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Expression analysis of the heat shock protein genes and cellular reaction in dojo loach *(Misgurnus anguillicaudatus)* under the different pathogenic invasion

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ABSTRACT

As molecular chaperones, heat shock proteins (HSPs) play essential roles in cells in response to stress conditions. Recent studies about immune functions of HSPs in fish have also been reported. In this study, based on the reported cDNA sequences of the four HSP genes, HSP70, HSC70, HSP90α and HSP90β, the temporal expression patterns of the four genes during embryonic development of dojo loach(*Misgurnus anguillicaudatus*) was assayed with qRT-PCR. All of the four genes were ubiquitously expressed in all detected embryonic developmental stages. Among of them, HSP70, HSC70 and HSP90β were highly expressed in the organ formation stage, while HSP90α was the highest expressed in myotome formation stage. Further, the immune responses of the four HSP genes were assayed when loach were infected with three different pathogens, bacterium (*Flavobacterium cloumnare* G4), parasite (*Ichthyophthirius multifiliis*) and fungus (*Saprolegnia*). All of the four genes were differentially expressed in four tissues such as skin, gills, spleen and kidney in response to the pathogenic invasion, but both HSP70 and HSP90α expressions were dramatically up-regulated. Further, the cellular responses of the loach skinand gill tissues were observed, in which the number of the skin goblet cells were significantly increased, and the gill lamellae became shorter and wider after infected. Thus, our work indicated that the HSPs may directly or indirectly involved in immune defense in fish, at least in the loach.

1. Introduction

Heat shock proteins (HSPs), an evolutionary conserved multigene family of proteins, exist in all organisms from bacteria to mammals [1,2]. HSPs are ubiquitously essential molecular chaperones whose expression are influenced by changes in temperature, dissolved oxygen levels, osmotic pressure, the presence of heavy metals and microbial infections or constitutively expressed in non-stressed cells as housekeeping proteins [3–5]. HSPs could be classified into the five major categories, including small HSPs family, HSP60, HSP70, HSP90 and HSP100 family according to molecular weights and functions [6]. Among of them, HSP70 and HSP90 families have been well studied and were reported to be involved in on immune regulation and signal transduction [7,8]. HSP70 family contains two genes, an inducible type HSP70 and a constitutive type HSC70 (heat shock cognate 70) [9,10]. HSP70 is highly induced upon stress stimulus, while HSC70 is actively expressed under non-stressed cells and remains unchanged or onlymildly induced during stress [11]. HSP70 and HSC70 share some similarities in protein folding/unfolding, assembly/disassembly, degradation and translocation. Interestedly, HSC70 exhibitssome unique functions such as regulating apoptosis, embryo development and innate immune reactions [12,13]. The HSP90 family have four major proteins in vertebrates, two major cytosolic isoforms referred to HSP90α and HSP90β, one endoplasmic reticulum isoform as HSP90β and the mitochondrial HSP90 homologue as TRAP [14,15]. Both HSP90α and HSP90β take part in cell proliferation and differentiation with specific

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functions. HSP90α is mainly associated with growth promotion, cell cycle regulation, and stress-induced cyto-protection, while HSP90β is mainly involved with early embryonic development, germcell maturation, cytoskeletal stabilization, cellular transformation, signal transduction, and long-term cell adaptation [16].

To date, studies on HSP70 and HSP90 have been carried out in various vertebrates including mammals, *Homo sapiens* [16,17] and *Rattus norvegicus* [18]; in avian, *Ansercygnoides* [19] and *Coturnix japonica* [20]; in reptiles, *Caretta Caretta* [21] and in amphibians, *Xenopus laevis* [22,23]. In teleost, HSP70 and HSP90 have been identified and assayed in such species as *Danio rerio* [24], *Cyprinus carpio* [25,26], *Oncorhynchus mykiss* [27], *Sciaenopsocellatus* [28] and *Solea senegalensis* [29] etc. However, the assays of HSP70 and HSP90 in response to the bacterial or fungous infection in fish, especially in dojo loach (*Misgurnus anguillicaudatus*) have not been reported.

The dojo loach (*Misgurnus anguillicaudatus*) is one of important commercial freshwater aquaculture species in Eastern Asian including China, Japan and Korea [30,31]. Recently, infectious diseases occurred frequently the intensive loach culture, which limit the profitability and development of aquaculture [32]. To reveal the potential roles of HSPs in loach development and their responses to foreign pathogen invasion, we firstly assayed the differential profiles of the four proteins, HSP70, HSC70, HSP90α and HSP90β at mRNA level during embryonic development. Then, we constructed three infection models (*F. columnare* G4, *I. multifiliis* and *Saprolegnia*) and sysmetically analyzed the mRNA expression of the four HSPs in different tissues of loach and the histological features of loach skin and gills upon infection. The obtained results demonstrated that the assayed HSPs exhibited deferentially expressed in response to bacterial or fungous invasion, suggesting their potential function in immune reaction in fish, or at least in dojo loach (*Misgurnus anguillicaudatus*).

2. Materials and methods

2.1. Fish and experimental samples

The healthy loaches (average body weight at 8.0 ± 1.0 g) used for the experiment were obtained from a farm in Wuhan (Hubei province, China) and acclimated in tanks suing recirculation water at 22–25 °C with continuous aeration. Loaches were fed with UV-irradiated frozen red worm twice a day (8:00 a.m. and 4:00 p.m.) and maintained for 2 months prior to treatments.

