Expending LSSU's Instruction and Research Capabilities By Using Zebrafish Model

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Abstract:

Zebrafish, a small freshwater fish, has becoming one of the most important vertebrate model systems, and been widely used in the studies on developmental biology, molecular biology, cell biology, genetics, neurobiology, oncology, immunology, marine biology, pharmacology, toxicology, and environmental protection. As the first aim of my sabbatical leave, I would like to create a Zebrafish model system on the campus at LSSU, and gain knowledge and skills to raise and maintain the zebrafish facility for expending LSSU's educational and research capabilities by integrating the model animal into research and instructional activities. Secondly, I would also like to contribute part of my sabbatical time in 2023/2024 on an ongoing collaborative research project (that was granted funding from Chinese Natural Science Fund for the period 2021-2024) to investigate the molecular mechanisms of cross-species transmission of shrimp convert- mortality nodavirus by using zebrafish model in the Yellow Sea Fisheries Research Institute, Qingdao, China. This sabbatical will not only help to make more scientific contributions, and strengthen national and international collaborations, but also can further promote undergraduate research at LSSU, raise the reputation of LSSU's faculty and publicized the institution as well.

Therefore, I am requesting a full year sabbatical leave, which will allow me to: 1) visit Zebrafish facilities (In the US and China) to gain experience, knowledge, and learn skills for raising and daily maintenance of zebrafish; 2) develop a new on-campus zebrafish model system for expending the educational and research capabilities for LSSU faculty/student, strengthen undergraduate research activities; 3) Participate and strengthen national and international collaborations, help generate basis to seek further external funding, and incorporate current research knowledge and skills into LSSU curricula. Successful completion of the proposed sabbatical activities will provide great benefit to LSSU's reputation in particular for the rapid growing animal biology, cannabis chemistry, fisheries and environmental science programs.

Background and Introduction:

Part I: Development of Zebrafish Model System

Model system plays essential roles in the scientific exploration. Application of various biological models (such as *E. coli*, yeast, fruit fly, mice etc.) has made great contributions for scientific discoveries. As a small freshwater teleost fish, zebrafish possesses unique features such as small size, short reproductive cycle, available of whole genome sequences, in vitro fertilization and embryo development transparency, easy to rear in large number in a small laboratory setting. Therefore, zebrafish has becoming one of the most important vertebrate model systems, and been widely used in the life science, biomedical and environmental toxicological studies. In our recent study, we applied zebrafish model to investigate the pathogenic mechanism of CMNV and it exhibits huge advantages than aquaculture species. As I discussed zebrafish model system with a professor (Dr. Jim Du from University of Maryland School of Medicine), who has over 30-year scientific experiences for using zebrafish model in his research. Dr. Du explained to me its broader applications on developmental biology, molecular biology, cell biology, genetics, neurobiology, oncology, immunology, marine biology, pharmacology, toxicology, and environmental protection (***Please see Dr. Du's letter***). He has expressed willingness to share his expertise with me, and kindly offered me the opportunity to visit his laboratory for 1-2 months to gain some working experience in the zebrafish model system. Therefore, as the major aim of my sabbatical leave, I plan to establish a small-scale zebrafish culture system on LSSU campus, and try to integrate the new organism model system into the rapid growing biology and chemistry programs such as Animal Biology and Cannabis Chemistry, as well as to inspire their scientific interests with more hand-on experiences and research opportunities for undergraduates at LSSU.

I also discussed the idea with my fellow faculty members, and realized that establishment of a new zebrafish model system will directly benefit for undergraduate educational classes, such as fertilization and embryo early development labs in Biol. 132, as well as associated class/labs in Biol. 202 Genetics, Biol. 208 Animal biology, Biol. 335 Animal Nutrition, Biol. 421 Advanced Cellular Biology and Molecular Biology, Chem. 351 Biochemistry and Chem. 353 Toxicology. The zebrafish model will also offer more

opportunities for senior research projects and allow our seniors to gain more technical skills/research experience with the popular animal model and benefit their future professional and graduate school study.

In addition, Cannabis Chemistry is an attractive field and the fast growing unique program at LSSU. However, the evaluation of toxicity of cannabis compounds, similar to other drug candidates, is very essential to know the endpoint of toxicity, dose-response relationships, and mechanism of toxicity, and also need to determine the toxico-dynamics of the drug before they are considered for commercial application. Due to the toxicity problem, many new drugs have been declined by the FDA. So far, zebrafish has become an ideal organismal model in drug discovery in particular for screening of lead compounds, target identification, target validation, and assay development. Moreover, the zebrafish system has also shown big potential in toxicological study of environmental contaminants. Thus, the zebrafish model system will expand the research capacity for our faculties especially in the fields of toxicity assay of drug/cannabis compounds on the organismal level, environmental pollutant analysis, and further studies of animal nutritional and physiological effects. (***See Dr. Mosey's letter***).

Part II. Collaborative Research

Since I joined LSSU as a faculty in 2009, my major scientific research has focused on exploring the pathogenic mechanisms of infectious diseases and host defense immunity in aquatic animals. Over the past several years, I have been collaborating with my collaborators on several research projects focusing on aquatic animal infectious diseases and their defense immunity mechanisms. These national and international collaborations has led me to make great scientific contributions with dozens of publications, and gain rapidly growing reputation in the field as a world recognized biology scientist as LSSU faculty (***See my CV***).

However, the Convid-19 pandemic imposed significant impacts on such collaborations during the past three years. As the second aim of my sabbatical leave, I would like to spend several months of my sabbatical leave (2023 summer and 2024 summer) to participate one of newly funded collaborative project with Dr. Qingli Zhang, from Yellow Sea Fisheries

Research Institute, Qingdao China (***See Dr. Zhang's Letter***). I have had a long-term collaboration with Dr. Zhang's Laboratory since 2008. In our recent studies about pathogenic agents of aquatic animals, we found more and more pathogens could frequently jump from their native susceptible hosts to unexpected host animals. Such as the newly identified covert mortality nodavirus (CMNV), it not only be able to infect most farming crustacean species, but also can infect wild aquatic species including crustacean, teleost fish, and even plant (tobacco) or mammal (mouse). The occurrence of CMNV has been widely recognized in the shrimp culture plants in coastal areas of China, Southeast and South Asian countries, as well as South and North America. Such findings attract our concerns on ecosystem biosecurity and risk of seafood production to our human health. For addressing above concerns, as a Co-PI, we recently received funds (670,000 Chinese Yuan, 2021-2024) to screen the potential susceptible host organisms and figure out its transmission routes, and associated cellular and molecular mechanisms for CMNV to make the case of cross-species transmission. (***See the attached Grant proposal***). For better understanding of the infection process of CMNV in fish, we applied zebrafish as a model animal to study its pathogenicity and it exhibited numerous advantages for our cellular, molecular and histopathological studies (**See the attached papers**). Continuous studies will help us better understand the molecular mechanisms of intraspecific transmission and uncover the reason for such large-scale epidemics of CMNV from the ecological view of pathogenesis, more importantly, this sabbatical research activities will offer me a good opportunity to gain more knowledge and experiences for using zebrafish as research model, and learn skills for maintenance of zebrafish facility.

So far, zebrafish has becoming an ideal model organism and been widely used in more than 800 research laboratories/centers worldwide in almost all aspects of life science, environmental toxicology, and novel drug discovery. I also get technical supports for using the zebrafish model system from my professional networking colleagues in the US, like UMich, MSU and University of Maryland, and in China, like Institute of Hydrobiology, Chinese Academy of Science (IHB). Therefore, the importance and its wide application of zebrafish model, as well as all above-mentioned resources and collaborations suggest a viable and fruitful future after successful completion of the sabbatical proposal. The zebrafish culture facility on campus will expand and strengthen LSSU's biology and

chemistry education and research capacity.

Outcomes:

1. **Gain new knowledge and skills for culture and maintain zebrafish and integrate the use of zebrafish model into the class instruction, and student/faculty research activities**
   A) Visit laboratories with zebrafish facilities in the US and China, and learn skills for maintenance and husbandry of zebrafish (2-4 weeks in Dr. Du's Lab at University of Maryland School of Medicine; 2-4 months in Dr. Zhang's Lab in Yellow Sea Fisheries Research Institute, China).
   B) Develop and Establish a zebrafish culture facility at LSSU, and incorporate the zebrafish model system into related courses (labs) in the Animal Biology and Chemistry programs (such Biol. 132, Biol. 220, Biol. 330, 335 and Chem. 353 etc.),
   C) Apply the zebrafish model system into faculty's research activities especially for the virulence study of pathogens, toxicological assays for environmental pollutants, toxicity activity of novel drug compounds/biological extracts, as well as developmental and molecular biology studies.
   D) Train students to take care the zebrafish facility, and provide 2-3 students' senior research projects. Students will gain new knowledge, and more cutting-edge lab skills and hand-on experiences at LSSU.

2. **To participate the funded on-going collaborative research project about the newly identified intraspecific transmission virus, covert mortality nodavirus (CMNV) with my Chinese collaborator (Dr. Zhang) from Yellow Sea Fisheries Research Institute in Qingdao, China.**
   A) To explore CMNV's susceptible host range and related cellular and molecular mechanisms for crossing species transmission by using the zebrafish model in 2023/2024, to gain more hands-on experience and related culture skills.
   B) According to the research progress, complete 1-2 peer-review publications, and present the new findings in scientific conference as measurable outcomes.
   C) Use research experience and skills for updating lab manual and improvement of

current lab instructions, especially the Biol. 433, Histology and Histopathology.

Timeline:

August – October 2023

1. Literature search on Zebrafish culture and scientific applications.
2. Visit Dr. Cunming Duan at University of Michigan and Dr. Jim Du at University of Maryland to gain suggestions and hand-on experiences for rearing zebrafish (work 2-4 weeks in Dr. Du's lab).
3. Search and collect the vendor information for zebrafish culture facilities and supplies.

November – December 2023

1. Visit China Zebrafish Resource Center (CZRC) in Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China. Attend the 7th Chinese Zebrafish PI meeting and related training course for zebrafish model system.
2. Participate the collaborative research with Dr. Zhang at Yellow Sea Fisheries Research Institute, Qingdao China to conduct the study of CMNV in zebrafish model system for the cellular and molecular pathogenic mechanisms.
3. Finish writing 1 manuscript and submit for peer-reviewed publication.

January – February 2024

1. Keep searching and collecting the vendor information for zebrafish culture facilities and supplies, and literature preparations particularly for zebrafish husbandry knowledge, such as water quality control of zebrafish model system, nutrition requirement, disease prevention, fertilization and early development of embryo etc.
2. Purchase and setup zebrafish culture facility and introduce about 50-100 zebrafish to test the new model system.

March – June 2024

1. Keep running the zebrafish model system and train LSSU students in Aquaculture Club for taking daily care of the zebrafish. Then introduce 100-200 new fish into the system.
2. Advise 2-3 senior students for their senior research projects.
3. Submit 1-2 collaborative proposals on aquatic animal health for requesting more internal or external funds to support my international collaboration and maintenance of zebrafish model system.

July – August 2024

1. Continue the collaborative research with Dr. Zhang at Yellow Sea Fisheries Research Institute, Qingdao China to conduct the study of CMNV in zebrafish model system for the cellular and molecular pathogenic mechanisms.
2. Preparation of 1-2 manuscripts for peer- reviewed publication.
3. Finalize the sabbatical leave project and write a final report.

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EDUCATION

1998-2002 Ph.D. Fish Pathobiology
Department of Biology, The Chinese University of Hong Kong.

Dissertation Title: *Vibrio alginolyticus: pathogenicity and its immunological control via vaccination in silver sea bream (Sparus sarba)*

1992-1995 M.S. Genetics (Molecular Virology concentration)
Institute of Hydrobiology, Chinese Academy of Sciences, China.

Thesis Title: *Development of a rapid diagnosis technique for hemorrhagic virus of grass carp (GCHV) based on reverse transcription-polymerase chain reaction (RT-PCR).*

1988-1992 B.S. Biology (Microbiology and Immunology Concentration)
Department of Microbiology, Wuhan University, China.

Thesis Title: *Isolation and identification of photosynthetic bacteria from Donghu Lake.*

PROFESSIONAL EXPERIENCE

8/2021-present	**Professor of Biology** & Manager of Fish Diseases Diagnostic Laboratory School of Science & Medicine; Lake Superior State University Sault Ste. Marie, MI, 49783
8/2014- 7/2021	**Associate Professor (Biology)** (Tenured) & Manager of Fish Diseases Diagnostic Laboratory School of Science & Medicine; Lake Superior State University Sault Ste. Marie, MI, 49783
2015- 2019	Adjunct Principal Investigator, Qingdao National Laboratory for Marine Sciences and Technology. Qingdao, China.
11/2009- 7/2014	**Assistant Professor (Biology)** & Manager of Fish Diseases Diagnostic Laboratory School of Biological Sciences; Lake Superior State University Sault Ste. Marie, MI, 49783
4/2002-10/2009	**Research Associate (Fish Immunology)** Department of Pathobiology, School of Veterinary Medicine University of Pennsylvania, Philadelphia, PA 19014
1/2001- 2/2002	**Postdoctoral Researcher (Cell Biology)**

Lab Biologie Cellulaire, Faculte de Medecine
Universite Paul Sabatier, Toulouse, France

7/1995-1/1998 **Assistant professor (Marine Microbiology)**
College of Marine Life Sciences, Ocean University of China, China.

TEACHING Experience:

11/2009-present, Teach the following courses for undergraduate students in the School of Biological Sciences at the Lake Superior State University.

Biol. 499: Senior Research Seminars
Biol. 495: Senior Project
Biol. 490: Independent studies (*for1-4students*) in:
- **Culture for Animal Cells**
- **Immunology**
- **Aquatic Animal Nutrition**
- **Ecology of Animal Diseases**
- **Histology/histopathology**
- **Microbiology**

Biol. 434: Histopathology*
Biol. 426: Ecology of Animal Diseases (lecture)*
Biol. 425: Virology (lecture & lab)*
Biol. 423: Immunology (lecture & lab)
Biol. 421: Advanced Molecular & Cell Biology (Lecture & Lab)
Biol. 400: Microbial Ecology *
Biol. 389: Biology Internships in:
- **Fish Aquaculture**
- **Fish Health ***
- **Marine Biology**
- **Animal Clinics***

Biol. 372: Freshwater Fish Culture (lecture & lab)
Biol. 232: Introduction of Aquaponics (co-teach)*
Biol. 208: Principles of Animal Biology and Health*
Biol. 204: Microbiology (lecture & lab)
Biol. 132: General Biology-Organism (lab)
Biol. 131: General Biology-Cell (lecture & lab)

*** New course at LSSU**

4/2002- 10/2009, **Fish Anatomy Lab** for VMD candidates at School of Veterinary Medicine, University of Pennsylvania, USA.

1/1998-12/2000, **General Microbiology Lab** and **Cell Biology Lab** for undergraduates at The Chinese University of Hong Kong, Hong Kong.

9/1995-12/1997 **General Microbiology** and **Marine Microbiology** (lecture & lab) for undergraduates at Ocean University of China, Qingdao, China.

HONORS

2021-22 Nominee for "LSSU Distinguished Teaching Award".

2019 Nominee for the membership to the North Central Regional Aquaculture Center's (NCRAC) Technical Committee/Research Subcommittee.

2018-present, Pearl River Scholar Professor, College of Animal Sciences, Zhongkai University of Agriculture and Engineering. Guangzhou, China

2017-2020, Adjunct Professor, Xiamen University, China

2014-2017, Member of the Consulting Committee for Aquatic Animal Medicine Program, Shanghai Ocean University, Shanghai, China.

2016-2017, **Adjunct Professor** in College of Animal Sciences, Zhongkai University of Agriculture and Engineer, Guangzhou, China.

2013 Nominee for the Distinguished Teacher Award at Lake Superior State University.

2011-2014 **Guest professor**, School of Biotechnology, East China University of Science and Technology, Shanghai, China.

2011- 2017 Selected as a member of External Peer-reviewer Panel for National Natural Science Foundation of China (like the NSF and NIH in the US).

2010-present **Visiting professor**, Institute of Oceanology, The Chinese Academy of Sciences, Qingdao, China

2009- 2013 Dr. Li was awarded a **"Taishan Scholarship"** (1,000, 000 Chinese Yuan) for recognized outstanding oversea scholar from the Government of Shandong Province, China for supporting the collaborative research in the area of "Infectious Diseases and Immunological Control in Marine Fish").

2001 National Innovation Award in Marine Sciences and Technology, State Oceanic Administration of China. (Second Prize)

2001 National Award for Advancement of Science and Technology, National Commission of Science and Technology of China. (Second Prize)

2000 International Outstanding Young Scientists Award (250,000 Franc), French Ministry of Research (as one of 50 awardees worldwide of year 2000)

GRANTS & CONTRATS

2022-2025 NSF- MRI: Acquisition of an Environmental Scanning Electron Microscope for the facilitation of research, education, and outreach in the Biological, Environmental, and Earth Sciences (Dr. Li, Co-PI, PI: Dr. Kolomyjec), awarded.

2021-2024 Chinese Natural Science Fund for "Study on cross-species transmission of shrimp convert- mortality nodavirus and its molecular mechanism". RMB670,000. **Co-PI**. (PI: Dr. Qingli Zhang, Yellowsea Fisheries Research Institute, Qingdao, China.)

2018-2021 "Pearl River Scholar" professorship of Zhongkai University of Agriculture and Engineering (ZKUAE), Guangzhou, Guangdong province. RMB 300,000/year for 3 years. PI, Co-PI Dr. Li Lin form ZKUAE.

2016-2017 Chinese Natural Science Fund for Oversea Researchers: Involvement of phagocytic B cells against infection in marine fish. RMB, 200,000. **PI.**

2015-2016 Chinese Natural Science Fund for "Protective Immunity of Inactivated *Edwarsiella tarda* Bacterins in Marie Fish. RMB 300,000. **Co-PI**, (PI, Dr. Guoshi Xie, Yellow Sea Fishery Research Institute, Qingdao, China).

2015-2017 Bureau of Indian Affairs-Great Lakes Restoration Initiative Funds (BIA-GLRIF). Monitoring fish movement and fish condition in tributaries of Whitefish Bay. $142,964. **Co-PI** ($ 80,033 for LSSU).

