

Article

Proteomic Characterization of the Salivary Microenvironment Associated with Mouthbrooding in Nile Tilapia (*Oreochromis niloticus*)

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Abstract

Mouthbrooding is a specialized parental care strategy that enhances offspring survival in many cichlid fishes; however, the molecular characteristics of the maternal buccal environment remain poorly understood. In this study, we compared embryonic performance between artificial incubation and natural mouthbrooding in Nile tilapia (*Oreochromis niloticus*) and characterized the buccal fluid proteome associated with mouthbrooding using LC–MS/MS-based proteomics. Naturally mouthbrooded embryos exhibited significantly higher survival rates and lower morphological abnormality rates than artificially incubated embryos. Proteomic analysis revealed extensive remodeling of buccal fluid protein composition during mouthbrooding and identified numerous differentially abundant proteins among mouthbrooding females, pre-mouthbrooding females, and males. Functional enrichment analyses showed that mouthbrooding-associated proteins were predominantly involved in immune regulation, epithelial organization, and host–pathogen interaction pathways. Proteins enriched during mouthbrooding were mainly associated with epithelial barrier maintenance and immune defense functions. These findings suggest that mouthbrooding females establish a distinct buccal microenvironment that may contribute to embryonic survival and developmental success. This study provides new insights into the molecular basis of parental care in mouthbrooding fishes and offers potential targets for improving artificial incubation strategies in tilapia aquaculture.

Keywords: Nile tilapia; mouthbrooding; embryo development; buccal fluid proteome; immune defense



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Key Contribution: Natural mouthbrooding enhances embryonic survival and developmental quality in Nile tilapia. This study identifies a mouthbrooding-associated salivary proteome that may provide epithelial and immune protection for developing embryos.

1. Introduction

As a major contributor to freshwater aquaculture and food security, Nile tilapia (*Oreochromis niloticus*) has gained considerable global importance. This status is closely

associated with its rapid growth, broad environmental adaptability, high reproductive capacity, and the sustained expansion of tilapia production over recent decades [1]. Successful reproduction and larval survival are critical determinants of production efficiency in tilapia farming [2–4]. Consequently, understanding the biological mechanisms that support early embryonic development has considerable ecological and aquacultural importance.

Unlike many teleost fishes that release fertilized eggs into the surrounding environment, Nile tilapia exhibit maternal mouthbrooding behavior [5–7]. Following fertilization, females actively collect and retain eggs within the buccal cavity, where embryos remain throughout early development until the yolk-sac larval stage. Mouthbrooding is widely recognized as an effective parental care strategy that increases offspring survival by protecting embryos from predators and environmental disturbances [8–10]. Recent comparative studies further suggest that mouthbrooding is associated with specialized reproductive investment, buccal morphology, and immune-related adaptations in teleost fishes [11,12]. As a result, naturally mouthbrooded embryos often exhibit higher survival rates than those produced through artificial incubation. Although the ecological significance of mouthbrooding has been extensively documented, the biological mechanisms responsible for its developmental benefits remain incompletely understood [13–15]. Across cichlid fishes, mouthbrooding represents a specialized form of parental care that protects developing offspring from predation and environmental disturbance while maintaining a relatively stable incubation environment. Although this strategy can enhance offspring survival, prolonged egg retention may impose energetic and reproductive costs on the parent, reflecting an important evolutionary trade-off [16].

Previous studies have primarily focused on the behavioral, ecological, and physiological aspects of mouthbrooding, including parental investment, oxygen transport, embryo ventilation, and reproductive trade-offs. The buccal cavity of mouthbrooding females is generally considered a protected incubation chamber that provides a relatively stable environment for embryonic development. However, embryos are continuously immersed in maternal buccal fluid throughout the incubation period, suggesting that saliva may represent an important but largely overlooked component of the mouthbrooding environment [17–19]. The teleost buccal cavity has also been recognized as an immune-relevant mucosal surface capable of mounting local immune responses [20]. In vertebrates, saliva contains a diverse array of proteins involved in tissue maintenance, immune defense, microbial regulation, and environmental adaptation [21–23]. Nevertheless, little is known about the composition of buccal fluid proteins in mouthbrooding fishes or their potential contribution to embryo development and protection. In the present study, the term “salivary microenvironment” is used as a conceptual description of the biochemical milieu associated with buccal fluid in the maternal buccal cavity.

Recent advances in high-throughput proteomics have provided powerful tools for characterizing complex biological fluids and identifying molecular pathways associated with physiological processes [24,25]. Proteomic approaches have been successfully applied to investigate immune responses, reproductive biology, and environmental adaptation in fish species [26–28]. However, comprehensive analyses of the buccal fluid proteome during mouthbrooding remain scarce. Characterizing mouthbrooding-associated buccal fluid proteins may provide important insights into the molecular basis of parental care and reveal factors that contribute to embryonic survival.

Therefore, the objectives of the present study were to (1) compare embryonic performance between artificial incubation and natural mouthbrooding, (2) characterize the buccal fluid proteome associated with mouthbrooding in Nile tilapia, and (3) identify molecular pathways potentially involved in embryo protection within the maternal buccal cavity. Our results demonstrate that natural mouthbrooding significantly improves embryo

survival and developmental quality and is accompanied by extensive remodeling of the buccal fluid proteome. Proteins enriched during mouthbrooding were primarily associated with epithelial barrier maintenance and immune defense, supporting the hypothesis that mouthbrooding females establish a distinct buccal microenvironment that may contribute to successful embryonic development.

2. Materials and Methods

2.1. Fish Husbandry and Experimental Design

A total of 12 sexually mature Nile tilapia (*Oreochromis niloticus*), comprising six females (XX) and six males (XY) forming six breeding pairs, were obtained from the Guangxi Academy of Fishery Sciences (Nanning, China). The broodstock had an approximate body weight of 1200 g and total lengths of 30–45 cm. All fish were healthy and exhibited normal external morphology. The broodstock were capable of normal spawning, and the females exhibited normal mouthbrooding behavior.

