

Mouthbrooding behavior and sexual immune dimorphism in Indian perch *Jaydia lineata*

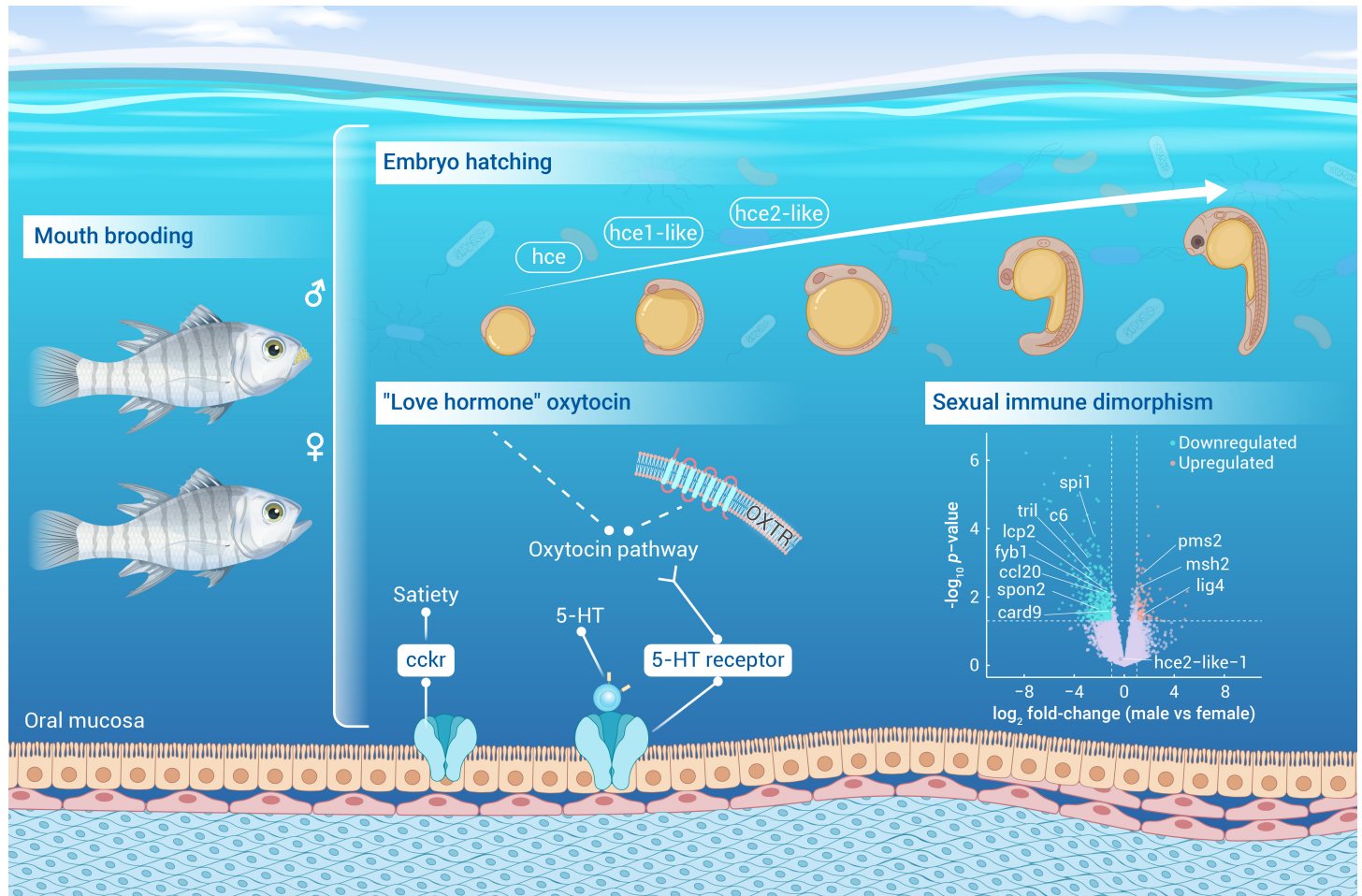
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Mouthbrooding teleosts, like *Jaydia lineata*, show unique parental care behaviors, revealing convergent evolution.
- Genomic analysis identifies genes associated with embryonic development, emphasizing parental care's importance.
- Adaptive convergence in oxytocin signaling pathway-related genes indicates the crucial role of hormonal regulation.
- The study reveals a trade-off between parental care and immune activation, with sexual immune dimorphism.



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Parental care enhances offspring survival in teleost vertebrates; however, its genetic basis and evolutionary traits remain unexplored. Therefore, we assembled the chromosome-level genome of the paternal mouthbrooder, *Jaydia lineata*, and observed convergent evolution in parental care fish. Genomic signatures revealed the association of certain genes with embryonic development, implying that parental behavior is conducive for offspring. Adaptive convergence occurred as an in evolutionary response oxytocin signaling pathway-related genes; thus, hormonal regulation is vital in this process. Specific neuropeptides and steroid hormones co-opted and primary immunodeficiency-related transcripts were enriched in Indian perch. Comparative transcriptome analysis revealed a trade-off between parental care and immunological activation in mouthbrooders. Sexual immune dimorphism, evident in the buccal mucosa, is characterized by the functional differentiation of immune-related genes in male mouthbrooders. Thus, we elucidated the genetic basis and unique physiological traits of mouthbrooding, offering insights into the independent evolution of complex parental care adaptations in teleosts.

INTRODUCTION

Parental care includes various aspects, such as time, energy, or resources, undertaken by parents to benefit their offspring.¹ This concept is central to comprehending the complexities of reproductive strategies in teleost fishes, which exhibit substantial diversity in reproduction modes (Figure S1). Additionally, parental care in teleost fishes offers an excellent opportunity to employ the comparative method in reproductive biology, shedding light on the evolutionary processes that have influenced these behaviors. Parental care among fishes is considerably diverse; factors contributing to this diversity are often linked to phylogenetic and ecological influences.² Evolutionary adaptation ensures that eggs produced by fish species displaying parental care are robust and exhibit rapid development and increased mass, thereby enhancing their likelihood of reaching maturity.³ However, despite these favorable outcomes, the genetic mechanisms driving the evolution of parental care in teleost fishes have largely remained unknown.

Current knowledge suggests that approximately one-quarter of all fish families exhibit parental care.³ This diversity in parental behavior prompts questions about the evolution of parental care, such as whether evolution acts on genetic loci that affect multiple forms of parental behavior independently and the mechanisms by which variation in these genes can influence behavior. Teleosts exhibit various reproductive strategies, including oviparity, viviparity, pouch brooding, and mouthbrooding. Among these, mouthbrooding is the most noteworthy parental care strategy, occurring in at least nine families of teleost fish (Figure S2). In mouthbrooding, eggs and/or larvae are protected inside the buccal cavity,^{2,4} in direct contact with the parental mucosa, for 4–6 weeks.⁵ Mouthbrooding, i.e., carrying offspring within the buccal cavity, requires substantial energy expenditure⁶ and often limits the feeding ability of the parents. A spectrum of physiological changes observed during mouthbrooding has the potential to disrupt the normal functioning of caregiving parents. Many mouthbrooding fishes cease feeding during brood-

ing and only resume foraging upon the conclusion of this phase.^{7,8} The act of nurturing offspring may require the redirection of stored resources, including macro- or micronutrients originally allocated for routine physiological processes and reproductive functions, including parental care activities. Brooding parents also experience reduced immune responses,⁹ heightened oxidative stress levels,¹⁰ and increased standard metabolic rates.⁶ Furthermore, mouthbrooding increases the risk of predation, parasitism, and infection for the brooders.⁹ This diversion can have adverse consequences for the overall well-being of the adult caregiver.¹¹

In nature, numerous instances of adaptive phenotypic convergence resulting from convergent genetic changes influenced by similar selective pressures have been observed among distantly related taxa.¹² Recent whole-genome comparisons of animals displaying convergent adaptive phenotypic evolution have revealed genome-wide evidence, including the presence of identical amino acid substitutions in orthologous genes, of this convergence.^{12,13} In this study, we aimed to elucidate the genetic basis of convergent evolution related to parental care, specifically focusing on mouthbrooding behavior. Accordingly, we assembled the chromosome-level genome of the Indian perch (*Jaydia lineata*), a notable example of male mouthbrooding teleost fish. Furthermore, we adopted a comparative genomics approach to identify the evolutionary drivers behind mouthbrooding. We also explored the potential trade-offs between parental care and immunological activation in mouthbrooding fish by conducting in-depth transcriptome analyses to identify the complex interplay between the expression of genes related to parental care and those involved in immune responses.

