Sex or sex role – unravelling the evolutionary route of sex-specific senescence

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Abstract

Sex-specific lifespan dimorphism is observed in many species, with females often outliving males. This is thought to be due to sex-specific and unequal resource allocation between reproduction and maintenance. However, most studies on ageing focus on animals with traditional sex roles, where females have the highest energy burden with both egg production and parental care. This leaves a gap in our understanding of how resource allocation and senescence are linked in species with non-traditional roles. To address this, we investigated the relationship between sex (biological) and sex role (behavioural) on resource allocation trade-offs in somatic maintenance in the broad-nosed pipefish *Syngnathus typhle*. This species is sex-role reversed, with males acting as the primary caretaker for offspring through their unique male pregnancy, providing the opportunity to decouple the roles of sex and sex role in resource allocation. Our results suggest that while some gene groups are influenced by sex roles (e.g., genome maintenance and immune defence), others depend on biological sex and hormones (e.g., energy and fat storage), with differences becoming more pronounced with age. These findings challenge traditional ageing theories and improve our understanding of how sex-specific differences in ageing are shaped.

1. Introduction

Ageing is manifested as progressive physiological deterioration accompanied by decreasing or ceasing fertility that correlates with an induced likelihood of mortality (Viña, Borrás, and Miquel 2007). Some species display accelerated or rapid ageing, for instance, killifish with 13 weeks lifespan or pacific salmon senesce rapidly after reproducing. Other species show slow or negligible senescence or have an extended lifespan such as rougheye rockfish (up to 200 years) or the greenland shark (roughly 400 years) (Stenvinkel and Shiels 2019; Trifonova et al. 2018). However, differences in lifespan vary not only across species but also between sex (Jones et al. 2014). Although the mechanistic pathways for ageing, also referred to as the “Hallmarks of Ageing”, are thought to be conserved (López-Otín et al. 2013), there is accumulating evidence that ageing leads to sex-specific phenotypes and age-related diseases (Gubbels Bupp 2015; Vom Steeg and Klein 2016; Zuk 2009). For example, a study

looking at 101 mammal species, revealed that female lifespan is on average 18.6% longer than that of males (Lemaître et al. 2020). The resource allocation theory predicts that the higher sex-specific lifespan dimorphism observed in Mammalia, Insecta, Reptilia, and Actinopterygii compared to Arachnida, Aves, Chondrichthyes, and Amphibia is due to selection acting differently on males and females, resulting in shifted investments for growth and reproduction based on the reproductive roles of the sexes in the species (Xirocostas, Everingham, and Moles 2020). 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 (Brooks and Garratt 2017; Janicke et al. 2016). On a similar line of thought Kirkwood's disposable soma theory states "longevity requires investments in somatic maintenance that reduces the resources available for reproduction" (Kirkwood and Rose 1991). Here, investment in reproduction is a traded-off with investment in somatic maintenance, thus reducing self-maintenance and determining lifespan.

Mammalian female reproductive requirements often include resource allocations for egg production, gestation, lactation and an overall higher parental investment as opposed to males' cheap sperm production, secondary sexual traits, and territory defence (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 lifespan, compared to males with a lower burden of parental care, allowing for multiple matings in a short period of time and thus focusing more on their reproductive success (Clutton-Brock and Isvaran 2007). This is, however, an example of stereotypical mammalian sex roles, where males compete for access to mates with their fitness being maximized by increasing mating rates, and where females are choosy with fitness being positively correlated with longevity (Bateman 1948; Rolff 2002; Williams 1957). In the animal kingdom, sex roles are not constrained and vary between species and even across an individual's lifetime based on different degrees of parental investment (Benvenuto et al. 2017; Janicke et al. 2016; Smith and Wootton 1995). For example, in the 85 teleost species that show parental care, the majority is dominated by paternal care, such as egg guarding, and oral brooding to full viviparity where the males will endure the largest amount of work (Smith and Wootton 1995; Wilson et al. 2001). Some of the most devoted male parents can be accredited to Syngnathids (seahorses, pipefishes and seadragons), which have evolved a range of brooding forms from simple attachment of eggs to the male tail to highly specialized male pregnancy with placenta-like structures analogous to those found in eutherian mammals, that provide embryos with nutrients, oxygen and immunological components (Beemelmanns and Roth 2016; Ripley and Foran 2009; Roth et al. 2011; Whittington and Friesen 2020; Wilson et al. 2001). Thus far experimental studies on parental investment have focused primarily on the mothers' fitness, linking it directly to the offspring's fitness, since females typically invest more not only in egg production but also in caring or nurturing (Wolf and Wade 2001). However, the range of sex role diversity in nature allows us to explore other avenues of resource allocation trade-offs and their effects on sex-specific senescence. Studying species with the varying spectrums of sex roles allows to test the

outcome of theories of ageing that were mainly developed for evolutionary consequences of conventional sex roles. Unfortunately, there is scarce literature on sex-specific ageing in teleosts and fish in general (Stenvinkel and Shiels 2019). Much of the gerontology research on the mechanisms of ageing is biased toward a few species such as roundworm (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), mouse (*Mus musculus*) and rat (*Rattus norvegicus domestica*) (Holtze et al. 2021; Mitchell et al. 2015). Studying the immense variety of species with sex lifespan dimorphism would further our understanding of the evolutionarily conserved mechanisms of ageing and improve the comparative approach between species (Holtze et al. 2021). Fish make up approximately half of all existing vertebrate species (over 20,000) and not only have various forms of mating systems and 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; Reznick, Ghalambor, and Nunney 2002). Nonetheless, while mammals and birds tend to have fairly constant fecundity across age groups, fish fecundity increases with age as they have indeterminant growth and thus can increase the number of eggs and offspring (Barneche, White, and Marshall 2018). This might cause different life-history choices and change the way they evolved ageing compared to mammals, making it worth investigating in fish.