2014-2015 Contract from *Algal Scientific Company*, Effect of Dietary beta-glucan Derived from Algae on Growth Performance, Disease Resistance and Immune Response in Atlantic salmon. $20,746. **PI.**

2014-2017 Great Lakes Fishery Trust. Re-Emergence of Epizootic Epitheliotropic Disease Virus: Potential Effects and Development of Improved Diagnostics &Control Measures. $446,492. **Co-PI.** (PI, Dr. Mohamed Faisal from MSU, East Lansing, MI). ($65,299 for LSSU) (extended to **8/2017**)

2014 The Great Lakes Council of the Federation of Fly fishers Research Grant, $400 (PI)

2014,1-2017,12 Key Project from Chinese Natural Science Fund for the study "Immuno-escape Mechanisms of *Edwarsiella tarda* in Turbot". Chinese Yuan (RMB) 3,000,000. **Co-PI.** (PI, Dr. Li Sun, from Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China)

2011-2014 Chinese Natural Science Fund for Application of Immuno-stimulants against Fish Diseases. RMB 500,000. **Co-PI,** (PI, Dr. Xiuhua Wang, Yellow Sea Fishery Research Institute, Qingdao, China).

2012 Superior Aqua-System Development for Michigan Clean Energy Venture Challenge. $39,000 **Co-PI,** (PI, Dr. Evans School of Biological Sciences, LSSU).

2012 Evaluation of the Relationship of Total Suspend Solids, Bacteria and Fish Health Status in Marquette Fish Hatchery. MDNR, $ 3999 (PI)

2011 The Great Lakes Council of the Federation of Fly fishers Research Grant, $400 (To support Dr Li's student for his collecting water samples from Marquette State Fish Hatcheries).

2011 LSSU Strategic Planning Initiative funding, $19,500. **(PI)**

2010 The Great Lakes Council of the Federation of Fly fishers Research Grant, $300 (To support Dr Li's student for her summer research in Platter River State Fish Hatcheries).

2009- 2019 Contracts with the Michigan Department of Natural Resources and Environment (MI-DNR): BKD and EMS diagnosis in Fish Gametes in MI State Fish Hatcheries. $3,500-5,000/year (PI)

2009-2013 **"Taishan Scholarship"** from Shandong Province, China to support Dr. Li's International Collaboration Research on Fish Infectious Diseases and Immunological Control. RMB 1,000,000 (PI).

THESIS COMMITTEES & ADVISORS

9/2014 -12/2017: Co-supervisor for a Ph.D. student (Megan Shavali) at Michigan State University, East Lansing, MI. USA

11/2009- present: Mentor for 30 LSSU senior students' Research Projects:

2010: 2 students (Heather Millard*; Renee Williams)

2011: 2 students (Jason Sauve; Katherine Marsh)

2012: 6 students (Charolette Niegoda; Trevor Asperger; Mike Caputo; Thomas Eitniear*; Jessica Phal; Adam Mackey)

2013: 1 student (Tyler Jackson*)

2014: 3 students (Lucas Bradburn*; Ashley Alexander; Shelby Stempky)

2015: 1 student (Angelina Walker) (Sabbatical leave, 2015F)

2016: 2 students (Scott Cooper*; Conner Workentine*)

2017: 1 student (Maranda Semig)

2018: 4 students (Jenay Andrews; Alysha Briglio; Nash Johnson*; Erica Stefano*)

2019: 5 students (Jacob Baker; Siyuan Ma*; Mitchell Burlingame; Allyson Glazier; Andrew Niemiec)

2020: 3 students (Ashley Gray; Michael Gills; Uttley Maxwell)

2021: 5 students (Rachel Farina, Zoe Lewis, Matthew Hansen, Jonathan Bramer; Jeremy Turnbow)

Under my supervision, some students won the following honors (with *)

- 3 students were awarded Michigan Fly Fishers Conservation Grants;
- 9 students received LSSU undergraduate research fund (URC grant);
- 3 students won the Fund for LSSU;
- 3 students received Michigan Sea Grant award;
- 3 students received "Best Poster award" of Biology Senior Research Symposium.

- 10+ students presented their research results in national/regional scientific conferences/meetings (AFS Meetings; AFS-Fish Health Meeting; Mid-West-F&W Conference etc.).

12/2010- present: Co-supervisor for 15+ Ph.D. students (Aquatic Animal Diseases &Immunology Program) in Institute of Oceanology, The Chinese Academy of Science, Qingdao, China; and Zhongkai University of Agriculture and Engineering, Guangzhou, China

MEMBERSHIPS

2006- present: International Society of Developmental and Comparative Immunology

2007- 2012: American Association of Immunologists.

2009-present: American Fisheries Society-MI Chapter

2013-present: International Society of Fish and Shellfish Immunology

PUBLICATIONS (*Corresponding author)

2022

1. Yin X, Li X, Mu L, Bai H, Yang Y, Chen N, Wu L, Fu S, Li J, Ying W, Ye J. (2022), Affinity-Driven Site-Specific High Mannose Modification Determines the Structural Polymerization and Function of Tetrameric IgM in a Primitive Vertebrate. *J Immunol*. doi: 10.4049/jimmunol.2100921.
2. Xu TT, Fan YD, Jia TC, Wang C,Wang W, Li J, Zhang QL and Yao CL (2022) Investigation on Natural Infection of Covert Mortality Nodavirusin Large Yellow Croaker (*Larimichthy scrocea*). *Front. Mar. Sci*. 9:789128. DOI: 10.3389/fmars.2022.789128.
3. Ma Z-Y, Liang J-X, Li W-S, Sun Y,Wu C-S, Hu Y-Z, Li J, Zhang Y-A and Zhang X-J (2022) Complement C3aEnhances the Phagocytic Activity of B Cells Through C3aR in a Teleost Fish. *Front. Immunol*. 13:873982. doi: 10.3389/fimmu.2022.873982.
4. Qin Z, Yang M, Lu Z, Babu VS, Li Y,Shi F, Zhan F, Liu C, Li J and Lin L(2022) The Oxidative Injury of Extracellular Hemoglobin Is Associated With Reactive Oxygen Species Generation of Grass Carp (*Ctenopharyngodon idella*). *Front. Immunol*. 13:843662. DOI: 10.3389/fimmu.2022.843662.
5. Wu LT, Li L, Gao AL, Ye JM, Li J. Antimicrobial Roles of Phagocytosis in Teleost Fish: Phagocytic B Cells vs Professional Phagocytes. *Aquaculture Fisheries* (2022). doi: 10.1016/j.aaf.2021.12.008.
6. Yang Y, Chen J, Lu L, Xu Z,Li F, Yang M, Li J, Lin L and Qin Z (2022) The AntibacterialActivity of Erythrocytes From Goose (*Anser domesticus*) Can Be Associated With Phagocytosis and Respiratory Burst Generation. *Front.*

Immunol.12:766970. DOI: 10.3389/fimmu.2021.766970.

7. ZJ Lu, ML Zhang, MZ Tang, YN Li, F Shi, FB Zhan, LJ Zhao, **J. Li**, L. Lin* and ZD Qin*. 2022. Heme oxygenase-1 protects against inflammatory and apoptosis induced by heme proteins in *Ctenopharyngodon Idellus* kidney cells. *Aquaculture*. 546 (2022) 737266.
8. Wang C., Liu S., Xu T.T., Li X.P., Li J. and Zhang Q.L. 2022. Pathogenicity study of covert mortality nodavirus (CMNV) infection in zebrafish model. *Aquaculture*. 546. DOI : 10.1016/j.aquaculture.2021.737378

2021

9. Wu L, Gao A, Li L, Chen J, **Li J**, Ye J (2021) A Single-Cell Transcriptome Profiling of Anterior Kidney Leukocytes From Nile Tilapia (*Oreochromis niloticus*). *Front. Immunol*. 12:783196. doi: 10.3389/fimmu.2021.783196.
10. ZZ Xu, YC Yang, V. Sarath Babu, JJ Chen, F Li, MX Yang, NQ Li, **J. Li**, L. Lin, ZD Qin, The antibacterial activity of erythrocytes from *Clarias fuscus* associated with phagocytosis and respiratory burst generation. *Fish & Shellfish Immunology,* 119 (2021) 96-104.
11. YN Li, XL Qiu, ZJ Lu, FB Zhan, MX Yang, V. Sarath Babu, **J. Li**, ZD Qin, L. Lin, Molecular and functional characterization of MST2 in grass carp during bacterial infection, *Fish & Shellfish Immunology*, 119 (2021) 19-30.
12. ZJ Lu, MZ Tang, ML Zhang, YN Li, F Shi, FB Zhan, LJ Zhao, **J. Li**, L. Lin*, ZD Qin*. Hemeprotein amplifies the innate immune receptors of *Ctenopharyngodon idellus* kidney cells through NF-κB- and MAPK-dependent reactive oxygen species generation. *Dev Comp Immunol*, 2021 Jul 14;104207.
13. ZJ Lu, MZ Tang, YN Li, F Shi, FB Zhan, ML Zhang, LJ Zhao, **J. Li**, L. Lin*, ZD Qin*. Molecular cloning and characterization of FADD from the grass carp (Ctenopharyngodon idellus) in response to bacterial infection. *Aquaculture*. 2021 542 :736829.
14. ZJ Lu, MZ Tang, ML Zhang, YN Li, F Shi, FB Zhan, LJ Zhao, **J. Li**, L..Lin*, ZD Qin*. Expression and Functional Analysis of the BCL2-Associated Agonist of Cell Death (BAD) Gene in the Grass Carp (*Ctenopharyngodon idellus*) During the Bacterial Infection. *Dev Comp Immunol*, 2021 Oct;123:104160.
15. AM.Hegazy，N Chen，HZ Lin，Sarath Babu V.，YCYang，ZD Qin，F Shi, **J. Li**，L. Lin. Induction of apoptosis in SSN-1cells by Snakehead Fish Vesiculovirus (SHVV) via Matrix protein dependent intrinsic pathway. *Fish & Shellfish Immunology*. 2021 Jun;113:24-34.
16. Yang MX, Lu ZJ, Li FL, Shi F, Zhan FB, Zhang YL, Zhao LJ, Li YN, **J. Li**, L. Lin*, ZD Qin*. Alginate oligosaccharide improves fat metabolism and antioxidant capacity in the liver of grass carp (*Ctenopharyngodon idellus*) *Aquaculture* 540 (2):736664. DOI: 10.1016/j.aquaculture.2021.736664.

17. Li YN, Lu ZJ, Zhan FB, Yang MX, Zhao LJ, Shi F, **J. Li**, L. Lin*, ZD Qin*. Nrf2 modulates host defense during antibacterial immunity response in grass carp (*Ctenopharyngodon idellus*). *Aquaculture* 536:736474. DOI: 10.1016/j.aquaculture.2021.736474.

2020

18. Shi F, Lu ZJ, Yang MX, Li FL, Zhan FB, Zhao LJ, Li YN, Li QQ, Li JT, **J. Li**, L.Lin*, ZD Qin*. Astragalus polysaccharides mediate the immune response and intestinal microbiota in grass carp (*Ctenopharyngodon idellus*). *Aquaculture* 534:736205. DOI: 10.1016/j.aquaculture.2020.736205.
19. Yang MX, Lu ZJ, Li FL, Shi F, Zhan FB, Zhao LJ, Li YN, Li QQ, Li JT, J. Li, L. Lin*, ZD Qin*. *Escherichia coli* induced ferroptosis in red blood cells of grass carp (*Ctenopharyngodon idella*). *Fish & Shellfish Immunol.* https://doi.org/10.1016/j.fsi.2020.09.036
20. Lu ZJ, Yang MX, Zhang K, Zhan FB, Li FL, Shi F, Li YN, Zhao LJ, J. Li, L. Lin*, ZD Qin*. 2021. *Aeromonas hydrophila* infection activates death receptor apoptosis pathway in the red blood cells of grass carp (*Ctenopharyngodon dellus*). *Aquaculture* 532 (2021)735956. https://doi.org/10.1016/j.aquaculture.2020.735956
21. Mu LL, Yin XX, Wu HR, Lei Y, Han KL, Mo JF, Guo Z, **J. Li** and Ye JM. Mannose-binding lectin possesses agglutination activity and promotes opsonophagocytosis of macrophages with calreticulin interaction in an early vertebrate. *J Immunol*. 205 (12) 3443-3455. https://doi.org/10.4049/jimmunol.2000256.
22. Shi F, Qiu XL, Nie LJ, Hu LY, Babu SV, Lin Q, Zhang YL, Chen LH, **J. Li**, Lin L., and Qin ZD. 2020. Effects of oligochitosan on the growth, immune responses and gut microbes of tilapia (*Oreochromis niloticus*). ***Fish & Shellfish Immunol***. 106:563-573. https://doi.org/10.1016/j.fsi.2020.07.049.
23. Shi F, Zi YJ, Lu ZJ, Li FL, Yang MX, Zhan FB, Li Y, **J. Li**, Zhao LJ, Lin L., and Qin ZD. 2020. *Bacillus subtilis* H2 modulates immune response, fat metabolism and bacterial flora in the gut of grass carp (*Ctenopharyngodon idellus*). ***Fish & Shellfish Immunol***. 106:8-20. https://doi.org/10.1016/j.fsi.2020.06.061.
24. Lu ZJ, Yang G. Qin ZD, Shen HY, Zhang ML, Shi F, **J. Li**, Babu SV, Lin L. 2020. Glutamate related osmoregulation of guanie nucleotide-binding protein G (I) α2 from giant freshwater prawn during molting and salinity stress. Aquaculture. 521:735000. https://doi.org/10.1016/j.aquaculture.2020.735000
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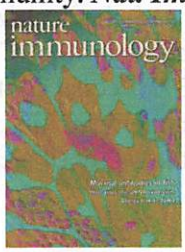

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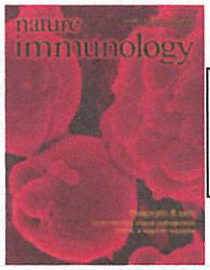

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BOOK CHAPTERS

The following research papers were included in the book ***"Diagnosis and Control of Bacterial Diseases in Penaeid Shrimps Hatcheries"***, which edited by Dr. Huai-shu Xu et al. and published by ***China Ocean Press*** in 1999, Beijing.

1: Feng J, Li Y, **Li J**, Qi ZZ, Wang XH, Ji WS, Xu HS, Yang XS and Ma JK. 1999. Studies on bacterial ecology in rearing water of *Penaeus chinensis* larvae. pp10-22.

2: Feng J, Li Y, **Li J**, Qi ZZ, Wang XH Ji WS, Xu HS, Yang XS and Ma JK. 1999. Studies on heterotrophic bacteria in rearing water of *Penaeus chinensis* larvae and its relationship with bacterial disease. pp23-36.

3: Li Y, **Li J**, Qi ZZ, Zhang XH, Wang XH, Ji WS, Xu HS, Vandenberghe J, Swings J, Yang XS and Ma JK. 1999. A study on aerobic heterotrophic bacterial flora and dominant vibrios composition in larvae and postlarvae of *Penaeus chinensis*. pp37-46.

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13: Wang XH, **Li J**, Ji WS and Xu HS. 1999. Application of probiotics in aquaculture (Review), pp121-126.

14: **Li J.**, Ji W.S. and Xu H.S. 1999. Advances on studies of diseases of penaeid shrimp larvae (Review), pp136-145.

15: Feng J, **Li J**, Ji WS and Xu HS. 1999. A review on the studies of bacterial ecology in hatchery-rearing waters of penaeid shrimp (Review), pp146-152.

PRESENTATIONS (*LSSU undergraduate, 2010-present)

Regional/National Conference or Workshop:

1. **Li J.** 2010. Thiamine Deficiencies Workshop, Ann Arbor, MI.
2. **Li J.** 2011. Annual Meeting of the Michigan Chapter of the American Fisheries Society. Petoskey, Michigan.
3. Williams R.* **Li J.**, 2011. Annual Meeting of the Michigan Chapter of the American Fisheries Society. Petoskey, Michigan. (*LSSU undergraduate)
4. Millard H.R.*, **Li J.**, 2011. Annual Meeting of the Michigan Chapter of the American Fisheries Society. Petoskey, Michigan. (*LSSU undergraduate)
5. **Li J.**, Williams R*. 2012. The joint Annual Meeting of the Wisconsin Chapter and Michigan Chapter of the American Fisheries Society. Marinette, WI. (*LSSU undergraduate)
6. Evans B., **Li J**. 2012. Immune Cells in the Brian of Larval Lake Sturgeon (*Acipenser fulvescens*). 2012 Great Lakes Lake Sturgeon Coordination Meeting. Sault Ste. Marie, MI.
7. **Li J.** 2014. Joint Annual Meeting for MI-OH Aquaculture Association. Toledo, OH.

8. **Li J.** 2014. Immune Evasion Mechanisms of *Edeardsiella tarda* and Application in Disease Control. The 39th Eastern Fish Health Workshop. April 28-May 2, 2014. Shepherdstown, WV.
9. **Li J. 2015.** Adjuvant Effect of *Quillaja Saponaria* Saponin (QSS) In Turbot (*Scophthalmus Maximus*) Upon Bath Vaccination. The 40th Eastern Fish Health Workshop. March 2-6, 2015. Charleston, SC.
10. Bradburn L*, Gao JZ. and **Li J.** *2016.* Health Status of Migrating and Resident Fishes in Three Tributaries of Whitefish Bay, Lake Superior. The *76th Midwest Fisheries and Wildlife Conference.* January 24-26, Grand Rapids, Mi. (* LSSU undergraduate).
11. **Li J.** 2017. Effects of Dietary beta-Glucan on Growth Performance, Disease Resistance and Immune Responses in Salmonid Fish. The Joint meetings of AFS-FHS and Great Lake Fish Commission FHC meeting and the 42nd Annual Eastern Fish Health Workshop. April 3-7. East Lansing, MI. USA.
12. **Li J.** 2018. **7th QIAGEN Investigator Forum** for Molecular Biology training. May 1-3, 2018. San Antonio, TX.
13. **Li J.** 2018. **MiTransfer Pathway Summit.** May 18, 2018. Lansing Community College (West Campus) Lansing, MI.
14. **Li J.**, 2018. **MiTransfer Pathways Summit** (*Biology Pathways II*). October 19, 2018. Adrian College (Tobias Center), Adrian, MI.
15. **Li J.** 2019, as an evaluation committee member, Dr. Li was invited to attend the meeting for "Aquaculture Curriculum Assessment" in Huazhong Agriculture University, July 19-20, 2019, Wuhan, China.
16. Jenay Andrews*, Siyuan Ma*, Nash Johnson* and **Li J.** 2020, Dr. Li presented their research results in **Michigan AFS meeting 2020** at Central Michigan University. March 12-13, 2020.)

