PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF *EDWARDSIELLA* SPECIES BACTERIA AND THEIR PATHOLOGIC AND IMMUNOLOGIC MANIFESTATIONS IN TELEOST FISH

by

ABIGAIL ROSE ARMWOOD

(Under the Direction of Al C. Camus and Matt J. Griffin)

ABSTRACT

The bacterial genus *Edwardsiella* affects fish species worldwide and contains two of the most important pathogens in catfish aquaculture. With the recent reclassification of the genus into five separate species, clarification is needed on pathogenicity, host-microbe relationships, and effectivity of preventative tools. Vaccination of cultured catfish with an orally delivered *Edwardsiella ictaluri* live-attenuated vaccine is a promising disease prevention method. An added benefit is the *E. ictaluri* vaccine also offers some cross-protection against a second *Edwardsiella* species of importance, the newly identified *Edwardsiella piscicida.* Studies on host-pathogen interactions and immune responses to the vaccine, as well as natural *Edwardsiella* spp. infections in catfish, are in their infancy. Historical overlap between *Edwardsiella tarda, Edwardsiella anguillarum*, and *E. piscicida* has obscured accurate documentation of pathogenicity to different catfish varieties and associated disease processes, with *E. anguillarum* and *E. tarda* seemingly causing minimal disease in cultured catfish. Though less significant in catfish aquaculture, *E. anguillarum*, the most recently recognized *Edwardsiella* sp., is an emerging fish pathogen with an expanding host range, particularly in cultured tilapia.

Information on *E. anguillarum*, most notably strategies for prevention and treatment, is limited due to previous mistaken identity and failure to recognize *E. anguillarum* as a discrete taxon separate from *E. tarda*. The overall objectives of the proposed research include: 1) characterizing intraspecific variability of *E. anguillarum* isolates in various fish species from different geographic locations, 2) comparing mortality and characterizing pathology associated with the recently reclassified *Edwardsiella* spp. (*E. tarda, E. piscicida, E. anguillarum*) in channel, blue, and hybrid catfish, and, lastly, comparing the susceptibility, immune responses, and pathologic changes between vaccinated and non-vaccinated, channel and hybrid catfish to experimental challenge with 3) *E. ictaluri* and 4) *E. piscicida*.

INDEX WORDS: Catfish, *Edwardsiella anguillarum, Edwardsiella ictaluri, Edwardsiella piscicida, Edwardsiella tarda*, Immune Response, Pathology, Vaccination

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DEDICATION

I dedicate this dissertation to my family including my husband Brandon Armwood, my son Aiden Armwood, my sister Stephanie Wisnet, my mother Deborah Wisnet-Hunter, my stepfather Jeff Hunter, my father Joseph Wisnet, and my stepmother Jan Wisnet.

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TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Genus *Edwardsiella* Taxonomy

The *Edwardsiella* are a genus of facultative intracellular, gram-negative, rod-shaped bacteria first identified in the 1960s. Originally placed in the family Enterobacteriacae along with other enteric bacteria, including *Escherichia coli, Salmonella* sp., *Klebsiella* sp., and *Shigella* sp., the genus was recently reclassified to the family Hafniaceae along with the genera *Hafnia* and *Obesumbacterium* (Adeolu et al., 2016; Ewing et al., 1965). *Edwardsiella tarda* was the first recognized species, isolated from the feces, wounds, blood, and urine of humans and animals (Ewing et al., 1965). Beginning in 1969, disease outbreaks in farm-raised channel catfish (*Ictalurus punctatus*) in Arkansas, Mississippi, Louisiana, and Texas were attributed to *E. tarda* (Meyer & Bullock, 1973), which is now a well-known, globally distributed pathogen of various fish species (Griffin et al., 2017). *Edwardsiella hoshinae*, the second species identified, was isolated as a commensal from birds and reptiles. It has been reported from water but is not associated with disease in fish (Grimont et al., 1980). A third species, *Edwardsiella ictaluri*, was initially isolated from diseased cultured catfish beginning in 1976 and is historically considered the causative agent of enteric septicemia of catfish (ESC) (Hawke et al., 1981).

The genus remained unchanged until advances in molecular techniques revealed three distinct intraspecific genetic groups within *E. tarda* that are phenotypically indistinguishable, including “typical motile fish pathogenic *E. tarda*”, “atypical non-motile fish pathogenic *E. tarda*”, and “fish non-pathogenic *E. tarda*” (Abayneh et al., 2013; Griffin et al., 2013;

Matsuyama et al., 2005; Sakai et al., 2007; 2009; Yamada & Wakabayashi, 1998; 1999). Isolates previously designated as "typical motile fish pathogenic *E. tarda*" have been reclassified as a separate species, *Edwardsiella piscicida* (Abayneh et al., 2013; Griffin et al., 2014; Reichley et al., 2017). Similarly, isolates previously categorized as "atypical non-motile fish pathogenic *E. tarda*" have been categorized as *Edwardsiella anguillarum* (Reichley et al., 2017; Shao et al., 2015); "Fish non-pathogenic *E. tarda*" now corresponds with isolates recognized as "true" or "bona fide" *E. tarda* (Griffin et al., 2014; Meyer & Bullock, 1973; Reichley et al., 2017).

Distinction between the five *Edwardsiella* species requires species-specific PCR (da Costa et al., 2022; Griffin et al., 2014; Reichley et al., 2015; 2017) or sequencing of discriminatory genes, such as the B subunit of the DNA gyrase gene (*gyrB*) or an internal fragment of the iron-cofactored superoxide dismutase gene (*sodB*) (Griffin et al., 2013; 2014; 2016; Reichley et al., 2017; Yamada & Wakabayashi, 1999). The 16S rRNA gene sequence shares ≥ 99.2% similarity between the *Edwardsiella* species and has been an inadequate target for intraspecific taxonomic differentiation (Griffin et al., 2016; Reichley et al., 2017). Consequently, historical reports of *E. tarda* predicated on phenotypic characters or 16s rRNA for identification likely represent a combination of true *E. tarda*, *E. piscicida,* and *E. anguillarum,* with a likely underrepresentation of *E. piscicida* and *E. anguillarum* in the literature (Griffin et al., 2017). In contrast, *Edwardsiella ictaluri* can be differentiated from *E. tarda, E. piscicida,* and *E. anguillarum* by phenotypic characteristics such as indole negativity, slower growth, varied colony morphology, and differences in temperature tolerance (Griffin et al., 2017). Still, despite this convoluted taxonomy, *E. tarda, E. ictaluri, E. piscicida,* and *E. anguillarum* are all recognized as globally distributed, fish pathogens of varying significance, affecting both wild and cultured fish (Griffin et al., 2020b). As the recent reorganization of *E. tarda* into three

discrete taxa obscures previous disease descriptions, Chapter 2 of this dissertation aims to clarify ambiguities regarding the virulence and pathology of *E. tarda, E. piscicida,* and *E. anguillarum* in US farm-raised catfish.

Importantly in terms of human health, *E. tarda* does pose low zoonotic risk to humans, although this is largely limited to immunocompromised individuals. Infections can occur via puncture, open wounds, or ingestion, leading to necrotizing skin lesions, gastroenteritis, or rarely, septicemia, meningitis, fasciitis, liver abscess, cholecystitis, or osteomyelitis (Hirai et al., 2015; Michael & Abbott, 1993; Mohanty & Sahoo, 2007; Suzuki et al., 2018; Wilson et al., 1989). Humans can acquire *E. tarda* infections from fish through ingestion, exposure to ornamental fish in home aquaria, and recreational or sport fishing (Clarridge et al., 1980; Suzuki et al., 2018; Vandepitte et al., 1980). Comparably, the zoonotic potential of *E. piscicida* and *E. anguillarum* is currently unknown, as it is unclear whether the *E. tarda* isolates implicated in human cases were accurately identified as true *E. tarda.* All three bacterial species are capable of growth at 37ºC and have potential to multiply in humans (Griffin et al., 2017; Reichley et al., 2017), although intraperitoneal injections of high doses of *E. tarda*, *E. piscicida*, and *E. anguillarum* into mice resulted in no outward signs of disease (Griffin et al., 2020b). Likely, *E. tarda,* and potentially *E. piscicida* and *E. anguillarum*, pose no greater threat to human health than other gram-negative fish pathogens (Griffin et al., 2020b).

Edwardsiella Microbiology

The *Edwardsiella* species can be cultured on common bacterial media, such as tryptic soy, Mueller-Hinton, and brain heart infusion agars, with or without supplementation with 5% defibrinated animal blood. Colonies are typically small, white to grey, and punctate. Colonies of *Edwardsiella tarda, E. piscicida,* and *E. anguillarum* are often visible within 24 to 36 hours at

temperatures between 28 and 37ºC, with optimal growth at 37ºC (Abayneh et al., 2013; Griffin et al., 2017; Reichley et al., 2017; Shao et al., 2015). In contrast, *E. ictaluri* grows best at 25-30ºC, producing colonies in 36-48 hours, and does not grow well >30ºC (Griffin et al., 2017). Phenotypic characteristics relevant to the four fish pertinent *Edwardsiella* species are summarized in Table 1. *Edwardsiella piscicida* and *E. anguillarum* have biochemical characteristics similar to *E. tarda*, further emphasizing the need for species-specific molecular differentiation (Abayneh et al., 2012; 2013; Griffin et al., 2013; 2014; Reichley et al., 2017). Additionally, intraspecific variation in phenotypic characters exists within the *Edwardsiella* species, such as variable motility and ability to produce hydrogen sulfide in triple sugar iron agar slants among *E. anguillarum* isolates (Reichley et al., 2017). Intraspecific phenotypic variation of the newly reiterated *E. anguillarum* is investigated in Chapter 3.

Edwardsiella tarda in Fish Disease

Edwardsiella tarda, first described in channel catfish, is historically considered one of the most important fish pathogens for which numerous pathologic descriptions exist in a large array of fish species (Meyer & Bullock, 1973). However, the recent segregation of *E. tarda* into multiple taxa has minimized its role as a major fish pathogen. Historical reports of disease caused by *E. tarda* prior to the recognition of *E. piscicida* and *E. anguillarum* likely represent a mixture of descriptions of the three taxa, obscuring accurate prevalence data and convoluting historical reports of *Edwardsiella* spp. pathology and virulence (Griffin et al., 2017). For example, many of the recent suspect *E. tarda* cases in the southeastern United States actually represent misidentified isolates of *E. piscicida*, further negating *E. tarda* as a significant pathogen in commercially grown ictalurids (Griffin et al., 2014; 2019). Limited virulence at naturally infective doses in *E. tarda* challenges in catfish further support its negligible role as a

pathogen of catfish aquaculture (Reichley et al., 2015; 2018). Unfortunately, inaccurate *E. tarda* descriptions arising from misidentified *E. piscicida* and *E. anguillarum* continue to be published, confounding accurate disease documentation (Griffin et al, 2014; 2017; 2020b).

While likely not as significant as previously thought, *E. tarda* can still cause opportunistic infections in fish, with infrequent outbreaks coinciding with stressful conditions, such as elevated temperature, maximum feeding and stocking levels, and high concentrations of organic wastes (Griffin et al., 2017; Meyer & Bullock, 1973; Reichley et al., 2018). While rare, outbreaks in catfish aquaculture typically affect market-sized fish with low mortality. These limited outbreaks usually occur late in the growing season when pond water temperatures and organic loads are highest (Hawke & Lester, 2004; Meyer & Bullock, 1973). Early descriptions of *E. tarda* infections in catfish include small cutaneous lesions, which progress to liquefactive bulla with foul smelling hydrogen sulfide gas (Darwish et al., 2000; Hawke & Lester, 2004; Meyer & Bullock, 1973; Reichley et al., 2017). The associated disease entity was named "emphysematous putrefactive disease", and the original isolate (Edwardsiella 9.1) has since been confirmed as true *E. tarda* (Meyer & Bullock, 1973; Reichley et al., 2017). More recent reports indicate *E. tarda* more commonly presents as a generalized septicemia (Darwish et al., 2000; Griffin et al., 2020b).

Treatment of *E. tarda* infection relies heavily on antibiotics and susceptibility profiles vary widely among isolates. Reichley et al. (2017) found genetically verified *E. tarda* isolates, including the first isolates from channel catfish, were all susceptible to drugs approved for US aquaculture, including florfenicol and oxytetracycline. In contrast, *E. tarda* isolates resistant to various antibiotics including ampicillin, amoxicillin, erythromycin, terramycin, chloramphenicol, tetracycline, and oxytetracycline have been reported from other studies (Akinbowale et al., 2006;

Hilton & Wilson, 1980; Jun et al., 2004; Loch et al., 2017). While many preventative vaccine candidates are available, efficacious vaccine development is challenged by delivery logistics, regulations surrounding the use of recombinant organisms in food animals, and intraspecific variation prior to the separation of *E. tarda* into multiple taxa (Griffin et al., 2020b). In brief, many of the initial frustrations in the search for an effective *E. tarda* vaccine are attributed to vaccine developers unknowingly working with multiple bacterial species.

Edwardsiella ictaluri in Fish Disease

Edwardsiella ictaluri, the causative agent of ESC in channel and hybrid catfish, is predominantly a pathogen of concern in United States catfish aquaculture, although outbreaks occur in other, primarily freshwater fish, including catfish in Asia and the Caribbean, tilapia cultured in Central America, zebrafish in the United States, and ayu in Japan (Bartie et al., 2012; Ferguson et al., 2001; Hawke et al., 1981; 2013; Nagai et al., 2008; Phillips et al., 2017; Soto et al., 2012). While isolates from US farmed catfish are largely genetically homogeneous, intraspecific variation exists between isolates from other fish species and geographic locations, despite analogous phenotypic traits (Aarattuthodiyil et al., 2020; Griffin et al., 2011; Purwaningsih et al., 2022; Sakai et al., 2009). Three distinct genotypes are recognized in catfish, tilapia, and zebrafish, respectively, based on repetitive-sequence-mediated PCR, gyrB sequences, and plasmid profiles (Griffin et al., 2016; Hawke et al., 2013). Important virulence factors include the type three secretion systems (T3SS), type six secretion systems (T6SS), transport proteins encoded by *tonB*, ferric uptake systems, and the urease system (Abdelhamed et al., 2013; 2017; Griffin et al., 2016; Santander et al., 2012; Thune et al., 2007).

Edwardsiella ictaluri is a primary pathogen in channel catfish and does not require predisposing conditions such as poor water quality, stress, or co-infections to cause disease,

though these can exacerbate losses (Griffin et al., 2017). Outbreaks in catfish often occur in late summer or early fall, coinciding with temperatures between 22 and 28ºC, often causing mortality in first-year fingerlings exposed to the pathogen for the first time (Francis-Floyd et al., 1987; Wise et al., 2004). Transmission occurs horizontally through water-borne exposure via ingestion or direct contact with mucosal surfaces including the intestinal mucosa and olfactory epithelium (Thune et al., 1993). Once *E. ictaluri* invades the olfactory epithelium, the bacterium can migrate to the brain along the olfactory tract, resulting in meningoencephalitis and subacute to chronic ulceration of skin overlying the fontanelle in the frontal bones causing the colloquial "hole-in-the-head" lesion (Hawke et al., 1981; Shotts et al., 1986). Clinical signs in affected catfish vary with disease time course and include sudden death, anorexia, erratic swimming, listless hanging in the water, lethargy, exophthalmia, and distended coeloms (Hawke & Khoo, 2004). Acute disease is characterized by rapid septicemia, with minimal clinical signs prior to death. Subacutely, fish can develop skin petechiation and ecchymoses, skin ulceration, hepatic and renal necrosis, and intestinal hemorrhage. Chronically infected fish can appear up to 30 days after the initial pond outbreak with low mortality rates, ulceration overlying the cranial fontanelle, and spiral swimming indicative of central nervous system disease (Hawke & Khoo, 2004). Fish surviving infection can clear the bacteria and become resistant, develop chronic disease, and/or become asymptomatic carriers (Hawke & Khoo, 2004; Klesius, 1992; Mqolomba & Plumb, 1992; Thune et al., 1993).