To assay the temporal mRNA expression patterns of the four genes, HSP70, HSC70, HSP90α and HSP90β, mature male and female loaches were injected with mixed oxytocin to produce germ and eggs as the method described in Zhou et al. [33]. Subsequently, developing embryos at different stages including unfertilized eggs, fertilized eggs, 2-cell, 4-cell, 8-cell, 16-cell, 32-cell and 64-cell stage, blastula, gastrula, myotome formation stage and organ formation stage were collected and frozen immediately in liquid nitrogen stored at −80 °C for further use.

2.2. Challenge experiment

2.2.1. Challenge with F. cloumnare G4

For the bacterial challenge experiments, *F. cloumnare* G4 were obtained from the institute of Hydrobiology, Chinese Academy of Sciences (Hubei, China) and it was cultured in Shieh broth for 24 h at 28 °C. Then, the bacterial concentration was measured by the plate count and adjusted to 1×10^5 CFU/mL. For infection group, 90 individuals were put in three replicate tanks and exposed to *F. columnare* G4 by immersion for 3 h at the concentration of 1×10^5 CFU/mL, and then removed to fresh water. For control group, another 90 individuals were treated with Shieh medium with no bacterial added. In each group, six loaches were randomly chosen for the collection of skin, gills, spleen and kidney at 12 h, 1 d, 2 d, 4 d, 7 d, 14 d, 21 d and 28 d after challenged. To obtain the gill blood, the gills was first perfused with PBS-heparin (12500 U/mL), and then its blood was collected. All of the collected tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C for total RNA isolation.

2.2.2. Challenge with I. multifiliis

Challenge with *I. multifiliis* (Ich) was performed using a reported method [34]. Briefly, *I. multifiliisw* asisolated and collected from heavily infected rainbow trout in the fishery research base at Huazhong Agricultural University (Hubei, China). For infection group, 60 loaches were divided into three replicate tanks (each tank contained ~200000 Ich theronts). For control group, the loaches were treated on tanks with normal water supply. Six loaches were randomly chosen for the collection of the tissues skin, gills, spleen and kidney at the time points same as described above. The collected samples were also stored at −80 °C for total RNA isolation.

2.2.3. Challenge with Saprolegnia

Saprolegnia was isolated from heavily infected rainbow trout in the fishery research base at Huazhong Agricultural University (Hubei, China). *Saprolegnia* cultures were maintained on a peptone-glu-coseagar (PGA) medium [35], and sporulation was performed according to the protocol described by Diéguez-Uribeondo et al. [36]. The *Saprolegnia* mycelium was first grown for 3 days at 25 °C on PGA medium covered with sterile rapeseeds, then the sporulation was induced followed by 2 d incubation in sterile water at 20 °C to allow release of the zoospores. Zoospores were counted using a hemocytometer and adjusted to 1×10^5 zoospore/ml. For infection group, 60 loaches were divided into three replicate tanks within 1 L of facility water at room temperature (each tank contained 1×10^8 zoospores). Fish from the control groups were treated in tanks with normal water. Samples were collected and treated similar to the time points as described above.

2.3. RNA extraction and cDNA preparation

Total RNA was extracted from the collected loach tissues using Trizol Reagent (Invitrogen Life Technologies, USA) following manufacturer's protocol. The quality of RNA was determined by electrophoresis and the concentration was measured with the Nanodrop 2000 (Thermo scientific, USA). One microgram of total RNA was reverse-transcribed into cDNA using a 5 × All-In-One MasterMix with AccuRT Genomic DNA Removal Kit (Abm, Canada).

2.4. Quantitative real-time PCR and expression analysis

The qRT-PCR was performed on a 7500 Real-time PCR system (Applied Biosystems, USA) using the EvaGreen 2 × qPCR Master mix (ABM, Canada). All samples were performed in triplicate wells, using the following conditions: 30 s at 95 °C followed by 40 cycles at 95 °C for 1 s and at 55 °C for 10 s. The dissociation curve was analyzed after thermal cycling to determine whether a specific-sized single amplicon amplified. Ct values determined for each sample were normalized against the values for EF1α. The relative fold change in expression to EF1α was calculated by $2^{-\triangle\triangle Ct}$ method [37]. The four pairs of primers were designed in the relatively conserved extracellular domain of HSP70, HSC70, HSP90α and HSP90β sequences, respectively. All primers used in this study are listed in Table 1.

2.5. Histological assays

To evaluate the morphological changes of skin and gills after challenged with bacterial and parasite, the dissected gills (~0.01 g) and skin (~0.25 cm^2) were fixed in 4% neutral buffered paraformaldehyde for 24 h. The fixed tissues were then dehydrated in a graded ethanol series, transparented with xylene, and embedded in paraffin for histological sectioning. Sections at 5–7 μm were obtained with a rotary microtome (HM 325 Manual Microtome, MICROM International GmbH,

Table 1
PCR primer sequences for the four HSPs members qRT-PCR from *M. anguillicaudatus*.

