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

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Research Article

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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 diseases [1–4]. 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 3ºC by 2050 compared to the second half of the 20th century [7] 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 species [1], 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 cultures[17], 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 IL1β1 ($p = 0.0001$), 200 fold for IL1β2 ($p = 0.0005$), 10 fold for TNFα1 ($p = 0.0090$), and 300 fold for TNFα2 ($p = 0.0470$) 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 (33ºC) than when kept at the normal rearing temperature (25ºC) (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, IL1β and TNFα 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 fishes [18], indicating that whether an animal is capable of inducing the expression of IL1β or TNFα 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 IL1β and TNFα [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 temperature [23], 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 25ºC 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 (25ºC). For the infection experiments and the sterile immune challenge experiments, goldfish were kept at either 25ºC or 33ºC 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 25ºC, and the harvested cells were cultured either at 25ºC and 33ºC.

Bacteria (*Pseudomonas aeruginosa*)

P. aeruginosa, strain PAO1 [24] was aerobically cultured overnight at 37℃ 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℃ 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℃ (normal rearing temperature) or 33℃ (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 121ºC 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 (25ºC) or at the high temperature (33ºC).

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 ($\geqq 2 \times 10^5$ 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 μg/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 ºC. 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 literature [21]. We chose the elongation factor 1α (EF1α) gene as an internal control as done in the preceding study in the goldfish [21].

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

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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.