Significant efforts have been to develop an industry applicable ESC vaccine with variable levels of success. Killed vaccines were largely unsuccessful because they did not elicit a protective cell-mediated immune response against *E. ictaluri* (Thune et al., 1997; 1999). While live attenuated vaccines stimulate sufficient protection against intracellular pathogens, these

vaccines are limited by logistical challenges associated with delivery in commercial ponds or lack of perceived financial benefit by producers (Bebak and Wagner, 2012; Griffin et al., 2020b; Wise et al., 2015). A live attenuated immersion vaccine developed for 7 to 12 day old fry has mixed results in the field and has failed to achieve widespread industry adoption, likely because the catfish immune system is typically not fully developed until at least 21 days post hatch (Bebak & Wagner, 2012; Petrie-Hanson & Ainsworth, 1999). More recently, an orally delivered, live attenuated *E. ictaluri* vaccine (U.S. Patent No. 8,999,319) has become available and confers exceptional protection against *E. ictaluri* in channel and hybrid catfish fingerlings (Aarattuthodiyil et al., 2020; Chatakondi et al., 2018; Greenway et al., 2017; Peterson et al., 2016; Wise et al., 2015; 2020). The parent strain (S97-773), isolated from an ESC outbreak on a catfish operation near Stoneville, MS, was attenuated by successive passage on TSA-blood agar plates with sequentially increased concentrations of Rifamycin SV sodium salt (Klesius & Shoemaker, 1999; Wise et al., 2015). The vaccine is mixed with feed pond side using an in-line mechanized delivery system and is delivered to first year fingerlings beginning approximately 30 days post-stocking, once catfish are consistently taking an offered feed and pond temperatures exceed the permissive ESC window. Oral delivery capitalizes on the natural route of infection and facilitates delivery to older, immune competent fish without the stress of handling or confinement that would be required for injectable or immersion immunization (Griffin et al., 2020b; Wise et al., 2015; 2020). The vaccine is widely used in commercial ponds in the southeastern United States, providing significant production and economic benefits (Hegde et al., 2022; Kumar et al., 2019; Wise et al., 2015; 2020). While live attenuation better stimulates adaptive immunity, the vaccine isolate can cause low levels of disease and mortality when fish experience stress, such as poor environmental conditions or other disease (Griffin et al., 2020b).

Additional details on ESC preventative and treatment strategies are discussed under "Contemporary *Edwardsiella* Management in United States Catfish Aquaculture".

Edwardsiella piscicida in Fish Disease

Edwardsiella piscicida, synonymous with "typical, motile, fish pathogenic *E. tarda*", was first recognized as a novel species in 2013 and has since been described in at least 30 different fish species (Abayneh et al., 2013; Griffin et al., 2020b; Matsuyama et al., 2005; Yamada & Wakabayashi, 1998; 1999). However, it is unclear whether the increasing number of reports represent true increased prevalence secondary to global aquaculture expansion, more resolute bacterial identification from improved molecular diagnostics, or, more likely, a combination of both. *Edwardsiella piscicida* accounts are probably underrepresented due to historic misidentification attributable to phenotypic and 16s rRNA ambiguity prior to the development of discriminating molecular techniques. Multilocus sequence analysis places *E. piscicida* isolates into five discrete phyletic groups, with intraspecific genetic variability highlighted by the representation of catfish isolates in all groups (López-Porras et al., 2021). Virulence factors include components of the T3SSs and T6SSs, some of which are plasmid mediated (Leung et al., 2019; López-Porras et al., 2021).

In the United States, *E. piscicida* has emerged as a pathogen of concern in farm-raised catfish. This emergence is largely attributed to the recent industry pivot towards increased production of channel x blue (*Ictalurus furcatus*) catfish hybrids (Griffin et al., 2019). The median lethal dose of *E. piscicida* isolate S11-285 was over 100 times higher than that of *E. tarda* isolate FL95-01 in all catfish, indicating *E. piscicida* is a more significant threat to commercial catfish production than bona fide *E. tarda* (Reichley et al., 2018). Of the 138 suspected cases of *E. piscicida* submitted to the Aquatic Research and Diagnostic Laboratory at

the Thad Cochran National Warmwater Aquaculture Center (NWAC) in Stoneville, Mississippi from 2013 to 2017, 89.1% were in hybrid catfish (Griffin et al., 2019). This trend has continued in recent years, with hybrids accounting for 91.5% of *E. piscicida* cases in 2020 and 95.0% of cases in 2021 (M.J. Griffin, unpublished data.). Trends are corroborated by increased susceptibility of hybrids to *E. piscicida* compared to channel catfish in experimental challenges (Reichley et al., 2018). Interestingly, *E. piscicida* is not typically problematic in fingerling catfish. Rather, *E. piscicida* outbreaks are predominantly an issue in stocker or market-sized catfish, often in the second growing season as fish approach harvest size. Losses acquired during this stage in production can be particularly costly, as producers have made significant investments into the crop that cannot be recovered if fish die prior to harvest (Griffin et al., 2019).

Gross lesions in naturally infected catfish are characteristic of bacterial septicemias, including dermal ulcerations, exophthalmia, abdominal distension, and splenomegaly (Griffin et al., 2019). Additionally, infections with *E. piscicida* can lead to cranial midline lesions exposing the frontal bone and open fontanel, described in *E. ictaluri* infections as “hole-in-the-head”. Histologically, naturally infected catfish develop a mononuclear meningoencephalitis, hemorrhagic branchitis, splenitis, ulcerative dermatitis, granulomatous interstitial nephritis, hepatitis, and/or hemorrhagic enteritis (Griffin et al., 2019). Intracoelomic challenges incite similar histologic lesions in catfish, primarily affecting the spleen, anterior kidney, posterior kidney, brain, gill, and spinal cord, with lesions ranging from acute necrosis with abundant bacteria to chronic and granulomatous with few bacteria (López-Porras et al., 2021). Similar lesions, including multiorgan necrosis and/or granulomatous inflammation with numerous gram-

negative bacteria, are described in other fish species (Camus et al., 2016; 2019; Fogelson et al., 2016; Shafiei et al., 2016).

Most *E. piscicida* isolates are susceptible to antimicrobials currently approved for use in food fish in the United States, namely: florfenicol, oxytetracycline, and Romet® (5:1 sulfadimethoxine and ormetroprim) (López-Porras et al., 2021; Reichley et al., 2017). Lopez-Porras et al. (2021) identified the plasmid genes, tetracycline resistances transcriptional repressor (*TetR*) and tetracycline efflux MFS transporter (*TetA*), within *E. piscicida* isolate S07-348, which are associated with resistance to oxytetracycline and tetracycline. Similar to *E. tarda* and *E. ictaluri*, numerous vaccine candidates exist and face similar logistical challenges. Fortuitously, the currently used live-attenuated *E. ictaluri* immersion vaccine (340X2; Wise et al. 2015) is also cross protective against *E. piscicida* related mortality in channel and hybrid catfish (Griffin et al., 2020a; Lopez-Porras et al., 2022). While the cross-protective nature of 340X2 can improve survival, the protection incurred is not as robust as seen against *E. ictaluri.* As such, additional research is needed to develop more effective *E. piscicida* vaccine candidates. This dissertation analyzes the potential cross-protective effects between the *Edwardsiella* congeners *E. piscicida*, *E. anguillarum*, and/or *E. ictaluri* (Chapters 2, 4, and 5).

Edwardsiella anguillarum in Fish Disease

Edwardsiella anguillarum was originally described from diseased eels (*Anguilla* spp.) in China (Shao et al., 2015), although archived *E. tarda* isolates reassigned to *E. anguillarum* have been identified back to 1994 (Reichley et al., 2017). While the name suggests the species is particularly pathogenic to eels, the bacteria is increasingly identified in other freshwater, brackish, and marine fish globally, including bream, grunt, perch, grouper, rohu, snakehead, bass, tilapia, and catfish (Armwood et al., 2019; Buján et al., 2018; Dubey et al., 2019; Griffin et

al., 2014; 2017; Katharios et al., 2019; Oh et al., 2020; Reichley et al., 2017; Shao et al., 2015; Ucko et al., 2016). Isolates have been identified across the globe in Central America, North America, South America, Israel, Asia, and Peru (da Costa et al., 2022; Griffin et al., 2020b; Sierralta et al., 2020). Similar to *E. piscicida*, historical misidentification as *E. tarda* has likely resulted in underrepresentation of the true host and geographic ranges of *E. anguillarum* (Griffin et al., 2017; 2020b). While synonymous with isolates once described as atypical non-motile *E. tarda*, motility reportedly varies within the species (Matsuyama et al., 2005; Sakai et al., 2007; 2009). Motility was observed in isolates described by Shao et al. (2015), Dubey et al. (2019), and Griffin et al. (2013) but not in *E. anguillarum* isolates EA011113 and EA181011 described by Katharios et al. (2019) and Ucko et al. (2016), respectively, where the bacteria reportedly lacked flagella. Notable virulence factors include outer membrane protein A (*OmpA*), T3SSs, and T6SSs (LiHua et al., 2019; Ucko et al., 2016). Further investigation is needed to understand the true significance of *E. anguillarum* intraspecific variation in relation to fish hosts and geographic locations.

E. anguillarum is a significant pathogen of grouper, sharpsnout sea bream, tilapia, and eel aquaculture (Armwood et al., 2019; da Costa et al., 2022; Katharios et al., 2015; 2019; Oh et al., 2020; Shao et al., 2015; Sierralta et al., 2020; Ucko et al., 2016). Affected grouper presented with severe suppurative nephritis and large abscesses in the adjacent musculature, liver, spleen, and heart (Ucko et al., 2016). Sharpsnout sea bream exhibited grossly visible nodules in the spleen and kidneys (Katharios et al., 2015; 2019). In tilapia, *E. anguillarum* causes sepsis with acute necrotizing to chronic granulomatous lesions primarily affecting the spleen, kidneys, and liver (Armwood et al., 2019; Oh et al., 2020). Mortality from natural infections in these fish species ranged from 5-30% (Armwood et al., 2019; Katharios et al., 2015; 2019; Oh et al., 2020;

Ucko et al., 2016). Reports of natural *E. anguillarum* infection do not exist in United States cultured catfish, and median lethal doses of *E. anguillarum* were too high to be calculated from experimental challenges in blue, channel, and hybrid catfish, suggesting *E. anguillarum* is of little significance to the catfish industry (Reichley et al., 2018).

While data is limited, recent studies of *E. anguillarum* antimicrobial sensitivity demonstrate susceptibility to common antibiotics, including tetracycline, oxytetracycline, and florfenicol, among tested isolates (Armwood et al., 2019; Reichley et al., 2017). Development of preventative methods, including vaccines, are in their early stages, although formalin-killed and recombinant *E. anguillarum OmpA* have demonstrated increased relative percent survival or antibody production in experimental trials in red sea bream (*Pagrus major*) and Japanese eels (*Anguilla japonica*), respectively (LiHua et al., 2019; Takano et al., 2011). Previous studies of similarities between O-serotypes and surface antigens among atypical non-motile *E. tarda* (*E. anguillarum*) and typical motile *E. tarda* (*E. piscicida*), suggest cross-protection may exist between these bacteria (Costa et al., 1998; Griffin et al., 2020a). Based on these earlier findings, investigations into potential cross-protection among the fish pathogenic *Edwardsiella* species induced by various vaccine candidates is a promising and justified line of research.

United States Catfish Aquaculture

Commercial catfish aquaculture is the largest food fish sector in the United States, with 323 million pounds of market-sized fish produced in 2021, yielding $398 million in sales (USDA/NASS 2022). Catfish aquaculture is primarily concentrated in the southeastern United States and is an important agricultural commodity for the economies of Mississippi, Alabama, Arkansas, and Texas (USDA/NASS 2022).

Catfish production is largely based on in-land pond systems that serve as brood/spawning, nursery, fingerling, and grow-out ponds (Tucker et al., 2004). Channel catfish, blue catfish, and channel (*Ictalurus punctatus*) ♀ x blue (*Ictalurus furcatus*) ♂ catfish hybrids are the catfish varieties utilized in US catfish aquaculture. To accommodate year-round demand and a consistent source of liquidity, channel catfish are predominantly produced in multi-batch systems using a "topping" strategy, wherein graded seine nets facilitate removal of market-size fish (0.45-0.8 kg), while smaller fish (<0.4 kg) escape the net and remain in the pond until they achieve market size. Fish removed at harvest are then replaced by smaller fingerlings (0.02-0.06 kg) or stocker sized fish (0.06-0.240 kg). These multi-batch systems may remain in continuous production for 10 years or longer (Hegde et al., 2022; Tucker et al., 2004). In contrast, hybrid catfish are typically raised in single-batch systems, as their body conformation leads to entanglement ("gilling") in the seine net, which prohibits efficient harvesting (Hanson et al., 2020; Kumar et al., 2019; 2021). The hybrid production cycle is accelerated compared to the channel catfish production cycle, largely due to hybrids voracious appetite and improved resistance to common catfish aquaculture pathogens, which translates to more efficient growth (Arias et. al., 2012; Griffin et al., 2020c; Hegde et al., 2022; Rosser et al., 2019; Wolters et al., 1996). Generally, eggs are produced in spring and fry stocked in nursery ponds for grow out as fingerlings in early summer. Stocks are typically thinned and spread across multiple ponds as fish grow, with market-size fish typically harvested approximately 18-24 months post-hatch, although this timeline is often shortened for hybrids. The all-in/all-out production for hybrids focuses hybrid harvest in October-January, which creates a seasonality to supply. By comparison, multi-batch production of channel catfish permits a continuous supply to processing plants throughout the year when hybrids are not available. While the total land acreage devoted

to catfish production has decreased in the last fifteen years, productivity of catfish ponds per unit area has increased due to recent technological advancements and improved production strategies (Hegde et al., 2022).

Historically, the US industry exclusively produced channel catfish until technological advancements removed logistical obstacles to hybrid production. Hybrids now represent greater than 60% of commercial harvests and are favored due to their improved feed conversion, superior growth, tolerance to crowding, and increased disease resistance (Dunham et al., 2014; Griffin et al., 2010; Hegde et al., 2022; Wolters et al., 1996). Additionally, the adoption of productivity enhancing technologies, such as intensively aerated and split pond systems, continues to drive the industry toward hybrid use (Hegde et al., 2022). Despite the superior growth of hybrids, 41% of farms surveyed in the three greatest producing states (Mississippi, Alabama, Arkansas) still raise only channel catfish in multi-batch systems to maintain a year-round fish supply (Hegde et al., 2022). Comparably, blue catfish have poor production characteristics and are therefore not cultured as a food fish, although they are essential for hybrid production (Dunham & Argue, 2000).

While it is the largest food fish sector in the US, the catfish industry has contracted significantly since 2003, when more than 660 million pounds of fish were produced (Hanson & Sites, 2003). Industry challenges include competition from foreign imports, higher feed and energy costs, more profitable land use alternatives and losses from disease. Diseases result in direct fish mortality, decreased growth performance, and necessitate additional financial investment in treatments that can minimize profit margins (Hawke & Khoo, 2004). Infectious disease prevention and mitigation through proper pond management, effective disease treatment, biosecurity, and vaccination are essential to maximize industry profitability. Understanding the

pathogens that impact production, their virulence in different catfish types, and methods for their prevention and control are critical to disease management and essential for industry solvency and sustainability.

Contemporary *Edwardsiella* Management in United States Catfish Aquaculture

As in the case for all intensive aquaculture systems, bacterial diseases are a significant obstacle to profitability in United States farm-raised catfish. Major bacterial pathogens include *Flavobacterium* spp., *Aeromonas* spp., and the *Edwardsiella* spp. (Griffin et al., 2019; LaFrentz et al., 2022; USDA/APHIS 2011). Antibiotic administration and feed restriction are management strategies employed during *Edwardsiella* spp. outbreaks. While the antimicrobials Aquaflor® (florfenicol) and Romet® (sulfadimethoxine and ormetroprim) are currently FDA-approved for use in medicated feeds to treat ESC, antibiotic administration requires a veterinary feed directive and comes with the drawbacks of higher producer costs, observance of required withdrawal times prior to harvest, failure to achieve therapeutic levels in fish, and the potential development of antibiotic resistance. On a practical level, reduced feeding activity during disease outbreaks complicates the delivery of effective antibiotic doses via medicated feed. The repeated use of antibiotics can also lead to bacterial resistance and strains of *E. ictaluri* and *E. piscicida* have developed resistance to available antibiotics (Abdelhamed et al., 2018; 2019). At present, no FDA-approved antimicrobials currently exist for *E. piscicida* treatment in catfish. Feed restriction, an efficacious method to minimize disease spread via fecal-oral transmission, is an alternative to the use of antibiotic medicated feeds but may also contribute to decreased production (Wise et al., 2008).

Additional preventative strategies utilized to negate the cons of feed restriction and antimicrobial use include the production of less susceptible fish strains and vaccination. Notably,

channel, blue, and hybrid catfish demonstrate varied susceptibility to economically important pathogens such as *E. ictaluri*, *E. piscicida,* and *Henneguya* spp. Channel catfish appear to be more vulnerable to disease induced by *E. ictaluri* and the causative agent of proliferative gill disease (PGD), *Henneguya ictaluri* (Bosworth et al., 2003; Griffin et al., 2010; Wolters et al., 1996). Though less significant to the industry, channel catfish may be more susceptible to channel catfish virus, Ictalurid herpesvirus I (IcHV1) than the other fish types, although there appears to be variability across strains (Plumb et al., 1975; 1978). Thus, relative disease resistance is an additional driver pushing producers toward increased hybrid production behind better production qualities. However, while incidence of some diseases (PGD, ESC) is reduced in hybrid systems, *E. piscicida* cases continue to rise concurrent with increased hybrid production. At present, *E. piscicida* is the only pathogen within the industry known to be more virulent in hybrid catfish than channel catfish (Griffin et al., 2019; Reichley et al., 2018). These developments warrant increased research investment into preventative methods to reduce *E. piscicida* losses in hybrid catfish.

