ABSTRACT

CORRALES, JONE. Antimicrobial Polypeptides, Piscidins and Histone-Like Proteins, in Important Aquacultured Fish and the Effect of Nutrition on their Expression and Susceptibility to Infection in Hybrid Striped (Sunshine) Bass (Morone saxatilis ♂ x M. chrysops ♀). (Under the direction of Edward J. Noga).

Innate immunity is the initial response of a host in defending against pathogen invasion (Beutler 2004, Janeway and Medzhitov 2002). The key features of an innate immune response are that it is: non-specific (broad-spectrum), rapid (often within minutes to hours) and conserved in its pattern of recognition. Antimicrobial polypeptides (AMPP) are a key component of the innate system and present in virtually all life forms (Zasloff 2002). One of the most widespread AMPP in fish are the piscidins. Piscidins have potent, broad-spectrum activity against viruses (Chinchar et al 2004), bacteria (Silphaduang and Noga 2001), fungi (Lauth et al 2002) and parasites (Colorni et al, Accepted). Another family of AMPP are histone-like proteins (HLPs), which are highly homologous to core nuclear histones (Robinette et al 1998, Noga et al 2002). Because disease is often the major cause of economic losses in fish farming, understanding how stress affects innate immunity (e.g., expression of AMPP) and thus disease susceptibility could be highly beneficial to the industry. One very important factor that can contribute to stress in aquaculture is inadequate nutrition. Feed is the single largest expenditure in semi-intensive and intensive farms (Lunger et al 2007, Twibell et al 2003, Mbahinzireki et al 2001). Thus, reducing feed costs while still maintaining optimal health is critical for successful production. As nutrient intake decreases, metabolic processes are impaired and susceptibility to disease increases. I anticipated that this would be reflected in the levels of piscidins and HLPs. The overall goal was to investigate the levels of these antibiotics that indicate fish are stressed prior to any
pathological signs. For this first, an ELISA for piscidin 4 was developed and validated and its application to assess stress was confirmed. Using this ELISA and various other independent assays the presence of piscidin 4 related AMPP were detected in important aquacultured fish. Next, I found that nutritional deprivation has a deleterious effect on piscidin 4 expression and that susceptibility to an ich increases in hybrid striped bass (Morone saxatilis ♂ x M. chrysops ♂). The nutritional deprivation also lead to dermatological condition in channel catfish.
Antimicrobial Polypeptides, Piscidins and Histone-Like Proteins, in Important Aquacultured Fish and the Effect of Nutrition on their Expression and Susceptibility to Infection in Hybrid Striped (Sunshine) Bass

(Morone saxatilis ♂ x M. chrysops ♀)

by

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DEDICATION

To my parents, my brother, and my sisters. I am forever grateful for their unconditional love.
BIOGRAPHY

Jone Corrales was born to Jose Luis Corrales and Maria Lourdes Izarzugaza on the ninth of February of nineteen seventy five. She grew up in a fishing town in the Basque Country, region between northern Spain and southwestern France. Growing up in the Bay of Biscay, she had an inherent interest in marine life. Thus, in 1994, she moved to the U.S. to study marine biology at the University of Miami, FL. When she was a sophomore, she had the opportunity to work studying morphological abnormalities of fish in Biscayne Bay at the Rosentiel School of Marine and Atmospheric Sciences under the direction of Dr. Michael C. Schmale. Her interest in aquatic animal health grew, and by the time she graduated with honors in marine biology, she gathered enough data to publish her first scientific paper. She then moved on to the field of environmental regulation and enforcement working at the Department of Environmental Resources Management in Miami-Dade County, FL. In 2002, she went back to academia to pursue her doctorate degree in aquatic animal medicine at the North Carolina State University, Raleigh, NC, under the direction of Dr. Edward J. Noga. In the summer of 2006, she was invited to attend a course in aquatic animal health and disease at the Hawaii Institute of Marine Biology, University of Hawaii, HI. She discovered her new interest in coral immunology and hopes to dedicate her upcoming postdoctoral years to this almost unexplored field in Hawaii.
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The work in this dissertation as well as many other studies would have never been possible without the guidance and teachings of my professor and the chairman of my advisory committee Dr. Edward J. Noga. One of Dr. Noga’s mottoes I should never forget as a scientist: be critical of your own work and you will be a better scientist; think beyond the obvious.

My sincere thanks to Dr. William L. Gordon whose expertise have been remarkable and invaluable to the completion of my work. Thanks also to Dr. Gordon for accommodating the late evening and weekend conference call discussions. I am honored to have had him in my committee. I would also like to thank Dr. Russell J. Borski, Dr. Gregory A. Lewbart, and Dr. R. Wayne Litaker for accepting to be part of my committee, for challenging me with questions that would only make a better scientist and for their interest in helping me take the
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I have spent most of my graduate school days in the lab which can often be frustrating and overwhelming. Thanks to the members of the Aquatic Medicine Lab Dr. Anirudh J. Ullal and Dr. Jung-Kil Seo (a.k.a. “JK”) for helpful discussions and for allowing me be a more well-rounded person by learning about their cultural backgrounds. In the last few months as I was writing my chapters, JK challenged me with daily questions that prepared me better for my defense and for future scientific endeavors, for that my special thanks to him. Many thanks to Mrs. Jeana Stephenson for her excellent assistance during sampling, performing assays, and most importantly for listening on those difficult and fruitless days.

I cannot forget all those friends who made the ugly days seem pretty, the long days seem short, the holidays away from home warm, and the gatherings full of laughter. For all of that and more, thanks to Everardo (Dr. Dic-el), The Zorrillas, Anne, Richard, Jon Zubi and the rest of the gang from the Basque Country. I could never forget to thank Randy for listening
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TABLE OF CONTENTS

| LIST OF TABLES | xi |
| LIST OF FIGURES | xiv |
| CHAPTER I. General Introduction | 1 |
| LITERATURE CITED | 13 |
| CHAPTER II. Development of an ELISA for Quantification of the Antimicrobial Peptide Piscidin 4 and its Application to Assess Stress in Fish | 22 |
| ABSTRACT | 24 |
| INTRODUCTION | 25 |
| MATERIALS AND METHODS | 29 |
| RESULTS | 40 |
| DISCUSSION | 43 |
| ACKNOWLEDGEMENTS | 50 |
| LITERATURE CITED | 51 |
| CHAPTER III. Detection of Antimicrobial Peptides Related to Piscidin 4 in Important Aquacultured Fish | 73 |
| ABSTRACT | 75 |
| INTRODUCTION | 76 |
CHAPTER IV.  Effects of Feeding Rate on the Expression of Antimicrobial Polypeptides and on Susceptibility to *Ichthyophthirius multifiliis* in Hybrid Striped (Sunshine) Bass (*Morone saxatilis* ♀ x *M. chrysops* ♀).

ABSTRACT...........................................128

INTRODUCTION....................................131

MATERIALS AND METHODS......................133

RESULTS..........................................141

DISCUSSION......................................146

ACKNOWLEDGEMENTS............................154

LITERATURE CITED..............................155
LIST OF TABLES

CHAPTER II.

Table 1. Recovery of piscidin 4 by ELISA from hybrid striped bass gill extracts spiked with synthetic piscidin 4..................................................................................58
Table 2. Reproducibility of the piscidin 4 dose-response with three gill extract pools as measured by ELISA..............................................................59
Table 3. Quantification of piscidin 4 via Western blotting and comparison to quantification via ELISA.................................................................60
Table 4. Percent homology of piscidins and piscidin-like AMP with the consensus N-terminal sequence of piscidins (amino acids 1-11) and the C-terminal sequence (from residue 12 onwards) of piscidin 4..........................61

CHAPTER III.

Table 1. Mean (± SE) piscidin 4 immunoreactivity in the gill, skin, blood, stomach and intestine of fish examined in this study..............................................115
Table 2. Comparison of mean gill piscidin 4 concentrations (μg/ml) of white bass, striped bass, European seabass, barramundi and red drum versus that of hybrid striped bass having either high, moderate, low or very low piscidin 4 levels.......................................................................................116
Table 3. Summary of antibacterial activity, expression levels and immunoreactivity of piscidin 4 in gill tissue of various fish.................................................117
CHAPTER IV.

Table 1. Relationship between condition factor, fish weight and fish length in each treatment group. N = 27 per treatment group…………………163

Table 2. Significant (Pearson, p < 0.05) correlations (not accounting for feed rate) or partial correlations (removing the effect of feed rate) between either condition factor, fish weight, or fish length, and piscidin 4 concentrations, HLP-1 concentrations, or antibacterial activity (ABA) in the tissues. For each tissue, the Day 80 data for all four treatment groups were combined (N = 36)……………………………………………………………………..164

Table 3. Significant (Pearson, p < 0.05) correlations (not accounting for feed rate) or partial correlations (removing the effect of feed rate) between either condition factor, fish weight, or fish length, and piscidin 4 concentrations, HLP-1 concentrations, or antibacterial activity (ABA) in the tissues. For each tissue, the data for all three sampling days and all four treatment groups were combined (N = 108)……………………………………165

Table 4. Significant (Pearson, p < 0.05) correlations (not accounting for day effect) or partial correlations (removing the effect of day) between either condition factor, fish weight, or fish length, and piscidin 4 concentrations, HLP-1 concentrations, or antibacterial activity (ABA) in the tissues. For each tissue, the data for all three sampling periods (Days 0, 50, 80) of each treatment group were combined (N = 27)…………..166
Table 5. Summary table of significant changes (indicated by *) in levels of piscidin 4, HLP-1 and antibacterial activity (ABA) at various time points and tissues

...168
## LIST OF FIGURES

### CHAPTER I.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Components of the innate immune system</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Proposed mechanisms of action for AMP: Peptide insertion and membrane permeability models (from Brogden 2005)</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Internal organs of bony fish</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>Model depicting health status and corresponding predicted AMPP levels</td>
<td>11</td>
</tr>
</tbody>
</table>

### CHAPTER II.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amino acid sequence alignments of piscidins and other AMP sharing homologous amino acid residues</td>
<td>62</td>
</tr>
<tr>
<td>2</td>
<td>Linearity of the piscidin 4 ELISA using gill extract pools (samples from 14 fish per pool) from hybrid striped bass</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>Comparison of linearity of the synthetic and native piscidin 4 dilution curves</td>
<td>66</td>
</tr>
<tr>
<td>4</td>
<td>Western blot analysis of piscidin 4</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
<td>Comparison of the western blotting pattern of previously frozen with freshly prepared synthetic piscidin 4</td>
<td>70</td>
</tr>
<tr>
<td>6</td>
<td>Western blot of synthetic piscidin 1 (P1), piscidin 2 (P2), piscidin 3 (P3), and piscidin 4 (P4)</td>
<td>71</td>
</tr>
</tbody>
</table>
CHAPTER III.

Figure 1. Amino acid sequence alignments of piscidins from Morone.................118

Figure 2. Bug blot of gill extracts...............................................................119

Figure 3. Western blot of piscidin 4 in gill extracts from fish examined
in this study.................................................................120

Figure 4. Immunohistochemical localization of piscidin 4 in normal gill tissue of
hybrid striped bass treated with monospecific, affinity-purified polyclonal
anti-piscidin 4 antibody (1:8000)..............................................121

Figure 5. Immunohistochemical localization of piscidin 4 in normal gill tissue of
striped bass treated with nonimmune serum or monospecific, affinity-purified
polyclonal anti-piscidin 4 antibody (1:8000)..............................122

Figure 6. Immunohistochemical localization of piscidin 4 in normal gill tissue of
white bass treated with nonimmune serum or monospecific, affinity-purified
polyclonal anti-piscidin 4 antibody (1:8000)..............................123

Figure 7. Immunohistochemical localization of piscidin 4 in normal gill tissue of
European seabass treated with nonimmune serum or monospecific, affinity-
purified polyclonal anti-piscidin 4 antibody (1:8000)...............124

Figure 8. Immunohistochemical localization of piscidin 4 in normal gill tissue of red
drum treated with nonimmune serum or monospecific, affinity-purified
polyclonal anti-piscidin 4 antibody (1:8000)..........................125

Figure 9. Taxonomic classification of fish that express piscidin 4.................126
CHAPTER IV.

Figure 1. Piscidin 4 concentrations (mean ± SE) in the gill, skin, blood and spleen of hybrid striped bass fed to satiation (high), half satiation (moderate), 0.6% BW three times a week (low), or not fed (fasting)………………………………169

Figure 2. Histone-like protein-1 (HLP-1) concentrations (mean ± SE) in the gill, skin, blood and spleen of hybrid striped bass fed to satiation (high), half satiation (moderate), 0.6% BW three times a week (low), or not fed (fasting)………172

Figure 3. Antibacterial activity Units (mean ± SE) in the gill, blood and spleen of hybrid striped bass fed to satiation (high), half satiation (moderate), 0.6% BW three times a week (low), or not fed (fasting)………………………………175

Figure 4. Qualitative antibacterial activity by the ‘bug blot’ assay in the gill of hybrid striped bass fed to satiation (high), half satiation (moderate), 0.6% BW three times a week (low), or not fed (fasting) on Day 80………………………...177

Figure 5. Condition factor (K), weight (g) and length (mm) of hybrid striped bass fed to satiation (high), half satiation (moderate), 0.6% BW three times a week (low), or not fed (fasting)………………………………………………...178

Figure 6. Mortality rates and secondary infection incidence in ich-challenged hybrid striped bass………………………………………………………………………………180

CHAPTER V.

Figure. 1. Gross and histopathologic LLD (HLLE) lesions in channel catfish……203

Figure 2. Electron microscopy of HLLD lesions in channel catfish…………………205
CHAPTER I

GENERAL INTRODUCTION
Disease is often the major cause of economic losses in fish farming. Traditional vaccines, although often effective, have numerous drawbacks, including: expense, being protective for only a specific pathogen, and often requiring delivery in impractical or undesirable ways (e.g., injection). Thus, optimizing innate defenses provides an important addition to enhance immunity.

Innate immunity is the initial response of a host in defending against pathogen invasion (Beutler 2004, Janeway and Medzhitov 2002). The key features of an innate immune response are that it is: non-specific (broad-spectrum), rapid (often within minutes to hours) and conserved in its pattern of recognition. Innate immunity is characterized by mechanisms and germline-encoded receptors (pattern recognition receptors) that adapt on an evolutionary timescale, rather than over a single lifespan. Innate immunity does not rely on the clonal expansion of antigen-specific lymphocytes, as does the adaptive immunity, which is based on receptors that are generated by somatic mechanisms during the ontogeny of each individual organism (Janeway et al 2001). Innate immunity can be constitutively expressed or inducible.

Because the innate immune response is first to respond to a pathogen, it plays a key role in a host’s ability to avoid the progression of a potential pathogen from a threat to an infection (Scott and Hancock 2000). Key components of the innate system include phagocytic cells (e.g., neutrophils and macrophages), tissue leukocytes (e.g, mast cells),
serum proteins (e.g., complement) and antimicrobial polypeptides (AMPP) (Hancock and Diamond 2000, Dixon and Stet 2001).

Figure 1. Components of the innate immune system.

Antimicrobial polypeptides (AMPPs) include antimicrobial peptides (AMP) as well as small proteins that are directly inhibitory to microorganisms. Proteins include lysozyme and histone. Antimicrobial peptides, which constitute the largest component of AMPP, are host defense effector molecules present in virtually all life forms (Zasloff 2002), including vertebrates, invertebrates, and plants. Antimicrobial peptides are also highly conserved emphasizing their evolutionary importance in host defense.
Antimicrobial peptides are typically 12-50 amino acids with a net positive charge of +2 to +7; hence, they are cationic due to their high percentage of basic amino acids (arginine, lysine, and histidine) (Hancock and Diamond 2000, Boman 2003, Boman 1998). Based on their three dimensional structure, although exceptions exist, they can be divided into three major classes: [1] linear peptides free of cysteines and often with an α–helical and amphipathic structure, [2] peptides with three disulfide bonds creating peptides with a flat, dimeric, β–sheet structure, and [3] peptides with a high percentage of certain amino acids (e.g., proline, arginine, glycine, tryptophan, or histidine) (Boman, 2003, Lauth et al 2002). Typically, half or more of an AMP’s amino acids are hydrophobic. Usually, the three dimensional configuration is either an amphipathic structure formed by hydrophobic and polar portions segregating from each or a cationic double-wing structure formed by two positively charged regions bracketing a hydrophobic core (Hancock and Scott 2000). To date, nearly 900 antimicrobial peptides have been identified in animals and plants (www.bbcm.units.it/~tossi/pag5.htm).

Antimicrobial peptides are encoded by single genes of highly homologous gene families. These gene families are located in clustered arrangements in the genome and mapped to syntenic (co-localization of genetic loci) chromosomal regions. For example, the human α- and β-defensins are on chromosome 8p21-23. The co-localization suggests co-evolution of different subclasses of AMP (Scott and Hancock 2000, Bals 2000). AMP target pathogens that have a membrane, such as enveloped viruses, bacteria, fungi, and parasites, lysing them upon contact. The lipid composition of the membrane determines AMP
“specificity” (targeting negatively charged, low cholesterol membranes) (Hancock and Diamond 2000, Yeaman and Yount 2003, Noga and Silphaduang 2003). AMP can also be synergistic with other antimicrobials. Examples include moronecidin and hepcidin (Lauth et al 2005), pleurocidin and salmon histone H1-derived peptides (Patrzykat et al 2001), magainin2 and tachyplesin (Kobayashi et al 2001), and indolicidin, bactenecin, protegrin 1, and LL-37 (Yan and Hancock 2001).

The first antimicrobial peptide to be isolated from an animal was cecropin, from the cecropia moth (Boman and Steiner 1981). Subsequently, a large array of AMP have been discovered in aquatic animals, including defensins and other cysteine-rich peptides in mollusks (reviewed in Seo et al 2005), tachyplesins and polyphemusins in chelicerates (reviewed in Bulet et al 2004), penaeidins in crustaceans (reviewed in Destoumieux et al 2000), and styelins in ascidians (reviewed in Tincu and Taylor 2004).

Antimicrobial peptides have also been isolated from primitive to advanced fish, including cathelicidins in Atlantic hagfish (Myxine glutinosa) (Uzzell et al 2003), misgurin in loach (Misgurnus anguillicaudatus) (Park et al 1997), and pardaxins in Moses sole (Pardachirus marmoratus) (Oren and Shai 1996). There is also genomic evidence for the presence of several AMP in fish, including cathelicidins in rainbow trout (Oncorhynchus mykiss) and Atlantic salmon (Salmo salar) (Chang et al 2006), defensins in zebrafish (Danio rerio) and pufferfish (Takifugu and Tetraodon) (Zou et al 2007) and NK-lysin in channel catfish (Ictalurus punctatus) (Wang et al 2006). A more comprehensive review of fish AMP is provided in Noga and Silphaduang (2003) and Bao et al (2006).
One of the most widespread AMP in fish are the piscidins. Although first isolated from mast cells of the commercially cultured hybrid striped bass (white bass, *Morone chrysops*, ♀ x striped bass, *Morone saxatilis* ♂) (Silphaduang and Noga 2001), there is evidence for the widespread presence of piscidins in higher teleosts (i.e., order Perciformes), having been immunolocalized in immune cells of six families (Moronidae, Serranidae, Sciaenidae, Cichlidae, Siganidae, and Belontidae)(Silphaduang et al 2006). Piscidins have potent, broad-spectrum activity against viruses (Chinchar et al 2004), bacteria (Silphaduang and Noga 2001), fungi (Lauth et al 2002) and parasites (Colorni et al, Accepted). Piscidins are thought to permeabilize the membrane of pathogens by toroidal pore formation rather than via the barrel-stave or carpet-like action (Fig. 2). The toroidal pore is formed by the lipid head groups of the membrane being inserted between the α–helices of the AMP (Campagna et al 2007).
In terms of small proteins, some of the first AMPP isolated from fish were histone-like proteins (HLPs), which are highly homologous to core nuclear histones (Robinette et al 1998, Noga et al 2002). Histone-like proteins were originally isolated from the skin of channel catfish (*Ictalurus punctatus*, family Ictaluridae)(Robinette et al 1998). Subsequently, histone-related AMPP have been linked to innate defense in a number of fish belonging to

Fish lack bone marrow and lymph nodes, and the thymus, kidney and spleen are their most important lymphomyeloid tissues (Patrzykat et al 2001)(Fig. 3). They are poikilothermic organisms whose internal temperature relies heavily upon the temperature of their environment. The adaptive immune system is temperature-dependent and suppressed if temperature decreases (Hoare et al 2002, Chen et al 2002, Scapigliati 1999). On the other hand, the innate immune system is relatively temperature-independent. Hence, innate immunity is especially important to fish.
The detrimental effects of aquacultural practices on fish are widely recognized. Such aquacultural practices include but are not limited to netting, grading, handling, transport, confinement, crowding, and stocking (Barton et al 1985, Maule et al 1989, Noga et al 1994, Wojtaszek et al 2002). Improper environment leads to stress, which in turn increases susceptibility to disease, decreases growth rate, and impairs reproduction (Pickering 1992). Because disease is often the major cause of economic losses in fish farming, understanding how stress affects innate immunity (e.g., expression of AMPP) and thus disease susceptibility could be highly beneficial to the industry.

One very important factor that can contribute to stress in aquaculture is inadequate nutrition. Feed is the single largest expenditure in semi-intensive and intensive farms (Lunger
et al 2007, Twibell et al 2003, Mbahinzireki et al 2001). Thus, reducing feed costs while still maintaining optimal health is critical for successful production. One mechanism that is currently under intense investigation in trying to optimize feed utilization is the use of compensatory growth. Compensatory growth is defined as growth acceleration that exceeds normal rates in animals or plants after growth-stunting conditions have been withdrawn (Ali et al 2003). Compensatory growth is often studied by comparing a population that receives ad libitum rations (control) during an experimental trial to a population that is feed-restricted part of the trial and then receives ad libitum rations for another part of the trial (treatment). For example, hybrid striped bass have been feed-restricted by feeding only once weekly during one month’s time. (Skalski et al 2005). Thus, not only has compensatory growth the potential to improve growth rate but also to reduce feed costs. However, whether such periods of feed deprivation have other, unintended consequences on AMPP levels are unknown.

The adverse effects of inadequate nutrition on health and immune function are widely recognized (Chandra and Kumari 1994, Calder and Jackson 2000). Cell-mediated immunity, phagocyte function, cytokine production, secretory antibody response, antibody affinity, and the complement system can be impaired (Chandra and Kumari 1994, Chandra 1996, Amati et al 2003). Feed deprivation increases susceptibility to infection and disease in many animals, including fish (Fletcher 1997, Gatlin 2002). Most of the work on the regulation of AMPP expression has focused on demonstrating their upregulation after acute challenge (Shike et al 2002, Lauth et al 2002, Shike et al 2004). However, very little is known about the effect of
nutrition on AMPP expression in any animal. In my thesis, I determined the relationship between nutrition, AMPP expression and susceptibility to infection.

**Hypotheses and research objectives**

As nutrient intake decreases, metabolic processes are impaired and susceptibility to disease increases. I anticipated that this would be reflected in the levels of piscidins and HLPs. In order to approach this work, I studied the model depicted in Fig. 4.

![Figure 4. Model depicting health status and corresponding predicted AMPP levels.](image)

The overall goal was to investigate the baseline levels of these antibiotics and establish highest and lowest “boundaries” that indicate fish are stressed prior to any pathological signs and to identify the best tool that will measure such levels. The main fish model was the hybrid striped bass, an important species in fish farming. The hybrid striped bass is now the fourth largest aquaculture industry in the U.S. with sales over $31 million in 2005 (USDA, National Agricultural Statistics Service). Hybrid striped bass is a cross between striped bass and white bass. Heterosis (hybrid vigor) explains the faster growth and
increased resistance to disease of the hybrid than the parental species (Harrell 1997, Rudacille and Kohler 2000).

