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De novo transcriptomic discovery and molecular characterisation of a caspase kinase gene in the elasmobranch, *Chiloscyllium griseum*

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Original Article

Abstract

Caspase kinases are the fundamental players of the apoptotic and immune signalling pathways in vertebrates. However, the molecular characterisation of this gene remains poorly delineated in elasmobranchs. The present study displays the first transcriptome-based approach in elucidating the molecular profile of the caspase kinase gene in *Chiloscyllium griseum*, a benthic shark inhabiting coral reefs and estuaries. The protocol follows the extraction of total RNA from immune-rich tissues such as the heart and liver with the aid of high-throughput sequencing technology. Functional annotation revealed a candidate caspase kinase transcript with an embedded serine/threonine kinase and caspase recruitment domains. The ORF prediction using TransDecoder encoded a 299 bp length amino acid that showed high homology with the caspase kinase from the vertebrate counterparts, suggesting evolutionary conservation. Gene expression profiling showed a significant level in the heart tissue rather than the liver ($P<0.001$), indicating its role in Cardiovascular apoptotic regulation. The dataset generated out of this investigation provides foundational molecular insights into apoptotic gene regulation in elasmobranchs, opening up new avenues in studies related to immune-apoptotic pathways in basal vertebrates.

Keywords: *Chiloscyllium griseum, caspase kinase, transcriptome, apoptosis, immune genes, elasmobranch*

Introduction

Apoptosis or programmed cell death is a highly orchestrated cellular process crucial for the development, immune-surveillance and stress adaptation in higher organisms (Kumar and Vaux, 2022). The key players of apoptosis are caspase-cysteine proteases. Phosphorylated caspase kinases have an additional role in innate immunity and cellular-stress signalling (Chen *et al.*, 2021; Zheng *et al.,* 2023). Caspase kinase belongs to a distinct subclass of protein kinases that plays a vital role in phosphorylating caspases, thereby fine-tuning their activity in apoptotic and inflammatory contexts. This group of kinases decide the timing, specificity, and magnitude of caspase-mediated signalling, thereby influencing cellular homeostasis, immune modulation, and responses to stress stimuli (Chen *et al.*, 2021; Zheng *et al.,* 2023). Recent advances in the caspase kinases studies often dealt with the regulation of mitochondrial outer membrane permeabilisation and inflammasome activation, during oxidative as well as Pathogen-induced stress (Tummers and Green, 2017; Lee *et al.*, 2022). The impairment of caspase kinase signalling has been shown to worsen pathological apoptosis, underscoring its therapeutic relevance, especially in the cardiovascular and neurodegenerative models (Wang and Wu, 2020; Nguyen *et al.*, 2023). Apart from the apoptotic regulatory role, caspase kinases are linked to other signalling cascades such as MAPK, NF-κB and JNK pathways, expanding their biological relevance in vertebrate physiology (Choi *et al.*, 2019; Gomez-Garcia *et al.*, 2021). Despite their established importance in mammals and teleosts, the molecular and functional identity of caspase kinases remains poorly characterised in cartilaginous fishes. Elasmobranchs, such as the grey bamboo shark (*Chiloscyllium griseum*), occupy a pivotal

phylogenetic position as early-diverging jawed vertebrates. Their slow-evolving genomes and distinct immunological features make them excellent candidates in the investigation of ancestral vertebrate immunity (Venkatesh *et al.*, 2021; Dooley and Flajnik, 2022).

The grey bamboo shark (*Chiloscyllium griseum*) is distinguished by several unique biological and ecological traits. Being benthic and nocturnal, it has an incredible way to cope with turbid as well as low-oxygen coastal waters (Ahmed *et al.*, 2021). A highly sophisticated sensory, olfactory system and dermal denticles contribute to its cryptic behaviour and immune-resilience. *Chiloscyllium griseum* is also equipped with a robust immune transcriptomic landscape containing myriads of Pathogen Recognition Receptors (PRRs) (Rao and Thomas, 2022; Prakash *et al.*, 2023). Sexual dimorphism and reproductive plasticity further accentuate the significance of this evolutionary distinct species (Nair *et al.*, 2023).

De novo transcriptomic approaches facilitate comprehensive gene discovery and functional annotation even in species lacking a reference genome (Conesa *et al.*, 2016; Love *et al.*, 2022). Earlier transcriptomic investigations in sharks have identified key immune and stress-related genes (Sundaravel *et al.*, 2024), yet caspase-related kinases remain obscure in these datasets.