In this study, we compared sex-biased gene expression between young and old broad-nosed pipefish *Syngnathus typhle*, a sex-role reversed species with male pregnancy, providing the unique opportunity of decoupling the role of the female sex (defined as the contribution of eggs) and pregnancy (body reshaping and energy allocation) (Roth et al. 2011). Male pregnancy has evolved only once in vertebrates and from there on has diversified across approximately 300 Syngnathidae species (Hamilton et al. 2017; Stiller et al. 2022). Syngnathids have also evolved a gradient of mating systems including sex-role reversal; e.g., in broad-nosed pipefish, their brood pouch is size-limited, and as the females produce on average more eggs than males can brood during the same period (Sagebakken, Ahnesjö, and Kvarnemo 2016), males end up being the sex that is more reproductively constrained (Mobley et al. 2018). 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) (Whittington and Friesen 2020). In such role-reversed species, males will be the choosy sex whereas females will be ornamented and actively court males (Mobley et al. 2018; Roth et al. 2011).

Using this unique species, we aimed to address two major gaps in our understanding of the evolutionary route to sex-specific senescence. First, how does sex (defined as the contribution of eggs or sperm) or rather sex-role (range of behaviours generally associated with being male or female) influence resource allocation trade-offs in somatic maintenance? Research studying resource allocation theory and the effects of senescence on males and females has been biased for animal models with conventional sex roles, where females generally have the highest

energy burden. Not allowing us to disentangle whether the evolution of a longer female lifespan is predominantly due to sex hormones and sex-chromosomes, or due to the resource allocation trade-off in sex-role behaviours such as parental investment. Second, if ageing theories are studied in animals where fertility declines with age to various degrees between males and females, how does senescence affect somatic and germline maintenance in teleost species where fertility increases proportionally with advancing age?

In this study, we used full transcriptome mRNA sequencing (RNA-Seq) to analyse gene expression in different tissues (brain, head kidney and gonads) between young and old female and reproductively active (pregnant) and non-reproductively active males. By identifying sex-biased gene expression and subsequent expression changes throughout life and reproduction activity, we aimed to understand the importance of sex and sex role in determining resource allocation in ageing. Has sex-role reversal as a consequence of increased male parental investment (i.e., male pregnancy) led to males investing more in longevity and females investing in reproduction? Alternatively, is biological sex a more important feature for lifespan dimorphism? We hypothesized that sex-lifespan dimorphism is more heavily influenced by sex-specific life-history traits, than by the biological sex. Our data revealed that while sex roles may influence some gene groups and invert their expression when moving from conventional to sex-role reversal, the activity of other gene groups may depend on the biological sex. This illuminates the importance of disentangling sex and sex roles when we aim to understand sex-specific life-history strategies, sex-biased gene expression and their consequences on longevity.

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. They were brought to GEOMAR institute, where body mass and total body length were measured. The animals were then euthanized with an overdose of MS-222 (500 mg/l; Sigma-Aldrich). Carcasses were dissected and different tissues (brain, head kidney and gonads) were sampled and were immediately preserved in RNAlater. Samples were kept at 4°C for three days, before transferring them to -20°C for long-term storage.

2.2 RNA isolation and Illumina sequencing

For our RNA-seq analysis, we extracted RNA from brain, head kidney, ovaries and testes, as gene expression is known to differ between tissues. Gonads are frequently used in sex-biased gene expression studies, as sex organs can reveal genes vital for sex-specific development or fertility regulation (Whittle and Extavour 2019). Also, the brain has shown sex and age-specific gene expressions (Hale et al. 2018; Mayne et al. 2016). We additionally used head kidney, which is unique to teleost fish but is analogous to the adrenal gland, producing both red blood cells for oxygen transport and white blood cells for immune defence (Geven and Klaren 2017). Its clear role in

immune regulation makes it the perfect organ to focus on genes displaying sexual immune dimorphism. The RNA extraction was performed using RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. As gene expression is known to differ between tissues, extraction yields were quantified using NanoDrop ND-1000 spectral photometer (Peqlab, Erlangen, Germany), RNA was then stored at -80°C. Library preparation (TruSeq stranded mRNA Kit by Illumina) and sequencing (RNA Seq NovaSeq6000 S4, 2x150bp, >17M reads per sample) were carried out at the Institute of Clinical Molecular Biology (IKMB).

2.3 Transcriptome analysis

The resulting reads were quality-controlled using FastQC v.0.11.9 (Andrews 2010) and trimmed using Fastp v.0.20.1 (Chen et al. 2018). Reads were aligned to a whole genome assembly of *Syngnathus typhle* (BioProject ID: PRJNA947442) using STAR v.2.7.9a (Dobin et al. 2013). Transcript abundance was quantified with TPMCalculator (Alvarez et al. 2019) in both raw read counts and transcripts per million (TPM), with counts normalised for library size and gene length. We used Orthofinder v.2.4.0 (Emms and Kelly 2019) to assign information to the annotated genes with these species lists (accession included): *Takifugu rubripes* (rubripes.fTakRub1.2), *Syngnathus rostellatus* (GCF_901709675.1, fSynAcu1.2), *Syngnathus accus* (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 analysis of the RNA-Seq data from Illumina was done in Rstudio v.3.4.0 (R Core Team 2022), using the limma:voom package v.3.54.1 (Ritchie et al. 2015) on *S. accus* orthology annotations. After scaling and normalizing with edgeR package v.3.14.0 (Robinson, McCarthy, and Smyth 2009), we discarded any genes with less than 10 counts in at least 3 libraries. To look at the gene expression differences between the organs we did a cluster analysis, detecting transcripts that were unique for each organ for subsequent enrichment and functional analysis. The limma:voom pipeline was run separately for counts in brain, head kidney and gonads, as the effects of other variables might be harder to detect with significant differences between tissues. Within a single organ a multi-level analysis with categorical paired comparisons was performed, to investigate differential gene expression for sex, age and pregnancy. This allowed to compute the contrasts between our single variables of interest, whilst still considering the variable interactions within the linear model analysis of limma:voom.

2.4 Statistical analysis

Morphological data comparison between groups was analysed by a two-way ANOVA, with statistical significance set at $P < 0.05$. To compare differentially expressed genes between the group variables (young and old females, young and old reproductively active thus pregnant and non-reproductively active males), we did a Principal Component Analysis (PCA) with the "regularized log transformation procedure" (rld) transformed expression values

to check for outliers and tissue variance (Figure S1). The few detected outliers were removed after further consideration, and subsequently, samples clustered according to tissue type (Figure S1). To estimate differences between factors such as sex, age and pregnancy, we separated the DEGs dataset for the three separate organs (Figure 1D and Figure S3). To check if transcript expression varied significantly, we also applied a PERMANOVA (Permutational Multivariate Analysis of Variance Using Distance Matrices) using function “adonis2” and method “bray”.

