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Short-term starvation and realimentation helps stave off *Edwardsiella tarda* infection in red sea bream (*Pagrus major*)

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ABSTRACT

Dietary regime modifications have been an integral part of health and healing practices throughout the animal kingdom. Thus, to assess the effects of periodic starvation and refeeding schedule on the physiological and immunological perturbations in *Edwardsiella tarda* infected red sea bream, we conducted a 20 day experiment using 4 treatment groups, namely, pre-fed placebo (PFP); pre-starved placebo (PSP); pre-fed infected (PFI); and pre-starved infected (PSI), wherein a 5 h *E. tarda* infection was done on the 11th day. In the present investigation, the pre-starved groups showed significant ($P < 0.05$) alterations in the liver Hexokinase and Glucose-6-phosphatase activity. The pre-starved fish also exhibited significant ($P < 0.05$) increment in the hepatosomatic index, along with increased hepatic glycogen content, in a time dependent fashion. The *PPAR* (peroxisome proliferator activated receptors)α transcription in the pre-starved group decreased significantly ($P < 0.05$) by 10dai, while the *PPAR*γ showcased a reverse pattern. The transcription of *Hepcidin1* and *Transferrin* (iron homeostasis related genes), and *Cathepsin D* and *Ubiquitin* (programmed cell death related genes) portrayed a time responsive decrease and increase in PSI and PFI groups, respectively. Additionally, in comparison to the PFI group, the PSI fish demonstrated substantially reduced oxidative stress level. Fluorescent Immunohistochemistry showed significant ($P < 0.05$) increase in p63 positive cells in the 10dai PFI fish in relation to the PSI group. Therefore, these findings provide new insight into the beneficial role of alternating starvation and refeeding schedule, preferably short-term starvation prior to an infection, in order to obtain better capability to battle against *E. tarda* infection in red sea bream.

1. Introduction

Dietary modulations, especially starvation or food restriction, are believed to beneficially increase resistance against several chronic and acute stressors, all throughout the animal kingdom (Mattson et al., 2003; Lara-Padilla et al., 2015). Starvation related metabolic stress initializes an allostatic mode in the body, resulting in the activation of several stress responses that actuates the production of acute phase proteins which in turn protects the animal from further oxidative and cellular damage (Mattson et al., 2003; Arjona et al., 2009). During starvation, a delicate homeostatic balance is maintained between the energy expenditure and energy stores, especially by the modulation in the carbohydrate, lipid and protein metabolism (Navarro and Gutierrez, 1995). Numerous studies have shown that, at the advent of starvation, liver mobilizes the stored hepatic glycogen and fats, activates the gluconeogenesis pathway, and reduces the rate of glucose utilization (Navarro and Gutierrez, 1995; Laiz-Carrion et al., 2012; Mohapatra et al., 2015) to maintain the bodily metabolic activities.

Food deprivation strategies, either calorie restriction or periodic starvation or moderate feed deprivation, can protract lifespan and enhance the disease fighting capability in vertebrates (Anson et al., 2003; Damsgard et al., 2004). The intricate balance between the nutritional condition, immunity and antioxidant capacity decides the physiological capability of the fish to fight against diseases (Feng et al., 2011). Several antioxidant enzymes (superoxide dismutase, glutathione peroxidase, etc.) are major players in the defense mechanism against the free radicals, thus works towards strengthening the immune condition of the animal. Experimental studies in fish and rodents have also demonstrated the influence of starvation (short or long term) on the immune system (Itoh et al., 2006; Caruso et al., 2011). Godinez-Victoria et al. (2014) reported that intermittently fasted mice showed better resistance to *Salmonella typhimurium* infection than the infected *ad libitum* fed mice. Starvation related transcriptional increase in various lipid and energy metabolism related genes is also responsible for the

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reduction of inflammation in the body (Jorgensen et al., 2008). It has widely been documented in several fish species that short-term starvation acts as an adaptive measure to fight against diseases by facilitating cellular degradation and removal of infection from the body, mostly by modulating the autophagic cell death defense mechanism (Harris, 2011; Bakowski et al., 2014).

Fish are ectothermic animals, hence behave very differently than mammals, in controlling their metabolic rate. Liver is one of the key organ which governs the physiological and immunological functions in the body, and plays a vital role during starvation as well as infection (Power et al., 2000; Sridee and Boonanuntanasarn, 2012). Red sea bream (*Pagrus major*), one of the most widely cultured marine fish in Japan, is a suitable candidate species for farming purposes in worldwide temperate climate. The relatively fast growth, trouble-free collection of fertilized eggs from mature cultured fish, easier adaptability to captive conditions (cages), and high market value, makes this species very lucrative for fish farmers (Dulcic and Kraljevic, 2007). However, various bacterial and viral infections have severely imperiled the red sea bream culture. Hence, the establishment of methods for early detection and prevention of the spread of epidemic diseases is essential to ensure a profitable culture system for the fish.

We have previously reported that, short-term starvation is beneficial in preventing *Edwardsiella tarda* infection in red sea bream (Mohapatra et al., 2015). Our prior work was designed to comprehend the starvation mediated physiological responses of *E. tarda* infected red sea bream. In our previous work, we continuously starved or fed the fish for 20 days, and exposed it to *E. tarda* or placebo midway (11th day) for 5 h, and found that starvation beneficially altered the glucose and energy metabolism, iron homeostasis, fatty acid metabolism, and programmed cell death responses. This starvation-associated multidirectional effect eventually improved the physiology of the fish and enhanced the disease resistance capacity. However, it was not clear whether the starvation-related benefits arose from the pre- or post-infection starvation. Additionally, we also wanted to discover whether alternate feeding-starvation regime, instead of continuous starvation, would be helpful in bringing about the necessary physiological changes in the fish to effectively fight the *E. tarda* infection. Thus to clarify further, in this paper, we aim to explore the effect of short-term starvation and refeeding schedule on liver enzymatic, transcriptional and structural changes upon *E. tarda* infection in red sea bream.