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Figures

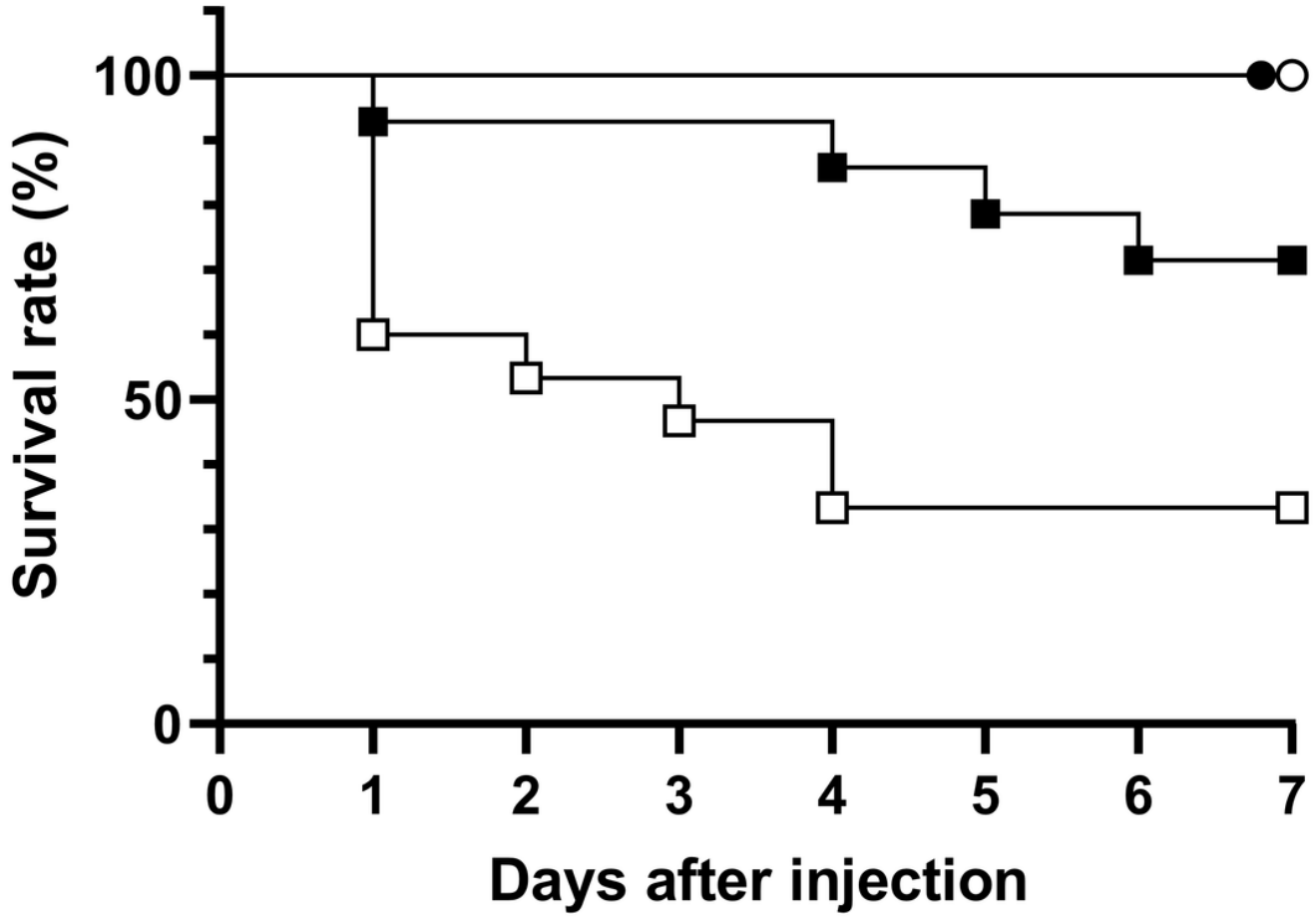

Figure 1

Figure 1

Temperature sensitivity of common goldfish for Pseudomonas infection. After the acclimation period, the common goldfish were divided into four groups as descried in the Materials and Methods section (two

infection paradigms and two temperature paradigms). The goldfish were either infected (P. aeruginosa; 3 x 107 CFU) or uninfected (saline) at either 25ºC or 33ºC. Survival curve for each group is shown in the figure over 7 days after the infection. Results from two experiments were combined and shown in the figure. Symbols: ● (saline, 25°C, n=8), ● (saline, 33°C, n=9), ■ (P. aeruginosa infection, 25°C, n=14) and □ (P. aeruginosa infection, 33°C, n=15). There was a statistically significand difference between the survival curves of P. aeruginosa infection (25ºC) and P. aeruginosa infection (33ºC) groups (P=0.021 by Log-rank test).