Before breeding, females and males were maintained separately in glass aquaria (85 cm × 40 cm × 40 cm) connected to a recirculating aquaculture system. The water temperature was maintained at 28 ± 1 °C, salinity at 1–5 ppt, and pH at 7.0–7.5, under a 14 h light/10 h dark photoperiod. Fish were fed a commercial diet twice daily.

The first experiment evaluated embryonic development and reproductive performance under artificial incubation and natural mouthbrooding conditions. The second experiment characterized buccal fluid proteins from three reproductive groups: sexually mature males (M), sexually mature pre-mouthbrooding females (PMF), and mouthbrooding females (MBF).

2.2. Embryo Collection, Artificial Incubation, and Evaluation of Reproductive Performance

Following natural spawning and fertilization, males were removed from the breeding tanks. Six independent fertilized egg clutches produced by six females were used in the incubation experiment. Each clutch contained approximately 600 fertilized eggs, with clutch sizes ranging from approximately 570 to 630 eggs. Approximately half of each clutch was gently removed from the maternal buccal cavity for artificial incubation, whereas the remaining eggs were retained in the buccal cavity for natural mouthbrooding. Because the retained eggs were not directly counted, the initial egg numbers in the two incubation groups were approximately, rather than exactly, equal. Each clutch was regarded as one biological replicate, yielding six paired biological replicates. For the artificial incubation group, freshly fertilized eggs were gently collected from the buccal cavity of females at 0 days post-fertilization (dpf) and transferred to commercial incubators (ZissAqua, Pyeongtaek, Gyeonggi-do, Republic of Korea). Eggs were incubated in aerated freshwater at 28 °C. Methylene blue (500 ppm) was added to the incubation water to reduce fungal contamination. Water was renewed twice daily, and continuous water circulation was maintained throughout the incubation period. For the natural mouthbrooding group, fertilized eggs were retained within the maternal buccal cavity throughout incubation, except for limited sampling for developmental observation. Embryonic development was monitored daily from 0 to 7 dpf. Artificially incubated embryos were observed directly, whereas a small number of embryos or larvae were gently removed from the maternal buccal cavity at each observation time point, photographed under a stereomicroscope, and not returned to the female. Major developmental stages were documented photographically. At 7 dpf, the survival rate was calculated as follows:

Survival rate (%) = (number of viable larvae at 7 dpf/number of eggs included in the survival assessment) × 100.

For the artificial incubation group, the initial egg number was recorded directly. For the natural mouthbrooding group, the initial egg number was estimated by subtracting the number of eggs assigned to artificial incubation from the estimated total clutch size. Embryos or larvae removed for developmental imaging before 7 dpf were excluded from the survival assessment. For deformity assessment, 50 surviving larvae were randomly selected from each incubation group within each biological replicate and were examined under a stereomicroscope. Larvae showing spinal curvature, lordosis, scoliosis, tail malformation, or other evident axial abnormalities were classified as malformed. Deformity rate was calculated as follows:

$$\text{Deformity rate (\%)} = (\text{number of malformed larvae} / 50 \text{ examined larvae}) \times 100.$$

2.3. Collection of Buccal Fluid Samples and Protein Extraction

Fish were fasted for 24 h and anesthetized with 0.05% tricaine methanesulfonate (MS-222) before sampling. Buccal fluid was operationally defined as a mixture of oral secretions and mucus aspirated from the buccal cavity. Samples were collected from mature males (M), pre-mouthbrooding females (PMF), and mouthbrooding females (MBF) using sterile pipette tips. MBF samples were obtained during the early mouthbrooding stage while embryos remained in the buccal cavity. The buccal cavity was not rinsed to avoid dilution or alteration of the native fluid composition. Buccal fluid was gently aspirated without scraping the oral epithelium, and direct contact with the embryos was minimized. Visible contaminants were removed before sample processing. Oral secretions and mucus were not physically separated.

Four buccal fluid samples were analyzed, comprising one mature male sample (M), one pre-mouthbrooding female sample (PMF), and two mouthbrooding female samples (MBF_1 and MBF_2). Because buccal fluid could be obtained only in limited amounts at defined reproductive stages, the proteomic analysis was designed as an exploratory comparison of physiological states. The M and PMF samples served as state-specific reference profiles, whereas two independent MBF samples were included to assess the consistency of the mouthbrooding-associated pattern. Immediately after collection, samples were snap-frozen in liquid nitrogen and stored at -80°C . For protein extraction, samples were thawed on ice, mixed with lysis buffer containing protease inhibitors, and sonicated on ice. After centrifugation at $12,000 \times g$ for 15 min at 4°C , the supernatants were collected, and protein concentrations were determined using a BCA assay according to the manufacturer's instructions. Approximately 50–100 μg of total protein was recovered from each 200 μL buccal fluid sample.

2.4. Protein Digestion by FASP

Approximately 30 μg of total protein from each sample was subjected to digestion using a modified filter-aided sample preparation (FASP) protocol. Samples were loaded onto FASP filtration units, washed to remove interfering substances, and digested overnight with trypsin. The resulting peptides were collected by centrifugation, desalted, dried, and stored before LC–MS/MS analysis.

