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Origin-associated transcriptomic signatures across the reproductive axis of *Solea senegalensis*

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Abstract

Understanding how captive conditions impair reproduction remains a major challenge in vertebrate biology and aquaculture. In Senegalese sole (*Solea senegalensis*), this problem is especially evident in captive-bred males, which are widely reported to show impaired mating behaviour and reduced production of functional sperm. To characterize gene expression variation in tissues involved in reproductive regulation, we performed RNA-seq profiling across the hypothalamic-pituitary-gonadal (HPG) axis in adult wild-bred and captive-bred males and females at the end of the spawning season. Tissue identity was the main driver of transcriptional variation, with sex-biased differences concentrated in the gonad and origin-associated differences more pronounced in central neuroendocrine tissues, particularly the pituitary. In males, integrated analyses identified candidate networks enriched in captive-bred individuals and containing genes related to

neuroendocrine signalling, steroid metabolism and reproductive regulation. These results provide a transcriptomic framework for understanding how origin-associated differences across the HPG axis may be relevant to the reproductive phenotype previously described in captive-bred Senegalese sole.

Keywords Integrated transcriptomics, reproduction impairment, domestic fish, steroidogenesis, neuroendocrine signalling, aquaculture

Introduction

Reproduction defines evolutionary success in natural populations and sets practical limits on productivity in animal production systems. In aquaculture, reliable reproductive performance of broodstock is essential to sustain production while maintaining genetic diversity and limiting inbreeding over generations [1]. Across many domesticated species, including numerous captive reared fish, reproductive performance is often compromised relative to wild conspecifics, although the magnitude of this effect varies among species [2, 3]. Despite its importance, the biological and environmental processes that give rise to reproductive impairment under domestic conditions remain poorly understood [4, 5, 6].

The Senegalese sole (*Solea senegalensis*), a flatfish species of high commercial value in Europe (2,500 tonnes, €19 million/year [7]), is a representative example of this phenomenon, as its reproductive performance is often disrupted under farming conditions [8, 9]. Captive-bred (CB) individuals of this species are widely reported to exhibit reproductive problems involving both behavioural and physiological components. These dysfunctions predominantly affect males and are associated with altered neuroendocrine coordination involving both reproductive behaviour and gonadal function [10]. CB males are frequently unable to perform courtship and mating, and

show a reduced production of functional sperm, compromising reproductive success on farms [11, 12, 13, 14]. As a result, production still relies on wild males acclimated to farm conditions (wild-bred, WB) and hampers the development of breeding programs [15].

Previous studies reported the low quality and amount of sperm in *S. senegalensis* [16, 17]. Moreover, recent transcriptomic analyses have identified consistent alterations associated with spermatogenesis and reduced reproductive performance of CB individuals [18]. At cellular resolution, single nucleus transcriptomics have further shown that CB males exhibit an incomplete progression of spermatogenesis, characterized by a depletion of advanced stages of germ cell differentiation, hypothetically associated with Sertoli-spermatogonia communication [19]. In parallel, functional experiments using gonadotropin-based treatments have demonstrated that hormonal stimulation, while capable of modulating key processes of spermiogenesis, is insufficient to fully restore the production of functional spermatozoa [15]. Consistent with the involvement of upstream regulatory mechanisms, environmental enrichment through sandy substrates has been shown to improve reproductive performance while inducing pronounced transcriptional changes in the brain, in addition to the testis [20]. Moreover, studies targeting the kisspeptin pathway have emphasized the role of central neuroendocrine control in regulating the reproductive axis in this species [21]. All these findings suggest that reproductive impairment in CB *S. senegalensis* may not be restricted to the gonad and may involve upstream components of reproductive regulation. However, how transcriptional differences are distributed across the hypothalamus, pituitary and gonad, and whether central and gonadal tissues show coordinated origin-associated patterns, remains poorly characterized. This highlights the need for an integrated transcriptomic view of the reproductive axis across tissues, sexes and origins.

Notably, WB individuals can reproduce successfully under captive conditions, whereas CB males often show reproductive failure [22]. This contrast suggests that origin-associated differences in gene regulation may contribute to the reproductive phenotype described in this species, although other origin-related factors, including developmental history, genetic background, age and reproductive experience, cannot be fully excluded. Such differences may involve changes in gene expression across the components of the reproductive axis [23], from its central neuroendocrine regulators, the hypothalamus and pituitary, to its peripheral effector organ, the gonad, together constituting the hypothalamic-pituitary-gonadal (HPG) axis. Accordingly, transcriptomic profiling across these tissues provides a suitable framework to characterize transcriptional variation across the reproductive axis.

This study uses RNA sequencing (RNA-seq) to characterize gene expression across the HPG axis of *S. senegalensis*, providing an integrated transcriptional view of the main regulatory system controlling reproduction. By comparing transcriptomic profiles across tissues, sexes, and origins, we aimed to define the molecular organization of the axis and identify transcriptional programs associated with different reproductive conditions. Because reproductive dysfunction in captive-bred Senegalese sole has been mainly documented in males [10, 11, 12], the male CB vs WB DEGs were further explored through network analysis to examine their potential functional relationships. Together, the results obtained provide a framework for understanding the molecular basis of reproductive dysfunction and may help guide future strategies for broodstock management and selective breeding in aquaculture.

Results

RNA-seq libraries quality

A total of 53 RNA-seq libraries were generated. After post-sequencing quality control, two libraries were excluded due to low mapping performance and inconsistent clustering. Therefore, 51 libraries were retained for downstream analyses, with an average of 59,504,052 reads per library and an average mapping rate of 92.3% to the *S. senegalensis* reference genome [14] (**Supplementary table 1**). Principal component analysis (PCA) clearly separated samples by tissue when plotting PC1 (47.8% of the total variance) and PC2 (18.8%) (**Fig. 1a**). Gonadal samples were further segregated by sex, reflecting strong sex-specific transcriptomic differences in this tissue. Hierarchical clustering yielded a similar pattern, with hypothalamus and pituitary, as in the PCA, grouping more closely together and gonads segregating by sex (**Fig. 1b**). No clear separation between WB and CB individuals was observed at this level, and although a certain

segregation by origin was detected within the pituitary cluster (**Fig. 1b**), this pattern did not result in fully clustering of samples by origin.

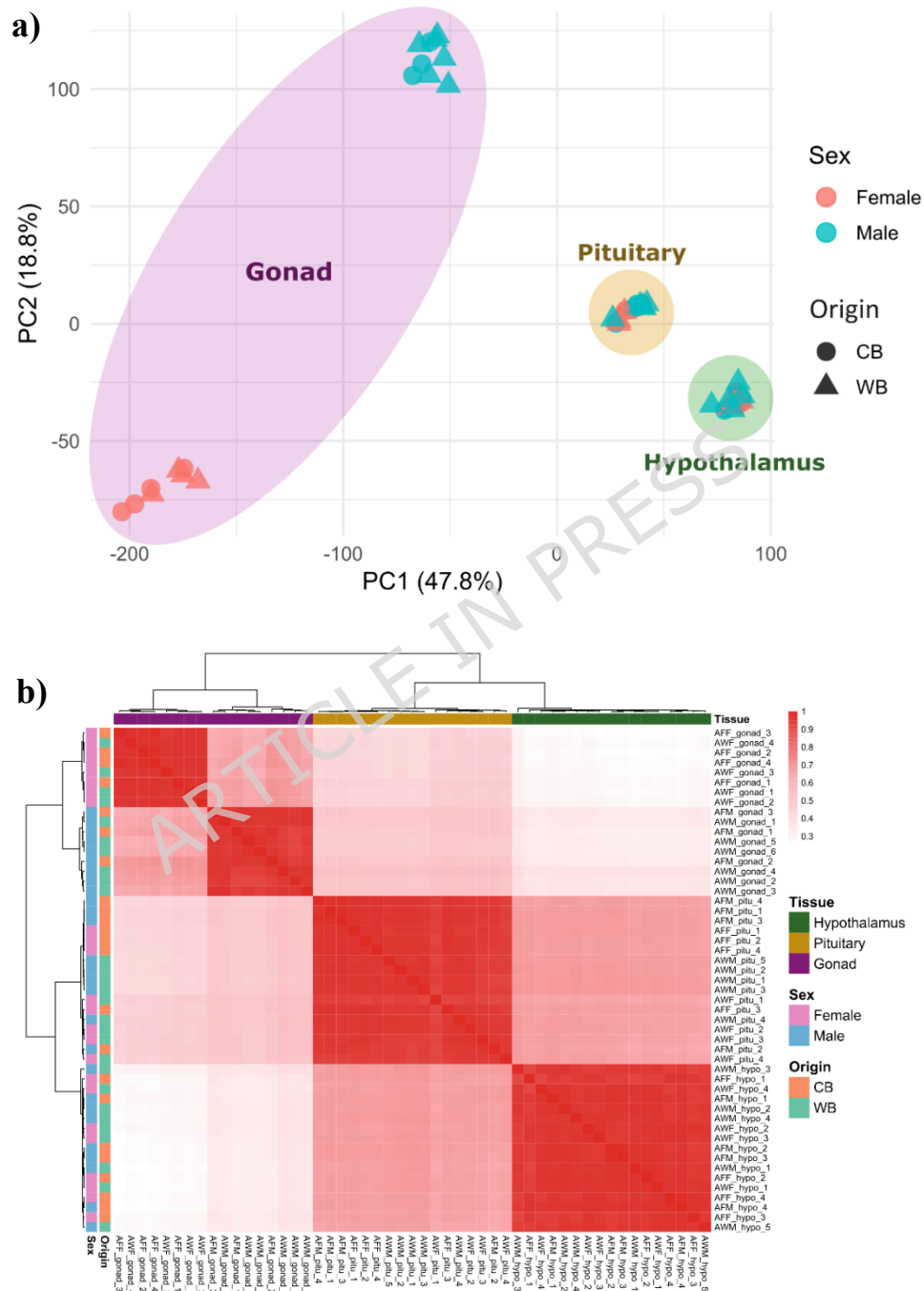


Fig. 1: Quality assessment of RNA-seq libraries across HPG axis samples. a) Principal component analysis (PCA) of all samples based on normalized expression values of genes retained in the filtered HPG axis transcriptome. **b)** Hierarchical clustering heatmap showing sample-to-sample similarity based on gene expression profiles. Clustering was performed using Spearman's correlation.