The specific aims were:

[1] To develop and validate an ELISA for piscidin 4 and use this assay to quantify piscidin 4 levels in cells and tissues

[2] To determine if piscidin 4 or related AMPP are present in several important aquacultured fish

[3] To determine the effect of nutrition on expression of antimicrobial polypeptides (piscidin 4, HLP-1) and the relationship of their levels to susceptibility to infection

[4] To document the effects on nutritional deprivation on the health of channel catfish (a case study).
LITERATURE CITED


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Antimicrobial activity in the skin of the channel catfish Ictalurus punctatus: characterization of broad-spectrum histone-like antimicrobial proteins. Cellular and Molecular Life Sciences 54: 467-475


CHAPTER II

DEVELOPMENT OF AN ELISA FOR QUANTIFICATION OF THE ANTIMICROBIAL PEPTIDE PISCIDIN 4 AND ITS APPLICATION TO ASSESS STRESS IN FISH
Development of an ELISA for Quantification of the Antimicrobial Peptide Piscidin 4 and its Application to Assess Stress in Fish

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Antimicrobial peptides (AMP) are an integral component of innate immunity. One of the most widespread AMP in fish are the piscidins, which have potent, broad-spectrum activity against viruses, bacteria, fungi, and parasites. The widespread phylogenetic distribution of piscidins suggests that they might play an important host defense role in many fish. Quantifying their expression is important in understanding how and where they function. Using a novel piscadin (piscidin 4) that we recently isolated from commercially cultured hybrid striped bass (white bass, *Morone chrysops*, ♀ x striped bass, *Morone saxatilis* ♂), we optimized the conditions for measuring this piscadin via sandwich ELISA. We used an antibody to the highly conserved amino terminus of all piscidins as the capture antibody and a peroxidase-labelled antibody specific for the carboxy terminus of piscidin 4 as the detecting antibody. Specificity of the detecting antibody was confirmed by lack of cross-reactivity with other piscidins in ELISA, as well as specificity for piscidin 4 in tissue extracts via western blotting. The accuracy of the test, defined as piscidin 4 recovery, was 96-103%. Precision, measured by the coefficient of variation, was 13-19%, and parallelism, determined by linearity of the response, had an $r^2 > 0.99$. The ELISA paralleled the results obtained via western blotting. Piscidin 4 levels expressed in gill tissue of healthy hybrid striped bass were well within concentrations that are lethal to important fish pathogens. Mean gill piscidin 4 in healthy hybrid striped bass was significantly greater than in either nutritionally stressed fish or in diseased (ectoparasite-infested) fish, suggesting that piscidin 4 can be significantly downregulated with stress or disease. These data suggest that the piscidin
4 ELISA might be a useful indicator of disease susceptibility, providing a new, sensitive tool for rapid screening of population health.

INTRODUCTION

Antimicrobial peptides (AMP) are an integral component of innate immunity and target a broad array of pathogens. The lipid composition of this membrane determines the mechanism of action of AMP (their preference for negatively charge and free of cholesterol membranes) which will lyse the membrane upon binding (Hancock and Diamond 2000, Yeaman and Yount 2003, Noga and Silphaduang 2003). An increasingly wide array of AMP have been discovered in aquatic animals: Examples include defensins and other cysteine-rich peptides in mollusks (reviewed in Seo et al 2005), tachyplesins and polyphemusins in chelicerates (reviewed in Bulet et al 2004), penaeidins in crustaceans (reviewed in Destoumieux et al 2000), and styelins in ascidians (Tincu and Taylor 2004). A number of antimicrobial peptides have also been isolated from primitive to advanced fish, including cathelicidins in Atlantic hagfish (*Myxine glutinosa*) (Uzzell et al 2003), misgurin in loach (*Misgurnus anguillicaudatus*) (Park et al 1997), and pardaxins in Moses sole (*Pardachirus marmoratus*) (Oren and Shai 1996). There is also genomic evidence for the presence of several AMP in fish, including cathelicidins in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) (Chang et al 2006), defensins in zebrafish (*Danio rerio*) and pufferfish (*Takifugu and Tetraodon*) (Zou et al 2007) and NK-lysin in channel catfish (*Ictalurus punctatus*) (Wang et al 2006). A more comprehensive review of fish AMP is provided
One of the most widespread AMP in fish are the piscidins. Piscidins have potent, broad-spectrum activity against viruses, bacteria, fungi, and parasites (Silphaduang and Noga 2001, Lauth et al 2002, Chinchar et al 2004, Colorni et al, Accepted). First isolated from mast cells of the commercially cultured hybrid striped bass (white bass, *Morone chrysops*, ♀ x striped bass, *Morone saxatilis* ♂) (Silphaduang and Noga 2001), there is evidence for their widespread presence in higher teleosts (i.e., order Perciformes), where they have been immunochemically localized to immune cells in six families (Moronidae, Serranidae, Sciaenidae, Cichlidae, Siganidae, and Belontidae), including striped bass (*M. saxatilis*), white bass (*M. chrysops*), snowy grouper (*Epinephelus niveatus*), spot (*Leiostomus xanthurus*), Atlantic croaker (*Micropogonias undulates*), rabbitfish (*Siganus rivulatus*), pearl gourami (*Trichogaster leeri*), Nile tilapia (*Oreochromis niloticus*) and European seabass (*Dicentrarchus labrax*) (Silphaduang et al 2006). The presence of a piscidin (called “dicentracin”) in European seabass (family Moronidae) was recently supported by the cloning of its gene and confirmation of its antibacterial activity (Salerno et al 2007). And the gene and antibacterial activity for a piscidin in mandarin fish (*Siniperca chuatsi*, family Percichthyidae) was also recently reported (Sun et al 2007).

Piscidins are linear, amphipathic, α-helical peptides that typically have a highly conserved N-terminus rich in histidine and phenylalanine (Fig. 1). Piscidins appear to adopt an amphipathic conformation upon binding to the cell membrane (Campagna et al 2007, Noga and Silphaduang 2003, N Park, U Silphaduang and E Noga, Unpublished Data), lysing the pathogen in a detergent-like fashion by toroidal pore formation in which the membrane
lipids are inserted between the α–helices. Hybrid striped bass, European seabass and mandarin fish express piscidins that are 22 amino acids long. In hybrid striped bass, these have been designated piscidins 1, 2 and 3 (Silphaduang and Noga 2001). All of these piscidins have highly homologous amino acid sequences in their mature peptide and vary among themselves by only a few, relatively conservative, amino acid substitutions, except for piscidin 3, which has a glycine substituted for histidine at position 17 (Figs. 1a,b).

At the genomic level, mandarin fish piscidin shares close homology to pleurocidins, linear AMP that have been identified in a number of flatfish (order Pleuronectiformes)(Murray et al 2003, Patrzykat et al 2003, Cole et al 1997), including winter flounder (*Pleuronectes americanus*), American plaice (*Hippoglossoides platessoides*), Atlantic halibut (*Hippoglossus hippoglossus*), yellowtail flounder (*Limanda ferruginea*), and witch flounder (*Glyptocephalus cynoglossus*) (see Patrzykat and Douglas 2003 for review). Although they do not express the highly conserved amino-terminus of mature piscidins from hybrid striped bass, European seabass, or mandarin fish, this close genetic relationship has indicated that pleurocidins are also members of the piscidin family (Sun et al 2007).

Recently, we have isolated a novel type of piscidin from hybrid striped bass. This AMP, which we have named piscidin 4, is 44 amino acids long and 5329 Da, making it twice the size of all other known piscidins (Noga et al, In Preparation). Piscidin 4 has an N-terminus that is highly homologous to other piscidins (Fig. 1b). Like the smaller piscidins, piscidin 4 has potent, broad-spectrum activity against both gram-positive and gram-negative bacteria, including important pathogens of *Morone*, such as the causes of pasteurellosis (*Photobacterium damsela* subsp. *piscicida*) and lactococciosis (*Lactococcus garvieae*) (Noga
et al, In Preparation). The widespread phylogenetic distribution of piscidins suggests that they might play an important host defense role in many fish. Quantifying their expression is important in understanding how and where they function, as well as what physiological processes affect their expression. A number of researchers have previously quantified gene expression of some AMP in fish, including piscidin 1 in striped bass and piscidin 2 in white bass (Lauth et al 2002), as well as dicentracin in European seabass (Salerno et al 2007), LEAP-2 (liver-expressed antimicrobial peptide-2) in channel catfish (Bao et al 2006), cathelicidins in rainbow trout and Atlantic salmon (Chang et al 2006), defensins in zebrafish and pufferfish (Zou et al 2007), NK-lysin in channel catfish (Wang et al 2006), and pleurocidin in winter flounder (Douglas et al 2001). However, there are very few validated assays for quantifying AMP protein expression. It is well-known that mRNA expression does not always directly reflect protein levels (Greenbaum et al 2003, Pandley and Mann 2000, Gygi et al 1999). Also, a single RNA transcript can be translated into more than one protein and not all transcripts are translated into functional proteins. Since proteins are the structural elements of metabolic and regulatory pathways, genomic expression is insufficient to elucidate biological function (Pandley and Mann 2000, Eisenberg et al 2000). Thus, knowing AMP protein levels is critical to understanding the mechanisms via which AMP function in vivo, as well as potentially providing a useful tool for assessing when an animal might be at significant risk of infection by many pathogens. The purpose of this paper is to describe the development and validation of an ELISA to quantify expression of the antimicrobial peptide piscidin 4.
MATERIALS AND METHODS

Tissue Collection

Hybrid striped bass (7 to 14 mo old, 39-94 g in weight, 167-200 mm total length) were obtained from a local producer. Fish were spawned on 5 June 05 and maintained in ponds until transport on 14 October 2005 to North Carolina State University. Specific conditions used for maintaining each stock of fish are described below (Piscidin 4 Concentrations in Healthy versus Stressed/Diseased Fish). For sampling, fish were euthanized one at a time in buffered tricane (500 mg/L tricane + 1000 mg/L sodium bicarbonate). Samples were collected from both clinically normal fish and from stressed/diseased fish (see Piscidin 4 Concentrations in Healthy versus Stressed/Diseased Fish). Each fish was weighed and measured. Gill samples were then collected as previously described (Noga et al 2001, Robinette and Noga 2001) to measure piscidin 4. Briefly, 50 ul of tissue was added to 150 μl of 1% acetic acid (1:4 dilution of tissue). After boiling for 5 min, the sample was homogenized and then centrifuged at 14,000 x g for 10 min at 4°C. The resulting extract (supernatant) was stored at -70°C until measurement of piscidin 4.

Tissue Extracts Used for ELISA Validation

Gill extracts from 63 hybrid striped bass having various concentrations of piscidin 4 were used in the ELISA validation assays. Gill extracts were arbitrarily divided into groups
having either high, moderate, low, or very low piscidin 4 levels. For some experiments, individual extracts were used. For other experiments, equal volumes of individual extracts were combined to make either high, moderate, low or very low pools. Details are provided in each validation assay below.

**Peptide Synthesis**

Piscidins 1 and 4, as well as an 11-mer fragment constituting the N-terminus of piscidin 1 (Fig. 1b), were synthesized using Fmoc chemistry on a Rainin Symphony instrument (Protein Technologies, Inc. Tucson, Arizona, USA) that provides on-instrument cleavage of the peptide from the resin. After synthesis, each peptide was purified via analytical reverse phase HPLC using a YMC C-18 column (4 mm x 50 mm, 3 μm particle size, 120 Å pore size support)(Waters Corporation, Milford, Massachusetts, USA) using an acetonitrile gradient that was eluted at 1 ml min$^{-1}$ where Buffer A was 0.05% trifluoro-acetic acid (TFA) in water and Buffer B was 80% acetonitrile in 0.05% TFA in water. Peptides were detected by their absorbance at 210 nm. Mass spectrometry of an aliquot of each peptide was carried out on a Micromass TofSpec SE mass spectrometer (Waters Corporation, Milford, Massachusetts, USA) that was operated in positive ion mode and that was equipped with a nitrogen laser (337 nm), a reflectron, delayed extraction and a post acceleration detector. Each purified peptide was lyophilized from 0.05% TFA/acetonitrile solution and stored desiccated under argon gas until reconstitution in solvent.
Preparation of Anti-piscidin Antibodies

For production of the secondary antibody (affinity-purified antibody to piscidin 4), two mg of the purified, 44-mer synthetic piscidin 4 peptide, constituting the entire sequence of piscidin 4 (Fig. 1) was conjugated to keyhole limpet hemocyanin (KLH) using maleimide chemistry, which linked the peptide to KLH via a cysteine added to the N-terminal phenylalanine. The conjugation via the terminal amino acid allows tertiary conformation of the peptide that may be expected to mimic that in the native peptide, thus eliciting anti-conformational antibodies important for recognizing the native peptide. Immunogen was mixed with Complete Freund’s adjuvant (1:1) and KLH conjugated peptide was injected into two New Zealand white rabbits biweekly at five subcutaneous sites (0.2 ml per site) using the following immunization schedule (100 μg injection–1): Days 0, 14, 28 and 42. Thirty ml of antiserum was collected from each rabbit on Days 35 and 45.

The antiserum was pooled and then affinity-purified using a column having piscidin 4 conjugated to cyanogen bromide-activated agarose as an immunosorbent (5 mg of piscidin 4 was reacted with 20 ml of agarose); 120 ml of antiserum (two 30 ml bleeds from 2 rabbits) was loaded onto the column (Uniflow 4, Sterogene, Carlsbad, CA). After washing, the affinity-purified antibody was eluted and concentrated. This method produces >0.1 mg of peptide-specific antibody ml⁻¹ of antiserum, as determined by recovered affinity-purified antibody. Antibody was >95% immunoglobulin G (IgG), as determined by immunoelectrophoresis using antibodies specific for rabbit IgG, IgM and serum proteins. The titer of the antibody was determined via ELISA, using piscidin 4 as the antigen coated onto a
microtiter plate. The piscidin 4 peptide (5 μg ml⁻¹ in phosphate-buffered saline [PBS], pH 7.2 to 7.5) was coated onto a microtiter plate at room temperature for 1 h. The plate was then washed and post-coated with 1% BSA in PBS for 30 min. The plate was washed and then dilutions of the antibody in 1% BSA/PBS/0.01% Tween 20 (Sigma #T6789, St. Louis, Missouri, USA) were added, beginning at 1 ug antibody ml⁻¹. After incubation for 1 h, the plate was washed, followed by addition of peroxidase-conjugated goat anti-rabbit IgG (h&l) in 1% BSA/PBS/0.01% Tween 20. After incubation for 1 h, the plate was washed and peroxidase substrate was added, incubated for 15 min, and then stopped with 1 N HCl (1:1). The absorbance was then read at 450 nm. The titer was read as the reciprocal of the antibody dilution (dilution of a 1 mg ml⁻¹ solution) that produced a net optical density of 1.0, compared to a blank (non-coated well), which had an optical density (OD) <0.1. The titer of this antibody was approximately 1:80,000. The peptide-specific antibody had less than 1% cross-reactivity by ELISA, where 1% cross-reactivity is 100 times more antibody than is required to produce the same optical density with either free KLH or free peptide that shares less than 3 amino acids in the sequence.

Following confirmation that a high titer of affinity-purified anti-piscidin 4 antibody had been generated, we then removed any antibodies having cross-reactivity with other piscidins by running the antibody preparation over a column in which an 11-mer peptide comprised of the N-terminus of piscidin 1 (FFHHIFRGIVH, Fig. 1a)(highly similar to the N-terminus of all other piscidins) was conjugated to cyanogen bromide-activated agarose as an immunosorbent (10.5 mg of the 11-mer fragment was reacted with 15 g of agarose). The pass-thru antibody (C-terminus anti-piscidin 4)(specific for piscidin 4) was then conjugated
to horseradish peroxidase activated by periodate oxidation.

The titer of this cross-adsorbed antibody used in all assays was approximately 1:18,000. This antibody had less than 1% cross-reactivity by ELISA, where 1% cross-reactivity is 100 times more antibody than is required to produce the same optical density with either piscidins 1, 2 or 3.

The primary (coating) antibody, an affinity-purified rabbit antibody raised against the N-terminus of piscidin 1 (FFHHIFRGIVH, Fig. 1a), was produced using a similar procedure (Silphaduang et al 2006). The titer of this antibody (called anti-FFHH) was approximately 1:18000. This antibody had less than 1% cross-reactivity by ELISA, where 1% cross-reactivity is 100 times more antibody than is required to produce the same optical density with either free KLH or free peptide that shares less than 3 amino acids in the sequence.

**Sandwich ELISA**

The sandwich ELISA that we developed was based upon using a "capture" (primary, anti-FFHH) antibody that specifically binds to the amino terminus of all piscidins, and a "detecting" (secondary) antibody that binds only to the capture antibody-piscidin 4 complex (Fig. 1a). To measure piscidin 4, ELISA plates (Nalge Nunc International, Rochester, New York, USA) were coated for one h at room temperature with 10 μg/ml of the rabbit anti-FFHH antibody. After washing, the plates were incubated with Stabilcoat (Surmodics, Inc., Eden Prairie, Minnesota, USA) for 20 min to eliminate non-specific binding and then stored at 4°C until use. Immediately before use, the plate was washed for re-hydration with 0.05 M
tris-buffered saline with 0.05% Tween 20.

Various dilutions of rabbit anti-FFHH antibodies (diluted in carbonate-bicarbonate) were coated onto ELISA microtiter plates and coated with Stabilcoat. Various dilutions of peroxidase-labeled rabbit anti-piscidin 4 antibodies were also tested. Optimal concentrations for all reagents were determined in a checkerboard fashion using doubling dilutions of all reagents.

After optimizing the assay, tissue extracts were diluted to the desired concentration (1:400 to 1:6000) in 0.05 M tris-buffered saline with 0.05% Tween 20 and 1% bovine serum albumin (BSA) (Sigma #T6789). All samples were added in duplicate to the ELISA plate and incubated for 1 h. The plate was washed and then the secondary antibody (peroxidase-labeled rabbit anti-piscidin 4) was added and incubated for 1 h. The plate was then washed and TMB peroxidase substrate was added and incubated for 15 min. The reaction was terminated with 3 M phosphoric acid. Absorbance was measured at 450 nm with an ELISA reader (uQuant Microplate Spectrophotometer, Model MQX200, Bio-Tek Instruments, Inc., Winooski, Vermont, USA). Pure synthetic piscidin 4 (Noga et al, In Preparation) was used to construct a standard curve using two-fold serial dilutions of synthetic piscidin 4 in sample diluent ranging from 5 to 0.156 ng/ml. Tissue concentrations of piscidin 4 were calculated by the ELISA software (KCjunior, Bio-Tek Instruments, Inc.) at 450 nm absorbance based on the optical density (OD) of the 4 parameter curve-fitting standards. All values were multiplied by 4 to account for the 1:4 dilution of tissue in 1% acetic acid (50 ul of tissue in 150 μl of 1% acetic acid) during sample collection. The positive control was gill extract from a pool of 14 hybrid striped bass strongly expressing piscidin 4. The negative control was diluent without
Recovery of Piscidin 4 and Reproducibility of the Assay

To test recovery and reproducibility, gill extracts from 21 fish having barely detectable piscidin 4 were spiked with synthetic piscidin 4 diluted in 0.01% acetic acid and prepared to a final concentration of 0, 0.25 or 1.25 ng/ml. Piscidin 4 concentration was independently measured (in duplicate) a total of three times for all 21 extracts. Percent recovery and coefficient of variation (CV) were then calculated. Percent recovery was calculated by dividing the mean of the three runs after spiking the sample with 0.25 or 1.25 ng/ml of piscidin 4 by the expected piscidin 4 concentration, and then multiplying the resulting value by 100. The expected value was the mean of the three runs without spiking plus 0.25 or 1.25 ng.

Linearity and Parallelism of the Response

Three pools of gill extracts (prepared for each pool by combining equal volumes of extract from 14 fish) having either a high, moderate or very low piscidin 4 concentration, were serially diluted. The high piscidin 4 pool was diluted 1:2000, 1:4000 and 1:6000; the moderate piscidin 4 pool was diluted 1:1000, 1:2000 and 1:3000; the very low piscidin 4 pool was diluted 1:400, 1:600 and 1:800. Each serial dilution was independently repeated a total of ten times. The mean piscidin 4 concentrations of the ten runs per dilution were plotted and
\[ r^2 \] values were determined for each dilution series and for each pool.

The CV and percent agreement (similarity of each set of dilutions for each pool) were calculated for each dilution for each pool. Percent agreement was calculated by dividing the lowest concentration detected in each pool by the mean of the pool and then multiplying this value by 100. Two-fold serial dilutions of pure synthetic piscidin 4 in sample diluent (5 to 0.156 ng/ml) was used to construct a standard curve independently repeated six times. Parallelism of the synthetic piscidin 4 was determined by plotting linear curves for each of the six runs. The linear curve of the three pools was then compared to that of the synthetic piscidin 4 and \[ r^2 \] values and linear equations were determined.

**Method Comparison: ELISA and Immunoblotting**

To further validate the piscidin 4 ELISA measurements in gill tissue, we compared them to those determined from Western blotting of identical samples. Samples tested were the three previously prepared gill extract pools with high, moderate, or very low piscidin 4 levels; in addition, we used another gill extract pool prepared from 10 fish that had a low piscidin 4 concentration. Piscidin 4 concentrations of all four pools were determined by simultaneously running dilution series of synthetic piscidin 4 (125, 100, 75, 50, 25, and 12.5 ng) to construct a standard curve. In most cases, we used synthetic piscidin 4 stock that had been stored at -70°C prior to use. However, we also compared the Western blotting pattern of previously frozen synthetic piscidin 4 with synthetic piscidin 4 that had been freshly prepared immediately before immunoblotting. In addition, to further validate the specificity of the
secondary antibody, 100 and 50 ng of synthetic piscidins 1, 2, 3, and 4 were also simultaneously immunoblotted.

All samples were heated to 70°C for 10 min in lithium dodecyl sulfate (LDS) sample buffer (Invitrogen #NP0007, Carlsbad, California, USA) and then electrophoresed on a Bis-Tris 4-12% gradient polyacrylamide gel (Invitrogen, #NP0322) at 20 mA for 3 h. After electrophoresis, the gel was transblotted onto a 0.2 μm PVDF membrane at 100 V for 1 h. The membrane was then incubated in 5% nonfat dry milk blocking buffer with Tween 20 (Sigma #T9039) on a shaker for 1 h at room temperature and incubated overnight at 4°C in horseradish peroxidase-labeled anti-piscidin 4 antibody at 1:1000 dilution in blocking buffer. After rinsing the membrane in tris-buffer-saline with Tween 20 on a shaker at room temperature for 30 min, ECL Western blotting reagents (Pierce, #32106, Rockford, Illinois, USA) were added and incubated for one min before detection using a monochrome CCD camera (UVP Laboratory Products, Upland, California) and quantification using a chemiluminescence densitometer (UVP Bioimaging Systems, Labworks Software, Upland, California).

**Piscidin 4 Concentrations in Healthy versus Stressed/Diseased Fish**

**Healthy Fish**

One group of 27 hybrid striped bass were held in three 60 liter freshwater aquaria at 26°C. These aquaria were part of a larger closed system consisting of 12 aquaria connected to
a central filtration system having a conditioned biofilter (bio cubes and bead filter [Aquadyne, Koi Camp Aquariology, Loganville, GA) and a titanium heater (Process Technology Co., Mentor, OH). Fish were fed at half the amount for satiation daily with a commercial 5 mm pelleted feed (Finfish Silver™, Zeigler Bros, Inc., Gardners, Pennsylvania, USA; 40% crude protein, 10% crude fat, and 4% crude fiber). A 75% water change was performed thrice weekly; water quality (pH, temperature, ammonia, nitrite, nitrate, and dissolved oxygen) was monitored twice weekly and remained within normal limits prior to sampling. After 80 days, nine fish (3 fish per aquarium) were sampled. At sampling, fish were ten mo old (80 ± 6 g, 190 ± 4 mm TL). Gill extracts were obtained from each fish as described above and piscidin 4 levels were measured by ELISA. All fish were also necropsied and blood cultures were taken for bacterial culture. Piscidin concentrations measured via ELISA were converted to tissue concentrations by the ELISA reader software (KCjunior, Bio-Tek Instruments, Inc., Winooski, VT) at 450 nm absorbance based on the optical density (OD) of the 4 parameter curve-fitting standards. All values were then multiplied by 4 to account for the 1:4 dilution of tissue in 1% acetic acid (50 ul of tissue in 150 μl of 1% acetic acid) during sample collection.

**Stressed Fish**

A group of 27 hybrid striped bass was held in separate aquaria in the same closed system as the healthy fish mentioned above. This group was fed 0.5% of body weight thrice weekly. After 80 days, nine fish (3 fish per aquarium) were sampled as described for the
healthy group. At sampling, fish were ten mo old (43 ± 3 g, 168 ± 3 mm TL).

**Diseased Fish**

A group of approximately 20 hybrid striped bass, maintained in a 550-l freshwater aquarium at 20°C, started to exhibit lethargy, flashing and clinical hypoxia. Clinical examination revealed a heavy infestation of the flagellate ectoparasite *Ichthyobodo necator* on both the skin and gills. The fish were subsequently treated with 25 ppm formalin as a prolonged immersion. After seven weeks, clinical signs recurred and the fish were re-treated with 25 ppm formalin prolonged immersion. Water quality (TAN, pH, and nitrite) remained normal during the epidemic and 75% water changes were performed weekly. Over the next three weeks, the fish developed more severe clinical signs, culminating in the deaths of nine fish. The next day, the remaining 12 fish were sampled as described for the healthy fish, except bacterial cultures were not taken. At the time of sampling, the fish were 14 mo old (60 ± 4 g, 200 ± 3 mm TL) and were confirmed to still have ichthyobodosis.

**Statistical Analyses**

Tukey’s pairwise mean comparison was used to determine if there were any significant differences between piscidin 4 concentrations of healthy versus stressed or diseased fish. All data were analyzed using SAS version 9 (SAS Institute, Cary, NC). Differences were considered significant if the p–value was ≤ 0.05.
RESULTS

Optimization of the ELISA

After examining various combinations of reagents, the optimized ELISA had the following characteristics:

Coated microtiter wells: Rabbit anti-FFHH affinity-purified antibody (titer 1:20,000) diluted in carbonate buffer to 10 ug/ml (= 1000 ng/well).

Postcoat: 1% BSA in tris buffer.

Sample: Fish gill extract diluted 1:100 to 1:6000 in Sample Diluent (= 1% BSA, tris buffer, 0.05% Tween 20).