Vaccination is one strategy shown to improve production efficiency and reduce bacterial disease in aquaculture. Significant research investment has been made to develop effective vaccines against ESC; however, most vaccines have failed to circumvent challenges of diminished immunocompetency in young fry, minimal return on investment (failure to meet producer expectations), and logistical hurdles to administration and delivery (Bebak & Wagner, 2012; Lawrence et al., 1997; Petrie-Hanson & Ainsworth, 2000; Thune et al., 1994; 1999). As previously mentioned, the US industry has widely adopted the use of an orally delivered, live attenuated *E. ictaluri* vaccine, with approximately 83% of fingerling farms vaccinating their stocks as of 2020 (Hegde et al., 2022; Wise et al., 2015). Net economic benefits to channel and

hybrid catfish fingerling production were estimated at $3199 and $6149 per hectare (Kumar et al., 2019), with relative percent survival ranging from 82% to 100% at a target dose of 10^7 CFU/g of feed (Chatakondi et al., 2018; Greenway et al., 2017; Peterson et al., 2016; Wise et al., 2015; 2020). The *E. ictaluri* vaccine is also thought to reduce *E. piscicida* related mortality on farms (Griffin et al., 2020a). Research has demonstrated that immunization by the live attenuated *E. ictaluri* vaccine decreases mortality in channel and hybrid catfish against subsequent *E. ictaluri* infection. Similarly, exposure to *E. piscicida* reduces mortality in subsequent *E. ictaluri* infectivity trials (Griffin et al, 2020a; López-Porras et al., 2022). If cross-protective effects exist as suggested in these studies, utilization of different *Edwardsiella* vaccines in a combinatory fashion may further enhance protection, specifically in hybrid catfish where the efficacy of the *E. ictaluri* vaccine can be overcome by an extremely high exposure dose (Griffin et al., 2020a). Further investigation is needed to better understand bacterial factors associated with attenuation, protective immune responses in fish, duration of immunity, and potential cross-protection between different *Edwardsiella* congeners, especially between *E. ictaluri*, *E. piscicida*, and *E. anguillarum.*

Catfish Immune Reponses to *Edwardsiella* species and Vaccination

Both natural *Edwardsiella* spp. infections and *E. ictaluri* vaccination alter expression of genes responsible for innate and adaptive immunity in catfish. Exposure routes (e.g., intraperitoneal injection bypassing mucosal immunity), stress, and temperature all have significant effects on the catfish immune response and outcome of infections (Moore & Hawke, 2004). Innate immunity is well conserved across vertebrae taxa and includes physical barriers (skin, gill epithelium, mucosa), nonspecific immune cells (macrophages, neutrophils, non-specific cytotoxic cells), complement, and other non-soluble factors such as chemokines and

cytokines. A number of genes involved in innate immunity have been identified in catfish and extensively reviewed by Gao et al. (2012). The following acute inflammatory responses are commonly observed in other teleost responses to bacterial infections (Grayfer et al., 2014) and are highly conserved across vertebrate taxa (Plouffe et al., 2005).

Pathogen recognition receptors (PRRs) are intra- or extracellular receptors on cells such as macrophages, dendritic cells, and neutrophils that detect tissue damage and/or infection. PRRs include toll-like receptors (TLRs), retinoic acid inducible gene 1 (RIG1)-like receptors, C-type lectins, and nucleotide-binding domain-leucine-rich repeat containing receptors (NLRs/NOD-like). Bony fish have at least 17 distinct TLRs, including several specific to fish (Grayfer et al., 2018). TLR3, TLR5, TLR5S, TLR20, and TLR21 have all been identified in catfish, in addition to other PRRs including NOD-like receptors and RIG1-like receptors (Baoprasertkul et al., 2007a; 2007b; Bilodeau & Waldbieser, 2005; Gao et al., 2012; Rajendran et al., 2012; Sha et al., 2009). TLR3, TLR5, and TLR21 expression is variably elevated in channel catfish after exposure to *E. ictaluri*, likely leading to downstream signaling and upregulation of inflammatory genes related to stimulation of both the innate and adaptive immune responses (Bilodeau & Waldbieser, 2005; Pridgeon et al., 2010).

Cytokines and chemokines are a diverse group of secreted pro- or anti-inflammatory factors that modulate and regulate immune responses. Interferons are a subset of these signaling proteins crucial to inducing responses against intracellular pathogens, including viruses and intracellular bacteria. Interferons are separated into three types, including type I IFN, type II IFN, and type III IFN. The Type I and Type II IFNs play significant roles against viruses, while the type II IFN family, of which there is only IFN-γ, is key for macrophage activation and killing bacterial, viral, and protozoal pathogens (Grayfer et al., 2018; Robertsen, 2006; Zou &

Secombes, 2011). Similarly, interleukins are another large group of cytokines, with interleukin-1 (IL-1) best described in catfish (Gao et al., 2012; Wang et al., 2014). IL-1β is a potent pro-inflammatory mediator, essential for host-defense against pathogens. Further, tumor necrosis factors (TNFs) are another group of cytokines that play important roles in inflammatory cell chemotaxis, priming reactive oxygen and nitrogen species responses, and cell life cycle regulation (Grayfer et al., 2018). Specific identified cytokines and chemokines in catfish are mentioned in Gao et al. (2012), Fu et al. (2017a), and Fu et al. (2017b), including IFN-γ, IL-1β, CXC subfamily chemokines (α-chemokines), and CC subfamily chemokines (β-chemokine). Transcription end-binding protein, β1-integrin, natural-resistance-associated macrophage protein, transferrin, and heat shock protein 70 were variably upregulated within 48 hours post-*E. ictaluri* infection in channel catfish (Elibol-Flemming et al., 2009). In yellow catfish, *E. ictaluri* challenge induces an increase in mRNA expression of the proinflammatory innate immune markers, IL-1β, TNF-α, and mannose receptor (MR), with rapid increases in IL-1β and MR in spleen and kidney one to three days post infection and TNF-α after 5 days (Chen et al., 2020).

Complement contains numerous soluble membrane-bound proteins, with diverse roles in inflammatory responses, microbicidal activity, phagocytosis, antibody production, and immune complex clearance. In mammals, complement can be activated through three different pathways: the classical pathway, alternative pathway, and mannose-binding lectin pathway. Like mammals, fish have comprehensive complement activation pathways, though teleost complement research is in its infancy (Gao et al., 2012). The complement components Cq1, C3, C4, Bf/C2A, Bf/C2B, DFI, CD59, and Df have all been identified in channel catfish, corroborating the existence of the classical and alternative macrophage pathways described below (Abernathy et al., 2009; Yeh et al., 2007; Zhou et al., 2012). While Zhang et al. (2012) identified the initiator of the mannose-

binding lectin pathway in channel catfish, confirmation of the full pathway requires additional investigation (Gao et al., 2012).

Non-specific inflammatory cells, such as macrophages and dendritic cells, are critical to initiating and modulating immune responses, as the key antigen processing cells (APCs) in fish. APCs are the crucial bridge between the innate and adaptive immune responses, but these links are poorly understood in teleosts. Macrophages present intracellular pathogens to cytotoxic T cells using the major histocompatibility complex I (MHCI) pathway, which is conserved in many fish lineages (Grayfer et al., 2018). In mammals, extracellular pathogens are presented to $CD8^+$ cytotoxic T cells similarly using MHCI and to $CD4^+$ T helper cells via the MHCII complex. MHCII components are absent in multiple teleost groups, but these fish build effective immune responses, suggesting they have developed alternative strategies (Grayfer et al., 2018; Star et al., 2011). Dendritic cells appear functionally parallel to those in mammals, often with strong antigen presentation and lymphocyte activation (Grayfer et al., 2018).

In mammals, macrophages polarize to produce distinct functional phenotypes in response to environmental conditions or specific cytokines. The mammalian phenotypes include M1/classically activated macrophages primed by IFNγ and TNFα and M2/alternatively activated macrophages stimulated by IL-4/IL-13 (M2a), immune complexes (M2b), or IL-10/transforming growth factor-beta (TGF-β)/glucocorticoids (M2c) (Zhou et al., 2014). M1 macrophages are vital to inflammatory and microbicidal responses, while M2 macrophages participate in anti-inflammatory and tissue repair processes. Teleost macrophage subsets parallel their mammalian counterparts but are less understood. Like mammals, M1 macrophages are essential in pathogen phagocytosis, restriction of available nutrients, and the production of cytokines, reactive oxygen species, and nitrogen intermediates (Grayfer et al., 2018). IFNγ and TNFα are consistent markers

of fish M1 macrophages (Grayfer et al., 2018). M1 macrophages contain high levels of inducible nitric oxide synthase (iNOS), which converts L-arginine to L-citrulline, resulting in production of nitric oxide. In contrast, M2 macrophages contain arginase which converts L-arginine to L-ornithine and urea, with L-ornithine contributing to tissue repair (Grayfer et al., 2018). Nitric oxide synthase genes are highly regulated after *E. ictaluri* challenge, with time-dependent expression patterns matching macrophage migration in the catfish skin, gills, and posterior kidney (Yao et al., 2014).

Specific immune responses include humoral and cell-mediated immunity. Humoral immunity is generally mediated by antibody responses. Catfish have a tetrameric homolog of the mammalian IgM as their main serum antibody as well as a homolog of IgD (Bengtén et al., 2006). Channel catfish can develop measurable antibody responses approximately 3-4 weeks after hatching (Petrie-Hanson & Ainsworth, 1999). Additionally, maternal antibody transfer occurs in catfish embryos and can interfere with early vaccination (Hayman & Lobb, 1993; Klesius, 1992; Petrie-Hanson & Ainsworth, 1999). Prior work has shown, antibody responses after *E. ictaluri* infection challenges were lowest in blue catfish, intermediate in hybrids, and highest in channels, and were negatively correlated with survival, likely related to the immunogenicity of *E. ictaluri* in channel catfish compared to hybrid and blue catfish (Wolters et al., 1996). Serum antibodies peaked around 14 and 21 days after *E. ictaluri* bath immersion and declined after 28 days (Klesius & Sealey, 1995).

Oral vaccination with a live attenuated *E. ictaluri* vaccine leads to increased anti-*E. ictaluri* antibodies, with an 18-fold increase in anti-*E. ictaluri* antibodies when compared to non-vaccinated controls (Wise et al., 2015). Seventy-five percent of vaccinated fish seroconverted, with a mean antibody titer of 13,954 compared to the mean antibody titer of 1,736 in non-

vaccinated fish (Wise et al., 2015). Early studies suggest re-exposure fails to stimulate a memory antibody response larger than the primary response to *E. ictaluri* immersion challenge (Klesius & Sealey, 1995). Kordon et al. (2018) examined the role of antibodies in the phagocytosis of *E. ictaluri* by channel catfish peritoneal macrophages, reporting that *E. ictaluri* opsonized with sera from fish vaccinated with a live attenuated vaccine were endocytosed more efficiently than those from non-vaccinated fish. Opsonization of *E. ictaluri* with inactivated sera led to decreased phagocytic uptake at 32ºC and nearly suppressed uptake at 4ºC, implying an important role of complement-dependent mechanisms for killing of *E. ictaluri* by peritoneal macrophages (Kordon et al., 2018). Griffin et al. (2020a) suggests the live attenuated *E. ictaluri* vaccine can also protect channel and hybrid catfish from *E. piscicida*, but the involvement of humoral immunity in this protection is undocumented.

The *Edwardsiella* spp. largely survive and replicate within macrophages, suggesting cell-mediated immunity is essential for protection (Booth et al., 2006; Shoemaker et al., 1997). T lymphocytes play a critical role in cell-mediated immunity through engagement of CD4 and CD8 leading to various signaling mechanisms. Historical understanding of catfish cell-mediated immunity against *E. ictaluri* is limited by a lack of specific markers, which advancing technologies such as RNA-sequencing can help overcome. Shortly after vaccination with a live attenuated mutant, IFN-γ gene expression is increased in the anterior kidney and spleen of channel catfish, correlating with increased L/CD207+ cells (Kordon et al., 2019). L/CD207+ cells are dendritic-like cells that resemble mammalian Langerhans cells and function in antigen presentation (Kordon et al., 2019). In this study, the response to wild-type bacteria and vaccination varied, with increased expression levels of MHC class II, CD4-1, and CD8α in response to wild-type bacteria but not the live attenuated (mutant) *Edwardsiella* strains,

suggesting stimulation of the adaptive immune response, especially by the high loads of wild-type bacteria. In blue catfish livers, two different MHC class I alpha chains and beta-2-microglobulin were upregulated 3 days post-*E. ictaluri* challenge, indicating active antigen processing and presentation were likely occurring as part of the cell-mediate immune response (Peatman et al., 2008).

Overall, information on immune responses to *Edwardsiella* spp. infections, especially those caused by *Edwardsiella* spp. beside *E. ictaluri*, and vaccination is limited. Transcriptomics allows for quantification of gene expression levels unveiling host responses to pathogens or vaccination using different techniques such as tiling microarray, cDNA Sanger sequencing, and high-throughput sequencing (Qian et al., 2014; Sudhagar et al., 2018; Wang et al., 2009). RNA-seq advantages over other technologies include the ability to detect transcripts without an existing genomic sequence, single base pair resolution, low background noise, and low required amounts of RNA (Wang et al., 2009). Several studies have employed detailed RNA-seq analysis in catfish, one revealing 1633 differentially expressed genes in *E. ictaluri* challenged catfish intestine (Li et al., 2012). Further research comparing immune gene expression by channel and hybrid catfish in response to *E. ictaluri* vaccination and infection by the commercially relevant E*dwardsiella* species, *E. ictaluri* and *E. piscicida*, is detailed in Chapter 4 and 5, respectively. Additionally, understanding of the duration of immunity and cross-protection between *Edwardsiella* species, as well as preliminary investigations into the cross-protective potential of *Edwardsiella* congeners, is described in Chapter 2.

The objectives of the project are as follows: 1) to describe the pathology and virulence of *E. tarda*, *E. piscicida*, and *E. anguillarum* in channel, blue, and hybrid catfish; 2) to document phenotypic and molecular intraspecific variation of *E. anguillarum* from non-anguillid fish from

varied geographic origins; and to characterize the immune response to experimental *3) E. ictaluri* and 4) *E. piscicida* challenges in cultured channel and hybrid catfish with and without vaccination with the live attenuated *E. ictaluri* vaccine.

Table 1.1. Relevant phenotypic characteristics of *Edwardsiella* species causing fish disease.

	Growth	Triple sugar iron reaction	Motility	Indole
E. tarda	28-37ºC, 24-36 hours	Alkaline over acid (K/A) with H_2S and gas[1,2]	Variable motility[1,2]	Positive[2]
E. piscicida	28-37ºC, 24 hours	Alkaline over acid (K/A) with gas and H_2S[1,3]	Motile[1]	Positive[3]
E. anguillarum	25-37ºC, 24-36 hours	Alkaline over acid (K/A) with variable gas and weak H_2S[1,4]	Variable motility[1]	Positive[4]
E. ictaluri	25-30ºC, 36-48 hours	Alkaline over acid (K/A) without gas production[1,5]	Non- to weakly motile[1,5]	Negative[5]

[1]Reichley et al., 2017
[2]Hawke & Khoo, 2004
[3]Abayneh et al., 2013
[4]Shao et al., 2015
[5]Hawke et al., 1981

CHAPTER 2

PATHOLOGY AND VIRULENCE OF *EDWARDSIELLA TARDA*, *EDWARDSIELLA PISCICIDA*, AND *EDWARDSIELLA ANGUILLARUM* IN CHANNEL (*ICTALURUS PUNCTATUS*), BLUE (*ICTALURUS FURCATUS*), AND CHANNEL X BLUE HYBRID CATFISH [1]

[1]Armwood, A.R., Griffin, M.J., Richardson, B.M., Wise, D.J., Ware, C., and A.C. Camus. 2022. *Journal of Fish Diseases,* 45, 1682-1698. Reprinted here with permission of the publisher.