HPG axis transcriptome profiling

Transcriptomes of the three tissues that constitute the HPG axis were first characterized jointly and subsequently analysed separately. The HPG axis transcriptome comprised 18,545 genes, representing 73.17% of the total annotated general transcriptome, with 15,613 genes detected in the hypothalamus (61.60%), 11,544 in the pituitary (45.55%) and 15,248 in the gonad (60.16%) (**Supplementary table 2**). A substantial proportion of genes (9,923) were active in the three tissues (**Supplementary figure 1**). Pairwise comparisons revealed that the hypothalamus shared more genes with the gonad (2,605) than with the pituitary (1,106), whereas the gonad and pituitary exhibited minimal overlap (285).

Functional enrichment analyses were also performed on the transcriptomes of the HPG axis as a whole and on each tissue separately taking as reference the *S. senegalensis* transcriptome [14]. A total of 323, 312, 370 and 443 significantly enriched GO terms were identified in the whole HPG axis, hypothalamus, pituitary and gonad transcriptomes, respectively (FWER < 0.05; **Supplementary table 2**), providing a global functional overview of the expressed gene sets. Across all datasets, the enriched terms were mainly related to general cellular and metabolic functions, including metabolic and biosynthetic processes, protein metabolism, translation, RNA processing, intracellular transport and protein localization. In addition, tissue-specific functional categories were detected. The hypothalamus was enriched for terms associated with intracellular signalling, neurogenesis and glutamate receptor activity, whereas the pituitary showed enrichment in vesicle mediated transport, oxidative phosphorylation, and protein targeting. The gonad presented the highest number of enriched terms and included categories related to cell cycle, chromosome organization, DNA replication and DNA repair.

Transcriptional identity of HPG axis components

Tissue-specific genes were identified through the calculation of the tissue specificity index (TSI; $\tau \geq 0.9$). In total, 1,817, 172 and 1,721 specific genes were detected in the hypothalamus, pituitary and gonad, respectively (**Supplementary table 3**). Genes with the highest TSI values in the hypothalamus were key neuroendocrine regulators, such as *oxl* (oxytocin/neurophysin I prepropeptide), *gnrh2* (gonadotropin-releasing hormone 2), *adcyap1b* (adenylate cyclase activating polypeptide 1b), *cart* and *cartpt* (cocaine- and amphetamine-regulated transcript), *nts* (neurotensin), and *pth4* (parathyroid hormone 4). In addition, neuronal markers such as *slc17a6a*, *slc6a1a*, *slc6a1b*, *grin1a* and *camk2a* and genes associated with myelination (*mbpa*, *plp1b*, *mpz*, *mag*) and synaptic function (*doc2a*, *stxbp6l*, *gjd1a*, among others) were also represented among the hypothalamus specific set. Together, these results confirm the strong neuroendocrine and neuronal identity of the hypothalamus transcriptome.

Among the 172 specific genes in the pituitary, those with the highest TSI values included several key endocrine regulators, such as the gonadotropin subunits *lhb* (luteinizing hormone beta) and *cga* (glycoprotein hormone alpha subunit), *tshb* (thyroid-stimulating hormone beta), *smtla* (somatolactin alpha), *soma* (growth hormone), and *prl* (prolactin). In addition, receptors involved in hypothalamic-pituitary signalling, such as *gnrhr2* (gonadotropin-releasing hormone receptor 2) and *nrbwr2a* (neuropeptide B/W receptor 2a), were also among the most tissue-specific genes. These findings are consistent with the central role of the pituitary in hormonal integration within the HPG axis. Notably, the gene annotated as *tshb* (ENSSSGG00010002420) in the Ensembl reference genome (GCA_919967415.2) corresponds to the follicle-stimulating hormone beta subunit (*fshb*), as confirmed by BLAST searches against the NCBI reference genome assembly of *S. senegalensis* (GCF_019176455.1) and by its assignment within the orthology analysis

performed in this study. This gene consistently clustered within orthogroups containing *fshb* genes across multiple teleost species, supporting its correct annotation as *fshb* rather than *tshb*.

Finally, among the 1,721 specific genes in the gonad, many were associated with meiosis, signalling, and transcriptional regulation, including *terb2* (telomere repeat binding bouquet formation protein 2), *lef1* (lymphoid enhancer-binding factor 1), *creb1* (cAMP responsive element binding protein 1) and *satb1* (special AT-rich sequence-binding protein 1). Histone-related genes such as *h2be1*, *h2a*, *hist1h4l* and *dpy30* were also prominently represented within this set.

Functional enrichment of tissue-specific genes

To further characterize the functional profiles of HPG tissues, GO enrichment analyses were performed using tissue-specific genes ($\tau \geq 0.9$) for each tissue (**Supplementary table 3**), revealing distinct functional profiles across the three components of the HPG axis with minimal overlap among the main Biological Process (BP) categories (**Fig. 2**).

In the

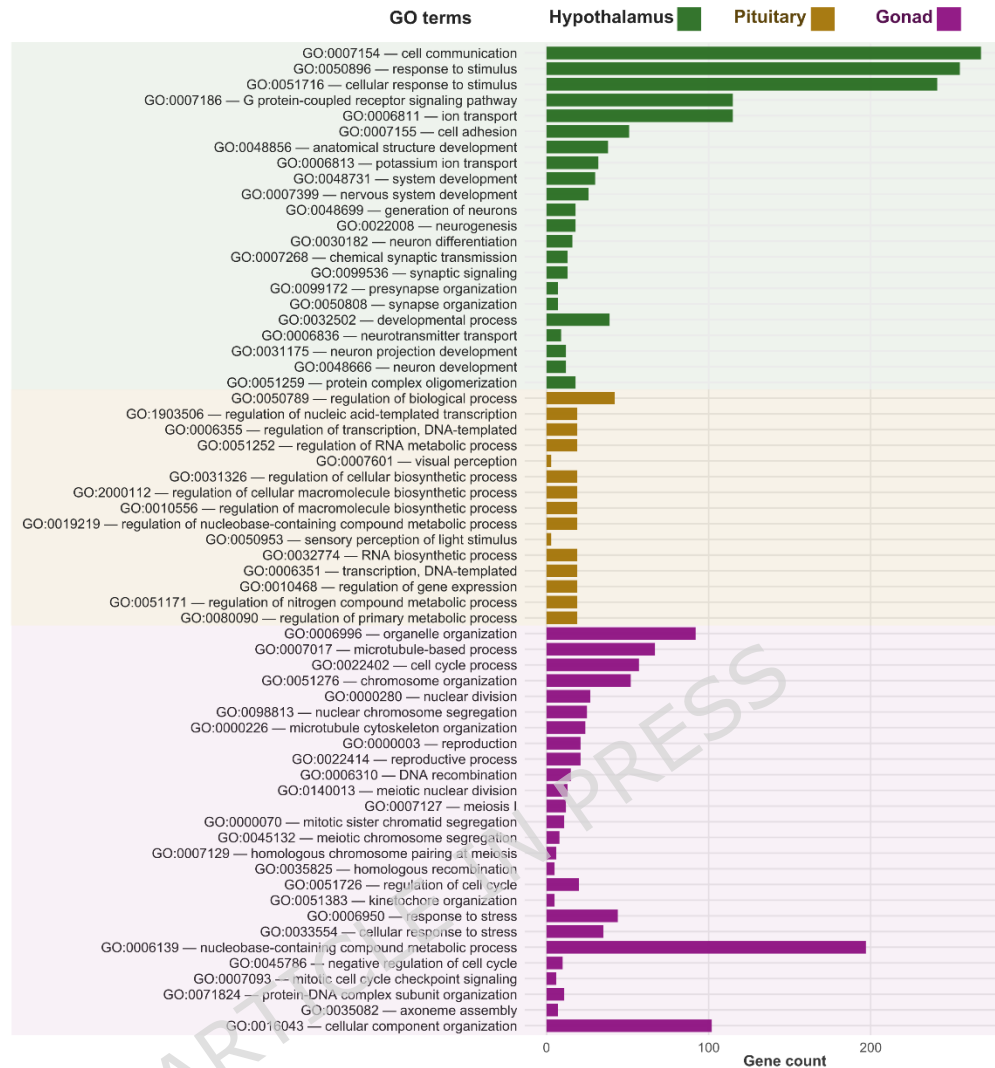


Fig. 2: Functional enrichment of tissue-specific genes in the HPG axis. Gene Ontology (GO) enrichment analysis of tissue-specific genes identified using the tissue specificity index (TSI; $\tau \geq 0.9$) in hypothalamus, pituitary and gonad. Representative Biological Process (BP) terms are shown after summarization of redundant categories. Bar length indicates the number of genes associated with each GO term. Only significantly enriched terms (FWER < 0.05) are displayed.

hypothalamus, a total of 126 significantly enriched GO terms were identified, 57 corresponding to Biological Process (BP). Among these, *cell communication*, *response to stimulus*, *G protein-coupled receptor signalling pathway*, *ion transport*, and processes related to *neurogenesis*, *synaptic signalling*, and *neuron differentiation* were the most represented and accounted for the highest gene counts within the hypothalamus specific gene set.