Primer	Sequences
qMaHSP70-F	TATCACAGTTCCCGCCTATTTC
qMaHSP70-R	TTTGCCTTTATCCAGCCCAT
qMaHSC70-F	CTGATTGGTCGCAGGTTTGA
qMaHSC70-R	GACTTGGTCTCGCCCTTGTAT
qMaHSP90α-F	ATTCCCAACAAACAAGACCG
qMaHSP90α-R	TAGAAACCCACGCCAAACTG
qMaHSP90β-F	AGAAGGCTGACGCAGATAAAA
qMaHSP90β-R	GGGCACATCTTCATCCTCATC

Waldorf, Germany) and stained with conventional haematoxylin and eosin (HE) for routine histology examination. The stained sections were examined under microscope (Olympus, BX53, Japan) analyzed with the Axiovision software.

2.6. Statistic analysis

All data were expressed as the mean ± SEM and checked for normality and homogeneity of variances before statistical analysis. A one-way analysis of variance (ANOVA) was conducted with SPSS 16.0 software (SPSS Inc., USA). Differences were considered significant when $P < 0.05$.

3. Results

3.1. Expression patterns of HSP70, HSC70, HSP90α and HSP90β at different embryonic development stages

Assayed with qRT-PCR techniques, the temporal mRNA expression profiles of the four genes, HSP70, HSC70, HSP90α and HSP90β were summarizedin in Fig. 1. All of the four genes were ubiquitously expressed in all detected embryonic developmental stages, but maintained a relatively lower expression from unfertilized eggs to blastocoel. However, their expressions were highly up-regulated from gastrula to organogenic stages. Among of them, HSP70, HSC70 and HSP90β exhibited the highest expression at the myotome formation stage, but HSP90α with an exception.

3.2. Effect of F. cloumnare G4 infection on histological structure and HSPs expression in M. anguillicaudatus

To reveal how the spatial expression of the HSP genes being influenced in response to *F. cloumnare* G4 infection on *M. anguillicaudatus*, the expression levels of the four HSPs, HSP70, HSC70, HSP90α and HSP90β, transcripts at the four tissues, gill, skin, kidney and spleen, were determined with qRT-PCR. As summarized in Fig. 2, the four HSP genes were differentially expressed in a tissue-specific manner and infection time dependent. Both HSP70 and HSP90α mRNA expression were detected in all of the four tissues with a gradual increasing from 12 h to 28 d post infection (Fig. 2B) and they reached the highest at 28 d. Compared with controls, HSP70 expression level was up to 4.08-fold in gills, 5.23-fold in skin, 11.98-fold in kidney and 10.54-fold in spleen. Similar to HSP70, HSP90α expression was increased to 1.51-fold in gills, 2.97-fold in skin, 4.52-fold in kidney and 3.97-fold in spleen at 28 d post infection. However, HSC70 and HSP90β were ubiquitously expressed in all the four tissues, but their expression levels were relatively lower and showed relatively constant at different infection periods.

To detect whether *F. cloumnare* G4 infection could induce cellular responses, the loach skinand gill tissues were histologically assayed with Haematoxylin/eosin staining. As showed in Fig. 3, the goblet cells in skin significantly increased at 12 h (Fig.3b) and 96 h (Fig. 3c) post *F. cloumnare* G4 challenge, compared to control group (Fig. 3a). By