2.5 Orthology and enrichment analysis

For gene ontology analysis, we annotated the differentially expressed genes using a homology-based search of *Danio rerio* with OrthoFinder. The overall number of DEGs ended up being reduced as it was not possible to find orthologues for all transcripts. *Danio rerio* orthologues of DEGs were uploaded to g:Profiler (last access date January 2023) (Raudvere et al. 2019) to examine the functional profiling of transcripts from the cluster analysis based on tissue type, and the DEGs for young male (M.y.), young female (F.y.), old male (M.o.) and old female (F.o). The thresholds used were: Organism “*Danio rerio* (Zebrafish)”, Statistical domain scope set at “All known genes”, significance threshold “g:SCS threshold” at 0.05, 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 S1). A more targeted approach was used to investigate the presence and expression pattern of immune-related genes. Following results from our gene enrichment analysis in the head kidney, with terms such as innate immune system (REAC:R-DRE-168249) and adaptive immune system (REAC:R-DRE-1280218) enriched, we searched in our DEGs dataset what immune genes males and females had differentially expressed. As various genes in these pathways had numerous overlaps with other non-immune related GO terms and some more unique immune transcripts had been missed out in the GO analysis, we opted to use immunological keywords (see Table S4 for keywords) based on literature reviews on the immune response in our whole dataset (Chaplin 2010; Yuan et al. 2014). The keywords that were found in our dataset were grouped under umbrella terms as adaptive or innate immune response, with which we then compared their differential expression for sex and age.

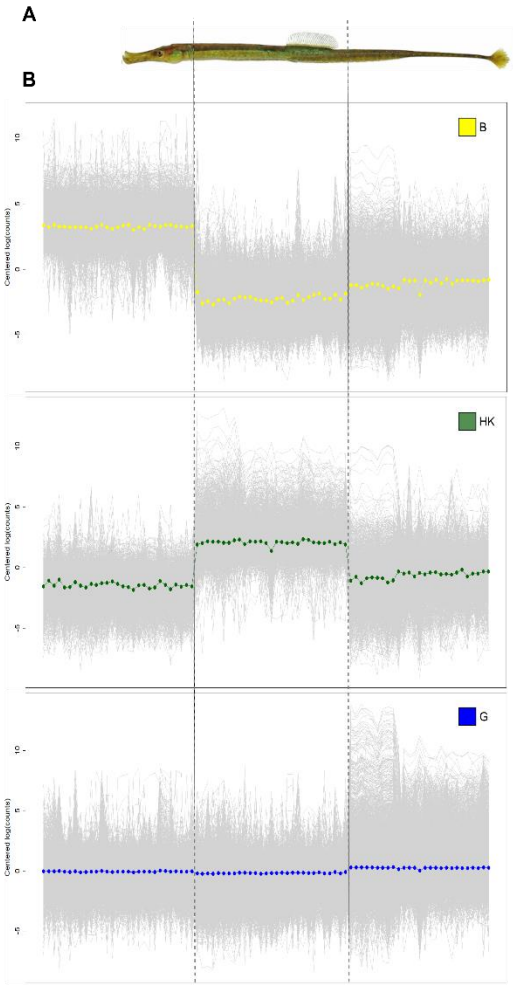
3. Results

3.1. Sample description and transcriptome analysis of differential gene expression in brain, gonads and head kidney

In our study we used 10 females and 20 males; the latter included 10 pregnant and 10 not mated yet. All fish were separated into old and young categories, based on morphological measurements (Figure S2). Body length and weight are frequently used in fisheries to roughly determine age, as the vast majority of fish have indeterminate growth with size and age being correlated (Barneche, White, and Marshall 2018). The average weight of the

sampled pipefish was 0.65 g (SD = 0.146) in young females (F.y.) and 0.6 g (SD = 0.082) in young males (M.y.), whilst 1.71 g (SD = 0.191) in old females (F.o.) and 1.63 g (SD = 0.243) in old males (M.o.). The average total body length was 12.3 cm (SD = 1.02) for F.y. and 11.56 cm (SD = 0.682) for M.y., 16.66 cm (SD = 0.764) in F.o. and 16.82 cm (SD = 1.41) in M.o. A two-way ANOVA confirmed that both total body length and weight were highly significant for age (length $P < 2.8e-12$ and weight $P < 5.65e-15$), but not sex ($P > 0.05$).

RNA sequencing expression profiles resulted in a total number of 26072 transcripts with an average of 1M reads per sample, of which 17200 remained after filtering out genes with less than 10 counts in at least 3 libraries. A cluster analysis (Figure 1B) revealed groups of genes expressed mostly in either brain, head kidney or gonads (Figure 1B). We then identified functional categories defining each organ of the pipefish based on clusters sharing similar expression patterns (Table S1). Genes involved in neuronal pathways were mostly localized in brain, as were immune response genes in head kidney (Figure 1C and Table S1). Gonads including both testes and ovaries had mostly pathways involved in genome maintenance and proteostasis regulation (Figure 1B). Additionally, all organs did have various metabolic related pathways enriched (Figure 1C). While there were strong differences between organs, a PERMANOVA analysis did not reveal any significant effect of pregnancy in any of the single tissues. Also, age did not quite reach significance in any of the tissues. However, sex was highly significant ($P < 0.001$) in both gonads and head kidney. Subsequent PCAs of each tissue confirmed these results (Figure S3 for brain and gonads), but for the PCA of head kidney there was also some indication that PC2 may reflect the size and thus age of the pipefish (Figure 1D).