MATERIALS AND METHODS

Sample collection for genome sequencing

A female Indian perch (*Jaydia lineata*) was collected from the Yellow Sea of China for genome sequencing and assembly. The fish was dissected following anesthetization with MS-222, and tissue samples were immediately preserved in liquid nitrogen for DNA and RNA extraction. White muscle tissue was collected for DNA extraction and subsequently used for PacBio and Hi-C sequencing. To assist in the annotation of genes, five additional tissues, including those from the brain, liver, kidney, spleen, and buccal mucosa, were collected for transcriptome sequencing.

Library construction and genome sequencing

High-quality DNA was extracted from the white muscle tissue of Indian perch using a Qiagen Genomic Kit (Qiagen, Hilden, Germany). The concentration and purity of the total DNA were assessed using NanoDrop™ 2000 (ThermoFisher Scientific, Waltham, MA, USA) and Qubit instruments (ThermoFisher Scientific), respectively. A paired-end library with an insert size of 350 bp was constructed and sequenced using the Illumina NovaSeq 6000 platform (Illumina Inc., CA, USA). For PacBio sequencing, a sequencing library was prepared using the TruSeq Nano DNA LT Library Preparation Kit (Illumina) incorporating the SMRTbell adapter. The library was assessed for quality using Thermo Qubit 3.0 (ThermoFisher Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Single-molecule sequencing was

performed using a PacBio Sequel platform to generate long-read data. Total RNA was extracted from the four tissues using the RNAiso Reagent, following the manufacturer's instructions. Equal amounts of RNA from the four tissues were mixed to create pooled RNA samples. An RNA library was prepared and sequenced using the Illumina NovaSeq 6000 (Illumina) by employing the PE150 approach. All sequencing procedures were conducted by Gooal Gene Technologies (Wuhan, China).

Genome size estimation and genome assembly

After filtering out reads containing adapter sequences, duplicated sequences, >10% N bases, and >50% low-quality bases (Phred quality scores ≤ 5) were filtered out. Approximately 72.95 Gb (table S1) worth of clean reads remained and were used for subsequent analysis. Illumina short reads from the 350 bp library were used for *k*-mer frequency analyses of the Indian perch genome.¹⁴ Genome size was estimated as follows: $\text{Genome Size} = K_{\text{num}}/K_{\text{depth}}$, where K_{num} is the number of *K*-mers and K_{depth} is the expected depth of *K*-mers.

The genome assembly was processed using canu¹⁵ and wtdbg2.¹⁶ Clean reads obtained from PacBio Sequel II sequencing were used as inputs (Table S3), and specific parameters (listed in Table S4) were applied during the assembly process. Subsequently, genome assembly was corrected in two steps. First, raw data from PacBio Sequel II sequencing were employed to refine the genome assembly sequences using blasr and arrows with default parameters. The genome assembly obtained from the first step was refined using Illumina sequencing data with bwa and Pilon default parameters. Purge_haplotigs was applied to remove redundancies after refinement, with the parameter break float 0.1 -a 60 (Table S4).

Hi-C sequencing and chromosome assembly

Hi-C sequencing was employed as an additional assembly step to achieve a chromosome-level assembly. The initial concentration of total DNA, insert size, and effective quantity (>2 nM) of the Hi-C libraries were determined using a Thermo Qubit 2.0, Agilent 2100, and Q-PCR, respectively. Raw reads obtained from the Illumina NovaSeq 6000 instrument were filtered, and only high-quality paired reads were retained for subsequent analysis. The percentage of non-repetitive valid pairs is an important index for evaluating library quality in the Hi-C analysis. The clean data (Table S6), obtained after data filtering (Table S7), mapped to the refined genome of Indian perch using BWA (version 0.7.17) software with default parameters.¹⁷ Chromosome-length scaffolds were generated using a 3D-DNA pipeline, which involved several steps, including preliminary filtration, misassembly correction, iterative scaffolding, and overlap merging. Interaction matrices were constructed using Juicer,¹⁸ with the "enzyme MboI" parameter, to identify errors in the order, direction, or assembly of the contigs. The JuiceBox tool was used to visualize the error correction process.¹⁹ Finally, the quality of the assembled Indian perch genome was evaluated using BUSCO (v4.0) with the lineage database actinopterygii_odb9.²⁰

Genome annotation

De novo repeats annotation of the Indian perch genome involved the identification of tandem repeats and transposable elements (TEs). Tandem repeat finder (TRF version 4.07b) was used for tandem repeat identification by employing default parameters.²¹ RepeatMasker and RepeatProteinMask (version 3.3.0, www.repeatmasker.org/) were used to identify the TEs. These tools perform homology searches against the RepBase library (Release 16.03; www.girinst.org/repbase/) to identify the TEs based on sequence similarities.²² RepeatModeler (version 1.08, <http://www.repeatmasker.org/RepeatModeler.html>) and LTR_FINDER²³ were applied for the ab initio prediction of repeat elements in the Indian perch genome with default parameters. Using the generated repeat library, the repeat content of the Indian perch genome was identified using RepeatMasker with the sensitive mode (-s) option.

De novo gene prediction was performed using Augustus²⁴ and Genescan with default parameters. GeMoMa v1.1.3 program²⁵ was employed for homology-based gene prediction by aligning the assembled genome with those of the spiny chromis, *Acanthochromis polyacanthus*, clown anemonefish, *Amphiprion ocellaris*, climbing perch, *Anabas testudineus*, Eastern happy,

Astatotilapia calliptera, Mexican tetra, and *Astyanax mexicanus*. To incorporate transcriptomic data, RNA-seq reads that passed quality control (QC) were mapped to the assembled Indian perch genome using HISAT2 aligner v2.0.4 with default parameters.²⁶ The resulting mapped transcripts were assembled using StringTie v2.2.0 software. To generate a non-redundant and more complete gene set, MAKER and HiCESAP tools were employed. The parameter used for this step was "-trans_cds_len 150bp." Functional annotation of the gene models was performed using a BLASTP search against several publicly available databases, including NR, TF, Swiss-Prot, TrEMBL, Pfam, KEGG,²⁷ and KOG. InterProScan¹⁸ was used to retrieve the associated GO terms²⁸ using default parameters. InterProScan 5 was used to predict the motifs and domains of each gene model against public protein databases.

For noncoding RNA annotation, tRNA was predicted using tRNAscan-SE v1.3.1, whereas rRNA was predicted using BLASTN, with rRNA from related species as reference sequences. Moreover, miRNA and snRNA were predicted using the Infernal v1.1 software based on Rfam ([ftp://ftp.ebi.ac.uk/pub/databases/Rfam](http://ftp.ebi.ac.uk/pub/databases/Rfam)).

Gene family identification and phylogenetic tree construction

Gene families were identified by comparing the protein sequences of Indian perch and 17 other species of ray-finned fishes (downloaded from the NCBI database) (*Cynoglossus semilaevis*, *Danio rerio*, *Gadus morhua*, *Gasterosteus aculeatus*, *Hippocampus comes*, *Larimichthys crocea*, *Microphis manadensis*, *Oreochromis niloticus*, *Oryzias latipes*, *Periophthalmus magnuspinnatus*, *Scleropages formosus*, *Seriola dumerili*, *Sphaeramia orbicularis*, *Takifugu rubripes*, *Thunnus maccoyii*, *Xiphophorus maculatus*, and *Lepisosteus oculatus* [used as an outgroup]). For the clustering of orthologous groups, the OrthoMCL software was employed with the following parameters: "identity cutoff 30 coverage cutoff 50 --abc -l 1.5 percent Match Cut off = 50, value Exponent Cut off = -5". This clustering approach helped identify orthologous gene groups based on sequence similarity. The longest transcript from each gene locus was selected as the representative gene during clustering. Genes that could not be clustered into any gene family and that were found exclusively in one species were considered species-specific. The one-to-one single-copy orthologous genes were extracted for maximum-likelihood (ML) phylogenetic tree construction using RAxML (v 8.2.12) with parameters "-f a -x 12345 -N 100 -p 12345 -m GTRGAMMA." In the ML analysis, the best-fit substitution model was determined using ProteinModelSelection. pl., which was provided by RAxML. The selected substitution model was then used for ML analysis. To assess the robustness of the phylogenetic tree, 1000 replicates were used for bootstrap support estimation. The divergence time among 18 species was estimated by r8s and MCMCTree, with parameter "--nsample <num> the number of samples for MCMC process 2000 --burnin <num> the number of samples to be discarded, default 2000; seed = -1 ndata = 1 model = JC69".