Conjugate: Rabbit anti-piscidin 4 affinity-purified antibody conjugated to horseradish peroxidase (titer 1:400,000), diluted in Sample Diluent to 1.25 ug/ml (= 125 ng/well).

Enzyme Substrate: TMB/H₂O₂

Stop: 3M H₃PO₄ Read: A450 nm

The positive control (hybrid striped bass gill extract, pool of 14 fish with high piscidin 4 concentration) consistently immunoreacted as expected. The negative (diluent only) control was consistently below detection limits.
Recovery and Reproducibility

The recovery efficiency of the optimized ELISA was determined by adding synthetic piscidin 4 to extracts. Concentrations of unspiked samples and expected recovery, actual recovery and percentage recovery of 18 samples spiked with piscidin 4 are shown in Table 1. Percentage recovery from gill extracts spiked with 0.25 ng/ml piscidin 4 ranged from 84-153% and from gill extracts spiked with 1.25 ng/ml piscidin 4 ranged 70-114%. Only two of the 36 tests (18 samples each spiked with 0.25 and 1.25 ng/ml) had a recovery rate of <80%. Samples had a slightly better recovery rate when 0.25 ng/ml of synthetic piscidin 4 was added.

Linearity and Parallelism

Coefficient of variation was 8%, 4% and 5% in the very low, moderate and high pools, respectively, indicating strong assay precision. The mean concentrations detected were 1.35, 6.98, and 12.11 μg/ml in the very low, moderate and high pools, respectively, and the three dilutions that yielded those levels had 93% agreement in the very low pool and 95% agreement in the moderate and high pools (Table 2). Also showing good linearity were the triplicate dilutions of each gill extract pool (r² ≥ 0.99 for each of the three curves and for all three curves combined) (Figs. 2a, b). The triplicate dilutions of the high, moderate, and very low pools had OD values that fell within those of the standard curve (Fig. 3). There was parallelism among the six curves independently generated with synthetic piscidin 4 (Fig. 3a)
and among the curves of the synthetic and the gill extract pools with similar linear equations, 
\[ y = 0.45x - 0.051 \] and \[ y = 0.49x - 0.076 \], respectively (Fig. 3b). The slope of the synthetic piscidin 4 linear curve (0.45) and that of native piscidin 4 linear curve (0.49) had a 96% agreement.

**Method Comparison: ELISA and Immunoblotting**

Immunoreactivity of piscidin 4 was detected by the peroxidase-labeled rabbit anti-piscidin 4 antibody in all hybrid striped bass gill extracts (Fig. 4a). As expected, piscidin 4 concentrations in the gill pools, calculated using the standard curve created with synthetic piscidin 4 (Fig. 4b), decreased proportionately from the high piscidin 4 pool to the very low piscidin 4 pool and closely corresponded with the piscidin 4 ELISA results (Table 3). Synthetic piscidin 4, as well as the high, moderate and low piscidin 4 gill extract pools had two bands: a lower predominant band at ~7 kDa and a higher molecular weight, less intense band near 14.4 kDa. The higher molecular weight band decreased in intensity as less synthetic piscidin 4 was loaded. This was also true for the gill extracts, as the very low piscidin 4 pool did not show the higher molecular band. When a dilution series of freshly prepared synthetic piscidin 4 was western blotted, the dimer was still present (Fig. 5). The most heavily loaded samples also revealed an additional band migrating at ~21 Da, suggesting that a multimer of piscidin 4 was also present. When piscidin 1, 2 and 3 were transferred onto the membrane and incubated with the piscidin 4 specific antibody (ELISA’s secondary antibody), no bands were detected (Fig. 6)
**Piscidin 4 Concentrations in Healthy versus Stressed/Diseased Fish**

Mean gill piscidin 4 concentrations in the healthy fish (32.5 ± 5 μg/ml), the feed-restricted fish (14 ± 3 μg/ml), the fasted fish (0.84 ± 0.2) and the diseased fish (5.9 ± 1 ug/ml) were significantly different from each other (Fig. 7).

**DISCUSSION**

We chose to develop an ELISA to piscidin 4 because its larger size (44 amino acids vs 22 amino acids for other piscidins) facilitated the production of two separate antibodies that had a greater likelihood of binding to opposite ends of this AMP without steric hindrance. In addition, it would be extremely difficult to make an ELISA that differentiated some of the other closely related piscidins from each other (e.g., piscidin 1 and piscidin 2 vary by only one conservative amino acid substitution). Piscidin 4 homology to the other piscidins is greatest in the N-terminus (defined as the first 11 amino acids), with piscidin 4 having six amino acids in common with piscidins 1 and 2 and dicentracin) (Fig. 1b). While piscidins 3 and 4 have five identical amino acid residues in the N-terminus, homology is somewhat less with sc-piscidin and chrysophsin-2 (four amino acids in common), chrysophsin-1 and epinecidin-1 (three amino acids in common), or chrysophsin-3 and pleurocidin (only one amino acid in common). The AMP which the primary antibody is likely to capture include piscidins 1-3, as well as dicentracin and sc-piscidin. Since piscidin 4 was also readily captured (even though it had only 55% homology with the FFHH antigen.
used to generate the primary antibody), it is likely that epinecidin-1 and chrysophsin-2, which have a similar percentage homology, would probably also be captured (Table 3). Thus, the primary antibody would probably also be effective in recognizing all those AMP via immunohistochemistry.

Due to the strong homology in the N-terminus among piscidin 4 and piscidins 1-3, cross-reactivity with the secondary antibody was a concern. This was mitigated by adsorbing cross-reacting epitopes by running the antibody over a column having the 11-mer peptide used to immunize the rabbits; this antigen was derived from a consensus sequence of the N-terminus of the piscidins (FFHHIFRGIVH). Although piscidin 4 shares homology with piscidins 1-3 in the amino-terminus, it is very different in the C-terminus, making the secondary antibody unlikely to cross-react with the other piscidins. This was borne out by (1) the lack of cross-reactivity of the anti-piscidin 4 antibody with other piscidins that were coated on an ELISA plate; and (2) our Western blotting data that showed no immunopositive band in the migration zone of piscidins 1-3 (Figs. 4 and 6). Also, our piscidin 4 secondary antibody is unlikely to cross-react with other piscidins because it has only very weak homology in the C-terminus (from residue 12 onwards) with other piscidin-related AMP (Table 4). It shares only five amino acid residues with epinecidin-1, four residues with piscidin 2, dicentracin, and chrysophsins 1-3, three residues with piscidin 1, sc-piscidin and pleurocidins, and only one residue with piscidin 3.

Validation of an ELISA entails confirming the reproducibility, precision and recoverability of the analyte from samples, as well as demonstrating specificity for the analyte using an independent measurement technique (Wild 2001a, b). Our ELISA was
validated by: 1) recovery of piscidin 4 from spiked samples (accuracy); 2) parallelism of curves generated using gill extract from pools with low, moderate, or high levels of piscidin 4 compared to curves generated with synthetic piscidin 4 (reproducibility and precision); and 3) comparison of piscidin 4 immunoreactivity via ELISA with that measured by Western blotting. The high recovery in our 21 independently run replications (Table 1), as well as the strong parallelism and linearity (Figs. 2, 3), indicate that the piscidin 4 ELISA is accurate and reproducible. Mean piscidin 4 recoveries were 103% and 96%, respectively, when 0.25 ng/ml or 1.25 ng/ml of piscidin 4 were added to gill extract. Optimal recovery typically falls within 90-110% of the expected concentration (Wild, 2001b).

The curves generated with gill extract pools showed excellent parallelism among themselves ($r^2 > 0.99$) (Fig. 2) and were also parallel to the synthetic piscidin 4 standard curves (Fig. 3b). High precision was shown with CVs of ≤ 8% and percentage agreement of ≥ 93% among the dilutions of each extract pool (Table 2). Since piscidin 4 is less homologous to the antigen used to generate the coating antibody than several other piscidins, it theoretically could be outcompeted in binding to the primary antibody. However, our high recovery rates, precision and linearity strongly indicate that this is not a problem.

The non-analyte constituents (the matrix) in a sample can greatly influence the performance of an ELISA by causing matrix effect, sample interference or cross-reactivity (Wild 2001a). Matrix effect is present when the dose-response curve of the standard differs from that of the test samples. Sample interference occurs when a substance in the matrix inhibits binding between the analyte and the antibody; cross-reactivity is due to structural similarities between an interfering molecule and the analyte, causing falsely higher readings.
In our ELISA, matrix effects, sample interference (false negative) or cross-reactivity (false positive) did not have a significant effect, since extracts that were spiked with synthetic piscidin 4 had a high and accurate recovery rate (Table 1) and were very similar to the standard curves of synthetic piscidin 4 (Fig. 3a). The most likely reason for lack of these interfering effects was the high sensitivity of the antibodies, especially the secondary antibody to piscidin 4, which allowed samples to be highly diluted, mitigating possible effects of interfering substances. We could detect low piscidin 4 concentrations when diluting gill extracts 1:400, but routinely tested extracts at higher dilutions (i.e., 1:800 or higher).

Performing two independent methods to measure an analyte greatly substantiates the results. The piscidin 4 concentrations measured by the ELISA paralleled the levels detected by Western blot, confirming the results obtained by the faster, higher throughput ELISA. The ELISA was also much more sensitive than Western blotting, as it detected concentrations as low as 0.156 ng/ml, which was equivalent to 0.00156 ng piscidin 4 in 10 μl (the volume that was loaded into a lane of the western blot), while 25 ng was the minimum detectable amount by the western blot. Western blotting is usually performed as an indirect immunoassay, using a primary antibody specific to the target protein and a secondary, peroxidase-labeled antibody that binds to the primary antibody. We instead used a direct assay, which, while faster than indirect and gave a good dose-response, is inherently less sensitive than indirect, which was reflected in the much lower sensitivity compared to our ELISA.

Piscidin 4 is highly positively charged (pI >11 [Noga et al, In Preparation]), which probably slowed its migration, making its estimated molecular weight via PAGE (~7 kDa)
larger than its nominal mass (~5.3 kDa). Highly basic proteins are well-known to migrate anomalously in PAGE gels due to their strong basic charge (Li et al 2007, Patat et al 2004, Richards et al 2001). Further evidence that the high positive charge of piscidin 4 affects its migration was that efficient transfer onto PVDF only occurred when the transfer buffer had a pH lower than the pI of piscidin 4 and the electrodes were reversed, thus allowing the peptide to migrate from the positive to negative pole. Besides the main immunoreactive band corresponding to piscidin 4, a second, much weaker band was present in the strongest gill extracts of hybrid striped bass, as well as in the lanes having the highest concentrations of synthetic piscidin 4 (Fig. 4a). This band migrated at a nominal molecular weight that appeared to be approximately twice the size of piscidin 4, suggesting that it might be a dimer of piscidin 4. The most heavily loaded samples also revealed an additional band migrating at ~21 kDa, suggesting that a multimer of piscidin 4 was also present. Non-covalent bonding between native protein chains has been observed, and some of these protein oligomers are highly stable, even under the strongly denaturing conditions used for dodecyl sulfate PAGE (Partridge et al 2002, Podlisny et al 1995). Freezing can cause some proteins to dimerize (Bennett et al 1994, Philo 2006). However, when a freshly prepared synthetic piscidin 4 sample was immunoblotted, the dimer was still present (Fig. 5), indicating that dimerization was not a storage artifact. Thus, the oligomers appear to form spontaneously in solution. The function of these alternative forms of piscidin 4 is unknown.

A few other ELISAs have been developed to measure AMP, including porcine cathelicidin PR-39 (Zhang et al 1997), human cathelicidin LL37 (Tao et al 2005), and human α-defensins 1-3 (HNP1-3) and human β-defensin 3 (Tao et al 2005). ELISA has also
occasionally been used to measure AMP in aquatic animals. Penaeidin and hemocyanin-derived AMP were measured after challenging shrimp (*Penaeus*) with a microbial cocktail (Destoumieux et al 2000, Destoumieux-Garzon et al 2001). However, sample recovery, reproducibility, assay precision, or parallelism was not reported for any of these studies. We have previously measured histone-like protein-1 (HLP-1), an antimicrobial polypeptide, by indirect ELISA. Analytical reverse phase high pressure liquid chromatography (RP-HPLC) supported the HLP-1 levels measured by ELISA. The ELISA results also correlated with a decrease in tissue antibacterial activity. However, this assay required partial purification of tissue samples prior to their measurement (Robinette and Noga 2001), making it a long and labor-intensive assay.

In our study, mean gill piscidin 4 concentration in the healthy hybrid striped bass was 32.5 ± 5 μg/ml, well within the levels that were inhibitory to important fish pathogens in vitro. For example, the minimum bactericidal concentration (MBC) of piscidin 4 for *Lactococcus garvieae* is 6.3 μg/ml and the MBC for *Photobacterium damselae* subsp. *piscicida* is 0.4 μg/ml (Noga et al, In Preparation). Pasteurellosis and lactococciosis are serious pathogens of hybrid striped bass and/or other important aquacultured fish (Austin and Austin 2007). Some have recently questioned whether many AMP in mammals actually function as antibiotics in vivo since the concentrations often measured in vivo are substantially lower than those apparently needed to inhibit pathogens under physiological conditions (Bowdish et al 2005). However, the concentrations that we measured in gill provide evidence that piscidin 4 functions as an antibiotic under normal physiological conditions. This is further suggested by our likely underestimation of the actual cellular
concentration of piscidin 4 in the animal, since it is present in certain immune cells, mainly mast cells (Corrales and Noga, In Preparation) and mast cells are also numerous in the gastrointestinal tract and skin (Reite 1998).

The feed-restricted hybrid striped bass expressed significantly less piscidin 4 than the healthy fish (Fig. 7), suggesting that even though these fish appeared clinically normal, they might be much more susceptible to infectious challenge. Histone-like protein in channel catfish skin is also downregulated after a chronic crowding stress (Robinette and Noga 2001). The *Ichthyobodo*-infested group also expressed significantly less piscidin 4 than the healthy fish (Fig. 7). *Ichthyododo* infestations can cause high mortalities, especially in young fish (Urawa 1995). During the feeding stage of their life cycle, trophonts feed on the skin and gill epithelium causing osmotic stress, respiratory impairment and can lead to secondary infections (Robertson et al 1981). Although mean piscidin 4 concentration in the *Ichthyobodo*-infested fish is cidal to some bacterial pathogens, *Ichthyobodo* might be more resistant, allowing it to proliferate at that piscidin 4 level.

In summary, we have developed and validated a sensitive and specific ELISA for measuring piscidin 4, an AMP that is present in *Morone* and also appears to be present in a number of other commercially important fish (Corrales and Noga, In Preparation). Preliminary studies suggest that piscidin 4 levels expressed in gill tissue are well within concentrations that are lethal to important fish pathogens and that levels can be significantly downregulated with stress or disease. ELISAs that measure antimicrobial polypeptides have the potential to be useful diagnostic tools in determining risk of infection of an individual or population (Robinette et al 2001, Tao et al 2005) and thus the piscidin 4 ELISA provides a
new, sensitive tool for rapid screening of fish population health.

ACKNOWLEDGEMENTS

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Table 1. Recovery of piscidin 4 by ELISA from hybrid striped bass gill extracts spiked with synthetic piscidin 4. Extracts having barely detectable piscidin 4 concentrations were spiked with a final concentration of either 0.25 or 1.25 ng/ml of synthetic piscidin 4.

Each sample (from a single fish) was run 3 times in duplicate. Mean piscidin 4 concentration prior to spiking, expected recovery, actual recovery and percent recovery were calculated for each sample.

<table>
<thead>
<tr>
<th>Fish #</th>
<th>Unspiked (ng/ml) (mean ± SD)</th>
<th>0.25 ng/ml</th>
<th>Expected Recovery (ng/ml)</th>
<th>Actual Recovery (mean ± SD)</th>
<th>% Recovery</th>
<th>1.25 ng/ml</th>
<th>Expected Recovery (ng/ml)</th>
<th>Actual Recovery (mean ± SD)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.88 ± 0.23</td>
<td>1.13</td>
<td>0.94 ± 0.22</td>
<td>84</td>
<td>2.13</td>
<td>2.21 ± 0.34</td>
<td>104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.04 ± 0.2</td>
<td>1.29</td>
<td>1.50 ± 0.13</td>
<td>116</td>
<td>2.13</td>
<td>2.42 ± 0.14</td>
<td>114</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.45 ± 0.16</td>
<td>1.70</td>
<td>1.69 ± 0.24</td>
<td>99</td>
<td>2.70</td>
<td>2.50 ± 0.48</td>
<td>93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.16 ± 0.14</td>
<td>1.41</td>
<td>1.42 ± 0.19</td>
<td>101</td>
<td>2.41</td>
<td>2.34 ± 0.47</td>
<td>97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.0 ± 0.06</td>
<td>1.25</td>
<td>1.21 ± 0.18</td>
<td>97</td>
<td>2.25</td>
<td>1.88 ± 0.67</td>
<td>83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.62 ± 0.39</td>
<td>1.87</td>
<td>1.89 ± 0.43</td>
<td>101</td>
<td>2.87</td>
<td>3.05 ± 0.42</td>
<td>106</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>1.04 ± 0.25</td>
<td>1.29</td>
<td>1.47 ± 0.36</td>
<td>113</td>
<td>2.29</td>
<td>2.63 ± 0.45</td>
<td>114</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.42 ± 0.32</td>
<td>1.68</td>
<td>1.74 ± 0.38</td>
<td>104</td>
<td>2.68</td>
<td>2.87 ± 0.34</td>
<td>107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>2.03 ± 0.18</td>
<td>2.28</td>
<td>2.32 ± 0.23</td>
<td>102</td>
<td>3.28</td>
<td>3.54 ± 0.34</td>
<td>108</td>
<td></td>
<td></td>
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<tr>
<td>10</td>
<td>1.37 ± 0.31</td>
<td>1.62</td>
<td>1.69 ± 0.43</td>
<td>104</td>
<td>2.62</td>
<td>2.74 ± 0.47</td>
<td>105</td>
<td></td>
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<tr>
<td>11</td>
<td>0.53 ± 0.2</td>
<td>0.78</td>
<td>0.69 ± 0.09</td>
<td>89</td>
<td>1.78</td>
<td>1.46 ± 0.14</td>
<td>82</td>
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<td></td>
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<tr>
<td>12</td>
<td>0.86 ± 0.18</td>
<td>1.11</td>
<td>1.11 ± 0.18</td>
<td>100</td>
<td>2.11</td>
<td>1.99 ± 0.28</td>
<td>94</td>
<td></td>
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<tr>
<td>13</td>
<td>0.35 ± 0.16</td>
<td>0.60</td>
<td>0.68 ± 0.15</td>
<td>114</td>
<td>1.60</td>
<td>1.72 ± 0.22</td>
<td>107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.24 ± 0.14</td>
<td>0.49</td>
<td>0.54 ± 0.15</td>
<td>110</td>
<td>1.49</td>
<td>1.40 ± 0.05</td>
<td>94</td>
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<td></td>
</tr>
<tr>
<td>15</td>
<td>0.43 ± 0.14</td>
<td>0.68</td>
<td>0.6 ± 0.1</td>
<td>89</td>
<td>1.68</td>
<td>1.22 ± 0.07</td>
<td>73</td>
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<tr>
<td>16</td>
<td>0.16 ± 0.14</td>
<td>0.41</td>
<td>0.62 ± 0.14</td>
<td>153</td>
<td>1.41</td>
<td>1.25 ± 0.1</td>
<td>89</td>
<td></td>
<td></td>
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<tr>
<td>17</td>
<td>0.98 ± 0.18</td>
<td>1.23</td>
<td>1.06 ± 0.16</td>
<td>86</td>
<td>2.23</td>
<td>1.56 ± 0.03</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>0.4 ± 0.24</td>
<td>0.65</td>
<td>0.57 ± 0.12</td>
<td>88</td>
<td>1.65</td>
<td>1.32 ± 0.15</td>
<td>80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean 103 96
Table 2. Reproducibility of the piscidin 4 dose-response with three gill extract pools as measured by ELISA. Pools (samples from 14 fish per pool) of hybrid striped bass gill extract with very low, moderate, or high piscidin 4 concentrations were serially diluted. Serial dilutions were performed 10 times (three dilutions per curve). Mean piscidin 4 concentration was calculated for each fold-dilution (10 replications). Mean piscidin 4 concentration was also calculated for each pool, as well as the coefficient of variation (% CV) and percentage agreement for each pool. The percentage agreement was the similarity of the mean concentrations of each dilution within a pool.

<table>
<thead>
<tr>
<th>Gill Extract Pool</th>
<th>Fold-Dilution</th>
<th>Mean (± SD) Piscidin 4 (µg/ml) per Dilution</th>
<th>Mean (± SD) Piscidin 4 (µg/ml) per Pool</th>
<th>% CV</th>
<th>% Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very low</td>
<td>400</td>
<td>1.34 ± 0.33</td>
<td>1.35 ± 0.11</td>
<td>8</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>1.26 ± 0.62</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>1.47 ± 0.61</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>1000</td>
<td>6.63 ± 1.61</td>
<td>6.98 ± 0.31</td>
<td>4</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>7.09 ± 1.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>7.23 ± 2.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>2000</td>
<td>11.48 ± 2.31</td>
<td>12.11 ± 0.56</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>4000</td>
<td>12.26 ± 2.91</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6000</td>
<td>12.58 ± 4.08</td>
<td></td>
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</table>
Table 3. Quantification of piscidin 4 via Western blotting and comparison to quantification via ELISA. Four pools of gill extracts from hybrid striped bass with either high, (H), moderate (M), low (L) or very low (VL) levels of piscidin 4 were immunoblotted. A dilution series of synthetic piscidin 4 was simultaneously run to generate a standard curve.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount added (ng)</th>
<th>Amount detected via western blot (ng)</th>
<th>Amount detected via ELISA (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gill extract H</td>
<td>62.4</td>
<td>39.40</td>
<td></td>
</tr>
<tr>
<td>Gill extract M</td>
<td>40.0</td>
<td>20.22</td>
<td></td>
</tr>
<tr>
<td>Gill extract L</td>
<td>35.9</td>
<td>11.90</td>
<td></td>
</tr>
<tr>
<td>Gill extract VL</td>
<td>21.7</td>
<td>4.74</td>
<td></td>
</tr>
<tr>
<td>Synthetic piscidin 4</td>
<td>150</td>
<td>150.2</td>
<td>-</td>
</tr>
<tr>
<td>Synthetic piscidin 4</td>
<td>125</td>
<td>124.4</td>
<td>-</td>
</tr>
<tr>
<td>Synthetic piscidin 4</td>
<td>100</td>
<td>95.1</td>
<td>-</td>
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<tr>
<td>Synthetic piscidin 4</td>
<td>75</td>
<td>82.9</td>
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<td>46.6</td>
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<tr>
<td>Synthetic piscidin 4</td>
<td>25</td>
<td>19.3</td>
<td>-</td>
</tr>
<tr>
<td>Synthetic piscidin 4</td>
<td>12.5</td>
<td>Undetectable</td>
<td>-</td>
</tr>
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Table 4. Percent homology of piscidins and piscidin-like AMP with the consensus N-terminal sequence of piscidins (amino acids 1-11) and the C-terminal sequence (from residue 12 onwards) of piscidin 4. Percent homology (= percent identity) was obtained using BLAST or calculated by hand by dividing the number of identical residues by the total number of residues and then multiplying by 100.