International Conference or Workshop:

17. D. Parra, **J. Li,** A. Rieger, YA Zhang, L. Randall, C. Hunter, D. Barreda, J. Oriol Sunyer. 2012. Peritoneal cavity B-1 B cells have phagocytic and microbicidal capacities, and present phagocytosed antigen to CD4+ T cells. *The 12th Congress of International Society of Developmental and Comparative Immunology,* July 9th-13th, 2012, Fukuoka, Japan.
18. L. Chen, C. Wang, L. Sun and **Li J.** 2012. Survivability of *Edwardsiella tarda* in fish serum relates to bacterial surface LPS. *The 12th Congress of International Society of Developmental and Comparative Immunology,* July 9th-13th, 2012, Fukuoka, Japan.
19. Chen L., C. Wang, L. Sun and **Li J.** 2013. Survival of *Edwardsiella tarda* in fish serum relates to bacterial surface LPS. *The 1st International Conference of Fish and Shellfish Immunology.* June 25-28, 2013. Vigo, Spain.
20. Wang Y., Wang X.H., Huang J. and **Li J.** 2013. Adjuvant effects of *Quillaja saponaria* saponins (QSS) on humoral immune responses in turbot. *The 1st International Conference of Fish and Shellfish Immunology.* June 25-28, 2013. Vigo, Spain.
21. **Li J.** 2013. The Involvement of Fish Complement in Innate and Adaptive Immune Responses. *2013 Fish Immunology Workshop*. July 18-20, 2013. Wuhan, China.
22. **Li J.** 2014. Infection and Immunity: Involvement of Innate and Adaptive Immune Responses in Fish Defense. In: *The Symposium on Infection and Immunity in Marine Organisms. July 25-26, 2014. Guangzhou, China(Keynote Speaker)*

23. **Evans B. and Li J.** Investigations on the function of immune-like cell clusters in the larval lake sturgeon brain. The 144th Annual Meeting of American Fisheries Society. August 17-21, 2014. Quebec city, Canada,
24. **Li J.** 2015. *Edwardsiella tarda* evades serum killing by preventing complement activation via the alternative pathway. *The 13th Congress of International Society of Developmental and Comparative Immunology,* June 28th-July 4th, 2015, Murcia, Spain.
25. **Li J.** 2015. Phagocytic B cells, crossroad of innate and adaptive immunity. International Conference on Marine Microorganisms. May 22-25, Qingdao, China. *(Keynote Speaker)*
26. **Li J.** 2016. Vaccination of Silver seabream against Vibriosis. *The 2nd International Conference of Fish and Shellfish Immunology.* June 26-30, 2016. Portland, Me, USA.
27. **Li J.** 2016. Evolution of phagocytic B cells, Innate or adaptive. International Workshop on Aquaculture Health. July 27-29th, 2016. Qingdao, China. *(Keynote Speaker)*
28. Li J. 2016. Application of Immunostimulants in Fish Aquaculture. Workshop on Immunology and Disease of Aquaculture Animals. July 24-25. 2016. Qingdao, China.
29. **Li J.** 2017. *2nd Fish Immunology Workshop*. July 17-19, 2017. Wuhan, Hubei, China.
30. Li J. 2017. Re-emerging of EEDV (Salmonid herpes virus 3) in Great Lakes: the biologic and pathogenic. State Key Laboratory Annual Workshop on Infection and Immunity. July 30, 2017. Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China.
31. Y.Q. Li, L. Sun & J. Li. "Internalization of Large Particles by Turbot (Scophthalmus maximus) IgM+ B Cells Mainly Depends on Macropinocytosis". 14th Congress of the International Society of Developmental and Comparative Immunology, June 17 - 21, 2018, Santa Fe, New Mexico, USA.
32. J. Li, 2018. "Endocytosis of Fish B cells: Pathways and its Regulatory Mechanisms in Flatfish". Aoshan Summit on Fish: Biology and Beyond. June 24-26, 2018, Qingdao, China.
33. Yiqun Li, Li Sun and J. Li. 2018. "Internalization of Large Particles by Turbot (Scophthalmus maximus) IgM+ B Cells Mainly Depends on Macropinocytosis" 8th International Symposium on Aquatic Animal Health. Sept. 2-6, 2018, Charlottetown, PEI, Canada.
34. J. Li.2018. "New Progress in the Study of Fish Phagocytic Cells". International Symposium of Fish and Shellfish Immunology and Disease Control, Oct. 9-12, 2018. Shanghai, China
35. J. Li, 2019. Dr. Li was invited to be the Section Chair (Fish Diseases and Immunology) for the 1st Hainan Young Scientist Form on Marine Science & Technology. June 30-July 2, 2019, Haikou, China.
36. J. Li, 2019. Dr. Li was invited as co-Chair to the 3rd Young Scientist Form of National Laboratory of Marine Science and Technology, and gave a presentation on "Effects of cell differentiation on the phagocytic activities of IgM+ B cells in a teleost fish". July 23-24, 2019. Qingdao, China.

INVITED SEMINARS (2010-PRESENT)

2020

Due to the Covid-19 pandemics, all academic activities were cancelled.

2019

1. August 1, 2019, Dr. Li invited to give a presentation on "Fish Molecular Immunology" in the College of Animal Sciences and Technology, Zhongkai University of Agriculture and Engineering, Guangzhou, China.

2. August 12, 2019. Dr. Li was invited to visit Yellow Sea Fisheries Research Institute and gave a presentation on "Effects of cell differentiation on the phagocytic activities of IgM+ B cells in a teleost fish".

2018

3. **Jun Li, 2018.** *Endocytosis of Fish B cells: Pathways and its Regulatory Mechanisms in Flatfish* Zhongkai University of Agriculture and Engineering, Guangzhou, Guangdong, China.
4. **Jun Li, 2018.** *Endocytosis of Fish Phagocytes: Pathways and its Regulatory Mechanisms*, Fujian Agriculture and Forestry University, Fuzhou, China.
5. **Jun Li, 2018.** *Endocytosis of Fish Phagocytic B cells: Pathways and its Regulatory Mechanisms.* Institute of Oceanology, Qingdao, Shandong, China.
6. **Jun Li, 2018.** *Internalization of Large Particles by Turbot (Scophthalmus maximus) IgM+ B Cells Mainly Depends on Macropinocytosis.* Huazhong Agricultural University, Wuhan, Hubei, China.
7. **Jun Li, 2018.** *Endocytosis of Fish B cells: Pathways and its Regulatory Mechanisms in Flatfish.* Shantou University, Shantou, Guangdong Province, China.
8. **Jun Li, 2018.** *Endocytosis of Fish B cells: Pathways and its Regulatory Mechanisms in Flatfish.* Xiamen University, Xiamen, China.
9. **Jun Li, 2018.** *New Progress in the Study of Fish Phagocytic Cells.* Yellow Sea Fishery Research Institute, Qingdao, China.
10. **Jun Li, 2018.** *Overview of salmonid fish aquaculture and diseases control in the North America.* Qinghai Minzhe Cold-water Fish Aquaculture Co. Ltd., Qinghai, China.

2017

11. **Jun Li, 2017.** Emerging and re-emerging fish infectious diseases in the Great Lakes. Zhongkai University of Agriculture and Engineering, Guangzhou, Guangdong, China.
12. **Jun Li,** 2017. Innate and Adaptive Immunity in Aquatic Animals. Hainan University, Haiko, Hainan, China.
13. **Jun Li,** 2017. Emerging and re-emerging fish infectious diseases in the Great Lakes. Zhongshan University, Guangzhou, Guangdong, China.
14. **Jun Li,** 2017. *New findings on Innate and Adaptive Immunity in Aquatic Animals.* Ocean University of China, Qingdao, Shandong, China.
15. **Jun Li, 2017.** *Re-Emergence of Epizootic Epitheliotropic Disease Virus affecting*

Lake Trout in the Great Lakes. Huazhong Agricultural University, Wuhan, Hubei, China.

16. **Jun Li, 2017.** *Re-Emergence of Epizootic Epitheliotropic Disease Virus affecting Lake Trout in the Great Lakes.* Yellow Sea Fishery Research Institute, Qingdao, China.

2016

17. **Jun Li,** 2016. New findings of fish mucosal immune functions and its application in fish vaccination. Huazhong Agriculture University, Wuhan, China.
18. **Jun Li,** 2106. Application of Immunostimulants in Aquaculture. Xiamen University, Xiamen, China.
19. **Jun Li,** 2016. New findings of fish mucosal immune functions and its application in fish vaccination. Shanghai Ocean University, Shanghai, China.

2015

20. **Jun Li,** 2015. Adjuvant effects of QS saponins in Turbot (*Scophthalmus maximus*) upon bath vaccination. **The Third Institute of Oceanography, SOA,** Xiamen, China.
21. **Jun Li,** 2105. Phagocytic B cells at the crossroads of innate and adaptive immunity. Xiamen University, Xiamen, China.
22. **Jun Li,** 2105. Recent Progress on the Study of Fish Immunity. Huazhong Agriculture University, Wuhan, China.
23. **Jun Li,** 2015. Innate and Adaptive Immunity in Aquatic Animals. Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China.

2014:

25. **Jun Li,** 2014. The Mucosal Immunity in Teleost Fish. The Third Institute of Oceanography, SOA, Xiamen, China.
26. **Jun Li,** 2104. Phagocytic B cells: Innate or Adaptive? Zhejiang University, Hangzhou, China.
27. **Jun Li,** 2104. Phagocytic B cells: Innate or Adaptive? Huazhong Agriculture University, Wuhan, China.
28. **Jun Li,** 2104. Phagocytic B cells: Innate or Adaptive? Agriculture University of China, Beijing, China.
29. **Jun Li,** 2014. Innate and Adaptive Immunity in Aquatic Animals. Shanghai Ocean University. Shanghai, China.
30. **Jun Li,** 2014. Recent Progress on the Study of Fish Immunity. Qingdao Agriculture University. Qingdao, China.

2013:

31. **Jun Li,** 2013. Evolution of phagocytic B cells. The Third Institute of Oceanography, SOA, Xiamen, China.

32. **Jun Li.** 2013. Survival of *Edwardsiella tarda* in fish serum relates to bacterial surface LPS. East China University of Science and Technology, Shanghai, China.

33. **Jun Li.** 2013. The Involvement of Fish Complement in Innate and Adaptive Immune Responses. Institute of Oceanology, The Chinese Academy of Sciences, Qingdao, China.

2012:

34. **Jun Li,** 2012. Evolution of phagocytic B cells. Institute of Hydrobiology, The Chinese Academy of Sciences, Wuhan, China.

35. **Jun Li.** 2012. IgT, IgT+ B cells and mucosal Immunity in Teolest Fish. East China University of Science and Technology, Shanghai, China.

36. **Jun Li.** 2012. Influence of complement and antibody opsonization in the uptake of particles by Rinbow trout phagocytes. Yellow Sea Fishery Research Institute, The Chinese Fishery Academy of Science, Qingdao, China.

37. **Jun Li.** 2012. New discoveries of fish B cells and immunoglobulins. Institute of Oceanology, The Chinese Academy of Sciences, Qingdao, China.

38. **Jun Li.** 2012. Influence of complement and antibody opsonization in the uptake of particles by Rinbow trout phagocytes. Ocean University of China, Qingdao, China.

2011:

39. **Jun Li,** 2011. Evolution of phagocytic B cell and macrophage. Shandong University, Jinan, China.

40. **Jun Li,** 2011. Evolution of phagocytic B cells. Invited seminar. East China University of Science and Technology, Shanghai, China.

41. **Jun Li.** 2011. IgT, IgT+ B cells and mucosal Immunity in Teleost Fish. Yellow Sea Fishery Research Institute, The Chinese Fishery Academy of Science, Qingdao, China.

2010:

42. **Jun Li.** 2010, Phagocytosis and phagocytic B cells in teleost fish. Ocean University of China, Qingdao, China

43. **Jun Li,** 2010. Evolution of phagocytic B cells. Yellow sea Fishery Research Institute, Chinese Fishery Academy of Sciences, Qingdao, China.

44. **Jun Li,** 2010. A New Discovery of Phagocytic B cells in Teleost Fish. The Hong Kong University of Sciences and Technology, Hong Kong, China.

45. **Jun Li,** 2010. Evolution of phagocytic B cells. School of Fishery and Life Sciences, Shanghai Ocean University, Shanghai, China.

University and Public Service: (2014-present)

Institutional and School Service

- LSSU COVID-19 task force team member and Subcommittee member (2020)
- LSSU Tenure & Promotion Committee member (2019)
- LSSU FA Executive Committee member (2016-2017).
- LSSU General Studies Advisory Committee member (2017-).
- LSSU Institutional Animal Care and Use Committee (IACUC) member (2014-2017)
- LSSU College of Science and Environment (CoSE) Laboratory Safety Committee member (2019- present)
- LSSU CoSE graduate program committee member for discussing the future graduate program @LSSU (2018).
- To be a **Search Committee member** for new faculty of Biology (2014-15)
- To help LSSU 's General Education Assessment activities for assessing the Nat. Sci Gen Ed. Courses.
- LSSU professional program coordinator/advisor committee member (Pre-vet advisor)
- To organize the Biology Senior Research Symposium (2017, 2018, 2019, 2020).
- As a LSSU representative to attend **Mi-Transfer Summit Meetings** for discussing the transfer pathway among Michigan community colleges and universities.

Profession service:

1.) Editorial service :

Associate Editor: ***Frontiers in Immunology***, Comparative Immunology
Guest Editor for: ***Frontiers in Immunology*** *Special topics.*
Guest-Editor for: ***International Journal of Molecular Sciences***
Special issue: ***Fish Molecular Biology*** (Edit 53 papers)

Editorial Board Committee member:

1) ***Marine Life Science and Technology***
2) ***Progress of Fishery Sciences***
3) ***Journal of Dalian Ocean University***
4) ***Water Biology and Security***

2.) Peer-reviewing manuscripts for the following scientific journals:

Aquaculture
Aquaculture Reports
Central European Journal of Biology
Developmental and Comparative Immunology
Diseases of Aquatic Organisms
Fish & Shellfish Immunology
Frontiers in Immunology
Hydrobiologica
General and Comparative Endocrinology

Journal of Aquatic Animal Health
Journal of Biological Chemistry
Journal of Immunology
Journal of Fisheries of China
Journal of Fish Diseases
Journal of General Virology
Journal of Invertebrate Pathology
Journal of Ocean University of China
Journal of Shellfish Research
Journal of Virology Methods
Journal of Visualized Experiments
Marine Genomics (2 manuscripts)
Molecular Biology Reports
North America Fish Aquaculture
Oceanologica et Liminologica Sinica
Reviews in Aquaculture
Plos One
PNAS
Scientific Reports
Veterinary Microbiology
Virology Journal
Viruses
Virus Research

LAKE SUPERIOR
STATE UNIVERSITY

14 September 2022

Re: Recommendation for **Jun Li**

To the LSSU Sabbatical Committee:

It is my pleasure to write to you on behalf of Dr. Jun Li to provide support for his application for sabbatical. As part of his sabbatical, Jun is proposing to develop capabilities for growing and maintaining populations of zebrafish at LSSU. Zebrafish are organisms often used in whole organism toxicology models, which provide critical data for understanding the viability of organic compounds as potential drugs and/or toxins. Access to such facilities and expertise on campus would be a boon to research efforts at LSSU in the areas of chemistry (drug development) and cannabis chemistry (preliminary toxicology data of individual compounds found in cannabis plant matter).

Jun has reached out to myself and other chemistry faculty to build potential collaborations that could effectively utilize zebrafish facilities for our ongoing research studies in these areas. Based on Jun's record of accomplishments, I have no doubt that he would be able to develop useful zebrafish research capabilities during his proposed sabbatical and would subsequently work with the LSSU research community to maximize the potential of zebrafish research at LSSU.

Sincerely,

R. Adam Mosey, Ph.D.
Associate Professor
Department of Chemistry
Lake Superior State University
Sault Ste. Marie, MI 49783
(906) 635-2284; rmosey@lssu.edu

UNIVERSITY of MARYLAND
SCHOOL OF MEDICINE

Shaojun (Jim) Du
Professor

Department of Biochemistry and Molecular Biology
701 E. Pratt Street
Baltimore, MD 21202
410-234-8854
sdu@som.umaryland.edu

September 12,2022

To whom it may concern,

It is my pleasure to write the letter on behalf of Dr. Jun Li in support of his application for a sabbatical research with the zebrafish model.

I am currently a full professor at University of Maryland School of Medicine. My major scientific expertise and research achievements lie in using transgenic and genetic approaches to elucidate the molecular regulation of embryonic development and muscle cell differentiation, and the application of transgenic technology in aquaculture. I have been conducting research by using the zebrafish model in the past 30 years of my academic career.

As a small freshwater teleost, zebrafish possesses unique features such as small size, short reproductive cycle, clear genetic background, in vitro fertilization, transparent embryos and well-established genetics approaches. Zebrafish has becoming one of the most important vertebrate model systems, and been widely used in the studies on developmental biology, molecular biology, cell biology, genetics, neurobiology, oncology, immunology, marine biology, pharmacology, toxicology, and environmental protection. Recently, zebrafish is also commonly applied to study human diseases since higher similarities of tissues and organs in zebrafish and human.

Zebrafish is also a very affordable and easy to maintain in large numbers in a relatively small laboratory space. As Dr. Li discussed with me, he wants to set up a small zebrafish culture facility in supporting of undergraduate education and related research at Lake Superior State University. There is no doubt such facility will be not only a great benefit for animal biology, genetics, cellular and molecular biology, and developmental biology education, but also a very useful model for related research activities, such as organismal toxicity assays and environmental pollutants protections. I would like to provide technical supports and share my experience and expertise in zebrafish with Dr. Li, and welcome Dr. Li to visit my laboratory to gain some work experience in the zebrafish model system. I strongly support Dr. Li's application for a sabbatical research in zebrafish. Should you have any questions, please feel free to contact me.

Sincerely,

Shaojun (Jim) Du, Ph. D.
Professor
Department of Biochemistry and Molecular Biology
School of Medicine, University of Maryland
701 East Pratt Street, Baltimore, MD 21202, USA
Email: sdu@som.umaryland.edu

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Dr. Jun Li

School of Science and Medicine
Lake Superior State University
Sault Ste. Marie, MI 49783, USA

9/14/2022

Dear Dr. Li,

I am very excited to know that you are applying sabbatical leave for 2023-24, and plan to come back Qingdao China for conducting our collaborative research project in person. I am writing to invite you to join my laboratory during the summer or fall season next year to participate the ongoing research project on viral covert mortality disease (VCMD) in cultured shrimp, and their pathogenic effects on fish and the marine ecosystems.