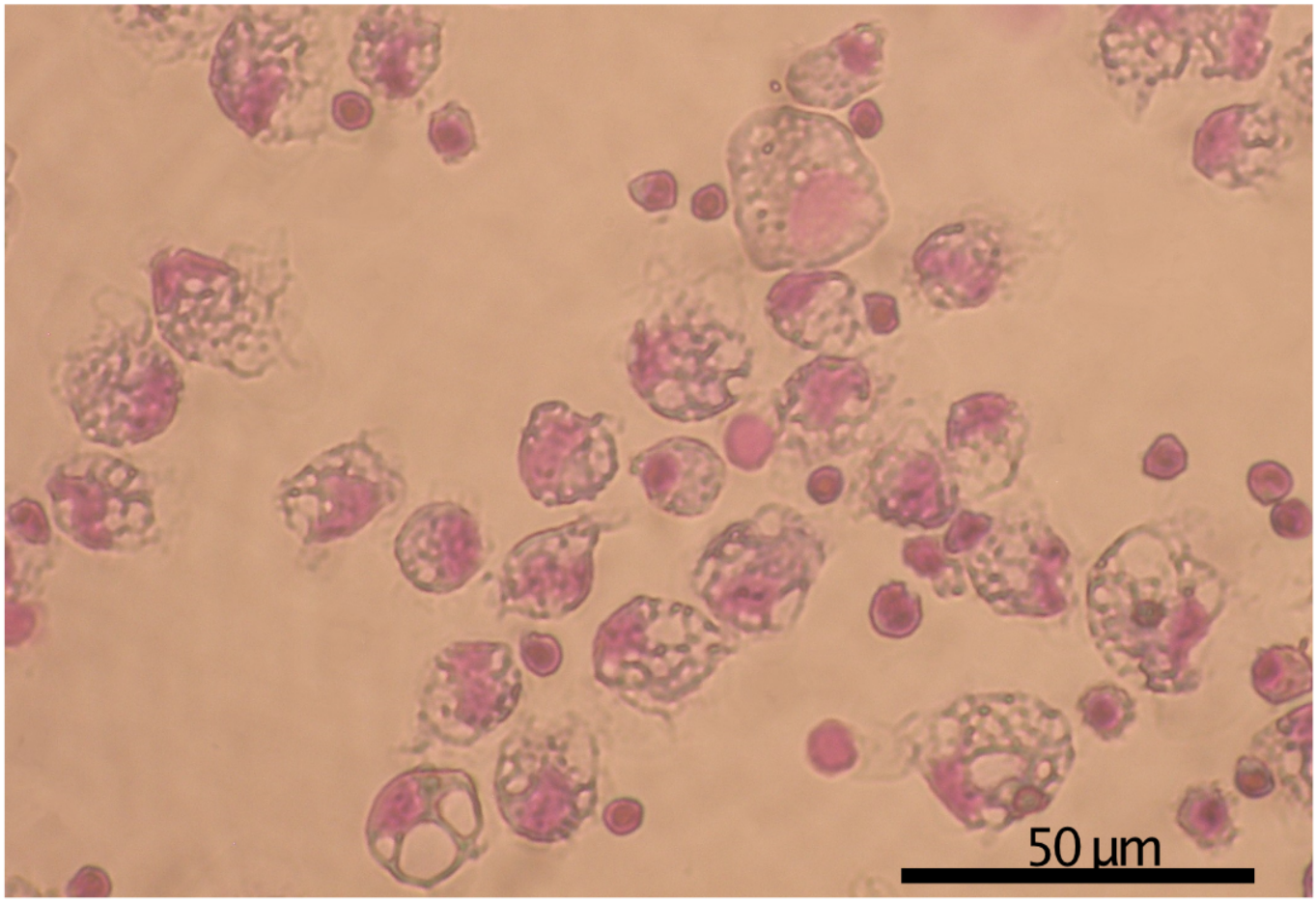

Figure 2

Figure 2

Microscopy image of the eye-sac cells from the bubble-eye goldfish. Eye-sac cells were harvested from eye sacs of untreated bubble-eye goldfish and stained with Giemsa. The scale bar represents 50µm.