<table>
<thead>
<tr>
<th>AMP</th>
<th>Homology with N-terminus of piscidin concensus</th>
<th>Homology with C-terminus of Piscidin 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piscidin 1</td>
<td>100 %</td>
<td>0 %</td>
</tr>
<tr>
<td>Piscidin 2</td>
<td>100 %</td>
<td>0 %</td>
</tr>
<tr>
<td>Piscidin 3</td>
<td>91 %</td>
<td>9 %</td>
</tr>
<tr>
<td>Piscidin 4</td>
<td>55 %</td>
<td>100 %</td>
</tr>
<tr>
<td>Dicentracin</td>
<td>100 %</td>
<td>0 %</td>
</tr>
<tr>
<td>Sc-piscidin</td>
<td>82 %</td>
<td>0 %</td>
</tr>
<tr>
<td>Epinecidin-1</td>
<td>46 %</td>
<td>0 %</td>
</tr>
<tr>
<td>Chrysophsin-1</td>
<td>36 %</td>
<td>0 %</td>
</tr>
<tr>
<td>Chrysophsin-2</td>
<td>46 %</td>
<td>0 %</td>
</tr>
<tr>
<td>Chrysophsin-3</td>
<td>10 %</td>
<td>11 %</td>
</tr>
<tr>
<td>Pleurocidin</td>
<td>18 %</td>
<td>7 %</td>
</tr>
</tbody>
</table>
Figure 1. Amino acid sequence alignments of piscidins and other AMP sharing homologous amino acid residues. (A) Amino acid sequences of piscidins 1, 2, 3 and 4 from Morone, and the 11-mer peptide fragment used to generate the antibody to the N-terminus of piscidins. In the piscidin 4 ELISA, the epitopes targeted by the primary (capture) antibody are underlined, while the epitopes targeted by the secondary (detecting) antibody are within the square. (B) Comparison of the amino acid sequences of the piscidins and piscidin-like AMP. Alignment of the mature amino acid sequences of pleurocidin (Cole et al 1997), chrysophsin 1, 2 and 3 (Iijima et al 2003), epinecidin-1 (Yin et al 2006), dicentracin (Salerno et al 2007), mandarin fish (Siniperca chiatsu) piscidin (Sc-piscidin)(Sun et al 2007), and piscidin 1, 2, 3 (Silphaduang and Noga 2001) and 4 (Noga et al, In Preparation). Gaps were introduced to maximize sequence similarities. Homologous amino acid residues are shaded. Numbers to the right of each sequence indicate the number of amino acids of the peptide. The peptide number shown above the alignment refers only to piscidin 4. The ‘*’ symbol indicates a single, fully conserved residue and the ‘:’ symbol indicates that residues with identical polarity (+/− charge) and hydrophobicity are fully conserved.
Figure 2. Linearity of the piscidin 4 ELISA using gill extract pools (samples from 14 fish per pool) from hybrid striped bass. The high piscidin 4 concentration pool was diluted 1:2000, 1:4000, and 1:6000; the moderate piscidin 4 concentration pool was diluted 1:1000, 1:2000, and 1:3000; and the low piscidin 4 concentration pool was diluted 1:400, 1:600, and 1:800. The correlation coefficient was determined for each pool (A) and for all pools combined (B). Each pool was independently serially diluted and tested a total of 10 times, for a total of 30 independent dilution curves.
Figure 3. Comparison of linearity of the synthetic and native piscidin 4 dilution curves. (A) A two-fold serial dilution of synthetic piscidin 4 (5 to 0.156 ng/ml) was performed independently six times. (B) Combined native piscidin 4 dilution curve (shown in Fig. 2b) were then plotted with the combined synthetic piscidin 4 standard curves of Fig 3a.
A) Synthetic piscidin 4 (ng/ml)

B) Piscidin 4 (ng/ml)
Figure 4. Western blot analysis of piscidin 4. (A) Serial dilutions of synthetic piscidin 4 (150 to 12.5 ng per lane) and pools of hybrid striped bass (HSB) gill extracts having either high, (H), moderate (M), low (L) or very low (V) concentrations of piscidin 4. The far left lane shows molecular weight markers. The short arrow to the right of the lanes indicates the area corresponding to the small piscidins. Each gill extract pool was prepared from 14 fish, except the low pool, which was prepared from 10 fish. (B) Standard curve of piscidin 4 prepared from a serial dilution run on western blot. Band volume (= densitometric intensity) was plotted against the amount of piscidin 4 (ng) and the curve was generated using a third order polynomial equation.
Figure 5. Comparison of the western blotting pattern of previously frozen with freshly prepared synthetic piscidin 4. Serial dilutions of synthetic piscidin 4 (150 to 50 ng per lane) were identically prepared for both stocks. The frozen stock was used in previous experiments to construct the standard curve and kept at -70°C between experiments. The fresh stock was prepared immediately before running the assay.
Figure 6. Western blot of synthetic piscidin 1 (P1), piscidin 2 (P2), piscidin 3 (P3), and piscidin 4 (P4). Either 100 or 50 ng of each piscidin were incubated with the piscidin 4 antibody (secondary antibody used in ELISA) after transfer. The far right lane shows molecular weight markers.
Figure 7. Mean (± SD) piscidin 4 concentrations in the gill of hybrid striped bass that were healthy (fed to satiation), feed-restricted (fed at 0.6% BW thrice weekly), or diseased (infested with *Ichthyobodo*). Lower case letters designate differences between groups (ANOVA, p < 0.05). N = 9, except for diseased fish, where N = 12.
CHAPTER III

DETECTION OF ANTIMICROBIAL PEPTIDES RELATED TO PISCIDIN 4 IN
IMPORTANT AQUACULTURED FISH
Detection of antimicrobial peptides related to piscidin 4 in important aquacultured fish

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Epithelial surfaces of fish, such as the gut, skin and gills, comprise a large surface area for possible pathogen invasion. Antimicrobial peptides, innate immunity components, play a significant role in protecting fish. Piscidins are a family of AMP that were first identified from mast cells of hybrid striped bass (\textit{Morone saxatilis} ♂ x \textit{M. chrysops} ♀) but have been recently identified in many other teleosts. In this study, we detected the presence of a new piscidin (piscidin 4) via bug blot, Western blot, ELISA and/or immunohistochemistry in striped bass (\textit{Morone saxatilis}), white bass (\textit{M. chrysops}), European seabass (\textit{Dicentrarchus labrax}), red drum (\textit{Sciaenops ocellatus}), and barramundi (\textit{Lates calcarifer}). All of these species belong to the order Perciformes, suborder Percoidei, and are members of the families Moronidae (basses), Sciaenidae (red drum) and Latidae (barramundi). Via bug blot, gill extracts from all species had antibacterial activity corresponding to the migration rate of piscidin 4. Western blotting showed that the greatest piscidin 4 immunoreactivity was in striped bass gill extract, but was also present in white bass, European seabass, barramundi and red drum. The Western blot data were corroborated via ELISA, where piscidin 4 concentrations were highest in striped bass (~20 μg/ml) and much lower in the other fish species (<1 μg/ml). The concentrations of piscidin 4 detected by the ELISA in striped bass gill were well within the levels that are inhibitory to important fish bacterial pathogens. Piscidin 4 was also detected via immunohistochemistry in gill of all fish except barramundi. Piscidin 4-positive cells were identified as mast cells (MC), but not all MC were piscidin 4-positive. This was especially evident in red drum, where the majority of
the MC were immunonegative. Species, age, size and physiological condition at sampling were some factors that might have affected piscidin expression in different species. Our data provide strong evidence that piscidin 4 isoforms are present in all these commercially important species.

INTRODUCTION

Epithelial surfaces of fish, such as the gut, skin and gills, comprise a large surface area for possible pathogen invasion. For example, the surface area of gill ranges from 90 mm² per cm body surface in flounder to about 5000 mm² per cm body surface in tuna (Gray 1954, Palzenberger and Pohla, 1992). This large surface area of the gill consistently provides a major route of entry for pathogen invasion, particularly if epithelial damage occurs. The initial response of a host in defending against pathogen invasion is via non-specific (innate) immunity (Beutler 2004, Janeway and Medzhitov 2002). Antimicrobial peptides (AMPs), host defense effector molecules present in virtually all life forms (Zasloff 2002), are a key component of this defense. Accumulative evidence is showing that AMPs play a significant role in protecting animals from infections, including fish (Jia et al. 2000). For example, transgenic Japanese medaka (Oryzias latipes) that express cecropin, an insect AMP, are more resistant to Pseudomonas fluorescens and Vibrio anguillarum (Sarmasik et al 2002); similarly, channel catfish (Ictalurus punctatus) expressing a cecropin transgene were more resistant to Edwardsiella ictaluri and Flavobacter columnare (Dunham et al 2002). Grass carp (Ctenopharyngodon idellus) expressing human lactoferrin, an antimicrobial protein,
were more resistant to *Aeromonas hydrophila* (Weifeng et al 2004) and grass carp hemorrhage virus (Zhong et al 2002), while transgenic zebrafish (*Danio rerio*) expressing chicken lysozyme were more resistant to *Flavobacter columnare* (Yazawa et al 2006).

An increasing number of AMPs have been isolated from fish (see Noga & Silphaduang 2003 and Bao et al 2006 for reviews): this includes cathelicidins in Atlantic hagfish (*Myxine glutinosa*) (Uzzell et al 2003), misgurin in loach (*Misgurnus anguillicaudatus*) (Park et al 1997), and pardaxins in Moses sole (*Pardachirus marmoratus*) (Oren and Shai 1996). There is also genomic evidence for the presence of several AMP in fish, including cathelicidins in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) (Chang et al 2006), defensins in zebrafish and pufferfish (*Takifugu* and *Tetraodon*) (Zou et al 2007) and NK-lysin in channel catfish (Wang et al 2006).

Antimicrobial peptides are typically present in leukocytes or in epithelial surfaces on the skin and the respiratory and gastrointestinal tracts. In fish, they have been identified in skin goblet cells (Cole et al 1997), in gill and intestine epithelial goblet cells (Pan et al 2007, Cole et al 2000) as well as immune cells, including mast cells (Silphaduang & Noga 2001, Murray et al. 2003), neutrophils (Salerno et al 2007) and rodlet cells (Silphaduang et al 2006), the latter which also appear to participate in immune defense (Reite and Evensen 2006).

Piscidins (GenBank Nos. Q8UUG0, AAL40409, P0C006), a family of linear amphipathic peptides, were first identified from mast cells of hybrid striped bass (*Morone saxatilis ♂ x M. chrysops ♀*) (Silphaduang and Noga 2001). Piscidins 1, 2 and 3 are 22 amino acids long and have a highly conserved, histidine-rich, phenylalanine-rich N-terminus
combined with a much more variable C-terminus (Silphaduang & Noga 2001, Fig. 1).
Piscidins 1, 2 and 3 have potent activity against many pathogens, including viruses, bacteria, fungi, and parasites (Chinchar et al 2004, Silphaduang and Noga 2001, Lauth et al 2002, Noga and Silphaduang 2003, Colorni et al, In Press). Piscidins are thought to permeabilize the membrane of pathogens by toroidal pore formation in which lipids of the membrane are inserted between the α–helices (Campagna et al 2007). The presence of a piscidin (dicentracin) in European seabass (Dicentrarchus labrax, f. Moronidae) was recently supported by the cloning of a piscidin gene and confirmation of its antimicrobial activity (Salerno et al 2007). And the gene and antibacterial activity for a piscidin in mandarin fish (Siniperca chuatsi, f. Percichthyidae) were also recently reported (Sun et al 2007). At the genomic level, piscidins are very closely related to pleurocidins, linear AMP that have been identified in a number of flatfish (order Pleuronectiformes)(Patrzykat et al 2003), including winter flounder (Pleuronectes americanus), American plaice (Hippoglossoides platessoides), Atlantic halibut (Hippoglossus hippoglossus), yellowtail flounder (Limanda ferruginea), and witch flounder (Glyptocephalus cynoglossus) (see Patrzykat and Douglas 2003 for review).

Although pleurocidins do not express the highly conserved amino-terminus of mature piscidins from hybrid striped bass, European seabass, or mandarin fish, the recent discovery of that mandarin fish piscidin shares close gene homology to pleurocidin has indicated that pleurocidins are also members of the piscidin family (Sun et al 2007).

Using an antibody raised against the highly conserved amino terminus of piscidins, we previously provided immunohistochemical evidence for the presence of piscidins in many perciform (o. Perciformes) fish, the largest and most evolutionarily advanced order of the
teleosts (Silphaduang et al 2006). This included immunoreactivity to mast cells, rodlet cells, and other unidentified cells that had atypical mast cell morphology in species belonging to the families Moronidae, Serranidae, Sciaenidae, Siganidae and Belontidae.

We have recently discovered another member of the piscidin family in hybrid striped bass. This AMP, which we have named piscidin 4, has an N-terminus that is highly homologous to other piscidins, but has 44 amino acids, making it twice as long as piscidins 1-3 (Noga et al, In Preparation). Like the other piscidins, piscidin 4 has potent broad spectrum antimicrobial activity; it also has the lowest hemolytic activity of all the piscidins (Noga et al, In Preparation). Since the C-terminal end of piscidin 4 is quite different from that of piscidins 1-3, it provided the opportunity to raise an antibody against piscidin 4 that could specifically recognize and differentiate this piscidin from piscidins 1-3. Prior evidence for the presence of the piscidin family across a wide phylogenetic range of taxa led us to attempt to specifically locate and quantify piscidin 4 isomers via Western blotting, ELISA and immunohistochemistry in the parental species of hybrid striped bass as well as several species of commercially important fish that are in the order Perciformes, including European seabass (f. Moronidae), red drum (*Sciaenops ocellatus*, f. Sciaenidae), and barramundi (*Lates calcarifer*, f. Latidae). Because previous studies showed evidence for the presence of piscidins in the families Moronidae and Sciaenidae, and the Latidae is also in the suborder Percoidei, we hypothesized that piscidin 4 is present in these commercially important fish.
MATERIALS AND METHODS

Experimental Fish

Six clinically normal white bass (6 mo old, mean weight 65 ± 13 g), striped bass (18 mo old, 393 ± 24 g), European seabass (12 mo old, 154 ± 13 g), red drum (3 to 12 mo old, 98 ± 46 g and barramundi (3 y old, 2378 ± 186 g) were used for this study. The white bass were spawned at the North Carolina State University Pamlico Aquaculture Field Laboratory (PAFL, Aurora, NC). At about three g, they were stocked into a pond (37,000 fish/ha) and fed a fine starter meal at 11 kg/ha/d at close to satiation thrice daily, five days/wk. Four mo later, six fish were netted, placed in a cooler supplied with air, and then sampled one at a time from a few min to several hr after collection. The striped bass were reared at PAFL like the white bass. In spring 2006 (at age 1+), they were stocked into a pond (~9,000 fish/ha) and fed close to satiation once per day for five days/wk. About 6 mo later, six fish were placed in a cooler and then sampled one at a time like the white bass immediately after collection. The European seabass and barramundi were spawned and cultured at the National Center for Mariculture (Eilat, Israel) in flow-through seawater (40.2 ± 0.5 ppt salinity, 23 ± 3°C) aquaria and fed daily with a commercial pelleted diet. The red drum were spawned at the Waddell Mariculture Center (Bluffton, South Carolina), stocked in ponds and fed close to satiation for four wk, when they were moved to a 2500 liter aquarium (18 ppt salinity, ~23°C) and continued to be fed at the same rate. About 5 wk later, fish were shipped to our laboratory (~24 hr) and then immediately sampled. Hybrid striped bass were obtained from a local
producer and were held in 60-liter freshwater aquaria at 26°C at a density of ~10.5 g/l in a close
system to be part of a feed rate experiment where each treatment group was fed with a
commercial pellet close to satiation (108 ± 11 g), half satiation (80 ± 6 g), 0.6% body weight
thrice weekly (43 ± 3 g), or fasted for 80 days (35 ± 3 g).

Tissue Collection

Fish were euthanized individually with buffered tricaine. A sample of gill was fixed in
10% neutral-buffered formalin (NBF) and then stored in 70% ethanol for
immunohistochemistry. Then, gill, skin, blood, stomach and intestine extracts were collected
as previously described (Robinette et al 1998; Robinette and Noga 2001). Briefly, 50 to 600
μl of tissue were collected (depending upon the size of the fish). Each tissue sample was
diluted in exactly three volumes of 1% acetic acid (HAc). After boiling for 5 min, the sample
was homogenized and then centrifuged at 14,000 x g for 10 min at 4°C. The resulting extract
(supernatant) was stored at -70°C until use. For electrophoretic assays, 250 μl of gill extracts
from European seabass, red drum and barramundi were lyophilized and reconstituted in 50 μl
of 1% HAc to prepare samples that were five times the original tissue concentration.

Peptide Synthesis

Piscidin 1 (both the entire peptide and an 11-mer fragment consisting of amino acids
1-10 of the amino terminus) and piscidin 4 were synthesized using Fmoc chemistry on a
Rainin Symphony instrument (Protein Technologies, Inc. Tucson, Arizona, USA) that provides on-instrument cleavage of the peptide from the resin. After synthesis, peptides were purified via analytical reverse phase HPLC using a YMC C-18 column (4 mm x 50 mm, 3 micron particle size, 120 angstrom pore size support) (Waters Corporation, Milford, Massachusetts, USA) using an acetonitrile gradient that was eluted at 1 ml/min where buffer A was 0.05% TFA in water and buffer B was 80% acetonitrile in 0.05% TFA in water. Peptides were detected by their absorbance at 210 nm. Mass spectrometry of an aliquot of each purified peptide was carried out on a Micromass TofSpec SE mass spectrometer (Waters Corporation, Milford, Massachusetts, USA) that was operated in positive ion mode and that was equipped with a nitrogen laser (337 nm), a reflectron, delayed extraction and a post acceleration detector. The purified peptides were lyophilized from 0.05% TFA/acetonitrile solution and stored dessicated under argon gas until reconstitution in solvent.

**Production of Anti-Piscidin Antibodies**

Anti-piscidin 4 antibody was produced by a commercial laboratory (Bethyl Laboratories, Montgomery, TX) using the company’s standard procedures. Briefly, 2 mg of piscidin 4 was conjugated to keyhole limpet hemocyanin (KLH) using maleimide chemistry, which linked the peptide to KLH via a cysteine added to the N-terminal phenylalanine. The conjugation via the terminal amino acid allows tertiary conformation of the peptide that may be expected to mimic that in the native peptide, thus eliciting anti-conformational antibodies.
important for recognizing the native peptide. Immunogen was mixed with Complete Freund’s Adjuvant (1:1) and KLH-conjugated peptide was injected into two New Zealand white rabbits biweekly at 5 subcutaneous sites (0.2 mL per site) using the following immunization schedule (100 µg/injection): Days 0, 14, 28 and 42. Thirty ml of antiserum was collected from each rabbit on days 35 and 45. The antiserum was pooled and was then affinity-purified using the piscidin fragment conjugated to cyanogen bromide-activated agarose as an immunosorbent (5 mg of piscidin fragment was reacted with 20 ml of agarose). One hundred and twenty ml of antiserum (two 30 ml bleeds from two rabbits) was loaded onto the column (Uniflow 4, Sterogene, Carlsbad, CA). After washing, the affinity-purified antibody was eluted and concentrated.

This method produces greater than 0.1 mg of peptide-specific antibody per mL of antiserum, as determined by recovered affinity-purified antibody. Antibody was greater than 95% IgG, as determined by immunoelectrophoresis using antibodies specific for rabbit IgG, IgM and serum proteins. The titer of the antibody was determined via ELISA, using the piscidin fragment as the antigen coated onto a microtiter plate. The piscidin 4 peptide (5 µg/mL in PBS, pH 7.2-7.5) was coated onto a microtiter plate at room temperature for 1 h. The plate was then washed and post-coated with 1% BSA in PBS for 30 min. The plate was washed and then dilutions of antibody in 1% BSA/PBS/0.01% Tween 20 were added, beginning at 1 µg antibody/mL. After incubation for 1 h, the plate was washed, followed by addition of peroxidase-conjugated goat anti-rabbit IgG (h&l) in 1% BSA/PBS/0.01% Tween 20. After incubation for 1 h, the plate was washed and peroxidase substrate was added, incubated for 15 min, and then stopped with 1 N HCl (1:1). The absorbance was then read at
450 nm. The titer was read as the reciprocal of the antibody dilution (dilution of a 1 mg/mL solution) that produced a net optical density of 1.0, compared to a blank (non-coated well), which had an OD < 0.1. The titer of the antibody used in all assays was approximately 1:80,000.

The peptide-specific antibody had less than 1% cross-reactivity by ELISA, where 1% cross-reactivity is 100 times more antibody than is required to produce the same optical density with either free KLH, conjugated KLH, or free peptide that shares less than 3 amino acids in the sequence.

Antibody against the conserved amino terminus of all piscidins (anti-FFHH antibody) was prepared as described in Silphaduang et al (2006).

**Acid Urea Polyacrylamide Gel Electrophoresis (AU-PAGE) Bug Blot**

Semi-quantitative analysis of piscidin 4 expression was performed using the “bug blot”. Gill extracts were electrophoresed via AU-PAGE (Harwig et al 1993) using the Mini-Protean II gel electrophoresis system (BioRad Laboratories, Inc., Hercules, California) according to manufacturer’s instructions. Briefly, samples were run in duplicate and after electrophoresis, the gel was divided in half. One half was stained with Coomassie Blue R-250 (Bio-Rad Laboratories, Inc., Hercules, California) and the other half was washed and “blotted” by overlaying it onto an agarose suspension of *Escherichia coli* D31. After incubation at 37°C for 18-24 h, clearing zones (indicating inhibition of bacterial and thus
antibacterial activity) in the agarose in lanes with tissue extracts were compared with antimicrobial polypeptide standards, as well as the stained half of the gel. Standards used included calf histone H2B (Roche Diagnostics Co., Basel, Switzerland), synthetic piscidin 1 and synthetic piscidin 4.

**Western Blotting**

Gill extracts were heated for 10 min at 70°C in lithium dodecyl sulfate (LDS) sample buffer (Invitrogen Cat. No. NP0007, Carlsbad, California, USA) and electrophoresed on a 4-12% gradient Bis-Tris polyacrylamide gel (Invitrogen, Cat. No. NP0322, Carlsbad, California, USA) at 20 mA for 3 h. After electrophoresis, the gel was either stained with Coomassie Blue R-250 or transblotted onto a 0.2 μm PVDF membrane at 100 V for 1 h. The membrane was then incubated in blocking buffer (5% nonfat powdered milk with tris-buffered-saline and 0.05% Tween 20 [Sigma# T9039, St. Louis, Missouri, USA]) on a shaker for 1 h at room temperature followed by overnight incubation at 4°C in horseradish peroxidase-labeled anti-piscidin 4 antibody at 1:1000 dilution in blocking buffer. Membranes were then rinsed in tris-buffered-saline with Tween 20 on a shaker at room temperature for 30 min. ECL Western blot reagents (Pierce, Cat. #32106, Rockford, Illinois, USA) were added to the membrane and incubated for one min before detection. Chemiluminescent signal was captured by a monochrome CCD camera (UVP Laboratory Products, Upland, California). Synthetic piscidin 4 and gill extract from hybrid striped bass known to have piscidin 4 were simultaneously run as positive controls.
ELISA

Gill, intestine and stomach extracts were collected from six striped bass, white bass, European seabass, red drum and barramundi. In addition, blood and skin were collected from striped bass, white bass and red drum. Piscidin 4 was measured via direct sandwich ELISA as described previously (Corrales et al, In Preparation). Briefly, microtiter plates were coated for one h at room temperature with an affinity-purified rabbit antibody to the highly conserved N-terminus of all piscidins (anti-FFHH) (Silphaduang et al 2006). After a washing step, the plates were incubated with Stabilcoat (Surmodics, Inc., Eden Prairie, Minnesota, USA) for 20 min to eliminate non-specific binding and stored at 4ºC until use. Immediately before using the plate, the Stabilcoat was removed by washing. Tissue extracts were diluted to the desired concentration (all extracts were diluted 1:100 except gill extracts from striped bass, which were also diluted 1:200) in sample diluent (0.05 M tris-buffered saline with 0.05% Tween 20 and 1% bovine serum albumin [Sigma #T6789, St. Louis, Missouri, USA]). All samples and controls were added in duplicate and incubated for 1 h. Plates were washed again before the secondary antibody (peroxidase-labeled rabbit anti- piscidin 4) was added and incubated for 1 h. The plate was then washed and TMB peroxidase substrate was added and incubated for 15 min. The reaction was terminated with 3 M phosphoric acid. Absorbance was measured at 450 nm with an ELISA reader (uQuant Microplate Spectrophotometer, Model MQX200, Bio-Tek Instruments, Inc., Winooski, Vermont, USA).

The positive controls were four pools of hybrid striped bass extracts known to have high, moderate, low, or very low levels of piscidin 4. Each pool was prepared by combining
equal volumes of extract from either 14 fish (high, moderate and very low pool) or 10 fish 
(low pool). The negative control was the sample diluent without tissue extract. Pure synthetic 
piscidin 4 was used to construct the standard curve. Tissue concentrations of piscidin 4 were 
calculated by ELISA software (KCjunior, Bio-Tek Instruments, Inc., Winooski, Vermont, 
USA) at 450 nm absorbance based on the optical density (OD) of the 4 parameter curve-
fitting standards. All values were multiplied by 4 to account for the 1:4 dilution of tissue in 
1% acetic acid during sample collection.

**Immunohistochemistry**

Gill tissue from three fish of each species was decalcified overnight in 10% formic 
acid. After embedding in paraffin, blocks were serially sectioned at a thickness of 5 μm. All 
sections were stained with hematoxylin and eosin (H&E). Hematoxylin and eosin staining 
distinguish differences in cell morphology: hematoxilin stains cellular basophilic structures 
(e.g., nucleus) blue and eosin stains cellular acidophilic structures (e.g., cytoplasm) pink. For 
immunohistochemistry, serial sections were deparaffinized in xylene and rehydrated. Slides 
were then blocked with goat serum for 20 min, and incubated with either nonimmune serum 
or monospecific, affinity-purified polyclonal anti-piscidin 4 antibody (1:8,000) for 30 min at 
room temperature. After washing with PBS, slides were incubated with biotinylated goat 
anti-rabbit serum (Biogenex, San Ramon, California, USA) for 20 min, followed by 
streptavidin-conjugated horseradish peroxidase (Biogenex, San Ramon, California, USA). 
Enzyme activity was detected with DAB (3,3’-diaminobenzidine). Slides were
counterstained with Meyer’s hematoxylin and Alcian blue. Photographs were made with an Olympus Vanox microscope. Slides were evaluated blindly (without bias or prior preference for a fish or tissue). Negative controls were produced by substituting anti-piscidin 4 antibody with nonimmune rabbit serum. The positive control was gill from hybrid striped bass, which was known to be positive in piscidin 4, as the peptide was isolated from this fish (Noga et al, In Preparation).