Abstract

In the mid-2010s, *Edwardsiella tarda* was reaffiliated into three discrete taxa *(E. anguillarum, E. piscicida,* and *E. tarda*), obscuring previous descriptions of *E. tarda*-induced pathology in fish. To clarify ambiguity regarding the pathology of *E. tarda, E. piscicida,* and *E. anguillarum* infections in US farm-raised catfish, channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and channel x blue catfish hybrids were challenged with comparable doses of each bacterium. The most severe pathology and mortality occurred in fish challenged with *E. piscicida*, supporting previous reports of increased pathogenicity in commercially important ictalurids, while *E. anguillarum* and *E. tarda* warrant only minimal concern. Acute pathologic lesions among bacterial species were predominantly necrotizing and characteristic of gram-negative sepsis but became progressively granulomatous over time. After 100 days, survivors were exposed to the approximate median lethal doses of *E. piscicida* and *E. ictaluri*, revealing some cross-protective effects among *E. piscicida*, *E. anguillarum,* and *E. ictaluri*. In contrast, no fish that survived *E. tarda* challenge demonstrated any protection against *E. piscicida* or *E. ictaluri*. This work supports reports of increased susceptibility of channel, blue, and hybrid catfish to *E. piscicida,* while highlighting potential cross-protective affects among fish associated *Edwardsiella* spp.

Introduction

Edwardsiella tarda, a cosmopolitan fish pathogen and causative agent of emphysematous putrefactive disease in channel catfish (*Ictalurus punctatus*), has historically been a bacterium of nominal concern in United States catfish aquaculture (Darwish et al., 2000; Meyer & Bullock, 1973). Until the 2010s, the genus *Edwardsiella* contained three species, *Edwardsiella hoshinae*, *Edwardsiella ictaluri*, and *E. tarda*, with only *E. ictaluri* and *E. tarda* associated with disease in US farm-raised catfish (Ewing et al., 1965; Grimont et al., 1980; Hawke et al., 1981). However, contemporary phylogenomic studies reorganized the genus, and *E. tarda* was reclassified into three species: *E. tarda*, *Edwardsiella piscicida*, and *Edwardsiella anguillarum*, corresponding to previous designations of "fish non-pathogenic *E. tarda*", "typical fish pathogenic *E. tarda*", and "atypical fish pathogenic *E. tarda*", respectively (Abayneh et al., 2012; 2013; Buján et al., 2018; Griffin et al., 2013; 2014; Reichley et al., 2017; Sakai et al., 2009a; 2009b; Shao et al., 2015; Yamada & Wakabayashi, 1999; Yang et al., 2012).

Pathologic descriptions of *E. tarda* infection in fish are numerous. However, many seminal studies occurred prior to the recognition of *E. piscicida* and *E. anguillarum*, when molecular sequencing was cost and technologically prohibitive for most fish diagnostic laboratories. While inferences can be made based on general designations of typical, atypical, or fish non-pathogenic *E. tarda*, it is often unclear which of the three new taxa are represented by historical reports (Darwish et al., 2000; Herman & Bullock, 1986; Miyazaki & Egusa, 1976; Padros et al., 2006; Uhland et al., 2000). For example, many isolates from US catfish aquaculture phenotypically identified as *E. tarda* were later confirmed by molecular phylogenomics to be *E. piscicida*, suggesting the reorganization of *E. tarda* obscured previous disease characterizations from US catfish (Griffin et al., 2014). Erroneous *E. tarda* descriptions, most often of

misidentified *E. piscicida* isolates, continue to be published in the contemporary literature, further confusing the associated diseases (Griffin et al., 2014; 2017; 2020b).

Early reports of *E. tarda* infection in catfish describe small cutaneous lesions progressing to liquefactive bulla containing hydrogen sulfide gas (Darwish et al., 2000; Hawke & Khoo, 2004; Meyer & Bullock, 1973; Reichley et al., 2017). However, recent reports indicate *E. tarda* more commonly presents as a generalized septicemia (Darwish et al., 2000; Griffin et al., 2020b), with several studies indicating limited pathogenicity for bona fide *E. tarda* in commercially important ictalurids (Reichley et al., 2015; 2018). As a result, recognition of *E. piscicida* and reaffiliation of isolates previously deemed "*E. tarda*" has diminished significance of true *E. tarda* as a catfish pathogen in the southeastern US (Reichley et al., 2017). While *E. tarda* has historical significance to catfish aquaculture, reports of *E. anguillarum* outbreaks in the US catfish industry do not exist, likely due to limited virulence in ictalurid species (Reichley et al., 2017; 2018).

In contrast to the negligible effects of *E. tarda* and *E. anguillarum* on catfish production, *E. piscicida* has emerged as a major pathogen concurrent with the industry's pivot to increased channel (*Ictalurus punctatus*) ♀ x blue (*Ictalurus furcatus*) ♂ catfish hybrid production (Griffin et al., 2019; Reichley et al., 2015; 2018). *Edwardsiella piscicida* can cause catastrophic losses, primarily affecting stocker or market-sized catfish hybrids (Griffin et al., 2019; 2020b). Lesions parallel other bacterial septicemias, producing petechiae and dermal ulceration, iridial hemorrhage, reddening of the mouth and vent, exophthalmia, ascites, splenomegaly, renomegaly, and occasional intestinal hemorrhage (Griffin et al., 2019; López-Porras et al., 2021; Reichley et al., 2018). Histopathologic examination of naturally infected fish reveals mononuclear

meningoencephalitis, granulomatous hepatitis and interstitial nephritis, splenitis, hemorrhagic enteritis, hemorrhagic branchitis, and ulcerative dermatitis (Griffin et al., 2019).

Historically, the US industry produced channel catfish exclusively, but improved techniques have removed logistical obstacles to hybrid production, which now represents nearly 60% of commercial harvests (Hegde et al., 2022). Producers favor their improved feed conversion at harvest, superior feed consumption and growth, tolerance to crowding, and increased disease resistance (Dunham et al., 2014; Griffin et al., 2010; Wolters et al., 1996). Comparably, blue catfish have less desirable production characteristics and are not grown commercially but are essential for hybrid production (Dunham & Argue, 2000).

Interestingly, the three catfish types have varied susceptibility to bacterial pathogens endemic to the southeastern United States, including the *Edwardsiella* spp. (Reichley et al., 2018; Wolters et al., 1996). *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), is a significant pathogen of the US catfish industry (Griffin et al., 2017; Hawke et al., 1981). While outbreaks occur in channel, blue, and hybrid catfish, channels are the most susceptible (Wolters et al., 1996). In contrast, hybrid and blue catfish are more susceptible to *E. piscicida* (Reichley et al., 2015; 2018). This is reflected in case submissions to the Aquatic Research and Diagnostic Laboratory in Stoneville, MS, where channels make up ~70% of ESC diagnoses (Lester Khoo, personal communication). In contrast, hybrids account for <50% of total case submissions but contribute >90% of the *E. piscicida* cases (Griffin et al., 2019).

Management of *Edwardsiella* spp. outbreaks typically involve feed restriction or antibiotic interventions (Wise et al., 2004). More recently, an orally delivered, live attenuated *E. ictaluri* vaccine (U.S. Patent No. 8,999,319) became available and confers exceptional protection against *E. ictaluri* in channel and hybrid catfish fingerlings (Aarattuthodiyil et al., 2020;

Chatakondi et al., 2018; Greenway et al., 2017; Peterson et al., 2016; Wise et al., 2015; 2020). This vaccine has been adopted widely by commercial fingerling producers in the southeastern US (Hegde et al., 2022). Live-attenuated *E. ictaluri* immersion vaccination is also cross-protective against *E. piscicida* related mortality in channel and hybrid catfish (Griffin et al., 2020a; López-Porras et al., 2022). Similarly, bath exposure to *E. piscicida* protects channel and hybrid catfish against *E. ictaluri* challenge (Griffin et al., 2020a; López-Porras et al., 2022). However, the duration of this protection remains undocumented. Potential cross-protection from *E. anguillarum* and *E. tarda*, against *E. ictaluri* and *E. piscicida* is currently unknown.

While some disease descriptions associated with bona fide *E. tarda* infection in fish are true (Meyer & Bullock, 1973; Reichley et al., 2017), the historical understanding of *E. tarda* related pathology likely represents a combination of diseases caused by *E. tarda*, *E. anguillarum*, and *E. piscicida*. This study clarifies discrepancies in pathologic changes and virulence associated with *E. tarda*, *E. piscicida*, and *E. anguillarum* in channel, blue, and hybrid catfish, building on previous work by Reichley et al. (2015; 2018). Additionally, this work provides preliminary data investigating potential cross-protection from wild-type *Edwardsiella* congeners against subsequent *E. ictaluri* and *E. piscicida* challenge.

Methods

Fish

All animal handling procedures were performed in compliance with the Mississippi State University Institutional Animal Care and Use Committee. Catfish fingerlings were 4 to 6 months old and reared indoors under specific pathogen free conditions at the National Warmwater Aquaculture Center at the Mississippi State University Delta Research and Extension Center in Stoneville, Mississippi. Average initial weights were 8.4 g (range: 4.0-17.1 g), 5.0 g (range: 1.9-

15.9), and 7.6 g (range: 2.4-15.2 g) for channel, blue, and hybrid catfish, respectively. For challenges, fish were arbitrarily distributed into 80-L glass aquaria with 22 L of aerated, flow-through (1 L/min) well water (~23°C to 25°C). Fish were acclimated for 72 h and fasted for 24 h prior to challenge. Fish were offered a commercial floating catfish feed containing 40% crude protein (Rangen, Buhl, Idaho) at ~1-2% body weight once daily.

Experimental Trial 1

Archived isolates of *E. anguillarum* (LADL05-105; host *Oreochromis* sp.; Louisiana, USA; Griffin et al., 2013; Reichley et al., 2015a; 2015c), *E. tarda* (FL95-0; host *I. punctatus*; Florida, USA; Griffin et al., 2013; Reichley et al., 2015b; 2015c), and *E. piscicida* (S11-285; host *I. punctatus*; Mississippi, USA; Griffin et al., 2013; Reichley et al., 2015c; 2016) from the collection of M. J. Griffin were used in challenges. Prior to archiving, isolates were passed through channel catfish (2 x 48 h passages), and identities confirmed by multiplex real-time polymerase chain reaction (mPCR) (Reichley et al., 2015c; 2017). Cryostocks were revived by isolation streaking on Mueller-Hinton II Agar (BBL™; Becton Dickinson and Company) plates supplemented with 5% sheep blood (MHBA) and incubated for 24 h at 37°C. Individual colonies were expanded overnight in static 9 ml porcine brain-heart infusion broth (BHIb) cultures (Bacto; Becton Dickinson and Company) at 37°C after which 1 ml aliquots were seeded into 1-L BHIb cultures and grown for 18 h at 200 rpm at 37°C.

To account for variable pathogenicity of *E. tarda, E. piscicida,* and *E. anguillarum* in channel, blue, and hybrid catfish (Reichley et al., 2015c; 2018), each fish type received two (*E. piscicida*) or three (*E. tarda* and *E. anguillarum*) dose levels of bacteria. Expanded cultures of each bacterium were diluted to achieve challenge targets based on previous estimations of median lethal and infectious doses based on fish size (Reichley et al., 2018). Actual doses were

estimated by standard plate counts (Table 2.1). Eight treatment groups, consisting of *E. piscicida* high dose [EPHD], *E. piscicida* low dose [EPLD], *E. tarda* high dose [ETHD], *E. tarda* medium dose [ETMD], *E. tarda* low dose [ETLD], *E. anguillarum* high dose [EAHD], *E. anguillarum* medium dose [EAMD], *E. anguillarum* low dose [EALD], and unexposed controls were used for each catfish type (Table 2.1). EPHD was targeted to be roughly equivalent to EALD and ETLD due to its high pathogenicity, and thus only one additional lower dose was examined for *E. piscicida.* For pathology assessments, treatments and controls consisted of three sampling tanks (20 fish/tank). An additional non-sampling tank (20 fish/tank) was included for each treatment and control group to estimate dose mortality.

During challenge, fish were anesthetized by immersion in tricaine methanesulfonate (MS-222; Western Chemical, Ferndale, WA; 100 mg/L), and respective bacterial doses administered by intracoelomic (IC) injection (0.1 mL/fish). Non-challenged controls received 0.1 ml of sterile BHIb. Doses were delivered through the ventrocaudal coelomic wall, immediately cranial to the vent. Fish were observed twice daily for 14 days, and mortality recorded. Select posterior kidneys from fresh dead and moribund fish suitable for bacterial culture were inoculated on MHBA for 24-48 h at 37°C to confirm infection. Identification of recovered bacteria was confirmed by *Edwardsiella* spp. mPCR (Reichley et al., 2015c; 2017).

Fish were sampled at seven time points: 1, 2, 3, 5, 7, 10, and 14 days post challenge (dpc). Sampling was non-random, with preference given to fish showing clinical signs (lethargy, circling, failure to maintain neutral buoyancy) and/or gross lesions (exophthalmia, coelomic distention, petechiation). Sampled fish were excluded from survival analysis and mortality assessments. Time points were based on reported mortality trends for each bacterium in channel and hybrid catfish (Reichley et al., 2015c; 2018). One fish was collected from each sampling

tank (n = 3 fish/treatment group/sampling day) unless mortality precluded adequate numbers for sampling. Fish were euthanized by immersion in MS-222 (300 mg/L), an operculum removed, abdominal flaps excised, and whole bodies fixed in 10% neutral buffered formalin for at least 24 h. Bodies were decalcified in Kristensen's solution for 24 h then serially sectioned transversely into tissue cassettes before routine processing, embedding in paraffin, sectioning at 5 µm, and staining with hematoxylin and eosin (HE). Select sections were stained with Gram and Giemsa stains. Fish from *E. piscicida* (EPLD, EPHD), *E. tarda* (ETMD, ETHD) and *E. anguillarum* (EAMD, EAHD) groups were examined histologically. Fish from *E. tarda* (ETLD) and *E. anguillarum* (EALD) were not examined due to negligible mortality and minimal gross lesions observed in those fish.

Experimental Trial 2

After 14 days, surviving ETMD, EAMD, and EPLD fish were maintained for an additional 86 days then challenged with either *E. ictaluri* isolate S97-773 (host *I. punctatus*; Mississippi, USA; GenBank: ASM305840v1), *E. piscicida* isolate S11-285, or BHIb (negative control). Fish from each group were redistributed into nine tanks (three/treatment or control group) in variable numbers according to the number of available survivors (Table 2.2). Cryostocks were revived, expanded, and their identities confirmed as described for Experiment 1, except *E. ictaluri* which was incubated at 28°C. For *E. piscicida*, fish were anesthetized by immersion in MS-222 (100 mg/L) and injected IC with 0.1 mL of bacterial culture approximating 7.8×10^4 CFU/fish. The IC route was chosen for *E. piscicida* challenges, because oral and immersion do not consistently cause disease in commercial catfish and IC injection provides the most consistent infection results (Reichley, 2017). Fish were challenged with *E. ictaluri* via immersion, because, unlike with *E. piscicida*, immersion is a reliable exposure route

and more closely mimics natural infection. For the immersion challenge, waterflow was suspended for 30 m while 50 ml of culture approximating a dose of $5.7x10^6$ CFU/ml was added to the tank. Following static exposure, waterflow was resumed. Two tanks (25 fish/tank) served as non-challenged controls for each delivery method. Injection controls received 0.1 ml of sterile BHIb/fish. Immersion controls received 50 ml of sterile BHIb. Fish were observed twice daily for 35 days, and mortality recorded.

Statistical Analysis

Kaplan-Meier survival curves were created and analyzed using the *survminer* (v0.4.9; Kassambara et al., 2021) and *dplyr* (v1.0.8; Wickham et al., 2022) packages in R statistical software (v4.0.2; R Core Team, 2021). Treatment effects were evaluated using Cox proportional hazards models, in the *survival* package (v3.2-13; Therneau et al., 2021), or log-rank tests in R. In instances where no mortality occurred for a given treatment (e.g., Figure 2.1b), the Cox proportional hazards model is unable to calculate mortality risk and will yield an infinite estimate. To remedy the infinite estimate in these cases, a single mortality was added across all treatments to force an estimate of risk. In cases where treatments resulted in both 0% and 100% mortality for different groups (e.g., Figure 2.2b), use of the Cox proportional hazard model was precluded, and analysis was performed using log-rank tests. For Trial 1, log-rank tests were used to assess differences in survival probability between each treatment group compared to non-exposed controls. For Trial 2, Cox proportional hazard models were used to evaluate all groups with the exception of EP survivors, which were analyzed by log-rank test due to 0% mortality in the *E. piscicida* re-exposure group.

Results

In sampling tanks, which included scheduled fish sampling (censoring), blue and hybrid catfish had reduced survivability ($p < 0.05$) at any given time point compared to channel catfish when exposed to the highest doses of *E. anguillarum* and *E. piscicida*. Similarly, risk of mortality in blue catfish was 4.73 times higher ($p < 0.05$) than in channel catfish exposed to the ETHD (Figure 2.1a). Findings from sampling tanks were congruent with observations in non-sampling tanks (Figure 2.1b). For EPLD, blue catfish and hybrid catfish had greater mortality risk in both sampling (blue hazard ratio: 10.5, $p<0.001$; hybrid hazard ratio: 6.9, $p<0.001$) and non-sampling (blue hazard ratio: 14.5, $p<0.001$; hybrid hazard ratio: 4.3, $p<0.05$) groups compared to channel catfish.