2.5. Label-Free LC–MS/MS Analysis and Identification of Differentially Abundant Proteins

For each sample, 500 ng of peptides was loaded for label-free liquid chromatography–tandem mass spectrometry (LC–MS/MS). Peptides were separated using an EASY-nLC1200 system (Thermo Fisher Scientific, San Jose, CA USA) equipped with a C18 analytical column and introduced into a high-resolution Orbitrap mass spectrometer through a nano-electrospray ionization source. Raw MS data were processed using MaxQuant software (version 1.3.0.5) and searched against the *Oreochromis niloticus* protein database. Pro-

tein abundance was quantified using label-free quantification based on iBAQ-derived FOT values. For pairwise comparisons, the mean abundance of MBF_1 and MBF_2 was used. Differentially abundant proteins (DAPs) were operationally defined as proteins with $|\log_2\text{FoldChange}| > 1$ to prioritize pronounced abundance differences. Because the M and PMF groups were each represented by a single sample, no inferential statistical testing was performed. Proteins with $\log_2\text{FoldChange} > 1$ or < -1 were considered to show increased or decreased abundance, respectively. The complete lists of DAPs for each pairwise comparison are provided in Tables S1–S3. Because Cluster 1 proteins showed higher abundance in MBF than in M and PMF, Cluster 1 was selected for detailed pathway visualization. These pathways were selected from enriched KEGG pathways related to epithelial barrier maintenance and immune defense, and representative proteins were selected from proteins mapped to these pathways. Their iBAQ-derived FOT abundance values were compared among the M, PMF, and MBF groups. The representative proteins are identified using the symbols of their corresponding genes and are presented in roman type.

2.6. Functional Annotation and Statistical Analysis

Functional categories, enriched biological processes, and pathway-level features of the buccal fluid proteome were systematically characterized. DAP abundance values were standardized as Z-scores and subjected to hierarchical clustering using Cluster 3.0 with Euclidean distance and average linkage. The dendrogram was divided into four clusters according to its major branch structure, representing the predominant abundance patterns across the M, PMF, and MBF groups. Identified proteins were annotated using eggNOG-mapper (version 2.1.12). Genes from Nile tilapia were converted to their homologous zebrafish counterparts. Subsequently, Gene Ontology Biological Process (GO-BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted in R (version 4.3.1) using the clusterProfiler package (version 4.10.1) with zebrafish annotation databases. All proteins among the 6603 identified proteins that were successfully annotated and mapped to zebrafish orthologs were used as the enrichment background. Enrichment significance was determined with Fisher's exact test, using $p < 0.05$ as the cutoff. Embryonic performance experiments included six paired biological replicates, and all quantitative results are shown as mean $\pm$ standard deviation (SD). GraphPad Prism (version 10.0) was used for statistical testing and data visualization. Differences between artificial incubation and natural mouthbrooding treatments were examined using a two-tailed paired Student's *t*-test, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Early Embryonic Development and Reproductive Characteristics of Nile Tilapia

To compare developmental progression between incubation modes, early embryos of Nile tilapia (*O. niloticus*) were followed from fertilization until 7 dpf under artificial incubation or maternal mouthbrooding (Figure 1A,B). Embryos successfully progressed through cleavage, blastula, gastrula, and organogenesis during this period. Fertilized eggs were oval-shaped and contained a large yolk reserve that supported embryonic growth. During 0–2 dpf, embryos underwent rapid cleavage and gastrulation, accompanied by epiboly and embryonic shield formation. Neural plate formation and somite differentiation became apparent during late gastrulation. By 4 dpf, cardiac contractions and blood circulation were visible. Subsequently, embryos hatched through coordinated mechanical activity and chorion degradation. Between 4 and 7 dpf, complete eye pigmentation was established, melanophores became distributed along the dorsal surface and yolk sac margin, and progressive yolk absorption coincided with increased swimming activity and the initiation of exogenous feeding.

There were distinct differences in the incubation environment between the two reproductive modes (Figure 1C). Under artificial incubation, fertilized eggs were continuously exposed to water flow and environmental fluctuations. In contrast, mouthbrooding females retained fertilized eggs within the buccal cavity, forming a relatively enclosed incubation chamber. Noticeable expansion of the oral cavity and lower jaw region was observed during mouthbrooding, providing a protected environment for embryonic development. Continuous respiratory movements maintained water circulation and oxygen exchange throughout the incubation period.