D

B	HK	G	REAC Pathways	Description
			Nervous system development	Neural pathways
			Transmission across Chemical Synapses	
			GABA receptor activation	
			Ion homeostasis	
			Innate Immune System	Immune response
			Neutrophil degranulation	
			Haemostasis	
			Platelet activation, signalling and aggregation	
			Integration of energy metabolism	Metabolism
			Regulation of insulin secretion	
			Metabolism of lipids	
			Metabolism of amino acids and derivatives	
			Fatty acid metabolism	
			Cytochrome P450 - arranged by substrate type	
			Metabolism of RNA	
			Metabolism of proteins	
			Mitochondrial biogenesis	
			Biological oxidations	ROS regulation
			RHO GTPases Activate NADPH Oxidases	
			DNA repair	Genome maintenance
			DNA Double-Strand Break Repair	
			TP53 Regulates Transcription of DNA Repair Genes	
			Transcriptional Regulation by TP53	Proteostasis regulation
			Post-translational protein modification	
			Antigen processing: Ubiquitination & Proteasome degradation	

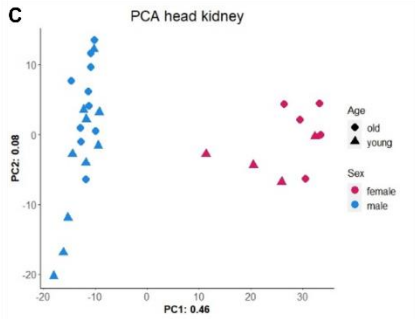


Figure 1: A) A graphical representation of *Syngnathus typhle* divided per body compartment. Picture modified from (Schneider, Woltering, Adriaens, & Roth, 2022). B) DEGs organised in clusters according to their expression in different tissues with B for brain, HK for head kidney and G for gonads. C) Summary table of REAC annotated functional pathways from the group of clusters sharing similar expression patterns. The colours correlate with the regression line for each cluster, with yellow corresponding to brain tissue, green to head kidney and blue to gonads (testis and ovaries together). D) Principal component analysis in head kidney for all individuals. The colours represent sex with blue for males and pink for females, while the shapes correspond to age category, with a circle for old and a triangle for young.

3.2. Age-dependent increase in sex-biased gene expression in head kidney: Metabolic and cell maintenance pathway analysis.

From the three distinct tissues, the differential gene expression analysis revealed that the head kidney had the highest count of significant (adj.P < 0.05) DEGs between males and females, with sex-biased gene expression differing even further when comparing young and old. The contrast M.y. vs. F.y. revealed 1919 DE transcripts, of which 1077 were upregulated in F.o. and 842 in M.y., and for M.o. vs. F.o. 3828, of which were 2114 upregulated in F.o. and 1714 in M.o. This suggests that sex-biased gene expression becomes more prevalent with age in pipefish. We wanted to understand how many of the significant DEGs were sex-specific across all ages, and which transcripts were biased only in young or old pipefish. We found that 569 transcripts were upregulated in males and 859 in females, irrespective of age (Figure 2A). On the other hand, 318 transcripts were only differentially expressed for sex in young pipefish, of which 182 were upregulated in M.y. and 136 if F.y. (Figure 2B), and 2059 DEGs in old pipefish, with 980 in M.o. and 1079 in F.o. (Figure 2C).

The following results of a functional enrichment analysis for both DEGs that were biased for sex, irrespective of age, and for those who were uniquely expressed in either sex for young or old, are summarised in Figure 2D for REAC and KEGG pathways (see supplements Table S1 for all results). There was a general enrichment in categories related to different metabolic pathways (metabolism of RNA - REAC:R-DRE-8953854, metabolism of lipids - REAC:R-DRE-556833, REAC:R-DRE-392499, fatty-acid metabolism - REAC:R-DRE-8978868) (Figure 2D). Both F.y. and F.o. had an overrepresentation of genes enriched for metabolic pathways involved in fat storage, such as fatty acid metabolism (REAC:R-DRE-8978868) and lipid metabolism (REAC:R-DRE-556833), peroxisomal lipid metabolism (REAC:R-DRE-390918) (Figure 2D). A closer look revealed upregulated genes such as *peroxisome proliferator-activated receptor gamma* (*pparγ*) a nuclear receptor important in the regulation of fatty acid metabolism balancing the storage and utilisation of fatty acids in the body (Rosen et al. 1999), or *adiponectin receptor 2* (*adipor2*) involved in the regulation of glucose (Table S2 and S3). F.o. differentially expressed genes such as hormone-sensitive *lipase* (*lipea*) and *lipoprotein lipase* (*lpl*), that encode enzymes that break down fats and regulate lipid metabolism.

Male pipefish had pathways such as metabolism of RNA (REAC:R-DRE-8953854), DNA metabolic process (GO:0006259), translation (REAC:R-DRE-72766), and ribosome (KEGG:03010) that were enriched (Figure 2D). M.y. and M.o. also both had an overrepresentation of genes involved in the non-sense mediated decay (NMD) (REAC:R-DRE-2990846) pathway (Figure 2D). This important pathway maintains the integrity of the genetic information by preventing the production of non-functional proteins, which is vital for correct intercellular communication (Hug, Longman, and Cáceres 2015). However, M.o. additionally had pathways enriched for DNA repair (REAC:R-DRE-73894), DNA double-strand break repair (REAC:R-DRE-5693532), and SUMOylation of DNA damage response and repair (REAC:R-DRE-3108214) (Figure 2D). For example, Mo's expressed more *rad* genes compared to any other group, such as *rad50*, *rad51b*, *rad52*, and *rad54* (Table S3). *Rad* genes are all involved in DNA repair, specifically in the process of homologous recombination (Henning and Stürzbecher 2003; Jalan, Olsen,

and Powell 2019). M.o. also differentially expressed *ATM serine/threonine kinase* and *checkpoint kinase 2* (*chek2*), both generally responsible for activating several downstream targets involved in repairing damaged DNA and preventing the development of cancer (Maréchal and Zou 2013; Mustofa et al. 2020). Whilst we found that M.y. differentially expressed *Sirtuin 2*, which is known to play a role in several processes from cell survival and DNA repair, to energy metabolism (Wang et al. 2019). M.o. had *mTOR kinase* upregulated, which can lead to increased cell growth and proliferation, but they also expressed *deptor*, which is a negative regulator of the mTOR signalling pathway acting as a feedback inhibitor (Catena and Fanciulli 2017). The downstream targets of mTOR are numerous (i.e., cell growth, metabolism, autophagy, translation initiation) and depend on sex hormones and environmental energy availability (Efeyan, Comb, and Sabatini 2015). Nonetheless, females also had various cell signalling pathways important for survival and maintenance, like FoxO signalling (KEGG:04068), with genes such as *forkehad box O4* (*foxo4*) and *peroxisome proliferator-activated receptor gamma, coactivator 1 alpha* (*ppargc1a*) homologous to *foxo1* upregulated in F.o.; or mTOR signalling (KEGG:04150), with express *lamtor1* and *lamtor2 also* upregulated in F.o., which act as scaffold-proteins by binding to mTOR complex1 promoting its activity (de Araujo et al. 2017); and autophagy (REAC:R-DRE-9612973), important in eliminating damaged proteins, with F.o. DE example *ATG5 autophagy related 5 homolog* (*atg5*) (Figure 2D).