EPHD resulted in reduced survivability ($p < 0.05$) compared to equivalent doses of *E. anguillarum* and *E. tarda* for all catfish varieties (Figure 2.2a). In both sampling and non-sampling tanks, the EPHD treatments approached or achieved 100% mortality in blue and hybrid catfish. Comparably, similar doses of *E. tarda* and *E. anguillarum* resulted in 0-5% mortality. In line with results from *E. piscicida* challenges, blues and hybrids were more severely affected, especially with *E. anguillarum.* In short, it required nearly 100X the *E. piscicida* dose to induce similar mortality with *E. tarda* and *E. anguillarum*.

In sampling tanks, *E. anguillarum* increased mortality risk of blue catfish at mid- and high doses compared to equivalent doses of *E. tarda* ($p < 0.05$). In contrast, mortality risk was 6.1 times higher ($p<0.05$) in channel catfish challenged with ETHD than those challenged with EAHD. In all catfish varieties, mortality curves for EPHD, EAHD, and ETHD were significantly different from non-exposed controls (log-rank test; $p < 0.001$). Similarly, challenge with EAMD reduced survivability in blue ($p < 0.001$) and hybrid ($p < 0.05$) catfish compared to controls

(Figure 2.2a). These findings are consistent with non-sampling tanks (Figure 2.2b). In sampling tanks receiving EPLD, survival probability was lower for channel (0.88, $p < 0.01$), hybrid (0.43, $p < 0.001$), and blue (0.38, $p < 0.001$) catfish than non-exposed controls (1.0). Similar trends were observed for hybrid and blue ($p < 0.001$) catfish in non-sampling tanks. No mortality was observed in any non-exposed control tanks.

While not all fish had gross lesions, when present, lesions were similar across all sampled fish varieties and were most frequent at the highest doses of *E. piscicida*, *E. tarda*, and *E. anguillarum.* Minimal gross lesions occurred in fish challenged with low doses of *E. tarda* and *E. anguillarum*, despite receiving a roughly equivalent dose as the EPHD group. No gross lesions were observed in non-challenged, control fish. Gross lesion development began in *E. piscicida* challenged fish 2 dpc, coinciding with the onset of mortality in the group, and lesions were not evident in surviving fish sampled 14 dpc. The most common change in all catfish types challenged with *E. piscicida* was mild, clear to pale red serosanguinous ascites, beginning 2 dpc. Additional findings included rare petechiation of the pectoral fins, reddened vents, and infrequent splenomegaly in hybrid and blue catfish 3 dpc. A single hybrid catfish also had a focally extensive, transmural body wall ulcer immediately caudal to the operculum exposing the coelomic cavity and swim bladder 3 dpc. One channel catfish presented with mild dorsal midline ulceration and swelling on day 10 (Figure 2.3a).

In *E. tarda* challenges, gross changes were limited to fish receiving the highest dose. Similar to *E. piscicida*, the most common alteration was mild, clear to pale red serosanguinous coelomic effusion from 1 to 5 dpc. Transmural body wall ulcerations with coelomic cavity exposure occurred in multiple channel and hybrid catfish from 2 to 5 dpc (Figure 2.3b, 2.3c).

Other mild changes included cutaneous petechiation and erythematous vents with rare mild splenomegaly and renomegaly. No gross lesions were observed after 7 dpc.

Similar to *E. tarda*, *E. anguillarum* challenge caused gross lesions only in fish receiving the highest dose. Fish most commonly exhibited mild to severe, often bright yellow coelomic fluid, extending 2 to 10 dpc (Figure 2.3d). Similar to the other bacteria, other sporadic lesions included reddened vents, pectoral girdle petechiation, and rare transmural body wall ulceration. No lesions were observed in surviving fish by day 14.

Histopathologic findings corresponded closely with gross lesions and mortality curves for each bacterial species. Consistent systemic changes occurred in fish challenged with ETHD (Table 2.3), EAHD, EAMD (Table 2.4), EPHD, and EPLD treatments (Table 2.5). In general, acute changes were typical of gram-negative sepsis, with day 1 and 2 lesions characterized by focal necrosis with abundant bacteria (Figure 2.4a). On day 5 and beyond, lesions became increasingly dominated by granulomatous inflammation and declining bacterial numbers (Figure 2.4b).

Lesions were most common in the coelomic cavity, followed by the spleen (Figure 2.4c) and liver (Figure 2.4d). The posterior kidney was frequently affected by *E. tarda* and *E. piscicida* challenge (Figure 2.4e) but only sporadically by *E. anguillarum*. Splenic lesions were predominantly acute necrosis, while renal and hepatic lesions progressed from acute necrosis to chronic granulomatous inflammation. Spleens were severely congested with diffuse necrosis of ellipsoids containing cellular debris and free gram-negative bacterial rods. Hepatic and posterior renal lesions consisted of multifocal necrosis and/or granulomatous inflammation. Hepatic necrosis was characterized by multifocal, random foci of hypereosinophilic hepatocytes with pyknotic nuclei surrounding a central area of cellular debris and abundant extracellular gram-

negative bacterial rods. Renal lesions consisted of necrosis of the hematopoietic tissue, with loss of cellular detail, cellular debris, and abundant extracellular bacterial rods. In all tissues, granulomatous inflammation was characterized by discrete aggregates of macrophages with rare phagocytized bacteria. Swim bladders, skin (Figure 2.4f), gills, anterior kidneys, and brains were affected only intermittently. Swim bladder and brain lesions were predominantly granulomatous, while anterior kidney and gill lesions were predominantly necrotizing. Non-inflammatory lesions included variable gill lamellar hyperplasia (Figure 2.5a), gastric glandular apoptosis (Figure 2.5b), gastric submucosal edema (Figure 2.5c), and exocrine pancreatic apoptosis (Figure 2.5d). The affected gastric glandular cells were shrunken, rounded, and brightly eosinophilic, with condensed nuclei. Exocrine pancreatic cells were similar with rounded shrunken cells surrounded by clear space and depleted of zymogen granules. No significant lesions occurred in the esophagus, thyroid, gonads, eyes, or heart of any fish. No histologic lesions were observed in control fish at any time point.

In *E. tarda* challenged fish (Table 2.3), lesions began 1 dpc, characterized primarily by necrosis, hemorrhage, and edema, most severe with the ETHD. Necrotic lesions often contained abundant free gram-negative bacterial rods. Inflammatory lesions were typically necrotizing in the internal viscera until 5 dpc when granulomatous inflammation predominated. Lesions were minimal to absent in fish 10 and 14 dpc, with nominal coelomic and perivascular inflammation. Skin and skeletal muscle abnormalities were rare, but included multifocal hemorrhages 1 dpc, occasional body wall ulceration (Figure 2.4f), and sporadic granulomatous inflammation by 5 dpc. Non-inflammatory findings commonly included gastric glandular cell apoptosis, exocrine pancreatic apoptosis, and gastric submucosal edema, which only occurred in fish 1 to 3 dpc. Apoptotic lesions occurred only in ETHD fish.

In contrast to *E. tarda*, histologic findings associated with EAMD and EAHD challenge occurred throughout the sampling period, beginning 1 dpc and continuing through 14 dpc (Table 2.4). The sporadic lesions were initially either necrotizing or granulomatous, with progression to solely granulomatous by 3 dpc. While inflammatory gill lesions were absent, lamellar epithelial hyperplasia was a frequent finding unique to the *E. anguillarum* challenges regardless of fish type. Gastric glandular cell and exocrine pancreatic cell apoptosis was common, beginning 5 and 2 dpc, respectively, and most frequent in the EAHD challenged fish.

Microscopic findings in *E. piscicida* challenged fish are summarized in Table 2.5. Systemic necrotizing and inflammatory lesions began 2 dpc, diminishing over time until present in only one of five fish 10 dpc, even in the EPHD treatment group. This affected channel catfish had disseminated granulomatous inflammatory foci throughout the liver, spleen, skeletal muscle, spinal cord, and brain. Granulomatous meningitis extended into the dermis overlying the cranial fontanel, approaching the colloquial "hole-in-the-head" lesion (Figure 2.6a,b). Similar to *E. tarda* challenged fish, transmural body wall ulceration occurred occasionally with *E. piscicida* infection. Gastric apoptosis and edema were commonly observed in fish 2 and 3 dpc, with sporadic exocrine pancreatic apoptosis. Lesions were minimal in fish surviving 14 days, though sampling was limited to only 3 channels and 1 blue catfish due to elevated mortality.

Cumulative percent mortalities for Experimental Trial 2 are listed in Table 2.2. Channel catfish that survived *E. piscicida* challenge had greater survival after *E. ictaluri* challenge than naïve fish (Figure 2.7). Similarly, catfish hybrids that survived *E. piscicida* or *E. anguillarum* challenge exhibited greater survival after *E. piscicida* challenge than naïve controls (Figure 2.8). Comparably, previous infection with *E. tarda* did not confer significant protection against

subsequent challenge with *E. piscicida* or *E. ictaluri* in channel, blue or hybrid catfish (Figures 2.7,2.8).

Discussion

Commercial catfish aquaculture is the largest food fish industry in the United States, producing 323 million pounds of market-size fish and $398 million in sales in 2021 (USDA/NASS, 2022). Despite this success, significant contraction has occurred since 2003, when the industry produced more than 660 million pounds of fish (Hanson & Sites, 2013). Increased feed and energy costs, more profitable land use alternatives, and foreign competition all jeopardize sustainability of US catfish aquaculture, driving a 40% reduction in water surface acres over the past decade (Hawke & Khoo, 2004; Hanson & Sites, 2013; USDA/NASS, 2022). In addition, infectious diseases also threaten industry stability. Bacterial infections cause substantial losses, specifically *Flavobacterium* spp., *E. ictaluri, Aeromonas hydrophila,* and *E. piscicida*, an emergent disease in hybrid catfish (Griffin et al., 2019; LaFrentz et al., 2022; USDA/APHIS, 2011). The recent reorganization of the *Edwardsiella* genus prompted investigations into disease pathology attributed to bacteria formerly classified as *E. tarda* to understand better risks associated with these newly recognized taxa and mitigate disease losses.

Well known as a fish pathogen since the 1970s, *E. tarda* causes disease in marine and freshwater fish worldwide (Griffin et al., 2020b; Meyer & Bullock, 1973). Following their official recognition in the 2010s, *E. anguillarum* and *E. piscicida* have been reported from at least 19 and 30 fish species, respectively (Griffin et al., 2020b; Leung et al., 2019). Currently insignificant to catfish aquaculture, *E. anguillarum* is increasingly important in cultured eels, tilapia, grouper, and sea bream (Armwood et al., 2019; Katharios et al., 2019; Oh et al., 2020; Shao et al., 2015; Ucko et al., 2016). However, it is uncertain whether the growing number of *E.*

anguillarum and *E. piscicida* reports represent true increased prevalence associated with global aquaculture expansion or more resolute bacterial identification attributable to improved molecular diagnostics.

In contrast to *E. tarda* and *E. anguillarum*, *E. piscicida* has emerged as a significant pathogen in US catfish aquaculture, predominantly catfish hybrids (Griffin et al., 2019; Reichley et al. 2018), an observation supported by data presented here. While mortality and pathologic changes occurred at high doses of *E. tarda* and *E. anguillarum*, it is doubtful fish would encounter bacterial loads this high in the environment, making natural infections unlikely in a non-compromised host. Moreover, fish were challenged via intracoelomic (IC) injection, circumventing innate host defenses, and limiting extrapolation of natural infection pathogenesis and disease onset timing. However, the IC route provides the most consistent infection results, as oral and immersion challenges with *E. tarda, E. piscicida,* and *E. anguillarum* bacteria do not reliably induce disease in cultured ictalurids (Reichley, 2017).

Herein, mortality risk varied between catfish types, with blue and hybrid catfish consistently more susceptible to *E. piscicida* than channels, supporting previous work and diagnostic patterns that suggest *E. piscicida* is more virulent in blue and hybrid catfish (Griffin et al., 2019; Reichley et al., 2018). In contrast to Reichley et al. (2018), blue and hybrid catfish experienced significant mortality to the high dose of *E. anguillarum* (~$1x10^6$ CFU/fish). Blue catfish were most susceptible to high doses of *E. tarda* (~$1x10^6$ CFU/fish). While poor production qualities preclude use as commercial food fish, male blue catfish are required for hybrid catfish fry production. Disease outbreaks among the small numbers of blue catfish broodstock used by the industry could be catastrophic to hybrid production. *E. piscicida* and, to a

much smaller extent, *E. anguillarum* and *E. tarda* should be considered potential pathogens in blue catfish culture, warranting biosecurity measures and possible vaccination to prevent losses.

Interestingly, the time course of mortality and disease was prolonged in *E. anguillarum* challenges compared to *E. piscicida* and *E. tarda*, with losses extending to 14 dpc. In contrast, *E. tarda* mortality occurred within the first 5 dpc, with most mortality occurring within the first 3 dpc. By 1 dpc, hemorrhage, necrosis, and rampant bacteria were evident in fish receiving the high dose of *E. tarda*, suggesting the rapid onset of mortality is associated with acute fulminating septicemia, rather than effects of released *E. tarda* products or poor water quality alone (Reichley, 2017).

Gross lesions caused by *E. piscicida*, *E. tarda*, and *E. anguillarum* challenge were indistinguishable. Lesions were typical of acute gram-negative sepsis and cannot be definitively distinguished grossly or histologically from *E. ictaluri* (Newton et al., 1989). However, *E. tarda* and *E. anguillarum* challenges at low doses ($<3x10^5$ CFU/fish), more reflective of natural exposures, did not induce significant lesions. As such, *E. tarda* and *E. anguillarum* are unlikely differentials for bacterial disease in cultured catfish. Objective quantification of gross and histopathologic lesions amongst the different bacteria and fish types was complicated by sampling bias for fish that were moribund or showing clinical signs. Gross examination of internal viscera was further limited by the small fish size but promoted complete histologic examination of tissues in serial cross-sections.

Natural *E. piscicida* infections in catfish reportedly progress from cutaneous petechiation to dermal ulceration. While rare petechiae and skin reddening were observed in this study, ulceration was not. Differences between natural infection and artificial challenge may reflect the IC challenge route and/or fish age. Fingerlings used in these studies were much smaller than the

stocker and market-size fish usually affected in commercial ponds. Consistent with previous descriptions of *E. piscicida* infection, the classic "hole-in-the-head" lesion often associated with *E. ictaluri* and chronic ESC was observed in a single EPHD channel catfish 10 dpc (Griffin et al., 2019; López-Porras et al., 2021; Newton et al., 1989).

Additional non-inflammatory lesions seen with all *Edwardsiella* spp. included gastric edema, gill lamellar hyperplasia, gastric glandular apoptosis, and exocrine pancreatic necrosis. While gastric edema has been associated with infection by other catfish bacterial pathogens, such as virulent *A. hydrophila* (Baumgartner et al., 2017), corresponding hemorrhage and lymphangitis did not occur in the *Edwardsiella* challenges. The lack of bacteria and associated inflammation suggests gill lamellar hyperplasia resulted from environmental causes rather than infection. Pancreatic changes were more frequent in *E. anguillarum* challenged fish but did occur sporadically following exposure to *E. piscicida* and *E. tarda*. However, changes were more consistent with apoptosis rather than necrosis as described previously for *E. anguillarum* (Reichley 2017). Similar findings with no evident pathogeneses were observed in previous *E. piscicida* studies, leading to speculation of soluble host inflammatory cell factor release, bacterial product release, or hypoxia due to blood flow alterations (López-Porras et al., 2021).