Expansion and contraction of gene families

The analysis of gene family expansion and contraction involved protein datasets from Indian perch and nine other ray-finned fish, namely *Danio rerio*, *Gasterosteus aculeatus*, *Hippocampus comes*, *Larimichthys crocea*, *Microphis manadensis*, *Oreochromis niloticus*, *Sphaeramia orbicularis*, *Takifugu rubripes*, and *Xiphophorus maculatus*. The gene family results obtained from the TreeFam pipeline and the estimated divergence times between these ten species were used as inputs for the analysis. The CAFÉ program (version 3.1) was employed to analyze the expansion and contraction of gene families based on the birth-and-death model. The parameters "-p 0.01, -r 10,000, -s" were set to search for the birth-and-death parameters (λ) of genes. This involved evaluating the probability of each gene family with the observed sizes using the Monte Carlo resampling procedure with 10,000 iterations. Gene families with $P < 0.01$ were considered for the birth-and-death parameter estimation. Gene families without homology in the SWISS-PROT database²⁹ were filtered out to reduce potential false-positive expansions or contractions caused by gene prediction. Furthermore, GO terms and KEGG pathway enrichment analyses of expanded/contracted genes in Indian perch were performed to identify the number of significantly expanded and contracted gene families and compare them to the common ancestor of cardinalfish.

Positive selection of genes (PSGs)

We identified PSGs in Indian perch and compared them to other background teleost fish. One-to-one orthologous genes were extracted from the same teleost selected for the gene family expansion/contraction analysis. Positive selection analyses were conducted using the branch-site model implemented in the Codeml program of PAML (version 4.9j).³⁰ We compared model A, where sites may be under positive selection ($\text{fix_omega} = 0$), with a null model, A1, which allows sites to evolve neutrally or under purifying selection ($\text{fix_omega} = 1$ and $\text{omega} = 1$). P -values were computed using the χ^2 statistic adjusted by the false discovery rate method (FDR adjusted $P < 0.05$) to facilitate multiple testing. The obtained PSGs were enriched in the GO and KEGG categories.

Convergent evolution analysis

To identify positively selected genes related to the emergence of parental behaviors, positive selection analyses were conducted using all five parental care species, including tiger tail seahorse, Southern platyfish, Nile tilapia, Indian perch, and Asian bonytongue, as foreground branches. Positively selected genes were intersected for further analysis. The Ka/Ks ratio of 9,489 orthologous genes from 11 species with a branch model under two assumptions: a one-ratio model that assumes the same Ka/Ks ratio for two clades (foreground and background branches) and a two-ratio model in which the two clades were assigned to different Ka/Ks ratios. To understand the mechanisms by which mouthbrooding fish achieve a trade-off between reproduction and survival, we performed another positive selection analysis using four datasets with the following rules: the orthologous genes (i) from all three mouthbrooding fishes, (ii) Nile tilapia, (iii) Indian perch, and (iv) Asian bonytongue were all set as foreground.

We used a previously published method³¹ to detect genome-wide signatures of convergent evolution related to parental behaviors in the lineages of five parental care species (tiger tail seahorse, Southern platyfish, Nile tilapia, Indian perch, and Asian bonytongue) and three mouthbrooding fish species (Nile tilapia, Indian perch, and Asian bonytongue). In group 1, the most recent common ancestor (MRCA) sequences were reconstructed for 9,489 orthologs among 11 species of bony fish using the Codeml program in PAML software. We identified convergent amino acid sites among five parental care fishes following these criteria: (i) identical amino acid residues among four of the five parental behavior species; (ii) inferred amino acid changes between the tiger tail seahorse lineage and its most recent common ancestor with the Southern bluefin tuna lineage; (iii) inferred amino acid changes between the Southern platyfish lineage and its most recent common ancestor with the medaka lineage; (iv) inferred amino acid changes between the Nile tilapia lineage and its most recent common ancestor with the flier cichlid lineage; (v) inferred amino acid changes between the Indian perch lineage and its most recent common ancestor with the giant fin mudskipper lineage; and (vi) inferred amino acid changes between the Asian bonytongue lineage and its most recent common ancestor with the African bonytongue lineage.

To filter out noise resulting from chance amino acid substitutions,^{32,33} a Poisson test was performed to verify whether the observed number of convergent sites for each gene was significantly greater than the expected number caused by random substitutions under the JTT-f gene model.³¹ Benjamini and Hochberg's approach was used to correct for multiple comparisons ($Q\text{-value} < 0.05$). Finally, a gene with non-random convergent substitution amino acid sites, which was also identified as PSG, was inferred to have undergone adaptive convergence.

Rapidly evolving genes (REGs)

The same orthologous genes and tree topologies used for the convergent evolution analysis were used in this analysis. The branch model in PAML was used with the null model ($\text{model}=0$), which assumed that all branches evolved at the same rate, and the alternative model ($\text{model}=2$) was used. LRT was conducted to examine whether the foreground branch (the parental care lineage) exhibited a significantly higher ω value than the background branch (the oviparous lineages).³⁴ Genes with $P < 0.05$ were considered to be evolving at a significantly faster rate in parental care group. GO and KEGG enrichment analyses of the REGs were performed.

Transcriptome analyses

We performed transcriptome analyses on the brain, buccal mucosa, liver, kidney, and spleen of the Indian perch. Each tissue sample was analyzed as three or four biological replicates for female and male individuals (in mouth-brooding stage). All samples were collected from the Yellow Sea of China. Additionally, we performed transcriptome sequencing specifically on the brains of giant fin mudskippers. Brain transcriptomes were sequenced for both male and female individuals with four biological replicates for each sex. Sequencing libraries were generated using the Hieff NGS Ultima Dual-mode mRNA Library Prep Kit for Illumina (Yeasen Biotechnology Co., Ltd., Shanghai, China), following the manufacturer's recommendations and index codes were added to attribute sequences for each sample. The libraries were sequenced on an Illumina NovaSeq platform to generate 150 bp paired-end reads, according to the manufacturer's instructions. Raw reads (raw data) in the FASTQ format were first processed using in-house Perl scripts. Clean reads were mapped to the Indian perch genome using the Hisat2. The gene expression levels were estimated as fragments per kilobase of transcript per million fragments (FPKM) mapped fragments. DEGs between the two groups were analyzed using DESeq2. The resulting P -values were adjusted using the Benjamini–Hochberg method to control for the FDR. Genes with an adjusted $P < 0.05$ and fold change ≥ 2 found by DESeq2 were assigned as differentially expressed. Both GO and KEGG enrichment analyses of the DEGs were performed.

Statistical analyses

Statistical analysis was conducted using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). All data were reported as the mean $\pm$ standard error of the mean (SEM). Significant differences were represented by P -value using Student's t -test, and a significance level of 0.05 was used.

RESULTS AND DISCUSSION

Evolution of reproductive patterns in teleosts

We used DNA from a female Indian perch and applied PacBio, Illumina, and Hi-C sequencing technologies to generate a high-quality genome assembly (Tables S1–S8). We estimated the Indian perch genome size is around 895 Mb by k -mer distribution (Table S2 & Figure S3). The assembled genome of *J. lineata* comprised 690 contigs, with a length of approximately 929 Mb and a contig N50 of 17.66 Mb (Table S5). Among these, 917 Mb were anchored into 23 chromosomes, with a scaffold N50 of 41.28 Mb, using Hi-C data (Tables S6–S9 & Figures S4–S5). The Indian perch genome demonstrates high completeness, with Benchmarking Universal Single-Copy Orthologs (BUSCO) analyses revealing 4,251 complete single-copy orthologs in the genome assembly, accounting for 91.47% of single-copy orthologs present (Table S10), providing a solid foundation for further research and functional exploration. A total of 23,169 protein-coding genes were predicted in the assembly by combining homology-based and *de novo* gene prediction methods, with 78.50% supported by RNA-seq transcripts (Table S11). Repeat elements account for approximately 48.86% (453.66 Mb) of the Indian perch genome, with class II DNA transposons being the most abundant class (32.27%; 299.61 Mb) (Table S14 & Figure S6).