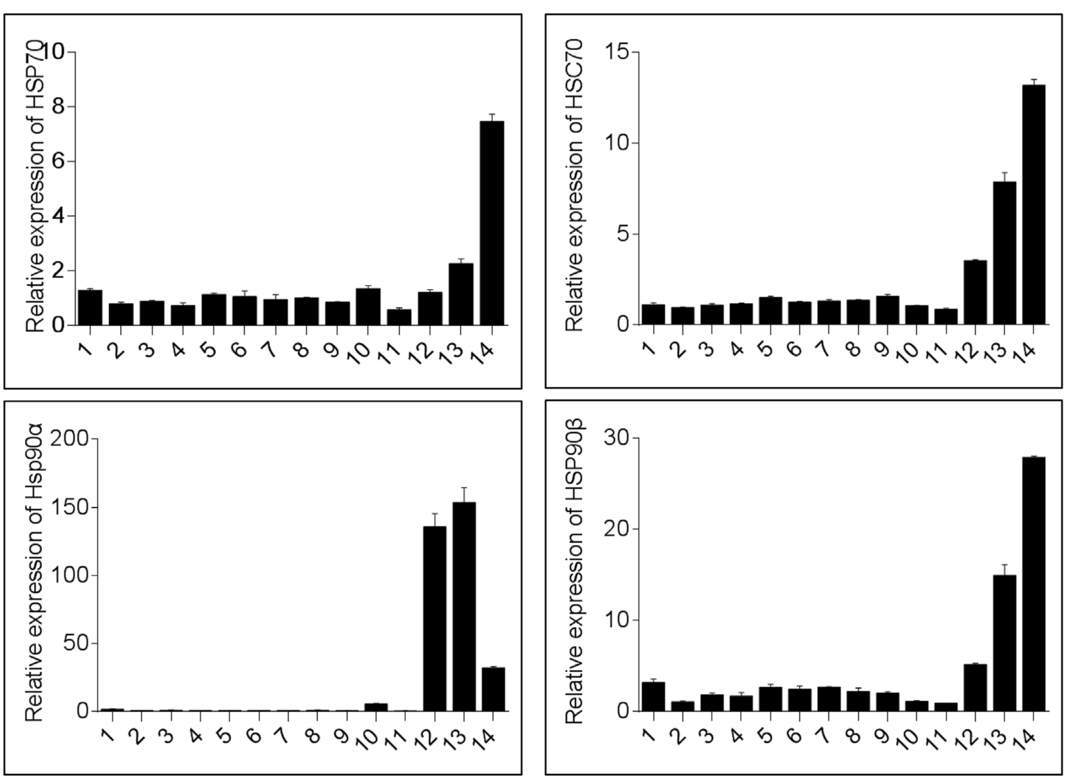

Fig. 1. Expression patterns of HSP70, HSC70, HSP90α and HSP90β genes at different embryonic developmental stages in *M. anguillicaudatus*(n = 4). 1, unfertilized eggs; 2, fertilized eggs; 3, 2-cell stage; 4, 4-cell stage; 5, 8-cell stage; 6, 16-cell stage; 7, 32-cell stage; 8, 64-cell stage; 9, multicellular stage; 10, blastula; 11, blastocoel; 12, gastrula; 13, myotome formation stage; 14, organ formation stage.

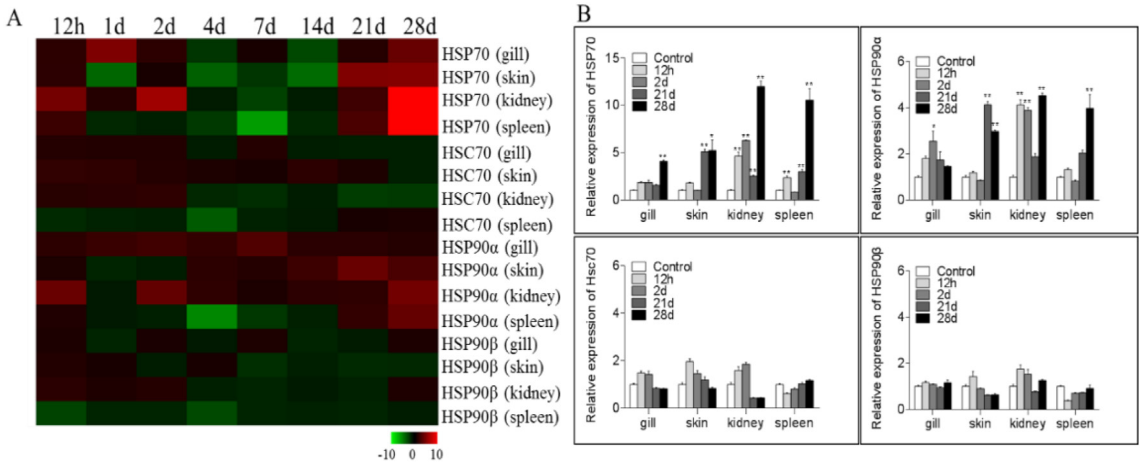

Fig. 2. The transcription level of HSP70, HSC70, HSP90α and HSP90β genes in different tissues responses to *F. cloumnare* G4 infection. (A) Heat map illustrates the fours HSP protein expression levels upon *F. cloumnare* G4 infected fish at 12 h, 1 d, 2 d, 4 d, 7 d, 14 d, 21 d and 28 d post infection (n = 6) in the skin, gill, spleen and kidney. Color value: $2^{-\triangle\triangle Ct}$ (fold change). (B) the four HSPs mRNA expression levels in skin, gill, spleen and kidney at 12 h, 2 d, 21 d and 28 d (n = 6). *0.01 < P < 0.05, **P < 0.01 (unpaired Student's t-test). Data are representative of three independent experiments (mean and SEM).

statistically analysis, the numbers of mucus cells per millimeter skin tissue was increased to about 5 folds at 12 h (Fig. 3g) and folds at 96 h (Fig. 3g) in the infected fish. For the gill tissue, the gill lamellae became shorter and wider after infected with *F.columnare* G4, at 12 h (Fig.3e) and 96 h (Fig. 3f) compared with controls (Fig. 3d). By analysis of the length-width ratio of gill SL between the control and infected fish confirmed that the gill lamellae were morphologically changed into a relative wider (Fig. 3).

3.3. Effect of *I. multifiliis* infection on histological structure and HSPs expression in *M. anguillicaudatus*

The expression levels of the four HSPs, HSP70, HSC70, HSP90α and HSP90β transcripts in gill, skin, kidney and spleen upon *I. multifiliis* infection were also evaluated by qRT-PCR (Fig. 4) of the four HSPs, HSP70 was expressed in all of the four tissues, but showed the highest expression in skin (25.78-fold), then came to and the gills (11.85-fold), and lowest expression in kidney and spleen tissues. HSP90α was relatively higher expressed in skin and kidney, and it was highest expressed at 12 h, then gradually decreased in skin, but the highest expression was detected at in kidney at post 28 day infection by *I. multifiliis*. However, expression of HSC70 and HSP90β did not show significant changes in the four tissues at different infecting time points.

