Fish & Shellfish Immunology 47 (2015) 902–912

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Humoral and mucosal defense molecules rhythmically oscillate during a light–dark cycle in permit, *Trachinotus falcatus*

Carlo C. Lazado [a, *], Ivar Lund [a], Per Bovbjerg Pedersen [a], Huy Quang Nguyen [b]

[a] Technical University of Denmark, DTU Aqua, Section for Aquaculture, The North Sea Research Centre, DK-9850 Hirtshals, Denmark
[b] Research Institute for Aquaculture No.1 (RIA1), Ministry of Agriculture and Rural Development, Dinh Bang, Tu Son, Bac Ninh, Viet Nam

ARTICLE INFO

Article history:
Received 11 September 2015
Received in revised form
22 October 2015
Accepted 23 October 2015
Available online 27 October 2015

Keywords:
Circadian rhythm
Clock
Innate immunity
Mucosal immunity
Skin mucus

ABSTRACT

Circadian rhythm provides organisms with an internal system to maintain temporal order in a dynamic environment. This is typified by a 24-h cycle for a number of physiological processes, including immunity. The present study characterized the humoral and mucosal defense molecules and their dynamics during a light–dark (LD) cycle in juvenile permit, *Trachinotus falcatus*. All studied defense molecules were constitutively identified in serum and skin mucus. Serum generally exhibited higher levels of these defenses than skin mucus, with the exception of anti-protease (ANTIPRO). The difference in ANTIPRO, lysozyme (LYZ), esterase (ESA) and catalase (CAT) levels between serum and skin mucus was not affected by the phase of the daily cycle. However, a clear phase-dependent difference was observed in protease (PRO), globulin (GLOB), myeloperoxidase (MPO), alkaline phosphatase (ALP) and glutathione peroxidase (GPX) levels. Activities of ALP and GPX displayed significant daily rhythmicity in both serum and skin mucus. Circadian profile of ALP was identical in both biofluids, but an antiphasic feature was exhibited by GPX. GLOB and MPO levels also exhibited significant daily oscillation but only in serum with acrophases registered at ZT 14.5 and 6.15, respectively. Mucus PRO and serum ANTIPRO demonstrated significant temporal variations during a daily cycle albeit not rhythmic. Cluster analysis of the defense molecules in serum and skin mucus revealed two different daily profiles suggesting a possibility of distinct circadian control between humoral and mucosal immunity. These observations indicate that LD cycle had a remarkable impact in the defense molecules characterizing the humoral and mucosal immunity in permit. Daily rhythmic patterns of these defense molecules contribute to our understanding of the barely explored interplay of immunity and circadian rhythm in teleost fish. Lastly, the results could be useful in developing aquaculture practices aiming at modifying the immune functions of permit for improved health.

1. Introduction

Circadian rhythm is a fundamental biological phenomenon enabling organisms to maintain temporal order in their physiological and behavioral processes [1,2]. A network of clock genes and their proteins operating through a transcriptional-translational feedback loop is the core machinery perpetuating the 24 h rhythmicity [3,4]. The molecular and biochemical basis of circadian time-keeping systems is highly conserved among organisms hence has been widely considered as a universal adaptive mechanism [5]. This evolutionarily conserved adaptation allows organisms to anticipate environmental changes and schedule their biological activities at the most optimum and advantageous time of the day [5,6].

Host immunity is one of the many physiological systems where circadian rhythm imposes regulatory control. Circadian effects on immune function indicate a partitioning of immune response into two fundamental states, namely a state of anticipation and enhanced immune activity, and the opposing state that is related to repair and regeneration [7]. In mammalian models, circadian rhythms have been observed in lymphocyte proliferation, antigen presentation, cytokine gene expression, inflammation, and

Abbreviations: ALP, alkaline phosphatase; ANTIPRO, anti-protease; CAT, catalase; ESA, esterase; GLOB, globulin; GPX, glutathione peroxidase; LD, light:dark; LYZ, lysozyme; MPO, myeloperoxidase; PRO, protease; SOD, superoxide dismutase; ZT, *Zeitgeber* time.

* Corresponding author. Technical University of Denmark, The North Sea Research Park, DK-9850 Hirtshals, Denmark.
E-mail address: carlolazado@yahoo.com (C.C. Lazado).

http://dx.doi.org/10.1016/j.fsi.2015.10.037
1050-4648/

phagocytosis, among many others [8–10]. In fact, circadian clocks are expressed in several subsets of immune cells such as mononuclear cells, macrophages, dendritic cells, B cells and natural killer cells [8,11–13], indicating their essential regulatory role in cellular immune functions. In the last decade, the status of circadian rhythm as an integral regulator of immune responses was accentuated with a growing body of evidence linking its significance in disease onset and therapies [14].

Components of the fish immune system also exhibit daily variations [15], though the information is very scarce thus relatively incomparable to the level of understanding in its mammalian counterpart. Daily rhythmicity is demonstrated by several components of fish innate immunity such as hemolytic complement, lysozyme, peroxidase, globulin, phagocytosis and reactive oxygen species [16–19]. Recently, it was demonstrated in zebrafish (*Danio rerio*) that migration of neutrophils to the site of injury was rhythmic and that melatonin regulates this response [20]. Current knowledge confirms the interactions of nervous and endocrine systems with the immune system in vertebrates, including fish [15]. Melatonin, a key hormonal output of circadian rhythm, has been documented to be a modulator of fish immunity [15,21].