In the pituitary, 29 enriched GO terms were detected, including 20 BP. Compared to the hypothalamus, the total number of enriched terms and gene counts per term were lower, consistent with the smaller number of tissue-specific genes identified. Remarkable BP categories included *regulation of transcription*, *regulation of gene expression*, *RNA biosynthetic process*, and *regulation of macromolecule metabolic process*. Notably, *hormone activity* (Molecular Function term) was exclusively enriched in the pituitary (**Supplementary table 3**).

Among the 126 enriched GO terms identified in the gonad, 76 corresponded to BP categories. The most representative BP terms were related to *organelle organization*, *chromosome organization*, *nuclear division*, *meiotic nuclear division*, *DNA recombination*, *cell cycle regulation*, and *reproduction* (**Fig. 2**; **Supplementary table 3**).

Transcriptional differences between conditions across the HPG axis

Sex-dependent differences

Differential gene expression analyses revealed pronounced sex-biased transcriptional differences across the HPG axis (**Fig. 3**; **Supplementary table 4-6**). The gonad exhibited by far the strongest transcriptional divergence between males and females.

In the overall sex comparison (pooled origins), 15,435 DEGs were detected in the gonad, of which

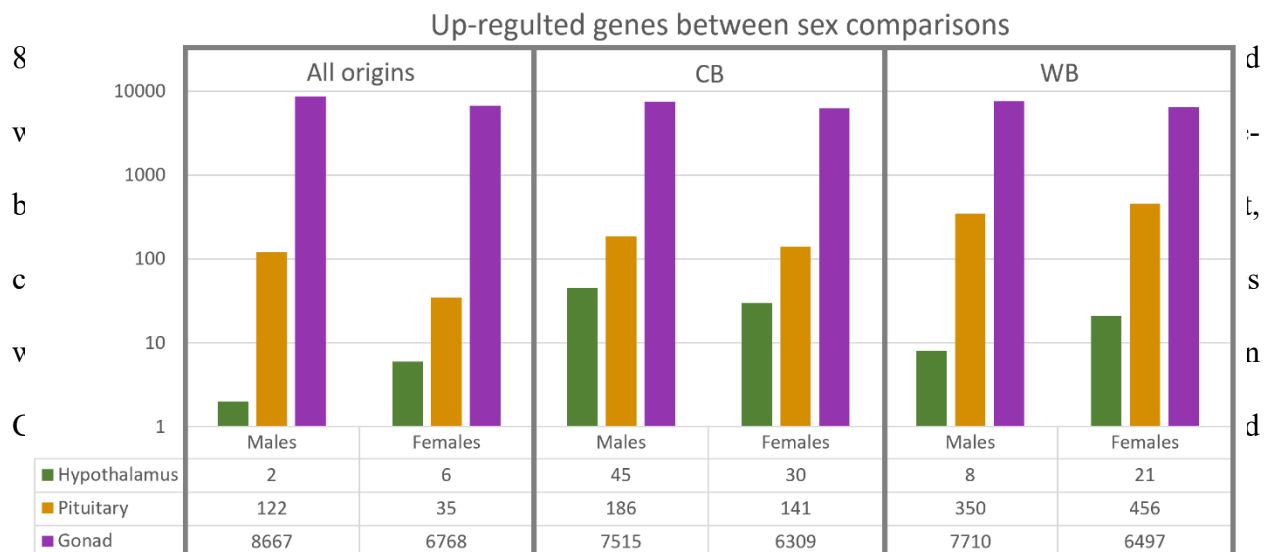


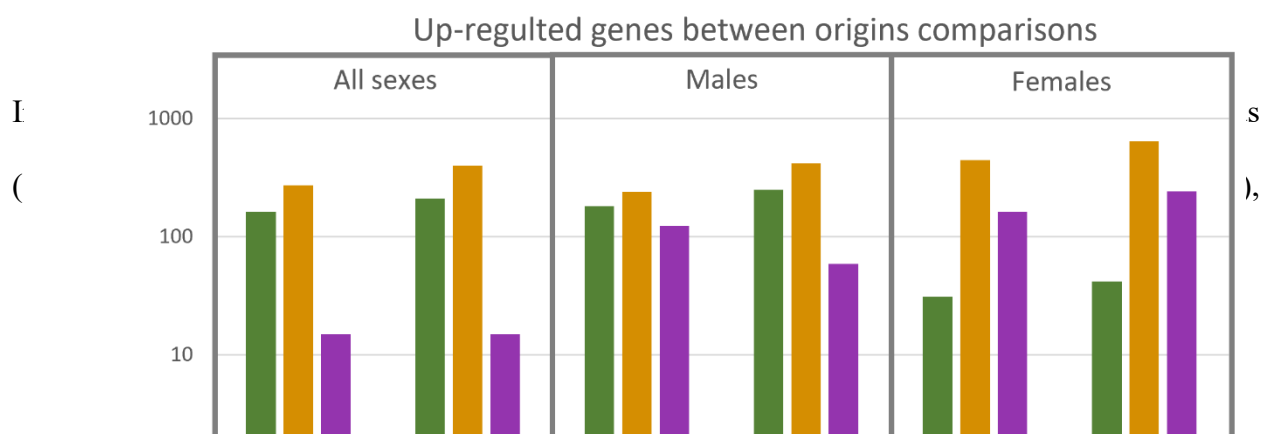
Fig. 3: Sex-based differential gene expression across the axis. Barplot showing the number of upregulated genes identified in hypothalamus, pituitary and gonad across male vs female comparisons, including all samples (All origins) and origin specific subsets (CB and WB). Bars represent the number of genes upregulated in each condition. The table below indicates the exact number of genes per tissue and comparison. DEGs were defined using a Benjamini-Hochberg adjusted p-value

and 21 female-biased). The pituitary showed a higher level of sexual divergence, with 157 DEGs in the overall comparison (122 male-biased and 35 female-biased), increasing to 327 in CB individuals (186 male-biased and 141 female-biased) and 806 in WB individuals (350 male-biased and 456 female-biased), suggesting a more pronounced sex-associated regulation in this tissue compared to the hypothalamus.

Functional enrichment analyses of sex-dependent DEGs (**Supplementary table 10**) were dominated by terms enriched in the gonad. Enriched categories were primarily associated with intracellular processes, including purine metabolism, nucleus and organelle-related functions. In the pituitary, enrichment profiles differed between origins, CB associated contrasts were enriched for signalling and response to stimulus categories, whereas WB associated comparisons were mainly related to mitochondrial and respiratory chain processes. In contrast, functional enrichment in the hypothalamus was limited, with no significant GO terms detected in the overall comparison or in the WB male versus female contrast.

Origin-associated differences

Origin-based comparisons (CB vs WB) revealed associated transcriptional differences across the HPG axis, with a higher number of DEGs detected in central neuroendocrine tissues than in the gonad in several contrasts (**Fig. 4; Supplementary table 7-9**). Because origin may be partially associated with other biological variables, including reproductive status, age and body size, these comparisons are interpreted as origin-related patterns rather than as isolated effects.



whereas the gonad displayed only 30 DEGs, evenly distributed between origins (15 CB and 15 WB). When analysed separately by sex, males showed 432 DEGs in the hypothalamus (182 CB, 250 WB), 657 in the pituitary (240 CB, 417 WB), and 183 in the gonad (124 CB, 59 WB). In females, the hypothalamus showed 73 DEGs (31 CB, 42 WB), the pituitary 1,086 (447 CB, 639 WB), and the gonad 406 (164 CB, 242 WB).

Functional enrichment analyses (**Supplementary table 10**) were more consistently detected in central neuroendocrine tissues than in the gonad. In males, hypothalamic and pituitary DEGs were enriched for signalling and response to stimulus categories. In females, the strongest enrichment was observed in the pituitary, where mitochondrial and respiratory chain related functions predominated. In contrast, gonadal comparisons showed limited or no significant enrichment in the overall and male specific contrasts, although some significant terms were detected in females (**Supplementary table 10**).

Functional gene networks associated with male origin

To explore the origin-related transcriptional differences in males [10, 11, 12], network analyses were focused on the CB vs WB male comparison. A subset of DEGs was successfully mapped to zebrafish orthologs using the orthology framework described above and used for network construction in Cytoscape. Of the 1,182 zebrafish orthologs, 1,130 were represented as connected nodes, generating 1,419 edges. MCODE analysis identified 27 subnetworks (**Supplementary table 11**), which were explored to identify modules with literature supported relevance to reproductive regulation. In the main text, we focused on two subnetworks because their gene composition showed the clearest relationship with reproduction. This selection was used to guide biological interpretation and does not imply that the remaining subnetworks are not biologically relevant.

A first integrated network (Network 1), constructed from DEGs identified between CB and WB males across hypothalamus, pituitary, and gonad (**Fig. 5**), revealed a strongly interconnected module spanning the three hierarchical components of the HPG system.

