age and an accumulation of germline mutations could reduce the mean fitness of older male offspring (Johnson et al. 2018; Monaghan, Maklakov, and Metcalfe 2020), and there may also be age-dependent changes in resources allocation to ejaculate (Hooper et al. 2018; Vega-Trejo et al. 2019). In some species having an older father has been shown to decrease the lifespan of his children and to induce more chronic illnesses (Arslan et al. 2017; Xie et al. 2018). Such effects have often been linked to genetic and molecular changes caused by ageing or due to environmental wear and tear (Fay et al. 2016). In spite of this reality, in some animal species, females are more attracted to bigger and older males, as their long survival in a hostile environment is proof of 'superior genes', which if passed on to their children will increase their average fitness (R. Brooks 2001; Vega-Trejo et al. 2019). Such mate preference may present certain advantages for her and her offspring, such as having a father who successfully protects the offspring from aggressors and/or provides higher quality paternal care, among other things (J. Lindström and H. Kokko 1996; Komers and Dhindsa 1989). Thus, choosing the right mate can be fundamental for the success of the following generation and such choice can vary depending on the respective reproductive trade-offs between males and females, and how senescence may act in that particular species.

It has nonetheless become evident that studies have predominantly centred on the female perspective due to the significant maternal investment in offspring. However, this focus presents a unique challenge: distinguishing the impacts of ageing on the physiological changes induced by pregnancy from those on an ageing germline. Furthermore, it is crucial to recognize that fertility reduction with increasing age, often observed in traditional animal models, does not universally apply across the animal kingdom. Intriguingly, species exhibiting indeterminate growth patterns may buck this trend, experiencing

heightened reproductive outputs as they age (Barneche, White, and Marshall 2018). To delve deeper into the complexities of somatic and reproductive ageing, it becomes imperative to broaden our research horizons. This entails exploring non-conventional model species, those featuring sex-role reversal or displaying varying rates of fertility throughout their lifespan. By doing so, we can unravel the intricacies of ageing's impact on both somatic and reproductive facets, shedding light on a more comprehensive understanding of the ageing process and its effects on progeny fitness and health.

Evolution of male pregnancy and sex-roles in Syngnathidae

Teleost fishes exhibit a remarkable range of variation in parental care, with approximately half of the existing species demonstrating exclusive paternal care (Gittlemann 1981; Gross and Sargent 1985). The Syngnathidae family, which includes seahorses, pipefish, and seadragons, is home to some of the most dedicated male caregivers, which have evolved a range of brooding forms analogous to those found in mammalian maternal systems; with the female transferring eggs to the male prior to fertilization (H. Hamilton et al. 2017; Stiller et al. 2022). Remarkably, the phenomenon of male pregnancy has its origins in a single evolutionary event, which then gave rise to approximately 300 distinct species of syngnathid (Figure 3A) (H. Hamilton et al. 2017).

Within this family, two distinct brooding strategies have evolved: trunk brooding (*Nerophinae*) and tail brooding (*Syngnathinae*) (Figure 3A) (H. Hamilton et al. 2017). The extent of paternal investment varies depending on the pouch morphology between different species, ranging from simple egg attachment to fully enclosed pouches (Figure 3B). For instance, *Syngnathus* has an inverted semi-sealed pouch (type 4, Figure 3B), while *Hippocampus* have evolved the most advanced sealed pouches (type 5, Figure 3B) (H. Hamilton et al. 2017; A. B. Wilson 2001). Besides safeguarding the embryos, the brooding structure serves multifunctional roles during pregnancy, including osmoregulation, gas exchange, aeration and in some more advanced species also nutrient provision (Stölting and Wilson, 2007; Whittington and Friesen, 2020).

On top of this extraordinary reproductive phenomenon, this colourful family of fish displays a range of sex roles from conventional (i.e., male-male competition and female choice) to reversed (i.e., female-female competition and male choice) or even plastic, and also mating systems including monogamy (one male mates only with one female, as in many seahorses), polygamy (multiple mating by both males and females, as in the genus *Syngnathus*) and polyandry (multiple mating by females, as in the genus *Nerophis*) (Rosenqvist and Berglund 2011; Anthony B. Wilson et al. 2003). In some species, males may experience prolonged gestational periods (can range from 9 days to 69 days depending on the species) (Sloman and Buckley 2011), and constraints on the size of the brood pouch (correlated to body size) and the energy invested (correlate to pouch type, Figure 3B) may predispose them to face limitations in their reproductive capacity compared to females (Anders Berglund, Widemo, and Rosenqvist 2005, 19; Rosenqvist and Berglund 2011). In these species, females typically use sexual signals for both mate attraction and female-female competition, while males may display temporary ornaments towards potential partners. For instance, in the sex-role reversed pipefish *Syngnathus typhle*, females will

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display temporary striped melanin-based patterns to attract mates and deter other females (Anders Berglund and Rosenqvist 2009). Both males and females exhibit a preference for larger mating partners, as reproductive success is linked to body size with larger females producing a greater number of larger eggs and males accommodating more offspring (A. Berglund, Rosenqvist, and Svensson 1986).

The Syngnathidae family can be found in a broad range of temperate and tropical environments, including rocky and coral reefs, sandy and silty seabeds, mangrove forests, seagrass beds, estuaries, and rivers (H. Hamilton et al. 2017). Among shallow-water fish, this family is notable for its exceptional biological diversity and the abundance of species it encompasses. The evolution of male pregnancy remains a captivating enigma, with the precise mechanisms and reasons behind it remaining a subject of ongoing exploration. One plausible hypothesis posits that paternal care could have arisen as a means to promote a more equitable sharing of the energetically demanding responsibilities of parental care between males and females. This cooperative effort could serve to alleviate the considerable time and energy burden placed on females, who typically must invest substantially in both the production of costly eggs and the subsequent protection and care of embryos (Janicke et al. 2016).

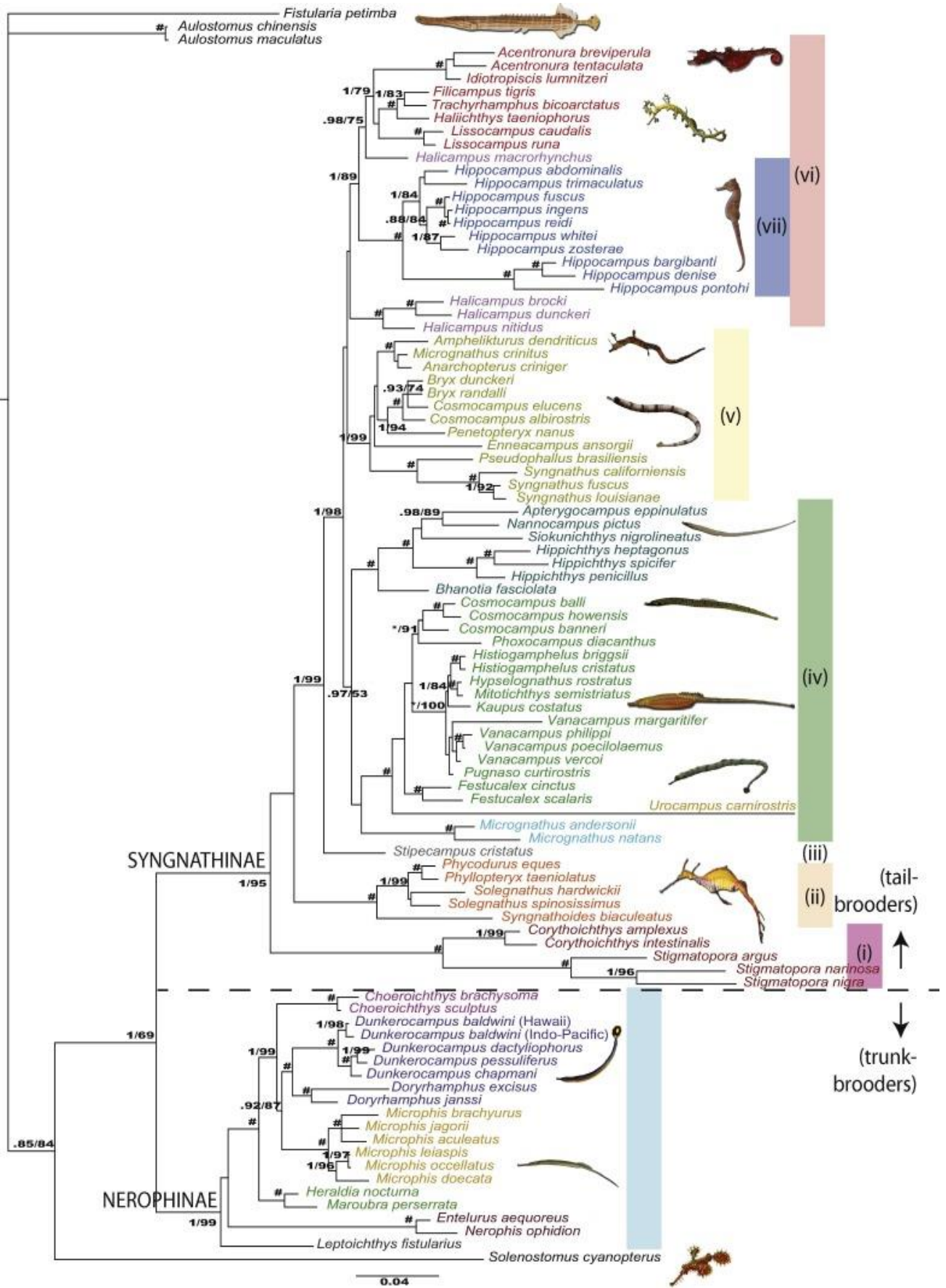
Additionally, in the realm of reproductive strategies, male pregnancy provides fathers with the opportunity to assert their paternity more effectively, thereby reducing the need for alternative strategies such as sperm competition or the formation of copulatory plugs (Stockley 2004; Sutter 2021). However, perhaps even more fascinating is the notion that the distinctive anatomical structures and genomic features of teleost may have played a pivotal role in enabling the evolution of male pregnancy and its diverse array of sexual roles. During the course of evolution, teleost have lost the müllerian duct, which has led to the development of reproductive structures entirely separate from the urinary system creating favourable conditions for physiological changes to occur (Adolfi et al. 2019). Teleost also possess distinct genetic characteristics, including whole-genome duplication (WGD) (Van De Peer, Maere, and Meyer 2009), possibly enhancing the capacity for the novel use of genes and remodelling of the immune system to facilitate pregnancy (Adolfi et al. 2019; Roth et al. 2020).

Species like seahorses and pipefish provide a unique opportunity to enhance our understanding of resource allocation dynamics across male and female reproductive strategies. Currently, the field of ageing research predominantly relies on a limited set of animal models, which not fully represent the diversity of resource allocation patterns and their connection to evolutionary theories of ageing. Disregarding the potential for evolutionary flexibility in diverse animals, which allows them to change or adapt over time based on shifts in environmental conditions and selective pressures, hinders our understanding of the intricate interplay between reproductive strategies and ageing mechanisms. Evolutionary innovations, such as the varying degrees of male pouch types, sex roles, mating systems, and sexual dimorphism among these species offer insights into the fundamental shift of resource allocation from reproduction to growth and maintenance. This approach allows us to explore resource allocation strategies and the intricacies of sex-specific senescence, revealing how different species adapt to varying life-history choices.

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Additionally, syngnathids propel us into a new research era aimed at distinguishing biological sex from gender roles and understanding the associated costs and trade-offs in resource allocation. They also serve as a valuable avenue to investigate trans-generational plasticity, an essential aspect of evolutionary biology often overshadowed by maternal-centric studies. This focus on maternal investment presents a unique challenge: distinguishing the effects of ageing on physiological changes induced by pregnancy from those on an ageing germline. Furthermore, while traditional models typically exhibit consistent fertility reduction with age, the Syngnathidae family features exceptions. Some species display indeterminate growth patterns, resulting in heightened reproductive outputs as they age, challenging established paradigms and underscoring the importance of exploring non-conventional model species (Barneche, White, and Marshall 2018). In essence, this family provides a valuable lens through which to explore the complex interplay of ageing and reproductive strategies, pushing the boundaries of our understanding of these fundamental biological processes.

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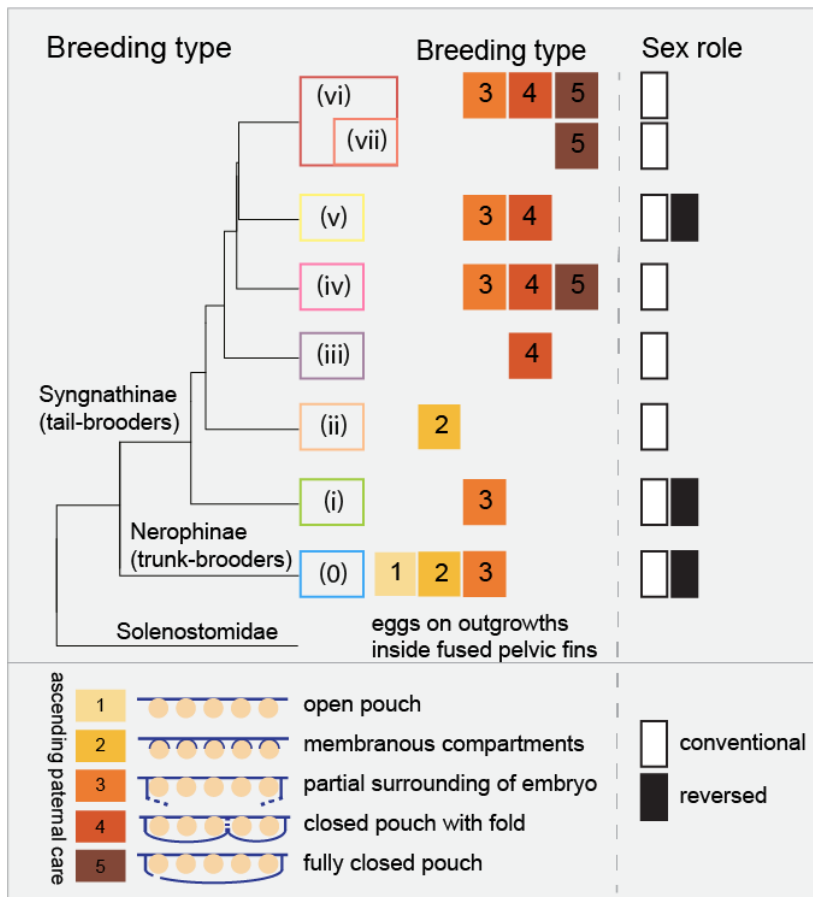


Figure 3: A) The phylogeny tree is taken from H. Hamilton et al. 2017 (copyright agreement Elsevier 5677060823770), showing the evolution of male pregnancy and the split off between *Nerophinae* and *Syngnathinae*. B) This diagram is also taken from Hamilton *et al* (2017) but has been adapted by Olivia Roth and Astrid Böhne, illustrating the various types of pouches involved in the evolution of paternal care, male pregnancy and the reversal of traditional sex roles.

THESIS OUTLINE

Sex roles, parental investment and reproductive strategies are fundamental aspects of animal biology, influencing various life history traits across species. Understanding these dynamics is crucial for comprehending the complexities of ageing and lifespan dimorphism, as they are tightly interconnected. However, current research predominantly relies on model species with conventional reproductive roles, limiting our insights into the broader spectrum of reproductive strategies found in nature.

This thesis aims to bridge the existing knowledge gaps by investigating the influence of reproductive strategies, parental age, and resource allocation on gene expression, development, and life history traits in non-model species. The lined seahorse and broadnosed pipefish, serve as primary study organisms, providing unique insights into the intricacies of reproductive biology and its implications for sex-specific trade-offs in resource allocation and life history.

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

Aim: To investigate how caloric restriction (CR) affects the growth, brood pouch formation, gene expression, and microbial composition in male and female lined seahorses, providing critical insights into resource allocation strategies in a species with male pregnancy.

This work helps to expand our understanding of resource allocation dynamics in the context of male pregnancy and unconventional reproductive roles. It also advances current knowledge by elucidating the trade-offs between investing in reproduction or longevity and highlighting the critical link between nutrient intake and reproductive strategies. These findings can inform future conservation efforts and provide insights into the sex-dimorphic responses to caloric restriction based on reproductive strategies.

Chapter II – Navigating sex and sex-roles: deciphering sex-biased gene expression in a species with sex-role reversal (*Syngnathus typhle*)

Aim: To explore sex-biased gene expression patterns in male and female broadnosed pipefish, highlighting the unique aspects of sex-role reversal and its implications for reproductive strategies and gene regulation.

This chapter advances current research by distinguishing between biological sex (egg or sperm producer) and sex-role (parental care investment and/or mate competition), shedding light on key factors that drive sex-biased gene expression. Understanding the context of sex-biased gene expression in relation to both gender context and sex-determination context is crucial for future research in this field, as it provides essential insights on the development of targeted treatments or medications based on gender and sex-related factors.

Chapter III - The effect of parental age on the quantity and quality of offspring in *Syngnathus typhle*, a species with male pregnancy

Aim: To examine the role of parental reproductive age in shaping pregnancy duration, offspring characteristics, and differential gene expression in offspring, emphasizing its relevance to reproductive strategies and ageing.

This research advances current understanding by addressing the understudied aspects of parental age effects in sex-role reversed species, offering insights into the complex interplay between parental age, resource allocation, and transgenerational phenotypic plasticity.

CHAPTER I

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

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ABSTRACT

Caloric restriction (CR) studies have traditionally focused on species with conventional reproductive roles, emphasizing females greater investment in costly gametes and parental care. The divergent impact of CR on males and females is evident across species, the factors driving this variation, i.e., resource allocation in reproductive elements vs. distinct life histories remain unclear. To address this, we investigated the effects of CR on growth, gene expression, and intestinal microbiota in the lined seahorse *Hippocampus erectus*, a species with male pregnancy and unique life history. Juvenile seahorses were subjected to ad libitum (AL) or CR for 5 months. CR stunted male (but not female) growth and brood pouch development, reflecting the energy demands associated with pouch development and parental care. Transcriptome analysis demonstrated organ- and sex-specific responses to CR with distinct lipid and energy-related pathways activated in males and female livers, indicative of survival enhancement strategies. Fasting had minimal impact on spermatogenesis, but downregulated lipid metabolic and inflammatory genes in ovaries, emphasizing the importance of pre-copulatory resource allocation in female gametes. While microbial diversity increased with fasting, no discernible sex-specific effect on hind-gut microbiota was identified. Our research indicates that nutrient limitation's impact on males and females is influenced by their allocation of resources to reproduction and parental investment, rather than being solely determined by biological sex. We underscore the significance of studying species with diverse reproductive strategies, sex roles, mating systems, and life-history strategies to understand the sex-specific effects of caloric restriction.

Keywords: caloric restriction, resource allocation, life-history, seahorse, male pregnancy

1. INTRODUCTION

Caloric restriction (CR) influences numerous signaling pathways involved in growth, metabolism, oxidative stress response, damage repair, inflammation, autophagy, and proteostasis, thereby shaping the aging process and yielding positive physiological outcomes. The latter encompass improved

cardiovascular health, reduced oxidative damage, enhanced DNA repair, promotion of autophagy, and mitigating gut dysbiosis (1–3). Lifelong CR increases lifespan in a range of species, including yeast (4), nematodes (5), fruit flies (6), and rodents (3,7). Rejuvenating properties were suggested for mice, rhesus monkey and humans fed on calorie-restricted diets who had DNA methylation patterns equal to their younger counterparts (8), while the proinflammatory gut of aged mice was reversed to a younger healthier microbial composition after two months of CR (9). The gut microbiome plays a crucial role in various aspects of 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 (10).

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 (11). According to Kirkwood's disposable soma theory, longevity requires investing in somatic maintenance reducing the resources available for reproduction (12). When animals lack sufficient energy for reproduction, they prioritize extending their lifespan until resources become abundant again, at which point they resume breeding (13). This strategy aims to maximize overall reproductive success (14). Some species naturally have long periods of fasting during hibernation periods when food is scarce, including many reptiles and fish (15,16). However, species with long lifespans, high caloric diets and high reproductive outputs, such as queen bees, ants and termites challenge this theory (17,18). If done excessively, CR can ultimately have negative health impact in humans (e.g. extreme weight loss, lethargy, diminished libido etc.) (3,14,19,20).

Female mice and fruit flies were suggested to respond better to CR with respects to increased health- and lifespan than their male counterparts (21,22). Fasting further extended lifespan of males, but not in female killifish, potentially facilitated by sex-specific expression changes in liver tissue (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 (20), variations in sex steroids (24), adiponectin/leptin levels, immune function, and body fat distribution have been proposed (21,25). Distinct reproductive strategies and specific energy allocation may serve as ultimate drivers explaining sex-specific responses to fasting. Females invest 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 parental care, such as pregnancy and lactation in mammals (27,28). As females are typically the limiting sex due to costly egg production and 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 flexibility across species (26,31,32), leaving a gap in our

understanding regarding whether the sex-specific effects of fasting originate from distinct resource allocation to reproductive elements or from distinct life history strategies. Understanding the ultimate drivers of sexual dimorphism in the effects of CR on health across different species holds significant implications for various fields and aspects of human health. This knowledge can offer valuable insights for CR interventions in sex- and gender-specific health or the development of CR mimetics, potentially revolutionizing approaches to enhance well-being and longevity (33).

To bridge the existing research gap, we designed an experiment focusing on how CR, implemented through intermittent fasting (IF), affects development, gut microbiota and aging pathways in a species with male pregnancy, the lined seahorse *Hippocampus erectus*. In this species the female transfers 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 (34,35). Their unique reproductive biology allows us to disentangle the cost of investment into the brooding structure (male) vs. investment into egg production (female), 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. In our study we took 52 three-month-old juvenile (not sexually mature) seahorses, which were divided equally into two feeding groups: Ad libitum (AL) and calorie-restricted (CR). The AL group was fed twice a day, while the CR group was fed every-other-day, resulting in a calorie restriction 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 at each food provisioning (36). This avoids any negative effects of overeating in the AL and ensures us that the fasting feeding group is truly undergoing a restriction in calories.

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 assess egg production. Using RNA sequencing we analyzed transcriptome-wide differential gene expression from liver, head kidney, and gonads, between AL and CR groups, and comparing male vs. female. We also sampled hind-gut to genotype microbial composition using 16S rRNA amplicon sequencing to investigate potential changes induced by fasting. In parallel to the main experiment, we also sacrificed four-month-old and three-year-old seahorses, both males and females, to compare potential rejuvenation or senescence effects of CR and AL treatments, providing insights into the hypothetical long-term effects of CR on aging and overall health.

2. MATERIAL AND METHODS

2.1 Study organism and design

Chapter I

Fifty-two three-month-old juveniles *Hippocampus erectus* from an aquarium bred 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 a CR regime. The tanks were divided into 9 AL and 9 CR, with alternating tanks placed next to each other to reduce tank location effects. 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 (8am) and in the late afternoon (4pm); 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. 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 reduce bacterial diseases in fish (37)), 15 µl of JBL Atvitrol 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 CR 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 CR 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). Additionally, female ovaries were weighed and the width and length of pouch were measured for 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.

To assess how CR influences aging compared to the AL treatment, we also took four-month-old juveniles labelled as control young (CY), and three-year-old adults labelled as control old (CO) that had been kept in the same 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 three tissue types.

To extract the microbiota from the hind-gut, DNA extraction was performed using the DNeasy Blood & Tissue Kit (QIAGEN, Germany) following the manufacturer's protocol including a pre-treatment for Gram-positive bacteria with ameliorations from (38). 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 dietary experiment with an additionally 10 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). Amplicon sequencing 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 Kiel.

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 (day 157). Among them, the survivors encompassed 16 individuals belonging to the AL group with 11 AL males and 5 AL females, while 22 individuals belonged to the CR group with 11 CR males, and 11 CR females. It is worth noting that at the beginning of the experiment

we could not divide the seahorses by sex as they were not sexually mature, resulting in a smaller number of AL females compared to the other groups.

For the purpose of the morphology analysis, we included only the 38 individuals which made it to the end of the experiment, with an additional separate analysis comparing the diet groups to the young and old age groups of seahorses (5 CY, 5 CO both sexes). To investigate the effect of treatment (AL, CR) and separately also group (AL, CR, CO, CY), on male's and female's weight (in grams [g]) and total body length (in centimetres [cm]), statistical analysis was done in Rstudio (v.4.2.2) (39), 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 for both weight and length. Following the rejection of the Kruskal-Wallis test, we performed 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 fasting effects were characterized by constructing differential expression signatures associated with each organ. 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) (40). The adapters had already been trimmed by BGI Tech Solutions. Regrettably, during the run, six samples failed, all of which were ovarian tissue (two AL, one CR, two OC, and one YC). For the alignment process, we used STAR (v.2.7.9)a (41) to map the reads to a genome assembly of *Hippocampus erectus* (BioProject: PRJNA347499). Subsequently, read counts were obtained using TPMCalculator (42). In order to investigate potential distinctions among different groups (AL, CR, CO, CY) within specific organs, we partitioned the dataset by organ type. 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, head kidney, ovaries and testes. We did a principal component analysis (PCA) with multiple PCs 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) (43), 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. We performed this process separately for each organ, resulting in different gene sets for each: 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) (44) 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 (45). 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 and corrected for multiple testing.

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) (46). 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 (47). 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 (48). 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 OTUs 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) (49). 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 ($x \sim \text{diet}*\text{sex}$, $\text{data}=\text{data}$, $\text{permutations}=999$), preceded by testing for normality with a Shapiro-Wilk normality test. To compare groups, pairwise.adonis2 v0.4 (50) 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 ($p.\text{val} < 0.001$).

Relative abundance was calculated for the phylum and genus taxonomic level (Figure S3E-F and also Figure 4A-B). 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 S3F). Despite *Vibrio* prevalence not showing significant differences across treatment (Fig. S3F) 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 analyses one including the genus *Vibrio* with results in supplementary material (Fig. S3A-F) and another analysis excluding this OTU (Fig. 4A-F). Following the removal, the data was re-normalized, and analysis and hypothesis testing repeated.

3. RESULTS

3.1 Calorie restriction reduces growth

The reduced caloric intake 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 $p = \text{NS}$). 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 $\chi^2 = 26.625$, Fig. 1B) and length (AL 13.75 ± 1.27 cm vs. CR 10.76 ± 0.37 cm, adj.p.val < 0.002 with $\chi^2 = 22.524$, 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 length (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 dietary 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. 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 thickness (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).