2. Materials and methods

2.1. Fish rearing and experimental design

Three hundred and twenty red sea bream (24 g ± 0.24 body weight) were obtained from the Yasutaka Suisan Company, Japan, and distributed randomly in 8 different tanks (tank 1–8) with two replicates, for a total of 20 fish per tank. The fish were acclimated to the experimental conditions for 1 month. During this period, the fish were fed to satiation twice a day with a commercial diet (Otohime EP_2, Marubeni Nisshin Feed Co. Ltd., Japan). The water quality parameters like temperature (25–26 °C), pH (7.4–7.6), salinity (30–32 ppt), dissolved oxygen (6.4–6.8 mg/L), ammonia-N (<0.01 mg/L), nitrite-N (<0.01 mg/L) and nitrate-N (0.12–0.15 mg/L) were maintained throughout the experimental period. The experimental tanks were supplied with a continuous flow of sea water, and maintained on a 12 h light/12 h darkness regime. Following the acclimation period, fish in tanks 1–4 were fed with the same commercial diet twice a day for 10 days, while the fish in tanks 5–8 were continuously starved for the same time period. At the end of 10 days, fish in tanks 3, 4, 7 and 8 were immersed in *E. tarda* infected sea water (10^9 cfu/mL), while fish in tanks 1, 2, 5 and 6 were immersed in sea water containing the respective sterilized media, for a time span of 5 h. After the infection/placebo exposure, fish in all the tanks were reared in pathogen-free sea water for another 10 days. However, during these later 10 days of treatment, the feeding regime was reversed, i.e. tanks 1–4 were not given any feed and were starved, while the fish in tanks 5–8 were fed to satiation. The different experimental tanks were named as pre-fed placebo (PFP; tank 1, 2), pre-starved placebo (PSP; tank 5, 6), pre-fed infected (PFI; tank 3, 4) and pre-starved infected (PSI; tank 7, 8). Four fish from each replicate of all groups were sampled immediately after 5 h of bacterial immersion (named as 0dai (days after infection)), and further subsequently sampled on the 5th and 10th day after immersion (named as 5 and 10dai, respectively). On the sampling days, the fish were anaesthetized using clove oil (50 μL/L) and blood was collected. Serum was obtained by allowing the blood to clot for 3 h, centrifuging at 3500 *g* for 15mins at 4 °C, and collecting the straw-colored supernatant. The serum was stored at −80 °C until further use. The anaesthetized fish were then killed by a sharp blow to the head and the livers were excised. The hepatosomatic index (HSI) was calculated as liver weight/fish weight × 100. Portions of the liver were kept in 0.25 M sucrose solution, RNAlater and Bouin fixative solution for enzymatic analysis, quantitative gene profiling, and histological observations, respectively.

2.2. Laboratory preparation of pathogenic bacteria

Virulent strain of *E. tarda* (obtained from the Fisheries Research Division of Ainan Town Office, Japan) was cultured, harvested and quantified according to the previously mentioned procedure (Mohapatra et al., 2015), and further added to the sea water at a bacterial concentration of 10^9 cfu/mL.

2.3. E. tarda concentration in the liver

DNA was isolated from the liver using DNeasy Blood and Tissue kit (Qiagen, USA), following the manufacturer's protocol, and quantified. 10 ng of each sample was analyzed by real-time PCR using SSO fast probe mix and *E. tarda* specific primers (Mohapatra et al., 2015).

2.4. Determination of enzyme activities

A 5% tissue homogenate was prepared by homogenizing the preweighed liver tissue in ice-cold sucrose solution (0.25 M). Homogenates were then centrifuged at 5000 *g* for 20 min at 4 °C, and the supernatant was collected carefully and stored at −30 °C, until further use.

The following enzyme activities were measured using an iMark microplate reader spectrophotometer (Bio-Rad, USA). Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX), and Total antioxidant capacity (TAC) were measured using SOD assay kit-WST (Dojindo, Japan), Catalase assay kit (Abnova, Taiwan), GPX assay kit (Biovision, USA) and Total antioxidant capacity assay kit (Biovision, USA), respectively, following manufacturer's protocol.

Metabolic enzymes were assessed, according to the procedures described in Yengkokpam et al. (2013), following the methods of Easterby and O'Brien (1973), Bergmeyer (1974), Wroblewski and Ladue (1955), Marjoric (1964) and De Moss (1955) for the measurement of Hexokinase (HK), Pyruvate kinase (PK), Lactate dehydrogenase (LDH), Glucose-6-phosphatase (G6Pase) and Glucose-6-phosphate dehydrogenase (G6PDH), respectively.

2.5. Quantitative analysis of target gene expression

cDNA sequences of various genes, such as Peroxisome proliferator-activated receptor α (*PPARα*, AB298547.1), *PPARγ* (AB298549.1), *Hepcidin1* (AY452732.1), *Transferrin* (AY335444.1), *Cathepsin D* (AY190689.1), *Ubiquitin* (AY190746.1), *Carnitine palmitoyltransferase 1* (*CPT1*, KY496625), *Fatty acid binding protein 3* (*FABP3*, KY484087), and *Fatty acid transport protein* (*FATP*, KY484086) and *L8* (internal control, AY190734.1) were obtained from the National Center for Biotechnology Information (NCBI) database, and PCR amplified from liver cDNA, using gene-specific primers (Mohapatra et al., 2015). Thereafter, they were