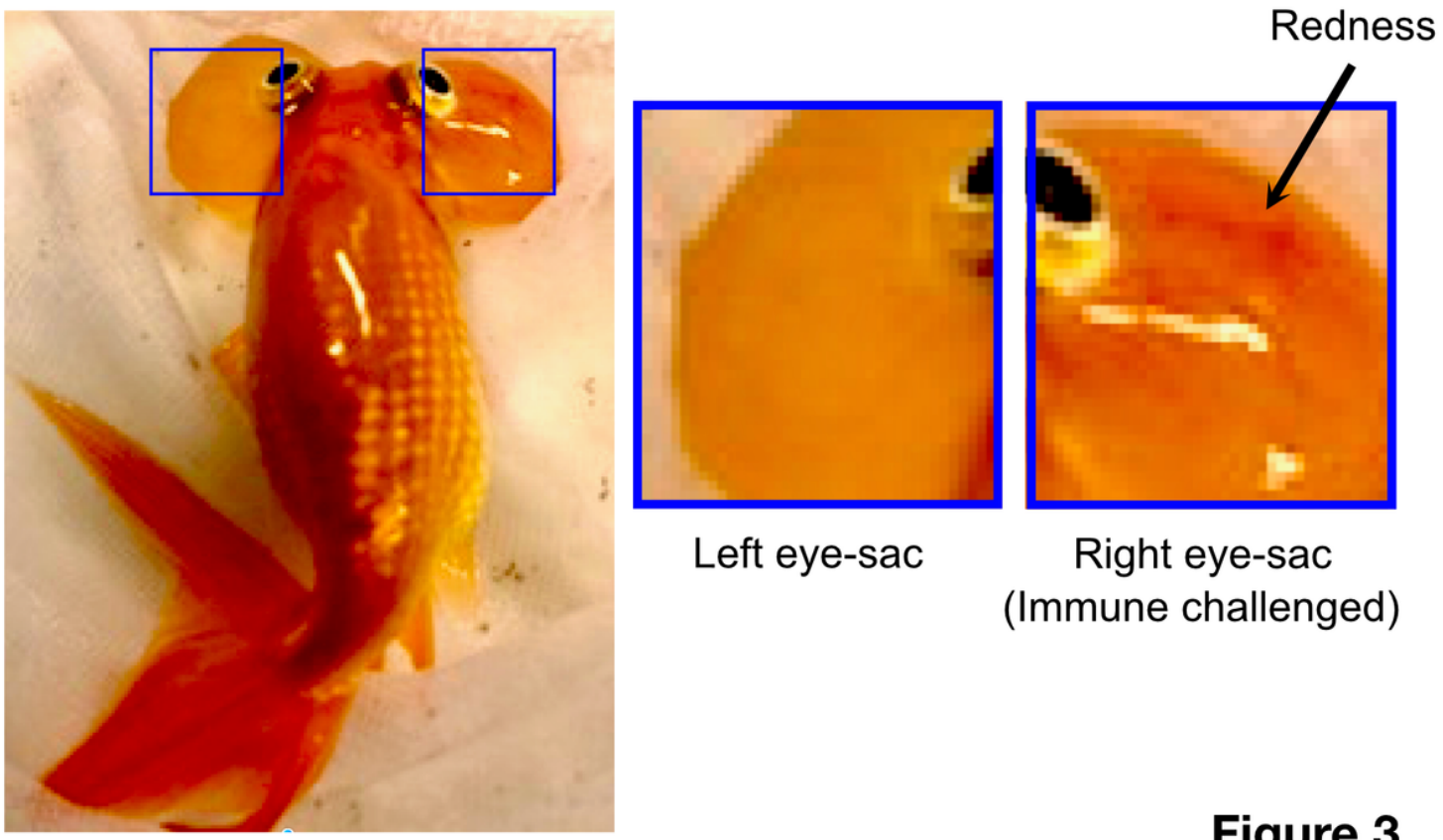

Figure 3

Figure 3

Inflammatory symptom (redness) of the eye sac. Heat-killed P. aeruginosa cells (50 µL of 10-fold concentration of full growth) were injected into the right eye sac of the bubble-eye goldfish, while saline (50 µL) was injected into the left eye sac. Redness was observed on the right eye sac as shown in the figure after 20 hours.

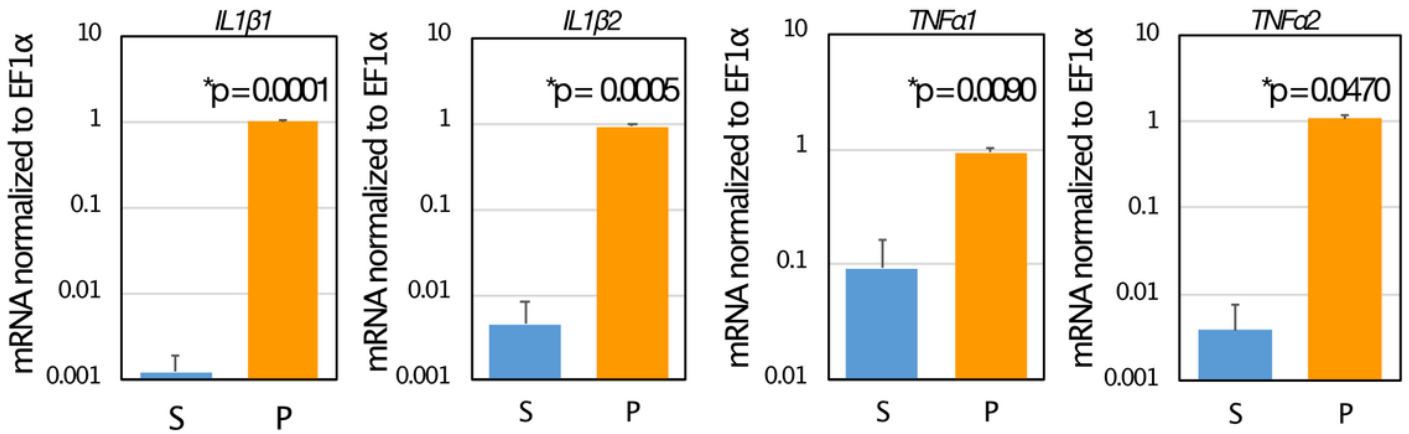

Figure 4

Figure 4

Pro-inflammatory cytokines are expressed in the eye-sac cells in the response to heat-killed P. aeruginosa cells. Twenty-four hours after the injection of heat-killed P. aeruginosa cells (50 μL of 10-fold concentration of full growth; P) or saline (50 μL; S) into eye sac, eye sac cells were collected and analyzed for gene expression (see Materials and Methods section for details). Levels of mRNA for IL1β1, IL1β2, TNFα1 and TNFα2 were shown in the figure (values were normalized by a housekeeping gene (EF1α)). A representative result from three replicates is shown.