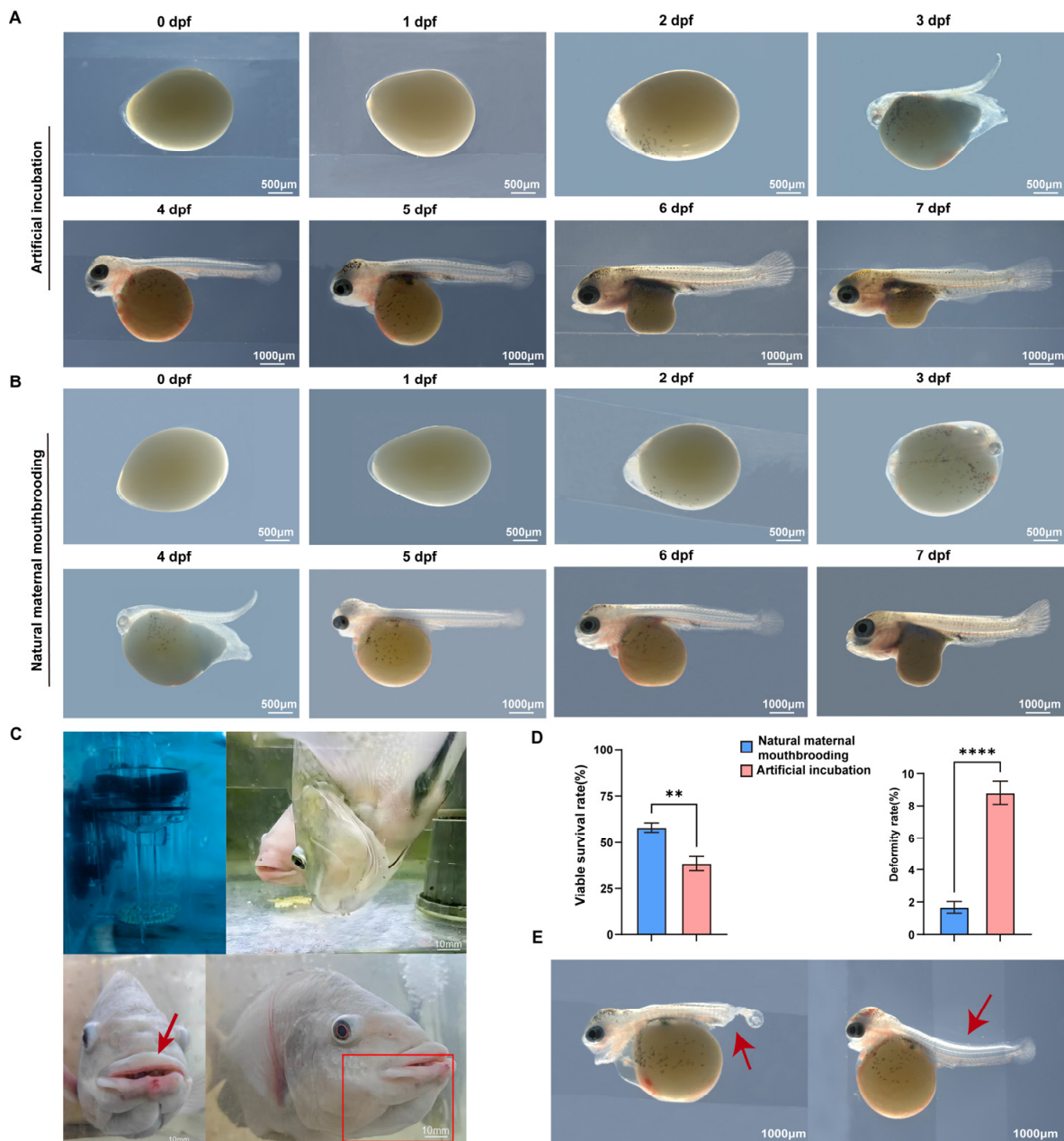


Figure 1. Embryonic development and reproductive performance under artificial incubation and natural maternal mouthbrooding in Nile tilapia. (A,B) Embryonic development from 0 to 7 days post-fertilization (dpf) under artificial incubation (A) and natural maternal mouthbrooding (B). (C) Representative images of artificial incubation and mouthbrooding females. Red arrows and boxes indicate the enlarged buccal cavity and lower jaw region. (D) Survival rate and deformity rate of embryos/larvae under the two incubation modes. (E) Representative live malformed larvae from the artificial incubation group under a stereomicroscope. Red arrows indicate tail malformation and spinal curvature. Data are presented as mean $\pm$ SD; ** $p < 0.01$, **** $p < 0.0001$.

3.2. Natural Mouthbrooding Improves Embryo Survival and Reduces Spinal Malformations

Incubation mode significantly influenced offspring survival and developmental quality (Figure 1D,E). Embryos incubated under natural mouthbrooding conditions exhibited significantly higher survival rates than those subjected to artificial incubation. Marked differences in spinal morphology were also observed between the two groups. Artificially incubated larvae displayed a substantially higher incidence of morphological abnormalities, including asymmetric tail curvature, spinal bending, and axial deformities. In contrast, larvae produced through natural mouthbrooding exhibited normal body morphology and a markedly lower frequency of developmental abnormalities. Together, these observations revealed substantially higher survival and lower skeletal malformation rates in naturally mouthbrooded embryos than in artificially incubated embryos.

3.3. Proteomic Profiling and Quality Assessment of Buccal Fluid Proteins During Mouthbrooding

To characterize the buccal fluid microenvironment associated with mouthbrooding, salivary proteins collected from males (M), pre-mouthbrooding females (PMF), and mouthbrooding females (MBF) were analyzed using label-free LC-MS/MS-based proteomics. A total of 414,101 spectra were generated, of which 105,804 matched spectra were successfully assigned. The analysis identified 43,167 peptides, including 35,520 unique peptides, resulting in the identification of 6603 proteins (Figure 2A). Protein sequence coverage analysis demonstrated satisfactory proteome depth and data quality. Proteins with sequence coverage greater than 10% accounted for 61.7% of all identified proteins, whereas proteins with coverage above 40% represented 11.4% of the dataset. Proteins exhibiting sequence coverage below 10% accounted for 38.3% of the identified proteins (Figure 2B). These results demonstrated broad proteome coverage and provided a basis for subsequent fold-change-based comparisons among reproductive states.

3.4. Identification of Differentially Abundant Proteins Among Reproductive Groups

Comparative analysis revealed differences in the number of detected buccal fluid proteins among mature males, pre-mouthbrooding females, and mouthbrooding females (Figure 2C). Using the criterion of $|\log_2\text{FoldChange}| > 1$, differential abundance analysis identified 3001 differentially abundant proteins (DAPs) in the comparison between mouthbrooding females and pre-mouthbrooding females (MBF vs. PMF), including 576 upregulated and 2425 downregulated proteins (Figure 2D; Table S3). In the comparison of MBF vs. M, 2569 DAPs were identified, including 969 upregulated and 1600 downregulated proteins (Figure 2D; Table S1). In the comparison of PMF vs. M, 2661 DAPs were identified, including 1866 upregulated and 795 downregulated proteins (Figure 2D; Table S2). The number and distribution of DAPs varied among comparison groups, indicating extensive changes in buccal fluid protein composition associated with sex, reproductive status, and mouthbrooding behavior.

3.5. Hierarchical Clustering Reveals Distinct Expression Patterns of Differentially Abundant Proteins

To investigate global expression patterns, hierarchical clustering analysis was performed using DAPs included in the clustering analysis (Figure 2E). The mean abundance profile of MBF_1 and MBF_2 was used to represent the MBF state in the clustering analysis, supporting the consistency of the mouthbrooding-associated pattern. Based on their relative abundance across the three reproductive states, the DAPs were classified into four major clusters (Clusters 1–4). Cluster 1 contained proteins exhibiting elevated abundance specifically in mouthbrooding females, whereas their abundance remained relatively low in males and pre-mouthbrooding females. In contrast, proteins assigned to Clusters 2–4 displayed reduced abundance during mouthbrooding or showed higher abundance in

non-mouthbrooding individuals. These results revealed distinct state-associated protein abundance patterns.