The cellular response in loach skin and gills upon infected with *I. multifiliis* was also histologically assayed (Fig. 5). The number of goblet cells in skin was significantly increased (Fig. 5b) and the gill lamellae in gills became much shorter and wider (Fig. 5e). As showed in Fig. 5c and f (arrow pointed), parasites (*I. multifiliis)* was accumulated in skin and gills at 96 h post infection, which suggested the successful infection of parasites on the loach tissues.

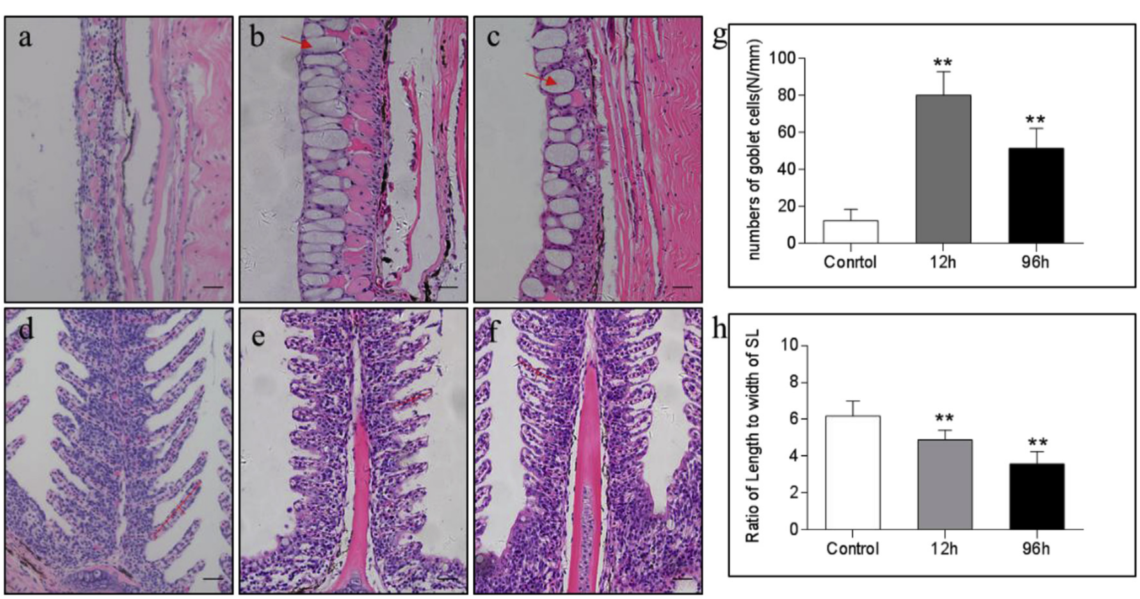

Fig. 3. Morphological changes in skin and gills of *M. anguillicaudatus* infected with *F. cloumnare* G4. (a–c): Haematoxylin/eosin staining on loach skin from uninfected fish (a), 12 h (b) and 96 h (c) post infection; (d–f): on loach gill, uninfected fish (d), 12 h (e) and 96 h (f) in infected fish. (g) The numbers of goblet cells per millimeter skin tissue in control and infected fish (n = 6 per group). (h) The length-width ratio of gill SL from control and infected fish (n = 6 per group). Arrowheads indicate gobletcells. Scal bar, 30 μm.

