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Identification of centrarchid hepcidins and evidence that 17β-estradiol disrupts constitutive expression of hepcidin-1 and inducible expression of hepcidin-2 in largemouth bass (*Micropterus salmoides*)☆

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ABSTRACT

Hepcidin is a highly conserved antimicrobial peptide and iron-regulatory hormone. Here, we identify two hepcidin genes (*hep-1* and *hep-2*) in largemouth bass (*Micropterus salmoides*) and smallmouth bass (*Micropterus dolomieu*). Hepcidin-1 contains a putative ATCUN metal-binding site in the amino-terminus that is missing in hepcidin-2, suggesting that hepcidin-1 may function as an iron-regulatory hormone. Both hepcidins are predominately expressed in the liver of largemouth bass, similar to other fish and mammals. Experimental exposure of pond-raised largemouth bass to 17β-estradiol and/or the bacteria *Edwardsiella ictaluri* led to distinct changes in expression of *hep-1* and *hep-2*. Estradiol reduced the constitutive expression of *hep-1* in the liver. Bacterial exposure induced expression of *hep-2*, suggesting that hepcidin-2 may have an antimicrobial function, and this induction was abolished by estradiol. To our knowledge, this is the first report of the regulation of hepcidin expression by estradiol in either fish or mammals.

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1. Introduction

During the last decade there has been increasing awareness and concern regarding endocrine disrupting chemicals (EDCs) [1]. These chemicals are virtually ubiquitous and have been identified in aquatic ecosystems around the world [2–5]. Of particular interest are estrogenic endocrine disrupting chemicals (EEDCs), which have been shown to modulate immune responses in addition to reproductive physiology [6–9]. In recent years, fish kills and fish lesions have occurred concurrent with a high prevalence of intersex in the Potomac and Shenandoah Rivers [10,11]. These fish kills primarily involve centrarchids and a number of opportunistic bacteria, fungi, and parasites have been isolated or observed in affected fish. Given that no single pathogen appears to be associated with these fish kills, a general suppression in immune function, perhaps due to the presence of EEDCs or other chemicals, has been suggested [10,11]. Impairment of the acute phase protein response due to putative exposure to EEDCs has not been examined in these fish.

Hepcidin is a small, cationic, disulphide-bonded peptide and a type II acute phase protein that has a critical functional role as an iron-regulatory hormone [12,13]. It was first identified in human urine and plasma [14,15] and has since been identified in other mammals, including mice and pigs. While originally described as an antimicrobial molecule that exhibits antibacterial and antifungal activity in vitro (Liver-Expressed Antimicrobial Protein; LEAP-1) [14,15], the in vivo antimicrobial function of hepcidin is not clear. It is possible that hepcidin acts as an antimicrobial peptide in macrophages: hepcidin localizes to the phagosome of mouse macrophages infected with mycobacteria and exhibits antimycobacterial activity in vitro [16].

Hepcidin is instrumental in the normal homeostatic regulation of iron levels in humans. This is clinically evident as hepcidin mutations are often associated with juvenile hemochromatosis, a condition in which there is a severe overload of iron in the liver and heart [17]. In addition, hepcidin is involved in the anemia of inflammation [12], in which iron sequestration purportedly increases resistance to microbial infection [18]. In its role as an

☆ GenBank accession numbers: *Micropterus salmoides hep-1*: EU502749, EU502754; *Micropterus salmoides hep-2*: EU502750, EU502755; *Micropterus dolomieu hep-1*: EU502751, EU502756; *Micropterus dolomieu hep-2*: EU502752, EU502756; *Micropterus dolomieu* 18S: EU502753.

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iron-regulatory hormone, hepcidin binds to the iron transporter ferroportin and induces internalization and degradation of ferroportin [19]. Ferroportin is expressed in the small intestine, in hepatocytes, and in macrophages. Hepcidin-mediated degradation of ferroportin blocks uptake of iron from the intestine and release of stored iron from macrophages [19,20], thus decreasing the abundance of available iron.

Hepcidin is primarily expressed in the liver [14,15] and its expression is induced by infection [12], inflammation [21], and excess iron [22]. Expression of hepcidin is stimulated by interleukin-6 [23] and interleukin-1 [24]. Regulation of hepcidin expression by estrogen has not yet been reported, but expression of interleukin-6, which stimulates hepcidin expression, is inhibited by estrogen [25]. Macrophages and neutrophils, but not hepatocytes, produce hepcidin in response to bacterial exposure in a TLR4-dependent fashion [26]. TLR4 is a toll-like receptor that is known to recognize lipopolysaccharides (LPS) in association with MD-2 in humans and mice.

Hepcidins have been identified in several species of fish, including hybrid striped bass [27], winter flounder and Atlantic salmon [28], zebrafish [29], Japanese flounder [30,31], red sea bream [32], channel catfish [33], and sea bass [34]. The structure and sequence of hepcidin genes are conserved between mammals and fish. Genes that encode this peptide consist of three exons and two introns, and are predicted to encode preproproteins with a signal peptide and acidic propiece that are cleaved to form the mature peptide. Fish hepcidins are predominantly expressed in the liver, similar to mammalian hepcidins, but are also detected in other tissues [27–29,31–34]. Several fish species encode multiple distinct hepcidin genes that display differential tissue expression [28–30]. In fish, expression of hepcidin is induced by bacterial infection; structural components of bacteria, *e.g.* lipopolysaccharides; and polyI:C (a double-stranded RNA molecule) [27,28,30,34–39]. Synthetic fish hepcidin peptides have been shown to be bactericidal and fungicidal in vitro [36,38]. Expression of hepcidin is also regulated by iron. In sea bass, expression is stimulated by iron overloading [34], while in Japanese flounder, expression of one hepcidin appears to be inhibited in the liver and expression of another hepcidin appears to be stimulated in kidney in response to iron overloading [30]. In zebrafish, hepcidin expression is reduced by anemia and induced by high levels of iron [40]. Hepcidin expression is also reduced by anemia in catfish [41] and by hypoxia in goby fish [42].

