

A Novel Application of Bubble-eye Strain of *Carassius Auratus* for Ex Vivo Fish Immunological Studies

Hiroto Nakajima

Teikyo University

Atsushi Miyashita

Teikyo University

Hiroshi Hamamoto

Teikyo University

Kazuhisa Sekimizu (✉ sekimizu@main.teikyo-u.ac.jp)

Teikyo University

Research Article

Keywords: *Carassius auratus*, ex vivo, bubble-eye, novel application, fish immunological studies

DOI: <https://doi.org/10.21203/rs.3.rs-273301/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

In this study, we investigated a new application of bubble-eye goldfish (commercially available strain with large bubble-shaped eye sacs) for immunological studies in fishes utilizing the technical advantage of examining immune cells in the eye sac fluid *ex vivo* without sacrificing animals. As known in many aquatic species, the common goldfish strain showed an increased infection sensitivity at high temperature, which we demonstrate may be due to an immune impairment using the bubble-eye goldfish model. Injection of heat-killed bacterial cells into the eye sac resulted in an inflammatory symptom (surface reddening) and increased gene expression of pro-inflammatory cytokines observed *in vivo*, and high rearing temperature suppressed the induction of pro-inflammatory gene expressions. We further conducted *ex vivo* experiments using the immune cells harvested from the eye sac and found that the induced expression of pro-inflammatory cytokines was suppressed when we increased the temperature of *ex vivo* culture, suggesting that the temperature response of the eye-sac immune cells is a cell autonomous function. These results indicate that the bubble-eye goldfish is a suitable model for *ex vivo* investigation of fish immune cells and that the temperature-induced infection susceptibility in the goldfish may be due to functional impairments of immune cells.

Introduction

Infection control is key in aquaculture (Lafferty et al. 2015). Failure to control infection can lead to colony collapse (i.e., death), which can be very damaging to the local industry. Despite significant investments in infection control, including the use of antibiotics, the aquaculture industry is still affected by infectious diseases¹⁻⁴. High temperatures, often caused by heat waves, increase the risk of infection through a combination of accelerated pathogen growth and suppression of host immune defenses^{1,5,6}. In addition, aquaculture will experience unprecedented impacts from climate change, as global average temperatures are likely to increase by 3°C by 2050 compared to the second half of the 20th century⁷ and the number of heat waves is likely to increase. Therefore, understanding how temperature increases will affect infectious diseases in cultured aquatic species is important to prevent potential deterioration of the industry.

Temperature alteration impairs fish immunity and develops infections as evidenced by increased infection rates and shortened lifespan in aquatic species^{5,8-14}. Most of those studies reported that a warm temperature is associated with increased infection resistance, while it is not always true for some contexts. Increased temperature triggers a stress response that suppresses the immune system of fish species¹, and such hyperthermia may impact the fishery. To prevent potential damage to the industry, immunostimulants and vaccination may be effective, if not complete^{15,16}. To overcome the effects of current and future infections, good laboratory models for screening immunostimulants are needed. For such immunostimulant screening, it is essential to use both live animals raised in laboratory tanks and *ex vivo* analysis using freshly harvested immune cells. *Ex vivo* systems allow high-throughput screening of potent immunostimulant candidates, while *in vivo* confirmatory experiments are required for extensive

testing at the industrial level. Lack of either approach will delay the research. In this study, we propose the use of bubble-eye goldfish to fulfill this requirement.

The bubble-eye goldfish is a commercially available strain of goldfish in East Asia. Bubble-eye goldfish have large eye sacs (ocular sacs) under each eye. The ocular sacs are filled with fluid containing lymph and immune cells and can be retrieved with a needle without sacrificing the animal. The ocular sacs can be sampled repeatedly, allowing for time-course studies that track the same set of individuals over time. The lymph of the eye sac contains growth-promoting factors that act on fish cell cultures¹⁷, and cells in the eye sac fluid may play an immunological role. In this study, we will demonstrate the expression of immune-related genes in eye sac cells in response to immune challenge with heat-killed bacteria to elucidate their immune function. We will also demonstrate how temperature elevation affects immune function in goldfish, both *in vivo* and *ex vivo*, using a bubble eye goldfish model.