Using single-copy orthologous genes, we constructed a phylogenetic tree of 18 ray-finned fishes to confirm the evolutionary placement of fish species exhibiting parental care behaviors (Figures 1A–B). Previous research has established that the ancestral state among bony fishes is characterized by the absence of parental care.³⁵ Concordantly, our phylogenetic findings revealed that parental care in teleost fishes exhibits a broad phylogenetic distribution. Notably, our findings highlight the substantial variation in parental care strategies, which have independently evolved on multiple occasions across this diverse taxonomic group (Figures 1A–B). Furthermore, mouthbrooding has independently evolved multiple times, especially within the families Cichlidae, Apogonidae, and Osteoglossidae.³⁶ Among them, the mouthbrooder *J. lineata* and *Sphaeramia orbicularis* diverged from *Periophthalmus magnuspinnatus* approximately 96.73 million years ago (Mya), coinciding with the emergence of the most inclusive lineages of acanthomorph fishes during the Cretaceous era; this is considered the most substantial evolutionary radiation observed in vertebrate history.^{37,38} To understand the histological organization of mouthbrooding fish, paraffin sections of buccal

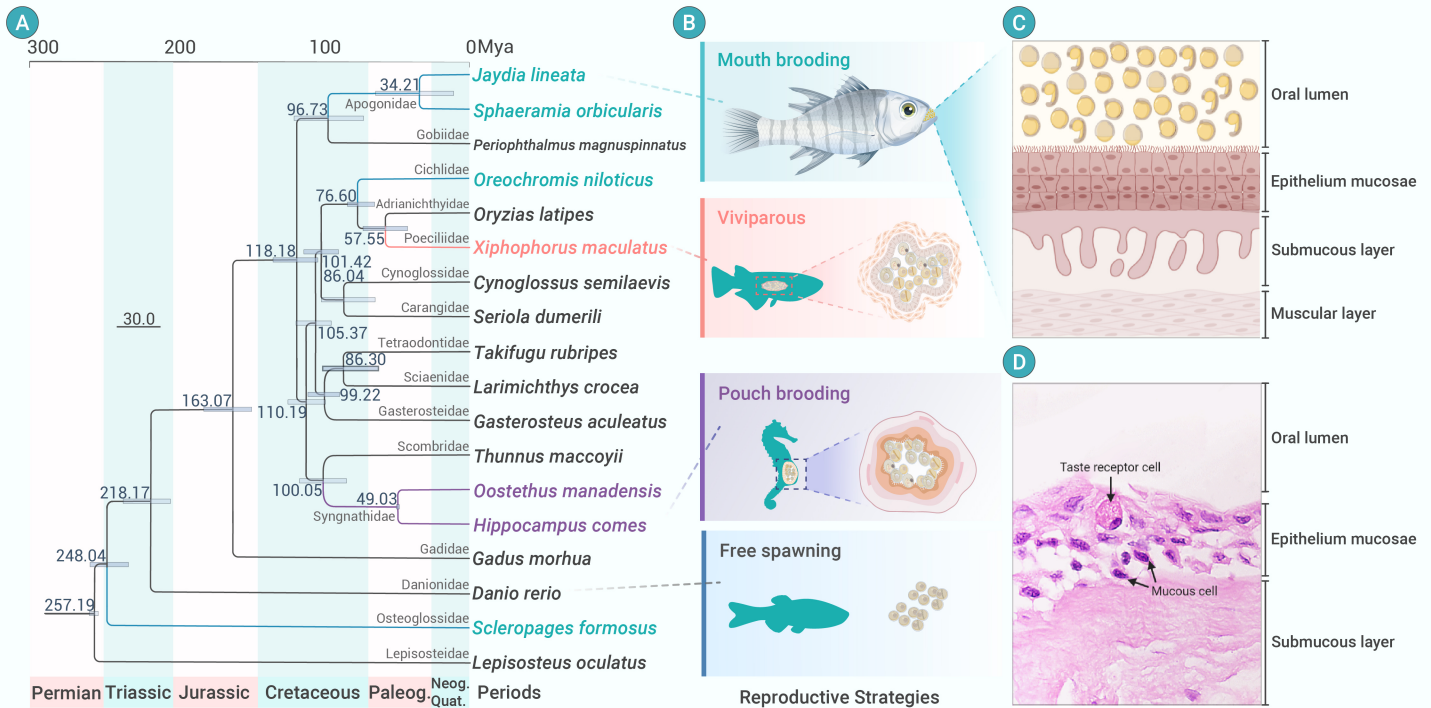


Figure 1. Phylogenetic position and reproductive strategies in fishes with parental behaviors (A) Phylogenetic relationships of Indian perch and 17 other representative ray-finned fishes. Turquoise, salmon, purple, and gray represent mouthbrooding, viviparous, pouch-brooding, and free-spawning species, respectively. The numbers on the tree denote the estimated divergence time. (B) Reproductive strategies in fishes. The branches of parental care fishes are colored. (C) Schematic diagram of buccal mucosa in mouthbrooders. (D) Morphological features of buccal mucosa in male Indian perch. Paleog: Paleogene; Neog: Neogene; Quat: Quaternary.

mucosa obtained from *J. lineata* were stained with hematoxylin and eosin (H&E). It was observed that the buccal mucosa of Indian perch contained a large number of mucus-producing cells in the buccal epithelium (Figures 1C–D). Additionally, high levels of expression of most immune markers were detected in the buccal mucosa of teleosts.³⁹ This suggests that mouthbrooding fish have the potential to create a protective microbial ecosystem for the offspring.

From a broader perspective, the divergence timeframe for major parental care patterns, including skin/pouch brooding in Syngnathidae, mouthbrooding in Cichlidae and Apogonidae, and viviparity in Poeciliidae, spans from 50–130 Mya, aligning with the Cretaceous period (Figure 1A). Incidentally, this timeframe coincides with the accelerated radiation of modern teleost fish groups during the Cretaceous era. We hypothesized that diverse parental care strategies evolved from the expanding diversity of acanthomorph fish lineages, which independently adapted to various global ecological conditions.

Genome-wide signatures of convergent evolution in fishes with parental behavior

Bony fishes represent the largest vertebrate group and exhibit unparalleled diversity in reproductive strategies.⁴⁰ Research into reproductive strategy has predominantly focused on unraveling the fitness-enhancing mechanisms, especially those increasing the chances of offspring survival.^{41,42} Within the realm of parental behaviors, we noticed independent evolutionary occurrences in fishes with male pregnancy, ovoviviparity, and mouthbrooding; therefore, we analyzed these three distinct lineages to detect molecular signals associated with the convergent evolution of this phenotype (Figure 2A). After selecting five parental care species (tiger tail seahorse, Southern platyfish, Nile tilapia, Indian perch, and Asian bonytongue) as foreground branches, we identified 43 genes displaying convergent signatures for positive selection (Table S16). Moreover, among the parental behavior-exhibiting fish groups, we identified 1,755 convergent amino acid substitutions distributed across 689 genes among four of the five parental behavior species (Figure 2B & Table S17).

We also estimated the ω (dN/dS) values of 9,489 orthologous protein-coding genes in parental care fishes and other teleosts, using spotted gar as

an outgroup and observed a significantly higher ω value within the caregiving group compared with that in the non-parental care group ($P < 2.2e-16$, Figure 2C). Subsequently, we identified 1,101 rapidly evolving genes (REGs) (Figure S9); Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses revealed that these REGs were significantly enriched in immune-related pathways, such as antigen processing and presentation, RIG-I-like receptor signaling pathway, and primary immunodeficiency (Figure 2D). These findings highlight the pivotal role of immunological adaptations in evolving unique reproductive strategies.

We focused on positively selected genes exhibiting non-random convergent amino acid substitutions to identify conservative signatures of adaptive convergence. This stringent approach helped identify 15 adaptively convergent genes (Table S16). Most of these genes are potentially linked to embryonic development in animals. These include Ras-related protein Rab34 (*Rab34*), with two specific convergent amino acid substitutions (H40N and I135V), consistently present across all five parental behavior species (Figures 2B & S11). Notably, the I135V substitution is located in the RAB domain. Rab GTPases are pivotal in various cellular processes, including membrane trafficking and intracellular signaling, which are crucial for optimal embryonic development.⁴³ In mammals, Rab34, which is localized on the cilia, plays a crucial role in several processes, including the successive fusion of preciliary vesicles to generate ciliary vesicles and the migration of the mother centriole from the perinuclear region to the plasma membrane.⁴⁴ The primary cilium is a microtubule-based organelle protruding from the cell surface and is vital in embryonic development. Rab34 mutant mice are non-viable owing to significant ciliogenesis disruption that results in the loss of their ability to respond to Hedgehog (Hh) signaling;⁴⁴ this pathway is vital in regulating the development of various structures in both embryonic and adult metazoans.