As what I informed you last year, the proposed project was granted 670,000 Chinese Yuan, from Chinese Natural Science Fund for the period of 2021 to 2024. As one collaborator of the project, your participation, your expertized knowledge and skills in the field of aquatic animal health are very important for us to finish the project with high scientific expectation. During your sabbatical research in my laboratory, we will supply you the necessary office space, and full access to the facilities and equipment in the Laboratory of Marine Aquaculture Animal Disease Control and Histopathology of Yellow Sea Fishery Research Institute. You will work together with 2-3 graduate students involved in the project, and hope you can supervise their research activities. We also expect that you can give a scientific presentation during the period you stay in Qingdao.

We look forward to your arrival and work with us in Qingdao. Please let me know if you have any questions or need any help.

Best wishes

Prof. Qingli Zhang, Ph.D.

Head of Key Laboratory of Maricultural Organism Disease Control
Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences
Qingdao, 266071, P.R. China .

Awarded 670,000 RMB by China NSF for the period of 2021, 1-2024, 12

Dr. Li as a Co-PI.

申请代码	C190602
接收部门	
收件日期	
接收编号	

国家自然科学基金

申 请 书

（2020版）

资助类别：面上项目

亚类说明：

附注说明：Study on cross-species transmission of shrimp covert mortality nodavirus and its molecular mechanism

项目名称：对虾偷死野田村病毒跨物种传播及其分子机制研究

申 请 人：张庆利　　电 话：0532-85823062-812

依托单位：中国水产科学研究院黄海水产研究所

通讯地址：山东省青岛市南京路106号

邮政编码：266071　　单位电话：0532-85836340

电子邮箱：zhangql@ysfri.ac.cn

申报日期：2020年04月16日

国家自然科学基金委员会

基本信息

申请人信息	姓名	张庆利	性别	男	出生年月	1979年01月	民族	汉族
	学位	博士	职称	研究员		每年工作时间（月）		6
	是否在站博士后	否		电子邮箱		zhangql@ysfri.ac.cn		
	电话	0532-85823062-812		国别或地区		中国		
	个人通讯地址	山东省青岛市南京路106号						
	工作单位	中国水产科学研究院黄海水产研究所/养殖生物疾病控制与病原分子生物学研究室						
	主要研究领域							

依托单位信息	名称	中国水产科学研究院黄海水产研究所		
	联系人	安青菊	电子邮箱	kyc@ysfri.ac.cn
	电话	0532-85836340	网站地址	www.ysfri.ac.cn

合作研究单位信息	单位名称
	滨州医学院

项目基本信息	项目名称	对虾偷死野田村病毒跨物种传播及其分子机制研究		
	英文名称	Study on cross-species transmission of shrimp covert mortality nodavirus and it's molecular mechanism		
	资助类别	面上项目	亚类说明	
	附注说明			
	申请代码	C190602.水产生物病原学与流行病学		
	基地类别			
	研究期限	2021年01月01日 — 2024年12月31日	研究方向：病毒病	
	申请直接费用	67.0000万元		

中文关键词	病毒；传染源；粘附；侵染
英文关键词	virus; source of infection; adhesion; infection

中文摘要	近年来，对虾偷死野田村病毒（CMNV）引起的病毒性偷死病（VCMD）给我国对虾养殖业造成了严重经济损失。最新研究证实，CMNV能跨越物种障碍感染多种鱼类，使我国海水养殖业面临新的威胁。然而受制于CMNV跨物种传播范围、对新宿主致病性以及其跨物种传播分子机制等关键信息的缺失，VCMD的防治面临无从着手的困境。本项目拟在发现CMNV可感染鱼类的基础上：（1）调查海水养殖系统中CMNV跨物种感染的生物种类，研究CMNV对重要宿主种类的致病性，分析不同宿主生物生态位并明确其在病毒传播中的作用；（2）筛查不同宿主中CMNV的潜在同源受体，通过病毒基因组测序、蛋白三维结构模拟以及体外和体内验证实验，解析病毒粘附和侵染过程中关键蛋白的结构与功能，阐释CMNV跨物种传播的分子基础。研究结果将揭示CMNV跨物种传播的分子机制和VCMD大规模流行的病原生态学起因，为VCMD有效防控提供强有力的理论支撑。
英文摘要	Abstract Viral covert mortality disease (VCMD), causing by covert mortality nodavirus (CMNV), resulted in serious economic losses of shrimp aquaculture in China in recent years. The latest research confirmed that CMNV could cross the species barrier to infect teleost fishes, and posed a new threat to China's marine aquaculture industry. Effective control measures of VCMD were scarce and hard to take for the reason that the people had no knowledge of host ranges of CMNV cross-species transmission, it's pathogenicity to new hosts, and mechanism of CMNV cross-species transmission in molecular level. Based on the confirmation of CMNV cross-species infection in fish, research work that we planned to do in this proposal included: (1) Focusing on the marine culture systems, to conduct survey of the novel hosts that were CMNV cross-species infected, evaluating the pathogenicity of CMNV to key hosts, and investigating the ecological niche of the novel hosts species population and their role in viral transmission; (2) Screening for potential homologous receptors for CMNV in different hosts, sequencing and analyzing the genomic characteristics of CMNV isolates from different hosts, exploring the structure and function of key protein molecules in the process of virus attachment and infection, to illuminate the molecular basis of CMNV cross-species infection, and finally to fully understand the molecular mechanism of cross-species transmission of CMNV. The research work in this proposal will uncover the mechanism of cross-species transmission of CMNV and the reason of large scale epidemics of CMNV from the view of pathogenic ecology, and finally help to the effective prevention and control of VCMD.

项目组主要参与者（注：项目组主要参与者不包括项目申请人）

编号	姓名	出生年月	性别	职 称	学 位	单位名称	电话	电子邮箱	证件号码	每年工作时间（月）
1	刘庆慧	1962-07-05	女	研究员	博士	中国水产科学研究院黄海水产研究所	0532-85823062-812	liuqh@ysfri.ac.cn	3**********7	4
2	李军 (JUN LI)	1968-12-21	男	副教授	博士	美国苏必利尔湖州立大学	+1-9066352094	jli@lssu.edu	G**********8	2
3	刘秀珍	1976-12-17	女	副教授	博士	滨州医学院	0543-3258367	liuxiuzhen0704@126.com	3**********2	2
4	万晓媛	1984-09-17	女	助理工程师	硕士	中国水产科学研究院黄海水产研究所	0532-858230622	wanxy@ysfri.ac.cn	3**********1	6
5	王崇	1991-04-06	女	博士生	硕士	中国水产科学研究院黄海水产研究所	0532-858230622	1834142471@qq.com	1**********4	10
6	李英瑕	1995-05-12	女	硕士生	学士	中国水产科学研究院黄海水产研究所	0532-858230622	1033913700@qq.com	3**********1	10
7	姚亮	1996-09-30	男	硕士生	学士	中国水产科学研究院黄海水产研究所	0532-858230622	yaoliang2019@163.com	5**********2	10
8	王伟	1997-11-28	女	硕士生	学士	中国水产科学研究院黄海水产研究所	0532-858230622	13173113773@163.com	3**********7	10

总人数	高级	中级	初级	博士后	博士生	硕士生
9	4	1	0	0	1	3

Aquaculture 546 (2022) 737378

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Pathogenicity study of covert mortality nodavirus (CMNV) infection in zebrafish model

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[a] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Function Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Key Laboratory of Maricultural Organism Disease Control, Ministry of Agriculture, Qingdao Key Laboratory of Mariculture Epidemiology and Biosecurity, Qingdao 266071, China

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ARTICLE INFO

Keywords:
Zebrafish model
Covert mortality nodavirus (CMNV)
Infection
Pathogenicity

ABSTRACT

Covert mortality nodavirus (CMNV), a new emerging shrimp virus, was capable of crossing species barriers to infect fish. However, impending studies in elucidating pathogenicity of CMNV in fish have been limiting due to the lack of appropriate animal disease models. An attempt of establishing an *in vivo* model of CMNV infection in zebrafish (*Danio rerio*) were conducted in present study. Further, CMNV infection in zebrafish important tissues was also investigated by using the methods of fatality rate statistics, histopathology, *in situ* hybrization (ISH) and transmission electron microscopy (TEM). Behavioral observations of the infected zebrafish showed that the infected individuals exhibited an abnormal spiraling swimming activity, and some individuals had exceptionally protruding eyes. The eye and brain tissues of artificially infected zebrafish were proved to be CMNV-positive by ISH, and pathological changes like vacuolation of nervous tissue were observed in this two tissues. Analysis of TEM also confirmed masses of CMNV-like virus particles in the brain and eye tissues of the infected zebrafish. CMNV positive signals of ISH were also detected in muscle, intestine and gill tissues, and myolysis in the muscle, dropping of epithelial cells in gill were found in the same site with positive signals. In addition, CMNV could cause 53.33% mortality of zebrafish within 14 days of post challenge injection. The establishment of the zebrafish infection model of CMNV offered a valuable tool for further investigations of the host-virus interactions, especially the underlying molecular mechanisms of CMNV for crossing species barrier to infect fish.

1. Introduction

Covert mortality nodavirus (CMNV), the pathogenic agent of viral covert mortality disease (VCMD), caused severe economic losses to the shrimp industry in China and the Southern Asian countries in recent years (Xing, 2004; Zhang, 2004; Zhang et al., 2017; Zhang et al., 2014). This new emerging virus could infect the major farming shrimp species including *Penaeus chinensis*, *Penaeus japonicas*, *Penaeus monodon* and *Macrobrachium rosenbergii* (Xing, 2004; Zhang, 2004; Zhang et al., 2017; Zhang et al., 2014). Other crustaceans species that co-exist in the shrimp farming ponds, such as *Parathemisto gaudichaud, Corophium sinense Zhang, Diogenes edwardsii* and *Parathemisto gaudichalldi* were susceptable to the CMNV too (Liu et al., 2018; Zhang et al., 2014).

CMNV is a novel species in the genus *Alphanodavirus*, one of the two genera in the family *Nodaviridae* (Briddon et al., 2005). *Nodamura virus* (NoV), the type species of *Alphanodavirus* genus, origianlly isolated from mosquitoes (*Culex tritaeniorhynchus*) (Johnson et al., 2003; Scherer and Hurlbut, 1967; Tesh, 1980), is lethal to mammals (Johnson et al., 2004; Scherer et al., 1968). Moreover, the latest research found that some fish species, including *Parallichthys olivaceus, Mugilogobius abei* and *C. auratus*, also could be infected by CMNV and resulted in apparent multiple organ damage in the affected individuals (Wang et al., 2019; Wang et al., 2018; Zhang et al., 2018). Overall, the above mentioned studies showed that CMNV was not only responsible for the new emerging diseases of shrimps, but also could cross species barriers and infect fish under natural conditions. However, there is no literature reported the pathogenicity of CMNV to fish and target tissues of virus proliferation currently.

As the most commonly used model animal, zebrafish (*Danio rerio*), possesses typical features such as small size, short reproductive cycle,

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https://doi.org/10.1016/j.aquaculture.2021.737378
Received 8 November 2020; Received in revised form 18 August 2021; Accepted 19 August 2021
Available online 21 August 2021

clear genetic background, *in vitro* fertilization and embryo development transparency (Tavares and Lopes, 2013). Thus far, zebrafish has been extensively used as model species in studies of developmental biology, genetics, drug screening, disease mechanism, toxicity testing, and environmental monitoring (Garnett et al., 2012; Hu et al., 2016; Liu and Dai, 2015; Ma et al., 2017; Xie et al., 2009; Yang et al., 2018; Zhang et al., 2018). Short reproductive cycle and abundance of genetic tools make the zebrafish an attractive model host to understand host-pathogen interactions (Briolat et al., 2014). After the whole-genome sequencing of zebrafish was completed, zebrafish has been effectively applied in the research to explore virus pathogenicity and virus-host interaction (Guerra-Varela et al., 2018; Johansen and Kremer, 2020; Rakus et al., 2020; Rakus et al., 2019). It has been successfully used in *Betanodavirus* and Infectious Hematopoietic Necrosis Virus (IHNV), in elucidating virus-host interactions (Lu et al., 2008; Ludwig et al., 2011). Therefore, zebrafish was chose to conduct more research on CMNV infecting fish in present study.

In order to better understand the pathogenicity of CMNV to fish, as well as to identify the primary target tissues of virus proliferation in fish, zebrafish were experimentally injected with CMNV in this study. The clinical signs of infected individuals were recorded and histopathological characteristics of major target tissues in the diseased fish were analyzed by using various techniques of molecular biology, histopathology (H&E), *in situ* hybridization (ISH), and transmission electron microscopy (TEM).

2. Materials and methods

2.1. Fish and virus preparation

Specific pathogen free (SPF) zebrafish (Wild-Type-AB Line, average length 3–4 cm) were purchased from an ornamental fish breeding center in Guangzhou, and the original source were traced from China Zebrafish Resource Center (CZRC), and Catalog ID was CZ1. The zebrafish was declared and tested to be negative for *A. hydrophila*, *A. sobria*, *A. veronii*, *Edwardsiella tarda*, *E. ictaluri*, *Flavobacterium columnare*, *Plesimonas shigelloides*, *Vibrio spp.*, *Mycobacterium* spp. and *Pseudoloma neurophilia*. The zebrafish were raised for acclimatisation in laboratory for 15 days before the infection experiment. However, no outside institution could provide CMNV detection currently, so the SPF zebrafish couldn't be ensured that they were free of CMNV. Therefore, before challenging experiment, 10 randomly selected zebrafish were collected to CMNV diagnosis by reverse transcription nested PCR (RT-nPCR) and ISH, and the results were all negative.

The maintenance of zebrafish was as reported previously (Avdesh et al., 2012; Kim et al., 2017). Briefly, clinically healthy zebrafish were reared and maintained at 26–28.5 °C in water tanks (volume of 20 dm^3) with continuous aeration. The 50% water in the tanks was changed daily. These zebrafish were fed 3 times daily with granulated feed. The CMNV virus particles used for the challenge was prepared according to the method previously reported (Pereiro, 2017; Zhang et al., 2014).

2.2. Experimental infection and sample collection

CMNV-infection group (virus infection) and control group were arranged in experimental infection. Each group included three replicates, and there were 10 zebrafish in each replicate. CMNV injection was conducted following the previous reports (Benard et al., 2012), and each individual in the CMNV-infection group was injected with 10 μL CMNV (5.6×10^5 copies/μL). Each individual in the control group was injected with 10 μL TN buffer (Tris-HCl 20 mM, NaCl 400 mM, pH 7, distilled water preparation, being used as dilution buffer in virus purification). Status and behaviors of fish were observed and recorded daily. Before tissue collection, fish were euthanized with an overdose of tricaine methanesulfonate (Sigma, St. Louis, USA) as reported previously (Wilson et al., 2009). The head of zebrafish (including eye, brain and gill tissues), intestine and muscle were collected based on the infectious characteristics of nodavirus, and preserved in 4% paraformaldehyde (4% PFA), RNAstore solution and 2.5% glutaraldehyde solution for further molecular biological, histopathological and electron microscopic analysis as reported (Zhang et al., 2018).

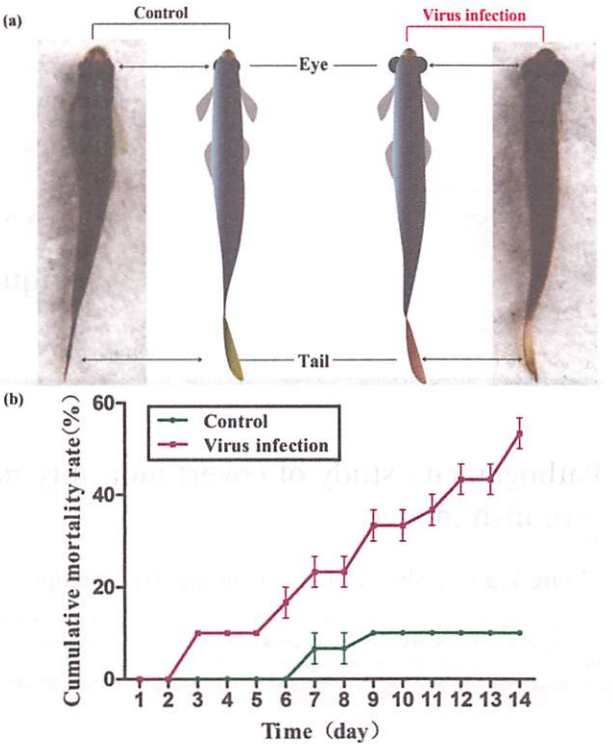

Fig. 1. The clinical signs observation and mortality statistical analysis of diseased zebrafish. (a) Clinical signs of zebrafish infecting from covert mortality nodavirus (CMNV). Shown on the left were the real and linear images of the zebrafish without CMNV infection in the control group, the right were the real and line graphs of the zebrafish in the infected group. Pay attention to compare the zebrafish in the infected group had abnormal protruding eyeballs and red-brown tails. (b) Statistical analysis of mortality in the artificial infection of zebrafish experiment. Cumulative mortalities of zebrafish were shown as means of data from three replicates for each experimental group (each replicate included 10 individuals). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Histopathological section

The tissue samples, including eye, brain, muscle, gill and intestine, in 4% PFA fixative were transferred to 70% ethanol after 24 h incubation, then embedded in paraffin blocks according to the methods reported by Bell and Lightner (Bell and Lightner, 1988). Each sample was prepared into triplicate paraffin sections (3 μm) for further histological and *in situ* hybridization (ISH) analyses according to the reported protocols (Lightner et al., 1996; Zhang et al., 2018).