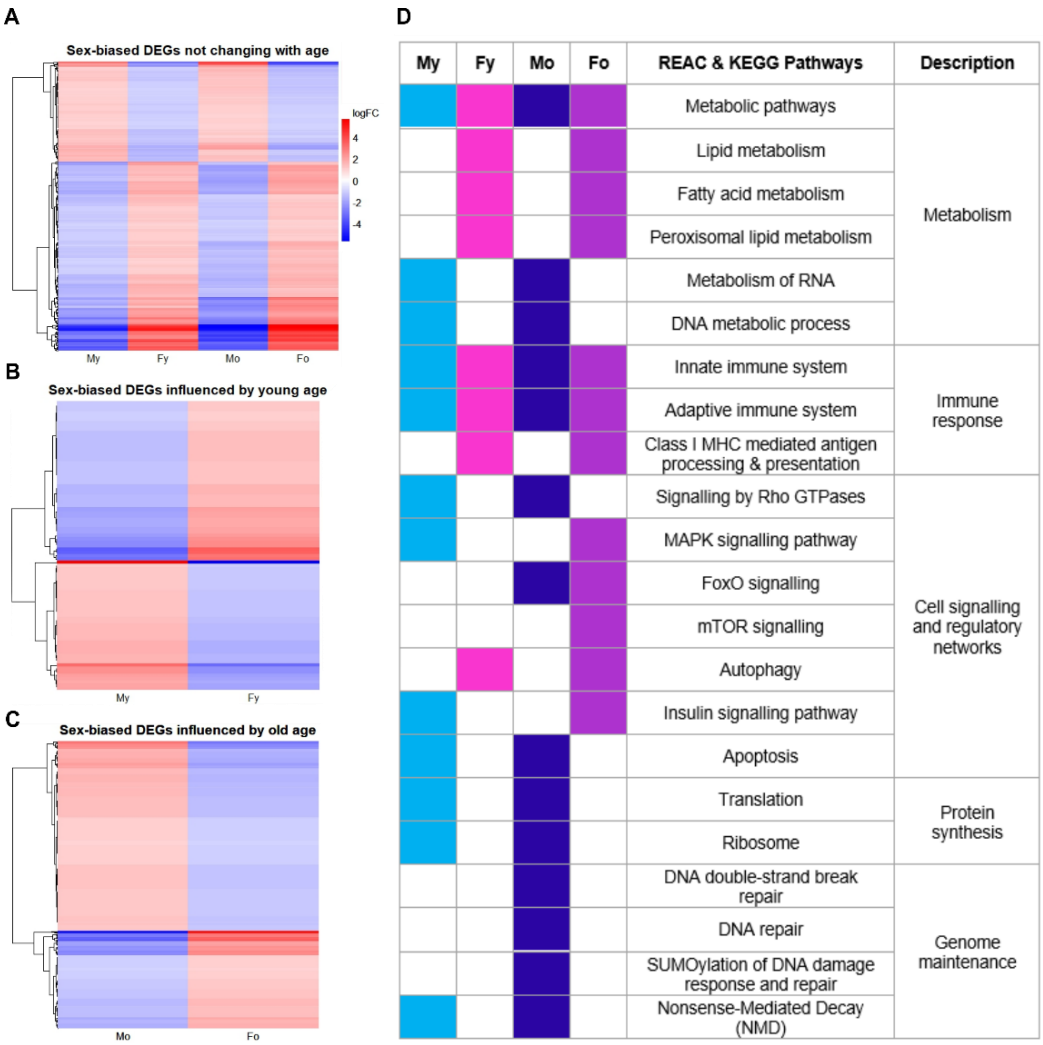


Figure 2: Heatmaps of DEGs and table annotating pathways of the transcripts from the heatmap. A) Heatmap of the 399 filtered genes (adj.P.val < 0.05, logFC < -1 and logFC > 1) that were differentially expressed for sex in both young and old pipefish, of which 138 upregulated and 269 downregulated in males. B) Heatmap of 87 filtered (adj.P.val < 0.05, logFC < -1 and logFC > 1) transcripts that were uniquely differentially expressed for sex only in young pipefish (39 M.y. and 48 F.y.). C) Heatmap of 527 filtered (adj.P.val < 0.05, logFC < -1 and logFC > 1) transcripts that were uniquely expressed for sex only in old pipefish (348 Mo and 179 Fo). D) Summary table of the annotated pathways from the functional enrichment analysis of DEGs in head kidney for DEGs from the heatmap.

3.3. Sexual immune dimorphism in head kidney reveals old male-biased gene expression

Many aspects of immune regulation in vertebrates are fairly well conserved and most of the major immune genes exist in teleost fish but may have multiple copies (paralogues) of particular genes (Bjørgen and Koppang 2021; Zou and Secombes 2016). The GO enrichment analysis found immune-related pathways, such as innate immune system (REAC:R-DRE-168249) and adaptive immune system (REAC:R-DRE-1280218) for each group (Figure 2D). Overall, we found that males had more upregulated immune genes compared to female, in both young and old (Table S5). Young pipefish differentially expressed 43 significant genes, of which 25 were upregulated in M.y. and 18 were upregulated in F.y., while old pipefish differentially expressed 71, of which 48 were upregulated in M.o. and 23 in F.o. (Table S5).