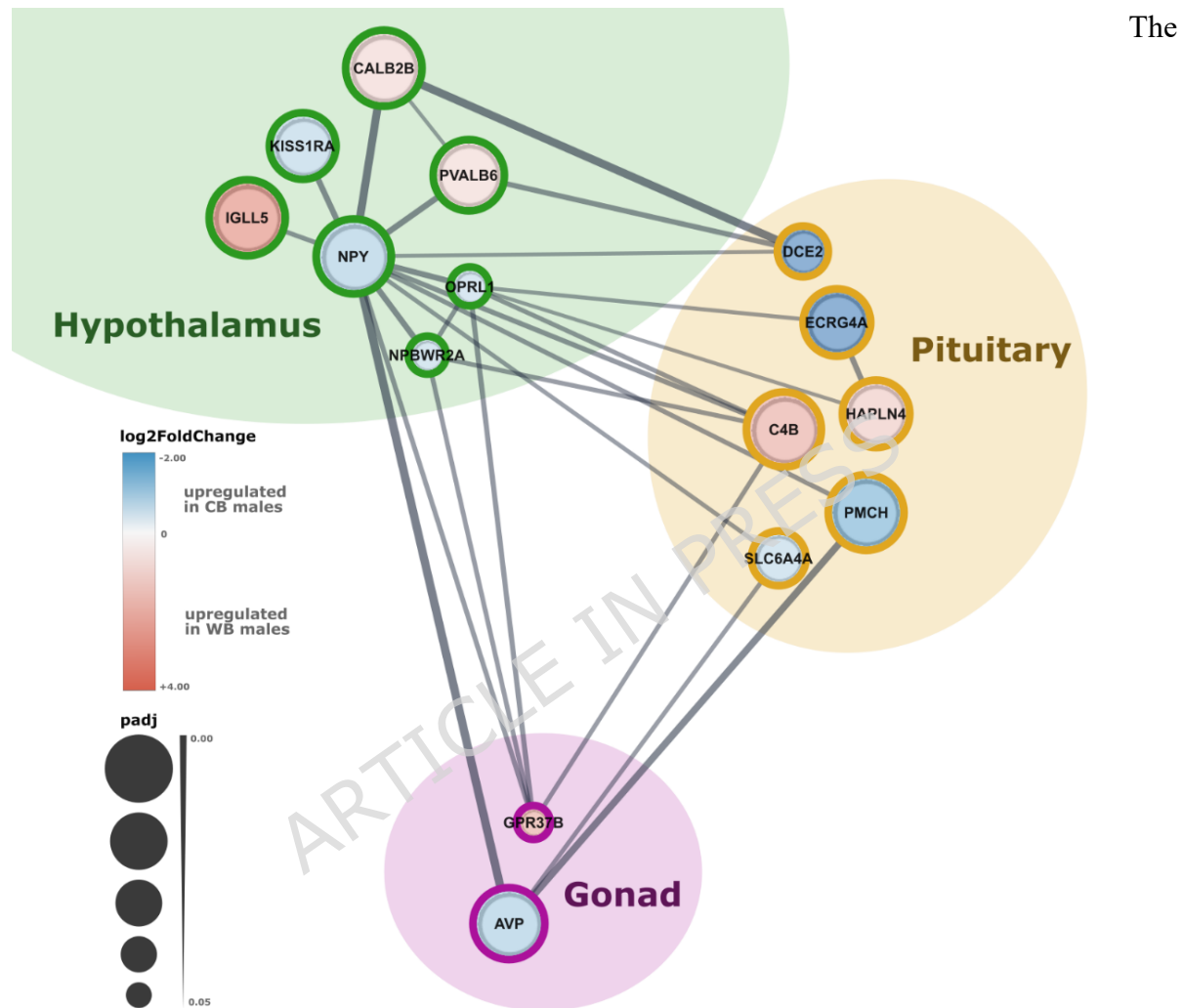


Fig. 5: Integrated interaction network 1 of DEGs between CB and WB males across the HPG axis. Network 1 constructed from DEGs identified in hypothalamus, pituitary and gonad. Nodes represent genes and are coloured according to expression changes (log2FC: positive values indicate higher expression in WB and negative values indicate higher expression in CB). Node annotation indicates tissue of origin.

network was organized around a central hypothalamic module, with the *npy* gene (encoding a neuropeptide regulating GnRH activity) displaying the highest connectivity and linking multiple neuroendocrine related genes across tissues. In the hypothalamus, *npy*, *kiss1ra* (encoding a

kisspeptin receptor that mediates HPG signalling), and other neuroendocrine factors, including *npbwr2a*, *opr11*, and *igll5* were upregulated in CB males and formed a tightly interconnected subnetwork (**Table 1**). The *npy* node exhibited multiple edges connecting directly to both hypothalamic and pituitary nodes, establishing a central position within the global topology. Likewise, *kiss1ra* and *npbwr2a*, both related to neuroendocrine signalling, were integrated within this central module. Pituitary genes upregulated in CB males, including *gad2*, *ecrg4a*, *pmch*, and *slc6a4a*, were incorporated into this reproductive network and displayed direct or indirect interactions with the hypothalamic module. These connections established a continuous interaction path linking central neuroendocrine components with downstream tissues.

At the gonadal level, *avp* was upregulated in CB males and remained connected to the central node through intermediate interactions. This gene encodes a vasopressin-like peptide related to oxytocin signalling. In addition, several relaxin- and claudin-related genes were also overexpressed in CB testes (**Supplementary table 8**). In contrast, genes upregulated in WB males, including *pvalb6* and *calb2b* in the hypothalamus, *c4b* and *hapln4* in the pituitary, and *gpr37b* in the gonad, were positioned more peripherally within the network and exhibited fewer connections compared to the CB associated subnetwork. Overall, the network topology highlighted a densely interconnected central module predominantly composed of genes upregulated in CB males, spanning hypothalamic, pituitary and gonadal compartments, whereas genes upregulated in WB males displayed a comparatively less integrated configuration across tissues.

A second network (Network 2) identified within the CB vs WB male interaction (**Fig. 6**), included genes predominantly associated with steroid metabolism and regulatory signalling processes. In contrast to the neuroendocrine centred structure described above, this network displayed a topology organized around interconnected steroid related nodes distributed across the HPG axis.

In the hypothalamus, *pgr* (encoding the nuclear progesterone receptor) and *fkbp5* (encoding a co-

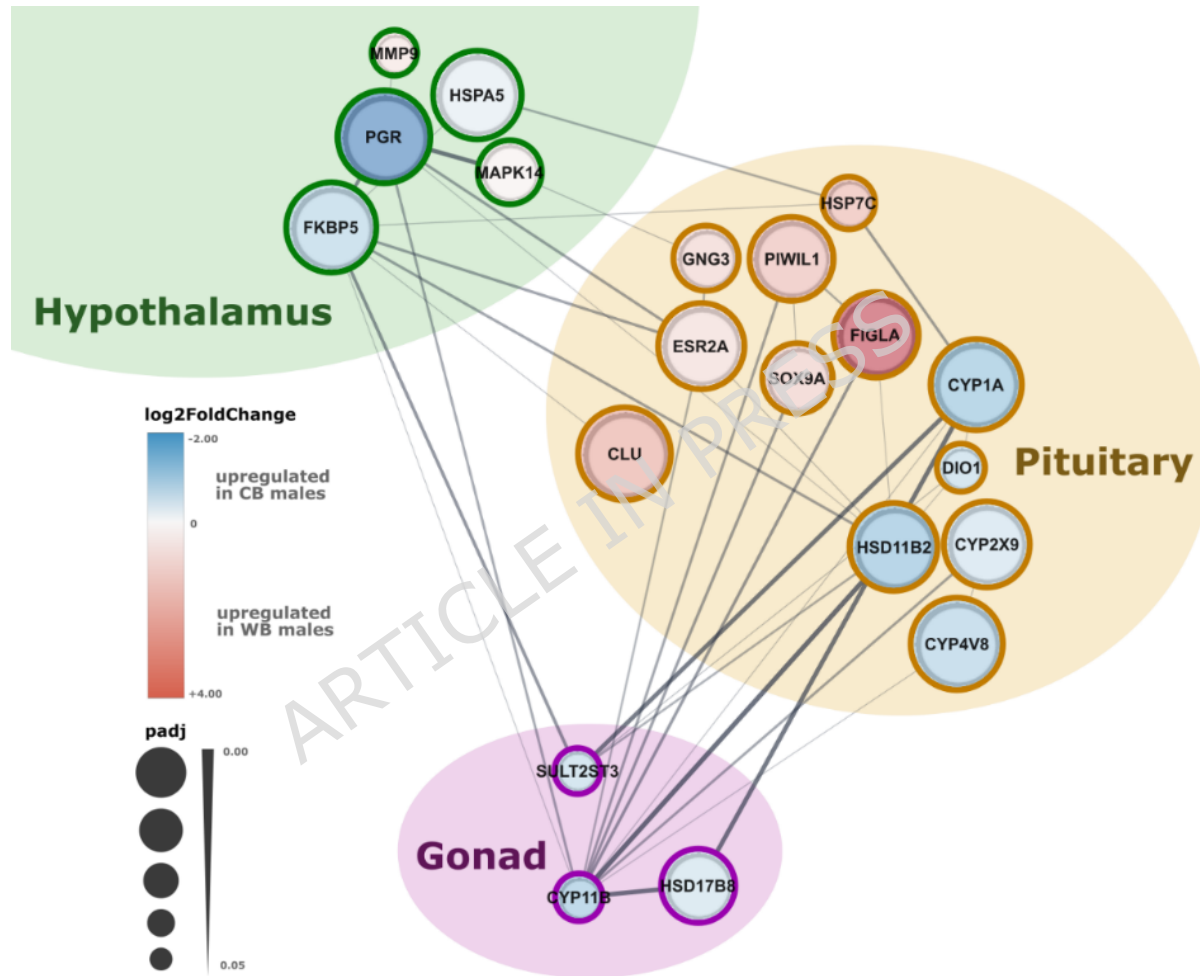


Fig. 6: Integrated interaction network 2 of DEGs between CB and WB males across the HPG axis. Network 2 constructed from DEGs identified in hypothalamus, pituitary and gonad. Nodes represent genes and are coloured according to expression changes (log2FC: positive values indicate higher expression in WB and negative values indicate higher expression in CB). Node annotation indicates tissue of origin.