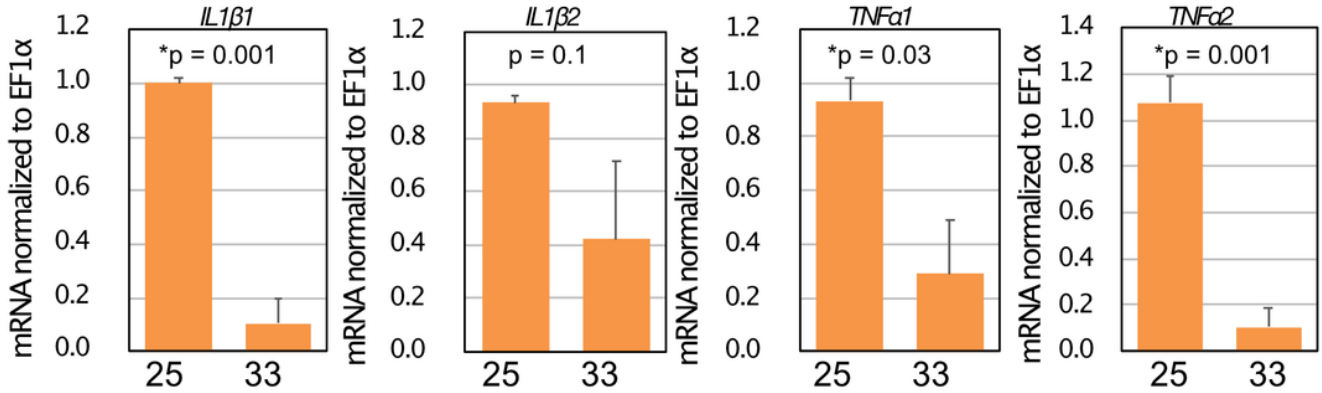

Figure 5

Figure 5

High temperature suppresses the expression level of pro-inflammatory cytokines in the eye-sac cells. Levels of mRNA for IL1β1, IL1β2, TNFα1 and TNFα2 were measured by qRT-PCR. Results from the high-temperature (33°C, n = 3) condition and the normal temperature condition (25°C, n = 2) are shown in the figure (values were normalized to EF1α). P values from Student's t-test are shown in the figure. The asterisks represent statistically significant differences between temperatures (significance levels were corrected by Benjamini-Hochberg procedure).