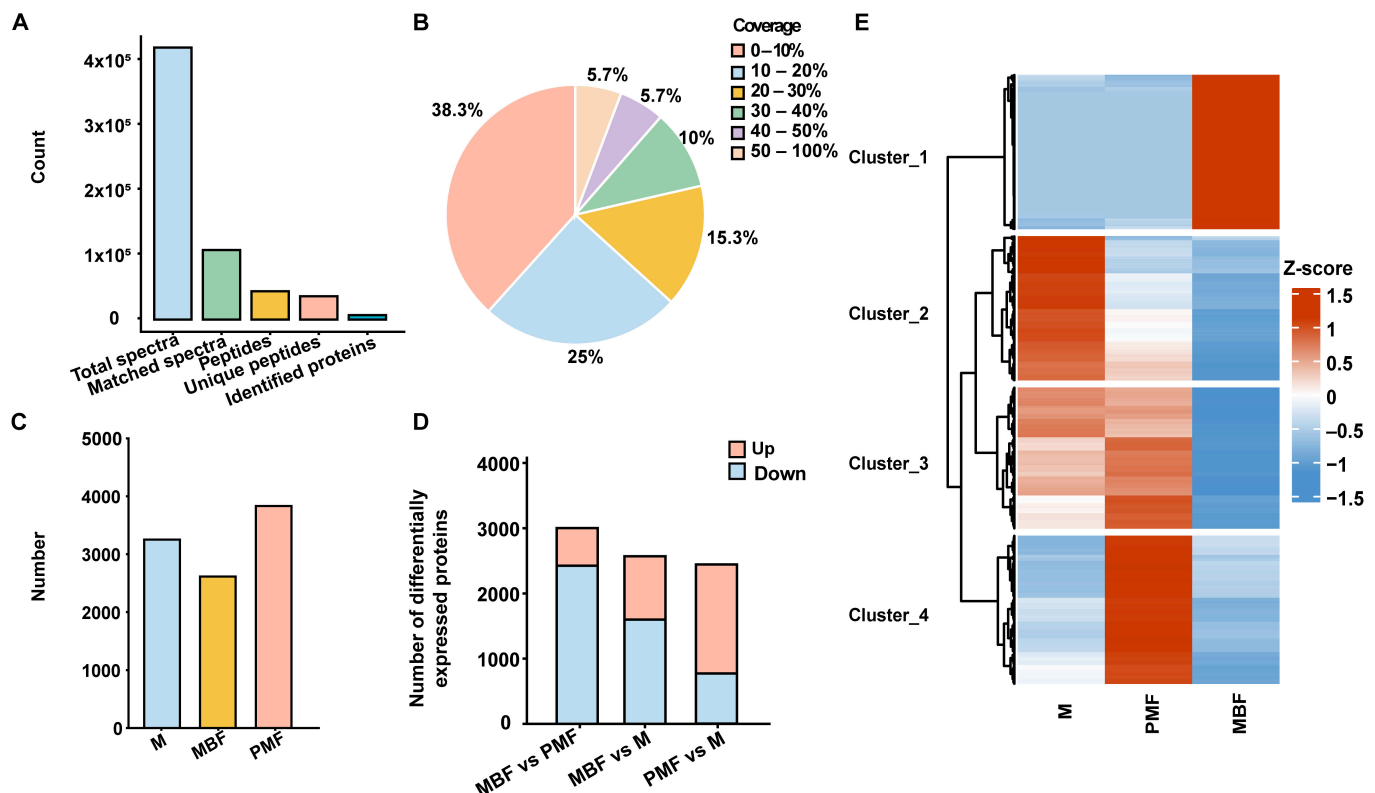


Figure 2. Overview of label-free buccal fluid proteomic analysis during mouthbrooding. (A) Summary of protein identification results. (B) Distribution of protein sequence coverage. (C) Number of identified proteins in males (M), pre-mouthbrooding females (PMF), and mouthbrooding females (MBF). (D) Numbers of differentially abundant proteins (DAPs) in pairwise comparisons. DAPs were defined as $|\log_2\text{FoldChange}| > 1$. (E) Hierarchical clustering of DAPs. Red and blue indicate relatively high and low abundance, respectively.

3.6. GO Biological Process Enrichment Analysis Reveals Functional Divergence Among Protein Clusters

Cluster 1 showed a clear enrichment of proteins involved in immune- and defense-associated biological functions. To characterize the biological functions represented by each abundance cluster, Gene Ontology Biological Process (GO-BP) enrichment analysis was performed. Representative enriched GO-BP terms included regulation of cytokine production, positive regulation of autophagy, and Wnt signaling-related processes (Figure 3). In contrast, Clusters 2 and 3 were enriched in pathways associated with nucleic acid catabolism, coenzyme biosynthesis, mRNA processing, and nucleocytoplasmic transport. Cluster 4 proteins were mainly enriched in biological processes related to Golgi vesicle transport, endomembrane system organization, and cytoplasmic translation. The four protein clusters therefore exhibited distinct functional enrichment profiles, suggesting substantial functional specialization within the buccal fluid proteome during mouthbrooding.