Identification of effective measures, such as vaccination, to prevent losses from *E. piscicida* and *E. ictaluri* are critical to industry profitability (Griffin et al., 2019; Kumar et al., 2019). Current management practices, including feed restriction and medicated feeds, contribute to lost production, antibiotic resistance, and economic losses (Griffin et al., 2017). This study supports previous work demonstrating improved survival in fish following exposure to *E. piscicida* and subsequent challenge with *E. ictaluri* (Griffin et al., 2020a). These findings, coupled with reports of protection conferred by a live-attenuated *E. ictaluri* vaccine against *E.*

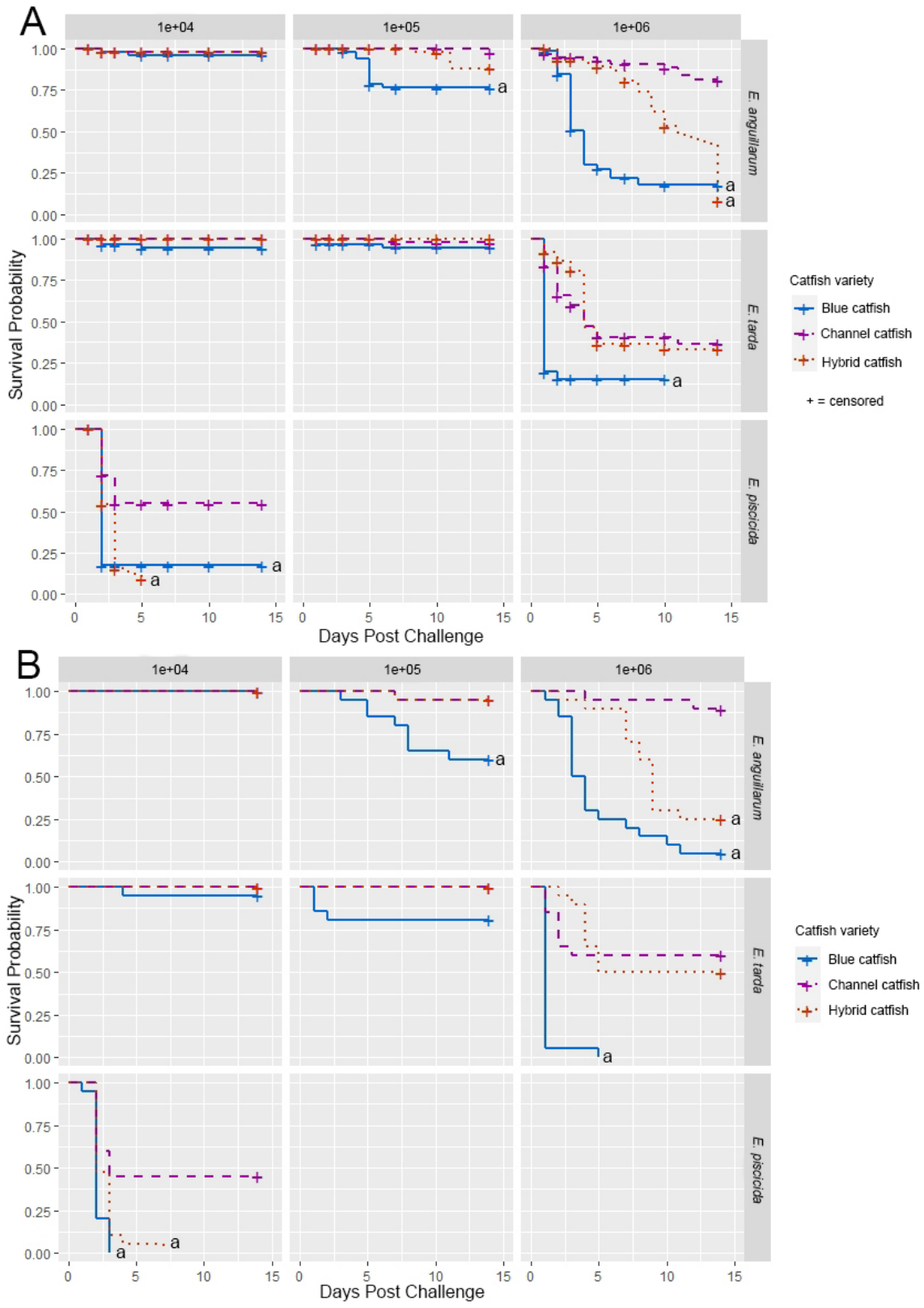
piscicida infection in channel catfish (Griffin et al., 2020a; López-Porras et al. 2022), suggest the various *Edwardsiella* congeners have potential as cross-protective vaccine candidates. While an effective live-attenuated *E. ictaluri* vaccine exists (Wise et al., 2015; 2020), utilization of other *Edwardsiella* species in a combinatory fashion may optimize protection, especially in hybrids where vaccine efficacy may wane against high doses of *E. piscicida* (Griffin at al., 2020a). Collectively, these studies highlight the potential of *E. piscicida* as a vaccine candidate. Given the difficulties of inducing disease by immersion or oral delivery of wild-type *E. piscicida* isolates, vaccination with *E. piscicida* may be less likely to induce clinical disease but immunogenicity with oral vaccination requires additional study (Griffin et al., 2020a; López-Porras et al., 2022; Reichley, 2017). Comparably, *E. anguillarum* may also confer some cross-protection but would require a significantly higher dose.

Although duration of immunity from *Edwardsiella* spp. exposure is not well studied, protective responses persisted for 100 days in this study. While fish were challenged IC to ensure a reliable and repeatable study model, translation of the observed immunity to natural infections, immersion baths, and oral delivery is unclear. Griffin et al. (2020a) reported immersion in wild-type *E. piscicida* produced 89-100% relative percent survival in channel catfish exposed to *E. ictaluri* 35 days post-immunization. López-Porras et al. (2022) reported similar protective effects afforded by heterologous *E. piscicida* isolates against *E. ictaluri*. While promising, results herein should be supported by more robust, replicated studies with larger sample sizes, particularly for blue catfish, which had the lowest survival in the initial challenges. While potential cross-protection from *E. anguillarum* and *E. piscicida* against the other *Edwardsiella* spp. warrants further investigation, *E. tarda* elicited no cross-protection against *E. ictaluri* and *E. piscicida* in any catfish types. This is not surprising given studies showing substantial phylogenetic distances

between *E. tarda* and the other *Edwardsiella* spp. fish pathogens (Griffin et al., 2013; 2014; Reichley, 2017; Shao et al., 2015).

Findings show *E. piscicida* is more pathogenic in channel, blue, and hybrid catfish than *E. anguillarum* or *E. tarda*, indicating the latter two pathogens to be of minimal concern to the southeastern United States catfish industry. Mortality risk varies between catfish species, with blues and hybrids having higher mortality to all three bacteria than channel catfish. This varied susceptibility between the three commercially important catfish types limits direct pathologic comparisons at equivalent bacterial doses. Challenge doses sufficient to induce disease in one host are inadequate to induce disease in another, which precludes direct comparisons and complicates analyses. While predominantly considered a hybrid catfish pathogen, *E. piscicida* has potential to evoke significant mortality in blue catfish, of which broodstock are essential for hybrid production. While disease time course, catfish type, and infective dose help distinguish lesions induced experimentally by the three bacterial species, histopathologic changes alone are not sufficient to reliably differentiate the similar associated diseases, especially in diagnostic settings where detailed clinical information is often lacking. Lastly, given their inability to induce severe disease in healthy fish by typical exposure routes, additional work exploiting the cross-protective potential of *E. piscicida* and *E. anguillarum* against *E. piscicida* and *E. ictaluri* in channel and hybrid catfish is a justified line of research.

Figures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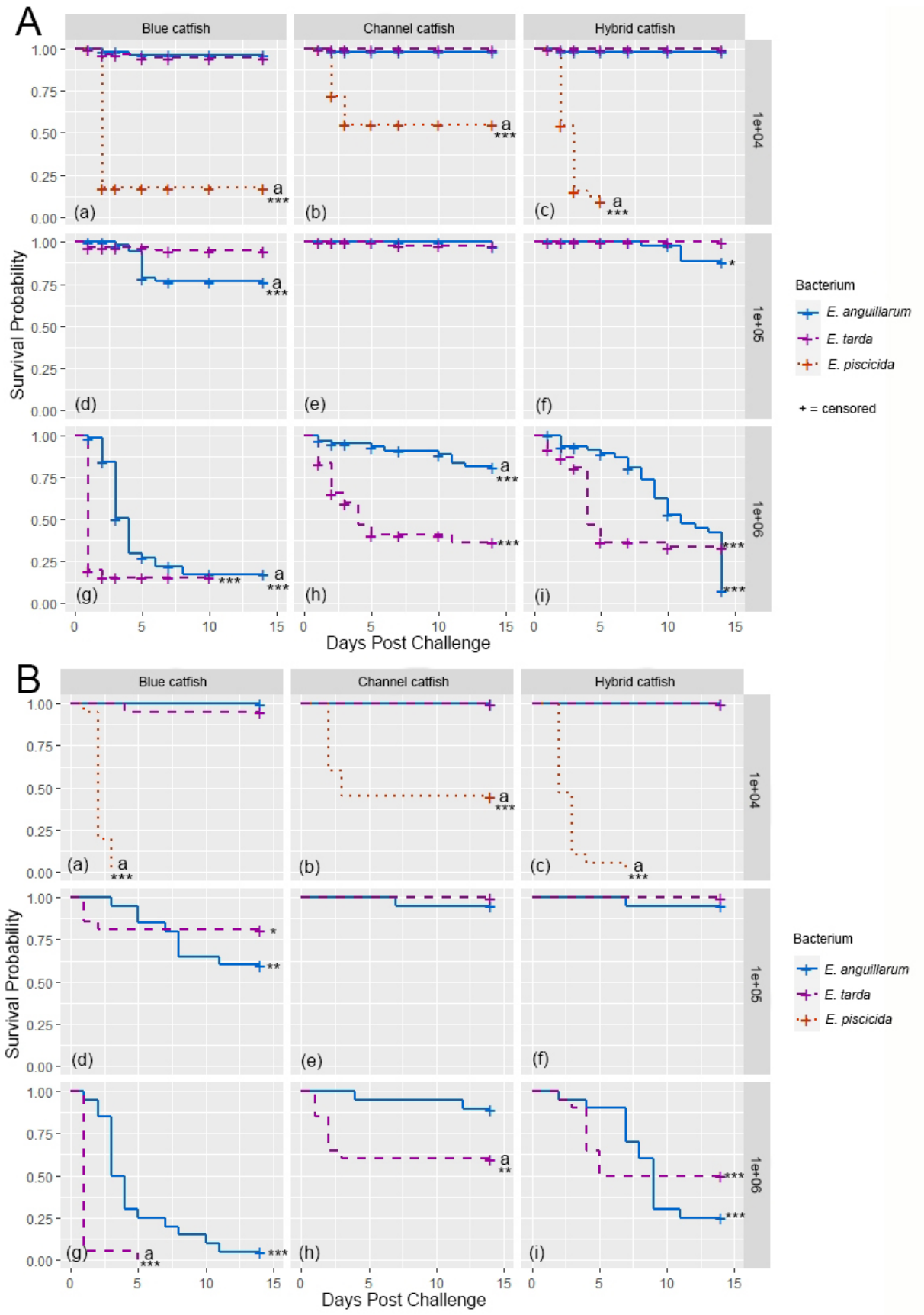
1e+04
1e+05
1e+06
E. anguillarum
E. tarda
E. piscicida
Survival Probability
Days Post Challenge
Catfish variety
Blue catfish
Channel catfish
Hybrid catfish
+ = censored
B
1e+04
1e+05
1e+06
E. anguillarum
E. tarda
E. piscicida
Survival Probability
Days Post Challenge
Catfish variety
Blue catfish
Channel catfish
Hybrid catfish

Figure 2.1. Kaplan-Meier survival curves comparing catfish variety. A) Kaplan-Meier survival curves with censoring from sampling tanks (n = 20 fish each tank; 3 tanks) for delivered doses of *Edwardsiella anguillarum*, *Edwardsiella tarda*, and *Edwardsiella piscicida* in channel, blue, and hybrid catfish. Controls (1.0 survival probability) are excluded from the figure. Survival probabilities for hybrid and blue catfish were compared to channel catfish using Cox proportional hazards models. Significant differences ($p < 0.01$) are indicated by "a". No challenges were performed at the 1e+05 or 1e+06 levels for *E. piscicida.* Doses approximate colony forming units (CFU) per gram of fish. B) Kaplan-Meier survival curves from non-sampling tanks (n = 20 fish each tank; one tank) for delivered doses of *E. anguillarum*, *E. tarda,* and *E. piscicida* in channel, blue, and hybrid catfish. Controls (1.0 survival probability) are excluded from the figure. Survival probabilities for hybrid and blue catfish were compared to channel catfish using Cox proportional hazards models. Significant differences ($p < 0.05$) are indicated by "a". Data was transformed when one or more treatment's mortality equaled 0%. No challenges were performed at the 1e+05 or 1e+06 levels for *E. piscicida.* Doses approximate colony forming units (CFU) per gram of fish.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Blue catfish
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E. piscicida
+ = censored
B
Blue catfish
Channel catfish
Hybrid catfish
1e+04
1e+05
1e+06
Survival Probability
Days Post Challenge
Bacterium
E. anguillarum
E. tarda
E. piscicida

Figure 2.2. Kaplan-Meier survival curves comparing *Edwardsiella* bacterial challenges. A) Kaplan-Meier survival curves with censoring from sampling tanks (n = 20 fish each tank; three tanks) for channel, blue, and hybrid catfish at comparable delivered doses of *Edwardsiella anguillarum*, *Edwardsiella tarda*, and *Edwardsiella piscicida*. Controls (1.0 survival probability) are excluded from the figure. Bacteria were individually compared to controls using log-tank tests (* = $p < 0.05$; *** = $p < 0.001$). (a-c) Kaplan-Meier survival curves for catfish exposed to *E. tarda* and *E. anguillarum* were compared to fish exposed to *E. piscicida* using Cox proportional hazards models. Significant differences ($p < 0.05$) are indicated by "a". (d-i) Kaplan-Meier survival curves for catfish exposed to *E. tarda* were compared to fish exposed to *E. anguillarum* using Cox proportional hazards models. Significant differences ($p < 0.05$) are indicated by "a". No challenges were performed at the 1e+05 or 1e+06 levels for *E. piscicida.* Doses approximate colony forming units (CFU) per gram of fish. B) Kaplan-Meier survival curves from non-sampling tanks (n = 20 fish each tank; 1 tank) for channel, blue, and hybrid catfish at comparable delivered doses of *E. anguillarum*, *E. tarda*, and *E. piscicida*. Controls (1.0 survival probability) are excluded from the figure. Bacteria were individually compared to controls using log-tank tests (* = $p < 0.05$; *** = $p < 0.001$). (a-c) Kaplan-Meier survival curves for catfish exposed to *E. piscicida* were compared individually to catfish exposed to *E. tarda* and *E. anguillarum* using log-rank tests. Significant differences ($p < 0.05$) are indicated by "a". (d-i) Kaplan-Meier survival curves for catfish exposed to *E. tarda* were compared to fish exposed to *E. anguillarum* using log-rank tests. Significant differences ($p < 0.05$) are indicated by "a". No challenges were performed at the 1e+05 or 1e+06 levels for *E. piscicida.* Doses approximate colony forming units (CFU) per gram of fish.

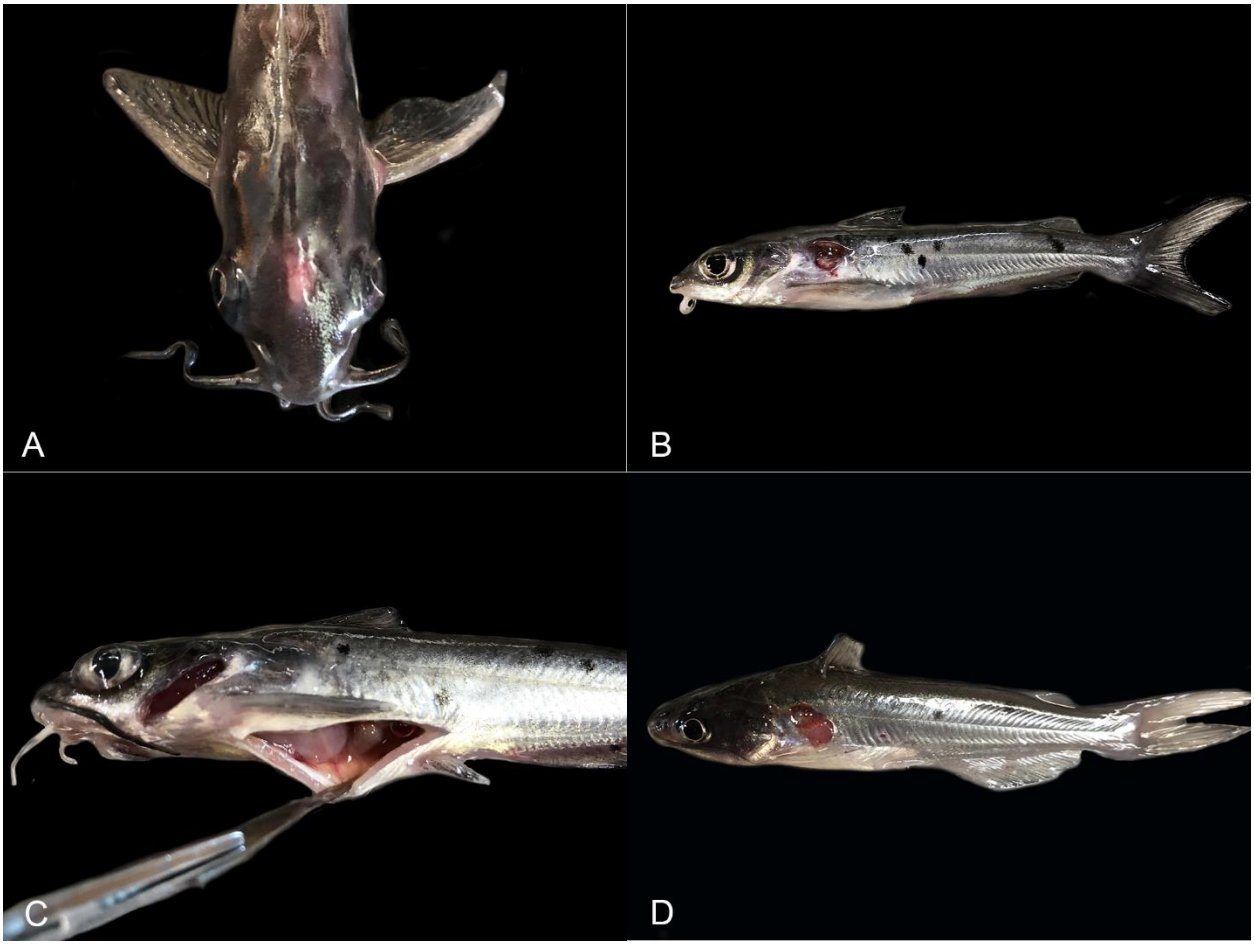

Figure 2.3. Gross lesions of *Edwardsiella* spp. infection. A) *Edwardsiella piscicida* infection, channel catfish, 10 days post challenge (dpc). The skin overlying cranial fontanelle is moderately raised, light pink, and minimally ulcerated. B) *Edwardsiella tarda* infection, channel catfish, 2 dpc. The lateral body wall is transmurally ulcerated caudal to the operculum. C) *E. tarda* infection, hybrid catfish, 2 dpc. The transmural body wall ulceration exposes the underlying swim bladder and coelomic viscera. D) *E. anguillarum* infection, channel catfish, 5 dpc. The coelomic cavity contains a moderate amount of light pink to yellow, serosanguinous, thin liquid.