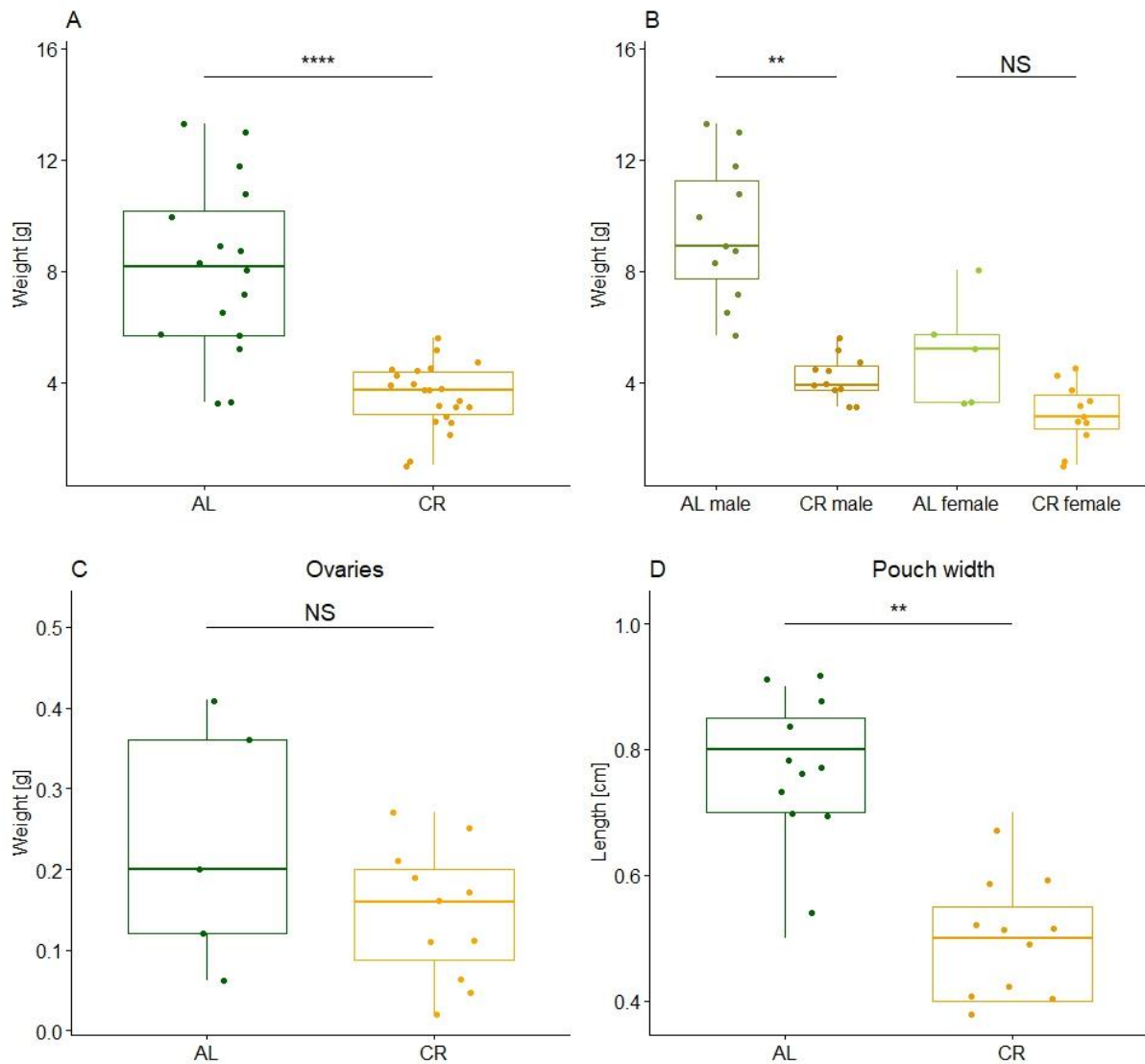


Figure 1: Boxplots depict morphological measurements of weight [g] on the y-axis, obtained at the end of a five-month diet treatment (day 157). The x-axis is divided into treatment groups: (A) showcasing weight differences between AL (16 individuals) and CR (22 individuals); (B) represents weight differences 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) shows difference in pouch width. Significance thresholds are indicated with asterisks (Kruskal-Wallis; ** < 0.001 and *** < 0.0001) to emphasize differences between treatment effects and within sex groups.

3.2 Fasting exerts stronger effects on female seahorse liver and gonads

The results from the statistical analysis on DGE revealed the strongest effects of the CR treatment in the liver (PERMANOVA; $p < 0.001$) compared to all other organs. This holds true after correcting for multiple testing with AL vs. CR showing an adj.p.val of 0.012 (Table S6). However, the interaction of dietary treatment and sex was not significant (Table S6). While we did find a strong effect of sex in the head kidney (PERMANOVA; $p < 0.001$), the effects of CR were only marginally significant in the liver (PERMANOVA; $p = 0.035$), and after Bonferroni correction there was no significant difference between

AL and CR seahorses (Table S6). In the gonads, we found no significant effects between the treatment groups in testes, compared to a significant difference in ovaries (PERMANOVA; $p < 0.001$) (full stats Table S6).

We were further interested in identifying potential organ senescence or rejuvenation patterns between the dietary treatment groups (AL and CR) and younger (CY) or older (CO) seahorses. Overall, we found that in liver all pairwise comparisons showed significant differences (adj.p.val < 0.006 , Table S6), likely reflecting the variation in this metabolic organ (Fig. 2). In the head kidney we found a marginal difference between AL vs. CO (adj.p.val < 0.05 , Table S6) compared to CR vs. CO (adj.p.val < 0.03 , Table S6), indicating potentially a stronger similarity between non-fasted seahorses and older seahorses. However, the same similarity was not reflected upon comparison of CR vs. CY (adj.p.val < 0.006 , Table S6), implying that head kidney did not show a potential rejuvenation upon fasting. The comparisons 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 comparing the dietary and control age groups can be found in the supplementary material in Table S1.

A PCA showed that the first PC reflected the different organs (see Fig. S2), PC4 reflects the differences between the dietary groups (AL and CR) and age groups (CO and CY), while PC5 separates CR and CY from AL and CO (Fig. 2). These differences are most clearly visible for the liver (Fig. 2). Significant results of the DGE contrast comparisons (limma:voom) between all interaction variables (e.g., AL vs. CO, CR vs. CY, etc.) are found in the supplementary material Table S1. The limma:voom DGE analysis revealed a high 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, Table S1). Female liver had 375 DEGs compared to 83 DEGs in male livers (adj.p.val < 0.05 , Fig. 3) (Table S1). In the gonads we found no DEGs between AL and CR in testes, but 366 in ovaries (Table S1). In the head kidney the DGE analysis did not reveal any significantly DEG in females and four not annotated genes in males supporting the non-significant statistical testing of fasting (Table S1). This 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.

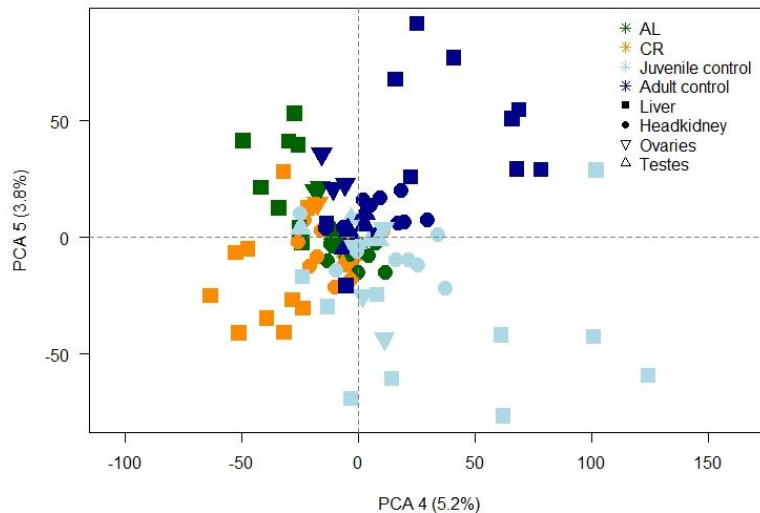


Figure 2: Principal Component Analysis (PCA) plots depicting organ separation and clustering of treatment groups. This particular PCA 4 and 5 depicts grouping of samples based on diet treatment and age groups (PCA 1 and 2 are in supplementary material Figure S2). The groups are color-coded as follows: CR (orange), AL (green), juvenile control (light blue), and adult control (dark blue).

3.3 Sex-specific fasting effects on liver energy and lipid metabolism

Gene enrichment analysis revealed that in the liver of females the top most significantly enriched pathways from GO:BP were sterol biosynthetic process and cholesterol biosynthetic process, for KEGG steroid biosynthesis and metabolic pathways, and for REAC also cholesterol biosynthesis and metabolism of steroids (Table S2, sheet A). A more in-depth comparison of the most significantly DEG ($\log_{2}FC \pm 1$, $\text{adj.p.val} < 0.05$) from these pathways that were either upregulated in AL or CR (and vice versa) is depicted in Figure 3 (and Table S3). Genes that were upregulated in CR females included cytochrome P450 family 51 (cyp51) crucial for the synthesis of cholesterol (51), squalene epoxidase (sqle) an enzyme important also in cholesterol synthesis (52), mevalonate kinase (mvk) important in sterol synthesis (53), hormone-sensitive lipase (lipe) hydrolyses triglycerides helping provide free fatty acids, and lipase hepatic A (lipca) involved in facilitating lipoprotein uptake from the cell (54).

Furthermore, other genes involved in ATP production and combating oxidative stress were upregulated by CR in the female liver, such as isocitrate dehydrogenase (NADP+) 2 (idh2), known for its importance in oxidative stress response and glucose sensing (55), or also peroxiredoxin 4 (prdx4) and peroxiredoxin 6 (prdx6) (56). Interestingly, autophagy-related 13 homolog (atg13) and acetoacetyl-CoA synthetase (aacs) (enzyme involved in degradation of ketone bodies) were also upregulated in CR, both play an important role in survival during nutrient depletion (57,58).

Downregulated genes in the liver of fasted females pertaining to metabolic pathways were particularly related to steroid synthesis, such as cytochrome P450 family 17 subfamily A (cyp17A2) important in synthesising progesterone and the progesterone receptor (pgr) itself androstenedione (59). Also noteworthy was the downregulation of STEAP family member 3 (steap3) and erythropoietin receptor

(epor) which could be synergistic, as the first has ferrireductase activity meaning it can reduce Fe³⁺ (ferric iron) to Fe²⁺ (ferrous iron) (60), and the latter regulates blood cell production (61).

Conversely, the DEGs in the liver of male seahorses showed that the top most enriched pathways were all related to energy production such as ATP biosynthetic process (GO:BP), Oxidative phosphorylation (KEGG); Mitochondrial biogenesis (REAC) (Table S2, sheet B). Although there were fewer genes affected by CR in the liver of males compared to females, the vast majority of those were upregulated in fasted seahorses (Fig. 3 and Table S3). The few CR downregulated genes belonging to these pathways were Coenzyme Q8A genome duplicate b (coq8ab), involved in coenzyme Q biosynthesis, which is essential for mitochondrial electron transport chain and energy production, and beta-carotene oxygenase 1 (bco11), playing a role in the cleavage of beta-carotene into retinal, a vital precursor of vitamin A (62). Some upregulated genes were the same as for female seahorses, like isocitrate dehydrogenase (NADP(+)) 2 (idh2) and lipase hepatic A (lipca) (Fig. 3). However, CR male seahorses seemed to react more acutely to glucose levels with upregulated genes such as lactate dehydrogenase D (ldhd), known for its role in converting pyruvate to lactate and oxidizing NADH to NAD⁺ at the end of glycolysis, fetuin B (fetub) a glycoprotein known to impair glucose metabolism and induce a form of insulin resistance (63). Other upregulated genes involved in regulating oxidative metabolism were thimet oligopeptidase (thop1) shown to regulate energy metabolism, with knockout thop1 mice displaying improvements in lipolysis and increased oxidative metabolism (64), and selenoprotein P (selenop2), one of numerous selenoproteins predominantly involved in oxidoreductase reactions (65). Interestingly, we also found significantly DEGs involved in regulating the circadian rhythm being upregulated in the liver of CR male seahorses. These included clock circadian regulator b (clockb) and cryptochrome circadian regulator 3 (cry3a). 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 acetyltransferase (HAT) activity (HAT function of CLOCK may be controlled by changing cell energy levels (66). 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 (67).

3.4 Enhanced anti-inflammatory response in ovaries

No significantly DEGs were found in the testes between AL and CR treatments, compared to ovaries which appeared far more affected through the diet with 356 being downregulated under CR and only 10 upregulated (Fig. 3). Enrichment analysis showed that the top pathways were lipid catabolic process for GO:BP, lysosome for KEGG, and innate immune system for REAC (Table S2, sheet C). Among genes pertaining to the lipid catabolic process, we found several involved in ATPase H⁺ transporting V1 and V0 (atp6v1f, atp6v1e1b, atp6v0cb) (Fig. 3 and Table S2). Furthermore, there were also several nutrient-sensing-related genes, which are crucial for regulating longevity pathways DE. Forkhead box O1 a (foxo1a) was downregulated in CR female ovaries, FOXO is a 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 (68). Also sestrin 2 (*sesn2*) was downregulated, which ultimately activates mTORC1 (69). Similarly, TELO 2 interacting protein 2 (*tli2*) was downregulated in CR, which binds to mTORC1, stimulating the downstream cascade for cell growth (70). These downregulated genes in CR equate to them being upregulated in AL female ovaries, implying that ovaries of AL females are more prone to having pathways activated for growth and cell proliferation. In such light AL female ovaries showed upregulation of claudin 7 (*cldn7b*), involved in controlling solute transfer across cell monolayers and possibly playing a role in cancer cell survival, growth, and invasion (71), and Rhophilin Rho GTPase binding protein 2 (*rhpn2*) acting as a scaffold protein with PDZ and Bro1 domains, participating in protein complex assembly, intracellular signalling, and endosome targeting (72).

Two acyl-CoA synthetase (ACS) enzymes related to lipid metabolic regulation, ACS bubble gum (*acsbg*) and long-chain ACS (*acsl*), were downregulated in the ovaries of 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 (73,74). In fact, all genes pertaining to the innate immune pathway were downregulated in the ovaries of CR females (Fig. 3 and Table S3 sheet D). These included, cytokine-like interleukin 12 receptor beta 2a (*ilrb2a*) playing a crucial role in controlling cell-mediated immunity by stimulating the production of interferon (IFN)- γ from T cells and natural killer cells (75); M17 a subfamily cytokine with similarities to mammalian IL-6, is known to induce the proliferation and activation of macrophages in goldfish (76); macrophage expressed 1 tandem duplicate 2 (*mpeg1.2*), this gene is induced by infection and has anti-bacterial functions (77); interferon regulatory factor 5 (*irf5*) playing a role in both antiviral and inflammatory responses (78); two chemokine receptors (*cxcr4a*, *cmklr1*) as well as a tumor necrosis factor (*tnf*), all of which are important in the inflammatory response (79). Furthermore, toll-like receptors (TLRs) 21 and 22 were downregulated, which are pattern-recognition receptors 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- κ B and MAPK inflammatory pathways (80,81).

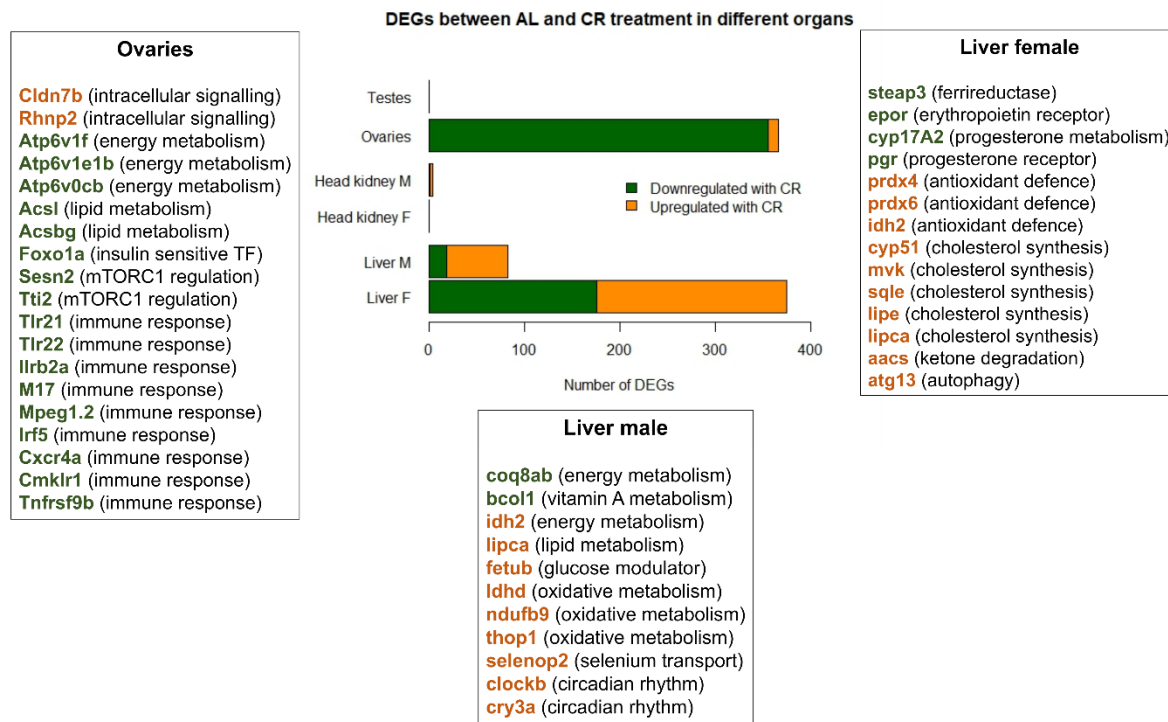


Figure 3: Barplot depicting DEGs between AL and CR-fed seahorses in different organs, categorized by sex (M = male and F = female). Upregulated genes in AL (downregulated in CR) are shown in green, while upregulated in CR (downregulated genes in AL) are shown in orange. Relevant DEGs with $\log_{2}FC \pm 1$ and $adj.p.val < 0.05$ discussed in the text are indicated in surrounding boxes. Supplementary material TableS3 provides an overview of the DEGs (with *Danio rerio* orthologues) with additional details.

3.5 Calorie restriction promotes greater gut microbial diversity

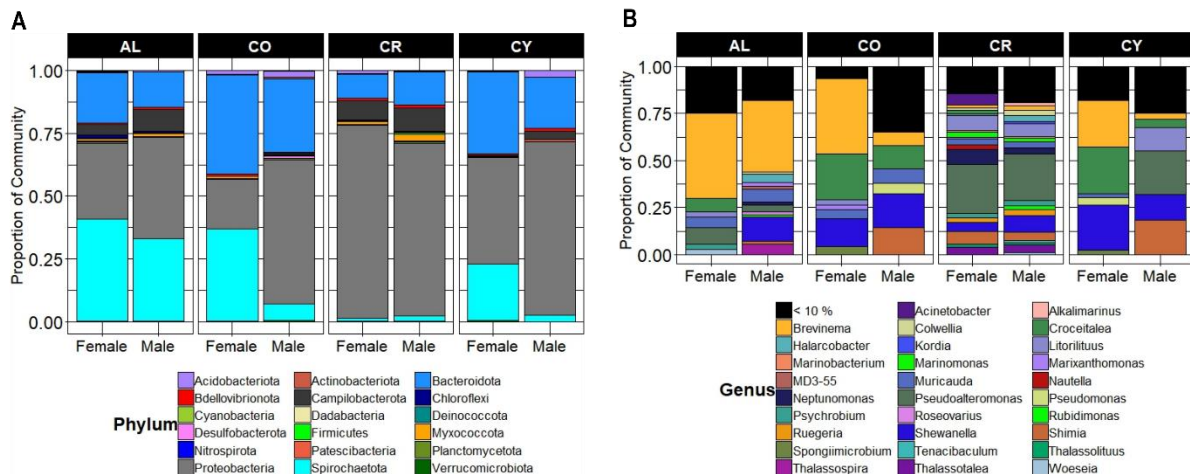
In the analysis of microbial 16S rRNAseq in seahorse hind-gut tissue – excluding here the *Vibrio*-inclusive dataset presented in the supplementary analysis (Fig. S3) - we uncovered a remarkably diverse and abundant bacterial community (genus level) in the fasted seahorses (Figs. 4B), with the most significant differences between the CR group and all others (AL, CY and CO; $adj.p.val < 0.2$), as demonstrated also in the α -diversity Shannon index analysis (Fig. 4D, Table S6). The AL group showed greater variation among individuals, with Shannon index values ranging from near zero (indicating few dominant taxa) to values nearing four (indicating higher diversity with several coexisting species) (Figs. 4C-D). Permanova analysis of α -diversity of only the treatment groups (CR and AL) and sex revealed a very strong dietary effect ($p.val < 0.007$), but no significant effect of sex or the interaction (Fig. 4C, Table S6).

Consistent findings were mirrored in the β -diversity analysis, encompassing similar results for both Bray Curtis and Jaccard metrics (refer to Table S6, only Bray Curtis figures are displayed below in Figs. 4E-F). Notably, CR individuals formed tighter clusters in comparison to all other groups (Fig. 4F), emerging as the most significantly distinct group (CR vs. AL or CY or CO, with adjusted $p.val < 0.006$). Even when

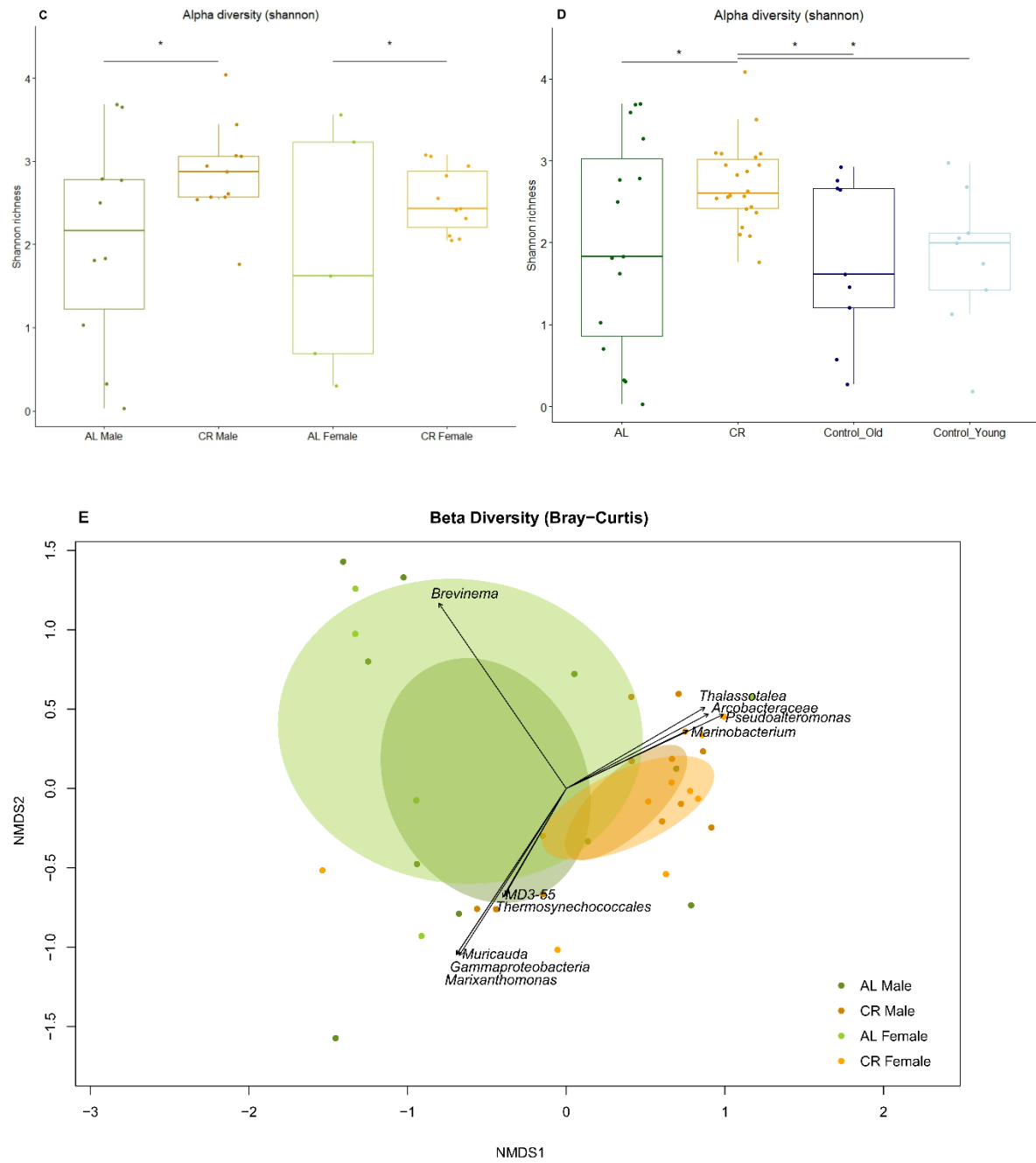
subjected to a permanova incorporating treatment groups (CR and AL) along with sex, the predominant effect persisted between CR and AL ($p.val < 0.001$), while significance remained elusive for sex or their interaction (Fig. 4E, Table S6). It is worth noting that despite discernible differences in relative abundance between males and females, as detailed in Fig. 4B, these variations did not attain statistical significance in the present analysis.

At the taxonomic phylum level, the dominant phyla in the CR seahorse gut microbiota were *Proteobacteria* (77% in females, 69% in males), followed by *Bacteroidota* (9% and 13%) and *Campilobacterota* (8% and 9%) (Fig. 4A). This differed from the AL seahorse gut microbiota, where *Spirochaetota* was the dominant phylum (41% and 33% in females and males, respectively) (Fig. 4A), followed by *Proteobacteria* (30% and 40%), *Bacteroidota* (20% and 14%), and *Campilobacterota* (4% and 9%). In CR seahorses, *Spirochaetota* (1% and 2%) was nearly absent, with *Proteobacteria* becoming the dominant phylum.

Genus-level analysis revealed that the dominant genus for *Spirochaetota* was *Brevinema* genus (family *Brevinemataceae*) (Fig. 4B). β -diversity NMDS plots, comparing dietary groups in Fig. 4E and treatment groups (AL, CR) and age groups (CO, CY) in Fig. 4F, indicated a strong association of the *Brevinema* with AL seahorses ($p.val < 0.001$, Table S6). While the gut of AL seahorses was mostly dominated exclusively by *Brevinema* (45% in females, 38% in males), the gut of CR seahorses exhibited a more diverse community (Fig. 4B), with some of the highest relative abundance belonging to *Proteobacteria*, such as *Pseudoalteromonas*, *Neptunomonas*, *Litorilittus*, *Shewanella*, *Marinobacterium*, *Marinomonas*, and *Thalassotalea* (Fig. 4B, 4E, and 4F). The gut microbial composition of the control age groups exhibited greater similarities between each other compared to the AL or CR dietary treatment groups, making a statement about the possibility of a rejuvenating or aging gut unlikely. Furthermore, both CO and CY displayed lower taxa diversity, featuring only a few dominant taxa that remained consistent across age groups but had distinct relative abundances between males and females (Fig. 4B).