In this study, we leverage high-throughput RNA-sequencing and integrative bioinformatics to unravel the molecular characteristics of the caspase kinase gene in *C. griseum*. Through structural domain analysis, expression profiling, and evolutionary comparison, we provide the first insights into the role of caspase kinase in this cartilaginous species. Our findings contribute to the growing understanding of apoptotic regulation in basal vertebrates and offer a new molecular perspective on immune evolution.

Material and methods

Sample collection and RNA extraction

Healthy live Specimens of *Chiloscyllium griseum* (Fig. 1) were obtained from the Neendakara Fishery Harbour, Kollam, Kerala (8°56'18.32"N and 76°32'33.78" E). Species identity was confirmed by both morphological characters and molecular analyses comprising including DNA barcoding. The shark sample used in the present study was carefully handled following the guidelines for the care and use of fish in research (De Tolla *et al.*, 1995). The protocols for animal experimentation were set up in compliance with the standards approved by the Institutional Animal Ethical Committee of the ICAR Central Marine Fisheries Research Institute (CMFRI), Kochi (MBT/GNM/25). These methods were also tested abiding by ARRIVE guidelines (http://arriveguidelines.org). Heart and liver tissues were excised immediately post-mortem, flash frozen in liquid nitrogen, and stored at –80 °C until further processing. Total RNA was extracted using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and purified by the nucleospin RNA clean-up kit. Isolated RNA was quantified using Nanodrop 2000 (Thermofisher Scientific, Massachusetts, USA). RNA integrity number (RIN) was assessed using an Agilent 2100 Bioanalyzer. Only samples with RIN >7 were selected for library preparation.

High-throughput RNA sequencing and de novo assembly workflow

cDNA libraries were prepared from those samples with RIN>7 (Harshan *et al.*, 2024) using TruSeq stranded mRNA library preparation kit following the manufacturer's protocol and subjected to sequencing on the Illumina HiSeq X10 platform (paired-end, 150 bp). Raw reads generated were scrutinised for quality check using FASTQC and trimmed out the adaptor sequences using Trimmomatic v 0.39 (Bolger *et al.*, 2014). De novo transcriptome assembly was carried out with the aid of Trinity v.2.8.5 (Grabherr *et al.*, 2011; Haas *et al.*, 2013) using preset parameters and redundant isoforms were reduced using CD-HIT-EST (Li and Godzik, 2006; Harshan *et al.*, 2024).

Transcript annotation and candidate gene characterization

The transcripts obtained after the de novo assembly (Harshan *et al.*, 2024) were functionally annotated using Trinotate (Bryant *et al.,* 2017). TransDecoder (Haas *et al.*, 2013) was used to extract the Open Reading Frames (ORFs). Both the BLASTx and BLASTp (Altschul *et al.,* 1990) were employed for inferring protein function, keeping the e-value threshold as 1e-5, ensuring high confidence matches to known

Fig. 1. Photograph of the grey bamboo shark (*C. griseum*) collected from Neendakara fishery harbour, Kerala

proteins. An investigation on the Pfam domains was done using HMMER, followed by protein functional classification using Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and orthologous gene clustering supported by eggNOG annotations (Cantalapiedra *et al.,* 2021). InterProScan (Jones *et al.,* 2014) further confirmed the presence of conserved domains.

In silico characterisation of functional domains

The presence of conserved domains, motifs and family signatures within the caspase kinase gene was predicted using the computational tool- InterProScan. This included an in-depth investigation of the caspase recruitment domains, serine/threonine kinase domains and MAP kinase-related features. Based on the domain confidence scores, hits were filtered and cross-checked with the known apoptosis and immunity-associated kinases. Results were further used to compare the structural and functional similarities with vertebrate homologs.

Orthology-based functional assignment and pathway analysis

The bioinformatics tool eggNOG-mapper was used to assign orthologous groups and retrieve GO terms and KEGG pathway associations related to the caspase-kinase gene, shedding light on its biological and evolutionary dynamics. Seed orthologs were derived using DIAMOND-based alignment against the eggNOG database. Gene Ontology terms corresponding to biological process, molecular function, and cellular component were derived, and the hits generated out of KEGG (Kanehisa *et al.,* 2021) pathway analyses were summarised to interpret potential roles in apoptosis, inflammation, and signalling cascades.