2.4. In situ RNA hybridization section

The DIG-labeled CMNV probe, a 244 bp fragment of the RdRp gene, was prepared according to the method reported by Zhang (Zhang et al., 2017). The sections of fish tissues were subjected to *in situ* hybridization according to the protocols previously reported (Chen et al., 2014; Piette et al., 2008), and the sections post ISH were stained with Nuclear Fast Red solution for counterstaining as described (Nuovo et al., 1999). *In situ*

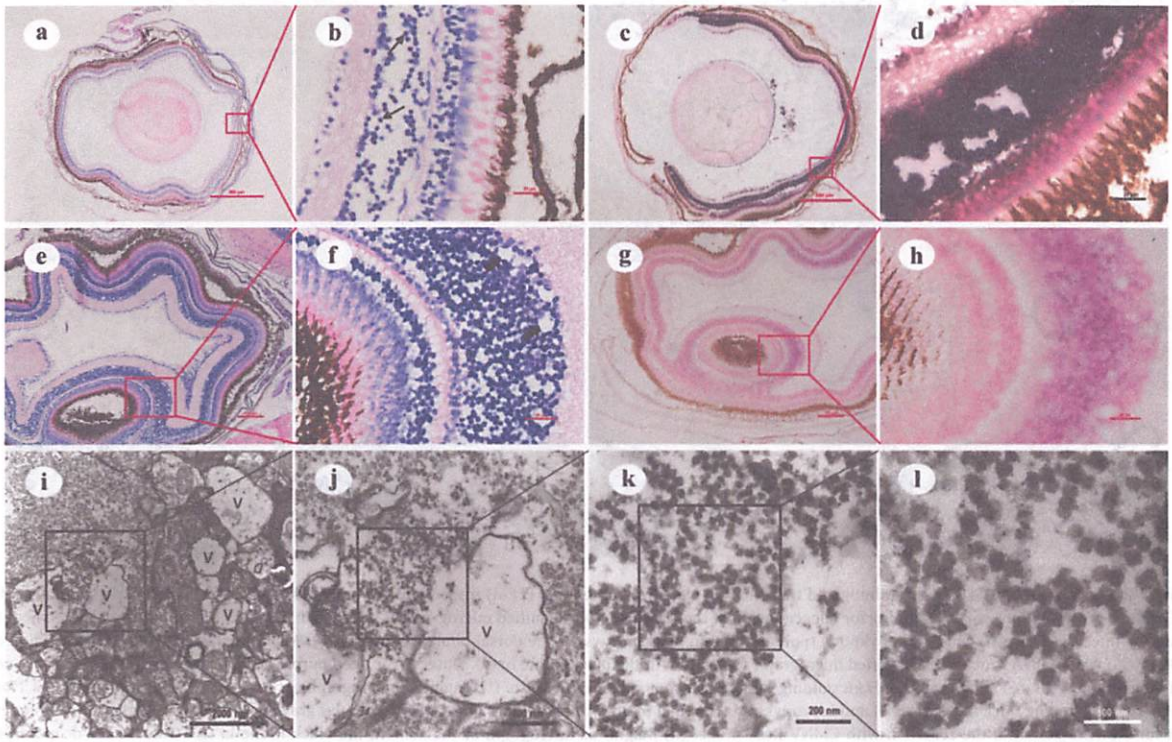

Fig. 2. *In situ* hybridization (ISH), H&E staining and transmission electron microscopic (TEM) inspections for eyes tissue of the zebrafish experimentally infected with CMNV. (a) Micrographs of H&E staining for eye retina of the zebrafish. (b) Magnified micrograph of the zone in the black frame in (a). (c) Micrographs of ISH for eyes of the zebrafish with CMNV RNA probe. (d) Magnified micrograph of the zone in the black frame in (c). (e) Micrographs of H&E staining for eye retina of the zebrafish. (f) Magnified micrograph of the zone in the black frame in (e). (g) Micrographs of ISH for eyes of the zebrafish with CMNV RNA probe. (h) Magnified micrograph of the zone in the black frame in (g). Observe serious vacuolation (pointed by thick black arrows) and karyopyknosis (pointed by thin black arrows) at the bipolar cells layer and ganglion layer of eyes, simultaneously note the dispersion of the ganglion layer. Observe the deep purple hybridization signals at the bipolar cells layer and light purple signals in the ganglion layer. (i-l) Micrographs of ultrathin section for eye of the experimentally infected zebrafish with CMNV visualized under TEM. Micrograph of (j),(k) and (l) showed the magnified zone in the black frame in (i)、(j) and (k), respectively. Note that the scattering CMNV-like particles in (i-l). Scale bars = (a and c) 500 μm, (e and g) 100 μm, (b, d, f and h) 20 μm, (i) 1000 nm, (j) 500 nm, (k) 200 nm, (l) 100 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hybridization sections were examined under the Nikon Eclipse E80i microscope (Nikon Co., Tokyo, Japan).

2.5. Transmission Electron microscopy

The samples (approximately $1mm^3$) were firstly preserved in TEM fixative for 24 h at 4 °C, and followed further fixation with 1% osmium tetroxide for 2 h, and then embedded in Spurr's resin according to the instructions (Fortunato et al., 2016; Zechmann and Zellnig, 2009). Then ultra-thin sections (50 nm) were prepared and stained with uranyl acetate and lead citrate as the previously reported method and examined by a JEOL JEM-1200 electron microscope (Graham and Orenstein, 2007; Panphut et al., 2011).

3. Results

3.1. Observation of clinical signs of infected zebrafish and cumulative mortality rate

In the group of zebrafish with CMNV infection, the infected fish exhibited obvious clinical signs, including decreased swimming ability, eyeball enlargement and abnormality, as well as "red-brown" colored tail (Fig. 1a). In addition, the infected zebrafish showed looping or spiral swimming in a belly-up position and loss of coordination. However, in the control group, all fish did not show any obvious symptoms as above mentioned. The cumulative mortality of CMNV infected group was up to 53.33% within 14 days post-injection (Fig. 1b). In contrast, the cumulative mortality of individuals in the control group was 13.33% (Fig. 1b), which might be caused by mechanical injury from the injection syringe. Taken together, after being infected by CMNV, the zebrafish exhibited typical clinical signs, and with a relatively high mortality.

3.2. Detection of CMNV in the eye by H&E, ISH and TEM

The histopathological effects of CMNV infection on the eyes of zebrafish were investigated through observation of H&E stained sections. Severe vacuolations were observed in the bipolar cell layer, and obvious diffusion and vacuolations were also appeared in the ganglion cell layer of CMNV infected eyes (Fig. 2a, b, e and f). Moreover, CMNV-positive hybridization signals could be detected in the bipolar cell layer and ganglion cell layer via *in situ* hybridization analysis (Fig. 2c, d, g and h). A large number of CMNV-like virus particles could be directly observed in the ganglion cells layer under transmission electron microscope (Fig. 2i-l).

3.3. Detection of CMNV in the brain by H&E, ISH and TEM

Regarding to the brain of CMNV infected zebrafish, the H&E and ISH results demonstrated that there were much serious vacuolation and karyopyknosis in the nerve cells region of dorsal olfactory nucleus of the

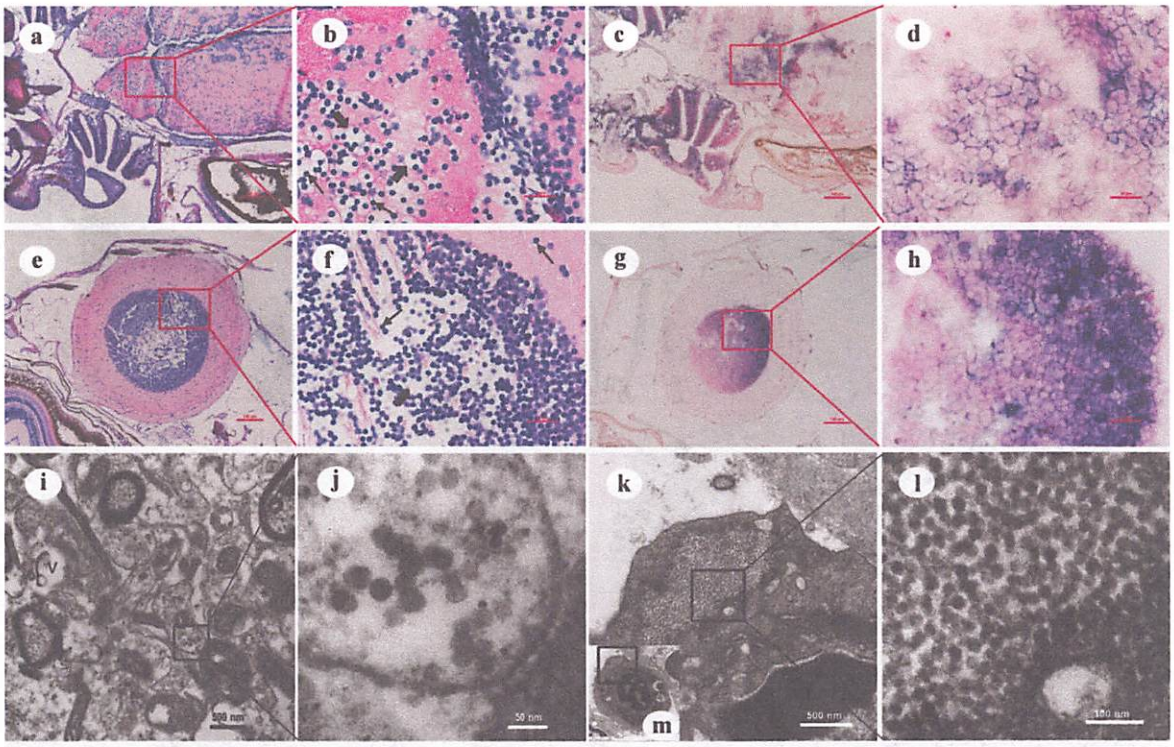

Fig. 3. *In situ* hybridization (ISH), H&E staining and transmission electron microscopic (TEM) inspections for brain tissue of the zebrafish experimentally infected with CMNV. (a) Micrographs of H&E staining for telencephalon of the zebrafish. (b) Magnified micrograph of the zone in the black frame in (a). Note the vacuolation (pointed by thick black arrows) and karyopyknosis (pointed by thin black arrows) at the dorsal olfactory nucleus. (c) Micrographs of ISH for telencephalon of the zebrafish with CMNV RNA probe. (d) Magnified micrograph of the zone in the black frame in (c). Observe the deep purple hybridization signals at the nerve cells of dorsal olfactory nucleus. (e) Micrographs of H&E staining for mesencephalon of the zebrafish. (f) Magnified micrograph of the zone in the black frame in (e). Note the vacuolations (pointed by thick black arrows) and karyopyknosis (pointed by thin black arrows) at the periglomerular gray zone of corpora bigemia. (g) Micrographs of ISH for mesencephalon of the zebrafish with CMNV RNA probe. (h) Magnified micrograph of the zone in the black frame in (g). Observe the deep purple hybridization signals at the periglomerular gray zone. (i-m) Micrographs of ultrathin section for brain and granular cells of the experimentally infected zebrafish with CMNV visualized under TEM. Micrograph of (j) showed the magnified zone in the black frame in (i), Micrograph of (l) and (m) showed the magnified zone in the black frame in (k) and (l), respectively. Note that the scattering CMNV-like particles in (j) and (l). Scale bars = (a, c, e and g) 100 μm, (b, d, f and h) 20 μm, (i and k) 500 nm, (j) 50 nm, (l) 100 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

telencephalon (Fig. 3a and b). Meanwhile, there were a large number of blue-purple CMNV-positive hybridization signals in the area of telencephalon (Fig. 3c and d). In addition, a large amount of vacuolation and karyopyknosis (Fig. 3e and f) and many hybridization signals (Fig. 3g and h) were found in the periglomerular gray zone of mesencephalon. Consistent with the above results, numerous CMNV-like virus particles were clearly observed in the zebrafish brain tissue (Fig. 3i and j) and its granular cells (Fig. 3k, l and m).

3.4. Detection of CMNV in the muscle, gill and intestine by H&E and ISH

In pathological sections of CMNV-infected zebrafish muscle tissue, severe dissolution-like necrotic muscle fibers and karyopyknosis were observed (Fig. 4a and b), and intensive deep purple CMNV-positive hybridization signals were also detected at the same regions with necrotic muscle tissues (Fig. 4c and d). In the pathological sections of gill, the secondary lamella became disordered and even distorted, some of its epithelial cells dropped, and the support function of column cells was lost (Fig. 4e and f). Furthermore, intense purple CMNV-positive hybridization signals were discovered in the same area of gill (Fig. 4g and h). In the pathological sections of intestine, karyopyknosis of infiltrated lymphocytes was obvious (Fig. 4i and j). Intensive positive hybridization signals of CMNV probe could be observed in the lymphocytes and the nucleuses of columnar epithelial cells (Fig. 4 k and l).

4. Discussion

Although CMNV is a new virus isolated originally from cultured shrimp, it had been demonstrated the capability of infecting wild or farmed fish (Wang et al., 2019; Wang et al., 2018). No study has, to our knowledge, been carried out to demonstrate that CMNV can infect fish artificially, so that the pathogenicity of CMNV to fish could be revealed. In the present study, we conducted the experimental challenge test to investigate the virulence and pathogenicity for CMNV to zebrafish. The results showed that CMNV could infect zebrafish, and showed virulence to zebrafish. In addition, the artificial infection of CMNV in zebrafish led to serious pathological changes that occurred in eye, brain, muscle, gill and intestine tissues.

The experimental challenge test revealed that CMNV could cause 53.33% mortality of zebrafish within 14 days of post challenge injection, which indicated that CMNV infection was virulent to zebrafish. Interestingly, CMNV infection could lead to abnormal swimming behavior of zebrafish, which mainly manifested in spiral-shaped vertical or horizontal swimming. Meanwhile, some infected individuals showed symptoms including abnormal prominent eyeballs and red-brown colored tail.

The histopathological analysis of the tissues of infected zebrafish indicated that obvious pathological changes had occurred in the eye retina, brain, muscle, gill and intestine. Subsequently, micrographs of

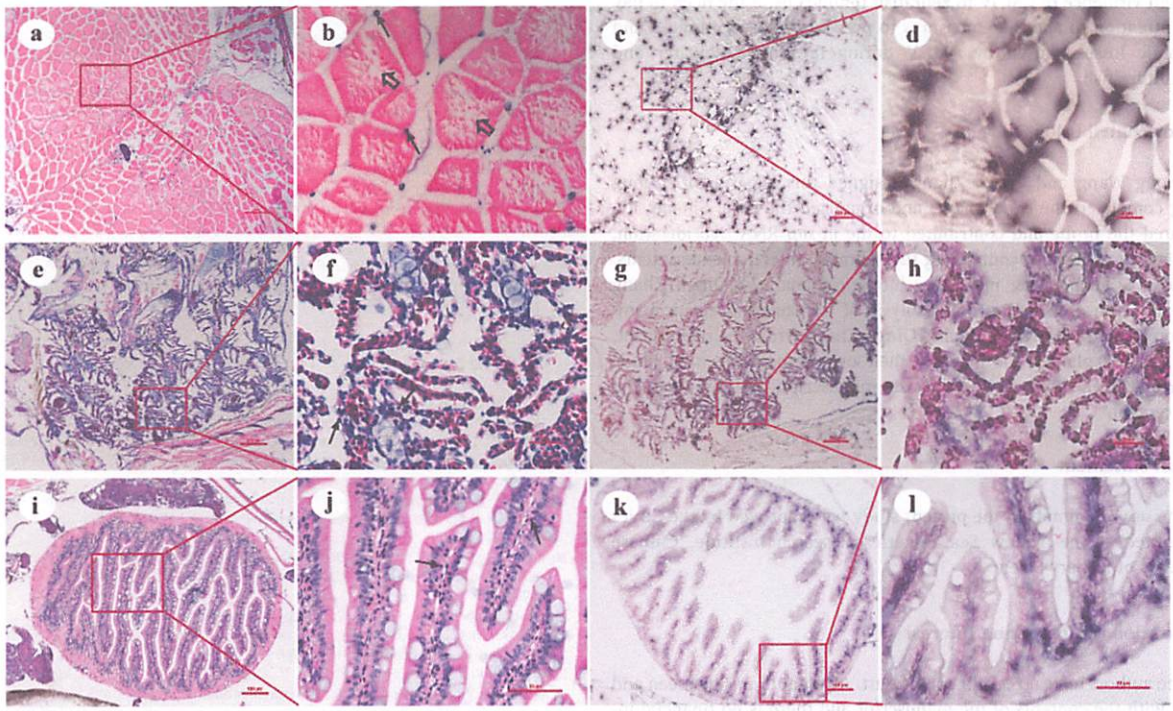

Fig. 4. *In situ* hybridization (ISH) and H&E staining for muscle, gill and intestine tissues of the zebrafish experimentally infected with CMNV. (a) Micrographs of H&E staining for muscle. (b) Magnified micrograph of the zone in the black frame in (a). Observe the severe dissolved necrosis (pointed by leary arrows) and karyopyknosis (pointed by thin black arrows) of the muscle. (c) Micrographs of ISH for muscle of the zebrafish with CMNV RNA probe. (d) Magnified micrograph of the zone in the black frame in (c). Observe the intense purple hybridization signals at the muscle tissue of zebrafish. (e) Micrographs of H&E staining for secondary lamella of the gill. (f) Magnified micrograph of the zone in the black frame in (e). Note the disorder and even distortion of the secondary lamella, the shedding of some epithelial cells and karyopyknosis (pointed by thin black arrows). (g) Micrographs of ISH for gill of the zebrafish with CMNV RNA probe. (h) Magnified micrograph of the zone in the black frame in (g). Observe the large number purple hybridization signals at the secondary lamella. (i) Micrographs of H&E staining for intestine. (j) Magnified micrograph of the zone in the black frame in (i). Note the karyopyknosis of lymphocytes (pointed by thin black arrows). (k) Micrographs of ISH for intestine of the zebrafish with CMNV RNA probe. (l) Magnified micrograph of the zone in the black frame in (k). Observe the large number purple hybridization signals at the lymphocytes, and few signals at the mucosa columnar epithelial cells. Scale bars = (a, c, e, g, i and k) 100 μm, (b, d, f and h) 20 μm, (j and l) 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ISH for infected samples showed that the intense purple positive hybridization signals of CMNV RNA probes were present in above-mentioned tissues, which occurred in sites of tissue with pathological changes. Vacuolation and nuclear shrinkage lesions had been found in the telencephalon, mesencephalon and retina of zebrafish and occuring of CMNV infection were also confirmed by ISH and TEM assays at these tissues.