We identified which innate and adaptive immune genes were upregulated in either M.y., F.y., M.o., or F.o. We found the following anti-inflammatory markers expressed both in young and old males: *interleukin 13 receptor alpha 1* (*il13ra1*); two copies of *interleukin-1 receptor type-1-like* (*il1rl1*), which acts as a decoy receptor for il1 by binding to it but not transducing the signal (Zou and Secombes 2016), thus neutralizing its activity; *tumour necrosis factor alpha-induced protein 8-like 2b* (*tnfaip8l2b*) downregulated the production of pro-inflammatory cytokines (NF-kB) (Liu et al. 2021) (Table S5). As for young and old females, they expressed *interleukin 11 receptor alpha* (*il11ra*), involved in the transduction of the signal of IL-11, an anti-inflammatory cytokine and interleukin 10 (*il10*), which suppresses the production of pro-inflammatory cytokines such as TNF-alpha, IL-1, and IL-6 also inhibit the activation of immune cells such as macrophages, which are important for the immunity against intracellular and extracellular pathogens (Zou and Secombes 2016). Pro-inflammatory cytokines expressed in males were: *interleukin 12 receptor beta 2a* (*il12rb2*), involved in the transduction of the signal of IL12 (Zou and Secombes 2016); *tumour necrosis factor receptor superfamily member 1B* (*tnfrsf1b*), involved in the transduction of TNF-alpha signal (Baker and Reddy n.d.); and several *chemokine receptors* (*cxcr1* and *cscr4a*) (Table S5). They also expressed multiple *neutrophil cytosolic factors (ncf1, ncf2* and *ncf4*), where NCF1 is considered a pro-inflammatory protein that is involved in the production of reactive oxygen species important for killing pathogens (Holmdahl et al. 2016).

However, older males had additional pro-inflammatory elements expressed: *tumour necrosis factor receptor superfamily* (*tnfrs*), *member 1a* and *21*; *interleukin 17 receptor C* (*il17rc*), which together with other IL-17 receptors (*il17ra* and *il17rd*) are involved in the transduction of IL-17A and contribute to an inflammatory response (Zou and Secombes 2016); toll-like receptors (TLR) were also only upregulated in old males, such as *tlr3* and *tlr18* (Table 1 and Table S5), the latter is a homolog of human *tlr1*, both important in initiating an immune response against viral infections by recognising double-stranded RNA (Kawasaki and Kawai 2014; Li et al. 2017). In comparison, the pro-inflammatory expression did not change all that much between young and old females, with them both expressing *il17ra1a*, part of the il17 receptor complex described above and *interferon-gamma receptor 1-like* (*ifngr1l*) (Table S5). Yet, F.y. did also have *interleukin 6 transducer* (*il6st*) upregulated, which is a transmembrane protein involved in the signal transduction of cytokines belonging to the IL-6 family (Grayfer and Belosevic 2012).

F.o. had several variants of major histocompatibility complex (MHC) class 1 upregulated compared to males (Table 1), 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). Nonetheless, M.o. in particular had a variety of upregulated clusters of differentiation (CD) genes, such as *cd7*, c*d44b*, *cd209*, *cd81*, *cd247*, and *cd79a* (Table 1). CD molecules encode surface proteins on white blood cells that are important in regulating the function of immune cells (Tian et al. 2022). M.o. also had an increased biased expression for immunoglobulins (Ig) (Table 1), also known as antibodies that help identify and neutralize bacteria and viruses, they are divided into heavy and

light polypeptide chains (Jung et al. 2006). M.o. expressed IgD, two Ig heavy variable genes *ighv1-4* and *ighv4-3* important in determining the antigen specificity of the Ig, two copies of Ig light 1 constant 3, and recombination signal binding protein for immunoglobulin kappa J region-like (*rbpjl*) (Table 1). The latter plays a critical role in the joining of the V, D, and J segments during the VDJ recombination process which is important in the generation of a diverse set of antibodies (Webb et al. 2021).

	GENE	My	Fy	Mo	Fo
adaptive	*c2cd2l*		■		
	cd9b				■
	leng9				■
	mal2				■
	sema3bl				■
	mhc1uka				■
	mhc1uka.1				■
	tcta				■
	rag1	■			
	cd99l2	■			
	cd44b			■	
	CR847844			■	
	jak3			■	
	ighv1			■	
	ighv4			■	
	cd81a			■	
	igl1c3			■	
	blnk			■	
	bcap29			■	
	rbpjl			■	
	cd247l			■	
	swap70a			■	
	igl1c3.1			■	
	cd79a			■	
	nfat5b			■	
	ighd			■	
innate	*nfil3*		■		
	irak1		■		
	nfil3.1		■		
	il6st		■		
	irf6		■		
	tnfrsf9b				■
	ifrd1				■
	maea				■
	il17rd				■
	c8a				■
	traf2b	■			
	cxcr1.1	■			
	cxcr1.2	■			
	crlf3	■			
	tlr18			■	
	unc93b1			■	
	cxcl12a			■	
	ifih1			■	
	irf2bpl			■	
	mpeg1			■	
	cxcr3			■	
	irf8			■	
	il1fma			■	
	il17rc			■	
	tlr3			■	

	tnfrsf21				
	ccl25b				
	tnfrsf1a				

Table 1: The following table displays immune genes that were significantly differentially expressed in the head kidney for sex exclusively either in young or old pipefish. Boxes coloured in red indicate genes upregulated for either M.y., F.y., M.o. or F.o.

4. Discussion

Our main aim was to understand how male and female sex-role reversed pipefish differentiate in allocating their resources for reproduction or soma maintenance for age. We analysed gene expression in three different tissues of young, old, female and reproductively active (pregnant) and non-reproductively active males in the pipefish *Syngnathus typhle*. We identified distinct expression profiles in brain, head kidney and gonads, with most annotated pathways being unique to the organ type (Figure 1D). Pregnancy and age did not appear to strongly influence gene expression in any of the tissues assessed. Sex had the strongest effect on gonads and head kidney, and not at all on brain (Figure 1C). The analysis also revealed that age had an impact on the types of genes that were differentially expressed between males and females in the head kidney.