RESULTS

Piscidin 4 Detection via Native Gel Electrophoresis and Western Blotting

Via AU-PAGE, multiple zones of antibacterial activity were detected in gill extracts of all five fish species (Fig. 2). The strongest clearing zone corresponded to the migration of the calf histone H2B standard, which was especially remarkable in the hybrid striped bass, European seabass, barramundi and red drum extracts. A second prominent clearing zone migrated parallel to the piscidin 1 standard, with hybrid striped bass, striped bass, white bass and European seabass extracts having a discrete clearing zone in this area. Although not as prominent, the clearing zones in barramundi and red drum extracts were suggestive of the presence of a peptide corresponding to the migration zone of piscidin 1. A third, less prominent clearing zone was present in extracts of hybrid striped bass, striped bass and white bass that paralleled the migration of synthetic piscidin 4. Hybrid striped bass, striped bass, white bass and European seabass another clearing zone between the migration areas
corresponding to piscidin 4 and calf histone H2B. The red drum extracts had multiple additional clearing zones in between those corresponding to piscidin 4 and calf histone H2B zones in addition to a clearing zone bellow the migration zone corresponding to the piscidin 1 standard.

Western blotting of gill extract showed that all five species had one or two immunopositive bands that migrated similarly to the piscidin 4 standards (hybrid striped bass and synthetic piscidin 4)(Fig. 3). The strongest immunoreactivity was in striped bass extract. Weaker but clearly positive immunoreactivity was also present in white bass, European seabass and barramundi extracts. Red drum extract had the weakest band. The migration rate of the band also varied among fish. While a band migrated at ~7 kDa in white bass, the immunopositive band in European seabass, barramundi and red drum migrated at ~14.4 kDa. Striped bass had bands that migrated at both ~7 and ~14.4 kDa, which was similar to the hybrid striped bass and synthetic piscidin 4 standards.

**Piscidin 4 Measurement via ELISA**

Via ELISA, numerous tissues were piscidin 4-positive. For all species, gill had the highest mean piscidin 4 concentrations (i.e., immunoreactivity)(Table 1). In general, the next strongest immunoreactivity was in stomach or intestine. Blood and skin had the weakest response. Gill piscidin 4 concentrations were highest by far in striped bass. Next highest, but considerably lower, were white bass and European seabass; red drum and barramundi had the lowest levels. In striped bass, gill piscidin 4 was about 38 to 190-fold higher than in the other
species. Striped bass also had the highest piscidin 4 levels in stomach, while white bass had the highest immunoreactivity in the intestine. On a relative basis, we compared gill piscidin 4 concentrations in these five species to those measured in hybrid striped bass having either high, moderate, low, or very low piscidin 4 levels (Table 2). Striped bass and white bass, the parental species of hybrid striped bass, had 2.1 and 77 times less piscidin 4 than hybrid striped bass expressing high piscidin 4 levels; striped bass had about 3 times more and white bass had 9 times less piscidin 4 than hybrid striped bass expressing very low piscidin levels. Red drum had the weakest piscidin 4 immunoreactivity.

**Immunolocalization of Piscidin 4**

Piscidin 4-positive cells were identified by comparing serial sections of tissues that were stained with either HE or anti-piscidin 4 antibody. Mast cells (MC) were consistently piscidin 4 positive, and were identified by their presence within solid tissues and the presence of large numbers of prominent, eosinophilic granules within the cytoplasm (Reite and Evensen 2006). As expected, piscidin 4-positive granules were abundant in hybrid striped bass MC, where piscidin 4 was isolated (Fig. 4). Large numbers of piscidin 4-positive MC were also present in striped bass, white bass and European seabass (Table 3, Figs. 5, 6, and 7). Because of the very small number of immunopositive cells in red drum (Fig. 8), a definitive identification of the specific cell type could not be made. Piscidin 4-positive cells were not detected in barramundi gill tissue (data not shown). The eosinophilic granules in red drum and barramundi MC also were not strongly eosinophilic compared to those of other fish.
(e.g., striped bass). The piscidin 4-positive MC were also distributed in specific regions in the tissue. Striped bass piscidin 4-positive MC were predominantly found in the tip of the primary lamellae, where they formed a dense layer of strongly positive cells. In white bass, piscidin 4-positive MC tended to form loose aggregates in the primary lamella, often in areas of mild focal lamellar hyperplasia, while in Europeans seabass, they were present in the base of the secondary lamellae like the piscidin 4-positive MC in white bass but more abundant and also in the tip of the primary lamellae like those in striped bass but less abundant.

DISCUSSION

A key to specifically detecting piscidin 4 in various fish species was the successful synthesis of piscidin 4 and the ability to use this peptide to raise an antibody that was highly specific to piscidin 4 and not cross-reactive with piscidins 1, 2 or 3. Producing these reagents using the native peptide would require a tremendously greater amount of work. Showing that the synthetic AMP works well as a substitute for native piscidin 4 provides the opportunity to use these piscidin 4 reagents in many applications where fish express this AMP. The four assays that we used to probe for piscidin 4 (bug blot, Western blot, ELISA and immunohistochemistry) were closely correlated with each other (Table 3) and together supported our overall conclusion that piscidin 4 isomers are present in all these species. The use of these multiple, complimentary assays was especially important when certain tests were negative (e.g., barramundi immunohistochemistry).

In the bug blot, where the peptides isolated from the various fish species tested were
electrophoretically separated on an agarose gel and overlaid on an agarose plate containing *E. coli*, all species had antibacterial activity (i.e., inhibition of bacterial growth) corresponding to multiple clearing zones. One of these clearing zones corresponded to the region of inhibition of bacterial growth corresponding to the migration of synthetic piscidin 4 and a clearance zone in the same migrating region was visible in all extracts examined (Fig. 2). A discrete clearing zone that corresponded to the migration rate of piscidin 4 was present in striped bass and white bass. In contrast, in European seabass, barramundi and red drum, the antibacterial activity corresponding to piscidin 4 migration was part of a larger, continuous clearing zone that extended from just below the HLP clearing zone to beyond the migration zone of synthetic piscidin 4. This suggested that there might be a number of antibiotic activities present, each migrating at a somewhat different rate. However, another possibility is that some activities might have been partially degraded yet still possessed some antibacterial activity. During acid-urea gel electrophoresis, proteins are separated while in their native conformation, and thus tissue preparation must prevent degradation for satisfactory polypeptide separation. The gill samples were boiled during collection to inactivate proteases. This procedure is usually fully adequate in preventing proteolytic degradation (Robinette et al 1998, Noga et al 2001) and, in our experience, is far superior to use of protease inhibitors for stabilizing AMPs in tissue extracts (A Ulla, JKSeo and EJ Noga, Unpublished Data). However, it is possible that this procedure is not completely effective for some fish species which we have not yet been validated (i.e., European seabass, barramundi, red drum), leading to partial degradation of the sample.

The strongest clearing zone corresponded to the migration of the calf histone H2B
standard, which parallels the migration of histone-like proteins (HLP) (Noga et al. 2002). Histone-like proteins are highly homologous to core nuclear histones (Robinette et al. 1998, Noga et al. 2002). Originally isolated from the skin of channel catfish (*Ictalurus punctatus*) (Robinette et al. 1998), HLPs were subsequently identified in skin, gill and/or spleen of hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female) and rainbow trout (*Oncorhynchus mykiss*) (Noga et al. 2001, Noga et al. 2002). Subsequently, related antimicrobial polypeptides (AMPP) have been linked to innate defense in a number of other fish including oncorhyninc II, a histone H1-derived 7.2 kDa protein from the skin of rainbow trout (*Oncorhynchus mykiss*) (Fernandes et al. 2004); parasin I, homologous to the N-terminal of human histone H2A and isolated from skin mucus of Asian catfish (*Parasilurus asotus*) (Park et al. 1998); hipposin, derived from histone H2A and isolated from skin of Atlantic halibut (*Hippoglossus hippoglossus*) (Birkemo et al. 2003); HSDF-1 (histone-derived fragment-1) derived from histone H1 and isolated from the skin of coho salmon (*Oncorhynchus kisutch*) (Patrzykat et al. 2001); and a proline rich histone H1-derived fragment from the skin mucus of Atlantic salmon (*Salmo salar*) (Lüders et al. 2005). The presence of histones and related AMPPs in a wide taxonomic range of fish species, from relatively primitive (e.g., *Ictalurus*, order Siluriformes) to advanced (e.g., *Morone*, order Perciformes) (Robinette et al. 1998, Noga et al. 2002), suggests that they might function as a host defense in most if not all teleosts.

Histone-like proteins and related polypeptides are inhibitory to many important fish pathogens, including a wide range of bacteria such as *Aeromonas hydrophila* and *Vibrio alginolyticus* (Robinette et al. 1998), *Aeromonas salmonicida* and *Vibrio anguillarum*. 
(Patrzykat et al 2001, Lüders et al 2005, Birkemo et al 2003), and *Yersinia ruckeri* (Birkemo et al 2003). In addition, water molds (*Saprolegnia*) (Robinette et al 1998), and the ectoparasitic dinoflagellate, *Amyloodinium ocellatum* (Noga et al 2002) are also highly susceptible. While further studies will be needed for confirmation, it is highly likely that the potent antibacterial clearance zone migrating parallel to calf histone H2B in European seabass, barramundi and red drum is also HLP.

A second prominent clearing zone in most fish paralleled the synthetic piscidin 1 standard. Piscidins 1 and 2 are 22 amino acid peptides which differ by only one amino acid at position 18; piscidin 3 is highly homologous to piscidins 1 and 2, but is more variable in its C-terminus. All three are present in hybrid striped bass (Silphaduang and Noga 2001). Because of their high sequence homology, piscidins 1-3 co-migrate and thus are indistinguishable on a bug blot. Recently, the nucleic acid sequence that appears to code for a 22 amino acid AMP (FFHHIFRGIVHVGKSIHKLVTG, Gene Bank No. AAP58960) (dicentracin) was cloned from European seabass head kidney leukocytes (Salerno et al 2007). The deduced sequence of dicentracin differs from piscidin 2 by only one amino acid at position 15 (serine for threonine) and from piscidin 1 by two amino acids (at positions 15 [serine for threonine] and 18 [lysine for arginine]), suggesting that it should migrate similarly to piscidins 1-3. Our bug blot data provides evidence that European seabass constitutively express this small piscidin, since a prominent clearing zone is present in European seabass extract that parallels the migration zone of piscidins 1-3 (Fig. 2). These data also confirm our prior discovery that immune cells positive with the anti-FFHH antibody to the piscidin family were present in European seabass (Silphaduang et al 2006). A very weak clearing
zone in barramundi extract was also suggestive of the presence of a small piscidin. In addition to the three main clearance zones, extracts of hybrid striped bass, striped bass, white bass and red drum showed other less discrete clearing zones, one between the piscidin 4 and calf histone H2B zones and another below the migration zone corresponding to the piscidin 1 standard. Whether these zones truly represent other novel antibiotic activities awaits confirmation.

The Western blot results mirrored the AU-PAGE data for piscidin 4 in most respects. The fish having the greatest piscidin 4 immunoreactivity via Western blotting was striped bass. White bass was much weaker but still positive. When European seabass, barramundi and red drum gill extracts were initially run, no piscidin 4 band was detected (data not shown). However, when these extracts were concentrated five-fold, a band was visible in all three species (Fig. 3). The specificity of the piscidin 4 antibody was further substantiated by the Western blot, which showed no evidence of cross-reactivity with the small (22 amino acid) piscidins, which are known to be present in hybrid striped bass, striped bass and white bass. There was also no evidence that dicentracin cross-reacts with our piscidin 4 antibody, since there was no immunoreactivity in this area in the European seabass extract.

The molecular weight of native piscidin is 5,329 Da, but the native and synthetic piscidin standards migrated at a level corresponding to a molecular weight of ~7 kDa. Piscidin 4 has an isoelectric point (pI) of about 11.2 (Noga et al, In Preparation), making it highly positively charged in physiological media. Although samples were mixed with LDS sample buffer, which adds negative charge to the peptide, the high pI of piscidin 4 probably slowed its migration, making its estimated molecular weight via PAGE larger than its
nominal mass. Highly basic proteins are well-known to migrate anomalously in PAGE gels due to their highly basic charge (Li et al 2007, Patat et al 2004, Richards et al 2001).

Besides the main immunoreactive band corresponding to piscidin 4, a second, much weaker band was present in gill extracts of both hybrid striped bass and striped bass, as well as in the lane having the synthetic piscidin 4 standard (Fig. 3). This band migrated at a nominal molecular weight that appeared to be approximately twice the size of piscidin 4, suggesting that it might be a dimer of piscidin 4. Non-covalent bonding between native protein chains has been observed, and some of these protein oligomers are highly stable, even under the strongly denaturing conditions used for dodecyl sulfate PAGE (Partridge et al 2002, Podlisny et al 1995). Freezing can cause some proteins to dimerize (Bennett et al 1994, Philo 2006). However, when a freshly prepared synthetic piscidin 4 sample was Western blotted, the dimer was still present, indicating that dimerization was not a storage artifact (Corrales et al, In Preparation).

All five species in our study belong to the order Perciformes, suborder Percoidei, and are members of the families Moronidae (basses), Sciaenidae (red drum) and Latidae (barramundi) (Fig. 9). In a previous study, we provided evidence for the widespread distribution of piscidins in the Perciformes (Silphaduang et al 2006), the largest and most evolutionarily advanced order of the teleosts. In that study, piscidins were identified via immunohistochemistry using an antibody raised against the conserved N-terminus of the piscidin family. Piscidins were localized to various tissues from fish in six families, including Moronidae (striped bass, white bass, European seabass), Serranidae (snowy grouper
Epinephelus niveatus), Sciaenidae (spot Leiostomus xanthurus) and Atlantic croaker Micropogonias undulates), Cichlidae (Nile tilapia Oreochromis niloticus), Siganidae (rabbitfish Siganus rivulatus), and Belontidae (pearl gourami Trichogaster leeri) (Silphaduang et al. 2006). Since spot and Atlantic croaker tissues were immunopositive, it led us to believe that piscidins might be present in red drum, also a sciaenid. Barramundi is also in the s.o. Percoidei. Barramundi tissue was negative via immunohistochemistry in our previous study (Silphaduang et al. 2006) and our anti-piscidin 4 antibody also failed to detect piscidin 4 in barramundi via immunohistochemistry. However, antibacterial activity was present in a zone similar to that of the piscidin 4 standard (Fig. 4), and the presence of piscidin 4 in barramundi was corroborated by the ELISA and Western blotting results. Since our antibody is highly specific for piscidin 4 (i.e., it does not cross-react with any other piscidins), immunopositive responses are not recognizing the piscidin family but rather the very different set of epitopes that are expressed in the C-terminus of piscidin 4.

The amino acid sequence of the piscidin 2 prepropeptide shares high homology (77% similarity) with that of the winter flounder pleurocidin prepropeptide in the N-terminal signal (Lauth et al. 2001, Sun et al. 2007). Also, the mature peptides of piscidin 2 and winter flounder pleurocidin have 63% similarity and 27% identity (Lauth et al. 2001, Sun et al. 2007). Furthermore, the amino acid sequence of the piscidin 2 prodomain in white bass and the piscidin 1 prodomain in striped bass both contain six repeats of the XQQ motif (Lauth et al. 2002), while there is only a single XQQ motif in the coding region of mandarin fish piscidin and this motif is absent in pleurocidin (Sun et al. 2007). At the mature peptide level,
the homology of mandarin fish piscidin with pleurocidin is weaker, while mandarin fish piscidin only differs by two amino acids with piscidin 1 and by three amino acids with piscidin 2, (Sun et al 2007). Phylogenetically, bass and mandarin fish belong to the order Perciformes and winter flounder to the order Pleuronectiformes. They are all in the superorder Acanthopterygii. Due to the homology between the coding region of the peptides and the phylogenetic relationship among species, Sun et al (2007) concluded that piscidins and pleurocidins appear to be derived from a common ancestor gene and thus pleurocidins belong in the piscidin family. Thus, piscidins appear to be the most common AMP family in fish.

The immunohistochemistry results indicated that all species except barramundi had piscidin 4-positive cells, and those piscidin 4-positive cells were identified as MC. This was expected, since we have previously reported that the MC was the predominant piscidin-positive cell type when probing with an antibody to the conserved N-terminus of the piscidins (Silphaduang et al 2006). Mast cells were identified as pleiomorphic, tissue-residing cells having prominent, eosinophilic granules (Reite and Evensen 2006). Not all MC were piscidin 4-positive. This was especially evident in red drum, where the majority of the MC were immunonegative. Variability in MC immunoreactivity was also observed when tissues were probed with the anti-FFHH antibody (Silphaduang et al 2006). Presence of piscidin 4-positive MC also varied spatially within the tissue. For example, striped bass piscidin 4-positive MC were predominantly found in the tip of the primary lamella. We previously found that abundance of piscidin-positive MC decreased from the tip of the primary lamellae (highly immunoreactive) to the connective tissue (weakly
immunoreactive)(Silphaduang et al 2006). Fish MC constitute a heterogeneous cell population with varied morphologies, granular content, sensitivity to fixatives, and response to stimuli (Reite and Evensen 2006). Thus, MC heterogeneity, along with different stages of cell maturation, might affect the immunoreactivity of MC populations. The AMPs chrysophsin from red sea bream (*Chrysophrys major*) (Iijima et al 2003) and winter flounder pleurocidin (Murray et al 2003) have also been immunolocalized to gill MC.

With inflammation, MC are activated, resulting in the secretion of vasoactive amines such as histamine (Janeway and Medzhitov 2002). Histamine release from human MC is induced by the defensin hBD-2 and the cathelicidin LL-37 (Niyonsaba et al 2001) and from rat MC by neutrophil defensins (Befus et al 1999). Fish MC were thought to lack histamine, but recently it has been detected in MC of gilthead seabream (*Sparus aurata*) and European seabass (Mulero et al 2007), both members of the order Perciformes. While histamine was detected via immunhistochemistry in gill and intestine from gilthead seabream and European seabass, it was absent in gill and intestine from turbot (*Psetta maxima*) and Senegalese sole (*Solea senegalensis*) (both in the o. Pleuronectiformes), rainbow trout (*Oncorhynchus myskiss*, o. Salmoniformes), European eel (*Anguilla anguilla*, o. Anguilliformes), zebrafish (o. Cypriniformes) and South American lungfish (*Lepidosiren paradoxa*, o. Lepidosireniformes). The restriction of histamine to the Perciformes led Mulero et al (2007) to speculate that there might be a relationship between the presence of both piscidins and histamine in MC. A number of flatfish in the o. Pleuronectiformes express pleurocidins (Patrzykat et al 2003). While Senegalese sole and turbot lack histamine, whether these flatfish express pleurocidin or other piscidins is unknown and it would be interesting to
determine if flatfish expressing pleurocidin were also histamine-positive. The possible functional relationship between piscidins and histamine in MC is also unknown. Although still speculation, piscidins might modulate histamine release from MC, as is the case of defensins and cathelicidins in mammals. Cationic peptides with an increasing net positive charge (more basic peptides) are more potent inducers of histamine release from MC (Befus et al 1999, Salmon et al 2001). The absence of histamine in the Pleuronectiformes, at least some of which might also lack pleurocidins, suggested that heterogeneity of MC populations and their mediators, as well as their overall relationship to AMP function, is evolutionarily complex (Mulero et al 2007).

The ELISA measurements showed that piscidin 4 concentrations in all five fish species were less than 1 µg/ml except for striped bass gill, which was much higher (nearly 20 µg/ml) (Table 2). The concentrations of piscidin 4 detected by the ELISA in striped bass gill were well within the levels that are inhibitory to important fish bacterial pathogens. For example, the minimum inhibitory concentration (MIC MBC) of piscidin 4 for Lactococcus garvieae is 6.3 µg/ml (Noga et al, In Preparation). Also, some pathogens are highly sensitive to much lower piscidin 4 levels (e.g., the MIC for Photobacterium damselae subsp. piscicida is 0.4 µg/ml) and thus, even the relatively low levels measured in most fish species in our study might be directly inhibitory. In addition, this does not take into account the possibility that piscidin 4 might also facilitate the killing of pathogens by other polypeptide antibiotics, such as small piscidins and HLPs, which also appear to be present (Fig. 2). It is also important to note that many AMPs possess immunomodulatory functions that are not tied directly to microbicidal action and these functions often occur at much lower concentrations.
than that needed for direct antimicrobial activity (Brown and Hancock 2006, Beisswenger and Bals 2005).

Interestingly, piscidin 4 levels not only varied considerably among tissues, but also among the fish species and hybrid that we examined. There are probably inherent, genetically-regulated differences in innate expression of piscidin 4 among different species and possibly among different strains of fish (i.e., hybrids). Heterosis (hybrid vigor) is characterized as superior qualities arising from the crossbreeding of genetically different plants or animals. It is produced by the differences in gene frequency between the two parental species. It has long been recognized that hybrid striped bass exhibit significant heterosis as reflected in faster growth rate, improved survival and greater disease resistance compared to the parental lines (Harrell 1997, Rudacille and Kohler 2000). To our knowledge, the relationship of AMP expression to heterosis has never been examined. Although additional studies are needed to determine if heterosis might affect AMP expression, striped bass, white bass and hybrid striped bass in our study varied considerably in their expression of piscidin 4. Additional evidence for a possible role of heterosis in affecting the expression of AMP is the presence of both piscidin 1 and piscidin 2 in hybrid striped bass (Silphaduang and Noga 2001), while piscidin 1 (but not piscidin 2) is present in striped bass while piscidin 2 (but not piscidin 1) resides in white bass (Lauth et al 2002). However, any of a number of other factors might be responsible for our observed differences in piscidin 4 among members of the Morone complex (see below).

Age, size, or health status/stress might also have affected piscidin 4 expression in the fish that we studied. The age and size of fish used in our study ranged widely, from 3 mo to 3
y and from 65 to 2378 g. Age and size can have a profound effect on immune function and susceptibility to disease (LaPatra 1998, Hrubec et al 2004). While little is known about this relationship for fish AMP, the winter flounder pleuorocidin transcript progressively increased from a very low level in the larval stage, where it was first detected at 13 d post-hatch, to maximum expression in adults (Douglas et al 2001). In adult winter flounder, a strong mRNA signal was detected in the skin but it was very weak in the intestine. Genes for pleurocidin-like peptides in other adults flatfish species were also expressed in a tissue-specific manner (greatest expression in the skin versus intestine) (Douglas et al 2001).

In aquaculture, the adverse effects of physiological stress on immune function are widely recognized (Wojtaszek et al 2002). While piscidin 4 levels in gill of hybrid striped bass and striped bass were similar (Table 2), the latter are much more stress-prone (Kerby 1986, Noga et al 1994). We have previously shown that environmental stress can cause a dramatic reduction in antimicrobial polypeptide levels (Robinette and Noga 2001). Thus, it is tempting to speculate that the greater disease resistance exhibited by hybrid striped bass might be at least partly due to the contribution of high piscidin 4 levels in striped bass combined with the less stress-prone behavior of white bass (Kohler et al 1994, Kerby 1993). Stress might also have had an effect on the expression of piscidin 4 in some of the individuals that we sampled. We sampled some of the fish in this study very soon after shipment and transport stress can be immunosuppressive (Barton 2000, Engelsma et al 2002, Small and Bilodeau 2005, Conte 2004). Other fish that we sampled might have been exposed to chronic stress, which is known to depress expression of other antimicrobial polypeptides (Robinette and Noga 2001). Others might not have been in optimal health. Although the
white bass appeared clinically normal at the time of sampling, histological examination of the gill showed some hyperplasia with lamellar fusion (Fig. 6) and these fish had much lower piscidin 4 levels than either hybrid striped bass or striped bass.

Another possibly very important reason affecting immunoreactivity among different fish species is that different isoforms of piscidin 4, which might vary in their affinity and thus immunoreactivity with our piscidin 4 antibody, might be present in different fish species. Possible variation in the amino acid sequence of these various AMP was also suggested by their different migration rates in LDS-PAGE (Fig. 3). While specific identity of these piscidin 4-immunoreactive proteins requires their isolation and sequencing from each fish, our data provide strong evidence that piscidin 4 isoforms are present in all these commercially important species. Our data also suggest that quantification of piscidin 4 might be useful as a diagnostic tool for measuring this AMP in several commercially important fish species, especially in the easily biopsied gill, which most strongly expressed piscidin 4. Based upon our data showing that piscidin 4 can be strongly downregulated with stress (Corrales et al, In Preparation), such a tool might be very useful in helping to predict risk of infection or disease.
ACKNOWLEDGEMENTS

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Table 1. Mean (± SE) piscidin 4 immunoreactivity in the gill, skin, blood, stomach and intestine of fish examined in this study.