It is known that viral species in genus *Betanodavirus* mainly target the eye and brain of their fish hosts, resulting in viral nervous necrosis or viral encephalopathy and retinopathy (Chi et al., 2001; Furusawa et al., 2007). CMNV showed similar pathogenicity to zebrafish, and resulted in encephalopathy and retinopathy as those viruses of *Betanodavirus* (Wang et al., 2019; Wang et al., 2018). According to previous studies (Blader and Strähle, 2000), the muscle, gill and intestine, especially brain and eye, the main component of the central nervous system, play vital roles to keep fish being survival in water. Therefore, retinopathy and damages of nervous system caused by CMNV infection were deduced to be responsible for the abnormal swimming behavior and eyeball protrusion of the infected zebrafish.

Furthermore, the behavioral and pathological changes of the zebrafish (model organism) infected with CMNV were pretty high similarity with those showed of the diseased shrimp (natural host). In behavior, the diseased moribund shrimp sank to the bottom of deep water and were rarely found in shallow water or swimming under the surface water (Zhang et al., 2014). Similarly, the infected zebrafish showed decreased swimming ability. Pathologically, the obvious pathological changes such as the dissolving necrosis of muscle, vacuolation and karyopyknosis appeared in the targeted tissues of the infected zebrafish, which was similar to the diseased shrimp (Zhang et al., 2017; Zhang et al., 2014). As a result, the comparison mentioned above might be better emphasise the usability of the zebrafish model for studying the mechanism of CMNV infection.

It has also been verified that three kinds of teleostean fishes, including *Mugilogobius abei, C. auratus* and *Paralichthys olivaceus*, could be infected by CMNV in natural condition (Wang et al., 2019; Wang et al., 2018; Zhang et al., 2018). *P. olivaceus* was a kind of mariculture fish, *M. abei* was a mariculture fish species but could grow in estuary brackish waters, however, zebrafish that used in this study was freshwater fish. Therefore, the results from the previous and present study demonstrated that both mariculture and freshwater fish could be infected by CMNV. The characteristic of broad host tropism of CMNV reminded it's potential risk threat to other fish species.

In addition, the CMNV infection in *M. abei* was occurred under the natural condition according to the previous report (Zhang et al., 2018). And the CMNV-free *M. abei* cohabitating in shrimp farming ponds started to be infected by CMNV after that CMNV-positive shrimp post-larvae were introduced into the ponds. The result of *M. abei* revealed the risk of CMNV transmission through waterborne. It can be speculated that the susceptibility of zebrafish to waterborne CMNV does exist, and the risk of CMNV infection natural in zebrafish deserves more attention in consideration the fish is widely used as a model fish in the world.

5. Conclusion

In summary, this study confirmed firstly that CMNV could infect freshwater species of zebrafish with intraperitoneal cavity injection and lead to a relatively high mortality rate. Meanwhile, the result showed that zebrafish could serve as an animal model for further study CMNV pathogenesis and neuron degeneration research in the future. Moreover, the high virulence of CMNV to zebrafish reminds us of the need to pay close attention to and take measures to prevent disease outbreaks and economic losses caused by CMNV during farming freshwater and mariculture fish.

Author contributions

Chong Wang and Qingli Zhang designed this experiment. Chong Wang completed the experiments and organized the data. Shuang Liu prepared the probe and contributed to the *in situ* RNA hybridization. Tingting Xu assisted in completing the zebrafish infection experiments. Xiaoping Li help doing molecular biological analysis. Qingli Zhang conducted the observation and analysis of transmission electron microscopy. Chong Wang, Jun Li and Qingli Zhang wrote the manuscript. All authors explained the data for their own responsible part, made important intellectual revisions to the manuscript, and approved the final version.

Ethical statement

The use of animals in the present study was approved by the Ethics Committee of the Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences.

Declaration of Competing Interest

The authors have no conflict of interest. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

Acknowledgements

This work was supported by the National R&D Program of China (2017YFC1404503), National Natural Science Foundation of China (32073016), Central Public-interest Scientific Institution Basal Research Fund, CAFS (NO. 2020TD39), Projects of International Exchange and Cooperation in Agriculture, Ministry of Agriculture and Rural Affairs of China-Science, Technology and Innovation Cooperation in Aquaculture with Tropical Countries along the Belt and Road, and Central Public-interest Scientific Institution Basal Research Fund, YSFRI, CAFS (NO. 20603022020005).

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frontiers in Marine Science

ORIGINAL RESEARCH
published: 11 March 2022
doi: 10.3389/fmars.2022.789128

Investigation on Natural Infection of Covert Mortality Nodavirus in Large Yellow Croaker, *Larimichthys crocea*

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Covert mortality nodavirus (CMNV) recently became more prevalent, severely affecting farmed shrimps, and also other invertebrates and teleost fish, in the coastal areas of China. For better understanding of its prevalence and susceptibility of large yellow croaker *Larimichthys crocea* to CMNV, an epidemiological survey was carried out in the main producing areas (Ningbo, Zhejiang, China, and Ningde, Fujian, China) and its offshore feeding grounds in the Southern Yellow Sea. The collected cultured *L. crocea* individuals showed clinical symptoms such as anorexia and abnormal swimming and diagnosed for CMNV infection by using RT-nPCR assay. The positive rates for CMNV in the collected *L. crocea* samples from Ningbo, Ningde, and the Yellow Sea were 14.29% (4/28), 20.00% (7/35), and 16.67% (1/6), respectively. Furthermore, CMNV diagnosis using TaqMan probe-based reverse transcription quantitative PCR (TaqMan RT-qPCR) yielded positive rates of 25.00% (7/28), 22.86% (8/35), and 16.67% (1/6) respectively in the above-mentioned samples, and with a range of 12.73–3,108.33 of CMNV copy numbers/μg total RNA. Phylogenetic tree analysis based on the RNA-dependent RNA polymerase (RdRp) and capsid protein genes showed that CMNV isolates from *L. crocea* samples were clustered tightly with the original isolate of CMNV from *Penaeus vannamei*. The histopathological studies indicated that severe cellular necrosis occurred in the heart, liver, spleen, eye, and gill of naturally infected fish. Stronger positive signals for CMNV-specific probes appeared in the necrotic cells and tissues in the *in situ* hybridization (ISH) analysis. Transmission electron microscopy (TEM) revealed the presence of numerous 30 nm diameter CMNV-like viral particles. The purified CMNV suspension could cause a significant cytopathic effect (CPE) in epithelioma papilloma cyprini (EPC) cells and resulted in the formation of typical inclusion bodies, and also visible CMNV particles around the lysed EPC cells under the TEM. Taken together, all results of this study demonstrated that *L. crocea* is susceptible to CMNV. The prevalence of CMNV and its potential impacts on the wild population of *L. crocea* see the need for further investigations.

Keywords: covert mortality nodavirus (CMNV), large yellow croaker (*Larimichthys crocea*), cellular necrosis, cytopathic effect, susceptibility

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Marine Science

Received: *05 October 2021*
Accepted: *09 February 2022*
Published: *11 March 2022*

Citation:
Xu TT, Fan YD, Jia TC, Wang C, Wang W, Li J, Zhang QL and Yao CL (2022) Investigation on Natural Infection of Covert Mortality Nodavirus in Large Yellow Croaker, Larimichthys crocea. Front. Mar. Sci. 9:789128. doi: 10.3389/fmars.2022.789128

INTRODUCTION

Covert mortality nodavirus (CMNV), an emerging alphanodavirus, severely impacted the shrimp aquaculture industries in China and Southeast Asian countries in the past decade (Zhang et al., 2014; Flegel, 2015; Thitamadee et al., 2016). Epizootic investigations recorded CMNV prevalence in farmed shrimp at the annual rates of 45.93%, 27.91%, 20.85%, 26.8%, and 16.3% respectively during the period of 2013–2017 in the major shrimp producing provinces in China (Zhang et al., 2017). Except for wide geographical prevalence, CMNV has also shown much broader host ranges. Previous studies had confirmed that CMNV could infect the main cultured crustaceans, including *Penaeus vannamei*, *Penaeus chinensis*, *Marsupenaeus japonicus*, *Penaeus monodon*, and *Macrobrachium rosenbergii*, and result in multiple tissue or organ damages (Zhang et al., 2014, 2017). Recent studies demonstrated that CMNV could cross species barriers to infect a variety of cultured and wild fish, such as cultured Japanese flounder *Paralichthys olivaceus*, wild gobiid fish *Mugilogobius abei*, and wild crucian carp *Carassius auratus*, which were collected from shrimp ponds or drainage channel affected by CMNV (Zhang et al., 2018; Wang et al., 2019a,b). More recently, varying prevalence (from 7 to 18%) of CMNV infection was reported in wild small yellow croaker *Larimichthys polyactis* sampled from coastal water of China in 2018–2019 (Xu et al., 2021). Furthermore, zebrafish (*Danio rerio*) also could be artificially infected with CMNV and the infected fish would exhibit abnormal spiraling swimming activity, with a cumulative mortality rate of 53.33% within 14 days of postchallenge injection (Wang et al., 2021). The above studies revealed that CMNV has been widely distributed in the farmed aquatic animals of coastal areas, and also the wild hosts from the offshore waters. The appearance of certain negative effects on teleost fishes has raised concerns about the prevalence of CMNV in both cultured and wild fish from the coastal areas.

The large yellow croakers (*Larimichthys crocea*) mainly distribute in the southeast coast of China, including the Southern Yellow Sea, the East China Sea, and the Northern South China Sea (Li and Yue, 2019; Wang et al., 2019). Due to overfishing, the natural resources of *L. crocea* have been seriously damaged and even on the verge of exhaustion (Chen et al., 2020). In recent years, with the success of artificial breeding, the *L. crocea* aquaculture industry has rapidly developed and *L. crocea* has become one of the most economically important marine fish species, especially in the provinces of Fujian (mainly in Ningde) and Zhejiang (mainly in Ningbo), the top two largest *L. crocea* producing provinces in China (Ministry of Agriculture and Rural Affairs of the People's Republic of China, 2021). However, more and more new emerging and reemerging infectious diseases, caused by bacteria, parasites, and viruses, have become the most important restricting factor for the healthy and sustainable development of *L. crocea* mariculture in China (Chen et al., 2003; You and Li, 2003; Shen et al., 2004; Wang et al., 2010; An, 2013; Xu et al., 2015). With big concern on the recent extremely high prevalence of CMNV in coastal aquaculture animals in China, in this study, we investigated the potential CMNV prevalence, and its histopathological characteristics in farmed and wild *L. crocea*

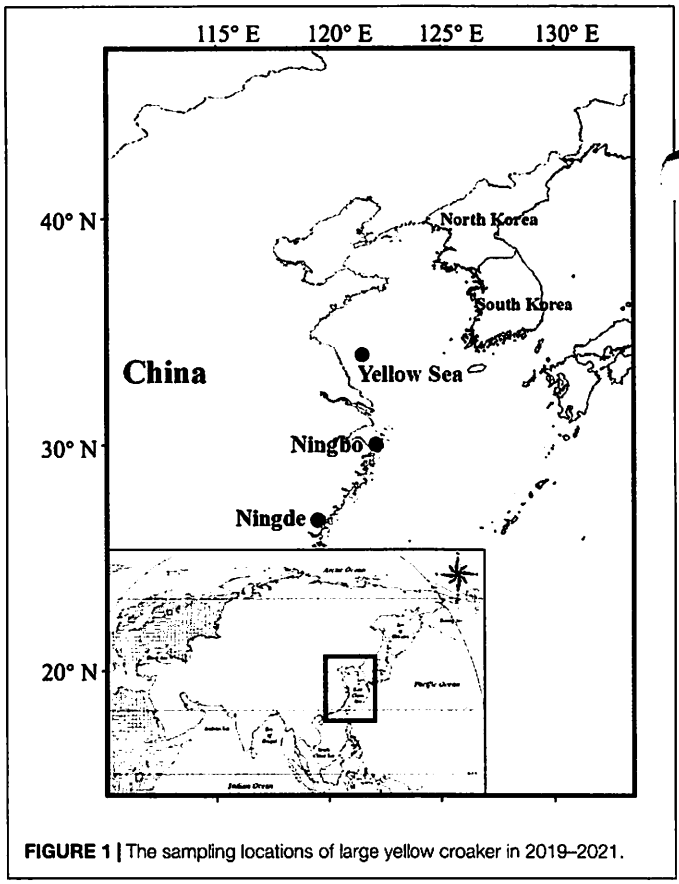

FIGURE 1 | The sampling locations of large yellow croaker in 2019–2021.

both in the main *L. crocea* farming areas (Ningbo, Zhejiang, China and Ningde, Fujian, China) and on offshore feeding grounds in the Southern Yellow Sea.

MATERIALS AND METHODS

Sample Collection

A total of 28 and 35 cultured *L. crocea* (20–30 cm in length) were collected from marine cages in Ningbo, Zhejiang, and Ningde, Fujian, respectively, during the period from 2019 to 2021 (**Figure 1**). Six wild *L. crocea* (10–14 cm in length) were collected from the southern Yellow Sea (34°N, 121.5°E) of China by bottom trawl in 2019 (**Figure 1**). The collected cultured *L. crocea* individuals showed clinical symptoms such as anorexia and abnormal swimming. The heart, liver, kidney, spleen, eye, and gill tissues were dissected from each individual, part of which was stored in 4% paraformaldehyde (4% PFA) solution (Sinopharm, Beijing, China) for *in situ* RNA hybridization and histopathological analysis, or in the RNAstore solution (Tiangen, Beijing, China) for molecular biological detection and 2.5% glutaraldehyde solution (Sinopharm, Beijing, China) for transmission electron microscopy (TEM) analysis, respectively. The remaining part was then stored in dry ice for virus purification. The collected wild *L. crocea* samples were stored in liquid nitrogen and brought back to the laboratory for further analysis.

Virus Isolation

The frozen tissues of *L. crocea* were first homogenized thoroughly in TN buffer (20 mM Tris/HCl, 400 mM NaCl, pH 7.4) and then subjected to the purification of viral particles according to a previously reported protocol (Xu et al., 2020). The purified virus particles were filtered through a 0.22-μm membrane prior to further use.

Cell Inoculation

The fish cell line [epithelioma papilloma cyprini (EPC)] was used for virus proliferation (Xiao et al., 2012). EPC cells were cultured in Eagle's minimal essential medium (EMEM, Sigma-Aldrich, United States) in a 25-cm^2 flask (Corning, United States) with 10% FBS at 25°C. One hundred milliliter of purified virus suspension was inoculated into a confluent monolayer of EPC cells. The same volume of EMEM was used as a mock infection in EPC cells as control. After adsorption for 1 h at 25°C, 5 mL of medium supplemented with 2% FBS was added into the flasks, and the infected cells were incubated at 25°C in the incubator (SANYO, Japan). The infected cells were examined daily under an inverted phase-contrast microscope (Nikon, Japan). When 70–80% of cultured cells showed cytopathic effect (CPE), the cultures were collected in 2.5% glutaraldehyde for TEM analysis or in an RNAstore solution for RNA extraction.

RNA Extraction

Approximately 40 mg tissues or cell cultures fixed in RNAstore solution and liquid nitrogen were used for total RNA extraction by TIANamp Marine Animal RNAprep pure Tissue Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. The concentration and purity of the extracted RNA was determined by Nanodrop 2000c (Thermo Scientific, Waltham, MA, United States).

Detection of Covert Mortality Nodavirus by RT-nPCR

By using the total RNA as template, the first-step PCR of the RT-nPCR was conducted by using the PrimeScript One Step RT-PCR Kit (Version 2.0) (TaKaRa, Dalian, China) in total of 25 μL reaction mixture, including 12.5 μL first-step buffer, 1 μL PrimeScript first-step enzyme mix, 1 μL forward primer (Noda-F1: 5′-AAATACGGCGATGACG-3′, 10 μM), 1 μL reverse primer (Noda-R1: 5′-ACGAAGTGCCCACAGAC-3′, 10 μM), 1 μL RNA template, and 8.5 μL ddH_2O. The programmed reaction was then performed for the reverse transcription at 50°C for 30 min, predenaturation at 94°C for 3 min followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 50 s, and the final extension at 72°C for 5 min. The second-step PCR of the RT-nPCR was carried out by using the TaKaRa Premix Taq™ (Ex Taq™ Version 2.0) in a 25 μL reaction mixture, which included 12.5 μL Premix Taq, 1 μL forward primer (CMNV-D-F1: 5′-TCGCGTATTCGTGGAT-3′, 10 μM), 1 μL reverse primer (CMNV-D-R1: 5′-TAGGGTCAAAAGGTGTAGT-3′, 10 μM), 1 μL product of the first-step RT-PCR, and 9.5 μL ddH_2O. The second-step PCR was performed in a similar way as the first-step PCR except for the incubation for the reverse transcription.

Two target CMNV-specific RNA-dependent RNA polymerase (RdRp) gene fragments of 619 and 413 bp (GenBank accession number KM112247) were amplified by the first- and second-step PCRs, respectively. The PCR products were sequenced by using the Sanger sequencing method.

Detection of Covert Mortality Nodavirus by TaqMan Probe-Based Reverse Transcription Quantitative PCR

All the samples for TaqMan probe-based reverse transcription quantitative PCR (TaqMan RT-qPCR) were analyzed and quantified in three duplicates for targeting the same gene as previously described (Li et al., 2018).

Histopathological Assay

The samples that were positive for CMNV from above TaqMan RT-qPCR assay were subjected to histopathological analysis with hematoxylin and eosin-phloxine (H&E) staining by using regular histological methods as previously described (Lightner, 1996).

In situ RNA Hybridization

The samples positive for CMNV in TaqMan RT-qPCR assay were subjected to *in situ* hybridization (ISH) analysis according to the previous procedures (Piette et al., 2008; Chen et al., 2014; Zhang et al., 2017). Nuclear Fast Red solution (Sigma-Aldrich, St. Louis, MO, United States) was added dropwise on the surface of the sections and dyed the nucleus for 2 min for counterstain postcolor development of BCIP/NBT in the ISH. The sections were visualized and photographed by using the Nikon Eclipse E80i microscope (Nikon Co., Tokyo, Japan).

Transmission Electron Microscopy

The samples preserved in 2.5% glutaraldehyde with the corresponding number were taken out and treated with 1% osmic acid for 2 h, and then dehydrated and embedded in Spurr's resin and stained with uranyl acetate and lead citrate following the protocols of Panphut et al. (2011). All specimens were visualized under JEOL JEM-1200 transmission electron microscopy at 80 kV.