Quantitative transcriptome analysis of candidate gene

Computation tool- 'Salmon' (Patro *et al.,* 2017) was used to check for transcriptome abundance with the aid of quasi-mapping. An experimental set-up with 12 sharks was divided into a 'control' group and 'thermal challenge/ heat-stressed' group, with each group comprising triplicates with 2 sharks in each replicate. The sharks were maintained at the marine hatchery in the Regional Station of ICAR-Central Marine Fisheries Research Institute, Calicut, Kerala, in a 1000 l concrete tank for a light-dark cycle of 12h:12h for a month before the treatment was given. The water quality parameters of the control group were dissolved oxygen (DO) 5 mg/l, pH 7.5, salinity 34 ppt and temperature = 28^{0}C. The set-up in the treatment group remained the same as the control group and differed only in terms of temperature, which was set as 32^{0}C, and the volume of water was reduced to 500 L. A submerged heater with thermostat and nearly four aeration tubes were supplied to the treatment tank. A gradient increase in the temperature of the treatment group with 0.8^{0}C was maintained for six days to create a 6^{0}C temperature gradient, thereby elevating the final temperature to 4^{0}C at the end of 30 day schedule to attain 34^{0}C, which was our desired temperature (stress). When the experiment was completed, three individuals from each triplicate were randomly sacrificed for the collection of desired tissues, *viz.,* liver and heart. The extracted tissues were immediately flash frozen in liquid nitrogen and stored at -80^{0}C until RNA isolation. Individual DGE libraries were generated using Illumina sequencing (150 bp), and differential expression between heart and liver tissues under both the 'control' as well as 'stressed' conditions was analysed with DESeq2. TPM (transcripts per million) values were calculated after the normalisation of expression values, and caspase kinase transcript expression levels were statistically compared using Student's t-test, keeping the $p < 0.001$ (significance level). Raw read counts generated by Salmon were imported into DESeq2 with appropriate experimental factors defined for tissue type (heart vs liver) and condition (control vs stress). Comparisons were made between tissues under the same conditions and across conditions within the same tissue. A transcript corresponding to the putative caspase kinase gene was examined for expression differences. Biological replicates confirmed consistent expression trends. The bar plots below illustrate normalised relative expression (TPM) in heart (Fig. 2) and liver tissues (Fig. 3).

Phylogenetic analysis

The evolutionary position of the caspase kinase gene was interpreted using phylogenetic analysis employing full-length amino acid sequences. The homologous amino

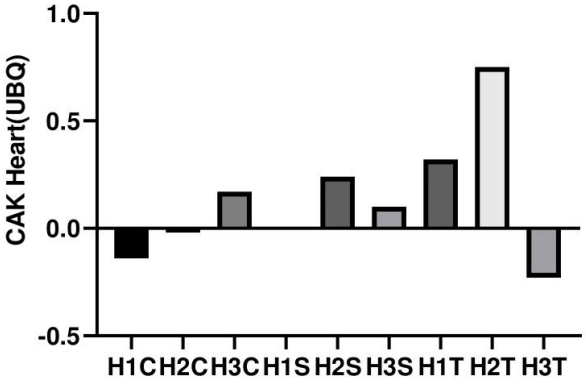

Fig. 2. Relative transcript expression (TPM) of caspase kinase gene in heart tissues under control and stress conditions

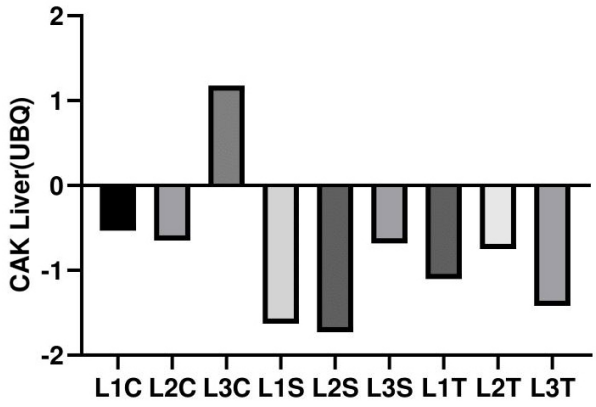

Fig. 3. Relative transcript expression (TPM) of caspase kinase gene in liver tissues under control and stress conditions

acid sequences from various vertebrate taxa were retrieved from NCBI (Sayers *et al.,* 2022) and UniProt (The UniProt Consortium, 2023) databases, such as early-diverging cartilaginous fishes, bony fishes, amphibians, reptiles, birds, and mammals. Multiple sequence alignment (MSA) was performed using 'MUSCLE' (Edgar, 2004) and alignment