N = 6 individuals per species. ND = not done

<table>
<thead>
<tr>
<th>Species</th>
<th>Gill</th>
<th>Skin</th>
<th>Blood</th>
<th>Stomach</th>
<th>Intestine</th>
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<tbody>
<tr>
<td>White bass</td>
<td>0.51 ± 0.24</td>
<td>0.06 ± 0.02</td>
<td>0.09 ± 0.02</td>
<td>0.08 ± 0.03</td>
<td>0.23 ± 0.07</td>
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<tr>
<td>Striped bass</td>
<td>18.78 ± 3.92</td>
<td>0.11 ± 0.03</td>
<td>0.12 ± 0.02</td>
<td>0.37 ± 0.02</td>
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<tr>
<td>European seabass</td>
<td>0.29 ± 0.05</td>
<td>ND</td>
<td>ND</td>
<td>0.06 ± 0.02</td>
<td>0.08 ± 0.02</td>
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<tr>
<td>Barramundi</td>
<td>0.13 ± 0.02</td>
<td>ND</td>
<td>ND</td>
<td>0.08 ± 0.02</td>
<td>0.07 ± 0.02</td>
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<tr>
<td>Red drum</td>
<td>0.10 ± 0.015</td>
<td>0.06 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.05 ± 0.004</td>
<td>0.06 ± 0.02</td>
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Table 2. Comparison of mean gill piscidin 4 concentrations (μg/ml) of white bass, striped bass, European seabass, barramundi and red drum versus that of hybrid striped bass having either high, moderate, low or very low piscidin 4 levels.

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>Mean piscidin 4 (μg/ml)</th>
<th>Relative amount in hybrid striped bass having piscidin 4 expression</th>
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<tr>
<td>White bass</td>
<td>6</td>
<td>0.51 ± 0.24</td>
<td>High 77X</td>
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<tr>
<td>Striped bass</td>
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<td>18.78 ± 3.92</td>
<td>Moderate 2.1X</td>
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<td>Low 0.6X</td>
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<td>Barramundi</td>
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<td>High 303X</td>
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<tr>
<td>Red drum</td>
<td>6</td>
<td>0.10 ± 0.015</td>
<td>High 394X</td>
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<tr>
<td>Hybrid striped bass – High</td>
<td>14</td>
<td>39.40 ± 2.52</td>
<td>Moderate 20.22 ± 0.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Low 11.90 ± 0.71</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Very Low 4.74 ± 0.91</td>
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Table 3. Summary of antibacterial activity, expression levels and immunoreactivity of piscidin 4 in gill tissue of various fish. ++++ = very strong, +++ = strong, ++ = moderate, + = weak, +/- = possibly positive, – = negative

<table>
<thead>
<tr>
<th>Fish</th>
<th>Bug blot</th>
<th>Western blot</th>
<th>ELISA</th>
<th>Immunohistochemistry</th>
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<tr>
<td>Hybrid striped bass</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
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<tr>
<td>White bass</td>
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<td>++</td>
<td>+++</td>
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<tr>
<td>Barramundi</td>
<td>+/-*</td>
<td>+*</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Red drum</td>
<td>+/-*</td>
<td>+*</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Tissue samples were concentrated five-fold before testing
Figure 1. Amino acid sequence alignments of piscidins from *Morone*. Amino acid sequences of piscidins 1, 2, 3 and 4, and the 11-mer peptide fragment used to generate the antibody to the N-terminus of piscidins. In the piscidin 4 ELISA, the epitopes targeted by the primary (capture) antibody are underlined, while the epitopes targeted by the secondary (detecting) antibody are within the square.
Figure 2. Bug blot of gill extracts. After electrophoresis by AU-PAGE, the gel was washed to remove the acid and urea, and then was laid on a lawn of *Escherichia coli* D31. The bacterial lawn was incubated overnight and then examined for inhibition of bacterial growth, as indicated by clearing (dark) zones on the plate. The control lane (A) was loaded with 3 antimicrobial polypeptides: calf histone H2B (3 μg, 13.5 kDa), synthetic piscidin 4 (0.5 μg, 5.3 kDa) and synthetic piscidin 1 (0.5 μg, 2.5 kDa). Clearing zones are present in Lanes B (hybrid striped bass moderate), C (striped bass), D (white bass), E (European seabass), F (barramundi), and G (red drum). The extracts of European seabass, barramundi and red drum are five-fold concentrates. A white circle surrounds the clearing zone that migrates parallel to pure piscidin 4 in Lane A. No zone is circled in Lanes E, F or G because the clearing zone was not discrete.
Figure 3. Western blot of piscidin 4 in gill extracts from fish examined in this study. Samples were separated on a 4-12% gradient Bis-Tris gel and transferred onto a PVDF membrane that was blocked for 1 h and incubated overnight at 4°C with horseradish peroxidase-conjugated anti-piscidin 4 antibody. Lane 1 - striped bass, Lane 2 – white bass, Lane 3- European seabass, Lane 4 - barramundi, Lane 5 - red drum, Lane 6 - hybrid striped bass having moderate piscidin 4 concentration, Lane 7 - synthetic piscidin 4 (50 ng). The extracts of European seabass, barramundi and red drum are five-fold concentrates.
Figure 4. Immunohistochemical localization of piscidin 4 in normal gill tissue of hybrid striped bass treated with monospecific, affinity-purified polyclonal anti-piscidin 4 antibody (1:8000). P – primary lamella, S – secondary lamella, M – mast cells. (A) Immunopositive cells that are present mostly at the tip of the primary lamella but also present at the base of the secondary lamella. (B) Nonimmune serum control of serial section of (A). (C) Immunopositive mast cells (arrows). (D) Hematoxylin and eosin (H&E) staining of a serial section of (C) showing mast cells that correspond to the strongly immunopositive cells in (C) (arrows).
Figure 5. Immunohistochemical localization of piscidin 4 in normal gill tissue of striped bass treated with nonimmune serum or monospecific, affinity-purified polyclonal anti-piscidin 4 antibody (1:8000). P – primary lamella, S – secondary lamella, M – mast cells. (A) Immunopositive cells that are most prevalent at the distal end of the primary lamella but are also present in more proximal areas at the base of the secondary lamella. (B) Nonimmune serum control of serial section of (A). (C) Immunopositive mast cells (arrows). (D) Hematoxylin and eosin (H&E) staining of a serial section of (C) with mast cells that correspond to the strongly immunopositive cells in (C)(arrows).
Figure 6. Immunohistochemical localization of piscidin 4 in normal gill tissue of white bass treated with nonimmune serum or monospecific, affinity-purified polyclonal anti-piscidin 4 antibody (1:8000). P – primary lamella, S – secondary lamella, M – mast cells. (A) Immunopositive cells that are most prevalent at the base of the secondary lamella (long arrows); note the focal hyperplasia and fusion of some secondary lamellae (short arrows). (B) Nonimmune serum control of serial section of (A). (C) Immunopositive mast cells (arrows). (D) Hematoxylin and eosin (H&E) staining of a serial section of (C) showing mast cells that correspond to the strongly immunopositive cells in (C) (arrows).
Figure 7. Immunohistochemical localization of piscidin 4 in normal gill tissue of European seabass treated with nonimmune serum or monospecific, affinity-purified polyclonal anti-piscidin 4 antibody (1:8000). P – primary lamella, S – secondary lamella, M – mast cells. (A) Immunopositive cells that are most prevalent at the base of the secondary lamella (arrows). (B) Nonimmune serum control of serial section of (A). (C) Immunopositive mast cells (arrows). (D) Hematoxylin and eosin (H&E) staining of a serial section of (C) showing mast cells that correspond to the strongly immunopositive cells in (C) (arrows).
Figure 8. Immunohistochemical localization of piscidin 4 in normal gill tissue of red drum treated with nonimmune serum or monospecific, affinity-purified polyclonal anti-piscidin 4 antibody (1:8000). P – primary lamella, S – secondary lamella, M – mast cells. (A) Very small number of immunopositive cells in the primary lamella (arrows). (B) Nonimmune serum control of serial section of (A). (C) Immunopositive mast cells (arrows). (D) Hematoxylin and eosin (H&E) staining of a serial section of (C) showing mast cells that correspond to the immunopositive cells in (C)(arrow).
Figure 9. Taxonomic classification of fish that express piscidin 4. Tissues were tested for the presence of piscidin 4 in members of the families Moronidae, Sciaenidae and Latidae (shaded and bold).
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CHAPTER IV

EFFECTS OF FEEDING RATE ON THE EXPRESSION OF ANTIMICROBIAL POLYPEPTIDES AND ON SUSCEPTIBILITY TO *ICHTHYOPHTHIRIUS MULTIFILIIS* IN HYBRID STRIPED (SUNSHINE) BASS

(*MORONE SAXATILIS♂ x M. CHRYSOPS♀*)
Effects of feeding rate on the expression of antimicrobial polypeptides and on susceptibility to *Ichthyophthirius multifiliis* in hybrid striped (sunshine) bass (*Morone saxatilis* ♀ x *M. chrysops* ♂)

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*Keywords: hybrid striped bass, nutrition, feed rate, antimicrobial peptides, parasitic susceptibility*
ABSTRACT

The single greatest expense in aquaculture is feed. To further optimize feed efficiency, culturists are attempting to improve feeding regimens. One method being intensively explored is restricting feeding for varying periods, which can induce compensatory growth (CG), an enhanced growth rate that occurs when restoring normal feeding after keeping fish in a suboptimal environment. However, inadequate nutrition is known to affect immune function and disease resistance and the effect of restricting feed on immunity is poorly understood. Among the most potent of the innate immune defenses are antimicrobial polypeptides (AMPP), comprising host-produced peptides and small proteins, that are broad-spectrum defenses. Among the most prevalent and potent AMPP in fish are histone-like proteins (HLP) and piscidins. In this study, gill, skin, blood and spleen samples were collected from fish fed either at a high (apparent satiation), moderate (half of the high feed rate), low (fed 0.6% of body weight three times per wk), or fasting (not fed) feed rate. Tissue antibacterial activity, HLP and piscidin levels were determined on Days 0, 50 and 80. At Day 50, there were no significant changes in AMPP expression in tissues of any treatment group. However, on Day 80, the fasting group had significantly less antibacterial activity and piscidin 4 concentration in the gill; piscidin 4 levels dropped well below the concentration that killed ich in vitro (25 μg/ml). Fasted fish also had the most rapid mortality rate after ich challenge. Our data indicate that prolonged fasting results in depressed levels of AMPP, which probably substantially contributes to increased disease susceptibility.
INTRODUCTION

The initial response to pathogen invasion is via non-specific (innate) immunity (Beutler 2004, Janeway and Medzhitov 2002). Antimicrobial polypeptides (AMPP), host-produced, broad-spectrum antibiotics comprising various peptides and small proteins, are a key component of this defense and are probably present in all animals (Zasloff 2002). Some of the first AMPPs isolated from fish were histone-like proteins (HLPs), which are highly homologous to core nuclear histones (Robinette et al 1998, Noga et al 2002). Histone-like protein-1 (HLP-1 is one of the most potent, broad-spectrum HLPs in fish, and can kill bacteria, water molds and parasites (Robinette et al 1998, Noga et al 2002). Histone-like proteins have been identified in skin, gill and/or spleen of channel catfish (*Ictalurus punctatus*) (Robinette et al 1998), rainbow trout (*Oncorhynchus mykiss*) (Noga et al 2001, Noga et al 2002) and hybrid striped bass (striped bass *Morone saxatilis♂* x white bass *M. chrysops♀*) (Noga et al 2001, Noga et al 2002). Histone-related AMPPs have also been linked to innate defense in Asian catfish (*Parasilurus asotus*) (Park et al 1998), coho salmon (*Oncorhynchus kisutch*) (Patrzykat et al 2001), Atlantic salmon (*Salmo salar*) (Lüders et al 2005) and Atlantic halibut (*Hippoglossus hippoglossus*) (Birkemo et al 2003).

Another family of AMPPs that is present in many teleosts are the piscidins, which have potent, broad-spectrum activity against viruses (Chinchar et al 2004), bacteria (Silphaduang and Noga 2001), fungi (Lauth et al 2002), and parasites (Colorni et al, In Press). First isolated from mast cells of hybrid striped bass (Silphaduang and Noga 2001), there is evidence for their widespread presence in higher teleosts (i.e., order Perciformes)
since piscidin genes have been identified in European seabass (*Dicentrarchus labrax*) (Salerno et al 2007) and Chinese perch (*Siniperca chuatsi*) (Sun et al 2007) and immune cells in six families (Moronidae, Serranidae, Sciaenidae, Cichlidae, Siganidae and Belontidae) are immunopositive for piscidin (Silphaduang et al 2006). Piscidins are linear, amphipathic, $\alpha$-helical AMPPs with a highly conserved N-terminus rich in histidine and phenylalanine. Hybrid striped bass express piscidins 1 and 2, as well as another isoform, piscidin 3, which varies significantly from piscidins 1 and 2 in the C-terminus. Piscidins 1-3 are 22 amino acids long. Recently, we have isolated a novel type of piscidin (piscidin 4) from hybrid striped bass, which is 44 amino acids long and 5329 Da, and thus twice the size of all other known piscidins (Noga et al, In Preparation).

There has recently been intense interest in optimizing feed efficiency in fish by taking advantage of compensatory growth, (CG), which is accelerated growth in an optimal environment after conditions causing growth depression have been withdrawn (Ali et al 2003). Compensatory growth has been documented in several fish (Skalski et al 2005, Turano et al 2007), including hybrid striped bass (Picha et al 2006). The growth depression that precedes CG is typically induced by a partial or complete feed deprivation (Ali et al 2003). However, feed deprivation increases susceptibility to infection (Fletcher 1997, Gatlin 2002). While several studies have shown a relationship between decreased expression of AMPPs and increased disease susceptibility in both spontaneous (Pütsep et al 2002, Islam et al 2001) and experimental models in mammals (Salzman et al 2003, Nizet et al 2001), relatively little is known about how inadequate nutrition affects constitutive AMPP expression in any animal.
Thus, the purpose of our study was to examine the effect of feed rate on the expression of HLP-1 and piscidin. We used as our model the hybrid striped (sunshine) bass, a major aquacultured fish in the United States that expresses high levels of both HLPs and piscidins. We also determined if there was any relationship between tissue levels of these AMPPs and resistance to challenge with *Ichthyophthirius multifiliis* (ich), one of the most important parasites of freshwater fish.

MATERIALS AND METHODS

**Experimental Fish**

Age-0 hybrid striped bass were obtained from a local producer, transported to North Carolina State University and then held in a 380 liter aquarium at 14°C for 67 d. During this time, fish were fed at a rate of 0.6% of body weight once daily with a 5 mm pellet (40% crude protein, 10% crude fat, and 4% crude fiber, Zeigler Bros., Inc., Gardners, PA). Ammonia, nitrite, and pH were monitored weekly. Fifteen days prior to stocking into experimental aquaria, the temperature in the holding aquarium was slowly increased from 14°C to 20°C over seven days. The fish were then transferred to the experimental aquaria while under sedation with 60 mg/L buffered tricane. After placing them in the experimental aquaria, fish were fed close to satiation.
Experimental Design

Fifteen fish were placed in each of twelve 60-liter aquaria (Total N = 180, treatment n = 45, density ~10.5 g/l). All aquaria were connected to a central filtration system having a conditioned biofilter (bio cubes and bead filter [Aquadyne, Koi Camp Aquariology, Loganville, GA] and a titanium heater (Process Technology Co., Mentor, OH). Fish began feeding normally almost immediately and were fed close to apparent satiation twice daily. After nine d in the aquaria, the temperature was increased from 20°C to 26°C over the next seven d. After 18 d at 26°C (day 0), all fish were weighed, some were sampled (see below) and the remainder were fed to apparent satiation for the next 7 d to allow recovery from any post-sampling stress. At 7 d, the fish were placed on the experimental feeding treatments.

Triplicate aquaria were randomly assigned to one of four feeding rates, with the daily ration split into two equal portions: high (fed to apparent satiation daily), moderate (fed half of the high feeding rate), low (fed 0.6% of body weight three times per wk), or fasting (not fed). To calculate the moderate feeding rate, the amount of feed eaten by the high feed rate group was weighed every two wk. The mean weight of all fish on day 0 was 42 ±15 g. During the experiment, 75% water changes were performed thrice weekly, and water quality was measured (ammonia, nitrite, nitrate, pH, and dissolved oxygen) twice weekly via spectrophotometric assays (DREL 2010, Model 26700, Hach Chemical Co., Loveland, CO).
Sample collection

Three fish from each aquarium were sampled at 0, 50 and 80 d. Each aquarium was sampled one at a time; the three fish were sedated with a low dose of buffered tricane (30 mg/L tricane + 60 mg/L sodium bicarbonate) and each fish was then euthanized one at a time in a separate container having a high dose of buffered tricane (500 mg/L tricane + 1000 mg/L sodium bicarbonate). Each fish was weighed and measured to calculate the Fulton’s condition factor, \( K = W \times 10^5 / L^3 \); \( W \) = weight in g, \( L \) = total body length in mm. Blood was then collected from the caudal vasculature with a heparinized syringe; a skin scraping and a gill clip were then taken and examined immediately for pathogens under a light microscope. Skin, gill, and spleen samples were then collected for AMPP levels. Gill, spleen and skin were processed for measuring AMPPs as previously described (Noga et al 2001, Robinette and Noga 2001). Briefly, 50 µl of tissue was collected to yield a total volume of 200 µl in 150 µl of 1% acetic acid (1:4 dilution of tissue). After boiling for 5 min, the sample was homogenized and then centrifuged at 14,000 x g for 10 min at 4°C. The resulting extract (supernatant) was used to measure antibacterial activity, piscidin 4 and HLP-1. As much blood as possible was collected from each fish, immediately mixed 1:1 with Alsever’s solution and placed at 4°C. Blood samples were then centrifuged for 1 min at 2,700 X g at 4°C. The plasma (supernatant) was separated and stored at -80°C. The packed erythrocytes were resuspended in 1% acetic acid (1:4 dilution of tissue), boiled and centrifuged like the gill, spleen and skin to collect the extract to measure antibacterial activity, piscidin 4 and HLP-1.
**Bacterial culture**

Blood from representative fish in each treatment (2 fish per aquarium) was collected from the caudal vasculature with a heparinized 1 cc syringe having a 23 GA needle. A drop was placed on a Columbia blood agar plate and then spread with a sterile swab (Mini-tip Culturette, Becton Dickinson, Franklin Lakes, NJ). Culture plates were incubated at room temperature and observed daily for 14 d.

**Antibacterial activity**

Antibacterial activity was measured using the radial diffusion assay (Noga et al 2002). Briefly, 2 mm diameter wells were punched in an *Escherichia coli* D31 agarose plate and 3 μl of tissue extract was pipetted into duplicate wells. The plate was then incubated at 37°C for 18 h, at which time the diameter of each clearing zone was measured with a vernier caliper to the nearest 0.1 mm. The radial diffusion assay clearing zone diameters were then converted to Units of activity by reference to a standard curve prepared by serially diluting calf histone H2B (Roche Diagnostics Co, Basel, Switzerland). The 1000 ug/ml concentration of calf histone H2B was considered to be 100 Units of activity.

Semi-quantitative analysis of AMPP expression was performed using the “bug blot”. Gill extracts were electrophoresed on acid-urea polyacrylamide gels (AU-PAGE) (Harwig et al 1993) using the Mini-Protean II (BioRad) electrophoresis system according to manufacturer’s instructions. Briefly, samples were run in duplicate in the AU-PAGE. After
electrophoresis, the gel was divided in half. One half was stained with Coomassie Blue R-250 (Bio-Rad Laboratories, Inc., Hercules, CA) and the other half was washed and “blotted” by overlaying it onto an *E. coli* D31 plate. After incubation at 37°C for 18-24 h, clearing zones in the agarose in lanes with tissue extracts were compared with AMPP standards as well as the stained half of the gel. Standards used included calf histone H2B (Roche Diagnostics Co., Basel, Switzerland), piscidin 1 and piscidin 4.

**AMPP detection via ELISA**

Piscidin 4 and HLP-1 were measured via ELISA as described previously (Corrales et al, In prep; Noga et al, In prep). For piscidin 4, microtiter plates were coated for one h at room temperature with a rabbit antibody to the highly conserved N-terminus of all piscidins (anti-FFHH antibody) (Silphaduang et al 2006). After a washing step, the plates were incubated with Stabilcoat (Surmodics, Inc., Eden Prairie, MN) for 20 min to eliminate non-specific binding and then stored at 4°C until use. Immediately before use, plates were washed with 0.05 M tris-buffered saline with 0.05% Tween 20. Tissue extracts (gill, skin, blood, and spleen) were diluted to the desired concentration (1:100 to 1:3200) in 0.05 M tris-buffered saline with 0.05% Tween 20 and 1% bovine serum albumin (BSA) (Sigma #T6789, St. Louis, MO). All samples were added in duplicate to the ELISA plate and incubated for 1 h. The plate was washed again before the secondary antibody (peroxidase-labeled rabbit anti-piscidin 4) was added and incubated for 1 h. The plate was then washed and TMB peroxidase substrate was added and incubated for 15 min. The reaction was terminated with 3 M
phosphoric acid. Absorbance was measured at 450 nm with an ELISA plate reader (KCjunior, Bio-Tek Instruments, Inc., Winooski, VT). Pure synthetic piscidin 4 (Noga et al, In Preparation) was used to construct a standard curve.

The HLP-1 ELISA was performed similarly but using primary antibodies that were homologous to the N-terminus and C-terminus of HLP-1 (anti-PDPA and anti-VSEG antibodies); the secondary antibody was affinity-purified, peroxidase-labeled antibody to calf histone H2B (Noga et al, In Preparation). The standard curve was prepared using a serial dilution of calf histone H2B, and the positive control was prepared from a synthetic antigen created by linking the N-terminus and C-terminus peptide antigens (used to prepare the coating antibodies) to BSA.

Tissue concentrations of piscidin 4 and HLP-1 were calculated by the software (KCjunior, Bio-Tek Instruments, Inc., Winooski, VT) at 450 nm absorbance based on the optical density (OD) of the 4 parameter curve-fitting standards. Samples with an OD ≥3 were diluted twofold and reran. All values were multiplied by 4 to account for the 1:4 dilution of tissue in 1% acetic acid (50 ul of tissue in 150 μl of 1% acetic acid) during sample collection.

**Ich challenge**

On Day 107, remaining fish were challenged with ich (*Ichthyophthirius multifiliis*). Eight fish from each group (high, moderate, low, and fasting) were placed in a separate plastic cage (48 cm in diameter, 38 cm deep) in a 1100 liter closed system fiberglass aquarium at room temperature (22°C). Three fingerling channel catfish, heavily infected with ich, were
then placed into the aquarium. This ich isolate had been obtained from a local aquarium store and had been serially propagated on channel catfish in our laboratory for the last 4 wk. The challenged hybrid striped bass were examined thrice daily for typical signs of ich (e.g., flashing, white foci on the skin); moribund or dead fish were immediately removed. Skin scrapings and gill and fin biopsies of all removed fish were examined; ich infection was confirmed via identification of trophonts with light microscopy.

**Antiparasitic activity**

Antiparasitic activity was tested against the pathogenic ciliate *Ichthyophthirius multifiliis*. The infective stage of ich is known as the trophont or feeding stage. When it matures (4-10 days under optimal temperature: 22°-25°C), the trophont falls off the host skin and enters the reproductive or dividing stage (tomont). Each trophonts leaving the host produces up to 1,000 new tomonts that will develop into theronts (infective stage) which can immediately infect a host. Ich was maintained in a channel catfish fingerling population by regularly adding naïve fish to an aquarium having infected fish. Ich trophonts were obtained by scraping parasites off the skin of infected fish and transferring them to two ml of filter-sterilized aquarium water in a polystyrene Petri dish. They were used immediately for testing. Eighty μl of filter-sterilized aquarium water was added to each well of a 96-well flat-bottom polystyrene microtiter plate (Costar, #3208) followed by 10 μl of each peptide dilution. Eight peptide dilutions were prepared in a two-fold dilution series (200 μg/ml to 1.507 μg/ml). Parasites in aquarium water and parasites in diluent alone (0.2% BSA in 0.01%
HAc) were negative controls. Using an inverted phase contrast microscope (Nikon, Tokyo, Japan), three trophonts in a total volume of 10 μl of aquarium water were added to triplicate wells. Observations were made every min for the first 15 min, every 5 min for the next 30 min and then every 10 min for the following 180 min. Test plates were again observed at 12 and 24 h. Parasites were observed for hyperactivity (sudden, rapid and random movement), decrease in ciliary movement and finally, death (i.e., cessation of all ciliary movement and lysis). The PCmin (minimum protozoacidal concentration) was defined as the lowest concentration where at least one parasite died and the PC100 (100% protozoacidal concentration) was the lowest concentration at which all parasites died. The experiment was replicated twice.