chaperone that regulates steroid receptors) were upregulated in CB males and positioned in the central region of the network. These nodes were connected to pituitary and gonadal genes within

the same interaction framework. In the pituitary, several genes upregulated in CB males were incorporated into this module, including *hsd11b2*, *dio1*, and multiple cytochrome P450 enzymes (*cyp11a*, *cyp4v8*, and *cyp2x9*) (**Table 2**). These pituitary nodes exhibited multiple connections linking them to hypothalamic regulators and downstream to gonadal components. At the gonadal level, *cyp11b*, *sult2st3*, and *hsd17b8* were upregulated in CB males and integrated into the same interconnected subnetwork. *Cyp11b* and *hsd17b8* are involved in steroidogenic processes, contributing to the functional coherence of the module across tissues.

In contrast, WB males showed higher expression of a distinct set of genes within this latter network. In the hypothalamus, *mapk14* and *mmp9* were upregulated and positioned peripherally relative to the CB associated core. In the pituitary, *esr2a*, *sox9a*, *piwill*, and *figla* formed a connected subcluster preferentially expressed in WB males. These WB enriched nodes were connected to the broader network but did not form the central steroid related module observed in CB males.

Overall, the topology of this second network revealed a densely connected steroid metabolic module predominantly composed of genes upregulated in CB males and extending across hypothalamus, pituitary and gonad, whereas genes upregulated in WB males formed a comparatively distinct regulatory substructure within the same interaction framework.

Tissue	Origin upregulated	Gene	Gene name	Description
Hypothalamus	CB	<i>npv</i>	neuropeptide Y	Hypothalamic neuropeptide regulating energy balance and GnRH neuronal activity.
		<i>kiss1ra</i>	kisspeptin receptor 1a	Kisspeptin GPCR mediating activation of GnRH neurons and HPG axis signalling.
		<i>npbwr2a</i>	neuropeptide B/W receptor 2a	Neuropeptide B/W receptor involved in central neuroendocrine regulation.
		<i>opr11</i>	opioid-related nociception receptor 1	Nociception GPCR modulating hypothalamic neuroendocrine signalling.
	WB	<i>pvalb6</i>	parvalbumin 6	Calcium-binding protein marking neuronal subpopulations in neuroendocrine circuits.
		<i>calb2b</i>	calbindin 2b	Calretinin calcium binding protein involved in neuronal signalling
		<i>igll5</i>	immunoglobulin lambda-like polypeptide 5	Immunoglobulin-like protein.

Pituitary	CB	<i>gad2 (DCE2)</i>	glutamate decarboxylase 2	GABA-synthesizing enzyme regulating inhibitory neurotransmission
		<i>ecrg4a</i>	oesophageal Cancer-Related Gene 4	Secreted peptide precursor with putative endocrine modulatory function.
		<i>pmch</i>	pro-melanin-concentrating hormone	Precursor of melanin-concentrating hormone involved in neuroendocrine regulation.
	WB	<i>slc6a4a</i>	solute carrier family 6-member 4a	Serotonin transporter regulating serotonergic signalling.
		<i>hapln4</i>	hyaluronan and Proteoglycan Link Protein 4	Extracellular matrix protein contributing to neuronal structural organization.
		<i>c4b</i>	complement component 4B	Complement component involved in immune related processes.
Gonad	CB	<i>avp</i>	arginine vasopressin	Vasopressin-like neuropeptide involved in neuroendocrine and reproductive regulation.
	WB	<i>gpr37b</i>	G protein-coupled receptor 37b	G protein-coupled receptor involved in neuromodulator signalling pathways.

Table 1

Table 1. Genes included in the network 1 associated with reproductive regulation. Gene lists corresponding to Network 1 (neuroendocrine related module) identified from the integration of DEGs across hypothalamus, pituitary and gonad. For each gene, tissue of origin, condition of upregulation (CB or WB), and functional description are indicated.

Table 2

Tissue	Origin upregulated	Gene	Gene name	Description
Hypothalamus	CB	<i>pgr</i>	progesterone receptor	Nuclear progesterone receptor mediating progestin signalling in reproductive neuroendocrine circuits and gonadal tissues.
		<i>fkbp5</i>	FKBP prolyl isomerase 5	Glucocorticoid receptor co-chaperone regulating stress reproduction axis interactions.
		<i>hspa5</i>	heat shock protein family A (Hsp70) member 5	Endoplasmic reticulum chaperone associated with cellular stress responses in endocrine tissues.
	WB	<i>mapk14</i>	mitogen-activated protein kinase 14	p38 MAP kinase involved in stress responsive signalling pathways that may modulate reproductive processes.
		<i>mmp9</i>	matrix metalloproteinase 9	Matrix metalloproteinase mediating extracellular matrix remodelling.
Pituitary	CB	<i>hsd11b2</i>	hydroxysteroid 11-beta dehydrogenase 2	Enzyme inactivating cortisol, modulating glucocorticoid effects that can influence reproductive axis function.
		<i>cyp1a</i>	cytochrome P450 family 1 subfamily A	Cytochrome P450 enzyme involved in steroid and xenobiotic metabolism.
		<i>cyp4v8</i>	cytochrome P450 family 4 subfamily V member 8	Cytochrome P450 linked to lipid and steroid metabolic pathways.
		<i>dio1</i>	iodothyronine deiodinase 1	Thyroid hormone activating deiodinase linking thyroid status with reproductive timing and maturation.
		<i>cyp2x9</i>	cytochrome P450 family 2 subfamily X member 9	Cytochrome P450 potentially contributing to steroid metabolism.
	WB	<i>esr2a</i>	oestrogen receptor 2a	Oestrogen receptor beta mediating oestrogen feedback on gonadotropin synthesis and reproductive tissues.
		<i>gng3</i>	G protein subunit gamma 3	G-protein gamma subunit participating in GPCR signalling, including hormone receptor pathways.
		<i>sox9a</i>	SRY-box transcription factor 9a	Transcription factor involved in gonadal differentiation and male reproductive development.
		<i>piwil1</i>	piwi-like RNA-mediated gene silencing 1	Germline associated protein essential for spermatogenesis and maintenance of germ cell integrity.
		<i>hsp7c</i>	heat shock cognate 70 kDa protein	Heat shock protein involved in protein folding and stress protection
		<i>clu</i>	clusterin	CLU Secreted chaperone implicated in cell survival and tissue remodelling.
		<i>figla</i>	folliculogenesis-specific bHLH transcription	Germline transcription factor involved in gametogenic regulation.
		<i>cyp11b</i>	cytochrome P450 family 11 subfamily B	Steroidogenic enzyme involved in corticosteroid and androgen biosynthesis pathways.
Gonad	CB	<i>sult2st3</i>	sulfotransferase family 2, cytosolic sulfotransferase 3	Sulfotransferase involved in steroid hormone inactivation and regulation of androgen availability.
		<i>hsd17b8</i>	hydroxysteroid 17-beta dehydrogenase 8	Hydroxysteroid dehydrogenase involved in steroid interconversion and local hormone regulation.

Table 2. Genes included in the network 2 associated with reproductive regulation. Gene lists corresponding to Network 2 (steroid metabolism related module) identified from the integration of DEGs across hypothalamus, pituitary and gonad. For each gene, tissue of origin, condition of upregulation (CB or WB), and functional description are indicated.

Discussion

This study provides the first integrated transcriptomic view of the hypothalamic-pituitary-gonadal (HPG) axis in *S. senegalensis*, establishing a framework to investigate how sex and origin shape

reproductive regulation. Transcriptional variation was strongly partitioned across the axis, with sexual divergence predominantly concentrated in the gonad, whereas origin-related differences were more pronounced in central neuroendocrine tissues, particularly the pituitary. However, origin-associated patterns should be interpreted cautiously, as origin could not be fully disentangled from other biological and life-history variables. In males, the group most affected by reproductive dysfunction, integrated differential expression and network analyses suggest coordinated alterations in neuroendocrine signalling and steroid related pathways, supporting an axis level regulatory reconfiguration.

Integrated transcriptional organization of *S. senegalensis* HPG axis

Reproduction in vertebrates is regulated by the HPG axis, a hierarchically organized neuroendocrine system in which environmental cues, metabolic status, and gonadal feedback converge to regulate gametogenesis and steroidogenesis [24]. At the apex of this axis, the hypothalamus integrates internal and external signals and modulates pituitary gonadotropin synthesis and release, primarily through GnRH dependent mechanisms, but also via additional stimulatory and inhibitory inputs [25]. The pituitary, in turn, translates these neural signals into endocrine output, while the gonad executes the final effector programs of steroid production and germ cell development. Importantly, sex steroids feed back onto both hypothalamic and pituitary levels, forming a tightly regulated loop that ensures reproductive homeostasis [26].