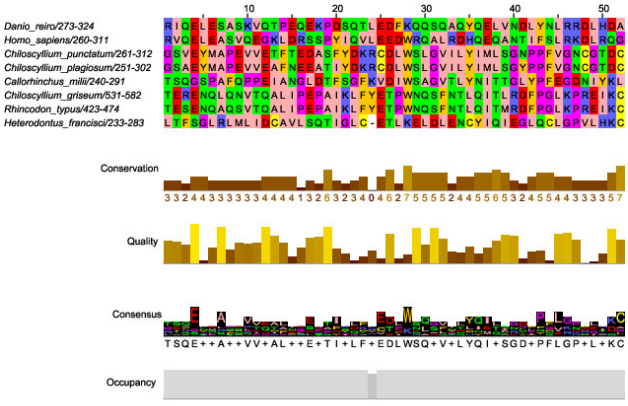

Fig. 4. Multiple sequence alignment of caspase kinase homologs, highlighting conserved motifs and domains, visualised using Jalview

quality was visually assessed using 'Jalview' (Waterhouse *et al.,* 2009) to identify conserved regions and domain arrangements (Fig. 4). Regions with excessive gaps were trimmed to enhance phylogenetic signal.

A maximum likelihood (ML) phylogenetic tree was constructed using iTOL (Letunic and Bork, 2021) as illustrated in Fig. 5. The resulting topology was interpreted to trace evolutionary relationships, with special emphasis on the clustering of *C. griseum* caspase kinase relative to homologs from other elasmobranchs and early vertebrates.

Results and discussion

Functional characterisation of caspase kinase signalling

Functional annotation of the candidate caspase kinase transcript in *Chiloscyllium griseum* revealed strong associations with several key immune and apoptotic signalling pathways, consistent with findings in early vertebrate studies (Shen *et al*., 2021; Duan *et al*., 2022; Zheng *et al*., 2023). KEGG and GO enrichment analyses indicated its involvement in the Apoptosis (ko04210) and MAPK signalling (ko04010) pathways, essential for regulating programmed cell death and cellular responses to stress. Additionally, the transcript was linked to the NF Kappa-B signalling pathway (ko04064), T cell receptor signalling (ko04660), Calcium signalling (ko04020) and cytokine-cytokine receptor interaction (ko04060). These functional associations emphasise the multifaceted regulatory role of caspase kinase in coordinating immune responses and maintaining cellular homeostasis, as similarly demonstrated in zebrafish and other model systems (Lee *et al*., 2022; Wang *et al*., 2023). Importantly, the functional annotation also revealed potential roles in pathogen recognition, inflammation modulation, and intracellular signalling, which are crucial to innate immunity. Their conservation in *C. griseum* underscores the evolutionary importance of these

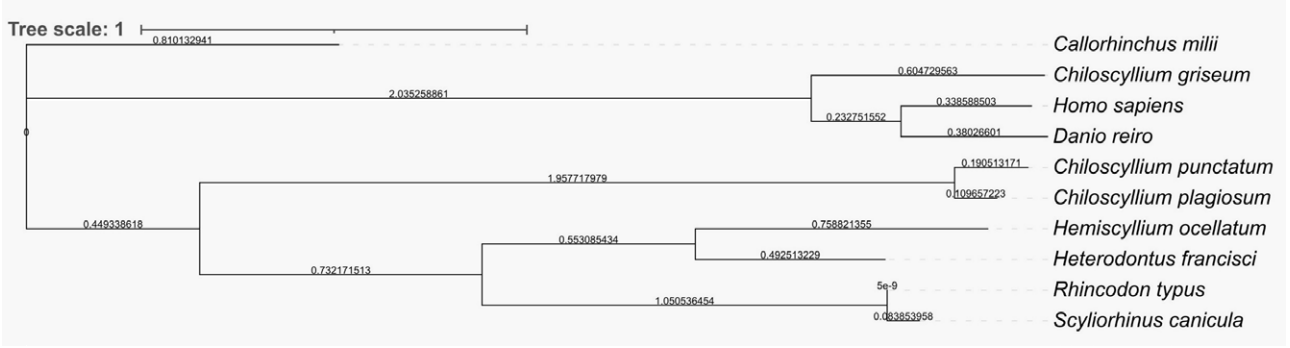

Fig. 5. Phylogenetic tree of caspase kinase genes constructed using the maximum-likelihood method in iTOL, illustrating evolutionary relationships among vertebrate taxa

signalling mechanisms across vertebrate lineages, suggesting that such multifunctionality in caspase kinases predates the divergence of cartilaginous and bony fishes.