**Statistical analyses**

All data were analyzed using SAS version 9 (SAS Institute, Cary, NC). The effect of experimental variables (feed rate, tank, sampling day) was tested by analysis of variance (ANOVA). Tukey’s pairwise mean comparison was run to determine if there were significant differences among means of piscidin 4 concentrations, HLP-1 concentrations, antibacterial activity Units, condition factor, weight and length in the fasting, low, moderate and high feeding rate groups. Prior to analysis, all data were tested for normality by determining the heterogeneity of variances and randomness via the linear additive model; when needed, data were log-transformed. Log transformation was required to analyze differences in antibacterial activity in the blood and spleen, HLP-1 levels in the skin and spleen, condition
factor, and weight. Correlations between condition factor, weight, or length and piscidin 4 concentrations, HLP-1 concentrations, or antibacterial activity were calculated. In addition, a partial correlation analysis was performed by removing the effect of feed rate to determine if fish size alone (i.e., condition factor, weight or length) correlated with piscidin 4 concentrations, HLP-1 concentrations, or antibacterial activity. The nonparametric Wilcoxon test was used to determine if there were any significant differences among the feed rate groups in either the number of bacterial colonies in blood or survival time after ich challenge. The Pearson correlation was used to determine if survival time after ich challenge correlated with levels of piscidin 4, HLP-1, or antibacterial activity. Differences were considered significant if the p-value was \( \leq 0.05 \).

RESULTS

Pathology and bacteriology examinations

Fish from all treatment groups did not have any gross lesions; no pathogens were detected in skin or gill biopsies at any time during the experiment. Blood cultures for bacteria were negative in all six fish of each of the four treatment groups sampled on Day 0 and Day 50. On Day 80, some fish in all four groups had a small number of bacteria (1 to 22 colonies). No predominant colony type was observed and in the fish with the largest number of colonies, six colony types were present. The number of colonies in blood did not significantly differ with feed rate and did not correlate with fish size, antibacterial activity Units, piscidin 4 levels or
HLP-1 levels (data not shown).

**Tissue concentrations of piscidin 4**

At Day 0, piscidin 4 concentrations were not significantly different among treatment groups. They were highest in the gill (11 μg/ml) and were much lower in the skin (0.24 μg/ml), blood (0.18 μg/ml) and spleen (0.36 μg/ml) (Fig. 1) (means for all four treatment groups combined) (Fig. 2). At Day 50, piscidin 4 did not vary among groups, but at Day 80, the fasting group had much lower gill piscidin 4 levels (0.84 μg/ml) than the moderate or high feed rate groups (Fig. 1b). The fasting group also had significantly decreased gill piscidin 4 levels at Day 80 compared to Days 0 or 50, while levels in the moderate group increased during this time (to 32.5 μg/ml) (Fig. 1a).

**Tissue concentrations of HLP-1**

At Day 0, histone-like protein-1 concentrations did not vary among treatment groups (Fig. 2) and were high in all tissues including blood (324 μg/ml), gill (140 μg/ml), spleen (132 μg/ml) and skin (36 μg/ml) (means for all four treatment groups combined). At Days 50 and 80, HLP-1 concentrations were not significantly different in any tissues among groups. However, gill HLP-1 concentrations significantly increased in the high (344 μg/ml) and moderate (358 μg/ml) feed rate groups from Day 0 to Day 80 (Fig. 2a). Skin HLP-1 concentrations also significantly increased in the low (70 μg/ml) and moderate (121 μg/ml) feed
rate groups from Day 50 to Day 80 (Fig. 2c).

**Antibacterial activity**

At Day 0 and Day 50, antibacterial activity did not differ among treatment groups for any tissue. No activity was detected in the skin. At Day 50, antibacterial activity was greatest in the gill (10 Units) followed by blood (6.3 Units) and spleen (2 Units) (means for all four treatment groups combined) (Fig. 3). However, on Day 80, gill antibacterial activity in the fasting group was significantly lower (5.3 Units) than that of the high (15 Units) or moderate (15.5 Units) feed rate groups (Fig. 3b). Between Days 0 and 80, spleen antibacterial activity significantly increased in the low feed rate group (to 9.8 Units) (Fig. 3e).

In the AU-PAGE “bug blot”, pooled gill extracts were run from fish sampled at Day 80. Extracts of the high, moderate and low feed rate groups had three distinct clearance zones, corresponding to the antibacterial activity of HLPs, piscidin 4, and piscidins 1-3 (Fig. 4). However, in the fasting group, the clearance zones for piscidin 4 and piscidins 1-3 were almost undetectable and the HLP zone was smaller than all other groups.

**Condition factor, weight and length**

From Day 0 to Day 80, condition factor (K) significantly increased in the moderate and high feed rate groups, remained unchanged in the low feed rate group, and significantly decreased in the fasting group (Fig. 5). By Day 50, K of the fasting group was significantly
lower than that of all other groups and remained significantly lower on Day 80 (Fig. 5b). At Day 50, K of the low and moderate feed rate groups was significantly lower than that of the high feed rate group, but by Day 80, K of the moderate feed rate group was the same as the high feed rate group. At Day 80, the moderate and high feed rate groups had significantly higher K values than the low and fasting feed rate groups.

From Day 0 to Day 80, the weight of the moderate and high feed rate groups significantly increased, while the low feed rate and fasting groups remained unchanged. By Day 50, the fasting group weighed significantly less than the moderate and high feed rate groups (Fig. 5) and the low feed rate group weighed significantly less than the high feed rate group. At Day 80, the low feed rate group and the fasting group weighed significantly less than the moderate and high and feed rate groups (Fig. 5d).

From Days 0 to 80, the mean length of the moderate and high feed rate groups significantly increased. At Days 50 and 80, the length of the fasting and low feed rate groups was also significantly less than that of the high feed rate group (Fig. 5f). As expected, K, weight and length were positively correlated (Table 1).

**Correlations between AMPP levels and either K or fish size**

In many instances, K, weight, or length were correlated with AMPP levels that were measured in various tissues (Tables 2, 3, 4). However, when the effect of feed rate was removed from the analysis (i.e., a partial correlation was performed), there were very few significant correlations (Tables 2, 3, 4).
Response to ich challenge

The three introduced, ich-infected channel catfish died in 24 h (Day 1) and were immediately removed from the challenge tank. On Day 1, flashing was observed in the fasting group. On Day 2, one fish each in the low and moderate feed rate groups was flashing, but all fish in the high feed rate group appeared normal. The fasting group died at a significantly faster rate than the high and low feed rate groups (Fig. 6a). The mortality rate was not significantly different between the low and moderate feed rate groups. The high feed rate group survived significantly longer than any other group. Gill clips, fin clips and skin scrapings of all dead or moribund fish consistently had heavy ich infections. At Day 2, two fish in the fasting group were diagnosed with water mold and ciliate ectoparasite (*Tetrahymena*) infections on the skin. In the fasting group, all eight fish developed concurrent *Tetrahymena* infection and six fish had water mold infection; four fish in both the low and the moderate feed rate groups, as well as two fish in the high feed rate group also had both *Tetrahymena* and water mold infections in addition to ich infections (Fig. 6b).

Antiparasitic activity of piscidin 4

All ich trophonts were lysed within one min of exposure to 100 µg/ml of piscidin 4 and the PC<sub>100</sub> was 50 µg/ml. The PC<sub>min</sub> was 25 µg/ml; at 12.5 µg/ml or less, all trophonts differentiated into tomonts and commenced dividing (formed theronts).
DISCUSSION

The importance of AMPP as an innate defense in fish has been supported by a number of studies that have shown that enhancing AMPP levels can greatly increase disease resistance. Transgenic Japanese medaka (*Oryzias latipes*) that express cecropin, an insect AMP, are more resistant to *Pseudomonas fluorescens* and *Vibrio anguillarum* (Sarmasik et al 2002); similarly, channel catfish (*Ictalurus punctatus*) expressing a cecropin transgene were more resistant to *Edwardsiella ictaluri* and *Flavobacter columnare* (Dunham et al 2002). Grass carp (*Ctenopharyngodon idellus*) expressing human lactoferrin, an antimicrobial protein, were more resistant to *Aeromonas hydrophila* (Weifeng et al 2004) and grass carp hemorrhage virus (Zhong et al 2002), while transgenic zebrafish (*Danio rerio*) expressing chicken lysozyme were more resistant to *Flavobacter columnare* (Yawaza et al 2006).

At the beginning of our experiment (Day 0), piscidin 4 concentrations in the gill averaged 11 µg/ml (Fig. 1) (means for all four treatment groups combined), which is well within the level that is inhibitory to fish pathogens. For example, the minimum inhibitory concentration (MIC) of piscidin 4 for *Streptococcus iniae* is 6.3-12.5 µg/ml, that for *Lactococcus garvieae* is 6.3 µg/ml and the MIC for *Photobacterium damselae* subsp. *piscicida* is as low as 1.5 µg/ml (Noga et al, In Preparation); all are serious pathogens of Morone and/or important aquacultured fish (Austin and Austin 2007). While piscidin 4 concentrations were much lower in blood, spleen and skin (< 0.5 µg/ml, ranging from 0.18 - 0.36 µg/ml, Fig. 1c, 1e, 1g), piscidin 4 might still be an important host defense in the latter tissues since it is present only in certain immune cells (i.e., mainly mast cells [Corrales et al,
Unpublished Data), and thus local tissue concentrations are presumably much higher.

Second, piscidin 4 might cooperate in the killing of pathogens by other AMPPs (i.e., other piscidins and HLPs). Synergism occurs among a number of AMPP (Lauth et al 2005, Patrzykat et al 2001, Kobayashi et al 2001, Yan and Hancock 2001). For example, the linear antimicrobial peptide pleurocidin from flounder synergizes with histone-related peptides in enhancing bacterial killing (Patrzykat et al 2001). Also, many AMPPs possess immunomodulatory functions that are not tied directly to microbicidal action and these functions often occur at much lower concentrations than that needed for direct antimicrobial activity (Brown and Hancock 2006, Beisswenger and Bals 2005). At Day 0, HLP-1 concentrations were high in all tissues (Fig. 2) and well within concentrations that are lethal to pathogens such as the protozoan ectoparasite, Amyloodinium ocellatum (lethal at 12.5 μg/ml) (Noga et al 2001), the bacterium Aeromonas hydrophila (inhibitory at 25 μg/ml), and the water mold Saprolegnia (lethal at 100 μg/ml) (Robinette et al 1998).

In contrast to our findings of cidal levels of AMPP in several tissues, some have recently questioned whether many AMPP in mammals actually function as antibiotics in vivo since their estimated tissue concentrations are often substantially lower than those apparently needed to inhibit pathogens under physiological conditions (Bowdish et al 2005). However, the cidal AMPP concentrations that we measured provide strong evidence that both piscidin 4 and HLP-1 can function as antibiotics in vivo.

At Day 50, piscidin 4 and HLP-1 concentrations were not significantly different among treatment groups in any tissue, nor did they change from Day 0, suggesting that both AMPPs are highly resistant to nutritional stress. Compensatory growth (CG) in hybrid striped
bass has been induced by feeding as little as two days in four weeks (Turano et al 2007) and in Arctic charr (*Salvelinus alpinus*) (Miglavs and Jobling 1989) and rainbow trout (*Onchorynchus mykiss*) (Weatherly and Gill 1981) by fasting fish for up to 60 and 91 days, respectively. Our data suggest that CG regimens that use even severe feed restriction might not affect this host defense, although this might not be true for all fish species or for all feeding regimens (see discussion of channel catfish below). At Day 80, gill piscidin 4 concentrations decreased dramatically (to 0.8 μg/ml). While gill HLP-1 concentrations were unchanged in the fasting group, they increased in the gill of the moderate and high feed rate groups, as well as the skin of the moderate and low feed rate groups. A summary table of all significant changes in AMPP and antibacterial activity levels at various times and tissues is shown in Table 5, and one can observe that the gill is the predominant tissue to monitor changes in AMPP levels and thus determine degree of immunosuppression.

Antibacterial activity (Fig. 3) and qualitative antibiotic levels in AU-PAGE bug blot (Fig. 4) reflected the changes that we measured in the piscidin 4 and HLP-1 ELISAs. Piscidins were undetectable in the fasting group (Fig. 4), including piscidins 1-3 which have potent activity against ich (PC$_{100}$ of 6.3 μg/ml) (Colorni et al, In Press; Ullal et al, Accepted), as well as bacterial pathogens such as *Aeromonas hydrophila* and *Streptococcus iniae* (MIC 0.8-3.1 μg/ml (Silphaduang and Noga 2001).

Evidence for an in vivo direct protective effect of AMPP in our study was also provided by the significant differences in survival time between feed rate groups after ich challenge (Fig. 6a). The low levels of multiple AMPP in the fasted fish probably played a major role in their increased susceptibility to ich challenge (Fig. 6). Since it was not possible
to nonlethally sample the fish immediately before challenge, we compared the mortality rate of ich-challenged fish to the AMPP levels in fish sampled at Day 80. A significant relationship was found between mortality rate and piscidin 4 concentrations, HLP-1 concentrations and antibacterial activity Units in all four tissues except skin HLP-1. The activity of piscidin 4 in vitro was similar to the mean gill piscidin 4 concentrations in the moderate and high feed rate groups (32.5 and 28.6 μg/ml, respectively), and was 30 times more than that measured in the fasting group.

Fasting fish, which had the lowest gill piscidin 4 concentration and antibacterial activity, also had the greatest incidence of water mold and *Tetrahymena* infections (Fig. 6b), two highly opportunistic pathogens that often take advantage of an immunocompromised host (Plouffe et al 2005, Udomkusonsri and Noga 2005). Skin damage by ich provides a portal of entry for such opportunistic agents. While we observed significant differences in mortality rates among various treatment groups, our challenge experiment probably underestimated the differences in susceptibility among the groups. All fish were initially exposed to exactly the same parasite dose, but as the most susceptible individuals (fasting group) became infected, many more parasites were produced, greatly increasing the challenge dose for the remaining, more resistant fish (i.e., high feed rate). If the fish had been in separate aquaria, herd immunity probably would have prolonged the latter group’s survival time. However, we chose this challenge method because it ensured that all fish received exactly the same initial challenge dose.

A few other studies in fish have shown a relationship between feed rate and disease susceptibility. These studies have mainly investigated feed restriction in channel catfish as a
possible means of reducing the severity of ESC (enteric septicemia of catfish; *Edwardsiella ictaluri* infection) outbreaks. Fingerling (22-43 g) channel catfish that were fasted for 6 mo were more susceptible to *E. ictaluri* than fish fed during this period. However, larger channel catfish (420-660 g) that were fasted for this same time period were more resistant than fish either fed during the entire time or only fed in alternate months (Okwoche and Lowell 1997, Kim and Lovell 1995). Fingerling (20 g) channel catfish that were fasted for 28 d were also more susceptible to *E. ictaluri* than fed fish. When unfed, challenged fish were fasted after the challenge, that group had the highest mortality (100%), while the lowest mortality (25.3%) was in fish fed to satiation throughout the experiment (both before and after challenge) (Lim and Klesius 2003). These studies suggest that the deleterious effects of feed rate on disease susceptibility can be size (or age) dependent. While little is known about the relationship between ontogeny and AMPP expression, mRNA expression of the flounder AMPP pleurocidin progressively increases from a very low level in the larval stage to maximum expression in adults (Douglas et al 2001). However, age was not a factor in our study because fish in all treatment groups were identical ages (same hatch day). Whether AMPP are involved in this increased susceptibility to *E. ictaluri* is unknown, but exposure of fingerling (25-45 g) channel catfish to chronic water quality stress for 4 wk causes a significant decrease in HLP-1 (Robinette and Noga 2001).

Feed deprivation increases susceptibility to infection in all animals including fish (Fletcher 1997, Gatlin 2002). However, our study does not prove that depressed AMPP levels are solely responsible for increased susceptibility to ich since a number of other changes in immune function can occur with inadequate feeding. Cell-mediated immunity, phagocyte
function, cytokine production, secretory antibody response, antibody affinity, and the complement system can all be depressed with nutritional deprivation (Chandra and Kumari 1994, Chandra 1997) in mammals, and fasting causes decreased phagocytosis (Okwoche and Lovell 1997), macrophage migration (Lim and Klesius 2003), and leukocyte counts (Lim and Klesius 2003) in fish.

Piscidin 4 appeared to be much more sensitive to feed rate than HLP-1. While the reason for this is uncertain, it is interesting that piscidins appear to be restricted to higher teleosts (i.e., perciform fish) (Silphaduang et al 2006) while HLPs appear to be more widely distributed, being present in relatively primitive (e.g., ictalurids, salmonids) as well as advanced (e.g., Morone) fish (Robinette et al 1998, Noga et al 2001). Thus, HLPs might be a more “ancient” defense than piscidins and if so, might be more resilient to stressors that could decrease its expression.

As expected, condition factor (K), weight and length were all positively correlated with feed rate (Table 1), and all three growth indicators significantly decreased in the fasting and low feed rate groups (Fig. 5). One concern was if changes in AMPP levels were due to the effect of feed rate (treatment effect) or if there were changing with fish size alone. At Day 80, correlations between K and AMPP were only explained by feed rate because there were no correlations were observed after subtracting the effect of treatment (Table 2). Combining all the fish of the experiment, correlations between K and AMPP were also only explained by feed rate (Table 3). Change in AMPP or antibacterial activity throughout the experiment could be affected by the correlation between weight and length and gill HLP-1 and gill antibacterial activity. However, the dramatic downregulation of gill piscidin 4 was only explained by the
treatment effect and not size. Within each feed rate group, there was no pattern between AMMP levels and K, weight or length. And after subtracting the effect of Day (partial correlations) which ignores time, the only was consistency appear to be that bigger fish had less AMPP levels in the spleen (Table 4). The spleen comprises the largest lymphoid tissue in teleosts and by removing foreign agents, it protects fish from blood-borne pathogens (Arma et al 2005). It is also a center for hematopoiesis, erythrocyte storage and antibody production; these different functions can be related to the ratio of red and white pulp (Quesada et al 1990). In our study we did not investigate the effect of feed rate on spleen structure, but one cannot help but speculate whether nutritional plane (feed rate) affects the ratio of red and white pulp and this in turn affects levels of AMPPs that would explain the negative correlation.

In our study, all four feeding groups remained clinically normal (i.e., had no evidence of skin, gill, or blood infection) throughout the entire experiment, before ich challenge. This emphasizes that even severely immunocompromised fish can remain disease-free, so long as pathogens capable of overcoming host defenses are excluded. We have also documented this previously in a model of acute stress, showing that maintaining fish in a specific pathogen-free environment prevents the development of secondary infection after severe, acute immunocompromise of the skin (Udomkusonsri and Noga 2005).

To our knowledge, our study is the first to examine the influence of feeding rate and thus overall nutritional status on the expression of antimicrobial polypeptides in any animal, and our data strongly suggest that nutritional status can significantly affect AMPP expression (Figs. 1-4). Since fasting reduces all nutrients, we do not know which specific nutrients are causing the decreased AMPP levels at Day 80. However, inadequate protein level or lipid
content can affect immune function and increase susceptibility to infections (Kiron et al 1995a,b; Miles and Calder 1998, Sheldon and Blazer 1991, Yin and Shiau 2003, Montero et al 1998). Little is known about how specific nutrients affect expression of antimicrobial peptides. The few studies performed have focused on how certain nutrients may induce upregulation, rather than constitutive expression. Butyrate, a short chain fatty acid, induced expression of the cathelicidin LL-37 in human colon epithelial cells (Schauber et al 2003). Vitamin D₃ induces LL-37 expression in cultured human keratinocytes (Schauber et al 2007) and myeloid cells (Gombart et al 2005), as well as defensin β2 expression in cultured human keratinocytes (Wang et al 2004). The essential amino acids isolucine (Fehlbaum et al 2000) and arginine (Sherman et al 2006), as well as the serum protein albumin (Sherman et al 2006), induce β defensin expression in cultured bovine kidney epithelial cells and human colon carcinoma cells, respectively.

While feed regimens have been focused on maximizing growth rate in aquaculture, there is increasing recognition of the need to establish feed regimes that allow optimal immunocompetence (Fletcher 1997). Here we demonstrated that feed rate can adversely affect AMPP expression and also affect resistance to infection. Future studies are needed to determine the precise relationship between feed regimen such as one leading to compensatory growth and AMPP expression.
ACKNOWLEDGEMENTS

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Table 1. Relationship between condition factor, fish weight and fish length in each treatment group. (+) positive correlation; (-) negative correlation (Pearson, p < 0.05). N = 27 per treatment group.

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Table 2. Significant (Pearson, p < 0.05) correlations (not accounting for feed rate) or partial correlations (removing the effect of feed rate) between either condition factor, fish weight, or fish length, and piscidin 4 concentrations, HLP-1 concentrations, or antibacterial activity (ABA) in the tissues. For each tissue, the Day 80 data for all four treatment groups were combined (N = 36). Only significant correlations are shown. (+) positive correlation; (-) negative correlation.

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Table 3. Significant (Pearson, p < 0.05) correlations (not accounting for feed rate) or partial correlations (removing the effect of feed rate) between either condition factor, fish weight, or fish length, and piscidin 4 concentrations, HLP-1 concentrations, or antibacterial activity (ABA) in the tissues. For each tissue, the data for all three sampling days and all four treatment groups were combined (N = 108). Only significant correlations are shown. (+) positive correlation; (-) negative correlation.

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Table 4. Significant (Pearson, p < 0.05) correlations (not accounting for day effect) or partial correlations (removing the effect of day) between either condition factor, fish weight, or fish length, and piscidin 4 concentrations, HLP-1 concentrations, or antibacterial activity (ABA) in the tissues. For each tissue, the data for all three sampling periods (Days 0, 50, 80) of each treatment group were combined (N = 27). Only significant partial correlations are shown. (+) positive correlation; (-) negative correlation.

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Table 5. Summary table of significant changes (indicated by *) in levels of piscidin 4, HLP-1 and antibacterial activity (ABA) at various time points and tissues. H = high feed rate group, M = moderate feed rate group, L = low feed rate group, F = fasting feed rate group.

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<th>Bar graphs (With a sampling day among treatment groups)</th>
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<td>Day 80 = H, M, and L * from F</td>
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<tr>
<td>HLP-1 Gill</td>
<td>H = 0 and 80* M = 0 and 80* L = 0 and 80*</td>
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<td>Skin</td>
<td>M = 0 and 80* also 50 and 80* L = 50 and 80*</td>
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<tr>
<td>ABA Gill</td>
<td>L = 0 and 80*</td>
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<tr>
<td>Spleen</td>
<td>L = 0 and 80*</td>
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168
Figure 1. Piscidin 4 concentrations (mean ± SE) in the gill, skin, blood and spleen of hybrid striped bass fed to satiation (high), half satiation (moderate), 0.6% BW three times a week (low), or not fed (fasting). In the line graphs (a,c,e,g), different letters within a treatment group indicate significant differences among sampling days (ANOVA, p <0.05). In the bar graphs (b,d,f,h), different letters within a sampling day indicate significant differences among treatment groups (p <0.05). N = 9 fish per treatment group per sampling time.
Figure 2. Histone-like protein-1 (HLP-1) concentrations (mean ± SE) in the gill, skin, blood and spleen of hybrid striped bass fed to satiation (high), half satiation (moderate), 0.6% BW three times a week (low), or not fed (fasting). In the line graphs (a,c,e,g), different letters within a treatment group indicate significant differences among sampling days (ANOVA, p <0.05). In the bar graphs (b,d,f,h), different letters within a sampling day indicate significant differences among treatment groups (ANOVA, p <0.05). N = 9 fish per treatment group per sampling time.
Figure 3. Antibacterial activity Units (mean ± SE) in the gill, blood and spleen of hybrid striped bass fed to satiation (high), half satiation (moderate), 0.6% BW three times a week (low), or not fed (fasting). In the line graphs (a,c,e), different letters within a treatment group indicate significant differences among sampling days (ANOVA, p <0.05). In the bar graphs (b,d,f), different letters within a sampling day indicate significant differences among treatment groups (ANOVA, p <0.05). N = 9 fish per treatment group per sampling time.
Figure 4. Qualitative antibacterial activity by the ‘bug blot’ assay in the gill of hybrid striped bass fed to satiation (high), half satiation (moderate), 0.6% BW three times a week (low), or not fed (fasting) on Day 80. Antibiotic standards included calf histone H2B (89% homologous to HLP-1), piscidin 4 (P4) and piscidin 1 (P1). Note that piscidin 1, 2 and 3 co-migrate on AU-PAGE. Clearance zones indicated antibacterial activity against *E. coli*. Encircled is activity in the high and fasting feed rates groups. HD80 = high feed rate group on day 80, MD80 = moderate feed rate group on day 80, LD80 = low feed rate group on day 80, FD80 = fasting feed rate group on day 80.
Figure 5. Condition factor (K), weight (g) and length (mm) of hybrid striped bass fed to satiation (high), half satiation (moderate), 0.6% BW three times a week (low), or not fed (fasting). In the line graphs (a,c,d), different letters within a treatment group indicate significant differences among sampling days (ANOVA, p <0.05). In the bar graphs (b,d,f), different letters within a sampling day indicate significant differences among treatment groups (ANOVA, p <0.05). N = 9 fish per treatment group per sampling time.
Figure 6. Mortality rates and secondary infection incidence in ich-challenged hybrid striped bass. (A) Mortality rates of fish fed to satiation (high), half satiation (moderate), 0.6% BW three times a week (low), or not fed (fasting) after exposure to ich. Lines with different lower case letters are significantly different (ANOVA, p < 0.05). N = 8 fish per group. (B) Number of fish that developed concurrent *Tetrahymena* and/or water mold infections.
A) 

Days post-infection

% mortality

0  25  50  75  100

0  2  4  6  8  10

High Moderate Low Fasting

A, B

B) 

No. of fish with concurrent infections

0  1  2  3  4  5  6  7  8

High Moderate Low Fasting
CHAPTER V

LATERAL LINE DEPIGMENTATION (LLD) IN CHANNEL CATFISH, *ICTALURUS PUNCTATUS* (RAFINESQUE)
Lateral line depigmentation (LLD) in channel catfish, *Ictalurus punctatus* (Rafinesque).