Chapter I



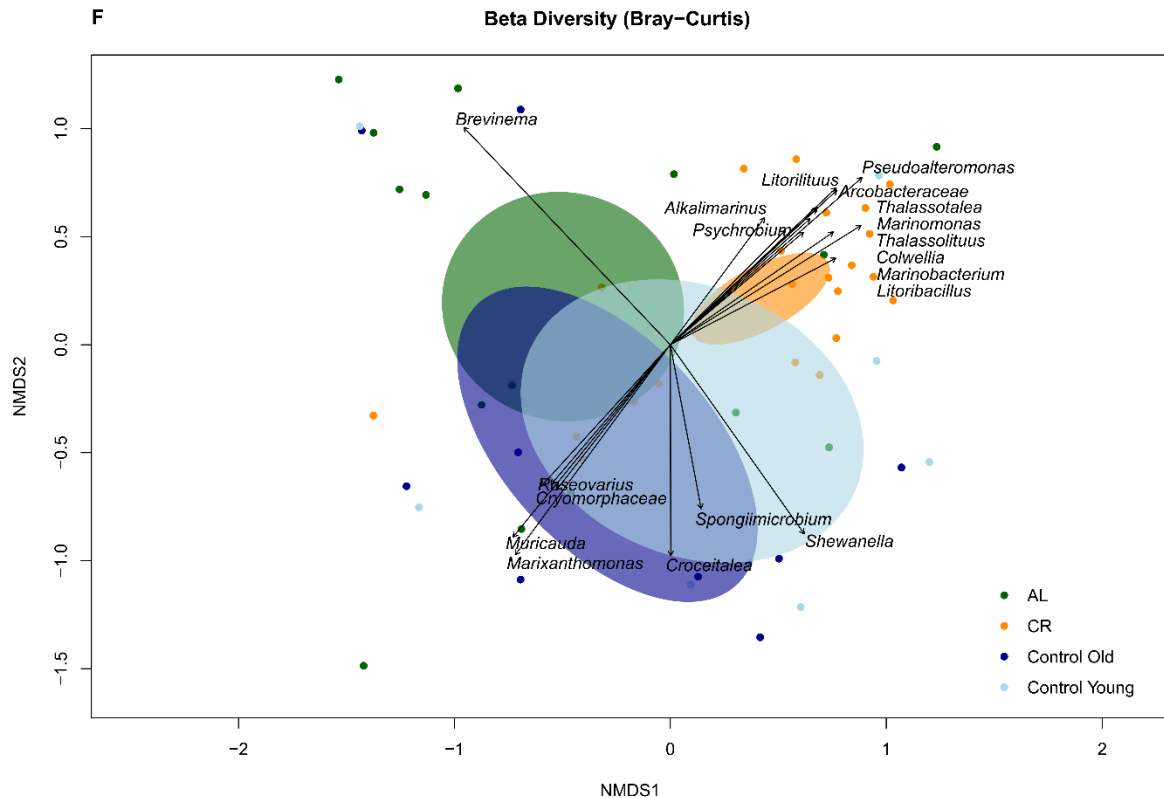


Figure 4: Microbial biodiversity analysis from hind gut tissue. A) Barplot with relative abundance of microbial taxa at the phylum taxonomic level. The panels are categorized based on diet, with AL (Ad Libitum) and CR (Caloric Restriction) groups, as well as control age groups CO (Control Old) and CY (Control Young). Furthermore, within each subcategory, panels are further divided to represent male and female seahorses. B) Relative abundance of microbial taxa at the genus taxonomic level with the analysis grouping low abundance taxa (< 10%) together. Similar to the phylum-level analysis, the panels are organized according to diet (AL, CR) and control age (CO, CY) groups, with additional subdivisions for male and female seahorses. C) 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 $* < 0.05$. D) Shannon richness comparing dietary treatment groups with age groups. P values represent adonis pairwise comparisons with $** < 0.005$. E) NMDS diversity plot for Bray-Curtis metric with AL and CR samples of males and females. F) 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 (82,83). This has led to the belief of evolutionarily conserved mechanisms of CR effects across the animal kingdom, which were challenged by a meta-analysis, revealing twofold stronger effects in model species than non-model species (84). This is potentially attributed to genetic responsiveness and publication bias favoring positive results in model organisms. Such bias underscores our constrained comprehension of how fasting impacts non-

model systems, which exhibit different evolutionary adaptations to food availability and varied evolutionary flexibility in reproductive systems, involving female and male-specific resource allocation trade-offs (21,82).

Our study aimed to elucidate the effect of CR, implemented through an alternating fasting regimen, on *Hippocampus erectus*. In this non-model species, males not only produce sperm but are the pregnant sex, incurring costly investment into development of brooding structure (85,86). Conversely, females produce energy-rich eggs. *H. erectus* unique reproductive biology allowed us to disentangle over sexual maturation, the cost of investment into the brooding structure vs. investment into egg production, two costly traits typically found in females. Seahorses further have simple digestive systems and lack a stomach leading to unknown responses to food deprivation as they typically eat at frequent intervals (36).

Fasting resulted in a slower growth trajectory (Fig. S1B), in line with prior studies, especially when initiated at a juvenile age (3). CR males were noticeably lighter and smaller compared to AL males, in contrast to female seahorses, who did not exhibit a comparable stunting effect (Fig. 1B and S1D). Additionally, female ovarian weight between AL and CR did not reach significance (Fig. 1C). Similar body weight, length, and ovarian weight observed in AL and CR females might reflect their reproductive strategy, where investing more energy in growth enhances their appeal to males. This is consistent with findings suggesting that larger females, potentially with bigger eggs, are favoured by males in mate selection (87). In contrast, we 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). This likely stems from the substantial energy investment required for developing the highly vascularised pouch tissue, potentially deferring the likelihood of pregnancy until more favourable conditions arise - consistent with the disposable soma theory. These results contrast with those observed in mammals, suggesting that differences in early-life history strategies in response to adversity, may prompt males to prioritize somatic growth while females allocate resources to other physiological adjustments (88). The reversal of energy allocation in male *H. erectus* away from somatic growth emphasizes that resource allocation is not solely dictated by the biological sex (egg or sperm producer) but is influenced by sex-specific life history costs, particularly parental care.

The cost of nutritional egg provisioning is highlighted by the stark contrast in gene expression between males and females in both gonadal and liver tissue, despite the absence of a significant difference in female ovarian weight between CR and AL treatments (Fig. 3). Fasting did not affect sperm production (no DEGs between AL and CR treatments in testes), consistent with findings in conventional model organisms (21), likely due to the lower production cost of sperm compared to resource-intensive egg formation. In the latter 356 DEGs were downregulated (opposed to only 10 upregulated) upon CR (Fig. 3), with top pathways relating to lipid metabolism and innate immune system (Table S2, sheet C). Also the liver female seahorses had more DEGs compared to males (Fig. 3) involving similar enriched

pathways pertaining to metabolism of steroids (Table S2, sheet A), however, in the liver DEGs were predominantly upregulated in CR seahorses. This could underscore the reliance of female reproductive functions on energy reserves in adipose tissue, where fat cells produce the hormone leptin, a process diminished during fasting with the loss of body fat (3). Decreased leptin levels correlate with lower progesterone concentrations (89), supported by downregulated *pgr* and *cyp17A2* in the liver of fasting females (Fig. 3), which could impair oocyte maturation.

Furthermore, the downregulation of numerous inflammatory genes (including TLRs 21 and 22, chemokines, *tnf*, *irf5*, and *M17*, known for their pro-inflammatory roles in fish (76,78,79)) in CR female ovaries suggests a potential limitation in their capacity to prime their eggs with immunological components for transgenerational plasticity (90,91). This aligns with prior studies (92,93), where research in *Syngnathus typhle*, a member of the Syngnathidae family, indicated that exposure to environmental stressors, like elevated temperatures, erases the effects of transgenerational immune priming, potentially driven by a resource allocation trade-off between survival and immune priming in times of limited resources.

We observed potential beneficial effects of fasting by upregulated genes for increased autophagy (*atg13*) and ketosis (*aacs*) in livers of CR seahorses, in line with other studies (3). The upregulation of both *sesn2* and *tti2* in AL seahorses compared to CR (ovarian tissue) results in an activation of mTORC1 and cell growth (69,70). Suppressing mTOR activity may promote longevity by shifting the body from a state of growth to autophagy, thus improving cell homeostasis (3,11). 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 (94). In some studies FoxO1a has been shown to have positive effects by promoting, among other things, DNA repair (94). A potential rejuvenation effect in the ovaries of fasting female seahorses, as the higher lipid stores and increased lipid synthesis in AL females ovaries promotes cell growth, while fasted females prioritize pathways for repair and regeneration, shifting from high reproductive output to increased somatic maintenance. This suggests a potential rejuvenation effect of CR (enriched pathways in CR vs. AL or vs. CO (Tables S1 and S2, sheet D)).

Overall, despite the absence of notable morphological effects of CR in female seahorses, there is a significant upregulation of lipid metabolic pathways in the liver, accompanied by a corresponding downregulation in the ovaries. The concurrent downregulation of numerous inflammatory genes in the ovaries highlights a nuanced balance between prioritizing individual growth and survival, potentially limiting their ability to prime eggs with immunological components for transgenerational plasticity. Simultaneously, they continue to strategically invest in pre-copulatory reproduction, capitalizing on their larger size to enhance attractiveness to potential mates.

Regardless of male seahorses showcasing only few DEGs upon CR, the liver displayed enriched pathways and genes important for aging, typically affected by fasting, including genes involved in

cellular respiration and oxidative phosphorylation (Table S2, sheet B) (3,11). In the liver of CR male seahorses genes controlling circadian rhythm (*ck1alpha* and *cry3a*), known to be impacted by daily nutrient level fluctuations (95,96), were upregulated. Studies in mice found sex-specific signaling of circadian clock genes, with *cry1* being significantly higher in the liver of CR males (97). A good regulation of the circadian clock has benefits for overall health.

We did observe a meaningful effect of the fasting treatment for both α - (Fig. 4D) and β -diversity (Fig. 4F) to all other groups (AL, CO, CY). 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 (98,99). Taxa richness (on genus level) was high in all CR seahorses, compared to the strong individual fluctuations of AL individuals and the CY and CO groups (Figs. 4B and 4D). Diversity of a microbial community correlated positively with stability and health (100). A recent systematic review of the impact of different fasting regimes in various animal models and humans, showed that most types of fasting led to an increase in α -diversity (10) as suggested by CR seahorses that exhibited an enhanced diversity of Proteobacteria at the expense of a reduction in Spirochaetota (Figs. 4A), in particular of the genus *Brevinema*. Notably, *Brevinema* has been identified as a key player in microbial communities associated with fish mucus (101) and gut microbiota (102), with a potential role in maternal microbial contribution to the next generation (103). Despite being found in both AL male and female *H. erectus* guts, a previous study in a related species (*Syngnathus typhle*) indicated that *Brevinema* may only be transferred to the next generation via the mother and not the pregnant father (103). However, the absence of *Brevinema* in the gut of CR seahorses raises intriguing possibilities, suggesting that fasting females may be unable to pass on this taxon to the next generation during reproduction.

The heightened relative abundance and taxonomic diversity within the Proteobacteria phylum (as illustrated in Fig. 4B, 4E, and 4F), aligns with findings from previous studies indicating an increased relative abundance of Proteobacteria in fasting vertebrates, including fish (99,104). Proteobacteria, recognized for their crucial role in preparing the gut for colonization by strict anaerobic bacteria, play a vital part in maintaining gut health (105). On the genus level, *Pseudoalteromonas* exhibited the highest relative abundance (approximately 25% in both males and females) compared to AL, comprising marine species with diverse activities including anti-bacterial, algicidal, anti-viral, and specific isolates hindering the settlement of common fouling organisms (106). This could potentially confer significant benefits to the immunological health of CR seahorses. Among the other prevalent taxa in CR seahorses, many are commonly found in marine organisms, but their correlation with specific metabolic roles in animals remains unexplored. However we did find *Neptunomonas*, which has potential nitrate reduction properties (107), and *Thalassotalea*, characterized as an aerobic, chemo-organotrophic genus of bacteria playing a crucial role in nutrient cycling and the decomposition of organic matter (108). The efficient utilization of various forms of energy from the environment by these microbes may contribute to their abundance, providing additional energy for the host during fasting. Additionally, a study revealed that *Litorilutius sediminis* was associated with tumor suppressive activity (109), that could also confer

some protective properties to CR seahorses. In summary, while the gut in CR conditions may exhibit potential for improved health compared to AL, a clear rejuvenation was not evident. The dominance of certain taxa in CO and CY, consistent across age groups with some variation between sexes, prompts us to consider that a younger gut does not necessarily equate to a healthier state in seahorses (Figs. 4A-B). Nevertheless, the observed effects of fasting suggest overall benefits.

In conclusion, our findings shed light on the intricate sex-specific responses to CR in seahorses, providing valuable insights into the adaptive strategies employed by males and females to cope with nutritional stress and maintain reproductive success. Importantly, our study emphasizes how the primary determinants of resource allocation are closely tied to sex-specific life-history strategies. In *H. erectus*, this is exemplified by the unique focus on male pregnancy and the allocation of resources post-copulation, contrasting with the pre-copulatory investment in female eggs, leading to distinct morphological and regulatory effects of fasting between males and females, that are opposed to what we have seen in typical model organism. Nonetheless, conserved mechanisms of CR were evident in both sexes, but to different degrees, with increased autophagy and ketosis pathways in CR females, and enrichment of pathways and genes important for cellular respiration, and circadian rhythm control in CR males. As we move forward, additional research in a diverse range of model species with varying reproductive strategies and sex roles will be essential to build upon our findings. Understanding the intricate connections between reproduction, resource availability, and fasting effects can contribute to a more comprehensive understanding of sex-specific health, lifespan dimorphism, and evolutionary adaptations in response to nutrient scarcity, particularly in the context of climate change. By adopting a multidisciplinary approach, researchers can make significant strides in translating the findings into practical applications for health and beyond.

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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 Digitalisierung (MELUND) (permit no. 1315/2021). No wild endangered species were used in this investigation.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflicts of interest.

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CHAPTER II

Navigating sex and sex-roles: deciphering sex-biased gene expression in a species with sex-role reversal (*Syngnathus typhle*)

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ABSTRACT

Sexual dimorphism, the divergence in morphological traits between males and females of the same species, is often accompanied by sex-biased gene expression. However, the majority of research has focused on species with conventional sex roles, where females have the highest energy burden with both egg production and parental care, neglecting the diversity of reproductive roles found in nature. We investigated sex-biased gene expression in *Syngnathus typhle*, a sex-role reversed species with male pregnancy, allowing us to separate two female traits: egg production and parental care. Employing RNA sequencing, we examined gene expression across organs (brain, head kidney, gonads) at various life stages, encompassing differences in age, sex, and reproductive status. While some gene groups were more strongly associated with sex roles, such as stress resistance and immune defence, others were driven by biological sex, such as energy and lipid storage regulation in an organ- and age-specific manner. By investigating how genes regulate and are regulated by changing reproductive roles and resource allocation in a model system with unconventional life-history strategy, we aim to better understand the importance of sex and sex role in regulating gene expression patterns, broadening the scope of this discussion to encompass a wide range of organisms.

Keywords: sex-biased gene expression, sex-role reversal, parental investment, male pregnancy, life-history trade-offs

1. INTRODUCTION

Males and females of the same species often display sexual dimorphism in morphological traits (1) stemming from sex-biased gene expression (2). Based on which sex displays higher expression levels, genes are categorized as male-biased or female-biased (2). The evolution of sex-biased gene expression can resolve conflicts between males and females, including sexual selection, sexual antagonism, relaxed selective constraint and conflicts in parental investment (1,2). The prevalence of sex-biased gene expression is notable across numerous species, although its variation is assigned to

differences across tissues and developmental stages (1–3), known to mostly be lowest during embryonic phases and highest in sexually mature adult stages (4) .

Sex-specific gene expression has predominantly been investigated in species characterized by conventional sex roles, encompassing male-male competition and female parental care (1). Given the evolutionary flexibility of sex roles, and their variation among species and across individual's lifetime, research going beyond conventional sex roles is tremendously required for understanding the evolution life-history strategies (5–7). Paternal care is the predominant parental care strategy in teleosts encompassing egg guarding, and oral brooding to full viviparity (7,8). The most devoted male parents can be accredited to the syngnathid family (seahorses, pipefishes and seadragons), which have evolved the unique male pregnancy ranging from simple attachment of eggs to the male ventral side to highly specialized brooding structures, analogous to those found in eutherian mammals, that provide embryos with protection, nutrients, oxygen and immunological components (8–12). Experimental studies on parental investment, however, have traditionally focused on mothers' fitness, as in classical sex roles, they invest more in egg production and nurturing offspring, directly impacting the offspring's fitness (13). Notably, in some syngnathid species, research has expanded to explore the father's fitness and investment in offspring, reflecting a broader understanding of parental roles (10,14–17). The wide diversity of natural sex roles ranging from female-biased parental care with male mating competition to male-biased parental care with strong female competition offers opportunities to explore resource allocation trade-offs as drivers for sex-specific life history strategy (18). Life-history strategy evolved with distinct energy investment into somatic maintenance and reproduction, resulting in differences sex-specific resource allocation for immune defence, metabolism and longevity (19–21). Unfortunately, we are lacking a comprehension of how patterns of sex-biased gene expression fluctuate in species featuring sex-role reversal with parental male-biased care. This basic research is crucial because gender dynamics are changing in human society and evolving over time. Currently, the concept of targeted medicine for sex-specific illnesses primarily relies on the binary classification of biological sex (egg or sperm producer) and tends to overlook the societal and environmental context of that sex. This approach creates a blind spot in biomedical research, as it fails to account for the costs of parental care and gender-specific settings that can result in shifts in gene expression.

To understand the importance of biological sex vs. sex-role in driving gene expression patterns, we compared sex-biased gene expression of young and older broadnosed pipefish *Syngnathus typhle*. The syngnathid family with their sex-role reversal and their unique male pregnancy evolution facilitates decoupling the role of the female sex (defined as the contribution of eggs) and pregnancy (body reshaping and energy allocation) (11). In the broadnosed pipefish, the females produce on average more eggs than males can brood (16,22), resulting in males being the more reproductively constrained sex (23). Larger brood size and limited resource availability influence the cost of male care, making embryo mortality inversely proportional to male condition (male mortality increases with a larger brood size) (12). Given their polygamous mating system and males being the limited sex, males result in being the choosier sex, preferring larger females, whereas females are ornamented and actively court males

(14,22–25). In the present study, we sought to investigate how these sex-specific expression patterns vary between young and older pipefish individuals and unravel the influence of age and resource allocation shifts on sex-biased gene expression across distinct organs and conditions. We hypothesized that, owing to the sex-role reversal in *S. typhle*, there would be a reversal in patterns of sex-biased gene expression compared to conventional model species. This shift is anticipated to be more strongly associated with sex roles rather than biological sex alone, and our expectation is that this reversed pattern would persist or even strengthen with increasing age. To test this, we conducted full transcriptome RNA sequencing (RNA-Seq) to analyse gene expression in three distinct organs: brain, head kidney, and gonads, across different age groups (approximately 1 year old and over 2 years old) and between female and pregnant/non-pregnant male pipefish. Our findings deepen our understanding of this unique system and contribute to discussions about sexual dimorphism, parental investment as drivers of gene expression.

2. METHODS

2.1 Sample collection

Broadnosed pipefish *Syngnathus typhle* were caught in the south-western Baltic Sea (54°39'N; 10°19'E) in spring right before their breeding season (26). They were brought to the GEOMAR institute, where body mass and total body length were measured. In our study we used 10 females and 20 males; the latter included 10 pregnant and 10 non-pregnant. We also separated the pipefish based on age, ca. 1 year old for young pipefish and above 2 years for old (their estimated lifespan in the wild is 3 years) and measured body size and weight (Figure S1). Given the limited availability of established models for studying non-model organisms like pipefish, our approach draws on years of fieldwork experience. Broadnosed pipefish engage in mating and breeding activities during spring, followed by migration to unknown areas during winter, before returning to the seagrass meadows of the south-western Baltic Sea for breeding in the subsequent year (26). The offspring cohorts from one breeding season typically only reach sexual maturation during the next breeding season, indicated in males over the development of a brood pouch that only becomes visible during the next breeding season (phenotypic signal for sexual maturation). This resulted in 5 individuals for each group young male (YM), young pregnant (YP), old male (OM), old pregnant (OP), young female (YF), and old female (OF).

The animals were sacrificed with an overdose of MS-222 (Tricaine methane sulfonate, 500 mg/l; Sigma-Aldrich). Carcasses were dissected, and different organs including brain, head kidney, and gonads were sampled and immediately preserved in RNA later. The samples were kept at 4 °C for three days, before being transferred to -20°C for long-term storage. Previous studies have highlighted substantial variability in sex-biased gene expression among organs (27–29). Gonads, exhibit the highest degree of sex-biased expression, with most biased genes involved in sex differentiation and fertility regulation (1,29). Notably, brain-specific dimorphism has demonstrated preliminary trends in *Syngnathus scovelli* (30), while the head kidney—a unique organ in teleost fish analogous to the adrenal gland—serves the

dual function of producing red and white blood cells, thereby supporting both oxygen transport and immune defense (31). Given its prominent role in immune regulation, the head kidney becomes a focal point for studying genes displaying sexual immune dimorphism in expression as identified in diverse species from insects to lizards, birds and mammals, with an overall stronger innate and adaptive immune response in females (32,33).

2.2 RNA isolation and Illumina sequencing

For our RNA-Seq analysis, we extracted RNA from brain, head kidney, ovaries and testes. The RNA extraction was performed using RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. Extraction yields were quantified using NanoDrop ND-1000 spectral photometer (Peqlab, Erlangen, Germany), RNA was then stored at -80°C (for information on quality and quantity of RNA for RNA-Seq refer to Table S5). Library preparation (TruSeq stranded mRNA Kit by Illumina) and sequencing (RNA-Seq NovaSeq6000 S4, 2x150bp, over 17M clean paired-end reads per sample) were carried out at the Institute of Clinical Molecular Biology (IKMB).

2.3 Data analysis

2.3.1 Morphological analysis

Statistical analysis was done in Rstudio (v.4.2.2) (34) and tests were considered significant when p-values were smaller than $p = 0.05$. Mean and standard deviations were calculated and in a separate two-way ANOVA the differences between weight [g] and length [cm] for sex and age were analysed, with Tukey's Honestly Significant Difference (TukeyHSD) post-hoc test for single interactions (boxplots for weight and length: Supplementary Material, Figure S1).

2.3 Transcriptome analysis

Two samples had to be excluded due to sequencing failure (one YM testes and one OM pregnant testes). The resulting reads were quality-controlled using FastQC v.0.11.9 (35) and trimmed using Fastp v.0.20.1 (36). Reads were aligned to a whole genome assembly of *Syngnathus typhle* (BioProject ID: PRJNA947442) using STAR v.2.7.9a (37). Transcript abundance was quantified with TPMCalculator (38).

Statistical analysis in Rstudio (v.4.2.2) (34) used the edgeR package (v.3.40.2) (39). We scaled the raw count data with 26072 genes to counts per million (cpm) and filtered it based on a minimum threshold of 10 counts in at least 20 libraries, resulting in 19348 genes. To control for composition biases, we normalized the data using the trimmed mean of values (TMM) with the calcNormFactors function.

We initially performed a principal Component Analysis (PCA) with the “regularized log transformation procedure” (rld) transformed expression values across all the samples to validate organ separation and check for outliers (Figures 1 and S2). Once we had assessed that organ type explained most variation in the dataset, we focused on single organ’s differential gene expression (DGE) analysis, to detect age, sex and pregnancy effects. The raw count data was separated in brain, head kidney, ovaries and testes. We reapplied filtering and normalisation steps and resulted in 18373 genes in brain, 16746 in head kidney, 16368 in ovaries and 18218 in testes. For each organ we plotted PCAs (Figure 1) and tested our hypotheses using a Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) using function “adonis2”, method “bray” and 999 permutations (adonis2 (counts ~ meta\$age*meta\$sex*meta\$pregnant, permutations = 999, method = "bray"). As a post-hoc we used the pairwise.adonis function with Bonferroni correction.

Subsequently, we employed the limma package (version 3.54.1) for conducting DGE analysis (40). limma employs a linear model approach coupled with empirical Bayes techniques and utilizes the voom method to transform count data into a continuous scale. This method is especially effective in addressing batch effects and reducing technical variability. The voom method is specifically adept at handling varying library sizes (41). To compare multiple groups (Table S2), we constructed a matrix containing independent contrasts. This matrix facilitated conducting a one-way analysis of deviance (ANODEV) for each gene. Following this, we calculated coefficients representing differences between groups to ascertain log fold changes (logFC) through the application of the contrasts.fit function. Then we implemented empirical Bayes moderation to shrink the estimated variance and conducted moderated t-tests to pinpoint genes with significant differential expression. For subsequent downstream analysis, our focus was exclusively on adjusted P-values below 0.05 and $\log\text{FC} \pm 1$. This ensured that the observed effects of treatments were statistically meaningful.

2.3.4 Gene ontology and enrichment analysis

For gene ontology analysis, we annotated the differentially expressed genes using a homology-based search of *Danio rerio* (GRCz11) with OrthoFinder (42). *Danio rerio* orthologues from the resulting DEGs between YM vs. YF and OM vs. OF in head kidney were uploaded to g:Profiler web tool (last accessed on 11th August 2023) (43) to perform gene enrichment analysis.

The settings used were: Organism “*Danio rerio* (Zebrafish)”, statistical domain scope set at “All known genes”, significance threshold “g:SCS threshold” at 0.01, numeric IDs treated as “ENTREZGENE_ACC”, the data sources were limited to GO biological processes and biological pathways the databases Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome (REAC) (Tables S4). To make our analysis more specific, we applied a maximum term size of 1000 to exclude overly broad categories and prioritize more informative outcomes.

3. RESULTS

3.1 Sex-biased expression between organs

The results illustrating the variations across distinct organs are depicted in the PCA plot shown in Figure 1A. The samples displayed strong clustering based on their organ types, and the brain exhibited the most distinct separation compared to the other organs. Given that the primary source of variance within the dataset arose from organ types, and our interest was focused on delving into the impacts of sex, age, and pregnancy status, we proceeded to conduct a more granular analysis within each individual organ.

Upon closer examination of the brain, hypothesis testing did not reveal any statistically significant disparities for the studied variables or their interactions (PERMANOVA, $P > 0.05$). This outcome is also visually evident in the PCA plot specifically dedicated to brain tissue (Figure 1B). While discernible differences did exist among the sex-specific gonadal tissues (Figure 1D), a detailed exploration within the testes and ovaries of the two distinct age groups (young vs old) failed to unearth any noteworthy effects. Nonetheless, a modest effect emerged from the interaction between age and pregnancy status (yes or no) within the context of testes (PERMANOVA, $P = 0.034$). Subsequent pairwise comparisons, however, did not yield any interactions that reached a statistically significant threshold. In terms of head kidney, neither age nor pregnancy displayed substantial impacts, whereas sex exerted a highly significant influence (PERMANOVA, $P = 0.001$). This distinction is depicted in the PCA plot presented in Figure 1C, wherein PC1 accounts for approximately 28% of the variance between samples. Additionally, PC3 appears to mildly differentiate between young and old individuals, with both OM and OP subjects clustering toward the upper left corner, and OF individuals gravitating in a similar direction on the right, albeit without achieving statistical significance.