Results

Temperature rise promotes infection in the common goldfish

As shown in Fig. 1, the common goldfish injected intraperitoneally with *P. aeruginosa* died earlier when kept at high temperature than when kept at the normal temperature ($p = 0.021$, Log-rank test). No death was observed for the saline-injected groups (Fig. 1).

Inflammatory responses to *P. aeruginosa* in the bubble-eye goldfish eye-sacs

Eye sac cells (Fig. 2) can be harvested and cultured *ex vivo*. In the response to immune challenge by heat-killed *P. aeruginosa*, redness (prominent blood vessels) was observed in the ipsilateral eye sac membrane, but not in the contralateral membrane (Fig. 3), which represents an inflammatory response induced in the eye-sac. We further examined the gene expressions of pro-inflammatory cytokines in the eye-sac immune cells (see Materials and Methods for technical details). As shown in Fig. 4, the mRNA level increased by 800 fold for IL1 β 1 ($p = 0.0001$), 200 fold for IL1 β 2 ($p = 0.0005$), 10 fold for TNF α 1 ($p = 0.0090$), and 300 fold for TNF α 2 ($p = 0.0470$) in response to the immune challenge (Fig. 4).

Temperature rise suppresses the pro-inflammatory cytokine expressions in the eye-sac immune cells both *in vivo* and *ex vivo*

In the eye-sac immune cells *in vivo*, mRNA levels of induced pro-inflammatory cytokine genes were lower when the fish were kept at high temperature (33°C) than when kept at the normal rearing temperature (25°C) (Fig. 5). We then harvested the eye-sac immune cells from untreated bubble-eye goldfish to obtain an *ex vivo* culture at 25°C (see Materials and Methods for details), where induction of pro-inflammatory cytokine genes by heat-killed *P. aeruginosa* was observed in adherent cells but not apparent in non-adherent cells (Fig. 6). Using the *ex vivo* culture, we examined the effect of temperature rise on the pro-inflammatory cytokine expressions in the eye-sac cells induced by heat-killed *P. aeruginosa* cells.

Compared with the normal temperature, high culture temperatures resulted in reduced cytokine expressions (Fig. 7, and Fig. S1-3).

Discussion

In the present study, we established a model system to study immune responses using the bubble-eye goldfish. As demonstrated in the present study, this model is suitable for both *in vivo* and *ex vivo* analyses of immune cells. For the *ex vivo* experiments, the eye sacs allow us to collect immune cells without sacrificing the animal, and the harvested immune cells can be cultured on a plastic dish. The *ex vivo* cultured cells expressed cytokine genes in response to the bacterial challenge, which is consistent with the cytokine gene expressions triggered by an immune challenge observed *in vivo*. The immune cells showed attenuated expressions of cytokine genes in response to temperature rise as observed *in vivo* and *ex vivo*, which sheds light on the underpinning mechanism of the increased infection risks in aquaculture upon temperature rises.

Among the cytokines, IL1 β and TNF α play important roles both in innate and acquired immunity, such as the activation of phagocytic cells and the promotion of immune-related gene expressions in a series of immunoreactive cells both in mammals and fishes¹⁸, indicating that whether an animal is capable of inducing the expression of IL1 β or TNF α in response to immune challenges reflects its infection resistance. In this sense, the attenuated induction of these cytokines upon temperature rise may explain the enhanced bacterial infection in the goldfish upon the experimental temperature rise tested in this study.

The microscopy suggests that most of the eye-sac cells are mononuclear cells, but heterogeneous in multiple histological properties such as cell size and nucleocytoplasmic ratio. It should be noted that the adhesion rate to the plastic dish was approximately 50%, and those cells that did not adhere to the dish may represent distinct population from the adherent cell populations. At least, the adherent population showed apparent expressions of pro-inflammatory cytokines in response to bacterial challenge, suggesting its role in the pro-inflammatory cytokine production. Also, mammalian studies revealed that dish-adhering cell populations are rich in monocyte/macrophage lineage cells^{19,20}, while goldfish macrophages express IL1 β and TNF α ²¹. These are consistent with our finding, and the dish-adhering eye-sac immune cells are most likely monocyte/macrophage lineage cells that respond to the immune challenge by expressing the pro-inflammatory cytokines.