Cloning and Analysis of Covert Mortality Nodavirus Genes

Five and two pairs of primers (**Table 1**) were designed based on the original sequences of CMNV RdRp and capsid protein genes, respectively (GenBank accession numbers MT270124 and MT270123). cDNAs were synthesized by using the extracted total RNA of *L. crocea* samples which were positive for CMNV with SMART® MLV-Reverse Transcriptase (TaKaRa) according to the procedure described by Xu et al. (2020). The reaction mixture and procedures for CMNV genes cloning PCR were referred to the previous report (Xu et al., 2020) with modifications of annealing temperatures and extension time (**Table 1**). The PCR product was subjected to electrophoresis on a 2% agarose gel and sequenced by the Sanger method (Sangon Biotech, Shanghai, China). Sequence analysis was conducted by using BioEdit (Ver.

TABLE 1 | PCR primer sequences, annealing temperature, and extension duration used to amplify the RdRp and capsid protein gene sequences of CMNV.

Genes	Fragment	Primer name	Annealing temperature	Primer sequence (5′-3′)	Extension time	Size of amplicons
RdRp	1	CMNV-R-F1	51°C	TCTGTAACATCTGACGTG	40 s	783 bp
		CMNV-R-R1		CAAACGCAATGGAAGC		
	2	CMNV-R-F2	51°C	ATTAAGGTCGGTGGCA	30 s	649 bp
		CMNV-R-R2		AGTGGGCTTCAGGGTT		
	3	CMNV-R-F3	53°C	CAGGTCAGTGGTGGTGGT	100 s	1683 bp
		CMNV-R-R3		TAAAGGGACGGAATGGTT		
	4	CMNV-D-F1	52°C	TCGCGTATTCGTGGAT	30 s	413 bp
		CMNV-D-R1		TAGGGTCAAAAGGTGTAGT		
	5	CMNV-R-F5	53°C	AACCATTCCGTCCCTTTA	50 s	881 bp
		CMNV-R-R5		CAGTGAAATCGGGTAGGC		
Capsid protein	6	CMNV-CP-F1	44°C	AGAACATCACGTAACAATC	50 s	662 bp
		CMNV-CP-R1		TCAATAGGGTCAGAAACT		
	7	CMNV-CP-F2	50°C	TACAGCGTCAAACCATTC	60 s	906 bp
		CMNV-CP-R2		TAGCCAAGTCTAGGAGGG		

7.1.3) and BLAST.[1] The phylogenetic tree based on the RdRp and capsid protein genes was generated using the neighbor-joining method of MEGA 7.0 (Tamura et al., 2011).

RESULTS

Detection of Covert Mortality Nodavirus by RT-nPCR and TaqMan Probe-Based Reverse Transcription Quantitative PCR

The results of RT-nPCR assay indicated that CMNV-positive rates of the collected *L. crocea* samples from Ningbo, Ningde, and the Yellow Sea were 14.29% (4/28), 20.00% (7/35), and 16.67% (1/6), respectively. The RT-qPCR test yielded 25.00% (7/28), 22.86% (8/35), and 16.67% (1/6) CMNV-positive rates in *L. crocea* collected from Ningbo, Ningde, and the Yellow Sea, respectively, with a range of 12.73–3,108.33 copy numbers/μg of total RNA (**Table 2**). The standard curve showed that the RT-qPCR had a high correlation coefficient (R^2 = 0.999) within the range of $2.21 \times 10^9 - 2.21 \times 10^0$ RNA copies/reaction (**Supplementary Figure 1**).

Detection of Covert Mortality Nodavirus in *Larimichthys crocea* Samples by *in situ* Hybridization

For further confirmation of the CMNV infection in the CMNV-positive *L. crocea* samples, ISH was conducted by using a CMNV-specific RNA probe. The results showed that positive purple hybridization signals of CMNV probe could be observed in the cardiac muscle (**Figures 2A,B**), hepatocytes (**Figures 2E,F**), spleen (**Figures 2I,J**), bipolar cell of the retina (**Figures 2M,N**), and chloride cells of gill filament (**Figures 2Q,R**). No positive hybridization signals appeared on the sections from the same samples without CMNV-specific RNA probes, or from the CMNV negative samples determined by RT-nPCR (**Supplementary Figures 2, 3**).

[1]http://www.ncbi.nlm.nih.gov/gorf/

Histopathology of *Larimichthys crocea* Infected by Covert Mortality Nodavirus

The impact of CMNV infection on the heart, eye, liver, spleen, and gill tissues of *L. crocea* was investigated by histopathology of H&E-stained sections as shown in **Figure 2**. Moderate dissolution-like necrotic muscle fibers and hemocyte infiltration in the fibromuscular stroma were observed in cardiac muscle (**Figures 2C,D**). Severe vacuolation and karyopyknosis were found in hepatocytes (**Figures 2G,H**) and bipolar cells layer of the retina (**Figures 2O,P**). Moderate vacuolation and degenerated erythrocytes were observed in the spleen (**Figures 2K,L**). Disorders of the secondary lamella were also viewed in the gills (**Figures 2S,T**).

Detection of Covert Mortality Nodavirus in *Larimichthys crocea* Samples by Transmission Electron Microscopy

Under the TEM, massive CMNV-like particles with approximate 28–32 nm in diameter and spherical to icosahedral shapes were observed in the blood cells in the kidney (**Figures 3A–C**) and cardiac muscle cells of the heart of the *L. crocea* (**Figures 3D–F**).

Nucleotide Sequence Analysis of RNA-Dependent RNA Polymerase and Capsid Protein Genes of Covert Mortality Nodavirus Isolated From *Larimichthys crocea*

Five amplicons of the nucleotide sequence of RdRp, with the sizes of 783, 649, 1,683, 413, and 881 bp were obtained by PCR (**Figure 4A**, lanes 1–5). Two amplicons of the nucleotide sequence of capsid protein gene, with the sizes of 662 and 906 bp, were generated by PCR (**Figure 4A**, lanes 6 and 7). Then, by sequencing and assembling the overlapping amplicons, the nucleotide sequences of RdRp and capsid protein genes of CMNV isolated from *L. crocea* were determined, which were 3,132 and 1,314 bp in length, respectively.

TABLE 2 | The results of RT-nPCR and TaqMan RT-qPCR assays.

Sample #	Sampling date	RT-nPCR	RT-qPCR CMNV copy/μg total RNA			
			Replicate 1	Replicate 2	Replicate 3	Mean
Ningbo						
LC190114001	14/01/2019	−	−	−	−	−
LC190114002		−	−	−	−	−
LC190114003		−	−	−	−	−
LC190114004		+	90	145.50	153.00	129.50
LC190114005		−	−	−	−	−
LC190114006		−	52	60.50	36.95	49.82
LC190114007		−	−	−	−	−
LC210612001	12/06/2021	−	6.65	24.30	18.65	16.53
LC210612002		−	13.75	24.45	−	12.73
LC210612003		+	359	189.50	190.00	246.17
LC210612004		+	3,275	3,860.00	2,190.00	3,108.33
LC210612005		+	850	1,180.00	1,140.00	1,056.67
LC211231001–LC211231016	31/12/2021	−	−	−	−	−
Ningde						
LC200820001	20/08/2020	+	91.93	290.00	34.61	138.84
LC200820002–LC200820008		−	−	−	−	−
LC210529001	29/05/2021	+	25.39	18.00	86.41	43.27
LC210529002		+	26.83	9.80	65.17	33.93
LC210529003		+	127.02	29.75	23.38	60.05
LC210529004		+	129.67	20.38	27.58	59.21
LC210529005		+	58.38	67.45	10.60	45.48
LC210529006		+	13.96	93.20	49.15	52.10
LC210529007		−	36.32	67.45	69.33	57.70
LC211231001–LC211231020	31/12/2021	−	−	−	−	−
Yellow Sea						
LC190521001	21/05/2019	+	31.35	96.50	111.00	79.62
LC100521002–LC190521006		−	−	−	−	−

+ is positive; − is negative.

Searching results of BLASTx showed that the obtained sequences of RdRp and capsid protein genes of CMNV isolated from *L. crocea* had 99% similarities to the corresponding gene sequences of CMNV isolates from *P. vannamei* (MT270124 and MT270123) and *L. polyactis* (MW625911 and MW625912). The phylogenetic analysis indicated that both the RdRp and capsid protein gene sequences of CMNV isolated from *L. crocea* were clustered tightly with the corresponding sequences of other CMNV isolates. However, it showed a high variation rate with the other members of *Alpha Nodavirus*, including *Drosophila melanogaster* American nodavirus (DmANV), flock house virus (FHV), black beetle virus (BBC), *P. vannamei* nodavirus (PvNV), and *M. rosenbergii* nodavirus (MrNV), etc. (**Figure 4B**).

Cytopathic Effect in Epithelioma Papilloma Cyprini Cells Infected With Covert Mortality Nodavirus

The CPE was first observed by light microscopy at 4 days postinoculation (dpi) of tissue filtrates. The cells shrank, their boundaries became invisible, and a degree of cell fusion appeared. The cell monolayer was destroyed on day four (**Figure 5A**). After three consecutive passages, the CPE became more consistent. Cell cultures were positive for CMNV by PCR assay (**Figure 5B**). The expected fragment with 619 and 413 bp in size of the CMNV partial RdRp gene was cloned and sequenced from the inoculated cells. The phylogenetic analysis showed that CMNV isolated from EPC cell cultures was clustered into the same branch with the known CMNV isolates (**Figure 5C**). Typical CMNV-like virus inclusion bodies could be observed around the lysed cells under the TEM (**Figure 5D**).

DISCUSSION

Up to now, except for members in the genus of *Betanodavirus*, CMNV was the only virus in the family of *Nodaviridae* that is able to infect fish naturally (Zhang et al., 2018; Wang et al., 2019a,b, 2021; Xu et al., 2021). In this study, the possibility and prevalence of CMNV infection in farmed and wild *L. crocea* were evaluated.

The total RNA samples extracted from the tissues of naturally diseased fish were positive for CMNV based on the RT-nPCR and TaqMan RT-qPCR assays. Meanwhile, the CMNV isolates from *L. crocea* showed highly homologous with that of CMNV

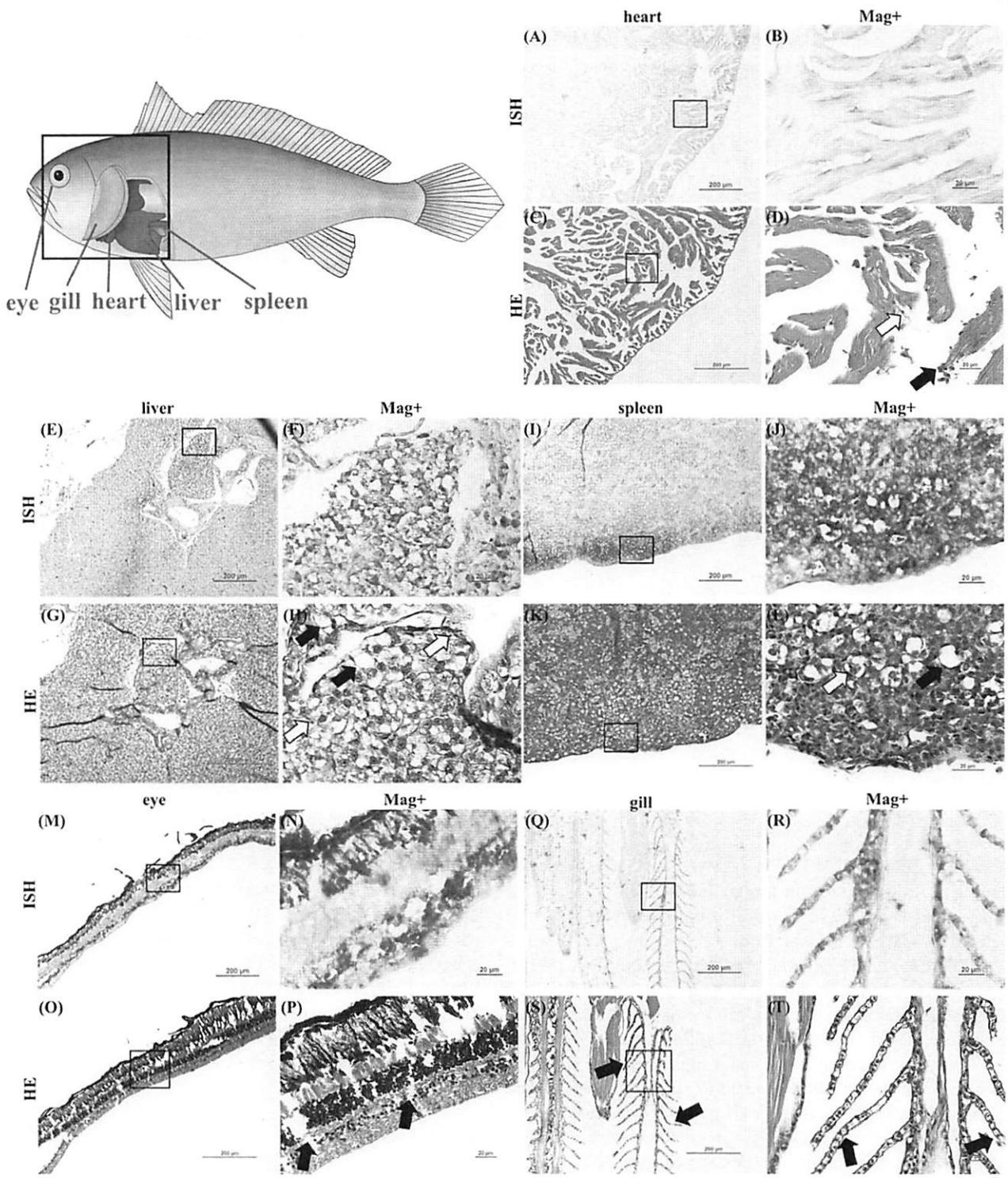

FIGURE 2 | Pattern diagram of *Larimichthys crocea* and micrographs of *in situ* hybridization (ISH) and HE staining for heart, liver, spleen, eye, and gill of the *L. crocea* naturally infected with CMNV. **(A)** Micrograph of ISH for the heart. **(B)** Magnified micrograph of the cardiac muscle in the black frame in **(A)**. Note the purple signals of CMNV probe in the necrotic cardiac muscle. **(C)** Micrographs of HE staining for the heart. **(D)** Magnified micrograph of the cardiac muscle in the black frame in **(C)**. Note the dissolved necrosis (pointed by white arrows) and mild hemocyte infiltration in the fibromuscular stroma (pointed by black arrows) of the cardiac muscle. **(E)** Micrograph of ISH for the liver of the *L. crocea*. **(F)** Magnified micrograph of the zone in the black frame in **(E)**. **(G)** Micrographs of HE staining for the liver. **(H)** Magnified micrograph of the zone in the black frame in **(G)**. Note the serious vacuolation (pointed by black arrows) and karyopyknosis (pointed by white arrows) of the hepatocytes. An intense CMNV probe hybridization signal could be observed at necrotic hepatocytes. **(I)** Micrographs of ISH for spleen. **(J)** Magnified micrograph of the zone in the black frame in **(I)**. **(K)** Micrographs of HE staining for spleen. **(L)** Magnified micrograph of the zone in the black frame in **(K)**. Note the

(Continued)

FIGURE 2 | moderate vacuolation (pointed by black arrows) and degenerated erythrocytes (pointed by white arrows) of the spleen. Intensive purple hybridization signals of CMNV probe could be observed at the spleen. **(M)** Micrographs of ISH for the eye. **(N)** Magnified micrograph of the zone in the black frame in **(M)**. **(O)** Micrographs of HE staining for the eye. **(P)** Magnified micrograph of the zone in the black frame in **(O)**. Note the serious vacuolation (pointed by black arrows) at the bipolar cells layer. Intensive purple hybridization signals of the CMNV probe could be observed at the bipolar cells layer. **(Q)** Micrographs of ISH for gill. **(R)** Magnified micrograph of the zone in the black frame in **(Q)**. **(S)** Micrographs of HE staining for gill. **(T)** Magnified micrograph of the zone in the black frame in **(S)**. Note the disorganized secondary lamella (pointed by black arrows). Intensive purple hybridization signals of the CMNV probe could be observed at the secondary lamella. Scale bars **(A,C,E,G,I,K,M,O,Q,S)**, 200 μm and **(B,D,F,H,J,L,N,P,R,T)**, 20 μm.