Several key genes, such as dystroglycan 1 (*Dag1*), significantly contribute to early embryonic development (Figure 2E). *Dag1*-null mice exhibit mortality at approximately E6.5–7.0 owing to the disruption of Reichert's membrane, an extra-embryonic basement membrane.⁴⁵ Vascular early response gene (*Verge*) is involved in angiogenesis and dynamically regulates endothelial cell signaling and vascular function.⁴⁶ Furthermore, *Ssbp2* regulates normal hematopoietic stem cell function during early development, with *Ssbp2*^{-/-}

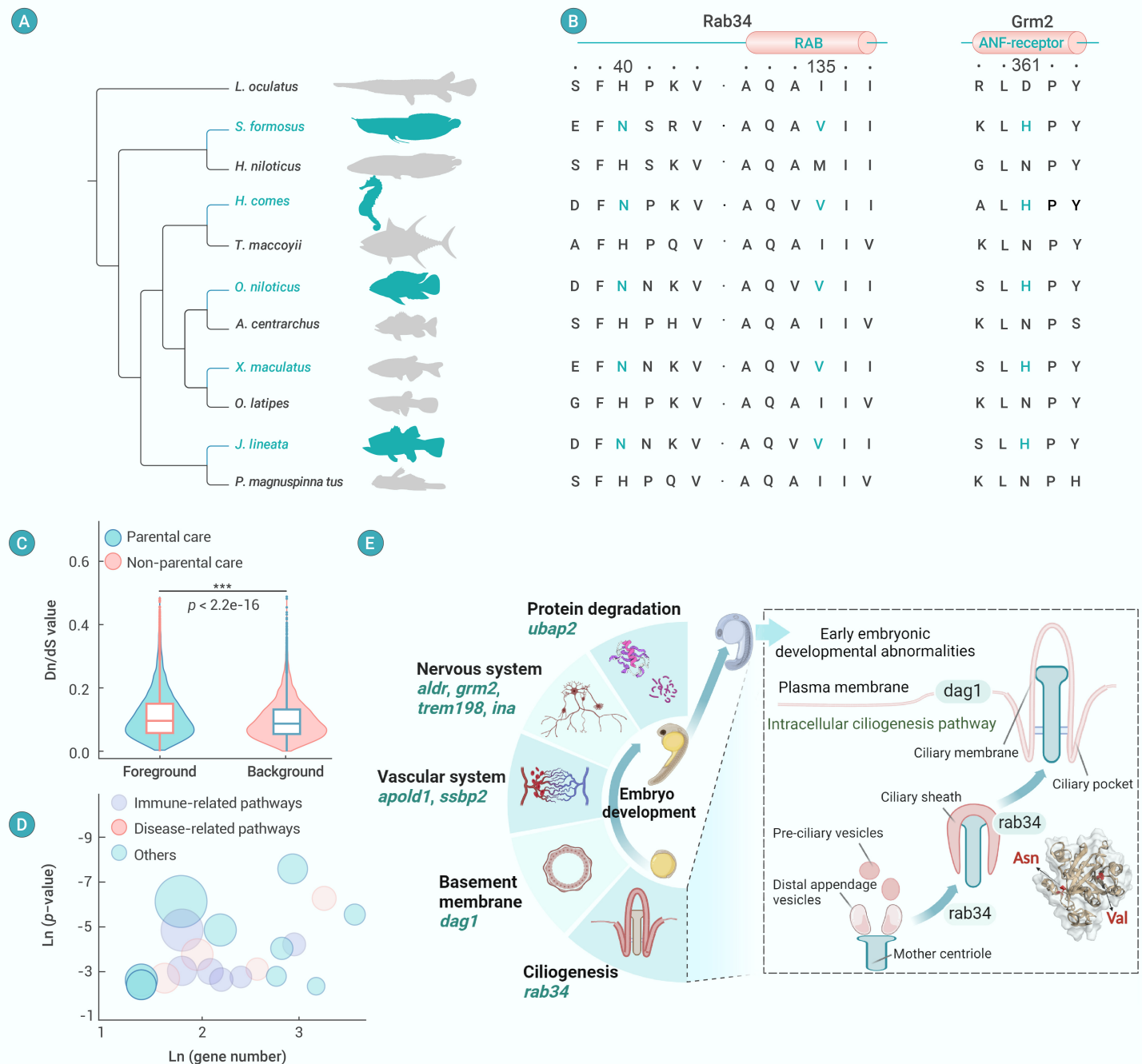


Figure 2. Genomic convergence analysis in fishes with parental behaviors (A) Phylogeny of parental care fishes and outgroups. Turquoise and gray denote fish with and without parental behavior, respectively. (B) Comparison of convergent amino acid substitutions in Rab34 and Grm2. Structural domains and the convergent amino acid residues are highlighted. The structural domain annotations were derived from the SMART database. (C) Comparison of the dN/dS ratios between parental and non-parental care groups. (D) KEGG enrichment analyses for rapidly evolving genes. (E) Adaptively convergent genes closely related to embryo development.

mice displaying hypoplastic hematopoietic tissues.⁴⁷ Spontaneous electrical activity during the embryonic period is fundamental for neural circuit development in the central nervous system,⁴⁸ driven by periodic membrane depolarization and the consequent increase in intracellular Ca^{2+} concentration in neurons.⁴⁹ Metabotropic glutamate receptors (*mGluRs/grm2*), with the N361H substitution, observed in all five parental behavior species (Figure 2B). *mGluR2* plays a principal role in modulating spontaneous Ca^{2+} spikes.⁵⁰ α -Internexin (*ina*), a neuronal intermediate filament protein, is expressed in most neurons at the beginning of differentiation and is implicated in neuroplasticity during development.⁵¹ Canonical Wnt/ β -catenin signaling is fundamental for embryonic development and tissue homeostasis in metazoans. *Tmem198* is required for Wnt-mediated neural patterning during *Xenopus* embryogenesis.⁵² Furthermore, the ubiquitin-associated protein 2 (Ubp2)

plays a role in embryonic development, particularly in protein quality control mechanisms and the ubiquitin-proteasome pathway (UPP), which is vital for protein degradation. In the early mammalian embryo, UPP may regulate the transition from maternal to embryonic developmental control, with disruptions in the UPP potentially compromising developmental potential.⁵³

Parental care includes aggressive and nurturing behavior; however, nurturing gains precedence in the species examined in this study. We observed that several genes associated with embryogenesis (Figure 2E) have undergone adaptively convergent evolution in different teleost lineages, suggesting that parental behaviors create more favorable conditions for embryo development.