Chapter II

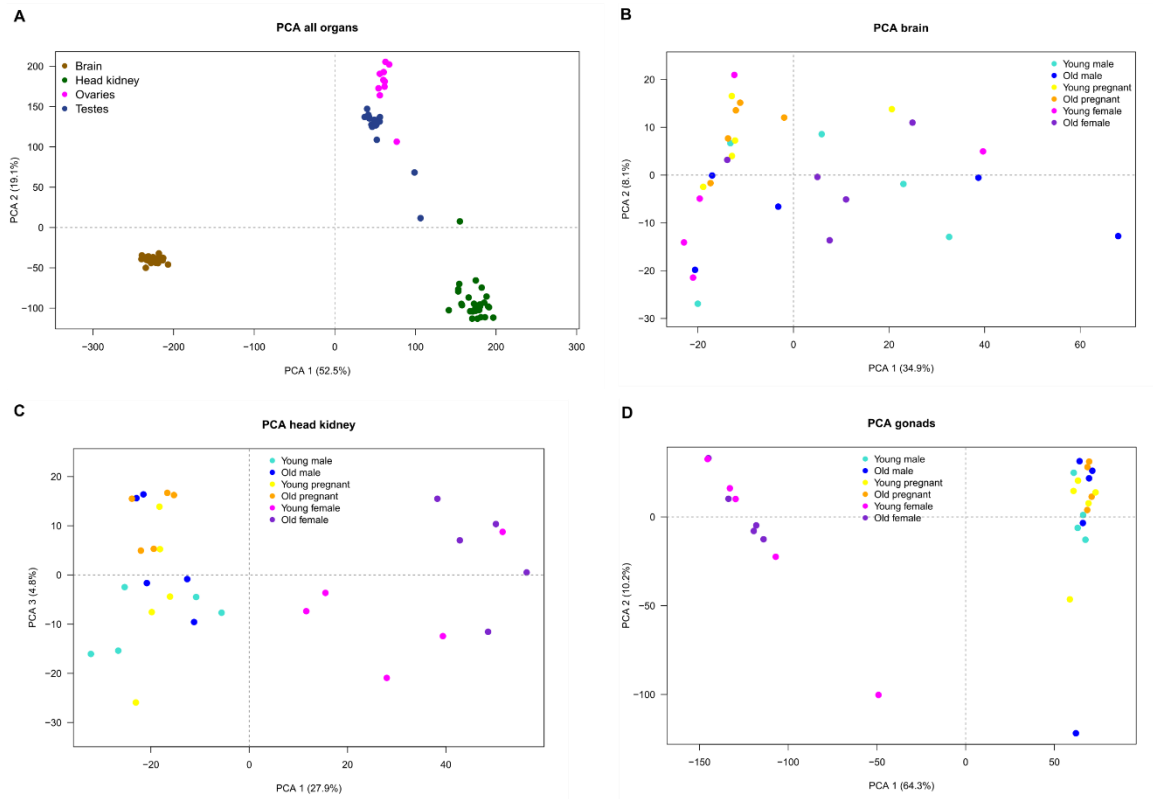


Figure 1: Principal Component Analysis (PCA) of gene expression patterns. A) PCA plot illustrating sample clustering based on organ type. Each organ is represented by distinctive colours: brain in brown, head kidney in green, ovaries in magenta, and testes in dark blue. Subsequent PCA plots provide detailed organ-specific analyses, incorporating distinctions for sex and life stage: young males in light blue, old males in blue, young pregnant individuals in yellow, old pregnant individuals in orange, young females in pink, and old females in purple. B) PCA plot for the brain, revealing no discernible clustering for sex, age or pregnancy. C) PCA plot for head kidney, focusing on PC1 and PC3. While PC1 clearly shows separation for sex, PC3 highlights a potential distinction between young individuals clustering more on the lower half and older individuals on the top half of the plot (additional insights into PC2 can be found in supplementary material, Figure S2). D) PCA plot depicting clear separation between ovaries and testes on PC1, with no discernible impact of life stages.

The differential gene expression analysis suggested that distinctions pertaining to sex and age were particularly pronounced within the head kidney (Tables S2 and S3). Notably, in the contrast comparison between YM and YF, 1930 significantly DEGs were identified ($\text{adj.p.val} < 0.05$), while the comparison between OM and OF yielded 3564 DEGs. Nonetheless, no differentially expressed genes (DEGs) were observed when comparing pregnant and non-pregnant males across all organs (Table S2). Results in the brain revealed only a couple of DEGs, which were discarded later in the analysis due to either low logFC or the absence of *Danio rerio* annotations (Tables S2 and S3).

Conversely, differential gene expression analysis within the respective gonadal organs (testes and ovaries) displayed an absence of significantly DEGs, when considering age or pregnancy (Table S2). Yet, analysis of testes vs. ovaries, revealed a high number of significantly DEGs for age (Tables S2). With a logFC threshold of ± 1 we found 6620 DEGs between YM vs YF, with 3370 upregulated in YMs

and 3250 in YFs, and 7504 for OM vs OF, with 3574 genes upregulated in OM and 3930 upregulated in OFs (Figure 2C). However, when checking for overlaps of DEGs between the two age groups, we found that the majority of DEGs did not change with increasing age (Figure 2D). Meaning that most genes were sex-biased irrespective of increasing age. Nonetheless, there were roughly 999 significant DEGs between YMs and YFs which were not DE between OM and OF and 1883 vice versa, making these age dependent (Figure 2D).

Figure 3B displays the most enriched pathways for gonads in different age groups, highlighting the pathways consistently biased by sex regardless of age. This encompasses overlapping genes from Figure 2D as well. There was a significant overlap between young and old males (Figure 3B and Table S4), including translation initiation, metal ion transport, and Nonsense-Mediated Decay (NMD). Including genes encoding for both TIMP and ADAM metalloproteinase, metalloproteinase, along with various types of ribosomal protein-encoding genes (Table S3 – gonads). In ovaries, we observed pathways enriched for ncRNA metabolic processes and several pathways related to RNA modification in older females' ovaries (Figure 3B and Table S4). The involved genes mostly encompass tRNA methyltransferases and zinc fingers (Table S3 – gonads).

3.2. Sex-biased gene expression in head kidney

Results from the differential gene expression analysis in head kidney revealed 912 significant DEGs (with logFC threshold of ± 1) in the contrast comparison between YM and YF, and 1556 DEGs between OM and OF (Figure 2A). In the first case, 500 genes exhibited upregulation in YM and 412 in YF, while for the OM vs OF comparison, 780 were upregulated in OM and 776 in OF. Overlaps between sex-biased expressed genes for young and old pipefish found that 291 DEGs were upregulated in both YF and OF, whereas approximately 223 were the same for both YM and OM (Figure 2B). GSEA on the latter, revealed that female-biased genes, regardless of age, displayed enrichment ($p < 0.01$) in processes such as transport, peroxisome, and the terminal pathway of complement (Figure 3A and Table S4). In contrast, male-biased genes exhibited enrichment in myeloid cell differentiation and hemopoiesis (Figure 3A and Table S4). Older females exhibited enrichment across categories linked to diverse metabolic pathways, encompassing lipid metabolism, fatty-acid metabolism, and peroxisomal lipid metabolism (Figure 3A and Table S4). Furthermore, heightened activities in cell respiration and oxidation processes, exemplified by pathways such as peroxisome, alpha-oxidation of phytanate, and biological oxidations (Figure 3A). In contrast, YF predominantly exhibited enrichment in the peroxisome pathway (Figure 3A).

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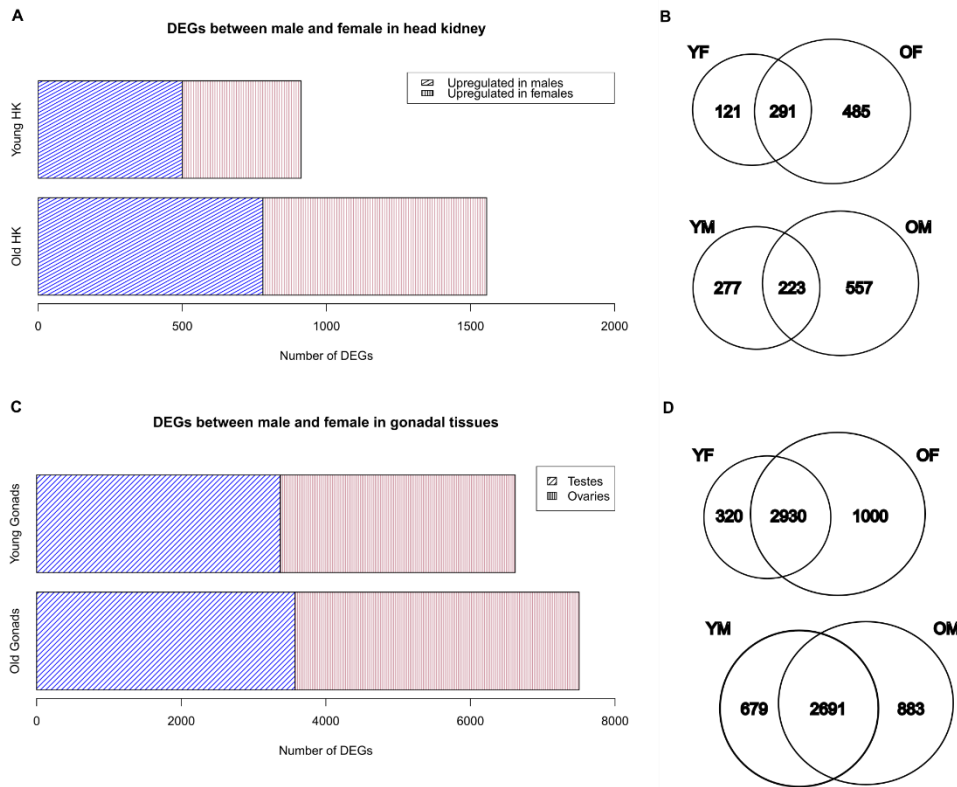


Figure 2: This figure presents the outcomes of the differential gene expression analysis in the head kidney ($\text{adj.p.val} < 0.05$; $\log\text{FC} \pm 1$), focusing young males (YM) vs. young females (YF), as well as old males (OM) vs. old females (OF). A) Bar plots illustrate the genes that exhibit upregulation in males compared to females, stratified across different age groups for head kidney. YM display overall 500 upregulated genes vs. 412 in YF, while OM showed 780 upregulated genes vs. 776 in OF. B) Venn diagram showing overlapping sets of DEGs between YF and OF, as well as between YM and OM. This helps us discern genes that exhibit sex-specific biased expression irrespective of age and those whose sex-biased expression changes particularly with age. C) Bar plots showing differential gene expression between testes and ovaries, for old and young pipefish. There were 3370 genes biased in YM testes vs. 3250 in YF ovaries, and 3574 in OM vs. 3930 in OF. D) Venn diagram showing overlapping sets of DEGs between males and females between young and old. Majority of DEGs are sex-biased irrespective of age.

Genes upregulated in OF compared to OM that were integral to metabolic pathways encompassed several cytochrome P450 enzymes such as *cyp1b1*, *cyp2r1*, *cyp4v7*, *cyp8b1* and *cyp19a1a*, constituting a superfamily of enzymes pivotal in metabolizing a spectrum of compounds, including drugs, toxins, and hormones (44). CYP1 localizes to the mitochondrial inner membrane, catalysing the critical initial step in steroid biosynthesis – the conversion of cholesterol to pregnenolone hormones (44). CYP2 enzymes, as recognized in mammals, metabolize vitamin D and arachidonic acid hormones (44). While CYP19, also known as aromatase, facilitates the conversion of C19 androgen to aromatic C18 estrogen (44). Further delving into lipid metabolism, several female-biased expressed genes stood out, including fatty acid amide hydrolase (*faah*), lipoprotein lipase (*lpl*), and apolipoprotein L 1 (*apol1*), A-II (*apoa2*), and apolipoprotein C-II (*apoc2*). Notably, *faah*, *apoc2*, and *lpl* also exhibited upregulation in YFs compared to YMs (Table S3). The peroxisome pathway displayed female-biased enrichment in

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both YF and OF (Figure 3A). These enzymes play pivotal roles in fatty acid oxidation and lipid metabolism at large, while also contributing to the detoxification of harmful compounds, including reactive oxygen species (ROS) (45). Particularly pronounced in OF were genes related to cell respiration and oxidation, such as various glutathione peroxidase forms (4a and 4b) and peroxiredoxin (prdx1, also observed in YF). Additionally, genes involved in respiration NADPH oxidase 1 (nox1) and 4 (nox4), acyl-CoA oxidase 3 (acox3, also evident in YF), ferredoxin reductase (fdxr), and ferredoxin 1 (fdx1, also observed in YF) exhibited upregulation (Table S3).

Numerous pathways exhibited enrichment in OMs relating to cell cycle, chromosome organization, DNA replication, and assorted cellular processes (Figure 3B and Table S4). Some DEGs that overlapped between these pathways belonged to the kinesin family (kifc1, kif11, kif14, kif15, kif20a, kif20ba, kif22, kif23, kif18a) or centromere proteins (cenpn, cenpe, penpk, cenpo, cenph, cenpt, cenpf, and numerous others) (Table S3). Additionally, still in OM pipefish, pathways concerning DNA repair (GO:0006281, $p < 0.002$) and DNA damage response (GO:0006974, $p < 0.0001$) exhibited enrichment (Table S4). Notable genes within these pathways encompassed damage-specific DNA binding protein 2 (ddb2) and DNA repair contributors rad51b and rad54, which play specific roles in homologous recombination-based DNA repair processes (46). Furthermore, ATM serine/threonine kinase (atm), checkpoint kinase 1 (check1), and checkpoint kinase 2 (chek2), with their overarching responsibilities in activating downstream targets implicated in DNA repair and cancer prevention (47,48), were also represented. Importantly, the timeless circadian clock gene exhibited upregulation in both young and old males compared to females (Table S3). Although fish-specific research in this context is lacking, studies in humans have revealed that its downregulation leads to telomere-associated DNA damage (49,49). Notably, intriguing male-biased expressed genes included deptor, a negative regulator of the Mammalian Target of Rapamycin (mTOR) signalling pathway that acts as a feedback inhibitor (50). Additionally, insulin-like growth factor 2 mRNA binding protein 2a (igf2bp2a) and, in older males, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (ppargc1a), a homolog of forkhead box O (FOXO) 1, all interconnected in nutrient sensing pathways and collectively influential in cell growth, metabolism, and lifespan regulation (51,52).

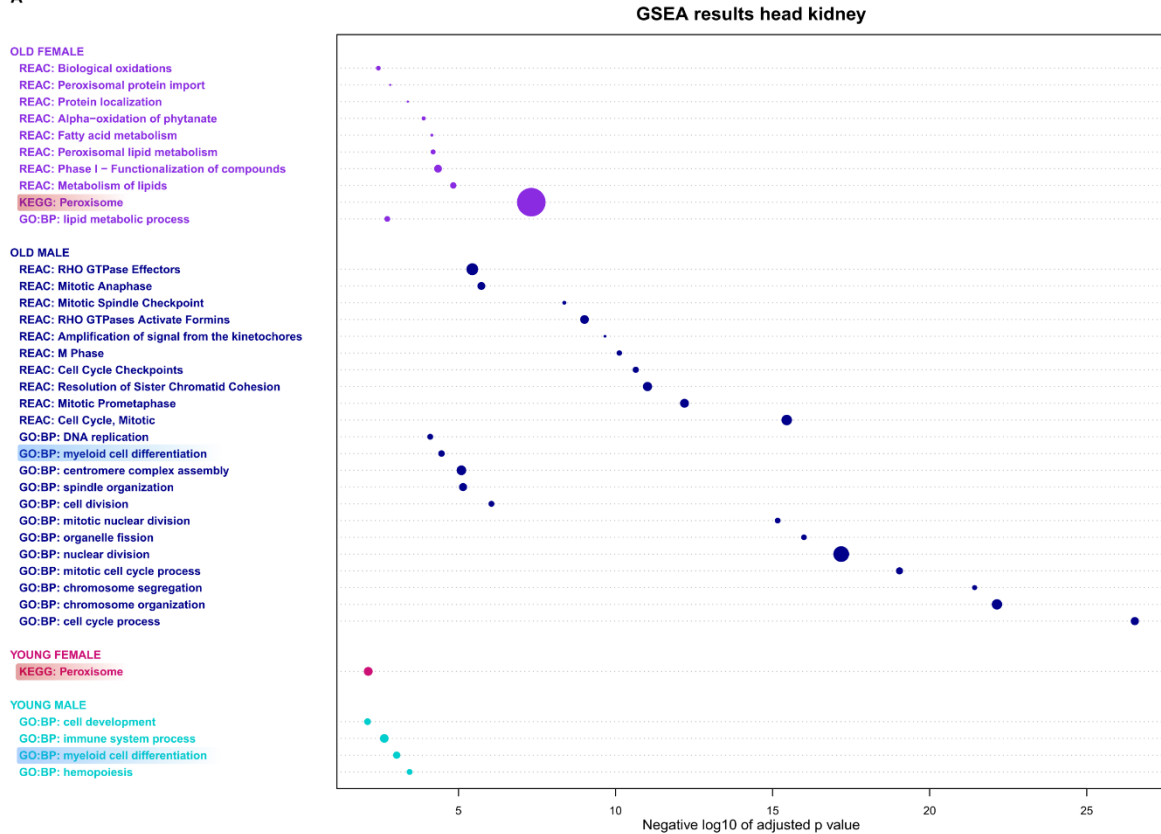
For genes particularly upregulated in YMs compared to YFs, we observed enrichment in hemopoiesis, cell development, and myeloid cell differentiation, although these also exhibited enrichment in OMs relative to females albeit to a lesser extent (Figure 3A and Table S4). These pathways' enrichment make sense, given the head kidneys' role in producing red and white blood cells (31). Interestingly, YMs uniquely exhibited enriched genes associated with immune system processes (Figure 3A and Table S4). Within this pathway genes included chemokine receptors 1 (cxcr1) and 4a (cxcr4a), interferon regulatory factor 5 (irf5), and toll-like receptor 8 (tlr8), the latter being a homolog of human tlr1 (53,54). There was also V-set immunoregulatory receptor (vsir), analogous to other immune checkpoint molecules, VSIR (VISTA) plays a role in modulating T cell responses, acting as a negative regulator of T cell activation to prevent excessive immune responses and maintain immune homeostasis (55,56). Additionally, the gene cluster of differentiation (CD) antigens, such as cd7al and cd247l, were identified,

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which participate in immune recognition processes (57). Notably, recombination activating gene 1 (rag1) was also found upregulated in YMs, encoding a protein crucial for V(D)J recombination, this process is fundamental for generating diverse antigen receptor molecules on immune cells, specifically B and T cells (58,59). Moreover, we observed pro-inflammatory cytokines expressed in males, including neutrophil cytosolic factors (ncf1, ncf2, and ncf4), where NCF1 stands out as a pro-inflammatory protein contributing to the generation of reactive oxygen species that are pivotal for pathogen eradication (60). Although females did not show enrichment in immune pathways, upon closer observation we found several immune-related genes notably upregulated in both young and old females compared to males (Table S3). These included interleukins and their receptors, such as il11ra, il17ra1a, il19l, and il10, the latter suppresses the production of pro-inflammatory cytokines such as TNF-alpha (61), and a major histocompatibility complex class I ZCA (mhc1zca) (Table S3), which are molecules present on the surface of all nucleated cells and bind to foreign antigens so that CD8+ T-cells can recognize and eliminate the invading pathogens (Garcia et al. 1996). Furthermore, interleukin 6 signal transducer (il6st) and interferon regulatory factor 6 (irf6) were elevated in YFs.

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A



B

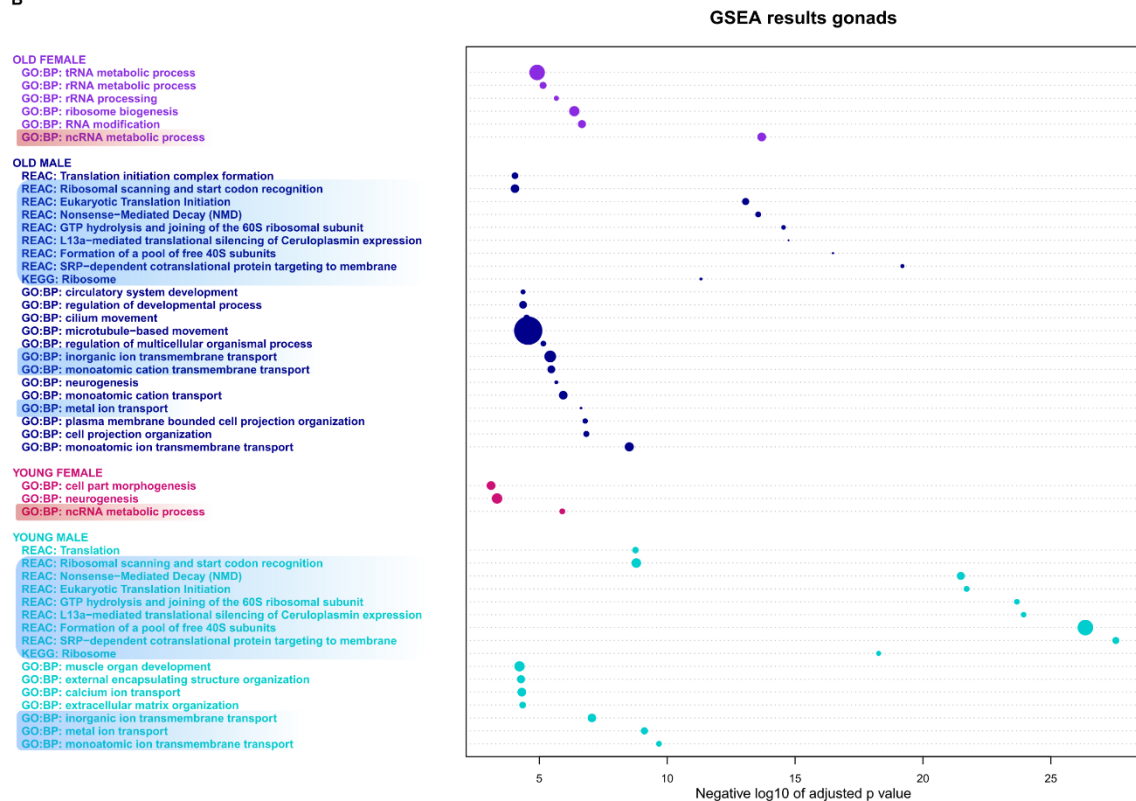


Figure 3: Gene set enrichment analysis results for *Danio rerio* annotated DEGs (logFC \pm 1, adj.p.val < 0.05). A) Results are for head kidney and B) gonads. In purple are pathways enriched for sex-biased expressed genes in

OF pipefish, in dark blue OM, in pink for YF, and in light blue YM. Highlights reflect pathways that were enriched irrespective of age, in light red we match YF and OF, and in light blue YM and OM. This is only within the respective organs. Data sources were limited to GO biological pathways, KEGG and Reactome, with a g:SCS threshold of 0.01. X-axis shows the negative log₁₀ of the adjusted p values. Dots are scaled to reflect proportions of genes within the specific category. As there were numerous pathways highly enriched in OM, we set a more stringent threshold of adj.p.val < 0.0001 and removed repetitive pathways for easier reading (full results are found in supplementary Table S4).

4. DISCUSSION

Our work presents a unique exploration into the intricate relationship between male and female life-history traits and their influence on gene expression patterns, particularly in the context of a sex-role reversed species featuring male pregnancy, *Syngnathus typhle*. By conducting comparative analyses of gene expression among males, females, young, old, pregnant and non-pregnant male pipefish across various organs (brain, head kidney, gonads), our aim was to uncover fresh insights into the roles of sex (i.e., egg or sperm producer) and sex-role (i.e., parental investment, mate competition) in shaping sex-biased gene expression patterns.

Our findings highlighted distinct gene expression patterns across different organs, whole brain, head kidney, and gonads. The most abundant DEGs were observed in the gonads and head kidney, with an occurrence of more sex-biased expressed genes between older pipefish, which aligns with research findings in other organisms (4). While examining the brain, no substantial evidence of sex differences emerged, aligning with findings in various teleost species, including East African cichlids (27), sparid sharpsnout seabream (*Diplodus puntazzo*) (28), the bluehead wrasse (*Thalassoma bifasciatum*) (62), and in the pipefish species *S. scovelli* (30), where only a limited number of sex-biased genes were detected. It is essential, however, to acknowledge the potential variability in sex-biased gene expression across species, as illustrated by studies in the tropical gar (*Atractosteus tropicus*) (63) and zebrafish (64). This emphasizes the significance of recognizing species-specific nuances within our study, providing valuable insights into the diversity of sex-related gene expression patterns in the teleost brain. Interestingly, pregnancy exhibited minimal or no effect on gene expression across all examined organs. This observation suggests that the physiological changes associated with pregnancy may not significantly affect the selected organs, considering that another study comparing pouch tissues and pregnancy gradients across four different syngnathid species found DEGs in immune pathways and metabolic processes (65,66).

Based on the results and the reproductive biology of *S. typhle*, we suggest that female pipefish may allocate a relatively higher proportion of their resources to pre-copulatory reproduction compared to males. This inference is drawn from the enrichment of female-biased pathways related to RNA modification in ovaries and the observation that older females display enrichment in metabolic pathways associated with lipid and fatty-acid metabolism in the head kidney (Figure 3). These patterns suggest a potential emphasis on oocyte development, maturation, and hormonal regulation, indicating a notable

allocation of resources, possibly towards pre-copulatory investment encompassing egg mass and physiological growth to attract more mates (Figure 4) (14).

The gonads of male pipefish also revealed pathways associated with sperm quality and ejaculate volume such as metal ion or calcium transport (Figure 3B), including metallo-peptidase and -proteinase genes (Table S3 - gonads) (67). However, there were not many other pathways specifically related to generating sperm and associated fluids/proteins. These results are consistent with research in *S. scovelli*, where a reduced expression of genes associated with sperm function was found, suggesting that pipefish may have evolved to produce a smaller number of sperm (67). The study also suggests that due to the selection for male pregnancy, this could potentially lead to a decrease in sperm count and shift energetic demands towards developing the brood pouch itself. Interestingly, the enriched NMD pathway also exhibited numerous male-biased genes in testes (Figure 3B). This pathway, crucial for RNA quality control and precise gene expression regulation, may play a vital role in maintaining the integrity of spermatogenesis (68).

In the head kidney, female-biased genes, irrespective of age, exhibited a notable enrichment primarily in the peroxisome pathway. These membrane-bound organelles play pivotal roles in lipid metabolism within cells, maintaining cellular oxidative equilibrium (69). Given the energy investment in processes like oocyte development by female pipefish, the heightened peroxisomal pathway enrichment suggests an augmented demand for lipid metabolism to fuel these activities. Notably, older females displayed enrichment in metabolic pathways pertinent to lipid and fatty-acid metabolism (in head kidney), including peroxisomal lipid metabolism. Enriched genes within these pathways, such as *faah* (70), apolipoproteins (71), and *lpl* (72), collectively influence lipid metabolism, lipase activity, and energy production. The key role of LPL, as a rate-determining enzyme for fatty acid supply to various tissues, hints at its potential to govern the allocation of dietary lipids towards storage or utilization (72–74).

Regulating dietary fats holds critical importance in fish, serving as both an energy reservoir and a fundamental source of essential fatty acids (EFAs) vital for growth, development, and reproduction (73,75). While basic metabolic processes align between males and females, variations in body composition, sex hormones, sex-specific energy expenditure, and age collectively influence nutrient metabolism and energy storage efficacy (76,77). Notably, oestrogen concentrations influence fat deposition and storage patterns in the body, aligning with findings in *S. typhle* where males produce testosterone and females produce oestrogen despite the reversed reproductive roles (78,79). The involvement of multiple cytochrome P450 enzymes, particularly OFs, such as CYP1 enzyme catalysing the conversion of cholesterol to pregnenolone hormones, which is the starting point in the production of cortisol, oestrogen, progesterone and testosterone (44) and CYP19 helping the conversion of androgen to aromatic oestrogen (oestrone and 17β -oestradiol) (44), implies potential links to oestrogen-driven processes. Thus, the interplay between energy allocation to eggs and ovarian oestrogen production likely contributes to the sex-biased expression of lipid metabolic-related genes (80). Considering the hormonal pathways inherent to females, an equally valid alternative hypothesis could

be that the increase in lipid and fatty acid metabolism may be due to a greater emphasis on fat storage in females compared to males. This would be consistent with the generally larger body size of female individuals within the species, highlighting the complex connection between hormonal control, energy allocation, and reproductive strategies (23,25,81).