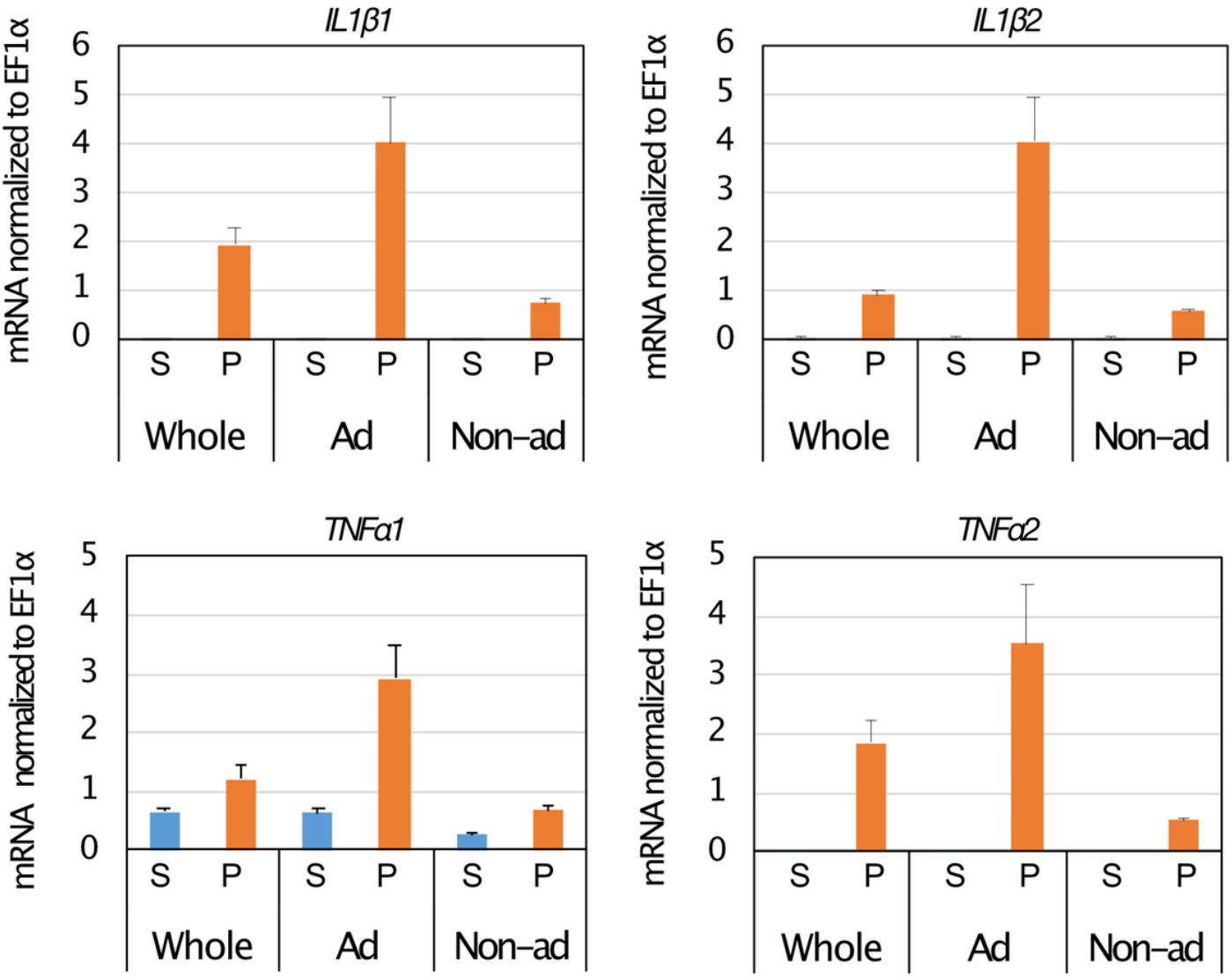

Figure 6

Figure 6

Ex vivo stimulation of eye-sac cells using heat-killed P. aeruginosa cells. The eye-sac cells of bubble-eye goldfish were suspended in medium (see Materials and Methods for details) and pre-incubated at 25°C in 24-well plate (8 x 104/0.8 mL/well) for two hours. Then, adherent (Ad) and non-adherent (Non-ad) cells, were separated by gentle pipetting and cultured. The result from unseparated cell population (Ad + Non-ad; Whole) are also shown in the panels. Gene expressions of pro-inflammatory cytokines in response to heat-killed P. aeruginosa (P) or saline (S) (four hours after adding of the Pseudomonas cells or saline into the culture media) are shown in the figure. The values were normalized to EF1α.

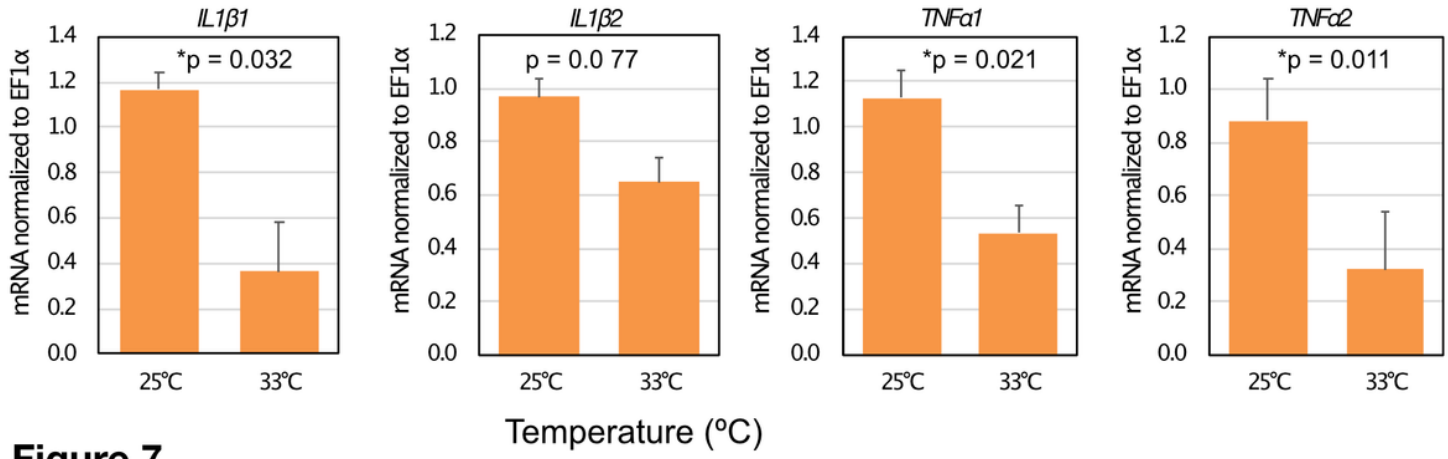

Figure 7

Figure 7

Temperature sensitivity of the ex vivo function of the eye-sac cells. Eye-sac cells, collected from three bubble-eye goldfish were cultured either at 25°C or 33°C. Gene expressions of pro-inflammatory cytokines in the presence of heat-killed P. aeruginosa cells (four hours after adding of the Pseudomonas cells) are shown in the figure. The values were normalized to EF1α. P values obtained from Student's t-tests are shown in the figure. The asterisks represent statistically significant differences (significance levels were corrected by Benjamini-Hochberg procedure).

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