Genetic basis of mouthbrooding evolution in teleosts

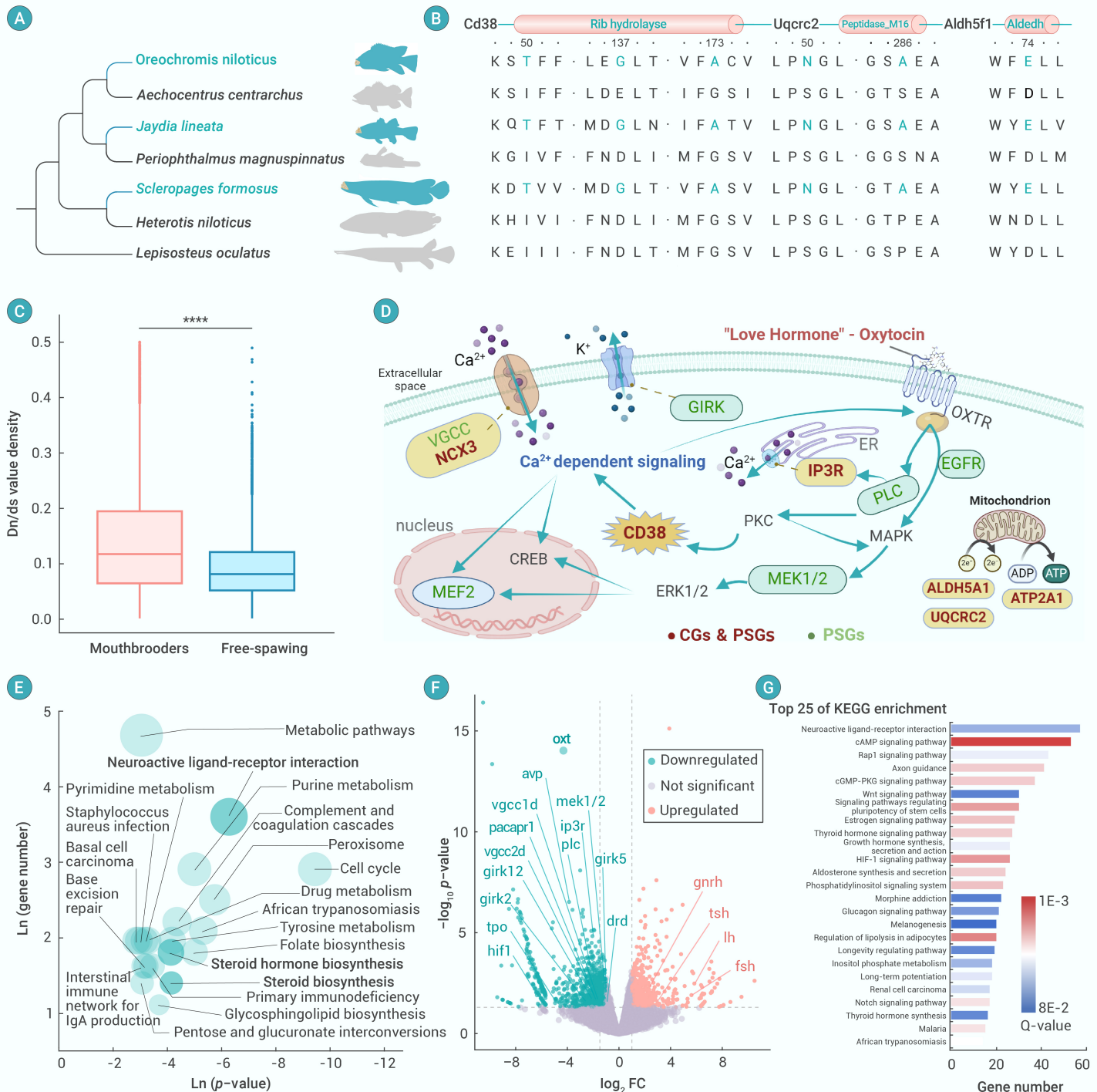


Figure 3. Evolutionary basis of mouthbrooding (A) Phylogeny of mouthbrooders (turquoise) and outgroups (gray). (B) Comparison of convergent amino acid substitutions in Cd38, Uqcrc2, and Aldh5f1. Structural domains and convergent amino acid residues are highlighted. (C) Comparison of the dN/dS ratios between mouthbrooding and free-spawning species. (D) CGs & PSGs and PSGs in mouthbrooders are involved in parental behaviors. (E) KEGG enrichment of DEGs in the brain of male Indian perch compared with that in the male giant fin mudskipper. (F) Volcano map of DEGs in the brains of male Indian perch compared with that in the brains of female Indian perch. (G) KEGG enrichment of DEGs between male and female brains of the Indian perch. CGs & PSGs: positively selected genes with non-random convergent amino acid substitutions; PSGs: positive selection of genes; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEG: differentially expressed genes.

To understand the mechanisms by which mouthbrooding fish strike a balance between reproduction and survival, we conducted comparative genomic analysis using a set of 10,899 orthologous genes from seven species, including Nile tilapia, Indian perch, and Asian bonytongue (Figure 3A). Initially, positive selection analyses were conducted with four different datasets from either Nile tilapia, Indian perch, Asian bonytongue, or all three mouthbrooders as foreground branches. Our findings revealed a genetic landscape comprising 3,657 genes that underwent positive selection during the evolution of mouthbrooding reproductive strategies (Table S18). Sub-

sequently, we identified 337 genes displaying convergent amino acid substitutions among two of the three mouthbrooding species (Figure 3B & Table S19). Finally, we identified 149 adaptively convergent genes characterized by positive selection and convergent amino acid replacements (Table S18). Among the 149 genes, several are involved in hormone regulation. Specifically, genes related to the oxytocin signaling pathway, thyroid hormone signaling pathway, prolactin receptor activity, and prolactin signaling pathway showed significant enrichment (Tables S20-S21). Dopamine biosynthetic processes and thyroid hormone pathways have been suggested

to be important in parental care.⁵⁴ Our findings highlight the vital role of hormonal regulation in mouthbrooding behavior, which is essential for parental care. Additionally, we found significantly higher dN/dS ratios in the three mouthbrooders (Figure 3C).

Ethological principles suggest that certain neuropeptides have been co-opted to modulate parental behavior.⁵⁵ We identified key genes and pathways related to oxytocin signaling (Figures 3B & D). Oxytocin (*oxt*) and its non-mammalian homologs are crucial in regulating various social behaviors. In fishes, isotocin (*ist*) is a homolog of mammalian oxytocin. OXT also contributes to the development of reproductive and social strategies, influencing some species or individuals to exhibit caregiving even in nonreproductive contexts.⁵⁶ CD38, a transmembrane glycoprotein with ADP-ribosyl cyclase activity, is vital in regulating social behavior by modulating OXT secretion. It catalyzes the formation of Ca^{2+} signaling molecules and has a key role in neuropeptide release, thereby critically regulating parental behaviors.⁵⁷ The plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), particularly the excitable cell-specific isoform 3 of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX3), contributes to the maintenance of Ca^{2+} homeostasis. It functions as a reversible transporter controlling Na^+ and Ca^{2+} transport.⁵⁸ Inositol 1,4,5-trisphosphate receptor (IP3R), a widely expressed channel that regulates calcium stores, is another key player. When activated by IP3 and Ca^{2+} signaling at a lower concentration, IP3R modulates the release of Ca^{2+} from stores into the cytoplasm, triggering downstream events.⁵⁹ Furthermore, several PSGs, including PLC, EGFR, MEK1/2, GIRK, VGCC, and MEF2, are considered to be involved in regulating parental care (Figure 3D).

Parental behavior-related gene expression likely occurs in brain regions governing caregiving.^{60,61} Therefore, we compared the transcriptome of the brains of the Indian perch and the giant fin mudskipper (*Periophthalmus magnuspinnatus*), a control species that does not exhibit caregiving (Figure S10). KEGG enrichment analysis of differentially expressed genes (DEGs) revealed gene signatures related to neuroactive ligand-receptor interaction, steroid hormone biosynthesis, steroid biosynthesis, and primary immunodeficiency in the brain of male Indian perch compared with that in the brain of mudskipper (Figure 3E & Table S22). Furthermore, significant differences were observed in the expression patterns of genes involved in hormone regulation between mouthbrooding fish and non-mouthbrooding fish, providing additional evidence supporting the role of hormones in the regulation of mouthbrooding behavior.

Additionally, comparison gene expression patterns of Indian perch revealed a higher number of DEGs (1,305 downregulated and 555 upregulated DEGs) in the brains of males compared with that in the brains of females (Figure 3F & Figure S10). KEGG enrichment analyses revealed DEGs in pathways related to parental behaviors, including neuroactive ligand-receptor interaction, thyroid hormone signaling pathway, thyroid hormone synthesis, dopaminergic synapse, and GnRH signaling pathway (Figure 3G & Table S24). Notably, several genes involved in the oxytocin signaling pathway, such as *oxt* (*ist*), *vgcc1d*, *vgcc2d*, *girk2*, *girk5*, *girk12*, *mek1/2*, *ip3r*, and *plc*, displayed significantly differentially expressed in the brains of male and female Indian perch (Figure 3F). These findings further confirm the pivotal role of the oxytocin signaling pathway in parental care.

Cellular metabolism involves the utilization of carbohydrates, fats, and proteins to produce energy. These molecules from metabolic processes are channeled into the tricarboxylic acid (TCA) cycle, generating substrates that enter the electron transport chain (ETC) for oxidative phosphorylation (OXPHOS). The ETC is situated within the extensive inner membrane of the mitochondrion, proximal to the mitochondrial matrix where the TCA cycle occurs. Among the critical genes for OXPHOS, aldehyde dehydrogenase 5a1 (*Aldh5a1*), ubiquinol-cytochrome-c reductase core protein 2 (*UQCRC2*), and sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (*ATP2A1*) exhibit convergent genomic variations and positive selection in all three mouthbrooders (Figures 3D & S12). *Aldh5a1* plays a role in the degradation of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), and *Aldh5a1*^{-/-} mice exhibited 100% mortality from generalized tonic-clonic seizures between the ages of 3–4 weeks.⁶² *UQCRC2* is a component of the ubiquinol-cytochrome c reductase complex within the mitochondrial ETC and has been linked to male fertility via its role in spermatogenesis.⁶³ *ATP2A1* is responsible for the enzymatic process of breaking down ATP while simultaneously

transporting calcium ions from the cytosol to the lumen of the sarcoplasmic reticulum. Notably, we observed 11 convergent substitutions in the functional domain regions of *ATP2A1* (Table S19) and identified 26 PSGs involved in OXPHOS (Figure S12). These genetic alterations in mouthbrooders may indicate enhanced physiological functions to meet the increased energy demands.

