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Expression of cytokines IL-1β and TNF-α in tissues and cysts surrounding *Didymocystis wedli* (Digenea, Didymozoidae) in the Pacific bluefin tuna (*Thunnus orientalis*)

Ivona Mladineo*, Barbara A. Block

Hopkins Marine Station, Stanford University, 120 Oceanview Blvd, Pacific Grove, 93950 CA, USA

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ABSTRACT

Tuna long distance migrations and exposure to wide range of ambient water temperatures facilitate infections with several parasitic groups. This is reflected in the remarkable diversity of tuna parasite communities, especially members of Didymozoidae superfamily (Poche, 1907) (Trematoda, Digenea). *Didymocystis wedli* is the most frequent species encountered in bluefin tuna parasitizing gill filaments, therefore suggested as a biological marker to differentiate between discrete tuna Atlantic stocks. Because of its high occurrence in gill tissue and inflammatory reaction as the consequence, the aim of our study was to asses if inflammatory madiation through expression of IL-1β and TNF-α is present locally at the site of *D. wedli* encystment, as well as if the systematic expression of cytokines can be detected in different tissues of infected versus uninfected fish. Quantification of localized cytokine expression was done on paraffine embedded gill sections by *in situ* hybridization, while quantitative PCR was used to mesured cytokine transcripts in skin mucus, kidney, spleen, gills and liver. Our results suggest that tuna constitutive expression of IL-1β and TNF-α in gills and skin implies a well-adapted innate immunity present at the barrier between the organism and environment. Upregulation of both cytokines in *Didymocystis*-infected gills not followed by a systematic response evidences the ongoing of an inflammatory process specific for the parasitation site. However, the lack of intensive cytokines response to *D. wedli* observed by molecular and histological data that fails to eliminate the parasite, could be related to the "old" age of the parasitic process.

1. Introduction

Bluefin tunas (Scombridae) include three species: Atlantic bluefin tuna (*Thunnus thunnus*), Southern bluefin tuna (*Thunnus maccoyii*) and Pacific bluefin tuna (*Thunnus orientalis*). They are the largest of the *Thunnus* species, characterized by endothermy, long life and the widest geographic distributions [1]. Currently, they represent the most valuable fisheries and finfish aquaculture product recognized and more than a half of the total world production is concentrated in the Mediterranean Sea [2]. Large-scale ranching operations are present also in North America and Australia [3,4]. It is considered that over 90% of the farmed bluefin tuna is designated for the Japanese sushi and sashimi market [5]. However, wild bluefin tuna fisheries have reached a plateau seriously endangering sustainability of present stocks and efforts to complete the aquaculture cycle of bluefin have rapidly expanded [6]. In order to limit bluefin tuna stocks being severed by overfishing, in 1982 International Commission for the Conservation of Atlantic Tuna (ICCAT) established fishing quotas for the Western Atlantic stock of the Atlantic bluefin, as well as for Eastern Atlantic and Mediterranean Sea stocks in the 1998.

Tuna long distance migrations and exposure to wide range of ambient water temperatures facilitate infections with several parasitic groups. This is reflected in the remarkable diversity of tuna parasite communities. Among these, the highest levels of prevalence and abundance are achieved by members of Didymozoidae superfamily (Poche, 1907) (Trematoda, Digenea) [7–9] both in the wild and reared tuna. Didymozoids show wide distribution, occurring in tropical and subtropical World oceans, while in the Mediterranean fish 23 species have been isolated so far [10]. They are gonochorists or hermaphrodites, typically encysted in pairs and showing wide localization and specific tissue tropism. In bluefin tuna they inhabit different tissues, parasitizing gills, cartilaginous, mucous and connective tissue in nose, mouth, ocular and gill cavity,

* Corresponding author. Present address: Institute of Oceanography & Fisheries, Laboratory of Aquaculture, P.B. 500 21000 Split, Croatia. Tel.: +385 21408047; fax: +385 21358650.
E-mail addresses: mladineo@izor.hr (I. Mladineo), bblock@stanford.edu (B.A. Block).

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as well as digestive organs, kidneys, gonads, skin and fins [11,12]. Throughout Didymozoidae genera there is enormous difference in body size and tissue localization that reflects in specific tissue alteration that can range from negligible host reaction to necrosis and sloughing of gill epithelium [13,14]. *Didymocystis wedli* is the most frequent species encountered in bluefin tuna parasitizing gill filaments [9], recently suggested as a biological marker to differentiate between discrete tuna Atlantic stocks that reach the Gulf of Mexico and the Mediterranean bluefin tuna population [15]. During *D. wedli* gill infections usually there is absence of a strong inflammatory response, however in some cases severe desquamation of primary lamellae complicated by secondary bacterial infections can develop [13]. Similar changes in form of lymphocytic aggregations limited to external wall of the Nemathobothriinae didymozoid cyst were previously reported [16], suggesting an inflammatory reaction that fails to reject the parasites. In bluefin tuna that have elevated needs for oxygen consumption necessary for their high metabolic turnover [1], parasitic processes in respiratory tissue can impose serious threats to normal functioning in reared conditions, already stressful for the animal.

Elevated levels of immunoglobulin E and eosinophile granulocytes as well as mast cell hyperplasia and secretion of inflammatory cytokines are the hallmarks of helminthic infection in mammals and lead to the development of more or less pronounced inflammatory reaction. In highly vascularized tissues, like gut or gill epithelium, parasites are more quickly detected by those immunity components circulating in the blood than species secluded in poorly vascularized tissues [17,18]. Cytokines as pleiotropic small-molecular-weight molecules, orchestrate complex regulatory networks that modulate immune response. Generally, they are operating in the vicinity where they are secreted, although some, such as interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α), circulate the blood to exert a systematic or endocrine effect. The cellular response of cells to cytokines usually occurs over a period of hours, requiring *de novo* production of mRNA and protein [19]. Upon interaction with a parasite, macrophages are the major mediators of inflamation, activated by proinflammatory cytokines IL-1β and TNF-α. Clear role of cytokines as innate immunity response to parasites was firstly evidenced in the epithelial cells of *Gyrodactylus derjavini* infected trout, where IL-1β, TNF-β and TGF-β (transforming growth factor) played an important role [20,21].