In teleost, this regulatory architecture is further distinguished by direct hypothalamic innervation of the pituitary and by the spatial segregation of endocrine cell types within it [27]. Such organization allows a strictly and context dependent modulation of gonadotropins, contributing to the remarkable plasticity of reproductive strategies observed across fish species [28].

Consequently, reproductive dysfunction in the HPG axis cannot be interpreted solely as a gonadal phenomenon, it may equally reflect altered neuroendocrine integration at its central levels [29].

In *S. senegalensis*, reproductive impairment has been predominantly described at the gonadal and chemo communication levels, particularly in CB males [10, 11, 12]. Focusing on gamete production, several studies have documented issues in spermatogenesis and alterations in germ cell-somatic cell interactions within the testis [19]. In parallel, CB males have also been reported to display disruptions in reproductive behaviour [12], which has already been explored in studies focused on sensory perception [13, 20]. However, gonadal dysfunction and altered reproductive behaviour have largely been investigated independently. Whether this phenotype results from intrinsic gonadal defects or from upstream dysregulation of hypothalamic-pituitary control remains unresolved. Addressing this question requires system level characterization of the complete HPG axis to complement tissue-specific studies.

Here, we provide the first integrated RNA-seq atlas of the hypothalamus, pituitary, and gonad in *S. senegalensis*, encompassing both sexes and origins. Tissue identity emerged as the primary driver of transcriptional differentiation, consistent with previous large-scale transcriptomic studies highlighting tissue type as the main determinant of gene expression patterns [30, 31] underscoring the intrinsic molecular specialization of each HPG component. The application of the tissue specificity index (TSI) further delineated coherent, biologically meaningful gene sets and functions that reflect the structural organization of the axis, neuronal and neuropeptide signatures in the hypothalamus, classical endocrine effectors in the pituitary, and meiotic and gametogenic programs in the gonad. Collectively, these results define the transcriptional landscape of the *S. senegalensis* HPG axis across its three components. Beyond framing the comparisons by conditions discussed below, this resource provides a necessary reference atlas to interpret how

alterations at different levels of the axis may converge to shape reproductive performance in this species.

Sex and origin conditions transcriptional divergence

Once tissue identity is established as the dominant source of transcriptional structure of the axis, the next relevant functional variation is driven by sex. As expected in a gonochoristic teleost, the gonad exhibits by far the strongest sexual divergence in our study at the transcriptomic level. The magnitude of the difference is striking, as more than 15,000 genes are differentially expressed between males and females in the global and within-origin comparisons, compared to only 157 and 8 genes in the pituitary and hypothalamus, respectively. This extensive divergence reflects the distinct genetic programs operating in testis and ovary, including differences between spermatogenesis and oogenesis, androgen vs oestrogen dominated steroidogenesis, and structural variation in germ cell organization and somatic support compartments [18, 32]. In teleost, the gonadotropins FSH and LH, differentially regulate gametogenic progression and steroidogenic pathways, thereby contributing to the establishment of sex-specific gonadal transcriptomes [29].

More informative, however, is the distribution of sexual divergence across the central components of the axis. While the hypothalamus shows minimal sex-biased transcription in the global comparison, the pituitary displays a clearer sexual dimorphism [33]. This asymmetry is biologically coherent given the nature of both tissues. The hypothalamus is a heterogeneous integrative centre in which reproductive neurons coexist with circuits regulating feeding, stress, circadian rhythms, and behaviour [25,29]. In this sense, the low number of DEGs detected in this tissue (8 genes) likely reflects a signal dilution effect inherent to bulk RNA-seq, where transcriptomic shifts in discrete neuronal populations, such as GnRH or kisspeptin neurons, are masked by the more stable expression of surrounding glial and non-reproductive neural circuits

[34, 35]. In contrast, the pituitary represents a more reproduction focused endocrine tissue, in which gonadotropins integrate hypothalamic input and gonadal steroid feedback. In teleost, the pituitary is directly innervated by hypothalamic projections, allowing fine regulation of LH and FSH secreting cells [29]. Because these endocrine cell populations directly translate sex-specific steroid feedback into hormonal output, differences between males and females are more likely to be reflected as transcriptional differences.

A different perspective emerges when examining sexual comparisons within each origin separately. In WB individuals, the pituitary exhibits a markedly stronger sexual dimorphism than in CB. In contrast, hypothalamic sex differences are somewhat more apparent in CB than in WB fish, although still limited in magnitude. This redistribution of sexual divergence across central tissues suggests that sex-associated transcriptional differences may vary across levels of the HPG axis and may be influenced by environmental, developmental or reproductive context [36, 37]. Rather than indicating a simple loss of sexual identity, these patterns suggest that the relative contribution of each tissue to sex-associated transcriptional variation may differ between origins. This interpretation is consistent with the known sensitivity of neuroendocrine pathways to environmental and physiological context, although functional validation would be required to determine whether these transcriptional differences translate into altered endocrine regulation.

Origin-based contrasts (CB vs WB) revealed a distinct distribution of transcriptional differences across the HPG axis. When sexes are pooled, more DEGs were detected in central tissues, particularly the pituitary, than in the gonad. This distribution suggests that origin-related transcriptional variation is not only restricted to gonadal tissue but also involves upstream neuroendocrine components. However, because origin cannot be fully disentangled from other biological and life history variables, including age, body size, reproductive experience and

reproductive status, these results should be interpreted as associative rather than causal. In teleost, reproductive function is tightly controlled by hypothalamic-pituitary signalling, which integrates environmental and physiological cues to regulate gonadal activity [35, 38]. Accordingly, origin-associated transcriptional differences may involve neuroendocrine regulatory pathways rather than being restricted to the gonad [28, 36, 37]. However, DEGs should be interpreted cautiously, as they reflect statistically differential expression rather than biological effect size.

When sexes are analysed independently, the picture becomes more layered. In males, the sex in which reproductive dysfunction has been most consistently described under captive conditions [10, 11, 12], origin-related differences are again more pronounced in pituitary and hypothalamus than in the gonad, although gonadal differences are clearly detectable. This pattern aligns with previous work in *S. senegalensis* showing that CB males exhibit impaired spermatogenesis characterized by altered Sertoli-germ cell interactions and incomplete progression of germ cell differentiation [19]. Importantly, such phenotypes may reflect changes in cellular composition or specific signalling rather than wholesale reprogramming of the entire gonadal transcriptome, which is consistent with the moderate number of gonadal CB vs WB DEGs relative to central tissues.

In females, the pituitary again stands out as the tissue with the highest number of DEGs. This result should be interpreted with caution because ovarian maturation status was estimated macroscopically and was not fully balanced between origins. Although the available metadata do not indicate a complete separation of reproductive status between CB and WB females, differences in ovarian maturation and GSI may have contributed to the transcriptomic variation observed in female pituitary and gonad samples. The functional enrichment patterns reinforce this observation, with origin comparisons in central tissues being enriched on signalling and stimulus response

categories in some comparisons, while mitochondrial and respiratory chain processes are enriched in others [18]. Notably, environmental enrichment in *S. senegalensis* has been shown to induce pronounced transcriptional changes in the brain alongside improvements in reproductive performance [20], supporting the idea that central regulatory tissues are particularly sensitive to environmental context [27, 39]. Together, these findings are consistent with the idea that central regulatory tissues may be particularly sensitive to environmental and reproductive context.

Finally, the predominance of central neuroendocrine origin effects suggests that early developmental conditions might imprint long lasting regulatory states in the reproductive axis. Epigenetic differences, including altered methylation profiles in gonadal tissue, have been reported in sole and linked to reproductive traits [40]. Although our study does not directly address epigenetic mechanisms, the axis wide redistribution of transcriptional differences between origins is compatible with developmental programming of neuroendocrine sensitivity and feedback integration.

Taken together, our results reveal a clear partitioning of transcriptional variation across the HPG structure: sex accounts for the strongest divergence at the gonadal level, whereas origin-associated differences are detected across the axis, with higher values of DEGs in central tissues. This hierarchical pattern provides a framework for exploring candidate pathways that may be relevant to the reproductive phenotype previously described in CB males. In the following section, we integrate male specific DEGs across tissues into interaction networks to identify candidate regulatory modules associated with origin.

Captive-bred vs wild-bred males: insights into reproductive dysfunction

Given the male bias of the reproductive impairment [10, 11, 12], we used the male CB *vs* WB contrast as the basis for a more detailed network-based exploration of origin-associated DEGs. These contrasts revealed distinct transcriptional profiles associated with origin. In CB males, DEGs were connected to candidate pathways related to metabolic cues, neuromodulators signalling and steroid metabolism, whereas WB males showed overexpressed genes associated with preserved endocrine feedback sensitivity and germline supporting programs. Considered together, these patterns suggest that reproductive dysfunction in CB males may reflect changes in genes related with regulatory balance rather than the absence of essential spermatogenesis related genes.

One of the most notable patterns in CB males is the overexpression of genes involved in neuroendocrine integration. Increased expression of *npv* is particularly informative in this context: in teleost, *npv* is not only a key regulator of feeding behaviour but also an important mediator linking energetic status to reproductive output [41, 42]. Its increased expression in CB males may suggest differences in the interaction between metabolic and reproductive signalling, that might be relevant in the context of the distinct environmental and physiological histories of CB and WB males. Within such framework, changes in *npv* signalling could reflect differences in hypothalamic regulatory pathways. The concurrent upregulation of *kiss1ra* and *npbwr2a* further suggest the differences in central neuroendocrine signalling. Kisspeptin signalling in teleost modulates reproductive circuits beyond classical GnRH activation and can influence dopaminergic systems involved in endocrine regulation [43]. In *S. senegalensis*, kisspeptin administration affects the reproductive axis and circulating microRNAs [21], indicating functional relevance of this pathway in the species. Similarly, *npb* signalling through *npbwr2a* can directly stimulate *fshb* and *cga* expression in pituitary cells [44], suggesting a possible link between hypothalamic signalling

changes the gonadotropins output. These alterations, together with the activation of *gad2* (DCE2), implicated in GABAergic modulation of LH release [45], may indicate differences in stimulatory and inhibitory signals at the hypothalamic-pituitary interface.