Regarding the temperature sensitivity of cytokine expressions, cytokine productions in human monocytes are reduced by high temperature *in vitro*²². However, empirical knowledge in fish immune cells is slim. A recent *in vivo* study in the crucian carp demonstrated that the host viability and the gene expression of pro-inflammatory cytokines after a bacterial infection was reduced by high temperature²³, supporting the present study in the bubble-eye goldfish. In the bubble-eye goldfish, the eye sac enables immune cell analyses as it provides up to 1 mL of lymphoid fluid from each individual, even without sacrificing the

animal. This feature is the major advantage of this model using the bubble-eye goldfish and will accelerate the molecular study of fish immunity and contribute to improved aquaculture productivities.

Methods

Goldfish strains (*Carassius auratus*)

The common goldfish ('Wakin') and the bubble-eye goldfish were obtained from a local supplier Kingyo-Zaka (Tokyo, Japan). Goldfish were fed with a commercial diet for goldfish (Kyorin, Hyougo, Japan). All experiments were done after an acclimation period (more than a week) in the laboratory after each purchase, where we kept the goldfish at 25°C in a fish tank (30 x 45 x 23 cm). The ranges of body weight were 7–10 g for the common goldfish, and 20–28 g for the bubble-eye goldfish upon the start day of each experiment. The research protocol was approved by Animal Welfare Ethics Committee of Genome Pharmaceuticals Institute Co., Ltd. and was conducted in compliance with all relevant guidelines and regulations applicable at the time and place of the experiments, including the ARRIVE guidelines.

Temperature rise paradigms

Temperature rise paradigms were given to the goldfish following the acclimation period at the normal temperature (25°C). For the infection experiments and the sterile immune challenge experiments, goldfish were kept at either 25°C or 33°C for 24 hours before treatments (i.e., infection or sterile challenge). For *ex vivo* experiments using harvested eye-sac immune cells, cells were harvested from bubble-eye goldfish reared at 25°C, and the harvested cells were cultured either at 25°C and 33°C.

Bacteria (*Pseudomonas aeruginosa*)

P. aeruginosa, strain PAO1²⁴ was aerobically cultured overnight at 37°C in LB10 medium. In infection experiments, the live *P. aeruginosa* cells were washed and suspended in saline (0.9% NaCl aqueous solution). For the heat-killed *P. aeruginosa* cells used in this study, we washed the live cells with saline, and then autoclaved the cells at 121°C for 20 min.

Infection experiments

To know the effect of temperature on the goldfish immunity, we intraperitoneally injected *P. aeruginosa* live cells (3×10^7 CFU/fish) to the common goldfish (Wakin). The injected fish were then kept at either 25°C (normal rearing temperature) or 33°C (high temperature) and monitored for their survival.

Sterile immune challenge to the eye-sac using heat-killed bacterial cells

An overnight culture of *P. aeruginosa* was spun and the pellet was resuspended in a tenth volume of saline. We autoclaved this suspension at 121°C for 20 minutes to obtain heat-killed *P. aeruginosa* cells. We injected 50 µL of the heat-killed *P. aeruginosa* cells into the eye-sac of one side, and 50 µL of saline

into the eye-sac of the other side. The immune challenges were done either at the normal rearing temperature (25°C) or at the high temperature (33°C).

Collection of eye-sac cells

Because the common goldfish is difficult to collect their immune cells without sacrificing the animal, we used the bubble-eye goldfish in the following part of this study to investigate the molecular responses of the goldfish immune system to temperature rises. Eye-sac cells floating in the eye-sac fluid can be easily collected from the eye sacs of bubble-eye goldfish ($\geq 2 \times 10^5$ cells/mL). Eye-sac cells consisted predominantly of mononuclear cells of various sizes, ranging from 5 to 20 μm , and different nucleocytoplasmic ratios (Fig. 2). Some of the cells showed adhesion to plastic dishes in vitro (data not shown). From the bubble eye-goldfish, the eye-sac fluid (containing the eye-sac cells) was collected from the eye sacs using a disposable plastic syringe with a 21-Gauge sterile needle.