Because of a high occurrence of *D. wedli* encysted in bluefin tuna gill tissue and inflammatory reaction as the consequence, the aim of our study was to asses if inflammatory madiation through expression of IL-1β and TNF-α is present locally at the site of *D. wedli* encystment, as well as if the systematic expression of cytokines can be detected in different tissues of infected versus uninfected fish.

2. Materials and methods

2.1. Fish and tissue sampling

Pacific bluefin tuna (*T. orientalis*) were captured by hook and line from the Mexican farming cages off San Diego, CA, USA, in waters of NW Mexico, held on board of the fishing vessel in wells filled with flowing seawater, and transported by a truck to the Tuna Research and Conservation Center (TRCC) in Pacific Grove, CA, USA (36°37′6″N, 121°54′10″W). Fish were held in two 109 m^3 circular tanks containing seawater. Fish were euthanased by pithing and necropsy was done immediately after death in order to isolate and identify didymozoids.

For gene expression study by Q-PCR, eleven fish (Lt = 23.6 ± 2.8 kg; Wt = 125 ± 8.5 cm, 2 + years) were sacrificed by a sharp blow in the frontal cranial bones, and gill tissue infected and uninfected with *D. wedli* as well as kidney, spleen and liver was immediately collected with scissors baked in oven overnight at 220 °C and cleaned with RNAase free solutions. For Q-PCR, tissue was immersed in RNAlater stabilization reagent at room temperature and (Quiagen), stored overnight on +4 °C and −80 °C until RNA extraction.

Parasitological examination was performed as previously described [9]. Briefly, didymozoid cysts from both tuna hosts were collected from gill and visceral organs, measured and ruptured with fine needles under the stereomicroscope. Individuals were flattened under coverslip pressure, stained in Borax carmine and mounted in Canada balsam. Identification was performed as previously described [11,12, 22,23].

2.2. Gene expression by Q-PCR

Total RNA was extracted from skin mucus, liver, spleen, kidney and gills, following standard protocol from RNeasy Fibrous Tissue Mini Kit (Quiagen), after disruption and homogenization with baked stainless steel beads in Mixer Mill MM 300 (Quiagen). The purity and quantity of total extracted RNA was measured on the Nanodrop in ng/μl. Poly A^+ RNA was purified from total extracted RNA by Oligotex Kit (Quiagen) and again checked for purity and quantity on the Nanodrop.

Total quantity of Poly A^+ RNA was reverse transcripted in cDNA, using iScript™cDNA Synthesis Kit (Bio-Rad), following manufacturer profile. Quantities of cDNA were normalized to 1 μg/μl.

Fragments of genes of interest were amplified in thermo-cycler using previously reported primers (IL-1β: F 5′ GGR SAG CGA CAT GGY RCG ATT TCT 3′, R 5′ GGT GCT GAT GTA CCA GTT G 3′; TNF-α: F 5′ CCA GGC RGC CAT CCA TTT AGA AG 3′, R 5′ CCG ACC TCA CCG CGC T 3′; β actin: F 5′ ATC GTG GGG CGC CCC AGG CAC C 3′, R 5′ GTC ATC TTC TCY CTG TTG GC 3′) [24]. Protocols for amplification included using 1.75–2.5 mM of $MgSO_4$, 25 mM of dNTP, 1 unit of Platinum *Taq* High Fidelity (5 U/μl) (Invitrogen) and 1 μg/μl of template was used for the reaction. Products were loaded on a 2–3% agarose gel and visualized by adding SYBR Safe (1%) directly in the gel. Products of adequate size were purified using QIAquick PCR Purification Kit (Qiagen) and cloned in chemically competent TOP10 vector cells using Invitrogen TOPO TA Cloning Kit. Clones were selected and screened for the insert using T3 and T7 primer set. The purified PCR product was sequenced on an ABI 3100 automatic DNA sequencer (Applied Biosystems), using the ABI PRISM BigDye Terminator Cycle Sequencing Kit, in both directions.

Sequences were aligned with sequences stored in GeneBank (β actin: EU30041; IL-1β: EU30045; TNF-α: EU300946) by Clustal X, implemented in the MEGA 3.1 software, using default parameters.

Real-Time PCR assays were performed using iQ SYBR Green Supermix in accordance with manufacturers procedure (Bio-Rad) on an iQCycler platform. Template cDNA was diluted 1:10 and run in triplicate. Control reactions without cDNA template (No-Template Control) with sampled RNA, were performed to check that amplified products were not a result of DNA contamination or due to primer-dimer effects. Primer (IL-1β: F 5′ CTC TTC TAC AAA CAG GAC ACT GG 3′, R 5′ AGT TGG GAA AAC GGG CAG 3′; TNF-α: F 5′ CTG GAG TGG AGA GTT G 3′, R 5′ GGC TGT AGA CCA AGT AG 3′) concentrations were optimized for each target and housekeeping gene in order to obtain the concentration that yields the lowest C_T values. During that run, raw data from a melting curve were collected after reaching 95 °C, while subjecting the products to different temperature increments, eventual primer-dimer formation was ruled out [25].

The threshold was calculated using maximum curvature approach, with per-well baseline cycles determined automatically. Data analysis window was set at 95.00% of a cycle, centered at the end of the cycle. Weighted mean digital filtering has been applied,