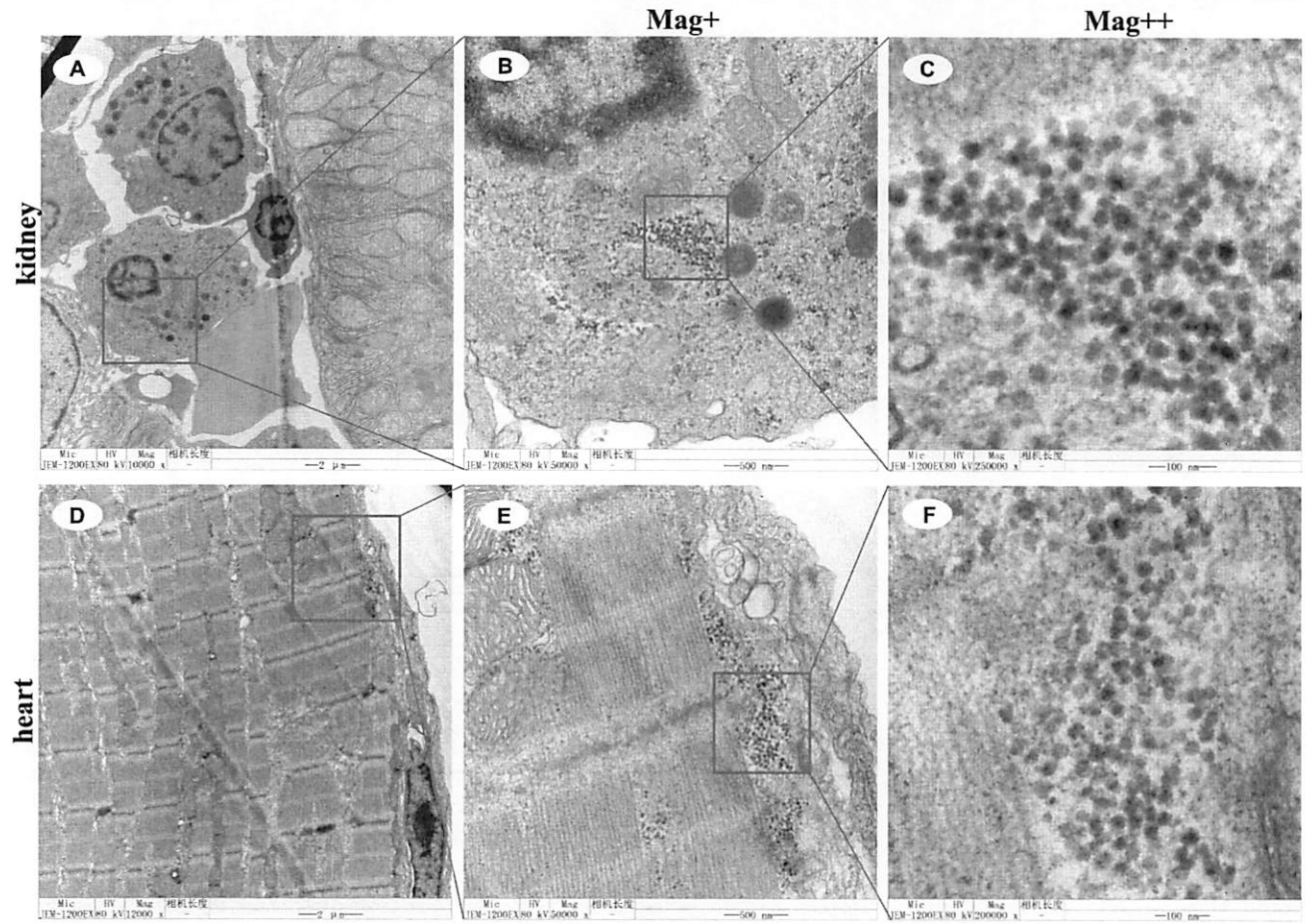

FIGURE 3 | Transmission electron microscopic micrographs of ultrathin section of kidney and heart of the *Larimichthys crocea*. **(A)** The blood cells of the kidney of the *L. crocea*. **(B)** Magnified micrograph of the blood cells in the red frame in **(A)**. Note mass CMNV-like particles distributed in the blood cells. **(C)** Magnified micrograph of the CMNV-like particles in the red frame in **(B)**. **(D)** The cardiac muscle of the heart of the *L. crocea*. **(E)** Magnified micrograph of the cardiac muscle in the red frame in **(D)**. Note mass CMNV-like particles distributed in the cardiac muscle. **(F)** Magnified micrograph of the CMNV-like particles in the red frame in **(E)**. Scale bars, **(A,D)**, 2 μm, **(B,E)**, 500 nm, and **(C,F)**, 100 nm.

isolates from cultured *P. vannamei* in the phylogenetic tree analysis. It suggests that CMNV-*L. crocea* isolates might originate from the aquacultured shrimp. In addition, CMNV-like virus particles appeared in the cytoplasm of cardiac muscle and kidney under TEM observations, roughly spherical in shape, without an envelope, and about 30 nm in diameter. The morphological characteristics and size of viruses were generally consistent with the previous findings (Zhang et al., 2014; Wang et al., 2019a,b).

Recent studies have found that CMNV could infect the crucian carp *C. auratus*, the gobiid fish *M. abei*, the Japanese flounder *P. olivaceus,* and the small yellow croaker *L. polyactis* under natural conditions (Zhang et al., 2018; Wang et al., 2019a,b; Xu et al., 2021). In this study, CMNV infection in *L. crocea* could cause histopathological alteration and lesions, including extensive cardiac muscle necrosis and severe vacuolation in the liver, eye, and spleen, which were similar to the clinical signs found in other fishes with CMNV infections. In addition to the above-mentioned tissue damages, severe tissue necrosis also occurred in the gills of CMNV-positive *L. crocea*. Meanwhile, the intense purple positive hybridization signals of CMNV-specific RNA probes were present in the necrotic tissues in the ISH analysis. Moreover, the purified CMNV suspension could cause significant CPE in EPC cells, and typical CMNV-like inclusion bodies could be observed around the lysed EPC cells under the TEM. Those results provided reliable evidence to support that CMNV was a novel pathogen of *L. crocea*.

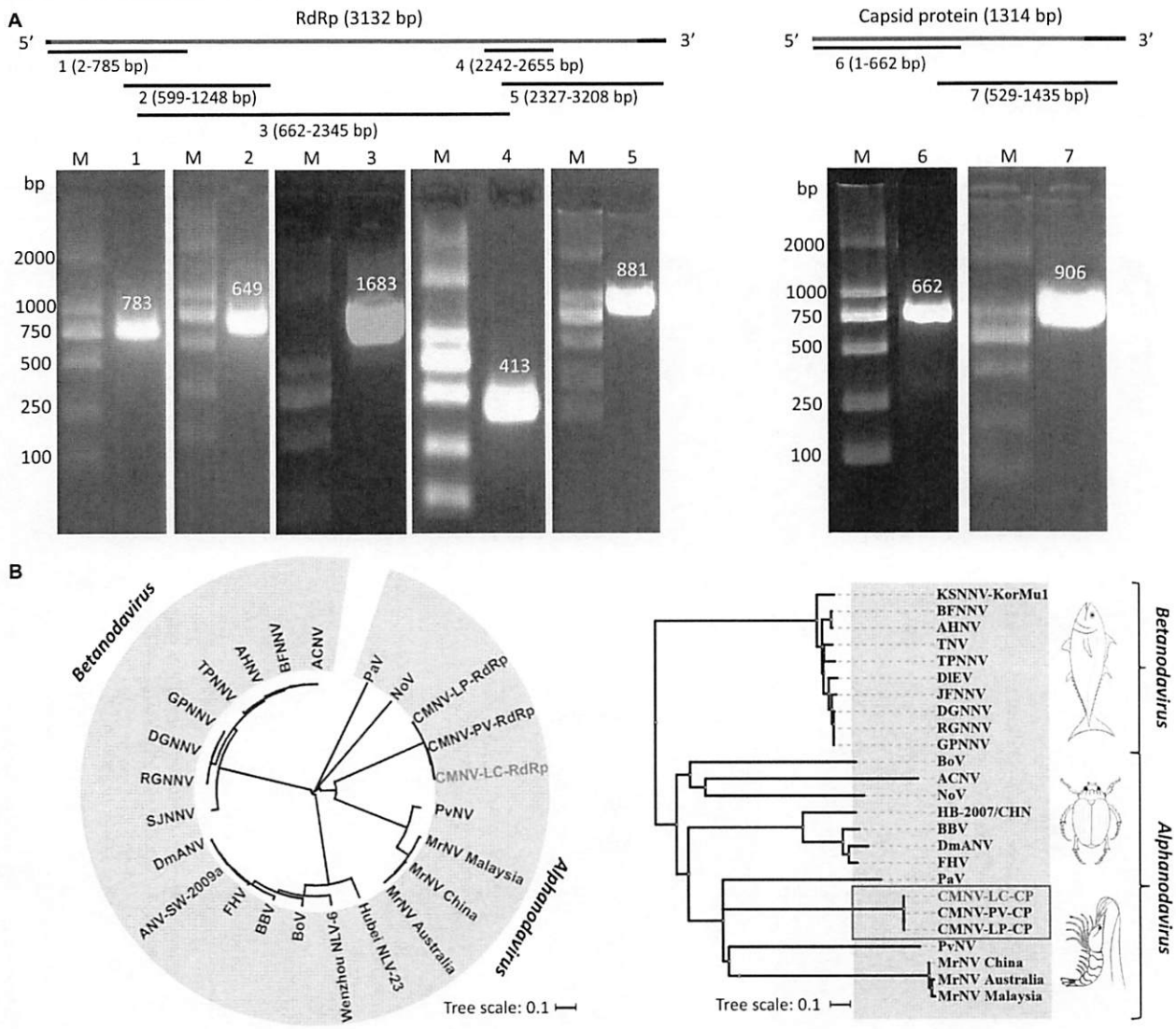

FIGURE 4 | The cloning and sequences analysis of RdRp gene and capsid protein gene of CMNV isolated from *Larimichthys crocea*. **(A)** Assembly strategy and agarose gel electrophoresis of PCR amplicons in cloning RdRp and capsid protein genes from *L. crocea*. The numbers 1–7 represent the seven PCR amplicons produced in cloning the CMNV genome. The images are cropped from the original image (**Supplementary Figure 4**). Lane M: 2000 bp marker; lanes 1–5: amplicons of the sequence of RdRp; lanes 6 and 7: amplicons of the sequence of capsid protein. **(B)** Phylogenetic diversity of RNA-dependent RNA polymerase (RdRp) and capsid protein genes from different CMNV isolates and other nodaviruses (for virus abbreviations, see **Supplementary Table 1**). CMNV-LC-RdRp and CMNV-LC-CP represented the RdRp and capsid protein gene of CMNV isolated from *L. crocea*, respectively. The phylogenetic tree was generated by the neighbor-joining method using the MEGA 7.0 program, and the scale bar was 0.1.

Larimichthys crocea is the fish of highest yield in mariculture in China (Ministry of Agriculture and Rural Affairs of the People's Republic of China, 2021). However, with the rapid expansion of the culture, *L. crocea* has been suffering from several serious diseases, in which large yellow croaker iridovirus (LYCIV) is a viral disease that can cause high mortality up to 75% of cultured *L. crocea*, which results in severe economic losses (Chen et al., 2003). CMNV can naturally infect large yellow croakers and cause multiple histopathological damages, which undoubtedly further increases the risk of disease outbreaking and also the difficulty of breeding healthy large yellow croakers, and might lead to more threat to large yellow croakers farming. Hence, the disease caused by CMNV infection in large yellow croakers deserves further epidemiological investigation and research.

Stock enhancement has become a positive approach to restore the natural resource of *L. crocea* (Lin, 2006; Xu, 2009; Zhang et al., 2010; Ma, 2017). Although stock enhancement could amplify the abundance of target species (Bartley and Bell, 2008; Jiang et al., 2014; Lorenzen, 2015), it may also pose some risks for the recipient aquatic ecosystem (Wang et al., 2006). It was reported that the release of cultured Atlantic salmon in Norwegian offshore waters caused infections of local wild populations of Atlantic salmon with *Gyrodactylus salaris* and *Aeromonas salmonicida*, respectively (Heggberget et al., 1993).

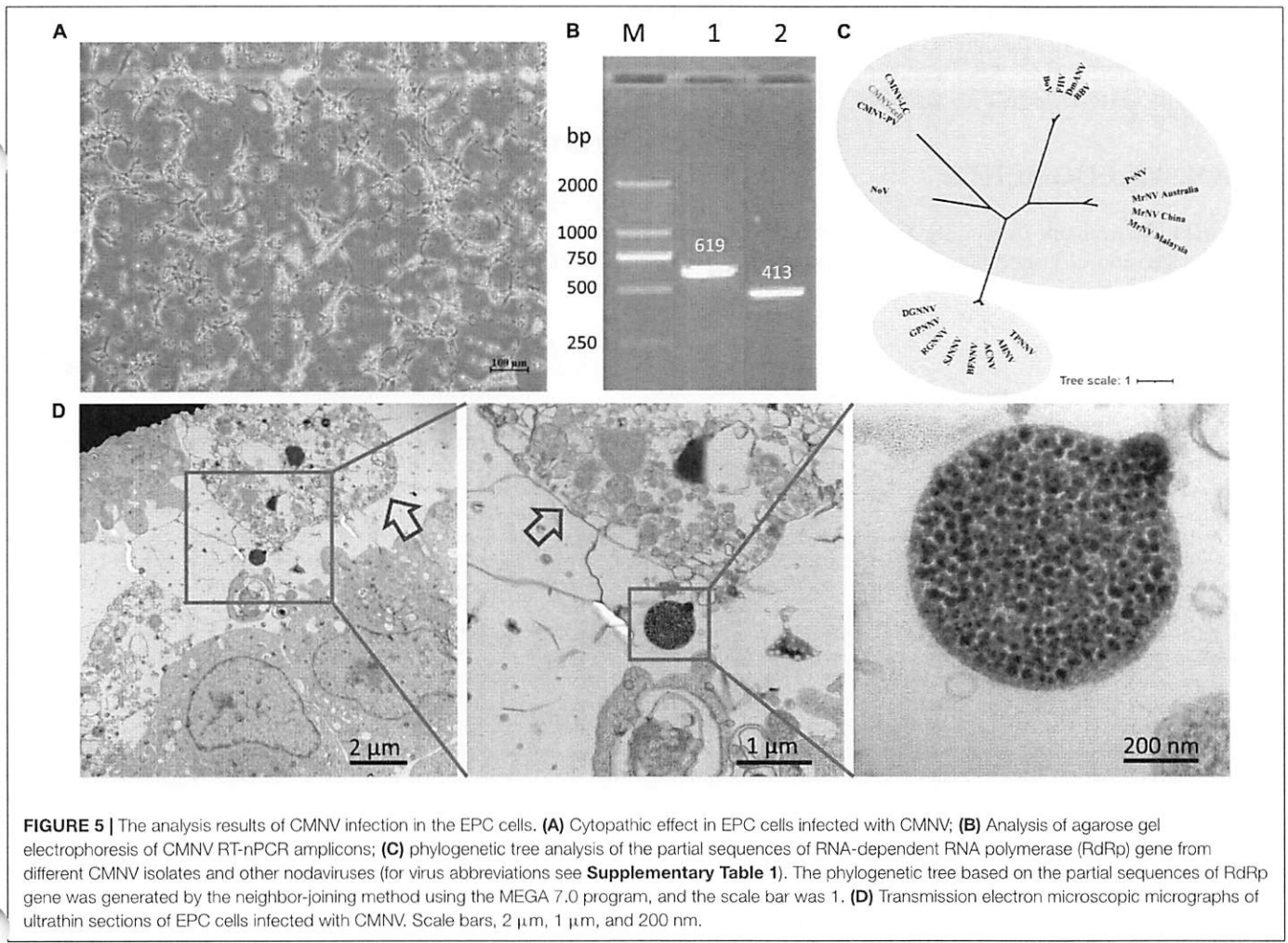

FIGURE 5 | The analysis results of CMNV infection in the EPC cells. **(A)** Cytopathic effect in EPC cells infected with CMNV; **(B)** Analysis of agarose gel electrophoresis of CMNV RT-nPCR amplicons; **(C)** phylogenetic tree analysis of the partial sequences of RNA-dependent RNA polymerase (RdRp) gene from different CMNV isolates and other nodaviruses (for virus abbreviations see **Supplementary Table 1**). The phylogenetic tree based on the partial sequences of RdRp gene was generated by the neighbor-joining method using the MEGA 7.0 program, and the scale bar was 1. **(D)** Transmission electron microscopic micrographs of ultrathin sections of EPC cells infected with CMNV. Scale bars, 2 μm, 1 μm, and 200 nm.

The present finding that CMNV naturally infected *L. crocea* and induced severe tissue necrosis in infected individuals suggests that CMNV should be regarded as a pathogen of serious concern to be screened before releasing *L. crocea* into the natural waters to minimize the adverse impact on stock enhancement.

CONCLUSION

In summary, the evidence of pathogen detection, histopathology, ISH, TEM, and cell inoculation consistently confirmed that *L. crocea* is a new susceptible host for CMNV, which highlights that threats due to CMNV infection should be seriously considered in the development of coastal aquaculture industry of *L. crocea* and the conservation of coastal ecosystems.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Yellow Sea Fisheries Research Institute.

AUTHOR CONTRIBUTIONS

QZ and CY designed the experiments. TX, YF, TJ, CW, and WW executed the experiments. TX and QZ analyzed the data. TX and TJ contributed to sampling. TX wrote the manuscript draft. QZ, JL, and CY revised the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents, and approved the final version.

FUNDING

This work was supported by the Marine S&T Fund of Shandong Province for Pilot National Laboratory for Marine Science and Technology (Qingdao) (2018SDKJ0303-1), National Natural Science Foundation of China (32073016), Central Public-Interest Scientific Institution Basal Research Fund, CAFS

(No. 2020TD39), Project of Species Conservation from the MARA-Marine Fisheries Resources Collection and Preservation, and Central Public-Interest Scientific Institution Basal Research Fund, YSFRI, CAFS (No. 20603022020005).

ACKNOWLEDGMENTS

We would like to thank Dr. Guoshi Xie and also the colleagues from the Division of Fishery Resources and Ecosystem, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences for sampling.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.789128/full#supplementary-material

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1. EXPRESS WARRANTY. Aquatic Enterprises Inc. (AEI) warrants that the components that it manufactures will be free from defects in material and workmanship for the life of the components while said components are owned by the customer. This warranty includes racks, sumps, tanks, gutters, drainpipes, pre-filters, and manifolds. AEI's sole obligation under this warranty is to repair or replace any defective parts for the customer, and customer may not under any circumstances seek any repayment of the purchase price for warranty repairs. Upon replacement, all replaced parts shall become the property of AEI, and shall be returned to AEI immediately. All components not manufactured by AEI shall not be covered by this warranty and shall be covered by the original manufacturer's warranty only, if any.

2. EXCLUDED CLAIMS. AEI shall have no obligation to repair or replace parts for the customer if repair or replacement of the parts is needed because of any cause external to the AEI product, including but not limited to being caused by a Force Majeure event, by a natural disaster (including but not limited to an earthquake, flood, hurricane, tornado, or other natural disaster), or by any other external cause.

3. LIMITATION OF LIABILITY. AEI shall not be liable for any lost profits, lost data, or other consequential, incidental, or punitive damages as a result of failure of the AEI product, including but not limited to loss of research animals, interruption of research, or any other consequential or incidental damages to the customer. AEI's liability to the customer in any action, whether in contract, tort, strict liability, breach of warranty, or any other type of action, is hereby limited to the actual damages of the customer, not including lost profits, data, or research, up to a maximum amount of the purchase agreement price for the actual components requiring repair or replacement.

4. DISCLAIMER OF WARRANTIES: Except as explicitly provided in Paragraph 1 of this Warranty Statement, AEI makes no representation or warranty, whether express or implied, of any kind whatsoever with respect to any goods or services provided to the customer, including but not limited to any Warranty of Merchantability or any Warranty of Fitness for a Particular Purpose.

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571 Elm Street, Unit E, Bridgewater, MA 02324
Phone 206-937-0392
www.aquaticenterprises.com

AQUATIC ENTERPRISES

Bringing your environment to life since 1990

We appreciate the opportunity to provide this proposal and look forward to working with you on your new system.

ACCEPTANCE:

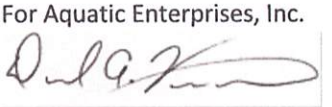
I accept the Terms & Conditions of this proposal.

For _____

SIGN:_____ DATE:_____

PRINT: _____ TITLE: _____

For Aquatic Enterprises, Inc.



_____ DATE:_____

Daniel A Vinci

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