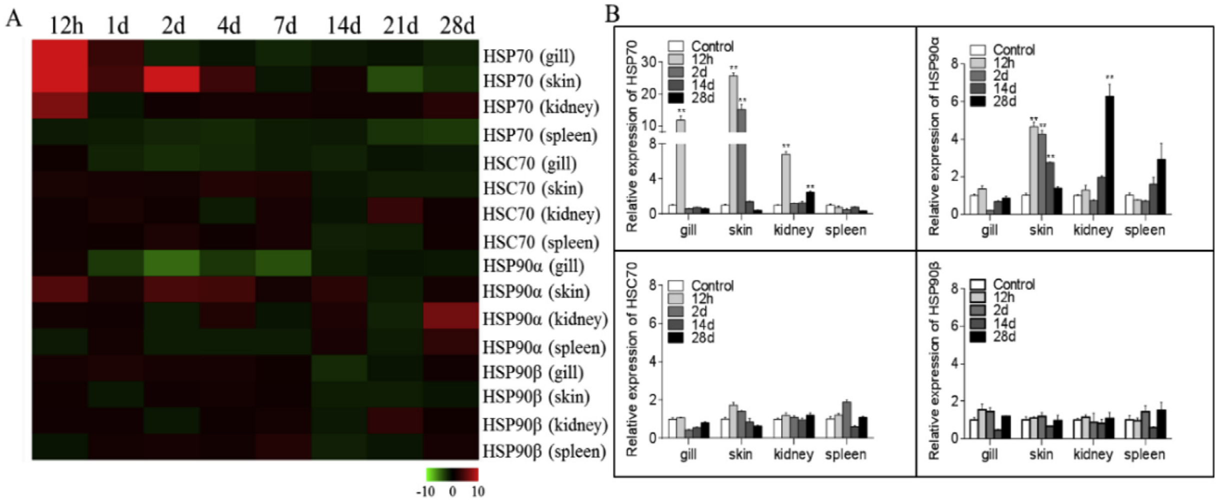

Fig. 4. The transcription levels of HSP70, HSC70, HSP90α and HSP90β genes in different tissues responses to *I. multifiliis*. (A) Heat map illustrates results from real-time PCR of HSP70, HSC70, HSP90α and HSP90β mRNA expression in *I. multifiliis* infected fish versus control fish measured at 12 h, 1 d, 2 d, 4 d, 7 d, 14 d, 21 d and 28 d post infection (n = 6) in the skin, gill, spleen and kidney. Color value: $2^{-\triangle\triangle Ct}$ (fold change). (B) HSP70, HSC70, HSP90α and HSP90β mRNA expression levels in skin, gill, spleen and kidney were detected at 12 h, 2 d, 14 d and 28 d from control and infected fish by qRT-PCR (n = 6). $^{**}P < 0.01$ (unpaired Student's t-test). Data are representative of three independent experiments (mean and SEM). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Effect of Saprolegnia infection on histological structure and HSPs expression in M. anguillicaudatus

Upon infected with *Saprolegnia,* white myceliums were observed to aggregated on the loach skin surface at day 1 post infection (Fig. 6), which caused an apparent body morphological changes and indicate an effective infection by the parasites. To reveal how the four loach HSP genes in response to *Saprolegni* invasion, the expression levels of HSP70, HSC70, HSP90α and HSP90β transcripts in gill, skin, kidney and spleen were also determined with qRT-PCR. The turnout data demonstrated that all of the four genes appeared up-regulated in the four tissues (Fig. 7), but with tissue-specific and infection time dependent manner. HSP70, HSC70, and HSP90α were highest expressed in skin and then came to kidney, but lower in gill. Temporally analysis, the highest expression of HSP70 occurredpost 24 d, HSP90α post 28 d and HSC70 post 1d in skin.

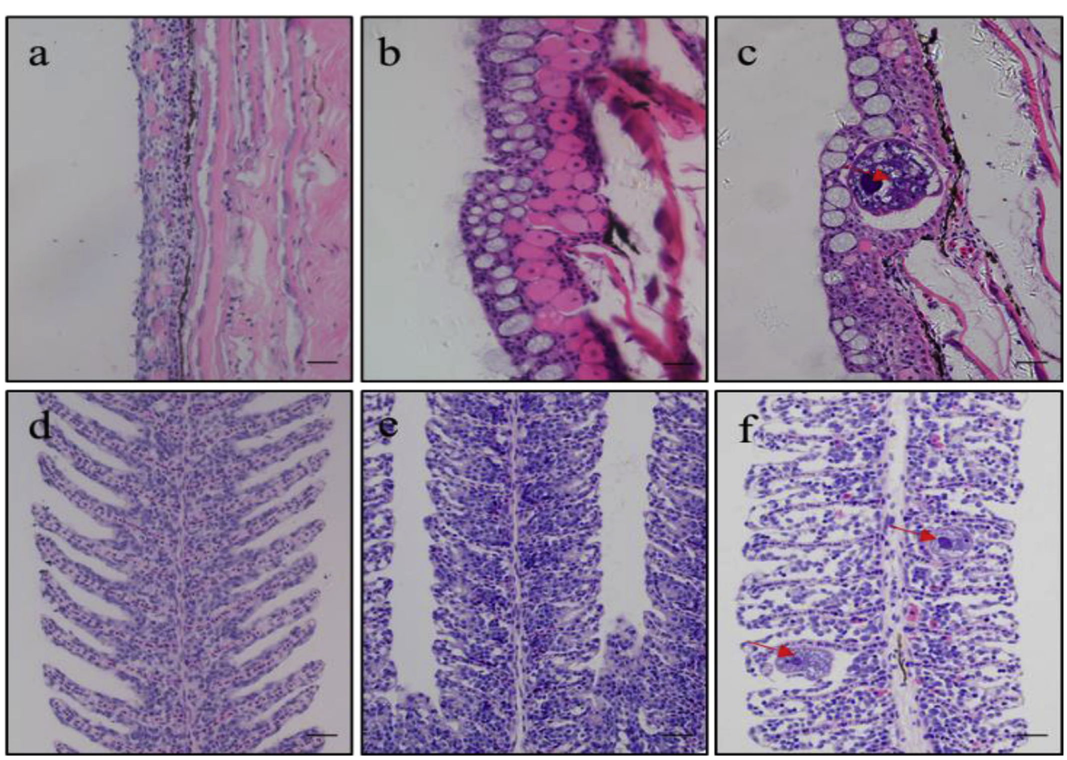

Fig. 5. Morphological changes in skin and gills of *M. anguillicaudatus* infected with *I. multifiliis*. (a–c): Haematoxylin/eosin staining on loach skin from uninfected fish (a), 12 h (b) and 96 h (c) infected fish; (d–f): Haematoxylin/eosin staining on loach gill from uninfected fish (d), 12 h (e) and 96 h (f) infected fish, respectively. Arrowheads indicate parasites. Scal bar, 30 μm.

Fig. 6. The surface of loach skin at day 1 post *Saprolegnia* infection. Arrowheads indicate white mycelium aggregated on the of loach skin surface.