Gene expression analysis of eye-sac immune cells

Twenty-four hours after the injection of heat-killed *P. aeruginosa* into the eye sac, we collected the eye-sac cells as described in the 'Collection of eye-sac cells' section. We then isolated the mRNA and performed qRT-PCR analyses (details describe in the following section).

Ex vivo culture of the eye-sac cells

We used a disposable syringe (5 mL) with needle (21 Gauge) to harvest the eye-sac immune cells. The harvested cells (typically 4 mL) were suspended in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated calf serum (SAFC Biosciences, USA, 3% autologous eye-sac fluid, 20 mM HEPES buffer and antibiotics (100 U/mL of penicillin and 100 $\mu\text{g}/\text{mL}$ of streptomycin) and cultured in 24-well plastic plate (catalog# 3820-024, AGC Techno Glass) (8×10^4 cells/0.8 mL/well) for at least 2 hours. The medium had been preincubated at 25 °C. In this condition, approximately 50% of the harvested cells adhered to the plastic dish. To give an immune challenge, 1.5×10^8 cells of heat-killed *P. aeruginosa* were added in the medium when the cells were suspended in the culture dish.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from eye-sac cells by using TRIzol Reagent (ThermoFisher Scientific), treated with DNase I (Promega), and reverse transcribed to obtain cDNA using the High Capacity RNA-to-cDNA Kit (ThermoFisher Scientific) following the manufacturer's protocol. Using the cDNA, gene expressions were analyzed by qRT-PCR. The qRT-PCR was performed using 7500 Fast Real-Time PCR System (ThermoFisher Scientific) using Fast SYBR® Green Master Mix (ThermoFisher Scientific). Primers for each target gene were described in the literature²¹. We chose the elongation factor 1 α (EF1 α) gene as an internal control as done in the preceding study in the goldfish²¹.

Statistical analysis

To test the differences between survival curves, log-rank tests were performed using GraphPad Prism version 8.4.3 (GraphPad Software Inc.). To test the differences between mean values, Student's t-tests were performed using Microsoft Excel 2013.

Declarations

Acknowledgements

We thank Genome Pharmaceuticals Institute and Kingyozaka (the local goldfish supplier) for providing materials, animals, and technical advice. This work was supported by JSPS KAKENHI for Early-Career Scientists for AM (Grant# 20K16253).

Author contributions

Study conception and design: KS

Acquisition of data: HN, KS

Analysis and interpretation of data: HN, AM, HH, KS

Drafting of manuscript: HN

Critical revision: HN, AM, HH, KS

Competing interests

The authors declare no competing interests.

References

1. Lafferty, K. D. *et al.* Infectious diseases affect marine fisheries and aquaculture economics. *Ann Rev Mar Sci* **7**, 471-496, doi:10.1146/annurev-marine-010814-015646 (2015).
2. Moran, J., Whitaker, D. & Kent, M. A review of the myxosporean genus *Kudoa* Meglitsch, 1947, and its impact on the international aquaculture industry and commercial fisheries. *Aquaculture* **172**, 163-196 (1999).
3. Vendramin, N. *et al.* Viral Encephalopathy and Retinopathy in groupers (*Epinephelus* spp.) in southern Italy: a threat for wild endangered species? *BMC Veterinary Research* **9**, 1-7 (2013).
4. Traxler, G., Roome, J., Lauda, K. & LaPatra, S. Appearance of infectious hematopoietic necrosis virus (IHNV) and neutralizing antibodies in sockeye salmon *Onchorynchus nerka* during their migration and maturation period. *Diseases of Aquatic Organisms* **28**, 31-38 (1997).
5. Jiravanichpaisal, P., Soderhall, K. & Soderhall, I. Effect of water temperature on the immune response and infectivity pattern of white spot syndrome virus (WSSV) in freshwater crayfish. *Fish Shellfish Immunol* **17**, 265-275, doi:10.1016/j.fsi.2004.03.010 (2004).