3.7. KEGG Pathway Enrichment Analysis of Differentially Abundant Protein Clusters

To further characterize the biological pathways represented by each cluster, KEGG pathway enrichment analysis was conducted (Figure 4). Proteins in Cluster 1 were significantly enriched in pathways associated with host defense and immune regulation, including the Wnt signaling pathway, focal adhesion, Salmonella infection, glycine, serine and threonine metabolism, and tryptophan metabolism. Cluster 2 proteins were primar-

ily enriched in metabolic pathways, including cofactor biosynthesis, purine metabolism, and nucleotide metabolism. Clusters 3 and 4 were enriched in pathways associated with intracellular transport and macromolecule turnover, including endocytosis, autophagy, lysosomal function, and RNA degradation. Collectively, these enrichment profiles revealed functional partitioning of buccal fluid proteins into pathways related to immunity, metabolism, intracellular trafficking, and cellular homeostasis.

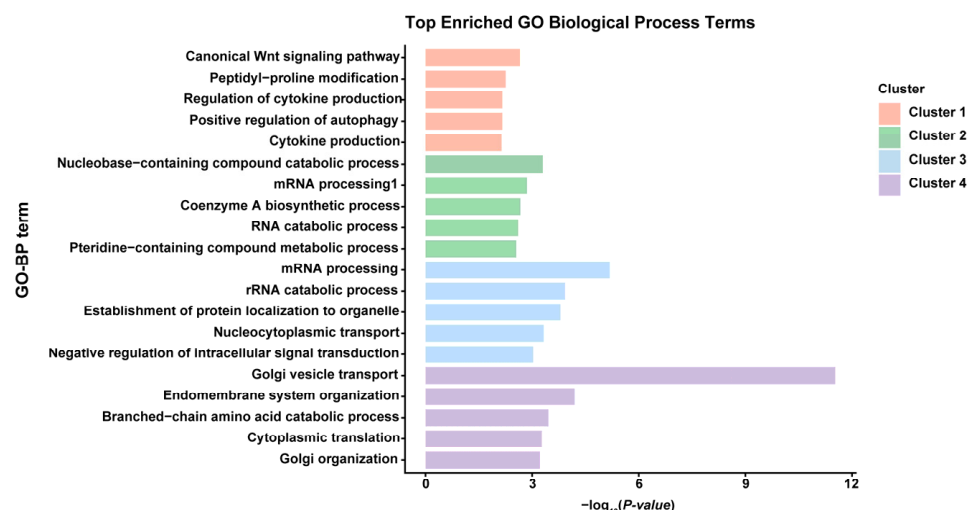


Figure 3. Gene Ontology Biological Process (GO-BP) enrichment analysis of differentially abundant protein (DAP) clusters. The x-axis represents enrichment significance [$-\log_{10}(p\text{-value})$], and the y-axis shows enriched GO-BP terms. Colors indicate different clusters.

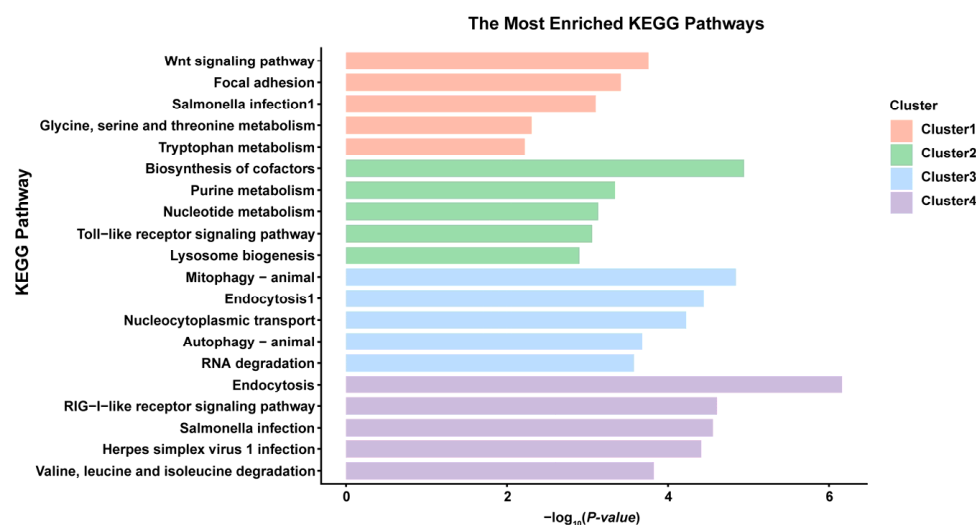


Figure 4. KEGG pathway enrichment analysis of DAP clusters. DAPs were classified into four abundance clusters and subjected to KEGG enrichment analysis. The x-axis represents enrichment significance [$-\log_{10}(p\text{-value})$], and the y-axis shows enriched KEGG pathways. Colors indicate different clusters.

3.8. Mouthbrooding-Associated Proteins in Epithelial Barrier and Immune Defense Pathways Show Increased Abundance

Because Cluster 1 proteins showed higher abundance in MBF than in M and PMF, Cluster 1 was selected for detailed pathway analysis. Four enriched pathways related to epithelial barrier maintenance and immune defense—Cadherin signaling, Wnt signaling, focal adhesion, and Salmonella infection—were selected for visualization in Figure 5. Representative proteins, corresponding to Fzd10, Krt98, Fat3a, Arf2a, and Arpc4, showed higher abundance in mouthbrooding females than in males and pre-mouthbrooding females

(Figure 5A,C). Proteins associated with immune regulation and host defense, including Taok3a, Ikbkg, and Gapdh, also displayed increased abundance in mouthbrooding females (Figure 5B,D). These abundance patterns suggest that mouthbrooding is associated with changes in proteins related to epithelial barrier maintenance and immune defense within the maternal buccal microenvironment.