4. Discussion

Heat shock proteins exert essential functions under stress conditions such as exposure to high temperature, oxygen radicals, toxins, microbes, and other factors, which may cause protein denaturation [38]. HSPs have been classified into six families according to their molecular size: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (15–30 kDa) including HSP27, and they play important roles in various physiological process from lower invertebrates to mammals [39]. Earlier studies have revealed the immune responding abilities of HSPs in various host [40]. In the present study, we firstly assayed the expression profile of the four HSPs in different embryonic developmental stages of *M. anguillicaudatus* (Fig. 1), and then we constructed three infection models with three pathogens, (*Flavobacterium cloumnare* G4), parasite (*Ichthyophthirius multifiliis*) and fungus (*Saprolegnia*) to infect the loach. Upon the pathogenic invasion, the expression profiles of the four HSPs, HSP70, HSC70, HSP90α and HSP90β were symertically assayed with qRT-PCR technologies, and the cellular modifications of the loach's skin and gill tissues were also analyzed with haematoxylin and eosin (HE) staining. The obtained results confirmed that HSPs expressions are differentially regulated and the morphological changes in loach skin and gills were also occurred. HSPs are constitutively expressed under certain physiological conditions and take part in many important life activities, such as metabolism, growth, differentiation, programmed cell death, and fertilization [41,42]. Thus, the obtained results suggested that the HSPs may directly or indirectly involved in immune defense in fish, at least in the loach. The mechanism behind this phenomenon might be that HSPs provides a rapid protective measure untilorganic osmolytes are fully accumulate [43]. HSPs are inducible and involved not only in protein folding and cytoprotection, but also in specific immune responses against infectious pathogens, thermal, and osmotic stress [44–46]. After being released from the cell, the HSPs act as messengers communicating the cells' interior protein composition to the immune system for initiation of immune responses against intracellular proteins [47].

Embryonic development in animals could be detrimentally affected by some environmental factors including unfavour temperature flux and chemical exposure. Thus an internal system to protect and maintain cell homeostasis could be established [48]. Heat shock proteins were reported to counteract the exposure of the external stimuli [49]. To date, there are few reports about HSPs expression patterns during different embryonic development in fish. Wang et al. (2015) reported that four HSPs, HSC70-1, HSC70-2, HSP90α and HSP90β in *Siniperca chuatsi* maintained unchanged during the early developmental stages, but significantly increased from gastrula until hatching stage [50,51]. In the present study, our work confirmed that the four HSPs, were also relatively stable during the early development stages from unfertilized eggs till blastocoels, but they showed increased expression at later developmental stages, especially highly expressed at the myotome formation stage and organ formation stage (Fig. 1). Thus, the results showed that different HSPs might play distinct roles during embryonic development, especially such as they regulates somatic and organic genesis in functionally.

In teleost, the skin and gill are the major mucosal immune tissues [52], acting as the first line of defense of pathogens and playing a vital role in the interaction between the external and internal environment. Similarly, kidney and spleen are also involved in immune systems in fish [53]. It is revealed that how the HSPs expression in the four fish tissues as well as their cellular responses upon pathogenic infection, which is beneficial to better understanding of their potential function in the immune response. In the present study, histological assays confirmed that the number of goblet cells was significantly increased in the loach skin tissues challenged with *F. columnare* G4 and *I. multifiliis,* while the gill lamellae were morphologically turned to become wider and shorter. Moreover, upon *I. multifiliis* parasite infection, lots of white mycelium were observed on the surface of loach skin. Thus, the obtained data revealed that once bacterial invasion, the immune related tissues, such as skin and gills in hosts could exert a response by