6. Ndong, D., Chen, Y.-Y., Lin, Y.-H., Vaseeharan, B. & Chen, J.-C. The immune response of tilapia *Oreochromis mossambicus* and its susceptibility to *Streptococcus iniae* under stress in low and high temperatures. *Fish & Shellfish Immunology* **22**, 686-694, doi:<https://doi.org/10.1016/j.fsi.2006.08.015> (2007).
7. Rowlands, D. J. *et al.* Broad range of 2050 warming from an observationally constrained large climate model ensemble. *Nature Geoscience* **5**, 256-260, doi:10.1038/ngeo1430 (2012).
8. Vidal, O. M., Granja, C. B., Aranguren, F., Brock, J. A. & Salazar, M. A profound effect of hyperthermia on survival of *Litopenaeus vannamei* juveniles infected with white spot syndrome virus. *Journal of the world aquaculture society* **32**, 364-372 (2001).
9. Guan, Y., Yu, Z. & Li, C. The effects of temperature on white spot syndrome infections in *Marsupenaeus japonicus*. *Journal of invertebrate pathology (Print)* **83**, 257-260 (2003).
10. Amend, D. F. Control of infectious hematopoietic necrosis virus disease by elevating the water temperature. *Journal of the Fisheries Board of Canada* **27**, 265-270 (1970).
11. Castric, J. & De Kinkelin, P. Experimental study of the susceptibility of two marine fish species, sea bass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*), to viral haemorrhagic septicaemia. *Aquaculture* **41**, 203-212 (1984).
12. OSEKO, N., YOSHIMIZU, M. & KIMURA, T. Effect of water temperature on artificial infection of Rhabdovirus olivaceus (hirame rhabdovirus: HRV) to hirame (Japanese flounder, *Paralichthys olivaceus*). *Fish Pathology* **23**, 125-132 (1988).
13. Kobayashi, T., Shiino, T. & Miyazaki, T. The effect of water temperature on rhabdoviral dermatitis in the Japanese eel, *Anguilla japonica* Temminck and Schlegel. *Aquaculture* **170**, 7-15 (1999).
14. Dorson, M. & Touchy, C. The influence of fish age and water temperature on mortalities of rainbow trout, *Salmo gairdneri* Richardson, caused by a European strain of infectious pancreatic necrosis virus. *Journal of Fish Diseases* **4**, 213-221 (1981).
15. Smith, V. J., Brown, J. H. & Hauton, C. Immunostimulation in crustaceans: does it really protect against infection? *Fish & shellfish immunology* **15**, 71-90, doi:10.1016/s1050-4648(02)00140-7 (2003).
16. Lillehaug, A., Lunestad, B. T. & Grave, K. Epidemiology of bacterial diseases in Norwegian aquaculture—a description based on antibiotic prescription data for the ten-year period 1991 to 2000. *Dis Aquat Organ* **53**, 115-125, doi:10.3354/dao053115 (2003).
17. Sawatari, E. *et al.* Cell Growth-Promoting Activity of Fluid from Eye Sacs of the Bubble-Eye Goldfish (*Carassius auratus*). *Zoological science* **26**, 254-258 (2009).
18. Zou, J. & Secombes, C. J. The function of fish cytokines. *Biology* **5**, 23 (2016).
19. Mosier, D. E. A requirement for two cell types for antibody formation in vitro. *Science* **158**, 1573-1575 (1967).
20. Nathan, C. F., Karnovsky, M. L. & David, J. R. Alterations of macrophage functions by mediators from lymphocytes. *The Journal of experimental medicine* **133**, 1356-1376 (1971).

21. Grayfer, L., Garcia, E. G. & Belosevic, M. Comparison of macrophage antimicrobial responses induced by type II interferons of the goldfish (*Carassius auratus* L.). *Journal of Biological Chemistry* **285**, 23537-23547 (2010).
22. Dinarello, C. A. *et al.* Inhibitory effects of elevated temperature on human cytokine production and natural killer activity. *Cancer research* **46**, 6236-6241 (1986).
23. Jiang, M., Chen, Z.-g., Zheng, J. & Peng, B. Metabolites-enabled survival of crucian carps infected by *edwardsiella tarda* in high water temperature. *Frontiers in immunology* **10**, 1991 (2019).
24. Miyashita, A., Takahashi, S., Ishii, K., Sekimizu, K. & Kaito, C. Primed immune responses triggered by ingested bacteria lead to systemic infection tolerance in silkworms. *PLoS One* **10**, e0130486 (2015).