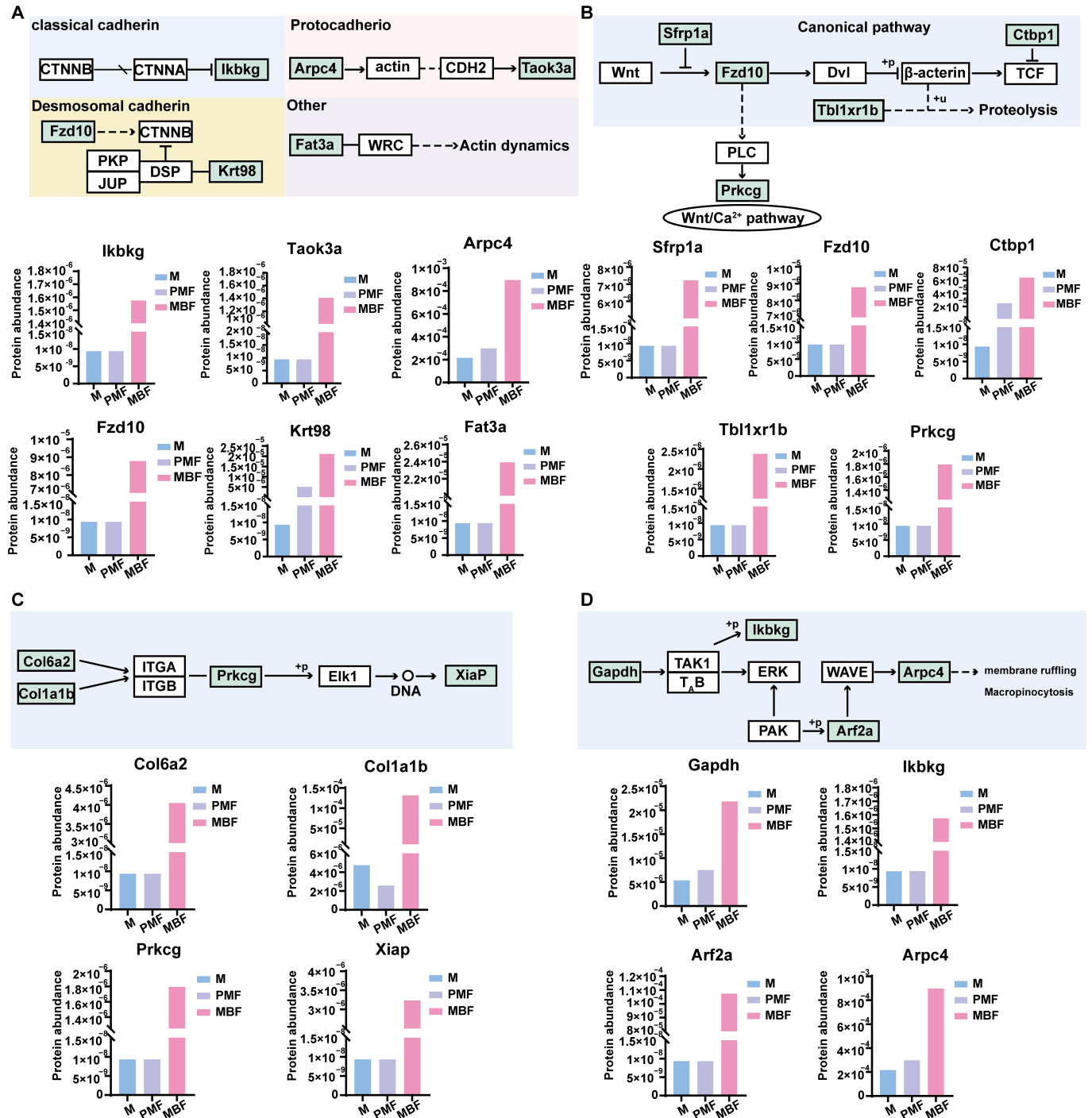


Figure 5. Selected KEGG pathways and abundance profiles of representative proteins associated with mouthbrooding. (A) Cadherin signaling pathway. (B) Wnt signaling pathway. (C) Focal adhesion pathway. (D) Salmonella infection pathway. Upper panels show KEGG pathway maps, and lower panels show the relative protein abundance among M, PMF, and MBF groups. Protein labels are shown in roman type using the symbols of their corresponding genes. Solid arrows indicate explicitly represented directional regulatory relationships, dashed arrows indicate indirect effects for which intermediate molecular details are not shown, and blunt-ended lines indicate inhibition.

4. Discussion

Mouthbrooding is a highly specialized reproductive strategy that has evolved in numerous cichlid species and is considered one of the key factors contributing to offspring survival in variable aquatic environments [7,13]. Although the behavioral and ecological significance of mouthbrooding has been widely recognized, the molecular characteristics of the maternal buccal environment remain poorly understood [15]. In the present study, we compared embryonic performance under natural mouthbrooding and artificial incubation conditions and characterized the buccal fluid proteome associated with mouthbrooding in Nile tilapia. Our results demonstrated that natural mouthbrooding significantly improved embryo survival and reduced morphological abnormalities. Proteomic analyses further revealed extensive remodeling of buccal fluid protein composition during mouthbrooding, with pronounced enrichment of proteins associated with epithelial maintenance, immune defense, and host–pathogen interaction pathways.

Natural mouthbrooding provided clear developmental advantages for Nile tilapia embryos [9]. Compared with artificial incubation, naturally mouthbrooded embryos exhibited substantially higher survival rates and lower incidences of morphological abnormalities. Similar observations have been reported in several cichlid species, where parental care behaviors increase offspring fitness by protecting embryos from predation, environmental fluctuations, and microbial infection [14]. The buccal cavity of mouthbrooding females creates a relatively stable developmental environment characterized by continuous water circulation, oxygen exchange, and physical protection [5]. However, the superior developmental performance observed under natural mouthbrooding suggests that factors beyond physical protection may contribute to embryonic success.