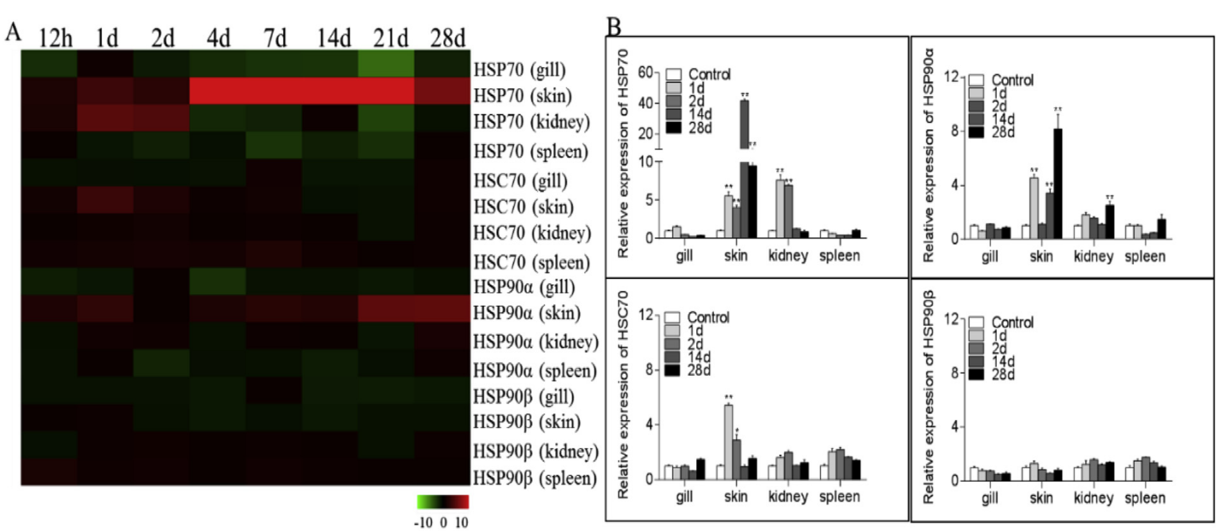

Fig. 7. The transcription level of HSP70, HSC70, HSP90α and HSP90β genes in different tissues responses to *Saprolegnia* invasion. (A) Heat map illustrates the mRNA expression levels of HSP70, HSC70, HSP90α and HSP90β infected fish at 12 h, 1 d, 2 d, 4 d, 7 d, 14 d, 21 d and 28 d post infection (n = 6) in the skin, gill, spleen and kidney. Color value: $2^{-\triangle\triangle Ct}$ (fold change). (B) HSP70, HSC70, HSP90α and HSP90β mRNA expression levels in skin, gill, spleen and kidney were detected at 1 d, 2 d, 14 d and 28 d from control and infected fish by qRT-PCR (n = 6). *0.01 < P < 0.05, **P < 0.01 (unpaired Student's t-test). Data are representative of three independent experiments (mean and SEM). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

changing their cellular morphologies.

In mammals, HSP70 could protect the organism against pathogens not only as a molecular chaperone, but also mediating the innate immune responses. Once exogenous “danger signal” occurred, HSP70 is released to promote immune responses against various pathogens [54,55]. Earlier studies reported that HSP70 mRNA synthesis was dramatically increased infected by *Vibrio anguillarum*, *Argopectenirradians Lamarck,*and its mRNA expression was reached to a maximum level at 8 h and lasted to 16 h in haemocytes, and then decreased gradually [55]. Similarly, hepatic HSP70 mRNA expression in *Megalobrama amblycephala* was significantly increased at first, reaching a peak at 6 h post infection and then decreasing after being challenged with *A. hydrophila* [3]. In the present study, once *F.columnare* G4 was infected, the loach HSP70 transcripts in gill, skin, kidney and spleen were also increased at first and decreased until day 14 post infection (Fig. 2). However, after day 21 post infection, HSP70 expressions were significantly up-regulated and maintained high levels until day 28 post infection, which suggested that HSP70 might not only take part in innate immunity, but also play roles in adaptive immunity. Different with bacterial infection, once infected by *I. multifiliis* (Fig. 4), the highest HSP70 mRNA expression was detected at 12 h and its expression levels in gill and skin were much higher than that in kidney and spleen, indicating that the gill and skin might be major immune tissues where HSP70 may be involved in. However, at day 7 post infection, down-regulated expressions were detected in gill, skin and spleen, which suggest that parasite infection incurs negative interference with the fish host immune system by producing a variety of molecules and enzymes damaging to host immune function and protection mechanisms as earlier reported [56,57]. HSP70 down-regulation following parasitic infection was also observed in other studies [58,59]. To better understand the role of HSP70 in fish for defensing pathogens, HSP70 expression in the four loach tissues, gill, skin, kidney and spleen upon infected with *Saprolegnia* were analyzed (Fig. 7). Interestingly, compared with three other tissues, HSP70 expression was always significantly up-regulated in skin from 12 h to 28 d, indicating that skin might be the primary site of immune response. Taken together, our work suggests that loach HSP70 gene may play essential roles in skin mucosal immunity against to bacteria, parasite and fungus invasions.

Several studies reported that HSC70 not only functions as the cellular molecular chaperone, but also involves in immune responsein aquatic animals [50,60]. In this study, HSC70 mRNA were also differentially expressed in the four loach tissues in response to bacteria, parasite and fungus infection, and it was significantly up-regulated in loach skin infected with *Saprolegnia.* However, the HSC70 mRNA expression level were relatively lower once infected with *F. columnare* G4 and *I. multifiliis.* These observations indicate that HSC70 could take part in immune response, but may be less active in defensing pathogens infection, compared to HSP70. Several studies reported that HSP90s also play essential roles in immune cells as a ligand for a variety of cell-surface receptors such as Toll-like receptors and CDs [61–63]. After being challenged with *A. hydrophila*, the HSP90α mRNA levels in both head kidney and spleen were markedly increased and the expression of HSP90β in the spleen was also up-regulated at 6 h in *Siniperca chuatsi* [51]. Similarly, our study observed that HSP90α expression was significantly up-regulated in gill, skin, kidney and spleen after challenged with *F. columnare* G4, *I. multifiliis* and *Saprolegnia*, but HSP90β expression was just slightly increased in the four tissues. These results indicate that HSP90α may modulate stronger immune responses than that of HSP90β.

In summary, based on the cDNA sequences reported previously, we firstly assayed the expression patterns of loach HSP70, HSC70, HSP90α and HSP90β genes at different early embryonic development stages. Then, three pathogens (bacteria, parasite and fungus) infection models were constructed. Upon their infection, the expression patterns of the four HSP genes in the four loach tissues, gill, skin, kidney and spleen as well their cellular responses were analyzed. The results revealed that four HSPs were differentially expressed in the assayed tissues with cellular morphological changes in the skin and gill tissues. Out of the four HSPs, HSP70 and HSP90α appeared acted as crucialroles in loach against pathogenic invasion. Thus, our work would contribute to a better understanding of the HSPs roles in immune response and provide some molecular insights into pathogen stress tolerance in fish, at least in the loach species.

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