Parental care influences sexual immune dimorphism in Indian perch

Providing parental care enhances the fitness of parents by increasing the survival rate of offspring; however, it may lead to trade-offs with other crucial traits, such as the immune system.⁶⁴ In most vertebrates, the parental care-associated elevated energy expenditure results in immunosuppression, depletion of energy reserves, loss of micronutrients, and heightened oxidative stress.^{65,66}

Comparative genomic analyses with other fish genomes helped identify PSGs and expanded/contracted gene families in Indian perch. We identified 500 genes with positive selection signatures ($P < 0.05$, FDR < 0.05 , Table S25). Among the top 20 enriched KEGG pathways, cytokine-cytokine receptor interaction, primary immunodeficiency, Jak/STAT signaling pathway, NF-kappa B signaling pathway, and TNF signaling pathway are associated with the immune system (Figure S13). We also identified 455 expanded and 2,920 contracted gene families in the Indian perch (Figure S14).

Peptidoglycan recognition proteins (PGLYRPs) are critical innate immunity molecules, possessing at least one carboxy-terminal PGRP domain. Teleost fish typically have three main types of *pglyrp* genes, including *pglyrp2* and teleost-specific *pglyrp 5/6*. In Indian perch, we also observed the presence of these *pglyrp* genes (Figure 4C); notably, we discovered two novel types of *pglyrp* genes, *pglyrp7* (*pglyrp7a*, *pglyrp7b*, and *pglyrp7c*) and *pglyrp8* (*pglyrp8a*, *pglyrp8b*, and *pglyrp8c*) (Figures 4C & S15). The duplication of PGLYRP1 in pteropods indicated that the copy number variation could aid in reducing inflammation.⁶⁷ The expanded *pglyrp* genes in Indian perch may help to regulate inflammation.

To investigate the trade-off between parental care and immunological activation in mouthbrooding fishes, we analyzed 32 tissue-specific transcriptomes. These datasets included the buccal mucosa, as well as the liver, kidney, and spleen from four female and four male Indian perch. Notably, gene expression profiles were more similar within organs than across organs, with significant differences observed between the sexes. Sex-related differences in gene expression were most evident in the kidneys, with 1,457 DEGs, whereas it was least evident in the liver (402 DEGs) (Figure S16). Furthermore, the buccal mucosa of the males exhibited more downregulated genes than that in the females (367 downregulated vs. 115 up-regulated DEGs) (Figure 4A).

GO enrichment analysis of DEGs in the liver between females and males revealed enrichment of genes related to essential processes such as intestinal lipid catabolic processes, proteolysis, lipid digestion, and intestinal lipid absorption (Figure S17), which are critical for energy supply. Notably, immune-related processes were the most enriched GO term associated with "biological processes" in the buccal mucosa compared with that in the kidney or spleen. These processes included the regulation of the immune system process, regulation of cytokine production, response to bacterium, and response to biotic stimulus (Figure S17). Moreover, most of the downregulated DEGs in the buccal mucosa were associated with immune responses, including functions such as the regulation of immune response and response to external biotic stimulus (Figure 4B). Several vital genes involved in the immune system, such as *c6*, *card9*, *ccl20*, *fyb1*, *lcp2*, *spi1*, *spon2*, and *tril*, were differentially expressed in the buccal mucosa (Figures 4A & S18). Upregulated genes in the buccal mucosa were associated with positive regulation of isotype switching to IgA isotypes, somatic recombination of immunoglobulin gene segments, and somatic diversification of immunoglobulins (Figure S19).

In maternal mouthbrooders, females typically exhibit a lower expression of immune-related genes.⁹ Considering that the oral mucosa is the initial point of contact with environmental substances before they enter the gastrointestinal tract, oral mucosal immunity plays a crucial role in neutralizing harmful foreign antigens, preventing colonization by pathogenic microorganisms, evoking protective immunoinflammatory responses against invading

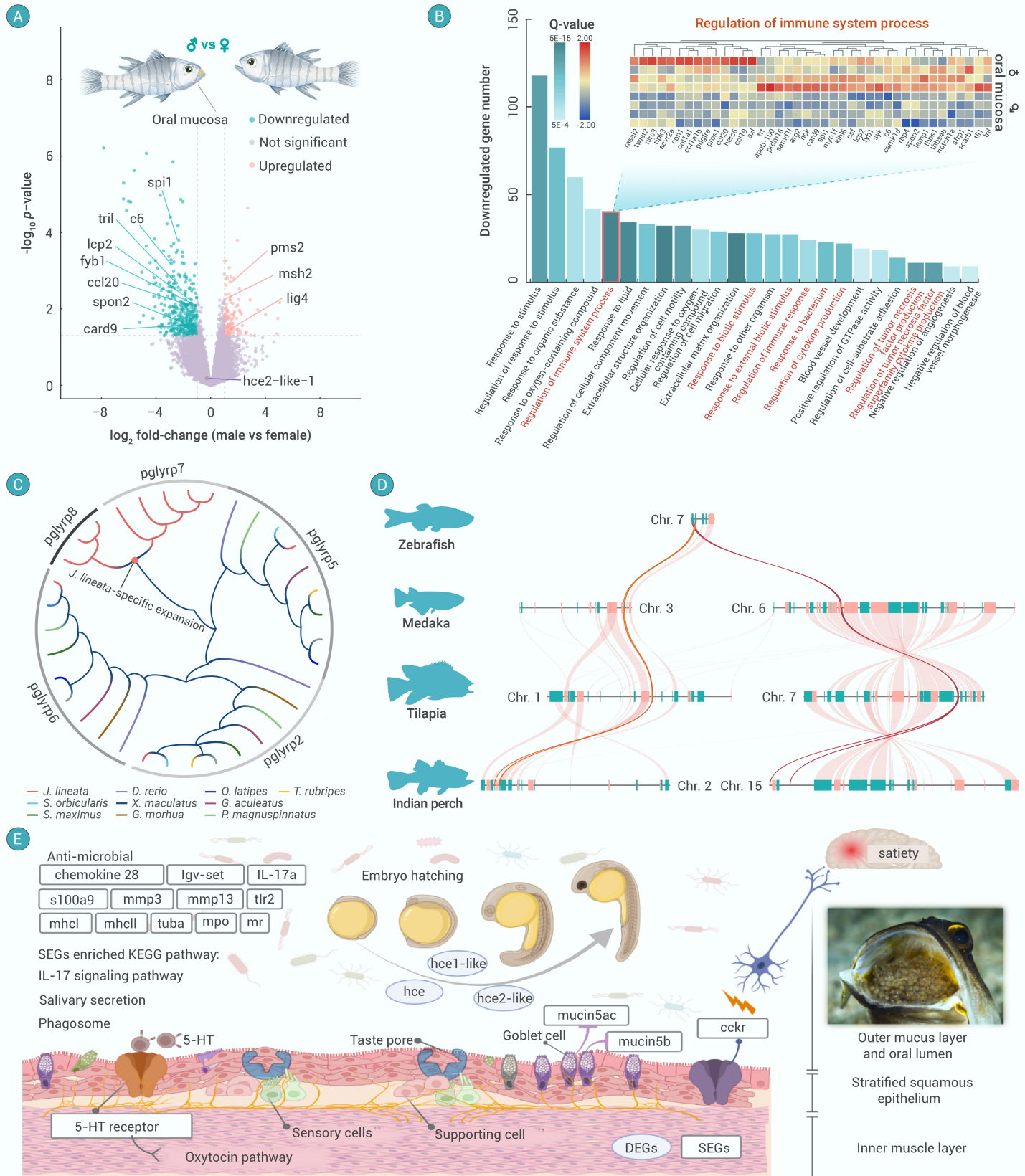


Figure 4. Unique biological function of buccal mucosa and specific immune responses in Indian perch (A) Volcanic map of DEGs in the buccal mucosa of male Indian perch compared with that in the female. (B) GO enrichment of downregulated DEGs between male and female buccal mucosa, focusing on genes associated with the regulation of the immune system process. (C) Phylogenetic relationships of pglyrp-indicated teleosts. (D) Identification of orthologous blocks shared among Indian perch, tilapia, medaka, and zebrafish, revealing expanded HCEs in the Indian perch. (E) DEGs, SEGs, and pathways in the buccal mucosa of the Indian perch, with associations to immune responses, satiety, and hatching functions. GO: gene ontology; HCE: high choriolytic enzyme; DEGs: differentially expressed genes; SEGs: organ-specific expression genes.