Mucosal immune system has become an integral component in the contemporary discussion of fish immunity [22]. Fish skin and its mucus layer constitute the biggest mucosal tissue that is rich in immunologically active cells and molecules [23,24]. Seasonal changes have been documented in skin mucus immune factors [25], but not on their daily rhythms. Unlike humoral and cellular immune factors, the skin and the associated mucus is directly exposed to light – one of the most powerful signals regulating circadian rhythm in fish [4].

Daily variations in immune function have direct implications on disease and health status [17]. It is important to highlight that immunological rhythms of many fish species are unknown, especially those with economic value. Therefore, the present study characterized the defense molecules in serum and skin mucus and their dynamics during a complete LD cycle in juvenile permit fish (*Trachinotus falcatus*). The model fish was selected because it is an emerging species being developed for tropical aquaculture and the results of the study will be essential in advancing its commercial scale domestication.

2. Materials and methods

2.1. Fish

Hatchery produced permit fish (*T. falcatus*) used in this study were provided by the Aquaculture Research Sub-Institute in North Central (ARSINC), Research Institute for Aquaculture No. 1, Cua Lo, Nghe An, Viet Nam. Upon arrival at the DTU Aqua facility in Hirtshals, Denmark, the fish fry (average size 1–2 g) were quarantined for two weeks. Thereafter, the fish were transferred to fiberglass holding tanks in a flow-through system. Water parameters were maintained as follows: temperature at 27–28 °C, dissolved oxygen levels were above 80% saturation; average salinity at 33 g L^{-1}; pH 7.3–7.4. The fish were fed with a high-protein commercial diet (EFICO Sigma 841, BIOMAR, Denmark) and the daily feed ration was calculated based on the biomass, a fixed feeding level and an expected FCR, and adjusted after each routine weighing interval. Constant illumination was provided by fluorescent white light with an average intensity of 150 lux (Light Meter RS 180-7133, Northants, UK).

All fish handling procedures employed in the study complied with the bioethical standards for animal experimentation practiced at the Technical University of Denmark.

2.2. Entrainment to a daily light–dark (LD) cycle

Fish were selected from the holding stocks and only those that were apparently healthy and free from any physical deformities were sorted and weighed. One hundred fish with an average weight of 110 ± 15 g were selected for the 12 h light:12 h dark cycle experiment. Ten fish were stocked to each of ten 189-L, cylindrical–conical, thermoplastic tanks. Each tank was supplied with seawater at a flow rate of 40 L h^{-1} and was part of a recirculation system. Water parameters were maintained as stated above. To ensure minimal disturbance to the fish during sampling, two tanks were exclusively dedicated to a single sampling point. The transparent tanks were covered with black plastic to avoid tank wall collisions and bruises due to burst swimming activity of this species (Lund et al., unpublished data). White LED light with a maximum average intensity of 350 lux placed above each tank provided the illumination and was programmed to deliver a daily photoperiod of 12 h light and 12 h darkness. The fish received a commercial diet (EFICO Sigma 879, BioMar, Denmark) with a daily ration of 2% (w/w) body weight. Automatic feeders were employed to deliver the feed at random times of the day to avoid the influence of feeding entrainment. The fish were under these conditions for 25 days.

2.3. Sample collection and preparation

There were two sampling approaches employed in the study. In all cases, there was no feeding 24 h prior to sample collection.

Since there were no available data on the humoral and mucosal defense molecules in the model fish, the first sampling was designed to identify some key defense factors in serum and skin mucus under normal conditions, specifically without entraining photoperiod signal. This was achieved by taking 12 fish of equal size from the holding tanks that were exposed to constant illumination. Samples were collected between 10^{00}–11^{00} AM. The second sampling was performed with the groups exposed to a 12L:12D photoperiod for 25 days. Sampling was carried out at a 6-h interval (*Zeitgeber* time: ZT0, 6, 12, 18 and 24) for a period of 24 h. ZT0 and ZT6 constitute the light phase, ZT18 and ZT24 represent the dark phase and ZT12 is the transition phase. Samples at ZT0 were collected when light intensity reached its maximum (*circa* 350 lux) and those at ZT24 were collected just before dark phase transitioned to light phase. Three fish were taken from each of the two representative tanks of a particular sampling point and euthanized with an overdose of ethylene glycol (Merck, Darmstadt, Germany). Sample collection during the dark phase was conducted in a room with an illumination not greater than 3 lux and a 5-min timeframe was strictly followed in sampling each fish.

Skin mucus samples were collected first by placing the fish in polyethylene plastic bag [26]. Mucus was collected by gently rubbing the fish inside the plastic in a downward motion for at least 10–15 times. The collected mucus was immediately placed in ice. It was ensured that there was no fecal contamination during the whole process. Skin mucus extract was prepared by re-suspending the collected mucus with 1 volume of phosphate-buffered saline (PBS, pH = 7.4) followed by a vigorous shaking. After centrifugation at 1500 rpm for 10 min at 4 °C (SIGMA 3–18K, Osterode, Germany), the supernatant was collected and kept at −80 °C until analysis. Blood was collected from the caudal vein using a non-heparinized syringe. The blood was allowed to clot at room temperature (18 °C) for 2 h and then at 4 °C overnight. Serum was collected after centrifugation at 750 *g* for 10 min, divided into several aliquots and stored at −20 °C until use. Protein content of serum and skin mucus was determined using bovine serum albumin as a standard (Thermoscientific, Illinois, USA).