The gene ontology enrichment analysis comparing M.y., F.y., M.o. and F.o. revealed that females had many more upregulated transcripts relating to energy and lipid metabolism, whilst males had several upregulated pathways involved in the translation and genome maintenance process. Basic metabolic processes in males and females are similar in nature. Howbeit, different body composition (fat and muscle mass), sex hormones, sex-specific energy expenditure and age are all known to influence the effectiveness of how they metabolise nutrients and store energy (Krumsiek et al. 2015; Quirós Cognuck et al. 2020). Often a higher muscle mass correlates with a higher metabolic rate and in male mammals, sexual traits, such as body size or body colouration, are under stronger sexual selection (Halsey et al. 2022). Both F.y. and F.o. had enriched metabolic pathways involved in fat storage, such as fatty acid metabolism (REAC:R-DRE-8978868) and lipid metabolism (REAC:R-DRE-556833). Additionally, females had both *pparγ* and *adiponectin* upregulated, the first a nuclear receptor important in the regulation of fatty acid metabolism which balances the storage and utilisation of fatty acids in the body (Rosen et al. 1999), found to be mostly higher expressed in the adipose tissue of females (Vidal-Puig et al. 1997), while the latter is involved in the regulation of glucose and was found to increase in older female mice (Gui, Silha, and Murphy 2004). These results match with research in conventional sex-role mammalian models (Figure 3), where females tend to have higher expression of genes involved in fat storage, consistent with the fact that they tend to store more body fat than males and are more efficient at using it as a source of energy (Shi, Seeley, and Clegg 2009). In mammals, increased lipid storage helps to ensure that enough energy is there to support herself and her offspring during development and provides a buffer against periods of food scarcity (Heldstab, van Schaik, and Isler 2017; Rödel et al. 2016). In the sex-role reversed broad-nosed pipefish this seems to remain a maternal trait and thus likely only

related to egg production, but not necessarily pregnancy. Furthermore, also sex-hormones play an important role in metabolism and particularly oestrogen concentrations can alter how and where fat is stored in the body (Rubinow 2018). In *S. typhle* males produce testosterone and females produce oestrogen (Figure 3), despite the male pregnancy and the sex-role reversal (Eens and Pinxten 2000; Mayer et al. 1993). Interestingly, a recent study in medaka revealed that females turned into males upon a starvation period, the inhibition of the fatty acid synthesis was suggested to have induced the sex reversal (Sakae et al. 2020). In *S. typhle* female would appear to rely more on lipids as a source of energy for growth and egg production (Valdebenito, Gallegos, and Effer 2015), fat deposits being possibly regulated by oestrogen. Male pipefish pregnancy is energetically costly, so they may be utilizing other preferred sources of energy such as carbohydrates or proteins.

Male pipefish expressed genes from pathways involved in NMD, RNA, DNA metabolic process and ribosomes, which could indicate changes in the rates and stability of translation and transcription processes in males compared to females (Hug, Longman, and Cáceres 2015; López-Otín et al. 2013). This could be due to testosterones promoting cell growth and more rapid division, which would possibly lead to a shorter lifespan (Ma et al. 2015; Park et al. 2016). However, testosterone levels change with age and the levels usually drop during male pregnancy (Eens and Pinxten 2000; Mayer et al. 1993; Scobell and Mackenzie 2011). Old male pipefish in particular had an overrepresentation of pathways relating to DNA repair (Figure 2D). This may be due to exposure to environmental damage or increased oxidative stress, with the cell trying to adapt and maintain genomic stability. From the perspective of somatic maintenance, these results could be very beneficial, as an accumulation of DNA damage over time is one of the major causes of ageing (López-Otín et al. 2013) and an efficient DNA repair system mechanism is crucial in resisting oxidative stress and maintaining genomic integrity. This suggests a sex-role-dependent selection for male pipefish to be investing more energy in protein and genome maintenance pathways (Figure 3). Nonetheless, there were many more genes involved in autophagy that were upregulated in F.o. (Figure 2D). Autophagy is vital to recycling and/or degrading damaged proteins and organelles (López-Otín et al. 2013). There is some research (primarily in rodents and humans) that indicate that there may be sex-specific differences in the regulation of autophagy through sex hormones (Park et al. 2016). Testosterone has been shown to decrease the expression of autophagy-related genes in men, while oestrogen increases them (Ma et al. 2015; Park et al. 2016). As previously mentioned, a sex-role reversal in *S. typhle* did not evolve with a reversal of sex hormones (Mayer et al. 1993; Scobell and Mackenzie 2011). Thus, it would appear that sex, specifically sex hormones, may be the determining factor for the regulation of autophagy. Moreover, it is important to note that the upregulation of autophagy genes is beneficial to a certain degree, as it has been found to lead to cell death and cancer if overexpressed (Finnegan et al. 2022).

Our differential gene expression analysis in head kidney also showed sex-specific immune gene expression, with the differences between males and females becoming even stronger with age. Sex immune dimorphism is known

in diverse species from insects to lizards, birds and mammals, with an overall stronger innate and adaptive immune response in females (Roved, Westerdahl, and Hasselquist, 2017; Klein and Flanagan 2016a). Theories suggest that this could be a result of being either the heterogametic or homogametic sex, as the same sex chromosomes help compensate for recessive mutations (Regan and Partridge 2013; Xirocostas, Everingham, and Moles 2020), or sex-steroid hormones where, for example, oestrogens can act as antioxidants (Borrás et al. 2021), boost telomere repair mechanism (Bayne et al. 2008), and testosterone can increase aggression providing an advantage in the competition so by increasing the extrinsic hazard risk (Brooks and Garratt 2017) and additionally acting immunosuppressive (Trigunaite, Dimo, and Jørgensen 2015). Generally, sex-dependent selection to optimise energy investment in either soma maintenance or reproduction may have led to differences in resource allocation in immune defence, metabolic requirements and ultimately mortality risk (Bronikowski et al. 2022; Kirkwood and Rose 1991; Pennell, Galligan, and Fish 2012). From our results, we found a sex-specific differential expression of immune genes, with males expressing more pro-inflammatory molecules, TLRs, CD genes, and immunoglobulins (Table 1 and Table S5). These results are in contrast to studies in humans and other mammals (Figure 3), 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 (Capone et al. 2018; Klein and Flanagan 2016). Furthermore, sex-hormone-specific research has shown that androgens typically induce an anti-inflammatory response (Klein and Flanagan 2016; Taneja 2018), increasing also *il10* production, which conversely, was significantly upregulated in female pipefish in our dataset, with also numerous pro-inflammatory markers being upregulated in males (Table 1 and Table S5). Although female *S. typhle* did not have such a strong immune variation with age, they did have MHC class 1 variants upregulated (Table 1). These molecules are a critical component of the adaptive immune system and are responsible for presenting viral peptides, among others, to CD8+ T cells for destruction (Garcia et al. 1996). This could mean that females are better equipped to mount a cytotoxic T-cell response against infected cells, which could be the result of certain pathogens having a higher prevalence in females compared to males. 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 (Richards, van Oosterhout, and Cable 2010). 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 broad-nosed pipefish (Beemelmanns and Roth 2016). Potential future studies could investigate if parents are also priming their offspring to sex-specific pathogens.