pathogens, and promoting tolerance.⁶⁸ Mouthbrooding fish, including the Indian perch, face the challenge of balancing parental care with immunologi-

cal defense, as both demand the allocation of limited resources. In response to the presence of semi-allogenic embryos as an external biotic stimulus,

male mouthbrooders downregulate the expression of immune-related genes to achieve immunological tolerance. Notably, pregnant male seahorses modulate the expression of immune-related genes to support the immunological tolerance of the embryos.⁶⁹ In caregiving fishes, this trade-off involves defending against potential pathogens while ensuring immunological tolerance toward developing embryos. However, unlike tetrapod species, where secretory IgA plays a key role in adaptive immune responses against oral pathogens,⁷⁰ teleost fish, such as mouthbrooding species, lack salivary glands in their buccal mucosa, which are instead populated with abundant mucus-secreting cells.⁷¹ Fish IgT and mucus-producing cells are reportedly evolutionarily distinct from mammalian IgA and salivary glands, respectively.³⁹

Parental investment drives sexual immune dimorphism.⁷² Among cichlids, maternal mouthbrooding species exhibit elevated immune cell activity in females compared to males, while no sexual immune dimorphism was observed in biparental mouthbrooding species.⁷² We detected effects of parental care behavior on sexual immune dimorphism in immune gene expression in paternal mouthbrooding species. We indeed found that sexual immune dimorphism is present in the buccal mucosa of Indian perch. Males of Indian perch have a lower expression of immune-related genes, possibly due to a resource allocation trade-off between investment in the immune system and expenditure during longer phases of parental care. Additionally, the most important immunocompetent organs and tissue of fish include the kidney, thymus, spleen, liver, and gut-associated lymphoid tissue.⁷³ However, among the top 20 significantly enriched GO terms, we did not find processes related to immunity in kidney, spleen or liver.

Distinctive biological functions of buccal mucosa in mouthbrooding fishes

To investigate whether the buccal mucosa exhibits distinctive biological functions, we identified characteristic gene sets for each organ and performed KEGG pathway enrichment analyses using organ-specific expression genes (SEGs). The results revealed that 553 genes were exclusively expressed in the buccal mucosa (Table S30). KEGG enrichment analysis helped identify gene signatures related to the IL-17 signaling pathway (*IL-17a*, *S100A9*, *muc5ac*, *muc5b*, *mmp3*, and *mmp13*), phagosome (*mhcl*, *mhcll*, *tlr2*, *tuba*, *mpo*, and *mr*), salivary secretion (*dmbt1*, *ae2*, and *plcβ*), and neuroactive ligand-receptor interaction (*cckr*, *prlr*, and *p2rx*) (Table S31). Lymphocytes primarily produce IL-17 and transmit signals to non-hematopoietic cells, linking the immune system to inflamed tissues, particularly mucosal tissues. IL-17/TH17 responses are essential for maintaining barrier integrity and preventing pathogenic infections in the oral mucosa.⁷⁴ S100A9, a calcium- and zinc-binding protein, is pivotal in regulating inflammatory processes and immune response,⁷⁵ as well as serves as a leptin-induced cue to exert several protective responses against metabolic imbalance.⁷⁶ Collectively, our findings suggested that the immune-related genes exclusively expressed in the buccal mucosa play a crucial role in protecting the mucosa and the developing embryo from potential pathogens.

The C6ASTs of astacin metalloproteases include hatching enzymes, nephrosin, pactacin, and patristacin. Enzymes, such as high choriolytic enzyme (HCE) and low choriolytic enzyme (LCE), are involved in lysing the chorion surrounding the egg. The Indian perch genome contains four pactacin genes (*hce111*–*hce114*), which is a higher number compared with one or two in other teleosts (Figure 4D & S20A). We observed that *hce*, *hce113*, and *hce211* were expressed in the buccal mucosa (Figure S20B), suggesting a potential role of the oral cavity in partial brooding function. Additionally, *hce1*-like is commonly expressed in the pancreas of *Hippocampus abdominalis* and *Oryzias latipes*, indicating its potential role as a novel digestive enzyme in teleosts.⁷⁷ Filial cannibalism is prevalent in the Apogonidae family, particularly among paternal mouthbrooders. In this behavior, male fish consume their eggs as a source of energy, with eggs being consistently detected in their stomachs.⁷⁸ The higher copy number of *hce1*-like genes in male Indian perch suggests its potentially significant role in digesting eggs in the stomach.

Several other key genes were also identified in the buccal mucosa (Figure 4E). Cholecystokinin (CCK) is a peptide hormone that regulates appetite and food intake primarily through CCK-A receptors (CCKAR), which

is present in smooth muscles, enteric neurons, and vagal afferent neurons in humans and animals.⁷⁹ The presence of *cckr* in the buccal mucosa may be involved in regulating the satiety of mouthbrooders and reducing food intake during brooding. Despite its name suggesting a crucial role in mammalian lactation, the interaction between prolactin and its receptors (PRLRs) is vital in parental care in fishes. In cichlids, a combination of PRL and estrogen can enhance the tendency for oral egg-carrying behavior.⁸⁰ Recent research suggests that serotonin (5-HT) acts as a central regulator influencing neuropeptides involved in social behaviors in mammals.⁸¹ Considering that parental care is a common social behavior in the animal kingdom, the identification of the expression of a 5-HT receptor in the buccal mucosa is noteworthy. Furthermore, our findings indicate that this receptor is highly expressed in males engaged in mouthbrooding behavior compared with that in females. Admittedly, the direct contribution of these genes to parental care remains unclear, and further functional validation experiments are needed to elucidate their precise functions. Combining genetic research with detailed behavioral studies would be valuable in understanding the interactions between genetics and behavior in mouthbrooding. Such investigations would provide a more comprehensive understanding of the role of these genes in parental care.

In conclusion, our findings demonstrate the significant convergence of genetic adaptations in parental care species, highlighting the importance of certain genes, such as *rab34* and *cd38*, and pathways in facilitating these behaviors. Notably, we identified that genes related to immune system regulation, hormonal control, and energy metabolism play crucial roles in mouthbrooding. Furthermore, our results provide insights into the trade-offs that mouthbrooding species face when allocating limited resources between caregiving and immune defense. We also observed that the buccal mucosa, a critical site for protecting developing embryos, exhibited unique gene expression patterns related to immune responses. Notably, we identified the presence of sexual immune dimorphism in Indian perch, emphasizing the influence of sex-specific factors on immune-related gene expression and their potential implications for reproductive strategies. Overall, our findings provide novel insights into the genetic mechanisms underlying the evolution of mouthbrooding in teleosts, emphasizing the roles of genes in embryogenesis, neuropeptide signaling, and immune adaptations.

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AUTHOR CONTRIBUTIONS

Q.L. designed the scientific objectives. Q.L. and T.G. oversaw the project. B.S., Y.Z. and H.Z. collected the samples. Y.H. Z., H.Y. and J.Z. performed genome analyses. Y.H.Z., X.W. and H.Y. performed transcriptomic analysis. G.Q., L.H. and Z.M. participated in the discussions related to data analysis. Y.H.Z., X.W. and Q.L. wrote the manuscript with input from all other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AND CODE AVAILABILITY

The whole-genome assembly of the Indian perch has been deposited at DDBJ/ENA/GenBank under the accession JAULSP000000000. RNA-seq reads for *J. lineata* and *P. magnuspinnatus* have been deposited in the NCBI Sequence Read Archive under accession numbers SRR25335882-SRR25335921 and SRR26319591-SRR26319598, respectively. The data generated or analyzed during this study are available within the published article and its supplementary information. Any further information required to reanalyze the data reported in this paper can be obtained from the lead contact upon request.

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-life.2024.100066>

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