In contrast, our results suggest that male pipefish, especially older individuals, may exhibit a strategic investment in their somatic maintenance. In the head kidney of OMs, we found pathways pertaining to DNA repair and damage response, indicating potentially a more robust DNA repair machinery in older males. Male-biased genes such as *depor*, *igf2bp2a*, and *ppargc1a*, known for nutrient sensing and pathways related to mTOR, insulin-IGF regulation and FOXO transcription factors, suggests a focus on cellular maintenance, stress resistance, maintenance of cellular homeostasis and potentially, longevity (50–52). The presence of the timeless circadian clock gene, associated with genomic integrity and potential telomere-associated maintenance (46), further reinforces the idea of male investment in health. Together, these findings may reflect male pipefish investing in mechanisms that promote their own health and somatic well-being (Figure 4).

An intriguing result was the heightened enrichment of processes related to haematopoiesis and the immune system in YMs as compared to YFs, encompassing genes linked to chemokine receptors, interferon regulatory factors, toll-like receptors, and immune checkpoint molecules. Considering that male pipefish undergo pregnancy and harbour embryos in their pouches, the increased emphasis on haematopoiesis and immune cell development in YMs might signify an evolutionary adaptation aimed at bolstering their own immune system (9). There are inherent trade-offs associated with balancing the reproductive system with the immune system. In species with internal fertilization and gestation, such as pipefish, there are specific demands placed on the immune system, including the need to avoid sexually transmitted diseases and prevent rejection of the developing embryo (82,83). Moreover, the upregulation of *rag1* is noteworthy, as this gene is pivotal for V(D)J recombination—a process generating diverse antigen receptor molecules on immune cells (59). This broader repertoire of immune receptors could effectively recognize a wider array of pathogens. This divergence might reflect evolutionary adaptations that cater to distinct immune system demands between the two sexes (65). We know that sex-specific pathogens exist in other fish species due to sex-specific behaviours, such as shoaling behaviour in female guppies' increased ectoparasite loads (84). Preliminary in house data suggests that there is a difference in ectoparasite prevalence between male and female pipefish and there is evidence to suggest that parent-specific transgenerational immune priming does occur in broadnosed pipefish (9). The increased number of YM-biased immune genes are in contrast to studies in mammals (Figure 4), where it has been shown that cell-mediated immunity seems to be more pronounced in females with both DC antigen-presenting genes, TLRs and immunoglobulins proliferating at higher rates (33,85). Furthermore, sex-hormone-specific research has shown that androgens typically induce an anti-inflammatory response (33,86), increasing also *il10* production, which conversely, was significantly upregulated in female pipefish (having higher oestrogen levels) in our dataset, with also numerous pro-inflammatory markers being upregulated in males (with higher

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testosterone) (Table S3). The enriched immune pathway in YM might indicate a sex pattern reversal of inflammatory response compared with conventional sex-role species (Figure 4), thus making pregnancy and the life-history trade-offs play a more significant role here. It was surprising however, that we did not find the same immune system pathway enriched in OM and that there were fewer sex-biased immune genes between OM and OF. Aging is associated with a decline in immune function, often referred to as immune senescence (87). This decline tends to affect both males and females similarly and can lead to a more uniform immune response among older individuals (87,88). This could in part explain our results, but further research is needed.

Our results illuminate that sex-biased gene expression is a dynamic interplay influenced by both biological sex and sex-specific life history traits. Within this intricate framework, resource allocation trade-offs into parental care can exert profound impacts on the patterns of sex-biased gene expression. Notably, our study suggests that some genes may be more influenced by biological sex, while others are shaped by sex roles. While these findings provide insights into potential differences in resource allocation between the sexes, it is important to note that direct quantification of reproductive investment requires additional research, such as assessments of reproductive effort or energy allocation. Moreover, to gain a more holistic understanding of the molecular processes governing sex-specific gene expression, it is crucial to consider post-transcriptional events, including the influence of non-coding RNAs on protein synthesis. Our research reveals that the gene expression landscape undergoes significant changes with age. Males and females exhibit distinct gene expression profiles, with some pathways converging (e.g., in the pipefish immune system) and others diverging (e.g., in anti-aging mechanisms) over time. These age-related shifts underscore the importance of studying sex-biased expression also in context of senescence and its distinct physiological changes between males and females. The interplay between biological sex, sex roles, age, and organ-specific factors can yield diverse and context-dependent gene expression patterns. In future research, we aim to extend our research beyond a single species of syngnathid, given the diverse array of pregnancy gradients, sex roles, and mating systems present within the family (24,89). We hope to have underscored the importance for a nuanced approach to the study of sex-specific gene regulation, accounting for the multifaceted nature of sex and sex roles in shaping gene expression dynamics.

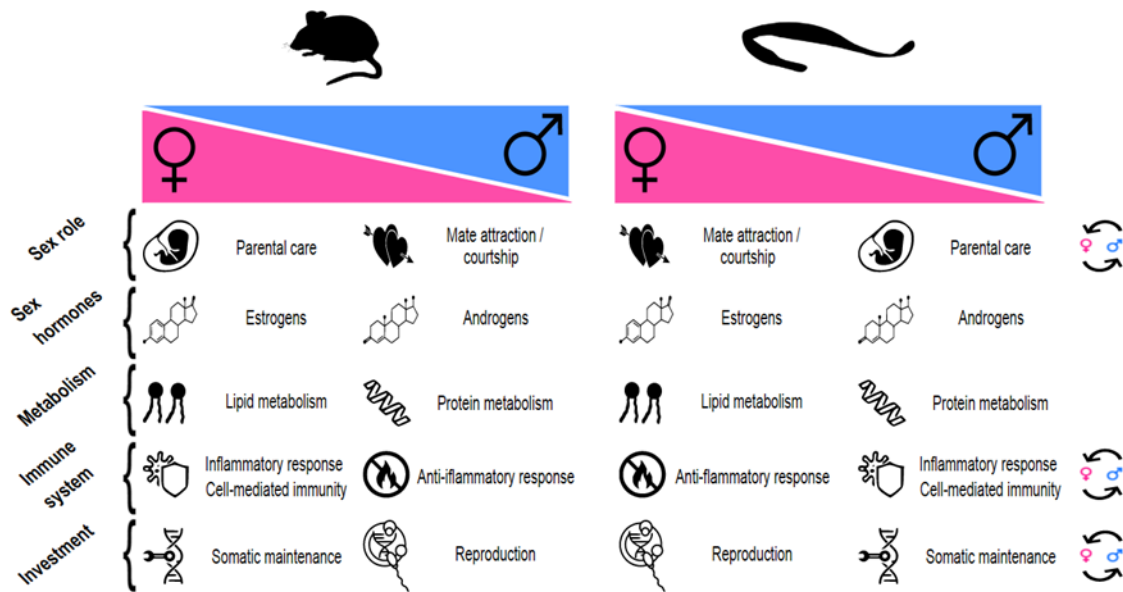


Figure 4: This section provides a summary comparison of traits between a conventional sex-role mouse model and the broadnosed pipefish, which displays sex-role reversal. The diagram highlights the differences in these traits based on biological sex (magenta for females and blue for males). Circular arrows on the far right indicate potential reversals in life-history traits compared to the conventional model species, based on known literature (sex role (24,25,65), sex hormones (79,90), and immune system (11,66,91)) and results presented in this paper (including metabolism, immune system and investment). The relevant literature supporting key points in mammals includes references for sex roles (92), sex hormones (93), metabolism (77,94), immune system (33,88), and investment (77,95).

ETHICS STATEMENT

Work was carried out in accordance with German animal welfare law and with the ethical approval given by the Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Digitalisierung (MELUND) Schleswig-Holstein (permit no. V24257982/2018). No wild endangered species were used in this investigation.

AUTHORS' CONTRIBUTIONS

F.A.P. and O.R. initiated and designed the study. F.A.P. carried out the experiment, analysed the data, interpreted the results and wrote the article. G.G.T. helped develop the DEGs limma:voom pipeline analysis. A.D. performed the orthology analysis and provided help with the GO profiling. O.R. helped during the field work, supervised the project and supported the writing of the manuscript.

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CONFLICT OF INTEREST DECLARATION

The authors declare no conflicts of interest.

DATA ACCESSIBILITY

Supplemental information include additional figures (Figure S1-S2), morphology measurements (Table S1), the significant differentially expressed genes (Table S2-S3), the gene set enrichment analysis results as tables (Table S4) and information on quality and quantity of RNA for RNA-Seq (Table S5). The raw sequencing data and metadata used in this study is available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject ID PRJNA943164 with submission ID SUB12940929. The whole genome assembly of *Syngnathus typhle* can be found under BioProject ID PRJNA947442 with submission ID SUB12974183.

DECLARATION OF AI USE

We have not used AI-assisted technologies in creating this article.

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CHAPTER III

The effect of parental age on the quantity and quality of offspring in *Syngnathus typhle*, a species with male pregnancy

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ABSTRACT

Parental age impacts offspring quantity and quality, with most studies focusing on the mother's age, who produces costly eggs and often carries out pregnancy, hampering the distinction between trans-generational age effects due to egg quality or physiological deterioration. To address this, we investigated the ramifications of parental age on the offspring in *Syngnathus typhle*, a pipefish species with male pregnancy, allowing us to separate these two female traits. By mating young and old parents we examined the impact of parental age on offspring number, size and gene expression. Our results revealed insights into differential gene expression patterns in offspring, strongly influenced by paternal lineage but minimally affected by maternal age. Offspring from old fathers exhibited notable changes in gene expression profiles, related to cell cycle regulation, protein synthesis, stress response, DNA repair and neurogenesis. Our findings provide valuable insights into the role of pregnancy in shaping offspring physiology. Moreover, we recognize the value of assessing a broader range of species that have evolved with sex-specific differences in parental investment vs. gamete provisioning, as the age of either the mother or father may hold greater significance than the other in influencing offspring fitness.

Keywords: parental investment, sex role, pregnancy, ageing, pipefish, gene expression

1. INTRODUCTION

The burden of parental care and the energy investment necessary for reproduction are often on different scales between males and females. In many species, energy investment differs based on the size of the gametes (known as anisogamy), with eggs typically much larger than spermatozoa and therefore regarded as more expensive to produce ¹. This primary difference is supposedly at the root of sexual selection, with females having a higher cost in offspring investment compared to males and, in turn, being the choosy sex ². Furthermore, in numerous animals, females also have the burden of pregnancy, and in mammals lactation and child-rearing. This is no small feat, as live-bearing, also known as viviparity, is considered one of the most significant forms of parental investment ³. This reproductive

strategy enables the embryo to remain inside the womb until it has fully developed, providing protection from external threats, and ensuring the best possible conditions for embryogenesis. Viviparity has evolved independently more than 150 times in vertebrates, including fishes, amphibians, reptiles, and once in mammals ³.

Across many taxa, parental age at the time of copulation affects the number of offspring produced, their fitness, and sometimes their ultimate lifespan ^{4,5}. With advancing age there is an increased likelihood in many species that genetic mutations and epigenetic variations accumulate, telomeres are shorter, the immune system weaker, and altered cell communication leads to cancer, increasing the likelihood of having offspring with birth defects or decreased lifespan ⁶. In the common shag (*Phalacrocorax aristotelis*), an avian species, chicks produced by older parents experienced greater telomere loss during their growth period ⁷. Older parents may inadvertently contribute to shorter offspring lifespans due to factors like increased frailty and accelerated ageing ^{4,7}. Studies in mice or Asian elephants revealed that the offspring from older mothers or even grandmothers had a shorter lifespan ^{8,9}.

This process represents a constraint on parental influence, as it is largely beyond the control of the parent. Conversely, there is a hypothesized mechanism known as transgenerational plasticity, where parents could potentially adapt their offspring's phenotype to the expected future environment ^{10,11}. This mechanism is believed to facilitate the adaptation of embryos to their expected environment, potentially enhancing their overall fitness ¹⁰. This rapid acclimatisation primarily relies on epigenetic modifications, as opposed to genetic changes that require the accumulation of mutations over multiple generations ¹². Some epigenetic modifications can disrupt cell communication and molecular processes, potentially affecting future generations as they are passed down to the embryo ¹³.

Experimental studies on parental investment and brood quality have focused primarily on maternal characteristics, seeing them as a major component of the offspring's own fitness, as females typically invest more not only in egg production but also in carrying and nurturing ¹⁴. Nonetheless, the designation of sex (egg or sperm producer) and their respective sex role (i.e., parental care, courting, etc.) can be more ambiguous in the animal kingdom ¹⁵. The realm of bony fish has seen the evolution of many forms of paternal care, from egg guarding, to mouth brooding, and varying degrees of pregnancy ^{16,17}. Some of the most dedicated male parents can be accredited to the *Syngnathidae* family, which has evolved a range of male pregnant brooding forms from eggs carried on the males' tails (e.g., *Nerophis ophidion*) to fully formed male pregnancies in brood pouches equipped with placenta-like system (i.e., *Hippocampus erectus*), providing the embryo with nutrients, oxygen and immunological components ^{18–21}. Furthermore, this diverse family of colourful fish exhibits a remarkable range of reproductive behaviours, including various sex roles (from conventional to reversed), mating systems (monogamy, polygamy, polyandry), and even prolonged gestational periods in some males (ranging from 9 to 69 days) ^{22–24}. Despite the fascinating array of reproductive strategies in syngnathid, our understanding of how these diverse sex roles and associated life history trade-offs influence offspring fitness remains limited. Gaining a deeper understanding of these dynamics holds significant implications for various fields, including evolutionary and reproductive biology, life history theory, and even conservation strategies aimed at managing populations with unique reproductive strategies.

For this reason, we used the broad-nosed pipefish *Syngnathus typhle*, as our study species. This species not only features male pregnancy with an inverted semi-sealed brood pouch²⁵, but also exhibits sex-role reversal, where females compete for access to males during the breeding season²⁶. In this unique system, males are more reproductively constrained due to the size limitations of their brood pouches, while females produce more eggs on average than males can brood simultaneously²⁷. The strength of sexual selection is reversed in this instance, with males being the choosier sex seeking out larger and more attractive females²⁶. We aim to investigate how maternal and paternal age, affect offspring quantity and quality in this sex-role-reversed species with male pregnancy, with a focus on whether the offspring's phenotype is more influenced by the mother or the father. This unique model species allows us to disentangle the effects of egg and pregnancy, typically female-specific traits, and explore the value of the eggs provided by the mother, as well as the role of the pregnant father in shaping the next generation.

This study aimed to address several key questions related to the impact of parental age and sex roles on offspring phenotype at birth in the unique reproductive system of *Syngnathus typhle*. Specifically, we investigated how increased paternal investment and paternal age influence offspring outcomes compared to maternal egg provisioning. One central question was whether the genetic history provided by the mother in the egg still outweighs the potential effects of the father's costly time and energy investment throughout pregnancy. Additionally, we sought to explore whether somatic age, often considered detrimental to reproduction and offspring health, holds true in a species where fertility increases with age. To address these questions, we conducted controlled mating experiments involving different combinations of parental age, including old males with old females (OMxOF), old males with young females (OMxYF), young females with old males (YFxOM), and young females with young males (YMxYF). We gathered quantitative data, such as the duration of pregnancy, the total offspring count, and morphological measurements at birth. In parallel, we performed full transcriptome mRNA sequencing (RNA-Seq) on at least five offspring from each mating pair to identify differentially expressed genes in the offspring based on the parental mating combination.

We hypothesise that the effects of paternal age may surpass those of maternal age in *S. typhle* due to the resource-intensive nature of paternal investment, which is expected to vary based on the father's body size and life history. The findings from this study are of paramount importance as they provide insights into factors that could significantly impact reproductive biology and offspring fitness in non-conventional sex-role species. By investigating the intricate dynamics of parental investment and age, our study contributes to a deeper understanding of the reproductive strategies and evolutionary biology of *S. typhle*, shedding light on how these factors may be crucial in shaping the fitness of the next generation in this unique fish species.

2. METHODS

2.1 Sample collection

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Syngnathus typhle was collected in late spring from two locations: the Limfjord in Trudstø, Denmark (56°47'N; 8°66'E) and the southwest Baltic Sea (54°39'N; 10°19'E). The pipefish were brought in separately to the GEOMAR Institute where they were divided by sex and placed in 100-liter tanks at either 30 ppt (Limfjord) or 18 ppt (Baltic) salinity, maintained at a temperature of 14°C. The fish received two anti-parasite treatments to avoid importing detrimental parasites into our laboratory environments. This consisted of a combination of Malachitgrün oxalat (0.18g/100mL) and Formaldehyde (2.06g/100mL), applied once on the first day and then once again on the third day. After which, to keep experimental conditions the same and to acclimate the pipefish collected from Trudstø to Baltic seawater, a slow filtered inlet drip was used over the course of a week till 18ppt was reached for all pipefish. After which, all pipefish were kept for another week with the temperatures very slowly being increased to 18°C, simulating environmental temperature conditions optimal for mating.

Pipefish that were roughly one year old were categorized as young, and those above 2 years as old. This was done by relying on our extensive fieldwork expertise since there aren't established models for studying non-model organisms like pipefish. Broadnosed pipefish are active in mating and breeding during spring, migrate during winter, and return to the seagrass meadows of the southwestern Baltic Sea for breeding in the following year ²⁸. It's noteworthy that offspring from one breeding season typically don't reach sexual maturity within that season, especially for male pipefish who need a functional brood pouch to carry female eggs.

Before the experiment began, the body mass and length were measured. One non-pregnant male and one female from the same population were then placed together in 30 individual tanks based on their age, where they were kept for a 2-week mating period. This time is more than sufficient to allow for the fish to mate, seeing as their typical reproductive window is quite brief. There was a higher number of pairs from Trudstø (23 pairs: 5 OMxOF, 6 OMxYF, 6 YMxOF, 6 YMxYF) compared to Falkenstein (7 pairs: 2 OMxOF, 1 OMxYF, 2 YMxOF, 2 YMxYF). The difference in the number of pipefish from each site was due to fewer fish being available at Falkenstein. Subsequently, the duration of male pregnancy was recorded. Once the offspring were born, five individuals from each parent were sampled and euthanized with an overdose of Tricaine mesylate (MS-222; 500 mg/l; Sigma-Aldrich), measured for total body length and weight. The whole body was immediately preserved in RNA later, as their organs were too small and translucent for individual dissection. The samples were stored at 4 °C for three days before being transferred to -20 °C for long-term storage.

2.2 RNA isolation and mRNA sequencing

To extract RNA from whole-body tissues, the RNeasy Mini Kit from Qiagen (Venlo, Netherlands) was used following the manufacturer's instructions for a total of 98 samples, roughly five offspring from five different parents and when pooled 25 for each parental age group (25 OMxOF, 23 OMxYF, 25 YMxOF, and 25 YMxYF). The quantity of extracted RNA was measured using a Pqlab NanoDrop ND-1000 spectral photometer (Erlangen, Germany) and the samples were stored at -80°C. Library preparation was performed at BGI Tech Solutions in Hong Kong using the DNBSEQ Eukaryotic Strand-specific

mRNA library, and then stranded mRNA sequencing was performed on a DNBseq platform (150bp reads, 25M clean paired-end reads per sample).

2.3 Statistical analysis

Statistical analysis was done in Rstudio v.4.2.2²⁹. To analyse the collected data on body size, pregnancy duration, and offspring quantity, we initially assessed the normal distribution of the data and calculated the mean and standard deviation. Next, we conducted separate two-way ANOVAs for the length and weight of the adult pipefish that successfully mated and gave birth to determine how significantly different young and old parent pipefish were (11 OM, 14 OF, 13 YM, 10 YF). The same test was done to assess any differences in pregnancy duration between various mating groups. Additionally, for the total offspring count and their morphological measurements at birth, we performed a one-way ANOVA test [e.g., `aov(offspring-quantity ~ Mating-age-combinations, data = data)`], followed by a Tukey's Honest Significant Differences (HSD) test. The HSD test allowed us to conduct a closer pairwise comparison between the different groups. With regards to the RNA-Seq data, we performed a PERMANOVA (Permutational Multivariate Analysis of Variance Using Distance Matrices) using the "adonis2" function, "bray" method and family as strata. We did this analysis on logarithmically transformed count data to determine whether there were significant differences in gene expression between parental mating combinations and their sampling location. After normalizing for differences in library size, filtering out low counts, and scaling to counts per million (cpm) as described in point 2.4, we performed a regularized log transformation procedure (rlog) on the data for a principal component analysis (PCA) to detect group clustering and possible outliers (Fig.S3). Two outliers (Fig. S3) identified during PCA analysis were distorting the data distribution, affecting our analysis. We removed them to ensure robust data normalization and improve differential gene expression analysis accuracy.

2.4 Gene expression analysis

Quality control of the resulting reads from the Illumina sequencing was carried out using FastQC v.0.11.9³⁰, and trimming was performed using Fastp v.0.20.1³¹. Subsequently, the reads were aligned to a whole genome assembly of *Syngnathus typhle* (BioProject ID: PRJNA947442) using STAR v.2.7.9a³². Transcript abundance was calculated using TPMCalculator³³. We used Orthofinder v.2.4.0³⁴ to assign functional information to annotated genes with a species list including the following accessions: *Takifugu rubripes* (rubripes.fTakRub1.2), *Syngnathus rostellatus* (GCF_901709675.1, fSynAcu1.2), *Syngnathus acus* (GCF_901709675.1, GCF_901709675.1_fSynAcu1.2), *Nerophis ophidion* (unpublished), *Oryzias latipes* (ASM223467v1), *Hippocampus erectus* (PRJNA613176), *Hippocampus comes* (H_comes_QL1_v1, GCA_001891065.1), *Lepisosteus oculatus* (LepOcu1), *Gasterosteus aculeatus* (BROADS1), and *Danio rerio* (GRCz11). Differential gene expression (DGE) analysis was performed on the RNA-Seq data obtained from Illumina in Rstudio v.4.2.2²⁹, using *S. acus* orthology annotations. The edgeR package v.3.40.2³⁵ was used for scaling to counts per million (cpm) and normalising for differences in library size, and genes with less than 10 counts in at least five libraries were discarded. We then normalised for composition bias by trimmed mean of M values (TMM) with `calcNormFactors` function, which calculates a set of normalization factors for each sample thereby

eliminating composition biases between libraries. After this, we used the *limma* package v.3.54.1³⁶, as a linear model-based method that uses empirical Bayes approach and *voom* method (converts count data to continuous scale) to identify differentially expressed genes (DEGs). *Limma* is a software package useful in accounting for batch effects and mitigating other sources of technical variation, while *voom* has the best power of methods that control the type I error rate and is specifically designed to handle library sizes that vary by an order of magnitude or more³⁷. This capability was crucial in this experiment because we wanted to reduce any batch effects and type I errors in DGE results due to pooling five individuals per parent and thus not being able to entirely avoid direct parental effects. The differential expression analysis comparing several groups was done by creating a matrix of independent contrasts, this way it was possible to perform a one-way analysis of deviance (ANODEV) for each gene. After which, we estimated coefficients of interest between groups to have log fold changes (logFCs) with the function *contrasts.fit*, and performed an empirical Bayes moderation of the estimated variance which shrinks sample variance and applies a moderate t-test to identify DEGs. For further downstream analysis, we only used the resulting adjusted P values below 0.05 to ensure that the observed treatment effects were real.

2.5 Orthology and enrichment analysis

A homology-based search of *Danio rerio* was used with OrthoFinder to annotate DEGs for gene ontology analysis. The *Danio rerio* orthologues of the DEGs per contrast group were then uploaded to g:Profiler (last accessed in March 2023) to conduct functional profiling based on the offspring's parental mating age combination (OMxOF vs. YMxYF, OMxOF vs. YMxOF, OMxOF vs. OMxYF, YMxYF vs. YMxOF, YMxYF vs. OMxYF). We set the statistical domain scope to "Only annotated genes" and the significance threshold to "g:SCS threshold" at 0.05. We also treated numeric IDs as "ENTREZGENE_ACC" and limited the data sources to GO biological processes and biological pathways from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Reactome (REAC) databases. The results of the analysis are presented in table S1.

3. RESULTS

3.1. Quantitative differences between offspring of different parental age combinations

Morphological measurements of length and weight of the parents showed a difference in total body size between young and old females ($P = 5.2\text{e-}12$) and for weight ($P = 3.69\text{e-}10$), as well as young and old males (length, $P = 4.6\text{e-}05$; weight, $P = 0.00413$). OFs had a mean size of 19.36 cm (SD = 0.72), YFs 14.56 cm (SD = 1.05), OMs 17.05 cm (SD = 1.47) and YMs 14.38 cm (SD = 1.11). Supplementary material Figures S1 A-D provide additional boxplot results of body weight and length of male and female parents.

Of the original 30 *S. typhle* couples, 24 successfully mated, males accepted and fertilised the eggs and followed through with the pregnancy. We removed the five couples who did not successfully mate from any further analysis, these included 1 OMxOF, 2 YMxYF, 1 OMxYF (Trudsø population) and 1 YMxYF,

1 OMxYF (Falkenstein population). Of the 24 pregnant males, we tracked their gestational duration and found no significant difference between the four different groups of mating combinations (Fig.1A). The average pregnancy time for each group was as follows: OMxOF 24.7 d (SD = 1.97), OMxYF 23.8 d (SD = 2.39), YMxOF 23.5 d (SD = 2.14), and YMxYF 25 d (SD = 1.58). There were however differences in offspring number, with the strongest significant difference ($P = 0.0013$, HSD test) between OMxOF and OMxYF (Fig.1B) and less so between YMxYF and OMxOF ($P = 0.027$). OMxOF had the highest number of offspring with an average of 68 (SD = 25.5), and OMxYF had the lowest average with 15.4 (SD = 9.1), while the other two groups were somewhere in between with YMxOF 46 (SD = 18.4) and YMxYF 31.4 (SD = 20.8). From the resulting offspring that we collected (five per family) right at birth for gene expression analysis, we found no significant difference in weight but a significant difference in body length between the fry having older parents (OMxOF) and all other groups (Fig.1C).