Signature domains and evolutionary features

The transcriptome-wide analysis of *C. griseum* revealed several caspase kinase-like transcripts featuring conserved domains characteristic of apoptosis and immune signalling regulation, as shown in Table 1. InterProScan annotation identified key domains such as CARD, STK, and MAPK-like regions in candidate transcripts. The presence of these motifs implies that the encoded protein may function at the intersection of signal transduction and apoptotic machinery. Notably, the CARD domain is associated with recruitment and interaction with other caspases and signalling proteins, while the serine/threonine kinase domain is indicative of phosphorylation-based regulatory control. These structural features suggest dual functionality-acting as both scaffold and catalytic protein in immune stress signalling. These transcripts show conserved features that align with caspase kinases seen in vertebrates, reflecting shared evolutionary constraints observed in basal vertebrates (Zhao *et al*., 2024).

Quantitative expression dynamics

Expression profiling supported by fold change data from qPCR (Table 2) demonstrated a variable expression pattern of the caspase kinase transcript in heart and liver tissues. Specifically, the transcript showed moderate expression under control conditions (28^{0}C) in the heart (2.56 ± 0.00 TPM) and was undetectable under thermal stress (34^{0}C). In liver tissue, expression was even higher under control conditions (4.83 ± 0.00 TPM) and also dropped to zero under thermal stress. The absence of expression in both tissues under stress may reflect transcriptional repression, degradation, or a regulatory silencing mechanism of caspase kinase under thermal challenge. These observations suggest that caspase kinase may contribute to maintaining cellular homeostasis under basal conditions, while its suppression during stress could relate to energy conservation or immune modulation. The tissue-specific expression pattern under control conditions highlights its physiological relevance, particularly in organs sensitive to apoptosis and immune

Table 1. Conserved domain architecture of caspase kinase candidates identified in silico using InterProScan

Transcript ID	Domain name	InterPro ID(s)	Pfam ID(s)	Functional annotation (GO Terms)
TRINITY_DN10851_c0_g1_i3	Caspase recruitment domain (CARD) family member 11	IPR001315	PF00619	Positive regulation of NF-κB transcription factor activity (GO:0051092); Regulation of apoptotic process (GO:0042981)
TRINITY_DN106_c0_g1_i3	MAP kinase-interacting serine/threonine-protein kinase 2-like protein	IPR011009 IPR000719 IPR017441 IPR008271	PF00069	Involved in stress-responsive kinase signalling (GO:0006950)
TRINITY_DN59731_c3_g2_i1	Caspase recruitment domain (CARD) family member 6	IPR001315 IPR011029	PF00619	Mediates protein-protein interaction in apoptosis (GO:0006915)
TRINITY_DN16650_c0_g1_i9	Caspase recruitment domain (CARD) family member 9	IPR001315 IPR011029	PF00619	Regulation of apoptotic process (GO:0004672; GO:0006915)
TRINITY_DN3050_c0_g1_i12	Caspase recruitment domain (CARD) family member 14	IPR001315 IPR011029	PF00619	Regulation of apoptotic process (GO:0045859; GO:0006915)
TRINITY_DN12087_c0_g1_i4	Caspase recruitment domain-containing protein 8	IPR011029 IPR000488	PF00531	Signal transduction (GO:0007165)
TRINITY_DN14696_c0_g1_i1	Serine protease	IPR009003 IPR008256 IPR001254 IPR018114	PF00089	Catalyses phosphorylation in signalling pathways (GO:0006468; GO:0007166)

Table 2. Relative Transcript Abundance (TPM) of caspase kinase in heart and liver tissues under control (28^{0}C) and stress conditions (34^{0}C)