One of the most notable findings of this study was the enrichment of pathways related to epithelial integrity and immune defense [23]. Consistent with these enriched pathways, representative proteins involved in cell adhesion and cytoskeletal organization, including those corresponding to Fzd10, Krt98, Fat3a, Arf2a, and Arpc4, showed increased abundance in mouthbrooding females. Cadherin signaling and focal adhesion pathways were selected for detailed examination because of their potential relevance to epithelial organization and barrier integrity. These pathways play essential roles in maintaining epithelial structure, regulating cell–cell interactions, and supporting tissue integrity [22]. During mouthbrooding, female tilapia continuously retain developing embryos within the buccal cavity for an extended period, exposing oral tissues to persistent mechanical stimulation. Fish mucosal barriers are highly responsive to environmental and husbandry-related conditions, and their integrity is closely related to local immune protection and epithelial homeostasis [29]. The increased abundance of epithelial organization-related proteins may therefore reflect adaptive remodeling of the oral mucosa to maintain barrier integrity during prolonged embryo incubation [26].

In addition to epithelial-related pathways, several immune-associated pathways were significantly enriched during mouthbrooding [30]. Wnt signaling, Toll-like receptor signaling, and pathways annotated as Salmonella infection exhibited strong enrichment among mouthbrooding-associated proteins [31,32]. Moreover, proteins corresponding to Taok3a and Ikbkg, which are involved in inflammatory signaling and immune regulation, displayed increased abundance in mouthbrooding females. Fish eggs and embryos are highly susceptible to microbial colonization because of their nutrient-rich surfaces and limited immune competence during early development [33,34]. Consequently, the maternal buccal cavity represents a potential interface between developing embryos and environmental microorganisms. Interactions between mucosal microbiota and host immune responses are increasingly recognized as important determinants of fish mucosal homeostasis under changing environments [35]. The enrichment of immune-related pathways, together with

the increased abundance of immune-associated proteins, suggests that mouthbrooding females may enhance local immune capacity within the oral cavity, thereby contributing to pathogen control during embryo incubation [36,37].

Based on these observations, we propose that the buccal cavity of mouthbrooding females forms a biologically active microenvironment that may support embryonic development through epithelial barrier maintenance and immune defense [38]. Unlike artificial incubation systems, where embryos are directly exposed to the surrounding aquatic environment, mouthbrooded embryos develop within a biologically active maternal niche containing proteins potentially associated with protective functions. This concept expands the traditional view of mouthbrooding as a purely behavioral adaptation and highlights the potential contribution of maternal molecular factors to offspring development [13]. In this context, buccal fluid proteins may represent an important but previously underappreciated component of parental care in mouthbrooding fishes [24,38].

The present findings may also have practical implications for tilapia aquaculture [3]. Artificial incubation is widely adopted in commercial hatcheries because it shortens the reproductive cycle and increases fry production efficiency. However, our results showed that artificial incubation was associated with lower survival and increased skeletal malformations compared with natural mouthbrooding. These observations raise the possibility that some beneficial factors present in the mouthbrooding environment are absent under artificial conditions. Identification of key buccal fluid proteins potentially associated with embryo protection may therefore provide opportunities to improve hatchery performance through the development of biomimetic incubation strategies [39,40]. Supplementation of artificial incubation systems with selected protective molecules, or the manipulation of microbial and biochemical conditions to better mimic the maternal buccal environment, may represent promising directions for future research.

Several limitations of this study should also be acknowledged. Although proteomic analyses identified numerous candidate proteins associated with mouthbrooding, the specific functions of these proteins in embryo protection remain to be experimentally validated. The proteomic analysis was designed as an exploratory comparison of defined reproductive states because only limited amounts of buccal fluid could be collected at specific mouthbrooding stages. The two MBF samples were averaged for the exploratory comparison, whereas the single M and PMF samples did not allow within-group biological variation to be assessed. Therefore, the identified DAPs should be regarded as exploratory candidates requiring validation in larger independent cohorts. Furthermore, the present study focused primarily on protein composition and did not investigate other potentially important components of the buccal microenvironment, including metabolites, microbial communities, and extracellular vesicles. Future studies integrating proteomics, metabolomics, microbiome analyses, and functional validation experiments will provide a more comprehensive understanding of the potential embryo-protective mechanisms associated with mouthbrooding [41].

5. Conclusions

The present study demonstrates that natural mouthbrooding significantly improves embryonic survival and reduces spinal deformities in Nile tilapia compared with artificial incubation. Proteomic analyses revealed extensive remodeling of the buccal fluid proteome during mouthbrooding and identified numerous proteins associated with epithelial organization, immune regulation, and host defense. Functional enrichment analyses consistently indicated that mouthbrooding-associated proteins were enriched in pathways related to epithelial barrier maintenance and immune protection. These findings support the concept that mouthbrooding females establish a biologically active buccal microenvironment that

contributes to successful embryonic development. Our results provide novel insights into the molecular mechanisms underlying parental care in mouthbrooding fishes and identify potential targets for the development of biomimetic incubation strategies aimed at improving larval production and survival in tilapia aquaculture.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fishes11080440/s1>, Table S1: Total proteins in MBF vs. M; Table S2: Total proteins in PMF vs. M; Table S3: Total proteins in MBF vs. PMF.

Author Contributions: Conceptualization, R.H. and J.L.; methodology, Y.Z., X.L. and S.H.; validation, Y.Z., X.L. and S.H.; formal analysis, Y.Z., X.L. and S.H.; investigation, Y.Z., X.L. and Y.L.; resources, J.L. and R.H.; data curation, J.L., Y.Z. and X.L.; writing—original draft preparation, Y.Z. and X.L.; writing—review and editing, R.H., J.L., Y.Z. and X.L.; visualization, X.L. and Y.Z.; supervision, R.H. and J.L.; project administration, R.H. and J.L.; funding acquisition, Y.Z., S.H. and J.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Ocean University (Approval Code: SHOU-DW-2025-014; Approval Date: 2 March 2025).

Informed Consent Statement: Not applicable.

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