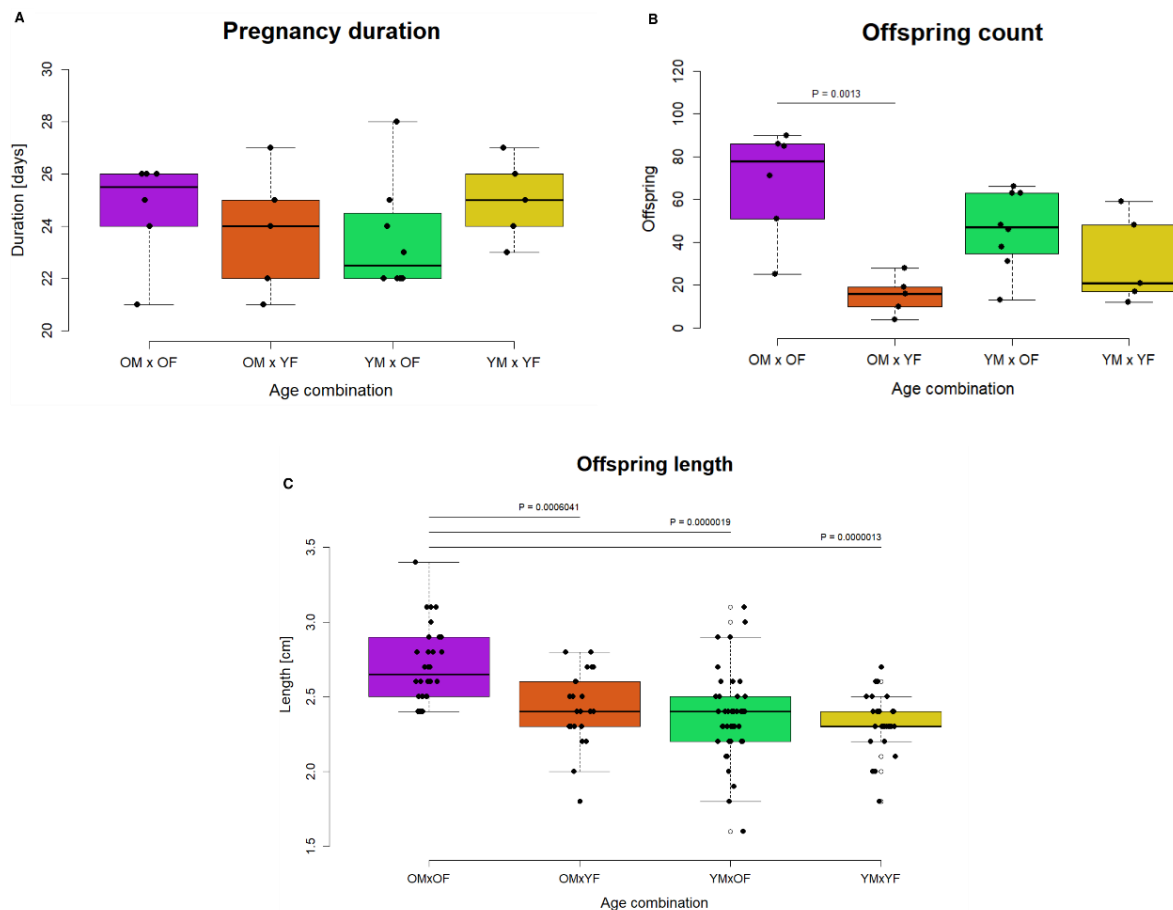


Fig.1: Boxplots of various quantitative measurements between different combinations of parental age of mating. In purple old male with old female, in orange old male with young female, in green young male with old female, and yellow young male with young female. Highly significant P values from HSD test are marked above the boxplots with a line designating the comparison groups. A) Boxplot of duration of the pregnancy in days. B) Boxplot of the offspring count per parental group. C) Boxplot of total body length immediately after birth of five offspring per parent in centimetres.

3.2. Age of the male parent plays a critical role in offspring DGE

Using a PERMANOVA, with factors being parental age combinations and location, on the 26072 transcripts from the sequenced *S. typhle* offspring, we found that parental age combinations were significant ($P = 0.013$), while the difference between sampling locations were not significant ($P = 0.116$), presumably as both parental populations were kept under the same conditions for several weeks before mating. With a PCA, we saw no differences between parental populations in numerous PCs (Fig.S2A-C) and were therefore confident that parental sampling location had no meaningful effect on the offspring and could group them together. When we then distinguished between the four different parental age groups we noticed that PC1 accounted for 20% of the variation, with the individual offspring clustering predominantly according to the father's age (Fig.2A). One can observe that on the left side of the plot individual offspring clustered based on parent's from OMxOF and OMxYF combinations, here old male being the common denominator, while on the right were offspring from YMxYF and YMxOF (Fig.2A), young males being the common denominator.

The analysis produced distinct numbers of DEGs for offspring from different parental age combinations (Fig.2B). When the age of both parents changed, 2874 genes were differentially expressed (DE) for OMxOF vs. YMxYF, and 1716 for OMxYF vs. YMxOF (Fig.2B). When the age of only the male (or father) changed, 1417 genes were differentially expressed for OMxOF vs. YMxOF, and 4630 DE for YMxYF vs. OMxYF (Fig.2B). In contrast, changes in female (or mother's) age produced the lowest number of DEGs, with 97 for OMxOF vs. OMxYF and 61 for YMxYF vs. YMxOF (Fig.2B). The direction of gene regulation for the different contrast groups is shown using log-fold change (logFC) in volcano plots in figure 2 from C to H, with the majority of the DEGs being between $\logFC \pm 0.5$. When the age of the female changed, very few genes exceeded this threshold (Fig.2D and G). In contrast, when the age of the father changed, many more genes had a higher logFC (Fig.2E and F).

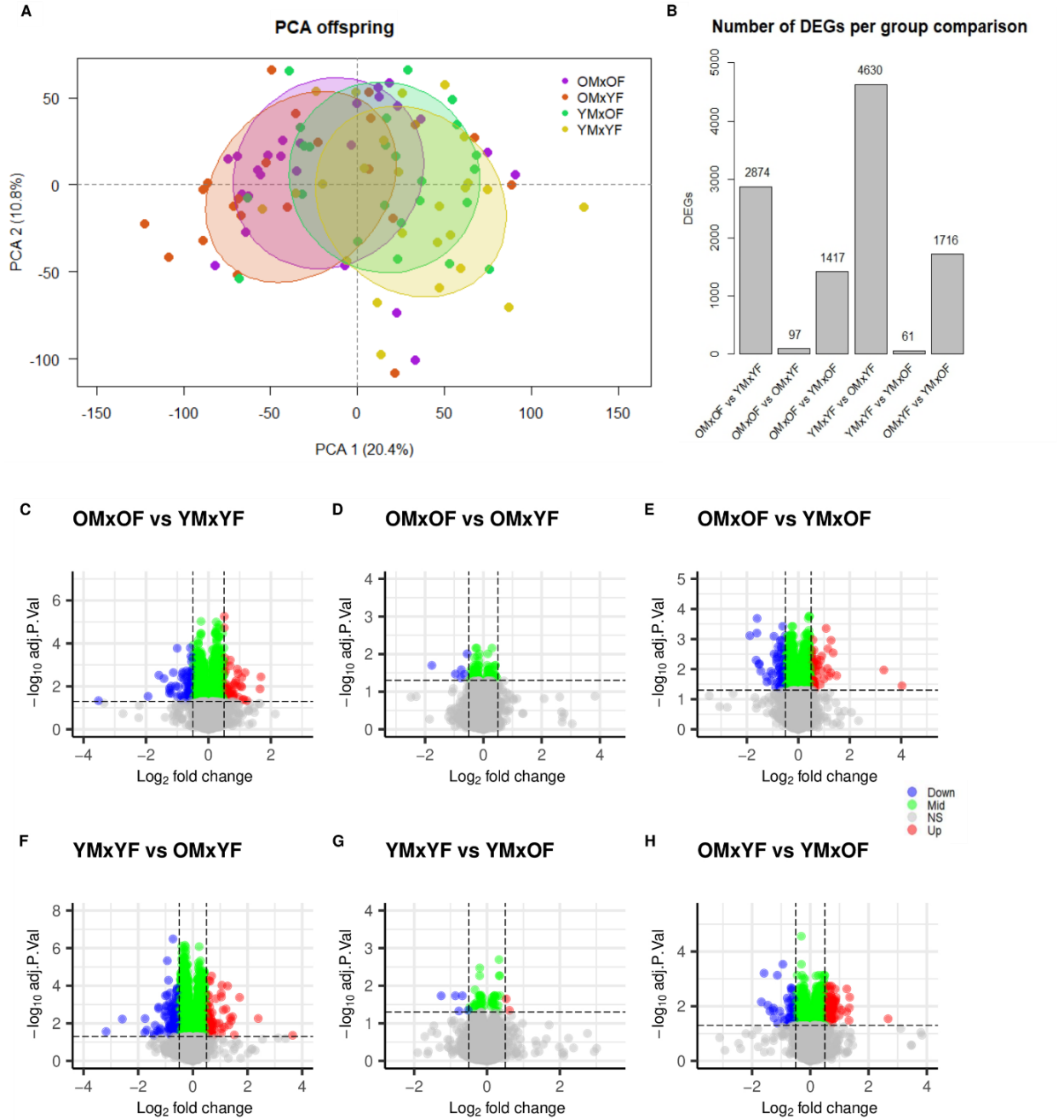


Fig.2: Results of the DGEs analysis. A) PCA of pipefish offspring grouped by parental age combination: Old Male x Old Female (purple), Old Male x Young Female (brown), Young Male x Old Female (green), and Young Male x Young Female (yellow). B) Bar graph showing the number of DEGs for each contrast comparison. C-H) Volcano plots showing the DEG results for different contrast groups. The x-axis represents the log2 fold change, and the y-axis represents the negative log10 of the adjusted P-value. Grey dots represent non-significant values (adj.p.val > 0.05), red dots indicate significant genes with a positive logFC above 0.5 (up-regulated), blue dots indicate significant genes with a logFC below -0.5 (down-regulated), and green dots are significant but with a small logFC (between -0.05 and 0.05).

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The results of the functional profiling of the DEGs in each comparison group showed no enriched or over-represented categories for the OMxOF vs. OMxYF and YMxYF vs. YMxOF groups due to the low number of DEGs. On the contrary, when the age of the father changed, we found pathways enriched in categories such as cell cycle, cell stress and response, protein synthesis and regulation, signal transduction, metabolism and more (Tables 1 and S2). The majority of overlapping pathways were found between OMxOF vs. YMxYF and YMxYF vs. OMxYF (Table 1). In the first group (OMxOF vs. YMxYF) it was complicated to disentangle what effects may be paternal or maternal, but we are confident that in the second group (YMxYF vs. OMxYF) effects observed are driven by male age. One of the few pathways that we found to be enriched in both OMxOF vs. YMxOF and YMxYF vs. OMxYF was the extension of telomeres, which activates telomere enzymes to add new telomere repeats onto the ends of chromosomes³⁸. Upon examining the genes involved in the extension of telomere pathway, we found that out of a total of 26 genes belonging to this pathway, 8 DEGs were present in OMxOF vs. YMxOF and were all down-regulated in offspring from YMxOF, while for the other group, there were 15 DEGs which were all upregulated for OMxYF (Tables S1 and S2). However, these genes were either *replication factors* or *polymerase subunits*, and we found that some overlapped with other pathways such as DNA replication and DNA repair, as frequently such genes are involved in a complex network of molecular processes contributing to the maintenance of genomic integrity.

There were enriched pathways pertaining to metabolism, protein synthesis, cell signalling and transduction that occurred only when the offspring had an old father (Tables 1 and S2). Over-represented genes in pathways such as translation, protein folding, metabolism of RNA, amide metabolic process, regulation of *PTEN* (*Phosphatase and Tensin Homolog*) stability and of *RAS* (*Rat Sarcoma*) by GAPs (GTPase-Activating Proteins), signalling by *Hedgehog* (*Hh*), and *MAPK* (*Mitogen-activated protein kinase*) signalling were predominantly, if not exclusively, up-regulated in offspring from older father's (Tables S1 and S2). Although there were some overlaps in the genes involved in these pathways, many of them encoded for mitochondrial ribosomal proteins, heat shock proteins, *cullin*, chaperones, prefolding subunits, proteasome activators or subunits, splicing factors, and *MAPK* (Tables S1 and S2).

Furthermore, the pathway for Orc1 removal from chromatin was enriched in OMxOF vs. YMxYF and YMxYF vs. OMxYF, with approximately 27 and 43 genes, respectively. Notably, 25 of the 27 Orc1-related genes were upregulated in offspring from OMxOF, while all 43 genes were upregulated in OMxYF (Table S2). Orc1 is a crucial protein in the initiation of DNA replication in eukaryotic cells and it has been found to have a strong impact on gene expression³⁹. On a similar note, offspring with an old male as father had enriched p53 pathways, which are involved in the response to DNA damage and other cellular stressors. The p53 protein is a transcription factor that regulates the expression of genes involved in cell cycle arrest, DNA repair, apoptosis, and senescence⁴⁰. The majority of genes involved in this pathway were up-regulated in offspring with an older father, regardless of the mother's age (Table S2). These included replication factors, cyclin-dependent and checkpoint kinases, and tp53-regulating kinases (Table S1). Furthermore, *lamtor3-5* were upregulated, which are important components of the

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Ragulator-Rag complex, which is responsible for regulating cell metabolism, trafficking in eukaryotic cells, and also the mTOR pathway, as it responds to amino acid levels in the cell ⁴¹.

We found GO terms for neurogenesis, neuron differentiation, neurotransmitter transport and so forth (Table 1 and S2), only for DEGs between YMxYF vs. OMxYF. In particular, for neurogenesis alone (pathway includes 900 genes) there were 200 DEGs associated with this pathway (in YMxYF vs. OMxYF), of which 177 were up-regulated in offspring from YMxYF (Tables S1 and S2).

OMxOF vs. YMxYF	OMxOF vs. YMxOF	YMxYF vs. OMxYF	Pathway	Term ID	Description
Yes	Yes	Yes	DNA replication	REAC:R-DRE-69306	Cell cycle
Yes	Yes	Yes	Mitotic G1 phase and G1/S transition	REAC:R-DRE-453279	
Yes	Yes	Yes	S Phase	REAC:R-DRE-69242	
Yes	No	Yes	G1/S Transition	REAC:R-DRE-69206	
Yes	No	Yes	Orc1 removal from chromatin	REAC:R-DRE-68949	
No	Yes	Yes	Extension of Telomeres	REAC:R-DRE-180786	
Yes	No	Yes	Amide metabolic process	GO:0043603	Metabolism
Yes	No	Yes	Metabolism of RNA	REAC:R-DRE-8953854	
Yes	No	Yes	Macromolecule catabolic process	GO:0009057	
Yes	Yes	Yes	Cellular response to stress	GO:0033554	Cell stress response
Yes	No	Yes	p53-Dependent G1 DNA Damage Response	REAC:R-DRE-69563	
No	No	Yes	Transcriptional Regulation by TP53	REAC:R-DRE-3700989	Protein synthesis and regulation
Yes	No	Yes	Translation	REAC:R-DRE-72766	
Yes	No	Yes	Protein folding	GO:0006457	
Yes	No	Yes	Deubiquitination	REAC:R-DRE-5688426	
Yes	No	Yes	Regulation of RAS by GAPs	REAC:R-DRE-5658442	Cell signal transduction
Yes	No	Yes	Regulation of PTEN stability and activity	REAC:R-DRE-8948751	
Yes	No	Yes	Signalling by Hedgehog	REAC:R-DRE-5358351	
Yes	No	Yes	Signalling by WNT	REAC:R-DRE-195721	
Yes	No	Yes	FLT3 Signalling	REAC:R-DRE-9607240	
Yes	No	Yes	MAPK6/MAPK4 signalling	REAC:R-DRE-5687128	
No	No	Yes	Neurogenesis	GO:0022008	Neuron development
No	No	Yes	Neuron differentiation	GO:0030182	
No	No	Yes	Neurotransmitter transport	GO:0006836	

Table 1: Summary table of most notable pathways from the gene set enrichment analysis. In the first three columns are the different parental group matches, where yes or no indicated if they had genes that were over-represented in a particular pathway.

4. DISCUSSION

Our study aimed to research how maternal and paternal age at reproduction affects offspring quantity and quality in *Syngnathus typhle*, a sex-role reversed species with male pregnancy. We observed major differences in the number of offspring at birth based on parental age (Fig.1B), despite male and female age not affecting pregnancy time or duration (Fig.1A). Having both parents being older led to the highest number of offspring, which can be attributed to the larger brood pouch of male pipefish and the ability of larger females to produce more eggs. Interestingly, the lowest number of fry came from couples where the father was old and the mother was young, rather than from couples where both parents were

young (Fig.1B). This finding suggests that older males may be less willing to accept eggs from younger females and may wait to fill their brood pouches to capacity when they encounter a larger and higher quality mate ⁴². Due to their polygamous mating behaviour, male *S. typhle* mate with multiple females, and embryos from different mothers can be found in a single pregnancy ²⁸. We hypothesize that sexual selection, in this case male pipefish's larger selection of partners, may explain the low number of offspring observed in couples with an old male and a young female (OMxYF). We also observed a significant difference in body size measurements of the newly born juvenile pipefish between OMxOF and all other groups (Fig1.C). As egg size correlates positively with female body size in pipefish, having an older mother may likely provide a developmental advantage, as she will provide larger and more nutrient-rich eggs, resulting in larger embryos ⁴³. In the wild, bigger is generally better, as the offspring will be less vulnerable to predators, can swim faster for resources and are more competitive ^{44,45}.

Examination of transcriptome-wide DGE revealed that the numerical disparity in DEGs among offspring from various combinations of parental age is primarily driven by the paternal lineage, with exceptionally many DEGs found when comparing offspring with both young parents (YMxYF) with those differing only in an older father (OMxYF) (Fig.2B). Interestingly, a PCA showed that the offspring grouped according to their father's age (Fig.2A), regardless of the age of the mother. This suggests that paternal age and his experienced life history, influence more strongly gene expression in *S. typhle* offspring, compared to the information provided by the mother in the egg. This may imply that the pregnant individual having longer contact with the developing embryos could influence epigenetic changes during embryogenesis and transmit non-genetic information, such as immune history and microbial communities, impacting offspring phenotype and possibly influencing their survival and reproductive success in the future ^{10,11,25,46}. Therefore, pipefish offspring may generally profit from the increased size and age of the mother by starting with a larger embryo, but their gene expression at birth and phenotype are likely more heavily influenced by the father. Research in species with female pregnancy has shown that the mother has a stronger effect on offspring phenotype, as information passed from her ecological environment to the offspring is a more truthful signal (i.e. salinity, temperature, food availability etc.) compared to the phenotype passed down from the father ^{47,48}. Yet, in such cases, one cannot differentiate whether it is due to the information provided in the egg or truly the increased parental care (i.e., pregnancy), as the two are intermingled within the female. Our results suggest that in *S. typhle* the reverse is happening, as developing embryos cannot predict their future environment, they will likely align their developmental trajectory with the paternal phenotype, thus making pregnancy a crucial element in transgenerational plasticity. Offspring DGE being driven by paternal age is an indication that pregnancy, and thus increased time and energetic investment in progeny, has a stronger effect on offspring gene regulation compared to the germline information alone. Unfortunately, from the RNA-Seq data we still cannot completely disentangle if these paternally inherited effects are predominantly epigenetically controlled and/or due to vertical transferral of immunological components or even due to the microbial pouch environment being transferred to the offspring.

As the paternal age appears to count for more in affecting offspring gene expression, are older *S. typhle* fathers truly detrimental to offspring health? Functional profiling of DEGs revealed that the father's age

was mostly influencing cell cycle, stress response, protein synthesis and regulation, signal transduction, and metabolism of the offspring (Table 1). However, we found that all 43 DEGs included in the Orc1 removal from chromatin pathway were up-regulated in offspring with older male parents (Table S1 and S2), and as removal of Orc1 has been shown to cause defects in DNA replication and loss of integrity of chromatin structure ³⁹, these changes can affect negatively gene expression and other cellular processes that depend on chromatin organization. Moreover, we also found many DEGs between YMxYF vs. OMxYF, that were involved in various cellular processes, such as protein synthesis and regulation, signalling by Hh, MAPK6/MAPK4 signalling (aka ERK3/ERK4), regulation of *PTEN* stability, and regulation of *RAS* by GAPs, majority of which were upregulated offspring from OMxYF (Table 1 and Table S2). These pathways are primarily involved in growth and developmental processes and could indicate that the age of the father at copulation negatively influences the rate of cell division in the offspring or possibly trying to mitigate DNA damage due to response to external stressors, which could both be linked back to telomerase activation ^{38,49}. Studies have shown that de novo mutations in germ cells that occur at higher rates in males, are then inherited and could subsequently lead to dysregulation of numerous cellular functions ⁵⁰. Unfortunately, limited research has been conducted specifically on the influence of parental age on these pathways. However, their biological role is known and could be relevant in offspring development. Hh signalling is particularly crucial in embryonic development, organogenesis, and the renewal of various stem cell populations ⁵¹. The commonly denominated atypical MAP kinase ERK3/ERK4 pathway is lined to having both pro- and anti-oncogenic properties in various animal models ⁵². ERK/MAPK pathway is also known to play an important role in the regulation of neuronal survival and synaptic plasticity ⁵³. The latter fit together with the results from the enrichment of pathways involved in neurogenesis and neuron differentiation between YMxYF vs. OMxYF (Table 1), in which 200 DEGs involved in the neurogenesis pathway alone had the vast majority (177 genes) upregulated in YMxYF (Table S1 and S2).

Nevertheless, we found that out of the 35 DEGs involved in the *PTEN* pathways between YMxYF vs. OMxYF, there were 33 up-regulated in offspring with OMxYF (Table S2). *PTEN* encodes for a protein that plays a critical role in regulating cell growth and survival by inhibiting the PI3K/AKT signalling pathway ⁵⁴, which in turn is crucial for regulating genetic stability and factors such as activating mTOR kinase inducing growth and translation, or by inhibiting transcription factors like FOXO family members thereby activating cell cycle progression and apoptosis ⁵⁴. Activation of members of the mTOR and inhibition of FOXO family have a negative impact on health and longevity ^{55,56}. Therefore, PI3K/AKT is involved in driving tumour progression and up-regulation of *PTEN* leads to its suppression, and in this instance, offspring from young fathers are at a disadvantage compared to those from old fathers. Furthermore, we found that several downstream targets of TP53 were up-regulated in offspring with young fathers compared to offspring from old fathers, in particular two *inducible tp53 proteins* and two *apoptosis-stimulating p53 proteins* (Table S1). TP53 is a transcription factor regulating the expression of a large number of genes involved in various cellular processes, including apoptosis, DNA repair, and cell cycle arrest ⁴⁰. There are synergistic interactions between *PTEN* and *p53*, with the latter binding to *PTEN* promoter regions and regulating their expression ⁵⁴. Also, the enriched pathway for regulation of *RAS* by GAPs, in DEGs from YMxYF vs. OMxYF, can function both as either a tumour suppressor or

as an oncogene depending on the genetic variants within the gene, seeing as *RAS* gene uncontrolled activation can lead to the development of tumours ⁵⁷ and GAP can downregulate said *RAS* genes ⁵⁸. It would appear in this instance, that offspring from young males may have a propensity for developing tumours and have an upregulated cell cycle activity. Unfortunately, we cannot confirm this with biopsies thus limiting assumptions on possible negative health effects being passed on by either older or younger pregnant fathers. In the future, we aim to follow up on the morphological development of the offspring and possible health deterioration or tumorigenesis.

Overall, seeing as many genes involved in Orc1 removal from chromatin were upregulated in offspring from older fathers, leading to a potential increase DNA replication defects and overall loss of chromatin integrity, we think this would result in long-term negative health effects. Even though the offspring of older fathers also had an up-regulation of PTEN-related transcripts, possibly leading to an inhibition of the PI3K/AKT pathway and reducing the risk of tumorigenesis being ultimately beneficial. Particularly, there is a growing body of literature showing that higher *PTEN* activity can influence neuronal survival and growth, contributing to various neurodegenerative disorders ⁵⁹. As we also found several neuronal developmental pathways enriched for DEGs between offspring from YMxYF and those from OMxYF, with many genes down-regulated in offspring having both old parents, we should also consider the possibility that the *PTEN* may negatively embryonic neurogenesis and further research is needed to confirm such effects. We propose that the importance of pregnancy in transgenerational plasticity in pipefish may be primarily attributed to the fact that older fathers have likely experienced a distinct life history compared to younger fathers, including potential exposure to pathogens or environmental stressors (i.e., nutrient availability, temperature fluctuations), which can then be modulating offspring phenotype.

This study delves into the less-explored domain of parental age effects in sex-role reversed fish species, contributing valuable insights into the intricate relationship between parental age, resource allocation, and transgenerational phenotypic plasticity. Our findings highlight the important role of pregnancy (i.e., parental care investment) and the parental life history, encompassing potential exposure to pathogens or environmental stressors, in influencing gene expression and the ultimate phenotype of the offspring. This observation underscores the evolutionary strategy of offspring striving to verify the accuracy of parental signals, thereby mitigating the risks associated with environmental incongruity, as proposed by Bell and Hellmann in 2019 ⁶⁰. These findings expand our current understanding of how parental characteristics shape the next generation in species with reversed reproductive roles, enriching the broader field of evolutionary biology. Moreover, we highlight the importance of moving beyond traditional animal models that oversimplify ageing as a binary division between male and female reproductive systems, and instead, we must acknowledge the complexity of nature, where gender roles can be fluid and ageing can be influenced by trade-offs in life history.

ETHICS STATEMENT

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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 Digitalisierung (MELUND) (permit no. 1288/2021). No wild endangered species were used in this investigation.

AUTHORS' CONTRIBUTIONS

F.A.P and O.R. planned the project. F.A.P and D.K. carried out the experiment. F.A.P. analysed the data, interpreted the results and wrote the article. A.D. performed the orthology analysis and provided help with the GO profiling. O.R. helped during the fieldwork and supervised the project. O.R. supported the writing of the manuscript.

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CONFLICT OF INTEREST DECLARATION

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Supplemental information includes both additional figures (Fig.S1-S3), the *Danio rerio* orthologues of the significant DEGs in offspring pipefish between the various contrast groups (Table S1) and the gene set enrichment analysis results (Table S2). The raw sequencing data and metadata used in this study are available from the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject ID PRJNA973859 and accession number SUB13263866 .

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SYNTHESIS

This thesis consists of three chapters focused on understanding how sex-specific life history strategies affect resource allocation to reproduction or growth and maintenance in species with male pregnancy. Each chapter delves a little deeper at exploring how males and females have evolved distinct life-history strategies to maximize their respective fitness. The chapters emphasize pregnancy's significant energy costs, driving essential shifts in physiological traits (e.g., immune system, DNA repair, metabolism) to ensure the pregnant sex's survival.

Throughout the thesis, attention was directed towards two syngnathid fishes, the lined seahorse (*Hippocampus erectus*) and broadnosed pipefish (*Syngnathus typhle*), in order to depart from the typical focus on heavily researched model species with more "traditional" reproductive strategies towards unconventional model species with highly derived life-history strategies. These fish species display varying degrees of male pregnancy (fully enclosed pouch vs. inverted semi-sealed pouch), mating systems (monogamous vs. polygamous), and sex roles (conventional vs. sex-role reversed).

In **chapter I**, the impact of nutrient availability on *H. erectus* was explored, where males undergo significant energy expenditure for pouch formation while still producing sperm, as opposed to the costly egg production in females. Transcriptome-wide differential gene expression analysis between fasted and non-fasted male and female seahorses in different organs (i.e., liver, head kidney, gonads) and microbial analysis of the hind gut was done to provide insight into resource allocation strategies in a species with male pregnancy. **Chapter II** analysed sex-biased gene expression in the sex-role reversed species *Syngnathus typhle*, where females mainly compete for access to males. Transcriptome-wide differential gene expression analysis was done across a selection of organs, distinguishing primarily between two age groups and sex. **Chapter III** aimed to examine the effects of parental age on the phenotype of the next generation in *Syngnathus typhle*, with a focus on whether offspring's phenotype is more influenced by the mother or the father.