An additional dimension is introduced by *fkbp5*, whose sustained upregulation has been associated with early life cortisol exposure and long-term changes in glucocorticoid receptor feedback sensitivity [46]. While stress hormone levels were not assessed in this study, the transcriptional differences observed in CB males are compatible with differences in genes related to stress-reproductive interactions. Given that CB individuals complete development entirely under captive conditions, it is plausible that early environmental factors might program persistent neuroendocrine differences that continue to shape adult reproductive regulation [34].

At the gonadal level, CB males display overexpression of genes controlling steroid synthesis and interconversion. Upregulation of *cyp11b*, a key enzyme in the synthesis of 11-ketotestosterone (11-KT), the principal androgen driving spermatogenesis in many teleosts [47], together with altered expression of *hsd17b8*, which modulates the balance between active and inactive sex steroids [48], suggests that expression of genes related to androgen dynamics are not reduced. The presence of *pgr* within this regulatory landscape is particularly significant. In teleost, *pgr* is expressed in Sertoli cells, Leydig cells and spermatogonia within the testis and is differentially regulated by oestradiol, 17,20 β P and 11-KT [49]. Importantly, its function has been linked more strongly to spermatogonia renewal than to meiotic entry.

In addition, the upregulation of *avp* observed in CB gonads may indicate local differences in vasopressinergic signalling associated with origin. In teleost, vasopressinergic systems are not only centrally regulated but can also act locally within the gonad as modulators of reproductive processes [50], supporting a potential role for *avp* in gonadal reproductive regulation. This local

signalling framework may also be consistent with the overexpression of relaxin- and claudin-related genes, which have been associated with alterations in cell adhesion and impaired spermatid release in CB males [51].

Overall, the transcriptional configuration observed in CB males may be consistent with genes related to spermatogenic regulation and somatic support cell function. This interpretation aligns with previous reports of impaired Sertoli-spermatogonia interactions and incomplete spermatogenic progression in CB sole males [19]. In this context, the observed patterns do not point to a simple loss of expression of key HPG axis (genes such as *gnrh*, *lhb*, *fshb*, *cga* are not differentially expressed), but rather to differences in regulatory pathways. The prominence of genes such as *npv*, *kiss1ra* or *nrbwr2a*, which modulate neuroendocrine signalling without being core gonadotropins, supports the hypothesis that origin-associated differences in regulatory modules may influence how signals are integrated across the axis, that might be relevant to the incomplete spermatogenesis progression observed in CB males.

In contrast, WB males exhibit a transcriptional profile that appears less dominated by neuroendocrine reconfiguration and more consistent with preserved endocrine feedback and germline integrity. The overexpression of *esr2a* in WB pituitary supports the maintenance of classical steroid feedback pathways, which are central to HPG regulation in teleost [29]. Additionally, higher expression of genes such as *piwill* and *sox9a*, both associated with germline maintenance and male gonadal differentiation, is compatible with a stable spermatogenesis program. Although the presence of *figla* in WB males may initially appear unexpected given its classical association with ovarian pathways [52], it may reflect differences in cell composition, transcriptional plasticity, or endocrine state rather than direct feminization. Together, the WB gene expression profile is more consistent with a regulatory configuration that supports effective germ

cell progression and feedback balance under captive conditions. Importantly, several genes outside the core network modules reinforce these hypotheses. For example, altered expression of *cyp26a1*, which regulates retinoic acid metabolism and influences meiosis entry [53], provides an additional mechanism by which spermatogenic timing could be affected in CB males. Likewise, differential expression of neuromodulator receptors and metabolic regulators suggests that the CB-WB divergence extends beyond isolated pathways and reflects broader adjustments in endocrine coordination.

Overall, the male specific transcriptional landscape suggests that CB and WB individuals represent alternative regulatory states of the HPG axis. Although reproductive performance was not directly measured in the individuals analysed, the reproductive dysfunction of CB males has been previously documented in behavioural and broodstock-performance studies in Senegalese sole [12]. Therefore, the male CB vs WB transcriptomic differences reported here should be interpreted in the context of this previously described phenotype. CB males showed differential expression of genes related to neuroendocrine modulation, stress-related signalling and steroid metabolism whereas WB males showed higher expression of genes associated with endocrine feedback and germline supporting processes. This distinction provides a candidate framework linking developmental environment to adult reproductive phenotype, rather than a single disrupted gene, reproductive impairment in CB males likely emerges from subtle but coordinated shifts in how metabolic, stress and steroid signals are integrated and transmitted across the HPG structure.

Conclusions

This study provides the first integrated transcriptomic characterization of the HPG axis in *S. senegalensis*, revealing a strong tissue-specific organization of gene expression across hypothalamus, pituitary and gonad. Sexual divergence was mainly concentrated in the gonad,

whereas origin-associated transcriptional differences were more evident in central neuroendocrine tissues. These results suggest that transcriptional variation related to CB or WB origin is not restricted to the gonad but also involves upstream components of the reproductive axis. In males, the group in which reproductive dysfunction has been most consistently described, integrated differential expression and network analyses highlighted candidate pathways related to neuroendocrine signalling, steroid metabolism and reproductive regulation. These pathways may be relevant to the behavioural and gonadal alterations previously reported in CB males, although their functional role remains to be tested.

Importantly, the present study cannot disentangle origin from other biological and life-history variables, including age, body size, reproductive status, maturation stage and reproductive experience. Therefore, the observed patterns should be interpreted as origin-associated transcriptional differences rather than causal effects of captive origin. Overall, this axis level transcriptomic framework provides a basis for future functional studies aimed at validating candidate pathways and improving broodstock management in Senegalese sole aquaculture.

Materials and Methods

Ethical statement

All specimens were provided by the Instituto Español de Oceanografía (IEO-CSIC) (Santander, Spain). All animal procedures were conducted in accordance with the ethical regulations of the University of Santiago de Compostela and complied with European Directive 2010/63/EU for animal experiments. The study was supervised by the University of Santiago de Compostela, and the Instituto Español de Oceanografía. Experimental protocols were approved by the Animal

Experimentation Ethics Committee of the Instituto Español de Oceanografía, authorization no. 2022GCELCE105202.

Individuals and sample collection

The study included adult captive-bred (CB) and wild-bred (WB) *Solea senegalensis* males and females. CB fish were hatchery produced individuals born in 2015, whereas WB fish had been collected from the Atlantic Ocean between 2012 and 2016 and maintained under captive conditions for more than one year prior to sampling. All fish were kept in indoor tanks supplied with flow-through seawater and maintained under controlled photoperiod and temperature regimes. Fish were fed a natural diet commonly used for Senegalese sole broodstock, including polychaetes, squid and mussels. [11, 12] Sampling was carried out between 6 and 8 June 2022 corresponding to the late phase of the Senegalese sole spawning season, during the mornings. Each fish was treated as an independent biological replicate, with 3-6 biological replicates per tissue, sex and origin group (**Supplementary Table 1**). Whenever available, hypothalamus, pituitary and gonad samples were obtained from the same individuals, however, because of tissue availability and post-sequencing quality-control filtering, the final RNA-seq dataset was not fully paired across all HPG tissues. Differential expression analyses were therefore performed independently within each tissue. No technical replicates were included.

Fish were euthanized by decapitation, and the hypothalamus, pituitary and gonad were immediately dissected, snap frozen in liquid nitrogen and stored at -80°C until further processing. The hypothalamus and pituitary were identified using established anatomical landmarks described for teleost, the hypothalamus was in the diencephalic region, posterior to the optic chiasm and anterior to the mesencephalon, whereas the pituitary was identified as a reddish ventral structure connected to the hypothalamus via the infundibulum at the base of the diencephalon [28]. Gonads

were excised through an abdominal incision in the ocular side of the animal, and sex was determined based on macroscopic morphology, including gonadal size, shape and coloration. Ovaries appeared as elongated paired structures with a granular texture corresponding to developing oocytes, while testes were markedly smaller, whitish, smooth, and compact paired structures allowing clear discrimination between sexes based on gonadal morphology [55]. Body weight and gonad weight were recorded for each sampled individual, and the gonadosomatic index (GSI) was calculated as $\text{gonad weight/body weight} \times 100$. In females, ovarian maturation status was estimated macroscopically based on gonad size and external appearance. Histological staging was not available; therefore, ovarian stage assignments should be considered approximate. Individual IDs are provided in **Supplementary Table 1** to indicate samples derived from the same fish across tissues.

RNA extraction

Total RNA was extracted from tissue samples using the E.Z.N.A.® DNA/RNA Kit (Omega Bio-tek, R6731), following the manufacturer's instructions. Prior to extraction, tissues were mechanically homogenized using a TissueLyser II (Qiagen) with a single 5 mm stainless steel bead per tube. Homogenization speed and duration were adjusted depending on the tissue: neural tissues (hypothalamus and pituitary) were disrupted using two 30 s cycles at 20 Hz with a 30 s pause interval between cycles, whereas gonadal samples were disrupted using two 1 min cycles at 30 Hz with a 1 min interval between cycles. Although the kit allows simultaneous isolation of RNA and genomic DNA, only RNA was used for these analyses. RNA quantity and quality were assessed prior to library preparation using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.) and RNA integrity (RIN) was evaluated using a Bioanalyzer 2100 system (Agilent Technologies).