Here, we identify two distinct hepcidin genes (*hep-1* and *hep-2*) in both largemouth bass (*Micropterus salmoides*) and the closely related smallmouth bass (*Micropterus dolomieu*). We show that both *hep-1* and *hep-2* are primarily expressed in the liver of largemouth bass. Constitutive expression of *hep-1* is downregulated by the sex steroid 17β-estradiol (E2). *Hep-2* expression is induced by bacterial exposure and this induction is blocked by estradiol. In the spleen of largemouth bass, expression of *hep-1* and *hep-2* are not affected by estradiol; however, bacterial exposure induced *hep-1* expression and reduced *hep-2* expression in largemouth bass spleens. This work is the first demonstration of the regulation of hepcidin expression by physiological concentrations of estradiol and suggests that environmental estrogenic endocrine disrupting chemicals (EEDCs) may also modulate hepcidin.

2. Materials and methods

2.1. Laboratory fish care

Six-month-old largemouth bass ($n = 30$) raised in facility ponds at the Leetown Science Center were acclimatized to experimental research tanks for three weeks before experimental exposures. Smallmouth bass, approximately one-to-two years old, were collected from the South Branch of the Potomac River via electroshocking by West Virginia Division of Natural Resources biologists and transported to the Leetown Science Center, Leetown, WV. Fish were maintained in 150 L black, circular tanks supplied with two complete changes of aerated, 20 °C spring water daily. Fish were fed live fathead minnows every four days. Animal research protocol was approved by the Leetown Science Center Institutional Animal Care and Use Committee (IACUC).

2.2. Gene identification

Primers used for sequencing are listed in Table 1 and described below. Genomic DNA was extracted from largemouth bass and smallmouth bass liver tissue using a DNeasy Blood & Tissue Kit (Qiagen). RNA was extracted from the liver tissue of largemouth bass and smallmouth bass exposed to estradiol using RNeasy Mini Kits (Qiagen) and transcribed into cDNA using Taqman Reverse Transcription Reagents (Applied Biosystems). 5′ and 3′ end sequences were determined using the First Choice RLM RACE kit (Ambion), according to kit instructions for 5′ and 3′ RACE (Rapid Amplification of cDNA Ends) and using the gene-specific primers described below and in Table 1. For sequencing, PCR products were cloned using a TOPO TA cloning kit (Invitrogen); bacterial colonies were lysed, cellular debris pelleted, and plasmid inserts amplified using M13forward and M13reverse primers according to Estoup & Turgeon [43]; PCR products were labeled using the BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems); cycle sequencing products were cleaned using CleanSeq (Agencourt); cleaned products were sequenced on an ABI3130 (Applied Biosystems).

Hepcidin gene sequences available in GenBank were aligned to identify conserved sequence regions. Forward primers LRO-001 and LRO-002 and reverse primer LRO-003 were used to amplify conserved hepcidin regions from smallmouth bass cDNA; two distinct hepcidin genes (*hep-1* and *hep-2*) were identified.

Table 1
Primers used for sequencing *hep-1* and *hep-2* from largemouth bass and smallmouth bass and 18S from smallmouth bass and primers used for real-time PCR expression analysis in largemouth bass.

Gene	Primer	Sequence (5′ – 3′)
hep-1, sequencing	LRO-001	ATTCAGTGTTGCAGTTGCAGTGGC
	LRO-003	TCAGAACCTGCAGCAGATACCACA
	LRO-009	ATTCTGGAGAGCTCTGCCGTCCCATT
	LRO-010	TCAGAACCTGCAGCAGATACCACA
	LRO-011	AAGGAGAGGTGGCTTTGACGCTT
	LRO-046	GTGGTGCTCTTTTGGTGGCCTG
	LRO-073	TCACATCAGGCAGAAGCGTCA
	LRO-074	TGTGCCGCTGGTGCTGCAACT
hep-1, real-time PCR	forward	CATTCACCGGGGTGCAA
	reverse	CCTGATGTGATTTGGCATCATC
hep-2, sequencing	LRO-002	TGGCCGTCGTGCTCACCTTTATTT
	LRO-003	TCAGAACCTGCAGCAGATACCACA
	LRO-010	TCAGAACCTGCAGCAGATACCACA
	LRO-012	AGAGCTCCGCTGTCCCAGTCA
	LRO-013	ACATTTAATGCCGCGCTCCTGTCT
	LRO-043	CTGGAAGATGCCATATAACAAC
	LRO-048	AGACAGGAGAACTCAGAGGAGC
	LRO-073	TCACATCAGGCAGAAGCGTCA
	LRO-076	CTTTTGCTGTGGCTGCTGCAC
	LRO-082	ATCCTGGAAGATGCCATATA
	LRO-129	CATGTGGATGACGTGGTCAC
	LRO-130	GACAGTAGGTGGTAAAACTGC
hep-2, real-time PCR	forward	TCAGTGGAATCCTGGAAGATGC
	reverse	ACAGCAAAAGCGACATTTAATGC
18S, sequencing	LRO-018	CAAGACGGACGAAAGCGAAAGCAT
	LRO-019	TCCCTTTAAGAAGTTGGACGCCGA
18S, real-time PCR	forward	GCAAAGCTGAAACTTAAAGGAATTG
	reverse	TCCCGTGTTGAGTCAAATTAAGC