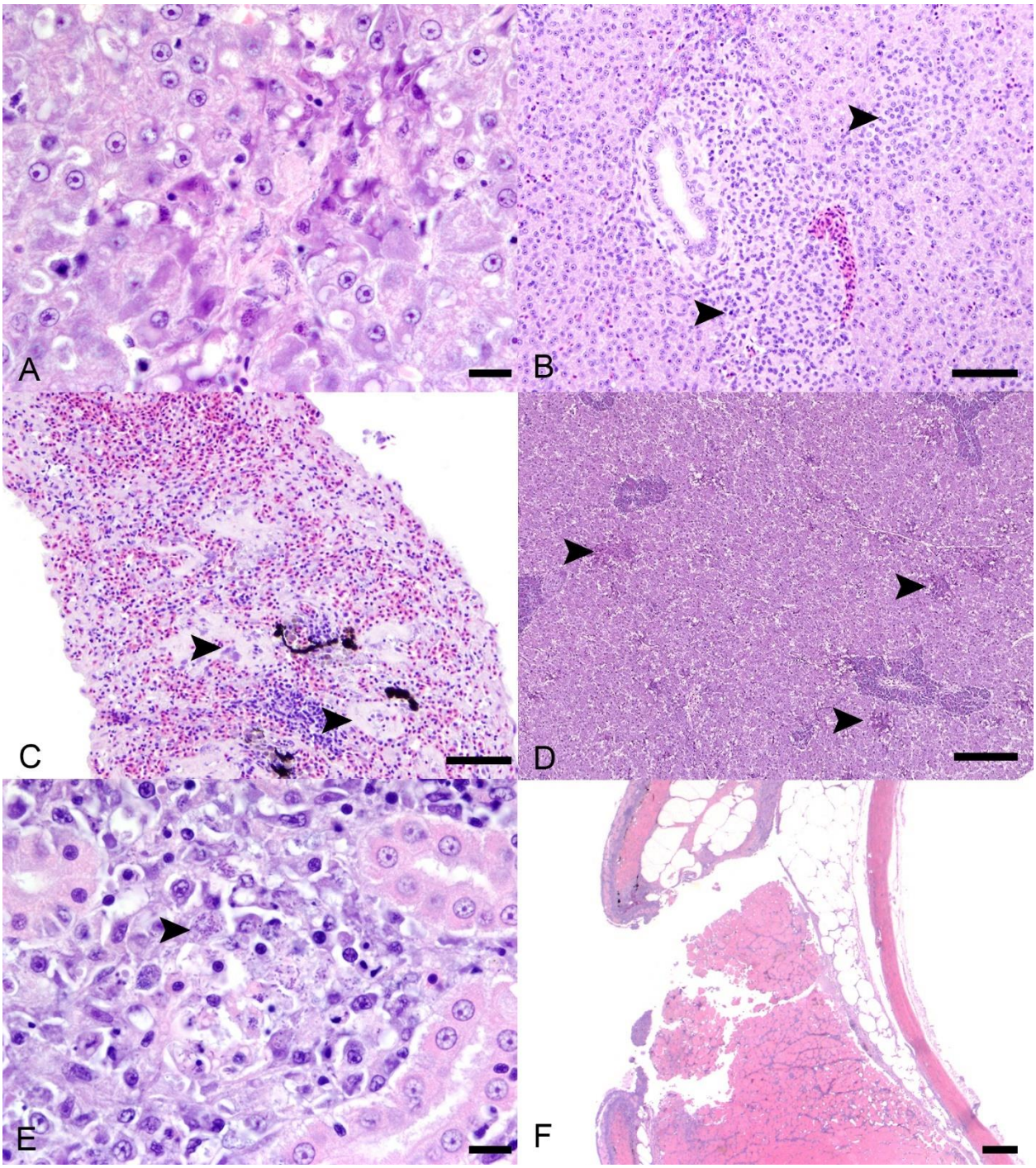

Figure 2.4. Histopathology of *Edwardsiella* spp. necrotizing and inflammatory lesions. A) Liver, hybrid catfish, *E. piscicida* low dose, 3 dpc. Acute necrotic areas characterized by hypereosinophilic cells with pyknotic nuclei, scattered cellular debris, and loss of cellular detail. Eosinophilic fibrin, abundant bacterial rods (arrowhead) and cellular debris are within the lesion

center. Haematoxylin and eosin stain (H&E). Bar = 10 µm. B) Liver, blue catfish, *E. anguillarum* middle dose, 14 dpc. Multifocal, well-demarcated aggregates of macrophages (arrowheads) infiltrate the parenchyma and contain rare intracytoplasmic bacterial rods better visualized in figure (a) above. H&E. Bar = 50 µm C) Spleen, channel catfish, *E. tarda* high dose, 1 dpc. Severely congested red pulp highlights necrotic splenic ellipsoids (arrowheads) containing abundant extracellular bacterial rods and karyorrhectic debris better visualized at higher magnification. H&E. Bar = 50 µm. D) Liver, blue catfish, *E. piscicida* high dose, 3 dpc. Multiple random foci of necrosis (arrowheads) with swollen, hypereosinophilic hepatocytes containing pyknotic nuclei. H&E. Bar = 150 µm. E) Posterior kidney, hybrid catfish, *E. piscicida* low dose, 3 dpc. Interstitial hematopoietic tissue is effaced by a focal area of necrosis, characterized by swollen hypoeosinophilic cells, loss of cellular detail, karyorrhectic debris, and abundant bacterial rods (arrowhead). H&E. Bar = 10 µm. F) Lateral body wall, hybrid catfish, *E. tarda* high dose, 3 dpc. Ulceration and transmural necrosis within an extensive area of body wall. Necrotic and adjacent intact skeletal muscle is expanded by hemorrhage, macrophages, lymphocytes, karyorrhectic debris, and small numbers of bacterial rods. H&E. Bar = 200 µm.

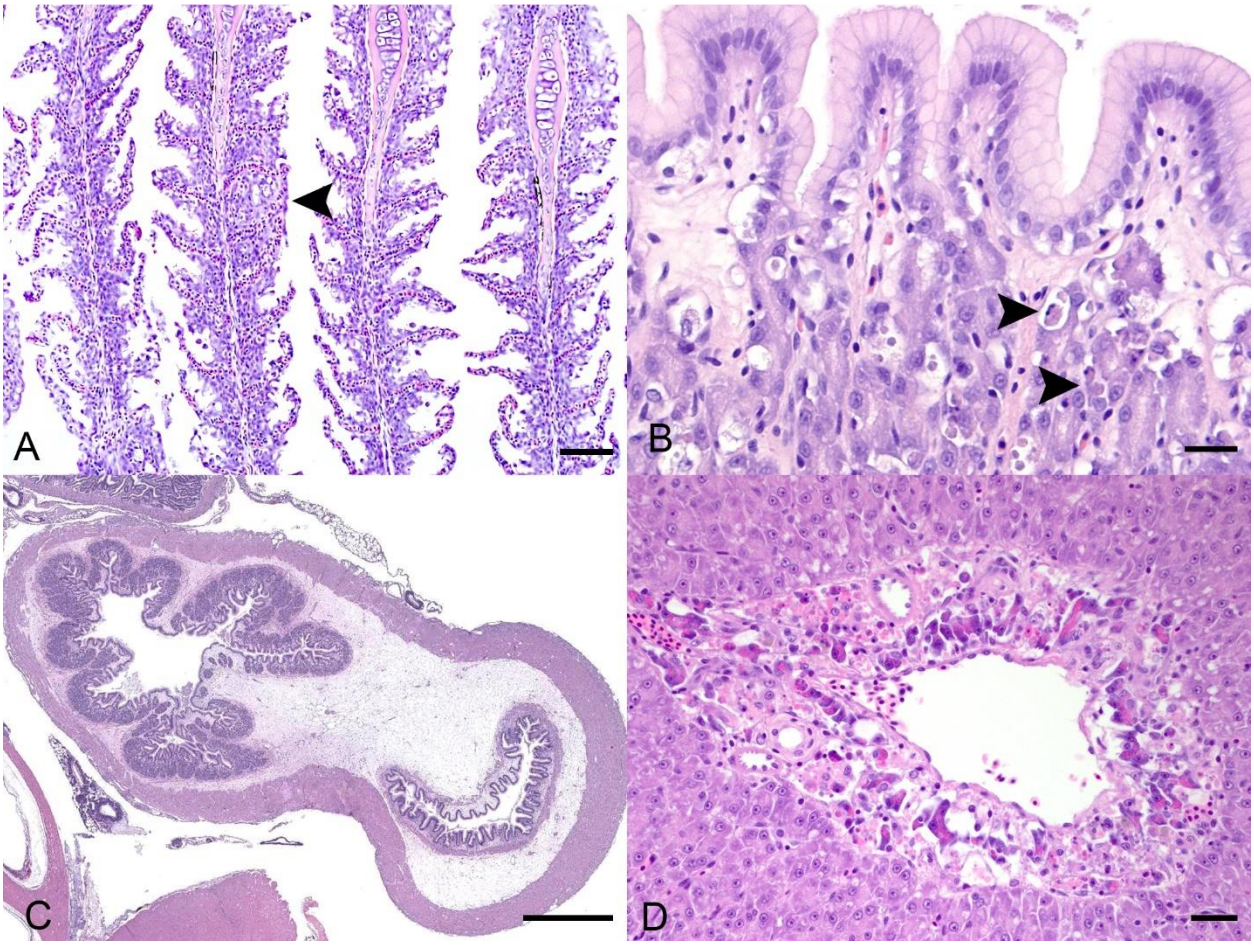

Figure 2.5. Histopathology *Edwardsiella* spp. non-inflammatory lesions. A) Gills, hybrid catfish, *E. anguillarum* medium dose, 5 dpc. Epidermal hyperplasia multifocally expands lamellae and fills interlamellar troughs (arrowhead) accompanied by low numbers of mucous and inflammatory cells. H&E. Bar = 50 µm. B) Stomach, channel catfish, *E. anguillarum* high dose, 2 dpc. Clear space surrounds apoptotic gastric glandular cells that are shrunken, rounded, and hypereosinophilic with pyknotic nuclei (arrowheads). H&E. Bar = 20 µm. C) Stomach, channel catfish, *E. anguillarum* high dose, 2 dpc. The submucosa is diffusely expanded by abundant clear space characteristic of severe edema. H&E. Bar = 1 mm. D) Intrahepatic exocrine pancreas, channel catfish, *E. tarda* high dose, 3 dpc. Pancreatic cells surrounding hepatic venules are often

shrunken and surrounded by clear space with decreased cytoplasm, decreased zymogen granules, and/or condensed nuclei. H&E. Bar = 20 µm.

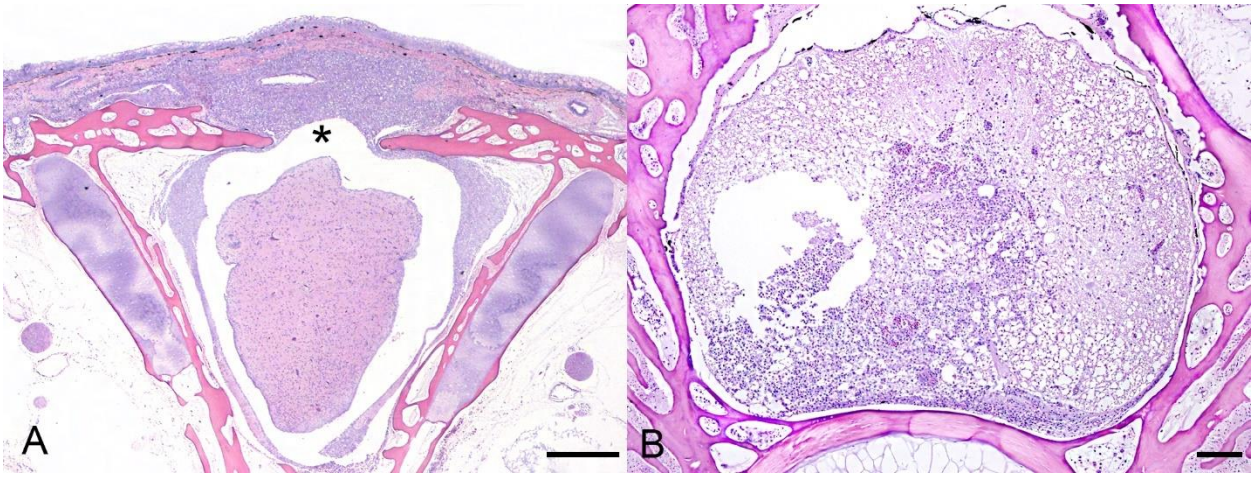

Figure 2.6. Central nervous system lesions in an *Edwardsiella piscicida* high dose challenged channel catfish 10 dpc. A) Brain. The forebrain meninges are markedly expanded by sheets of granulomatous inflammation, predominated by macrophages, that extends through the cranial fontanel (asterisk) to infiltrate the dermis and elevate the overlying epidermis. The neuroparenchyma is minimally affected. H&E. Bar = 500 µm. B) Spinal cord. Similar inflammatory infiltrates efface a focally extensive area of the spinal cord and ventral meninges, with marked vacuolation of the adjacent spinal tissue and dorsal displacement of the central canal. H&E. Bar = 100 µm.