RNA-seq library preparation and sequencing

RNA-seq library preparation and sequencing were performed by Novogene Europe (Germany). Messenger RNA (mRNA) was enriched from total RNA using poly(A) selection with oligo(dT) magnetic beads, followed by cDNA library construction according to the provider's standard protocol. Libraries were sequenced on an Illumina NovaSeq 6000 S2 platform to generate 150 bp paired-end reads. Raw sequencing data was delivered in FASTQ format and used for downstream analyses. A total of 53 RNA-seq libraries were initially generated. After post-sequencing quality control, two libraries were excluded due to low mapping performance and inconsistent clustering, resulting in a final dataset of 51 RNA-seq libraries with $n = 3-6$ biological replicates per group/tissue combination and ensuring robust transcriptomic inference (**Supplementary Table 1**)

Data processing

Raw RNA-seq data were processed using the nf-core/rnaseq 3.10.1 pipeline [56], executed under the singularity profile with default settings. The *S. senegalensis* Ensembl beta genome assembly and annotation (GCA_919967415.2 [14]) was used as reference for alignment and quantification. Briefly, initial quality assessment of sequencing reads was performed with FastQC [57], followed by adapter removal and trimming of low-quality bases using Trim Galore! [58]. Cleaned reads were aligned to the reference genome using STAR [59], and quantification was generated with RSEM [60] to obtain raw and normalized read counts (transcripts per million, TPM).

Data analysis

Statistics and Reproducibility

Each fish was treated as an independent biological replicate, and sample sizes for each tissue, sex and origin group are detailed in **Supplementary Table 1**. Principal component analysis was

performed in R using `prcomp()` function, and hierarchical clustering was based on Spearman correlation. Differential gene expression analyses were performed independently for each tissue using the DESeq2 v1.42.1 [61] statistical framework on gene-level expected counts imported with tximport v.1.30.0 [62]. Counts were modelled using negative binomial generalized linear models, and statistical significance was assessed using the Wald test. Multiple testing was controlled using the Benjamini-Hochberg procedure, and genes with an adjusted P value (p_{adj}) < 0.05 were considered differentially expressed. Gene Ontology enrichment analyses were performed using GOfuncR v1.22.2 [63], in an overrepresentation framework. Significantly enriched terms were defined using a family-wise error rate for overrepresentation ($FWER_{overrep}$) ≤ 0.05 . Detailed analytical parameters for each workflow are described in the corresponding subsections below. For each DESeq2 contrast, a specific sample sheet was generated including only the samples relevant to that comparison. Global comparisons included all individuals within each tissue, whereas sex-stratified origin comparisons and origin-stratified sex comparisons were performed using the corresponding sample subsets. No sex-by-origin interaction term was tested; therefore, these analyses were interpreted as stratified comparisons rather than formal interaction tests.

Transcriptome profiling

Following nf-core processing, gene level count matrices were filtered to retain expressed and consistently represented genes. Genes with five transcripts per million ($TPM \geq 5$) and represented in at least two replicates within the same condition were kept for downstream analyses. This filtering strategy was first applied to the complete dataset comprising all tissues to define the HPG axis transcriptome. The resulting filtered matrix was used for subsequent analyses, including tissue specificity index (TSI) calculations and differential gene expression analyses between experimental conditions. In parallel, the same filtering criteria were applied separately to the

specific subset of samples from each tissue to generate tissue transcriptomes, which were used primarily for characterize gene expression patterns within each organ.

Tissue specificity analysis

Tissue-specific gene expression was assessed using the tissue specificity index tau (τ) calculated as $\tau = \sum_{i=1}^n [1 - (x_i / \max(x))] / (n - 1)$, where x_i is the expression level in tissue i , n is the total number of tissues analysed, and $\max(x)$ is the maximum expression value across tissues for each gene. Tau values range from 0 for broadly expressed genes to 1 for highly tissue-specific genes [64]. The index was calculated using gene expression values (TPM) from RSEM outputs and averaged across biological replicates within each tissue (hypothalamus, pituitary and gonad). The τ index was calculated using normalized expression values across tissues, with values ranging from 0 (broadly shared genes) to 1 (highly tissue-specific genes). Genes with $\tau \geq 0.9$ were classified as tissue-specific. For these genes, the tissue with the highest mean expression was assigned as the specific tissue. These tissue-specific gene sets were subsequently used for Gene Ontology (GO) enrichment analyses to characterize their functional profiles using as reference the Senegalese sole HPG transcriptome [14].

Differential gene expression analysis

Differential gene expression analyses were conducted using the R/Bioconductor package DESeq2 v1.42.1 [61]. Gene expected counts and transcript abundance estimates generated by RSEM were imported into R using the tximport package v.1.30.0 [62]. Count matrices were constructed directly from RSEM gene files and used for downstream differential expression analyses. Analyses were performed independently within each tissue (hypothalamus, pituitary, and gonad). For each tissue, differential expression analysis was performed using a design including sex and origin as

explanatory variables. The following origin-based comparisons were conducted: (i) all WB vs all CB individuals, (ii) WB males vs CB males, and (iii) WB females vs CB females, where positive $\log_2$ fold change ($\log_2FC$) values indicate overexpression in WB individuals and negative values overexpression in CB individuals. Sex-based comparisons included: (i) all males vs all females, (ii) CB males vs CB females, and (iii) WB males vs WB females, where positive $\log_2FC$ values correspond to overexpression in males and negative values to overexpression in females. DEGs were defined based on statistical significance using a Benjamini-Hochberg adjusted p-value ≤ 0.05 , without applying an additional minimum $\log_2FC$ threshold; $\log_2FC$ values were used to assess the direction and magnitude of expression differences. Each comparison generated a list of DEGs per tissue. Reproductive stage, GSI, body weight and age were not included as covariates in the DESeq2 models because of the limited number of individuals per tissue/sex/origin group, the incomplete and macroscopic nature of ovarian stage classification, and the lack of exact age information for WB individuals. These variables were therefore considered in the interpretation of the comparisons rather than modelled as formal covariates.

Functional enrichment analysis

Gene Ontology (GO) enrichment analyses were carried out using the R/Bioconductor package GOfuncR v1.22.2 [63]. For enrichment analyses of the transcriptomes (both the HPG axis transcriptome and the individual tissue transcriptomes defined above), the full annotated *S. senegalensis* transcriptome was used as background. For enrichment analyses of tissue-specific genes identified using the tissue specificity index (TSI; $\tau \geq 0.9$), as well as for DEGs identified between experimental conditions ($p_{\text{adj}} \leq 0.05$), the background was restricted to the filtered HPG axis transcriptome defined in this study. GO terms were considered significant when the family wise error rate (FWER) for overrepresentation tests was below 0.05 ($FWER \leq 0.05$).

Gene networks construction and visualization

To integrate functional connectivity among DEGs between CB and WB males derived from hypothalamus, pituitary and gonad, network-based analyses were performed. As *S. senegalensis* is not represented in the STRING database [65], orthologous relationships with *Danio rerio* were inferred using OrthoFinder v2.5.4 [66] with default parameters. The analysis was performed using the proteomes of *S. senegalensis* and representative teleost species (*Cynoglossus semilaevis*, *Paralichthys olivaceus*, *Scophthalmus maximus*, *Oryzias latipes*, *Astyanax mexicanus*, and *D. rerio*) [67] retrieved from Ensembl databases. Orthogroups containing both *S. senegalensis* and *D. rerio* genes were used to generate a *S. senegalensis* to zebrafish mapping, which was subsequently restricted to the DEGs identified in this study. When multiple zebrafish ortholog candidates were recovered for a given *S. senegalensis* gene, the best annotated zebrafish gene was retained for downstream analyses. Then, the ortholog mapped DEGs list was imported into Cytoscape v3.10.4 [68] and queried against the STRING database using the stringApp v2.2.0 plugin [69].

PPI networks were constructed using a minimum interaction confidence score of 0.4 (medium confidence threshold; [65]), all additional stringApp parameters were kept by default. The resulting global network was subjected to topological clustering using the MCODE v2.0.3 algorithm [70] with default parameters, enabling the “fluff” option to identify densely connected subnetworks and expand cluster boundaries. Network visualization incorporated quantitative attributes derived from differential expression analyses. From the resulting clusters, subnetworks enriched in genes functionally related to reproductive processes were selected for detailed visualization and interpretation, where node colours represent fold change values (FC) (WB/CB: positive FC (in red) upregulated in WB, and negative FC (in blue) upregulated in CB) and node size adjusted p.adj values. Edge thickness reflected STRING interaction confidence scores.

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Authors' contributions

C.M.: Writing, original draft, Formal analysis, Data curation, Investigation. A.O. and T-S.D.: Writing, review & editing, Formal analysis, Investigation. R.F., N-P.R., R.R.C., HR: Writing, review & editing, Project administration. B.C. and M.P: Writing, review & editing, Supervision, Conceptualization.

Competing interests

The authors declare no competing interests.

Data availability

The raw RNA-seq data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) and are publicly available under BioProject accession PRJNA1466281 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1466281>).

Code availability

No custom software or mathematical algorithms central to the conclusions were developed for this study. Custom scripts used for routine downstream processing and visualization are available from the corresponding author upon reasonable request.

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