Tissue	Condition	Replicate 1 (TPM)	Replicate 2 (TPM)	Replicate 3 (TPM)	Mean ± SD (TPM)	Fold change (stress vs. control)	P-value
Heart	Control (28^{0}C)	2.56	2.45	2.70zz	2.57 ± 0.13	1.0 (reference)	3.75×10^{-6} (p-value <0.001)
	Stress (34^{0}C)	0.00	0.00	0.00	0.00 ± 0.00	0.00 (complete suppression)	
Liver	Control (28^{0}C)	4.83	4.60	5.00	4.81±0.20	1.0 (reference)	2.01×10^{-6} (p-value <0.001)
	Stress (34^{0}C)	0.00	0.00	0.00	0.00±0.00	0.00 (complete suppression)	

signalling, such as the heart. In the context of climate change, members of the MAP kinase family get stimulated in response to thermal stress, thereby activating the intrinsic apoptotic pathway. This, in turn, restores cell homeostasis by keeping a check on excessive apoptosis and eliminating damaged proteins (Dorion and Landry, 2002). Protein kinases β are crucial in maintaining cell integrity during oxidative damage/ heat stress due to climate change. Also, from our experiment, it is evident that caspase kinase pathways play a significant role in metabolic re-programming by integrating signals from energy sensors to assess the cell's resources and carefully re-allocating during thermal stress, thereby creating a prolonged phase of thermal resilience in the species. From the experiment, it was found that the caspase kinase gene is a subclass of the kinase family, which was involved in basal regulation and was completely switched off in response to acute stress. A two-sample t-test confirmed that caspase kinase expression was significantly higher in the liver compared to the heart under control conditions ($p<0.001$), consistent with previous observations in immune-active tissues (Huang *et al*., 2022; Chen *et al*., 2023), supporting tissue-specific transcriptional regulation.

Comparative phylogenetic insights

Phylogenetic analysis placed the *C. griseum* caspase kinase in a well-supported clade with other cartilaginous fishes, including *Hemiscyllium sp.*, *Heterodontus sp.*, and *Rhincodon typus*, suggesting strong evolutionary conservation within elasmobranchs. This cluster was clearly distinct from bony fish (*Danio rerio*) and more distantly from the human ortholog, which was used as an outgroup. The close grouping with *Callorhinchus milii* (a holocephalan) underscores the ancient origin of caspase kinase-like genes and reflects its conserved role in apoptosis and immune regulation among basal vertebrates. Bootstrap support values across major nodes reinforced the robustness of the tree topology, especially within the cartilaginous fish clade. This supports the idea that caspase kinase diverged early in vertebrate evolution and has retained key regulatory functions across lineages.

Conclusion

This study provides the first transcriptome-wide molecular insight into a Caspase Kinase gene in the grey bamboo shark *Chiloscyllium griseum*, a phylogenetically significant elasmobranch. The integration of *de novo* transcriptomic sequencing, functional annotation, domain analysis, and tissue-specific expression profiling revealed the presence of conserved apoptotic and immune-related features in the identified transcript. The candidate gene demonstrated enriched pathway associations, particularly in apoptosis and MAPK signalling, and possessed key conserved domains such as CARD and STK, consistent with vertebrate Caspase Kinases. Expression analysis indicated higher basal expression in liver and heart tissues under control conditions, with significant downregulation under thermal stress, suggesting regulatory sensitivity to environmental conditions. Phylogenetic clustering with other cartilaginous fishes further supported the evolutionary conservation of this gene. Collectively, these findings highlight the role of Caspase Kinase in immune-apoptotic regulation and stress adaptation in elasmobranchs and offer a valuable genomic resource for comparative immunological studies in basal vertebrates.

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Author contributions

Conceptualisation: SS; Methodology: SS; Writing Original Draft: PH; Data Collection: PH; Data Analysis: PH, SS; Supervision: SS.

Data availability

The de novo transcriptome data representing this study are deposited at the NCBI public repository (NCBI SRA: SRP338012).

Conflict of interest

The authors declare that they have no conflict of financial or non-financial interests that could have influenced the outcome or interpretation of the results.

Ethical statement

The protocols for animal experimentation were set up in compliance with the standards approved by the Institutional Animal Ethical Committee of the ICAR-Central Marine Fisheries Research Institute, Kochi (MBT/GNM/25). All these methods were also abiding by ARRIVE guidelines (http://arriveguidelines.org).

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