Sex-specific life history strategies determine resource allocation investment

It is widely observed across the animal kingdom that males and females adopt divergent reproductive strategies, in part contributing to distinct lifetime optima (Steven N. Austad and Fischer 2016; Maklakov and Lummaa 2013). In the typical framework, males tend to adopt a "live fast, die young" approach, favouring traits that enhance their secondary sexual characteristics and chances of rapid mating (Bonduriansky et al. 2008). In contrast, females, being the limited sex due to the time and energy required for egg production and gestation, prioritize longevity to maximize their overall fitness. This aligns with the disposable soma theory, which suggests that the body's maintenance and repair processes are metabolically costly (T. B.L. Kirkwood and Rose 1991). Alleles favouring increased resource allocation to reproduction often come at the expense of somatic maintenance, giving rise to sex-specific traits such as size dimorphism, immune dimorphism, and lifespan dimorphism (Van Den Heuvel, English, and Uller 2016b).

Furthermore, distinct reproductive investments can give rise to varying behaviours that increase exposure to environmental hazards, influenced by factors such as body size, conspicuous sexual traits, territory defence, and foraging patterns (Bonduriansky et al. 2008; Graves 2007). These differences in behaviour can result in varying levels of extrinsic mortality, ultimately leading to different rates of ageing (Steven N. Austad and Fischer 2016). For instance, in species such as killifish or three-spined stickleback, males tend to have shorter lives due to their conspicuous sexual traits and the resulting higher risk of predation, resulting in a faster rate of senescence (Candolin 1998; Reichard et al. 2022). This pattern is similarly observed in the kukri snake (*Oligodon formosanus*), where females exhibit higher territorial behaviour and engage more frequently in risky activities, ultimately leading to an overall shorter lifespan (Lee et al. 2019).

Nonetheless, much of the existing research has primarily focused on species conforming to traditional male and female reproductive sex roles. This focus has left a gap in our understanding of how the same theories apply to species with diverse reproductive strategies. In our rapidly evolving world, parental care roles are no longer exclusively defined by gender (Perrone, Wright, and Jackson 2009), and the traditional categories of biological sex based on sex-chromosome are evolving into a spectrum influenced by environmental and cultural factors (Lindqvist, Sendén, and Renström 2021). This phenomenon, which is observed in humans, has always existed in the animal kingdom, where both sex and sex roles can exhibit remarkable flexibility, driven by complex interactions involving genes, hormones, and responses to environmental cues (Anders Berglund, Widemo, and Rosenqvist 2005; De Mitcheson and Liu 2008; Houde 2001; Smith and Wootton 1995). Recognizing the need to explore the applicability of the disposable soma theory beyond traditional models, we turned our attention to non-model species, such as the syngnathid fish family, which includes males capable of pregnancy.

To gain insight into resource allocation in a syngnathid species, we conducted fasting experiments - an essential aspect of gerontology research that had not been explored in Syngnathidae species before (**Chapter I**). [Fasting experiments have been crucial in gerontology research because they offer the potential to extend lifespan, promote cellular mechanisms associated with healthy ageing, and investigate non-pharmacological strategies for combating age-related diseases (Mercken et al. 2012; Speakman and Mitchell 2011)]. We exposed juvenile lined seahorses, aged 3 months, to either a CR or an AL diet. *Hippocampus erectus*, known for its advanced brooding structure within the syngnathid family, invests heavily in gestational functions and brood pouch formation (Dudley et al. 2021). Males do, however, continue producing sperm and have increased testosterone levels, while females produce eggs and have higher oestrogen levels (Scobell and MacKenzie 2011). They exhibit sequential monogamy, with a fixed mate per breeding season, with similar selection imposed on males and females as they both must attract partners (Lin et al. 2021; Rosenqvist and Berglund 2011).

Our findings support the hypothesis that the sex investing more energy in body reshaping for pregnancy and facing a time constraint due to pregnancy duration will prioritize reproductive strategies aimed at maximizing reproductive output over time.

This phenomenon is evident in the observed effects of CR on male and female lined seahorses (**Chapter I**). Male lined seahorses subjected to CR exhibited more pronounced growth stunting compared to AL-fed seahorses, with delayed pouch formation likely due to the slower developmental trajectory and energy investment required for pouch tissue development. Conversely, females displayed less significant differences in body size and ovarian weight between the two dietary groups. Furthermore, gene expression analysis in gonads revealed that CR had negligible effects on testes but induced numerous differentially expressed genes (DEGs) in ovaries, primarily downregulated, associated with catabolic processes, lipid and energy metabolism, and the immune system. Furthermore, in the liver of CR female seahorses, numerous upregulated genes were linked to lipid, steroid, and cholesterol metabolic processes, as well as cellular respiration. CR male seahorses, on the other hand, displayed fewer upregulated genes associated with energy and lipid metabolism in their livers compared to females. Interestingly, CR in the liver of males also led to the upregulation of genes related to circadian rhythm, such as *clockb* and *cry3a*, which are closely tied to daily nutrient intake and metabolic control, suggesting improved insulin sensitivity and overall health outcomes (Longo and Panda 2016).

These results highlight the significant impact of nutrient deprivation on resources essential for reproduction and survival. CR females in the lined seahorse appear to adjust their nutrient allocation to enhance pre-copulatory outputs by promoting physiological growth and adapting metabolic pathways in the liver and ovaries to support egg production. In contrast, the reversal of these trends in male seahorses strongly indicates that they delay their reproductive efforts until more nutrients become available or until they can better sustain pregnancy. Based on the results presented in **chapter I**, it appears that when the costs of pregnancy are reversed, females tend to adopt a "live fast, die young" strategy. A similar strategy may have evolved in *S. typhle* females, presented in results from **chapter II**.

Chapter II of my thesis delves into the analysis of sex-biased gene expression in the sex-role-reversed broadnosed pipefish, *S. typhle*. In this species, males possess an inverted semi-sealed pouch, although may not invest as extensively in resource transfer as extensively as *H. erectus* (H. Hamilton et al. 2017). The polygamous mating style in *S. typhle* has led to males becoming the limited sex, with females primarily competing for access to males while also developing secondary sexual traits (Anders Berglund, Widemo, and Rosenqvist 2005; Rosenqvist and Berglund 2011).

Our RNA expression analysis (**Chapter II**) revealed enriched pathways in female ovaries governing gene expression, splicing, and RNA processing—crucial processes for oocyte development and maturation. In contrast, male-biased pathways in testes were related to translation and the MAPK signalling pathway. Analysis of sex-biased expressed genes in the head kidney indicated that reproductively mature females had more upregulated genes associated with peroxisomal, lipid, and fatty-acid metabolism. In head kidney of older males, there was an enrichment of processes related to cell cycle regulation, chromosome organization, DNA replication, and various cellular functions, suggesting a more robust DNA repair machinery and potential somatic anti-ageing effects in older males. Whereas, older female pipefish had pathways for maintenance of genomic integrity exclusively

enriched in the ovaries, potentially trying to counter age-related decline in the germline (opposed as to their own somatic self-maintenance). This contrast highlights that females invest more in reproduction, particularly in pre-copulatory processes such as oocyte development and related metabolic pathways. While we did not conduct a fasting experiment on *S. typhle* (this was performed in a separate experiment, regrettably not included here), we observed similar pathways affected in male and female pipefish and seahorse under fasting conditions, underscoring the significance of sex-biased gene expression in shaping responses to various environmental challenges.

These findings emphasize that considering sex-specific reproductive costs and their investments into the next generation provides a more dependable metric for assessing resource allocation between soma and germline, surpassing the significance of sex alone. We also underscore the importance of exploring species with non-conventional reproductive roles to gain deeper insights into the evolution of sex-specific age-related diseases and lifespan dimorphism, revealing that sex roles play a more pivotal role than previously assumed, challenging traditional assumptions.

Sexual immune dimorphism

Sexual dimorphism in the immune response is well document across various animal models and particularly in humans (Ghosh and Klein 2017; Pennell, Galligan, and Fish 2012; Taneja 2018). Females typically exhibit lower infection rates than males, but may manifest heightened amount of autoimmune diseases (Charlotte Jessica E. Metcalf, Roth, and Graham 2020; Pennell, Galligan, and Fish 2012). Compared to males, females exhibit more pronounced inflammatory, antiviral, and humoral immune responses. Sex hormones play a pivotal role in influencing the function of inflammatory cells and the control of the immune system (Taneja 2018). For instance, females, who possess estrogenic receptors on the surface of numerous immune cells, tend to exhibit more robust immune responses and increased vulnerability to autoimmune diseases (Bereshchenko, Bruscoli, and Riccardi 2018). Just as oestrogens and androgens have been observed to impact the immune system in mammals, similarly, in fish, there are significant variations in the immune responses between sexes (Campbell 2021).

This is in part thought to be due to sex-specific behaviours and possible parasite exposure, with a study done in Atlantic cod reporting differences in parasite populations affecting male and female based on their feeding behaviour (Hemmingsen, Halvorsen, and MacKenzie 2000). Or shoaling behaviour in female guppies leading to an increased ectoparasite load (Richards, van Oosterhout, and Cable 2010). However, trade-offs that come with balancing the reproductive system with the immune system can lead to varying immune capabilities between parents who provide care (e.g., pregnancy) and those who do not. There are particular demands placed on the immune system when internal fertilization and gestation occurs (i.e., avoiding sexually transmitted diseases, and rejection of the embryo) (Campbell 2021; Segner, Verburg-van Kemenade, and Chadzinska 2017), this has been particularly studied in mammals. Nonetheless, other classes of vertebrates would have to in a similar way, adjust their immune system interactions to match their particular reproductive strategies.

In such regard, in **chapter I** of the thesis we observed a significant downregulation of several immune-related genes in the ovaries of female seahorses under CR, suggesting a potential dampening of their

immune responses. These findings raise questions about the adaptive strategies employed by female seahorses when faced with limited caloric resources, possibly prioritizing energy allocation to reproductive functions over immune defence. Conversely, we found no DEGs in the testes of male seahorses under similar conditions. This lack of significant changes in immune gene expression in males hints at a sex-specific response to CR, emphasizing the presence of sex-biased immune dimorphism in seahorses. Further research is warranted to explore the underlying mechanisms behind these observed differences and their implications for the overall health and survival of seahorses under varying environmental conditions.

In **chapter II**, gene expression comparison between reproductively younger males and females, resulted in numerous upregulation of genes associated with the immune system in male pipefish. These included genes like chemokine receptors (cxcr1 and cxcr4a), interferon regulatory factor 5 (irf5), toll-like receptor 8 (tlr8), and immune checkpoint molecules like V-set immunoregulatory receptor (vsir). Additionally, genes related to immune recognition (CD antigens), V(D)J recombination (rag1), and pro-inflammatory cytokines (neutrophil cytosolic factors) were expressed in males. In females, both young and old, there was an upregulation of interleukins and their receptors, major histocompatibility complex class I ZCA (mhc1zca), interleukin 6 signal transducer (il6st), and interferon regulatory factor 6 (irf6). The overall increased upregulation of immune-related genes in young males compared to young females suggests a heightened immune response, possibly linked to the unique physiological challenges of male pregnancy at a younger age. Male pipefish may have a higher exposure and be more susceptible to microorganisms through females depositing their eggs in their pouch. Research has shown that microbial diversity is highest in the pouch during pregnancy and embryos are vulnerable to pathogenic attack (Tanger et al. 2023). Nevertheless, it was unexpected that we did not observe the enrichment of the same immune system pathway in older male pipefish, and we identified a smaller number of immune genes showing sex-biased gene expression between old male and female. In other model systems, including humans, it has been observed that immunosenescence tends to impact both males and females in a similar manner, potentially resulting in a more consistent immune response among older individuals (López-Otín, Carlos et al. 2023).

Our results highlights the importance of considering sex-specific immune responses in the context of both age and reproductive strategy, where males and females have different immune responses based on parental investment and sex roles. Rather than focusing solely on sex chromosomes and hormones, researchers should reconsider the role of male and female reproductive investment in shaping immune responses. This approach can provide a more comprehensive understanding of immune dimorphism and its implications for adaptation and survival in diverse species.

Offspring phenotype and parental investment

Chapter III builds upon the foundation laid in **chapters I** and **II** by investigating the implications of age, sex-specific life history strategies and reproductive roles on the next-generation of syngnathid fishes. In the first two chapters, we examined the immediate effects of nutrient availability and sex-biased gene

expression on male pregnancy and female competition for mates, respectively. Here, we found DEGs in gonads of males and females, that could potentially impact offspring health.

In **chapter I**, AL fed females showed upregulation of immune genes compared to CR females, which may relate to mothers investing in their offspring pathogen defence by trans-generational immune priming (TGIP) (Tetreau et al. 2019). One of the advantages of the adaptive immune response in vertebrates is the production of antibodies, which we know can be transferred via the placenta and/or milk in mammals, or via egg yolk in birds, reptiles and fishes (Hasselquist and Nilsson 2009). In particular, we found downregulation of TLRs 21 and 22 in the ovaries of CR females (so upregulated in AL), which play a major role in recognizing and responding to pathogen-derived ligands, thus linking adaptive immunity with innate immunity (Kawasaki and Kawai 2014; Rebl, Goldammer, and Seyfert 2010). This is interesting, as we know from previous research that stress exposure to parents, can abolish TGIP (Roth and Landis 2017), suggesting that CR female seahorses may allocate more resources to their own survival or that high cortisol levels may be acting both immunosuppressive while also changing their future offspring phenotype (e.g., making offspring smaller) (Sopinka et al. 2017).

In **chapter II**, testes from older pipefish exhibited enrichment in the MAPK signalling pathway, known for its role in growth, differentiation, and oxidative stress response (Hutton et al. 2017; Zhang and Liu 2002). This pathway may help sustain or adjust testicular functions with age-related stressors or changes.

From here, **chapter III** takes a broader perspective by investigating the effects of increased age at reproduction on the next generation in *Syngnathus typhle*. By examining the intergenerational consequences of increased energy allocation to male pregnancy or female competition, **chapter III** provides a deeper understanding of how these sex-specific life history strategies influence not only the current generation but also the evolutionary trajectory of the species.

Trans-generational plasticity (TGP) is a form of phenotypic plasticity spanning generations, wherein a parent's environment (e.g., temperature, day length, herbivory, toxins, light quality, food availability, and immune challenges) shapes their offspring's phenotypic development (Bell and Hellmann 2019). These environmental cues can affect parents either before (pre-fertilization, in ovo, or in utero effects) or after (parental behaviour) offspring are born. These cues are transmitted between parents and offspring and they can encompass hormones and/or protein content in eggs, miRNAs in gametes, chromatin structure in germ stem cells or mature sperm, and gut endosymbionts among other things (Bell and Hellmann 2019). Recent evidence underscores the variability of both maternal and paternal effects within TGP across different organisms (Beemelmanns and Roth 2016; Sopinka et al. 2017; Yehuda et al. 2008). Parents have the ability to convey these cues to their offspring through both pre-fertilization (e.g., via sperm) and postfertilization (e.g., through parental care) mechanisms. It stands to reason that the parent with the closest contact to the developing offspring for the longest duration (e.g., during gestation and/or extended parental care) becomes the more dependable source for the offspring to predict the future environment (Bell and Hellmann 2019). Consequently, the offspring may adjust their own phenotype more substantially in accordance with the signals from that particular parent. Sensitive timing windows

come into play in TGP; for example, the age at which a parent encounters an environmental signal and how they process it based on their own life history stage can significantly impact the transmission of these cues to their offspring (McNamara et al. 2016; Zannas and Chrousos 2017). As individuals age, their life history strategies can diverge between males and females, influenced by the environments they have encountered. This age-related divergence can, in turn, affect reproductive decisions, ultimately shaping the fitness and adaptability of the succeeding generation.

In the case of *S. typhle* offspring (**chapter III**), both maternal and paternal increases in body size led to slightly larger and more offspring compared to other parental combinations. This outcome aligns with expectations in teleost species, where increased reproductive output is often associated with advancing age (Barneche, White, and Marshall 2018). Nonetheless, a closer examination of offspring gene expression revealed a stronger influence from the paternal side. The most DEGs were found when comparing offspring with old fathers and young mothers to those with both young parents, and vice versa (i.e., offspring from young fathers and old mothers compared to those with both old parents), with maternal effects appearing to have negligible impact on gene expression (**chapter III**). Paternal age, in particular, exerted a noticeable influence on pathways related to cell cycle regulation, stress response, chromatin structure, and growth and development in offspring. While older fathers were associated with certain potential drawbacks, such as DNA replication defects and compromised chromatin integrity, they also exhibited the up-regulation of genes linked to the suppression of tumorigenesis, which could be advantageous for the health of their offspring.

These findings corroborate previous research, emphasizing that it is the life history, including potential exposures to pathogens or environmental stressors, of the parent with increased parental care and/or closer contact with the offspring (e.g., during pregnancy) that significantly impacts gene expression and the phenotype of the offspring. This phenomenon makes sense, as offspring strive to ensure the honesty of parental signals, thereby avoiding the costs associated with environmental mismatch (Bell and Hellmann 2019). The results underscore the intricate interplay between parental age, gender roles, and offspring development within this unique species, shedding light on the complex mechanisms at play in trans-generational plasticity.

Future perspective

The unique phenomenon of male pregnancy in the syngnathid fish family and its variety of species displaying different pouch types, sex roles, mating systems, sexual dimorphism, and ultimately sexual selection, makes them excellent model species for studying reproductive strategies, sex-specific life history trade-offs, parental investment, sexual selection, resource allocation, transgenerational plasticity, and inheritance. These diverse traits and behaviours in syngnathid fish offer a great opportunity to explore the complex interactions between the fundamental aspects of evolutionary biology and ecology, shedding light on broader principles that may apply to a wide range of organisms and ecological settings.

Synthesis

While research in this thesis represents only an initial exploration, it has unveiled a host of intriguing questions. For instance, what would be the impact of caloric restriction on the fitness of seahorse offspring (**chapter I**)? Do the biased-sex genes we identified in *Syngnathus typhle* also manifest in other syngnathid species (**chapter II**)? How does male pregnancy function at the molecular level in pipefish and what precisely gets transferred directly to offspring within the eggs and sperm, and how does this relate to TGP (**chapter III**)? These inquiries necessitate further experimentation, more comprehensive measurements, and potentially the examination of methylation patterns.

This thesis has allowed me to disentangle the roles of biological sex (egg and sperm) and sex roles (such as specific life history roles related to parental care or mate competition). While certain gene clusters appeared to be influenced by biological sex others appeared to be impacted by sex-role (**chapter II**), this has raised questions about whether they are regulated by specific sex-determining genes or by sex hormones. The influence of sex steroid hormones, such as testosterone and oestrogen, on secondary sexual traits, mating behaviour, courtship, aggression, the immune system, and pregnancy is somewhat documented (Cox, McGlothlin, and Bonier 2016; Klein 2000; Quirós Cognuck et al. 2020). Moreover, it has been suggested that these hormones remain conserved in species like *S. typhle* and *H. erectus*, where, despite male pregnancy, ovaries produce oestrogen and testes produce testosterone (Scobell and MacKenzie 2011). This prompts us to ponder how sex hormones have adapted to assume novel roles in response to the evolution of male pregnancy and differing life history trade-offs. Further exploration is imperative to comprehend the intricate mechanisms governing the regulation of sex-specific gene expression and to elucidate the potential roles of sex steroid hormones in a model species that defies conventional sex roles.

This research expands the discussion to encompass diverse organisms, trying to bridge the gap between evolutionary biology and biomedical science. The insights gained from these investigations hold immense potential for advancing our knowledge in not only evolutionary biology but also in the fields of biomedical research and gender-related health, opening new avenues for improved healthcare and a deeper understanding of gender-specific health disparities.

Author contributions

Chapter I

FAP and OR conceived the study. **FAP** dissected and processed the RNA-seq samples, while CFE processed the hind-gut samples. **FAP** analysed the RNA-seq data, while microbial 16S rRNA-seq was analysed by VAW and **FAP**. **FAP** wrote the manuscript, with input and editing from OR.

Chapter II

FAP and OR conceived the study. **FAP** and OR collected and dissected the samples. **FAP** did the tissue RNA extraction. **FAP**, GT, and AD analysed the data. **FAP** wrote the manuscript, with input and editing from OR, AD and GT.

Chapter III

FAP, OR and DK conceived the study. **FAP**, DK and OR collected and processed the samples at differing stages. **FAP** and AD analysed the data. **FAP** wrote the manuscript with input and editing from OR and DK.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit mit dem Titel:

Evolutionary Insights into Sex-Specific Life History Strategies, Resource Allocation, and Parental Investment in Male-Pregnant Syngnathid Fish: A Study of *Hippocampus erectus* and *Syngnathus typhle*

mit Hilfe meiner Betreuer und Co-Autoren nur unter Zuhilfenahme der angegebenen Hilfsmittel und Quellen sowie unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft verfasst habe.

Die Arbeit wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt.

Dies ist mein einziges und bisher erstes Promotionsverfahren.

Zusätzlich erkläre ich hiermit, dass mir in der Vergangenheit kein akademischer Grad entzogen wurde.

Teile dieser Arbeit (Kapitel I, II und III) wurden als Vorabdrucke auf Biorxiv hochgeladen.



Freya Pappert

Kiel, 11.10.2023

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APPENDICES

Appendix – Chapter I

Figure S1

The following figures showcase morphological measurement that could not be included in the main manuscript.

Figure A: This boxplot illustrates the comparison of seahorse weight (g) between the ad libitum (AL) and calorie-restricted (CR) dietary treatments over the course of 157 days. Prior to the dietary treatment, the seahorses exhibited a mean weight of 0.55 ± 0.29 g and a mean length of 4.58 ± 0.95 cm (26 individuals each). After 157 days, the AL group (16 individuals) demonstrated a mean weight of 8.09 ± 3.12 g, while the CR group (22 individuals) displayed a mean weight of 3.51 ± 1.17 g.

Figure B: This boxplot presents the progressive weight differences at different time points for both dietary treatments. At day 1, there were no significant differences observed between AL (0.49 ± 0.13 g) and CR (0.58 ± 0.36 g) groups. However, at day 55, the AL group (3.12 ± 1.29 g) showed significantly higher weight compared to the CR group (1.17 ± 0.44 g, Kruskal-Wallis $p < 0.001$). Similarly, on day 111, the AL group (7.66 ± 2.84 g) exhibited significantly greater weight compared to the CR group (2.88 ± 0.91 g, Kruskal-Wallis $p < 0.001$).

Figure C: This boxplot focuses on the length differences between the AL and CR dietary treatments. The AL group had a mean length of 12.93 ± 1.68 cm, while the CR group had a mean length of 10.28 ± 1.08 cm.

Figure D: In this boxplot, we analyse the size differences between the dietary treatments while considering the sex of the seahorses. For males, the AL group (13.75 ± 1.27 cm) displayed significantly greater length than the CR group (10.76 ± 0.37 cm, $p < 0.001$). In contrast, for females, there was no significant difference in length between the AL (11.11 ± 0.78 cm) and CR (9.81 ± 1.35 cm) groups ($p = \text{NS}$).

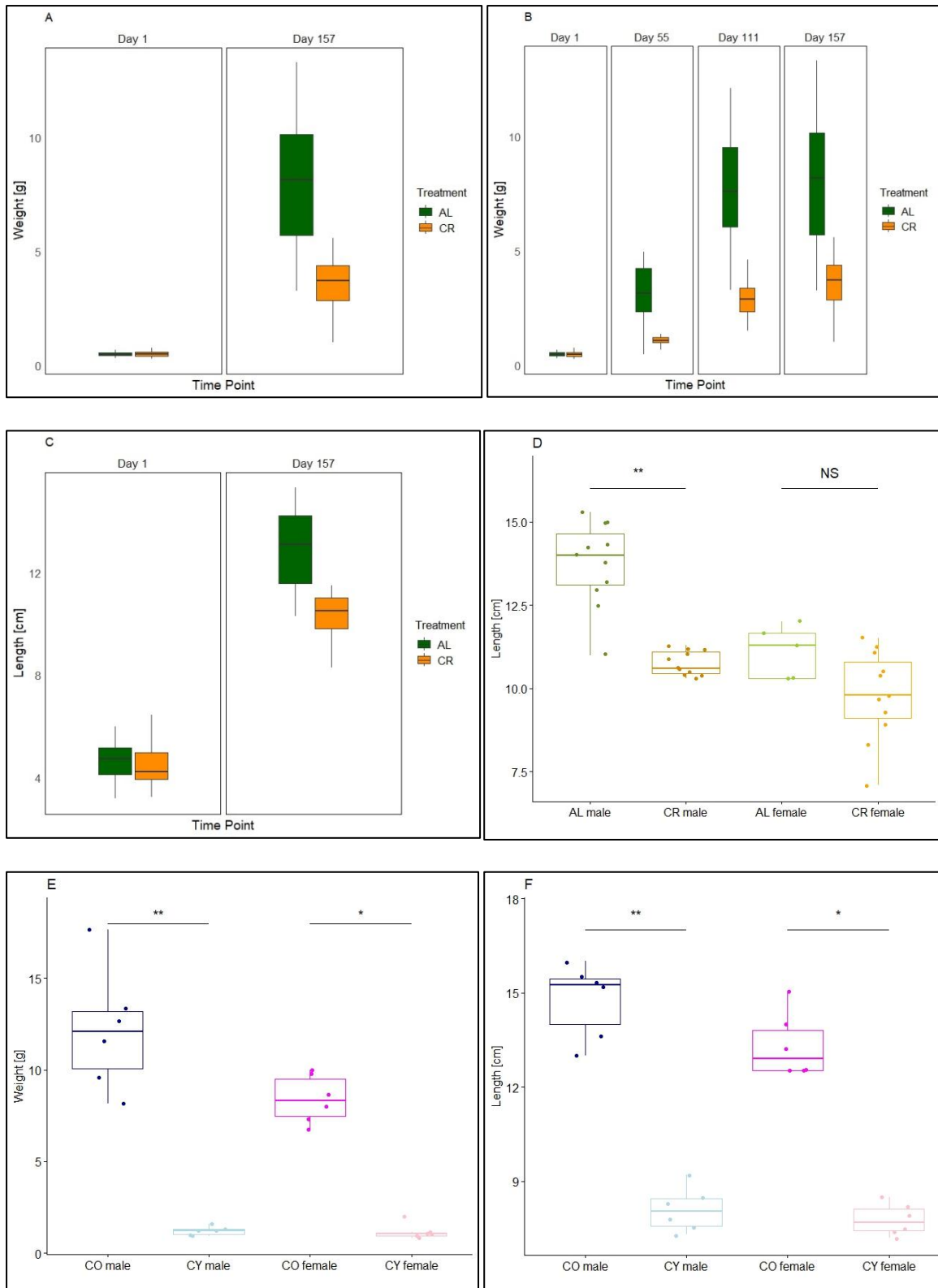
Figure E: This figure highlights the significant difference in weight between young (CY) and old (CO) control groups for sex. For males, CO had a higher mean weight of 12.16 ± 3.31 g compared to the CY with a mean weight of 1.2 ± 0.23 g. Similarly, for females, CO displayed a higher mean weight of 8.41 ± 1.31 g compared to CY with a mean weight of 1.16 ± 0.42 g. Males were on average heavier than females.

Figure F: Similar to Figure E, this figure presents the length differences between the older and younger females and males. For females, CO had a mean length of 13.3 ± 1.02 cm, while CY had a mean length of 7.78 ± 0.5 cm. For males, CO displayed a mean length of 14.77 ± 1.18 cm, while the CY exhibited a mean length of 8.1 ± 0.71 cm.