The fact that males have overall more upregulated immune genes, particularly innate genes in older individuals, might be the result of males requiring a more pronounced immune response to protect their health. In the sex-role revered pipefish *S. typhle* males have a greater investment in the survival of their offspring and themselves, as they are the sex that is reproductively constrained (Mobley et al. 2018). Also, males 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 (Tanger et al. 2023) and embryos are highly vulnerable to pathogenic attack (Wu et al. 2021). We also know from various animal systems that males and females respond differently to microbes and other immune challenges and that numerous factors contribute to sex-immune dimorphism from hormonal differences to behaviour-based pathogen exposure (Richards, van Oosterhout, and Cable 2010; Rubinow 2018). Such results would indicate a sex pattern reversal of inflammatory response compared with conventional sex-role species (Figure 3), thus making sex-role and the life-history trade-offs play a more significant role here.

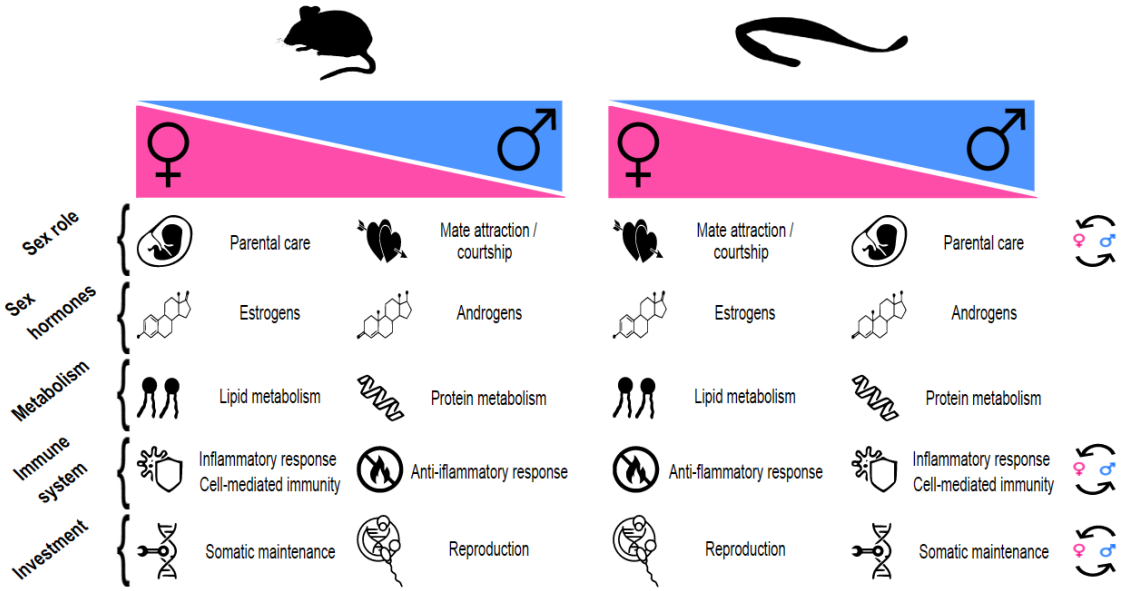


Figure 3: Here represented is a summary comparison of traits between a conventional sex-role mouse model and the broad-nosed pipefish, which exhibits sex-role reversal. Specifically, we examine how these traits differ based on biological sex (pink for females and blue for males). On the far right of the diagram, circular arrows indicate what life-history traits may be reversed compared to the conventional model species.

In summary, sex-biased gene expression was strongest between old male and female *S. typhle* indicating the importance of looking at sex traits in the context of senescence, specifically in species where fertility increasing proportionally with age. Biological sex seems to be responsible for regulating fatty acid and lipid metabolism in female pipefish, aligning with results from other species with conventional sex roles (Figure 3). Oppositely, our results suggest that sex-roles may be leading male pipefish to allocate more energy to genome stability and repair, and immune response as they are the sex that is time-constrained by the need to grow offspring in their pouch (Figure 3). Similarly how for female mammals, having a higher parental time cost may result in them increasing their reproductive time by upregulating their somatic maintenance and improving their health for the long haul.

Our study highlights the importance of considering the evolutionary context of senescence and the need to investigate a variety of species with non-conventional sex behaviours to comprehensively understand the evolution of sex lifespan dimorphism and sex-specific resource allocation. In future research, we aim to extend our studies on sexual dimorphism beyond a single species of Syngnathids, given the diverse array of sex role changes present within the family (Hamilton et al. 2017). By aggregating more datasets on sex-biased gene expression, we can gain a better understanding of what determines sex and sex role in Syngnathids. Furthermore, future research that involves comparing endocrine hormonal production and endocrinological manipulation could provide insights into how sex is decoupled from sex roles, while also addressing the impact of hormones on gene expression and the resulting phenotype.

5. Conclusion

Sex-specific lifespan dimorphism is found amongst many species and one prevalent hypothesis for this is that males and females have distinct life-history strategies, which lead to unequal resource allocation between somatic maintenance and reproduction, depending on how they acquire and optimise their fitness. In our study we analysed gene expression of the sex-role reversed broad-nosed pipefish and found sex-specific differential gene expression in head kidney varying with age. Specifically, females had many more upregulated transcripts relating to energy and lipid metabolism, while males had many upregulated pathways involved in translation, genome maintenance processes, and immune defence. Interestingly, while sex roles may influence some gene groups and reverse their expression (e.g. genome maintenance and immune defence) when moving from conventional to sex-role reversal, the activity of other gene groups may depend on biological sex and sex hormones (e.g., energy and fat storage). This study underscores the importance of incorporating a wider range of species that exist along a spectrum of sex roles, in order to enhance future research on sex-specific immune and lifespan-dimorphism, as well as the roles of biological sex and sex soles in shaping them.

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 Ditgitalisierung (MELUND) Schleswig-Holstein (permit no. V24257982/2018). No wild endangered species were used in this investigation.

Authors' contributions

F.A.P carried out the experiment, analysed the data, interpreted the results and wrote the article. 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 both additional figures (Figure S1-S3), the significant differentially expressed genes in head kidney and the gene set enrichment analysis results as tables (Table S1-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.

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