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**Short running title** Lateral line depigmentation

**Keywords:** head and lateral line erosion, lateral line canal, epidermis, depigmentation, melanocytes, antibacterial activity
ABSTRACT

Head and lateral line erosion (HLLE) is a chronic dermatopathy affecting a number of fish that presents as depigmented skin along the lateral line system of the trunk and head. We present microbiological, immunological and histopathological features of this lesion in channel catfish, *Ictalurus punctatus* (Rafinesque), that developed after exposure to a chronic nutritional stress. Depigmentation was limited to skin that was adjacent to the lateral line. The epidermis of affected fish was thin and reduced to a one-cell-thick layer over the lateral line. Melanocytes were depleted at the dermo-epidermal junction and formed aggregates in the epidermis. Innate immunity was weaker in affected fish than that previously measured in well-fed channel catfish. Because the pathology and apparent etiology of HLLE described in various fish species are highly variable, HLLE appears to be a clinical sign, rather than a disease or syndrome. Thus, we propose that this clinical sign be referred to as lateral line depigmentation (LLD), because this description more accurately encompasses all cases of this presentation reported in fish. Since nutritional requirements of channel catfish and lateral line neuroanatomy are well-known, the ability to reproducibly induce LLD in this species could provide a useful model for understanding its pathogenesis.
INTRODUCTION

Aquarium fish are commonly affected by a chronic dermatological condition that has been referred to as head and lateral line erosion (HLLE) or “hole-in-the-head”. It is especially common in ornamental marine fish of the families Acanthuridae and Pomacentridae and in tropical freshwater fish of the families Anabantidae, Belontidae and Cichlidae (Becker 1977; Ferguson & Moccia 1980; Gratzek 1988). Grossly similar lesions have also been observed in food fish such as Murray cod, *Maccullochella peelii peelii* (Mitchell) (Baily, Bretherton, Gavine, Ferguson & Turnbull 2005) and Atlantic cod, *Gadus morhua* (Linnaeus) (Möller & Anders 1986). Fish typically present with skin depigmentation along the lateral line canal and the cephalic canals where the neuromasts lay. While this clinical presentation appears to be very prevalent in certain fish, aside from cursory gross descriptions, there is very little information detailing the characteristics of the lesions and their possible etiology.

Here, we report microbiological, immunological and histopathological features of this dermatopathy in the important foodfish channel catfish, *Ictalurus punctatus* (Rafinesque), after exposure to a chronic nutritional stress. To our knowledge this is the first time that it has been reported in the family Ictaluridae.
MATERIALS AND METHODS

**Fish maintenance**

Clinically normal adult channel catfish were kept in a 1200 L aquarium having flow-through fresh well water (18 - 23 °C). Fish were fed a maintenance diet (0.6% BW thrice weekly) of a commercial feed (Zeigler Bros, Inc., Gardners, PA) containing 40% crude protein, 10% crude fat, and 4% crude fiber for over 12 mo. Three fish were then transferred to another 1200 L aquarium having the same conditions and feeding was halted. After fasting the fish for 12 mo, they were sampled. Three clinically normal fish from a 544 L closed freshwater system aquarium at 17°C that had been fed at 0.6% BW daily for 12 mo were also sampled at the same time. During the 12 mo trial, total ammonia nitrogen (TAN) and pH were tested daily and nitrite was tested weekly using commercial colorimetric assays (Aquarium Pharmaceuticals, Inc., Chalfont, PA); parameters were always within normal limits.

**Sample collection**

Each fish was individually sedated with buffered tricane, measured and weighed to calculate Fulton’s condition factor, \( K = \frac{W \times 10^5}{L^3} \); \( W \) = weight in g, \( L \) = total body length in mm). Then, skin and gill biopsies were collected for microscopic examination (wet mounts). To sample for antibacterial activity, skin and gill were collected in 1% acetic acid (1:4 dilution of tissue) as described previously (Noga, Fan, & Silphaduang 2002). After boiling for 5 min, each sample was homogenized and then centrifuged at 14,000 x g for 10
min at 4°C. The extract (supernatant) was used to measure antibacterial activity.

The fish was then euthanized and samples for bacterial culture were taken from two areas over the lateral line canal and kidney with a sterile swab (Mini-tip Culturette, Becton Dickinson, Franklin Lakes, NJ). Each sample was inoculated onto Columbia blood agar and incubated at room temperature; plates were observed for 14 d. The fish was then necropsied and wet mounts of stomach, anterior intestine and posterior intestine were examined. Skin, gill, heart, liver, spleen, anterior kidney, posterior kidney, stomach, anterior intestine and posterior intestine were fixed in 10% neutral-buffered formalin (NBF). Organs were embedded in paraffin and 5 µm sections were stained with hematoxylin and eosin (H&E). Skin was also fixed in 4F:1G fixative (McDowell's and Trump's) and stored at 4°C prior to electron microscopy. For transmission electron microscopy (TEM), tissues were trimmed to 1 mm thickness and post-fixed in 1% osmium tetroxide for 1 h and dehydrated in an ethanolic series culminating in two changes of 100% acetone. Tissues were then infiltrated with a 50:50 mix of acetone and spurr resin followed by 2 changes of 100% spurr resin. Tissues were then placed into molds and polymerized in fresh spurr. Semi-thin (0.5 µm) sections were stained with 1% toluidine blue-O in 1% sodium borate. Ultrathin (90 nm) sections were stained with methanolic uranyl acetate followed by lead citrate and examined with a transmission electron microscope (FEICO 208S, FEICO Morgagni, Hillsboro, Oregon). For scanning electron microscopy (SEM), tissues were rinsed in 0.1 M sodium phosphate buffer (pH 7.2), dehydrated in a graded ethanol series to 100% ethanol, critical point dried (Ladd Critical Point Dryer, Ladd Research Co., Burlington, VT), mounted on specimen stubs with colloidal silver, sputter-coated with gold-palladium and examined with a
scanning electron microscope (Model JSM-6360LV, JEOL Lt., Tokyo, Japan).

**Antibacterial activity**

Antibacterial activity was measured using the radial diffusion assay (Noga et al 2002). Briefly, 2 mm diameter wells were punched in a petri dish having a suspension of *Escherichia coli* D31 in agarose and 3 μl of tissue extract was pipetted into duplicate wells. The plate was then incubated at 37°C for 18 h, at which time the diameter of each clearing zone was measured with a vernier caliper to the nearest 0.1 mm. Clearing zone diameters were then converted to units of activity by reference to a standard curve prepared by serially diluting calf histone H2B (Roche Diagnostics Co., Basel, Switzerland). The 1000 μg/ml concentration of calf histone H2B was considered to be 100 Units of activity. Equivalents of HLP-1 were calculated by log-transforming the clearing zone diameters and their values in log units were then determined from the H2B standard curve. Values were then converted to arithmetic units of activity (Noga et al 2002). Units of activity were then multiplied by 4 to account for the four-fold dilution factor of tissue with 1% acetic acid during tissue collection. A t-test analysis was performed for statistical comparison using SAS version 9 (SAS Institute, Cary, NC). The significance level was considered to be p < 0.05.

Qualitative analysis of endogenous antibiotic expression was performed using the “bug blot” (Harwig Chen, Park & Lehrer 1993). Tissue extracts were electrophoresed on acid-urea polyacrylamide gels (AU-PAGE) using the Mini-Protean II (BioRad) electrophoresis system according to manufacturer’s instructions. Briefly, 30 μl samples were run in duplicate in the AU-PAGE. After electrophoresis, the gel was divided in half. One half
was stained with Coomassie Blue R-250 (Bio-Rad Laboratories, Inc., Hercules, CA) and the other half was washed and “blotted” by overlaying it onto an *E. coli* D31 agarose plate. After incubation at 37°C for 18-24 h, clearing zones in the agarose in lanes with tissue extracts were compared with polypeptide antibiotic standards as well as the stained half of the gel. Standards used included calf histone H2B, piscidin 1 and piscidin 4.

RESULTS

After 12 months of fasting, all three fish developed skin lesions that presented as multiple, depigmented, depressed foci that surrounded each neuromast pit (Figs 1a, b, c). Adjacent depigmented foci extended along the skin over the lateral line from the operculum to the caudal fin. In the most severely affected individual (Fish #1), the depigmented circular pits formed a continuous depigmented line (Fig. 1b), while in the moderate (Fish #2) and least affected fish (Fish #3), individual neuromast pits were distinguishable. The skin overlying the vertical row of neuromasts at the base of the caudal fin (Fig. 1b) was also depigmented in Fish #1. This vertical row is one of the four groups that comprise the superficial neuromasts of the trunk and is part of the lateral line canal (Northcutt et al 2000). Focal skin depigmentation was also present on the head (Fig. 1c), where it was unclear whether depigmentation affected the skin overlying the cephalic canals, the superficial neuromasts, the electrorceptors, or all three.

In all three affected fish, there was a discrete zone of epithelial loss adjacent to the neuromast pit (Figs. 1d-f, and Figs. 2a-d). The epidermis of all three affected fish was very
thin and often reduced to a one-cell-thick layer either over (the most affected fish) or proximal to but not directly above (the least and moderately affected fish, Figs. 1e, 2b) lateral line. The connective tissue appeared loose, especially in the most affected fish. Unlike in the clinically normal fish, where melanocytes formed a single layer of flattened cells mainly at the dermo-epidermal junction (Fig. 1d), melanocytes in affected fish were depleted from the dermo-epidermal junction and instead were rounded and formed aggregates in the epidermis (Figs. 1e, 1f). Histological sections of all other organs were unremarkable.

Wet mounts of skin, gill, stomach and intestine of affected fish had no parasites. Furthermore, no pathogens were seen in the skin lesions via histology, SEM, or TEM. Two of the four clinically normal fish had a very mild monogenean infestation (one in each fish on the gill biopsy; and one fish with one on the skin biopsy). Skin cultures of lesions were unremarkable; colony numbers in each sample from clinically normal fish (ranging from 1-103 colonies, 8 samples) and affected fish (ranging from 0-67 colonies, 7 samples) were similarly low, and skin samples from the most severely affected fish had the fewest colonies (0-2 colonies, 3 samples). One of the affected fish had a single bacterial colony from kidney culture.

The smallest fish (#1, 215 g, K=0.72) was most severely affected, the largest fish (#2, 602 g, K=0.71) was moderately affected and the fish weighing 390 g (#3, K=0.74) was least affected. While weight and length were not significantly different between the affected group (402 ± 194 g and 375 ± 65 mm, N = 3) and clinically normal group (553 ± 154 g and 378 ± 28 mm, N = 3), the condition factor was significantly lower in the affected group (0.72 ± 0.02 versus 1.01 ± 0.1).
Via radial diffusion assay, no antibacterial activity was detected in the skin extract of any affected fish, but activity was also very low (1 ± 1 Unit, equivalent to 30.8 ± 36 μg/ml of HLP-1, N = 3) in the clinically normal fish; mean values were not significantly different (p>0.05). Gill antibacterial activity was significantly lower in the affected fish (5 ± 3 Units, equivalent to 195 ± 115 μg/ml of HLP-1, N = 3) than in clinically normal fish (29 ± 6 Units, equivalent to 1145 ± 240 μg/ml of HLP-1, N = 3) (t-test 0.016). Via bug-blot, antibacterial activity was present in both skin and gill tissues of all affected and normal fish, but affected fish had consistently weaker activity than clinically normal fish. Activity was only present in the zone corresponding to histone-like proteins (HLPs). The Coomassie-stained gel confirmed the presence of bands corresponding to the antibacterial activity zone of HLPs in the bug-blot overlay.

DISCUSSION

The key diagnostic feature exhibited by channel catfish in our study was bilateral, focal, skin depigmentation over the lateral line. This gross depigmentation was due to the loss of expanded (spreading) melanocytes at the dermo-epidermal junction. Spreading of melanocytes, with formation of long cell processes, is responsible for the gross black appearance of skin (Kawauchi, Kawazoe, Tsubokawa, Kishida & Baker 1983). Contracted melanocytes, such as those present in the epidermis of affected fish, produces a blanched (“depigmented”) gross appearance. The melanocytes of affected channel catfish also formed aggregates in the epidermis (Fig. 1e, 1f). Melanocytes in similarly affected palette tang were also depleted in the dermo-epidermal junction and formed aggregates of contracted cells in
the epidermis (Blasiola 1989). Aggregates of other melanin-containing cells (melano-
macrophages centres) are common in haemopoietic tissues such as the kidney, spleen, and
hepatopancreas (Agius & Roberts 2003), but they have never been reported in the skin.
However, we found no melano-macrophages centers or any other pathology in the tissues of
affected fish.

The gross lesion we observed in channel catfish is identical to that observed in a
“disease” that has been most often referred to as “head and lateral line erosion” or HLLE. HLLE
most commonly affects certain tropical freshwater and marine aquarium fish of the families
Anabantidae, Belontidae, Cichlidae, Acanthuridae and Pomacentridae (Gratzek 1988), but a
grossly identical lesion has also been observed in some food fish (Baily et al 2005; Möller &
Anders 1986). Despite the widespread prevalence of this “disease”, aside from our study,
there have been only two published histological descriptions of HLLE. Bailey et al (2005)
described a similar gross lesion in Murray cod, (which they termed “chronic erosive
dermatopathy” or CED), where the skin over the sensory canals of the head and trunk was
depigmented. The gross lesion in palette tang, Paracanthurus hepatus (Lineaus), was also
identical (Blasiola 1989). Numerous descriptions of this same gross lesion have also been
reported anecdotally elsewhere (Paull & Matthews 2001; Varner & Lewis 1991).

While the gross HLLE lesion is apparently identical in all these fish, the pathogenesis
of the lesion can be quite different. Blasiola (1989) described very thin epidermis in HLLE-
affected palette tang, with the relatively few inflammatory cells in the epidermis being
present only in advanced cases. The severe epithelial loss, melanocyte aggregation in the
epidermis, and lack of inflammation were similar to our clinical findings in channel catfish.
However, in Murray cod, the epithelium was not thin, but rather was severely hyperplastic, with inflammation and necrosis overlying the sensory canals (Baily et al 2005). The hyperplasia appeared to originate within and adjacent to the canal and then radiate outward towards the epithelium (J. Baily, pers. commun.). The variable pathology, along with the diversity of risk factors associated with the development of the HLLE lesion (see below), indicate that the clinical signs presented by these various fish are probably not due to a single disease. Thus, we feel that it would be more appropriate to refer to fish exhibiting HLLE as instead being affected by lateral line depigmentation (LLD), since this is a more accurate description that encompasses the gross presentation of all affected fish. Thus, LLD is a clinical sign in response to any of a number of stressors that can lead to this gross lesion and is analogous to a skin ulcer (i.e., a gross manifestation of a general host response) rather than being a specific disease or even a syndrome.

Despite the high prevalence of LLD in aquarium fish, the etiology(ies) of the lesion remains unknown. Numerous accounts in the aquarium literature attribute LLD to hexamitid flagellates (e.g. *Hexamita, Spironucleus*) (Bassleer 1983). While *Spironucleus vortens* has been isolated from LLD lesions and from the intestine of discus, *Symphysodon discus* Heckel, and angelfish, *Pterophyllum scalare* (Lichtenstein) (Paull & Mathews 2001), hexamitids are common gut parasites of many fish species that are affected by LLD (e.g., cichlids and anabantids) and can spread to other tissues from the gut (Becker 1977; Ferguson & Moccia 1980). The only other pathogen that has been associated with LLD is a reovirus-like agent isolated from a liver-spleen homogenate of a single moribund marine angelfish, *Pomacanthurus semicirculatus* Lacepède (Varner & Lewis 1991). However, there was no
evidence presented that the virus caused LLD. In addition, neither we, Blasiola (1989), nor Baily et al (2005) found any evidence that an infectious agent was responsible for the LLD lesion.

In addition to our induction of LLD by fasting channel catfish, inadequate nutrition has been associated with other LLD lesions. Three palette tangs fed a commercial dry (flake) diet developed LLD after three wk, while three control fish fed the same diet but allowed to graze on benthic algae remained healthy (Blasiola 1989). Supplementing the diet of the three affected fish with vitamin C and green algae began to heal the lesions within 10 d. Induction of LLD was also linked to poor water quality. Bailey et al (2005) induced LLD in Murray cod, a freshwater fish native to Australia’s Murray-Darling River system, when fish were maintained in certain sources of groundwater. Lesions spontaneously resolved when fish were transferred to river water.

While the pathogenesis of LLD is poorly understood, the anatomical distribution of the lesion (i.e., intimate association with the lateral line) suggests that it might be linked to changes in the function of this neurosensory organ. The lateral line system is composed of mechanoreceptive neuromasts and electroreceptors (Webb 1989; Northcutt, Holmes & Albert 2000; Northcutt 2003). The neuromasts are either superficial (i.e., solitary) or housed in a canal (consisting of a single bilateral trunk canal and five bilateral cephalic canals). The trunk canal runs from posterior to the operculum to the caudal peduncle. The neuromasts detect water movement (Webb 1989; Northcutt 2003) and thus participate in predator-prey interactions, rheotaxis, and obstacle avoidance (Bleckmann 1993; Faucher, Fichet, Miramand & Lagardère 2006). Electroreceptors, which are also solitary, detect electric fields, allowing
intraspecies and interspecies communication. Although electroreceptors disappeared with the evolution of most teleosts, they later re-appeared in channel catfish (Northcutt, Holmes & Albert 2000).

Aminoglycoside antibiotics (e.g., gentamicin or streptomycin, also known to be ototoxic) or cadmium exposure impair neuromast function, preventing detection of hydrodynamic stimulation, which in turn alters swimming behavior (Faucher et al 2006). Since no behavioral changes have ever been reported in LLD-affected fish, this suggests that LLD neuromast and electroreceptor function in terms of their neurosensory and electoreceptive roles, respectively, are not impaired, although functional assessment of the neuromasts and electroreceptors in LLD-affected fish is needed to confirm this. Conversely, there might be a change in intercellular signaling from neuromasts or nervous tissue to epidermis. In mammals, nerve endings innervate the cutaneous epithelium and make synaptic contact with epidermal melanocytes (Hara, Toyoda, Yaar, Bhawan, Avila, Penner, & Gilchrest 1996). Epidermal cells of mice lacking neurohormonal stimulation have abnormally thin epidermis (Hsieh, Lin, Chiang, Huang, Ko, Chang & Chen 1997). And neurohormonal stimulation induces melanocyte proliferation (Hara et al 1996).

Thus, a decrease in neuronal signaling between skin and the adjacent neuromasts or nervous tissue might impair normal epidermal function. This suggests that the decreased epidermal thickness in LLD might result from atrophy rather than erosion. Epidermal erosion is defined as the loss of the epithelium down to but not including the basement membrane. When there is loss of the basement membrane, the skin is ulcerated. In some diseases, such as Cushing’s syndrome (due to elevated corticosteroid hormone levels), the skin in mammals
is thinner than normal but this is not due to erosion.

Fish can withstand long periods of starvation; for example, Atlantic cod, *Gadus morhua* L., can survive more than 195 d, bowfin, *Amia calva* L., survive for 20 mo, and common eel, *Anguilla anguilla* L., can survive for 3 y without feed (Love 1970). Fish are also now being routinely fasted for long periods of time as part of research to enhance compensatory growth (Ali et al 2003). Fasting fish for extended periods (up to 6 mo), has even been practiced routinely in commercial aquaculture operations (Kim & Lovell 1995). However, inadequate nutrition has deleterious effects on immune function (Chandra & Kumari 1994; Calder & Jackson 2000) and increases susceptibility of fish to disease (Fletcher 1997; Gatlin 2002). For example, channel catfish fasted for 6 mo had impaired phagocyte function (Okwoche & Lovell 1997). However, these same fish had increased resistance to infectious challenge compared to fish that were fed during this time (Okwoche & Lovell 1997), so the effects of feed deprivation on immune function in fish are not entirely clear.

The channel catfish that developed LLD were part of a study intended to induce low levels of polypeptide antibiotic defenses in skin and gill so that these tissue extracts could be used as controls for the development of improved tests to measure histone-like proteins. We had previously found that fasting was the most reliable means to induce low HLP levels in tissue, and thus used the least number of fish possible to obtain this extract. Histone-like proteins (HLPs), which are highly homologous to core nuclear histones, are the major polypeptide antibiotic defenses in channel catfish skin and gill (Robinette, Wada, Arroll, Levy, Miller & Noga 1998). Histone-like protein-1 (HLP-1), the predominant HLP in skin
and gill, is highly inhibitory to important fish pathogens. In vitro, as little as 12.5 μg/ml of HLP-1 kills over 80% of the highly lethal ectoparasite, *Amyloodinium ocellatum*, and at 100 μg/ml, it is 100% lethal (Noga, Fan & Silphaduang 2001). HLP-1 is also lethal to the infective zoospores of the water mold *Saprolegnia* at 100 μg/ml and is inhibitory to the bacterium *Aeromonas hydrophila* at as low as 25 μg/ml (Robinette et al 1998).

Preliminary observations had suggested that feed deprivation induced low HLP levels in fish (J Corrales and E Noga, Unpublished Data). As expected, the bug blot confirmed that HLPs were the only polypeptide antibiotic present in skin and gill extracts. Gill HLPs were significantly decreased in the fasted (LLD-affected) channel catfish compared to fed (clinically normal) fish (equivalent to 956 μg of calf H2B/ml vs. 195 μg/ml in affected fish). Gill HLP levels in healthy adult channel catfish are typically 800-1200 μg/ml (A Ullal, D Robinette and E Noga, Unpublished Data). While gill HLP levels in the clinically normal fish were as expected, skin HLP levels were very low in both LLD-affected and apparently healthy groups (0 μg/ml and 30.8 ± 36 μg/ml, respectively), and much lower than previously measured in healthy channel catfish (240-320 μg/ml) (A Ullal, D Robinette and E Noga, Unpublished Data). The clinically normal fish might have had abnormally low HLP levels since they were fed a maintenance (low feed rate) diet rather than a higher feeding rate. Although gill HLP levels of affected fish were within the cidal range for many pathogens, the decreased expression would be expected to make them more susceptible to infection if they were exposed to a pathogen at an appropriate concentration.

Despite the low HLP levels in skin of the healthy fish, there was a very mild, chronic monogenean infestation present on at least one fish. If HLP is important in protecting against
monogenean infestations, one would expect a more severe infestation in fish having depressed HLP levels. While the depressed levels still might be sufficient to inhibit the parasite, it also might be possible that other host defenses might be more important in protecting channel catfish against this parasite. The relationship, if any, between immune suppression and LLD is uncertain. While the mean condition factor of LLD-affected fish was abnormally low, that of the control fish was similar to what has been reported for channel catfish (Perkins, Griffin, Hobbs, Gollon, Woldford & Schlenk 1997). Thus, condition factor did not reflect the lower innate immune status of the clinically normal fish.

In summary, we have induced LLD (also known as HLLE) in channel catfish after feed deprivation. Since channel catfish is a readily available, relatively inexpensive species whose nutritional requirements, lateral line neuroanatomy and genome are well-known compared to other fish affected by LLD, the ability to induce LLD in this species could provide a useful model for understanding the pathogenesis of this common yet enigmatic clinical presentation.

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LITERATURE CITED


response behaviour of the sea bass (\textit{Dicentrarchus labrax} L.; Teleostei, Moronidae).

\textit{Aquatic Toxicology} \textbf{76}, 278-294.


Figure. 1. Gross and histopathologic LLD (HLLE) lesions in channel catfish. A = alarm cells, M = melanocytes, E = epidermis, D = dermis, C = lateral line canal. (a) Fish with moderate LLD showing depigmentation along the lateral line of the flank (arrows). Bar = 6.5 cm. (b) Fish with severe LLD showing wide, overlapping foci of depigmentation overlying the lateral line of the flank (arrow) and the caudal peduncle (arrowhead). Bar = 6 cm. (c) Fish with LLD depigmentation over cephalic neuromast pits (arrows). Bar = 5 cm. (d) Normal skin over the lateral line canal. Melanocytes (M) located at the dermo-epidermal junction. Bar = 250 μm. (e) Skin above the lateral line canal of LLD-affected fish. Inset, representative location of Fig.1f showing thinning of epidermis. Bar = 250 μm. (f) Periphery of an LLD lesion showing thin epidermis and melanocyte aggregation. Bar = 250 μm.
Figure 2. Electron microscopy of HLLD lesions in channel catfish. (a) SEM of normal skin. P = sensory pit. Bar = 500 μm. (b) SEM of skin with HLLD. Note the prominent depression (arrowheads) surrounding the sensory pit Bar = 500 μm. (c) TEM of the epidermis (E) and dermis (D) of normal skin. Bar = 3 μm. (d) TEM of epidermis and dermis of skin with HLLD. Note that the epidermis (E) is reduced to a single cell layer and melanocytes (M) are aggregated in the epithelium. Bar = 3 μm.