Figure G: This figure depicts the ovarian size differences between the experimental dietary treatment groups and the age control groups. No significant results were found between the AL (0.23 ± 0.15 g) and CR (0.15 ± 0.08 g) groups ($p = \text{NS}$). However, as expected, older female seahorses above age three displayed significantly heavier ovaries with a mean weight of 0.43 ± 0.13 g, compared to the much smaller and less developed ovaries of the younger females with a mean weight of 0.02 ± 0.02 g.

Figure H: This plot shows the pouch size length (diagonally from the anal fin to the end of the brood pouch) between CR and AL treated groups at the end of the experiment (AL 2.6 ± 0.47 vs. CR 1.82 ± 0.17 cm, Kruskal-Wallis $p < 0.005$).

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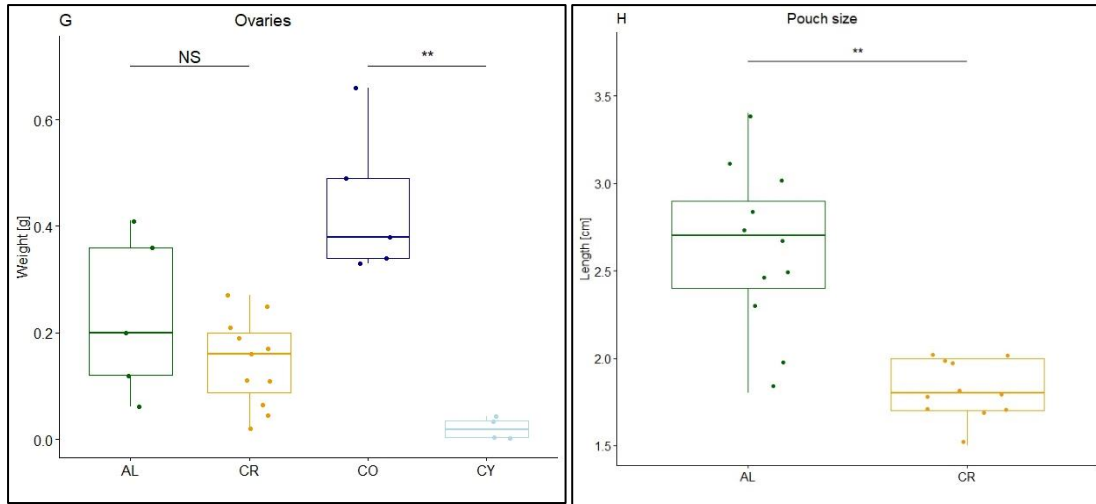


Figure S2

Principal Component Analysis (PCA) plot depicting organs and treatment groups. The plot illustrates the separation of organs with different symbols (liver, head kidney, ovaries, testes) based on principal components PC1 and PC2 on the left plot and PC2 and PC3 on the right plot. The groups are color-coded as follows: CR (orange), AL (green), juvenile control (light blue), and adult control (dark blue).

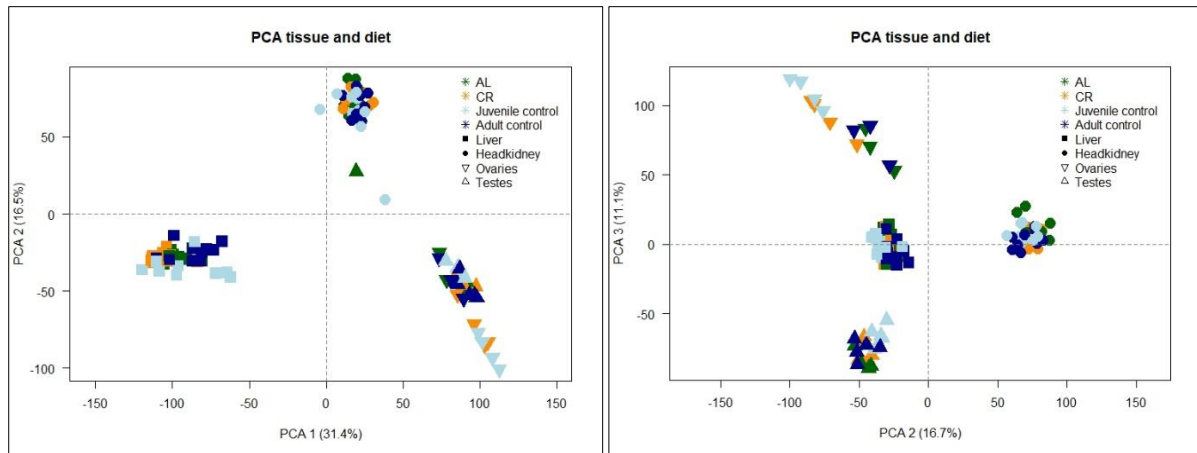


Table S1

Quantitative results from the differential gene expression (DGE) analysis (using *limma:voom*) comparing different groups. Only differentially expressed genes below an adj.p.val of 0.05 are showed in the table (one-way analysis of deviance).

Contrast comparison	Tissue	Counts (adj.p.val < 0.05)
AL vs CR (female)	Liver	375
AL vs CR (male)	Liver	83
AL vs CR	Liver	940
AL vs CO	Liver	3195
AL vs CY	Liver	5408
CR vs CO	Liver	5003
CR vs CY	Liver	4292

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AL vs CR (female)	Head kidney	0
AL vs CR (male)	Head kidney	4
AL vs CR	Head kidney	67
AL vs CO	Head kidney	1566
AL vs CY	Head kidney	2981
CR vs CO	Head kidney	2392
CR vs CY	Head kidney	4106
AL vs CR	Testes	0
AL vs CO	Testes	0
AL vs CY	Testes	646
CR vs CO	Testes	0
CR vs CY	Testes	345
AL vs CR	Ovaries	366
AL vs CO	Ovaries	0
AL vs CY	Ovaries	4966
CR vs CO	Ovaries	218
CR vs CY	Ovaries	0

Here as follows we explain some of the results from the differential gene expression analysis (*limma:voom*), showing contrast comparisons of the one-way analysis of deviance between the young and old control groups to the dietary treatment groups. We found slightly more DEG between AL vs CY (5408) compared to AL vs CO (3195), and equally when comparing CR vs CY (4292) with fewer DEG and CR vs CO with more DEGs (5003). Trends in head kidney followed for AL vs CO (1566) and AL vs CY (2981), but not so much when comparing CR vs CO (2392) and CR vs CY (4106). Interestingly, in the testes we found no DEGs between AL vs CO and CR vs CO, but differences between AL vs CY (646) and CR vs CY (345). This is likely due to the young control males having just reached gonadal maturation around 4 months when they were sampled, meaning all other seahorses were sexually more developed thus showing no differences between other groups. In ovaries, there were no significant DEGs between CR and CY seahorses, but 218 DEGs were identified between CR and CO. Same but reversed for AL vs CY showing many DEG (4966) and none between AL vs CO (Table S1). Interestingly, similar pathways were enriched for DEG between CR and CO with also here the vast majority of genes being downregulated in the CR compared to the older control group (Table S2, sheet D).

Figure S3

Results from 16S rRNA amplicon sequencing on hind gut tissue of *Hippocampus erectus*, including genus *Vibrio* are found from Figures A to E. The genus *Vibrio* made up most of the abundance across all samples, which was somewhat expected as it is an opportunistic marine bacteria found in warm waters that commonly infects Syngnathids and other fish. Its high prevalence across all samples caused background noise which reduced statistical differences between treatment groups and so was removed after careful consideration (results in main manuscript).

Figure A: The left side of the panel displays alpha-diversity with Shannon richness, with boxplots for treatment groups AL and CR along with age groups Control Old and Control Young. The x-axis represents the groups, while the y-axis shows the Shannon richness. Hypothesis testing for α -diversity for Shannon, before removing the *Vibrio* genus, indicated no significant differences for sex but a slight significance between treatment groups (PERMANOVA, $p.val = 0.03696$), but after post-hoc (pairwise.adonis) there was no significant difference between the factors.

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Figure B: The left side of the panel displays alpha-diversity with Simpson richness, with boxplots for treatment groups AL and CR along with age groups Control Old and Control Young. The x-axis represents the groups, while the y-axis shows the Simpson richness. Hypothesis testing for α -diversity for Simpson, indicated no significant differences for sex or diet groups.

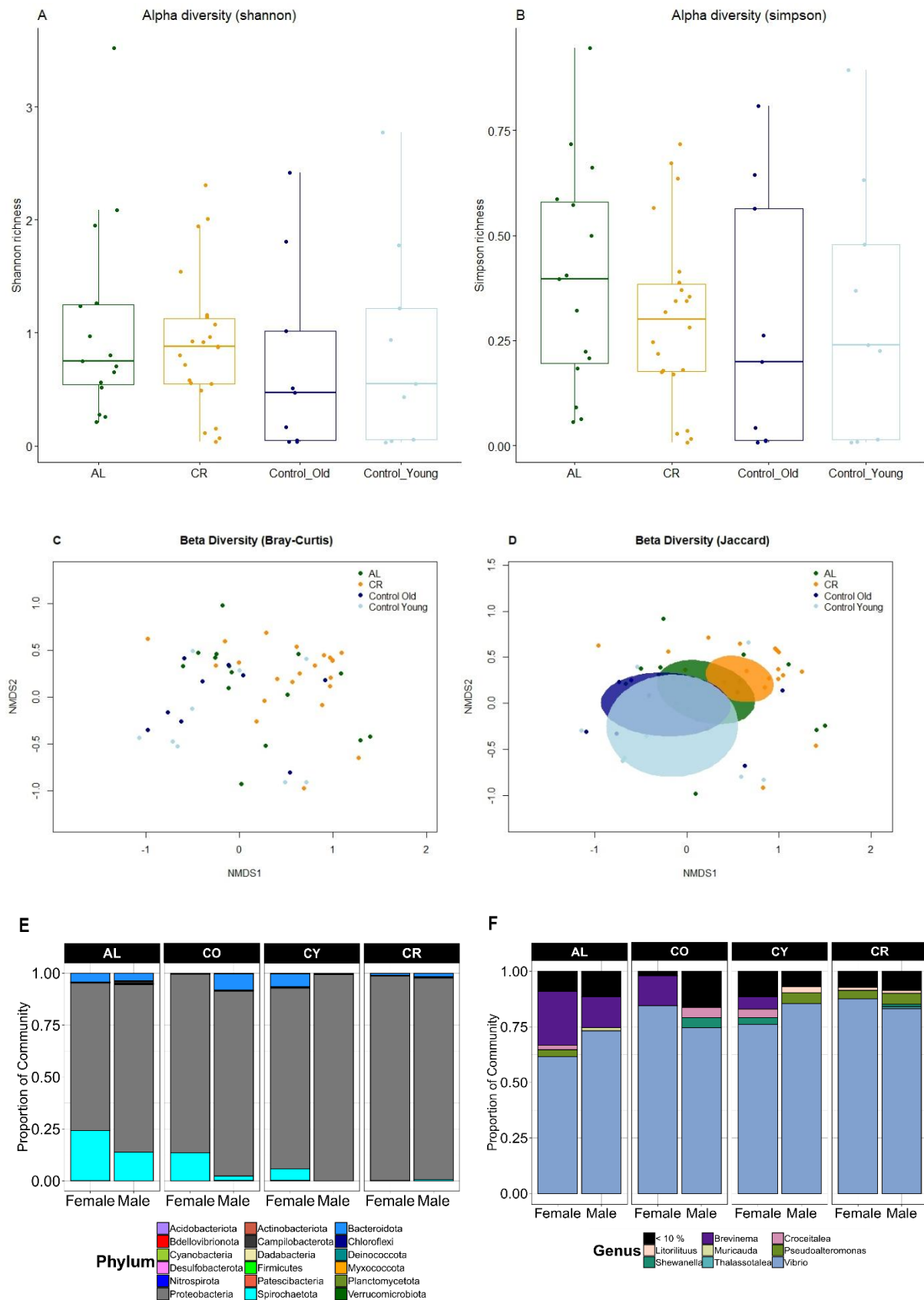
Figure C: NMDS beta diversity plot with Bray-Curtis dissimilarity matrix comparing all group variables, treatment groups AL in green, CR in orange, and age groups with CO in dark blue and CY in light blue.

Figure D: NMDS beta diversity plot with Jaccard distances comparing all group variables, treatment groups AL in green, CR in orange, and age groups with CO in dark blue and CY in light blue. Additionally, 95% confidence ellipses were added to aid in interpretation (this was not done in Fig. S3C just to compare the two plots, as results are very similar for both Bray-Curtis and Jaccard).

Figure E: Barplot with relative abundance of microbial taxa at the phylum taxonomic level including the most abundant genus *Vibrio*. The panels are categorized based on diet, with AL (Ad Libitum) and CR (Caloric Restriction) groups, as well as control age groups CO (Control Old) and CY (Control Young). Furthermore, within each subcategory, panels are further divided to represent male and female seahorses.

Figure F: Relative abundance of microbial taxa at the genus taxonomic level with the analysis grouping low abundance taxa (< 10%) together. Here too the genus *Vibrio* is included, showcasing how irrespective of treatment group or sex it is the most abundant taxa.

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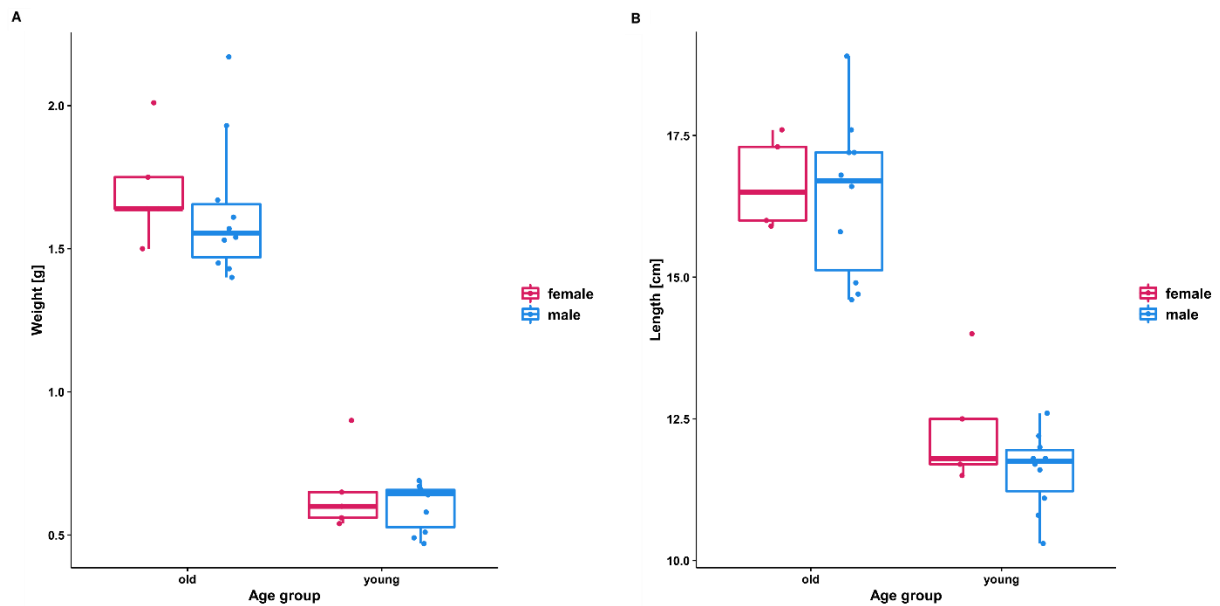
Supplementary tables S2 to S6 - can be accessed online.

Appendix – Chapter II

Supplementary Figure S1

Morphological measurement comparison between males (blue) and females (pink) of wild-caught *Syngnathus typhle*. (A) Weight in grams on y-axis and age group on x-axis. (B) Total body length in centimeters on y-axis and age groups on x-axis.

There were significant size differences between young and old. The average weight of the sampled pipefish was 0.65 g (SD = 0.146) in young females (YF) and 0.6 g (SD = 0.082) in young males (YM), whilst 1.71 g (SD = 0.191) in old females (OF) and 1.63 g (SD = 0.243) in old males (OM.). The average total body length was 12.3 cm (SD = 1.02) for YF and 11.56 cm (SD = 0.682) for YM., 16.66 cm (SD = 0.764) in OF and 16.82 cm (SD = 1.41) in OM. A two-way ANOVA of body weight and also of length, for sex and age, showed a significant difference for age ($P < 0.0001$), but not for sex or the interaction.



Supplementary Table S2

Quantitative results from the differential gene expression (DGE) analysis (using *limma:voom*) using contrast comparisons between the different variables for the various organs. Only differentially expressed genes with an adjusted P value smaller than 0.05 were considered (one-way analysis of deviance).

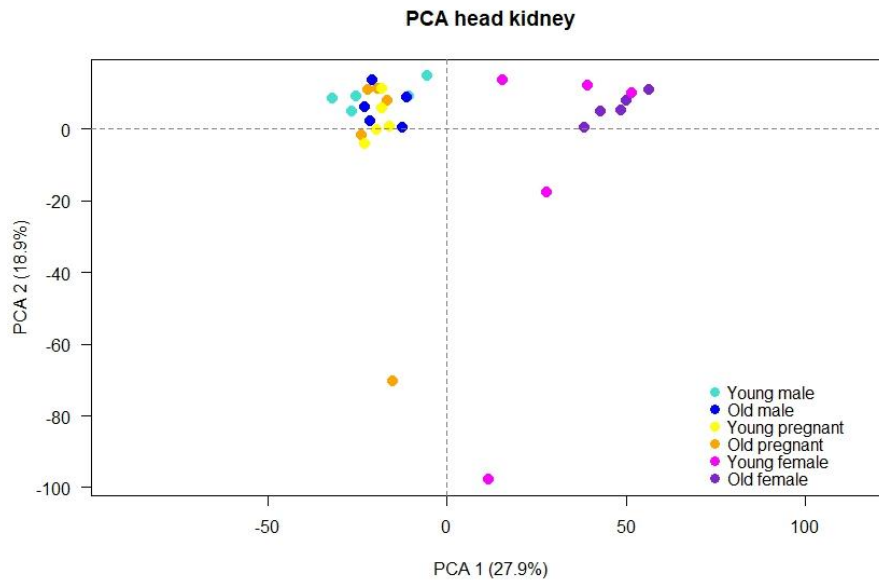
Contrast comparison	Tissue	Counts (adj.p.val < 0.05)
YM vs OM	Brain	0
YF vs OF	Brain	0
YP vs OP	Brain	0
YM vs YF	Brain	2
OM vs OF	Brain	2
YM vs YP	Brain	0
OM vs OP	Brain	0
YM vs OM	Head kidney	0
YF vs OF	Head kidney	4
YP vs OP	Head kidney	0
YM vs YF	Head kidney	1930

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OM vs OF	Head kidney	3564
YM vs YP	Head kidney	0
OM vs OP	Head kidney	0
YM vs OM	Testes	0
YP vs OP	Testes	0
YM vs YP	Testes	0
OM vs OP	Testes	0
YF vs OF	Ovaries	0
YM vs YF	Gonads	9166
OM vs OF	Gonads	9493

Supplementary Figure S2

Principal Component Analysis (PCA) results of PC1 and PC2 for the head kidney. The plots portray the relationships among different groups within the respective organ. Six groups are highlighted: young males in light blue, old males in dark blue, young pregnant individuals in yellow, old pregnant individuals in orange, young females in pink, and old females in purple.

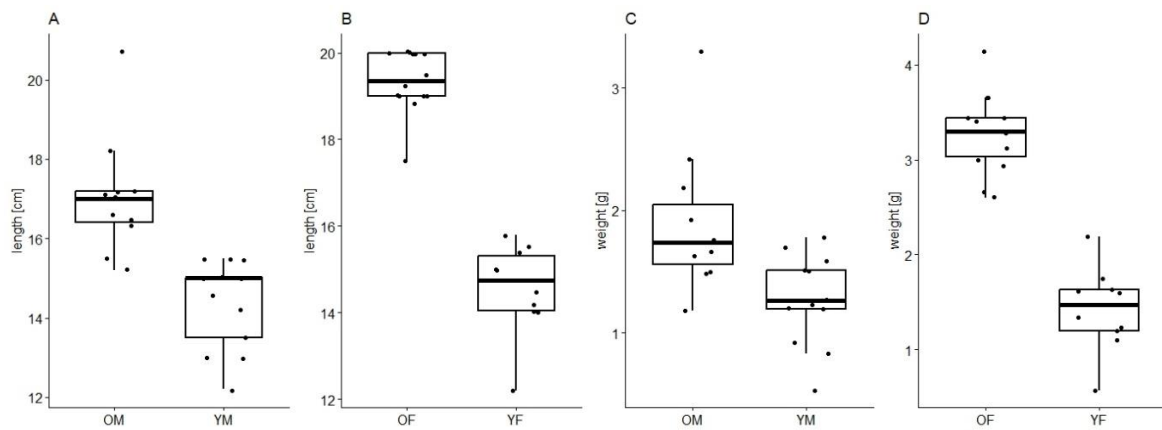


Supplementary tables S1, S3 and S4 - can be accessed online.

Appendix – Chapter III

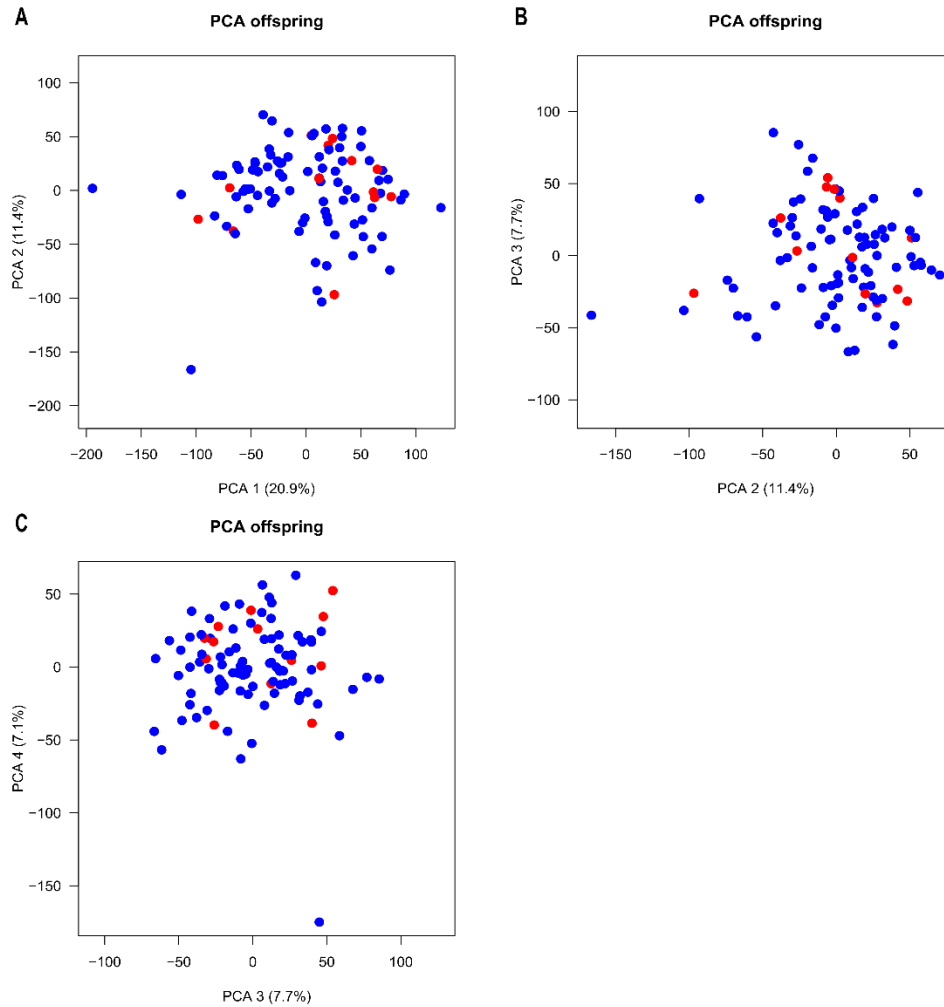
Supplementary Figures

Fig. S1: Represented are boxplots of morphological measurement results of length and weight of pipefish parents. The x-axis represents the age groups, with old male (OM), young male (YM), old female (OF), and young female (YF). The y-axis represents the measured parameters. In A) and B), the length of the pipefish is represented in centimeters [cm] with panel A) depicting males and panel B) females, while in C) and D), the weight is represented in grams [g], with equally C) including males and D) including females. A) the mean total body size for OM was 17.05 cm (SD = 1.47) and for YM 14.38 cm (SD = 1.11), (OM vs. YM [cm] ANOVA, $P = 4.6e-05$). B) OF total body size average was 19.36 cm (SD = 0.72), and for YF 14.56 cm (SD = 1.05), (OF vs. YF [cm] ANOVA, $P = 5.2e-12$). C) OM had a mean body weight of 1.88 g (SD = 0.58) and YM of 1.27 g (SD = 0.36), (OM vs. YM [g] ANOVA, $P = 0.00413$). D) OF had an average weight of 3.28 g (SD = 0.41), and YF of 1.42 g (SD = 0.44), (OF vs. YF [g] ANOVA, $P = 3.69e-10$).



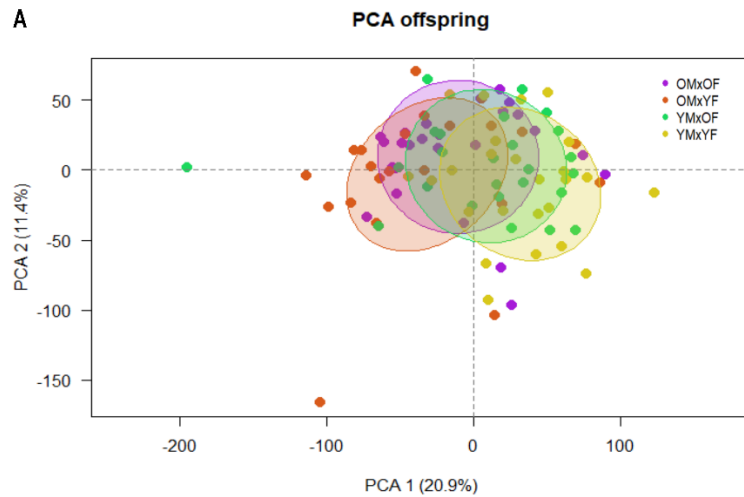
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Fig. S2 (A-C): PCA plots showing PC1 to PC4 on log transformed count data of all the offspring individuals based on if their parents came from Falckenstein (red) or Trudsø (blue). No obvious clustering was detected between offspring from different parental locations, which corroborated our PERMANOVA analysis ($P > 0.05$) that parental population didn't have an effect.



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Fig. S3: The figure shows a principal component analysis (PCA) of rld transformed counts of pipefish offspring, grouped by parental combination: OMxOF (purple), OMxYF (brown), YMxOF (green), and YMxYF (yellow). PCA1 in all likelihood reflects the difference between offspring from old and young fathers. However, we detected two distinct outliers, one from OMxYF and one from YMxOF. These samples were distorting the distribution of the data and we decided to remove them for differential gene expression analysis to facilitate data normalization, enabling better analysis and modeling.



Supplementary tables S1 and S2 - can be accessed online.

Science never solves a problem, without creating ten more.

- George Bernard Shaw