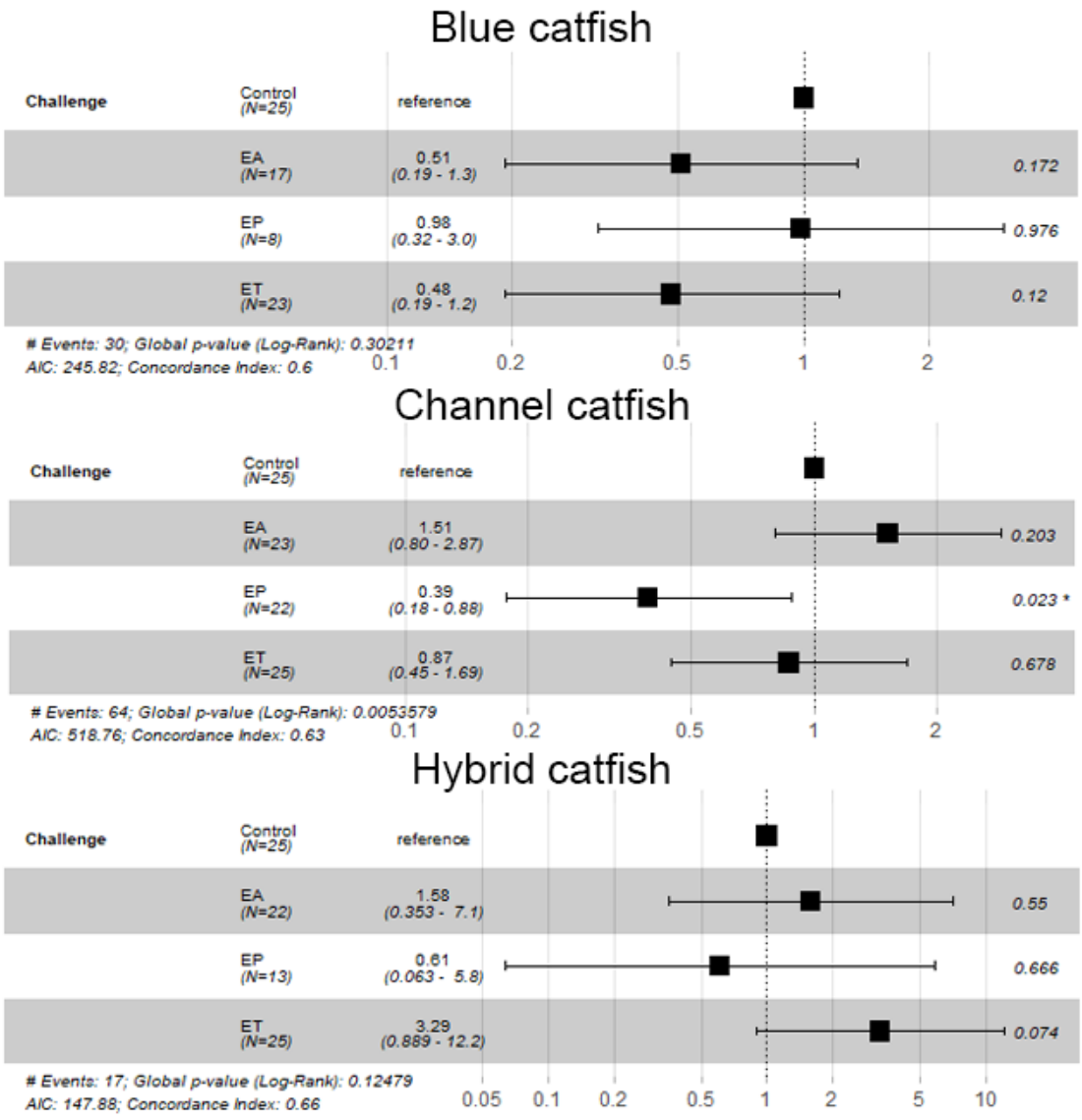

Figure 2.7. Cox proportional hazard models for blue, channel, and hybrid catfish initially exposed to *E. anguillarum* (EA), *E. piscicida* (EP), or *E. tarda* (ET) and subsequently exposed to *E. ictaluri* via immersion bath or a sham challenge. Results indicate probability of mortality compared to naïve controls. Risk ratio of 1 (dotted lines) indicate risk of mortality equal to naïve controls. Treatments with whiskers lying fully to the left or right of the dotted line indicate a significant reduction or increase in risk of mortality, respectively. * = $p < 0.05$. Concordance index is a goodness-of-fit metric with values > 0.5 indicating a model with discriminatory power.

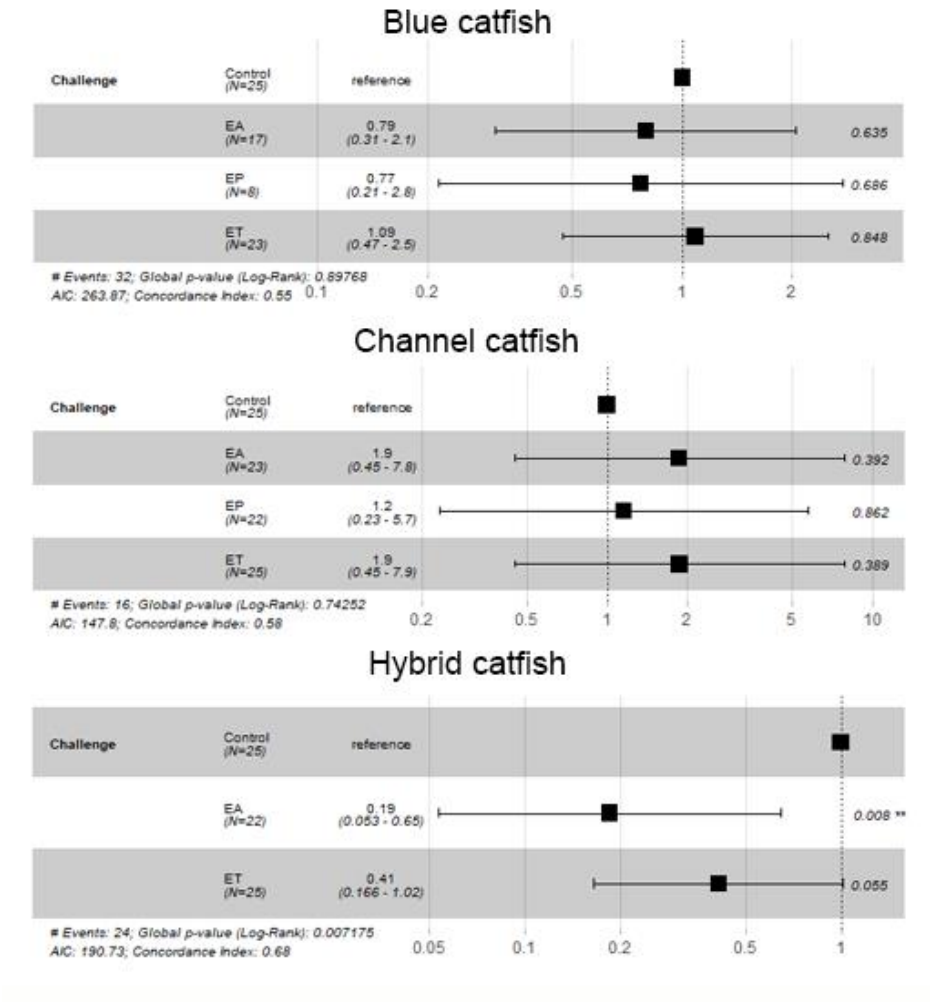

Figure 2.8. Cox proportional hazard ratios comparing blue, channel, and hybrid catfish initially exposed to *E. anguillarum* (EA), *E. piscicida* (EP), or *E. tarda* (ET) and subsequently exposed to *E. piscicida* via intracelomic injection or a sham challenge (** = $p < 0.01$). Risk ratio of 1 (dotted lines) indicate risk of mortality equal to naïve controls. Treatments with whiskers lying fully to the left or right of the dotted line indicate a significant reduction or increase in risk of mortality, respectively. *E. piscicida* in hybrids is excluded from the figure based on 0% mortality in hybrids previously challenged with *E. piscicida* but was significantly different ($p < 0.01$) than naïve controls based on a log-rank test. Concordance index is a goodness-of-fit metric with values > 0.5 indicating a model with discriminatory power.

Tables

Table 2.1. Bacterial isolates and respective doses used in experimental trial one.

Bacterial isolates	**CFU/g of fish**	**Abbreviations used in paper**
Edwardsiella anguillarum Isolate LADL05-105	1.60-1.67x10^4 (EALD)	*E. anguillarum* low dose (EALD)
	1.39-1.93x10^5 (EAMD)	*E. anguillarum* medium dose (EAMD)
	1.60-1.67x10^6 (EAHD)	*E. anguillarum* high dose (EAHD)
Edwardsiella piscicida Isolate S11-285	4.38-5.04x10^3 (EPLD)	*E. piscicida* low dose (EPLD)
	2.31-2.40x10^4 (EPHD)	*E. piscicida* high dose (EPHD)
Edwardsiella tarda Isolate FL95-01	1.28-1.33x10^4 (ETLD)	*E. tarda* low dose (ETLD)
	1.57-2.17x10^5 (ETMD)	*E. tarda* medium dose (ETMD)
	1.28-1.33x10^6 (ETHD)	*E. tarda* high dose (ETHD)
BHIb control	0	

BHIb = brain-heart infusion broth
CFU = colony-forming units

Table 2.2. Pooled cumulative percent mortality for fish initially exposed (1st exposure) to *E. anguillarum* (EAMD; 1.39-1.93x10^5 CFU/g fish), *E. piscicida* (EPLD; 4.38-5.04x10^3 CFU/g fish), or *E. tarda* (ETMD; 1.57-2.17x10^5 CFU/g fish) and subsequently challenged with intracoelomic (IC) injections of *E. piscicida* or by bath immersion (IM) in *E. ictaluri*. Statistical analysis is presented in Figs. 9 and 10.

1st Exposure	**2nd Exposure Cumulative Mortality**		
Blue catfish			
	E. piscicida	*E. ictaluri*	Sham
E. piscicida (n=8 fish/tank)	37.5%	50.0%	0.0%
E. anguillarum (n=17 fish/tank)	41.2%	17.6%	0.0%
E. tarda (n=23 fish/tank)	47.8%	30.4%	0.0%
Naïve (n=25 fish/tank)	44.0%	52.0%	IC 4.0% IM 0.0%
Channel catfish			
	E. piscicida	*E. ictaluri*	Sham
E. piscicida (n=22 fish/tank)	13.6%	40.9%*	0.0%
E. anguillarum (n=23 fish/tank)	21.7	87.0%	0.0%
E. tarda (n=25 fish/tank)	20.0%	68.0%	0.0%
Naive (n=25 fish/tank)	12.0%	72.0%	IC 0.0% IM 0.0%
Hybrid catfish			
	E. piscicida	*E. ictaluri*	Sham
E. piscicida (n=13 fish/tank)	0.0%†	7.7%	0.0%
E. anguillarum (n=22 fish/tank)	13.6%**	18.2%	0.0%
E. tarda (n=25 fish/tank)	28.0%	36.0%	0.0%
Naive (n=25 fish/tank)	68.0%	12.0%	IC 0.0% IM 0.0%

IC = intracoelomic injection
IM= immersion exposure
* = $p < 0.05$; ** = $p < 0.01$; Cox proportional hazard ratios in comparison to naïve controls
† = $p < 0.01$; Log-rank test in comparison to naïve controls

Table 2.3. Summary of tissue distribution and other lesions in *E. tarda* medium dose and high dose challenges.*

		Day 1			Day 2			Day 3			Day 5			Day 10			Day 14		
	Dose	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**
								Inflammatory/necrotizing/hemorrhagic lesions											
Coelom	Med	3/3	3/3	3/3	2/3	3/3	3/3	2/3	1/3	2/3	1/3	1/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3
	High	3/3	3/3	2/3	3/3	3/3	1/2	3/3	3/3	0/1	3/3	2/3	0/1	2/3	2/3	0/0	1/3	1/3	0/0
Spleen	Med	0/3	0/3	1/3	0/3	0/3	0/3	0/2	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/2	0/3	0/3	0/3
	High	1/3	2/2	0/1	0/3	3/3	0/1	2/3	1/2	0/1	2/3	1/3	0/1	0/2	0/3	0/0	0/3	0/3	0/0
Liver	Med	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	0/3	1/3	0/2	1/3	1/3	0/1	3/3	2/3	0/1	0/3	0/3	0/0	0/3	0/3	0/0
Brain	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	2/3	0/3	0/3	0/3	1/3	0/2	0/3	0/3	0/1	0/3	0/3	0/1	0/3	0/3	0/0	0/3	0/3	0/0
Anterior kidney	Med	0/3	0/1	0/0	0/2	0/1	0/2	0/2	0/2	0/2	0/2	0/1	0/1	0/2	0/1	0/3	0/2	0/2	0/1
	High	1/3	1/2	0/2	0/3	0/2	0/2	0/2	1/2	0/1	2/2	1/2	0/1	0/3	0/3	0/0	0/2	0/3	0/0
Posterior kidney	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	2/3	1/3	0/3	0/3	0/3	0/2	2/3	3/3	1/1	2/3	2/3	0/1	1/3	0/3	0/0	0/3	0/3	0/0
Spinal cord	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	0/3	0/3	0/2	0/3	0/3	0/1	0/3	0/3	0/1	0/3	0/3	0/0	0/3	0/3	0/0
Gill	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	1/3	1/3	1/3	0/3	0/3	0/2	0/3	0/3	0/1	0/3	0/3	0/1	0/3	0/3	0/0	0/3	0/3	0/0
Skin	Med	0/3	0/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	1/3	0/3	1/3	1/3	0/2	0/3	0/3	0/1	0/3	2/3	0/1	0/3	0/3	0/0	0/3	0/3	0/0
Swim bladder	Med	1/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	1/3	0/3	2/3	0/3	0/2	1/3	0/3	0/1	1/3	0/3	0/1	0/3	0/3	0/0	0/3	0/3	0/0
								Other lesions											
Gill lamellar hyperplasia	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	0/3	0/3	0/2	0/1	0/3	0/1	0/3	0/3	0/1	0/3	0/3	0/0	0/3	0/3	0/0
Gastric apoptosis	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	2/3	2/3	0/3	3/3	0/3	0/2	2/3	1/3	0/1	0/3	0/3	0/1	0/3	0/3	0/0	0/3	0/3	0/0
Gastric edema	Med	0/3	1/3	3/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	3/3	2/3	0/3	3/3	2/3	0/2	2/3	3/3	0/1	0/3	0/3	0/1	0/3	0/3	0/0	0/3	0/3	0/0
Pancreatic apoptosis	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	2/3	1/3	0/2	2/3	1/3	0/1	3/3	0/3	0/3	0/3	0/3	0/0	0/3	0/3	0/0

Ch = channel catfish; Hy = hybrid catfish; Bl = blue catfish

*With sample size less than three, fish were either not available for sampling due to mortality or tissue was not captured in histologic section.

Table 2.4. Summary of tissue distribution and other lesions in *E. anguillarum* medium dose and high dose challenges.*

		Day 1			Day 2			Day 3			Day 5			Day 10			Day 14		
	Dose	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**
Inflammatory/necrotizing/hemorrhagic lesions																			
Coelom	Med	2/3	1/3	1/3	1/3	0/3	0/3	2/3	2/3	2/3	1/3	2/3	3/3	1/3	2/3	2/3	2/3	0/3	1/3
	High	3/3	3/3	2/3	3/3	3/3	2/3	1/3	3/3	3/3	3/3	1/3	1/3	3/3	2/3	0/1	1/3	2/3	1/1
Spleen	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/2	0/3	0/2	0/3	0/3	0/3	0/3	0/2	0/3	0/3	0/2	0/2
	High	3/3	1/1	2/3	1/3	0/1	2/3	0/2	0/3	0/3	2/3	0/2	0/3	3/3	2/3	1/1	1/2	0/2	0/1
Liver	Med	0/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3	1/3	0/3	1/3	0/3	0/3	0/3	1/3	1/3	0/3	1/3
	High	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	1/3	2/3	0/3	1/3	1/3	0/3	1/1	2/3	0/3	0/1
Brain	Med	0/3	0/3	0/3	1/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	1/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/1
Anterior kidney	Med	0/2	0/2	0/2	0/2	0/2	0/3	1/2	0/1	0/3	0/3	0/1	0/1	0/3	0/3	0/2	1/3	0/3	0/2
	High	1/2	0/3	1/2	0/2	0/1	1/2	0/2	0/2	0/3	0/2	0/2	0/2	1/1	0/3	0/1	2/2	0/3	0/1
Posterior kidney	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3
	High	1/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	0/1	2/3	0/3	0/1
Spinal cord	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/1	0/3	0/3	0/1
Gill	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/1	0/3	0/3	0/1
Skin	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	2/3	1/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	0/3	2/3	0/3	0/3	0/3	0/3	2/3	0/3	0/3	1/3	0/3	0/1	1/3	0/3	0/1
Swim bladder	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	1/3	0/3	0/3	1/3	0/3	0/3	1/3	0/3	0/3	2/3	0/3	0/3	1/3	0/3	0/1	2/3	0/3	0/1
Other lesions																			
Gill lamellar hyperplasia	Med	0/3	0/3	0/3	2/3	0/3	0/3	1/3	1/3	3/3	1/3	3/3	3/3	0/3	0/3	0/3	0/3	0/3	1/3
	High	1/3	1/3	0/3	0/3	0/3	2/3	1/3	3/3	3/3	3/3	3/3	1/3	2/3	2/3	0/1	2/3	0/3	0/1
Gastric apoptosis	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	2/3	0/3	0/3	0/3	0/3	0/3	2/3	1/3	0/3	0/3	1/3	0/1	1/3	0/3	0/1
Gastric edema	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	1/3	3/3	2/3	2/3	1/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/1	0/3	0/3	0/1
Pancreatic apoptosis	Med	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	1/3	2/3	0/3	0/3	1/3
	High	0/3	0/3	0/3	1/3	0/3	1/3	1/3	0/3	0/3	3/3	3/3	0/3	3/3	2/3	1/1	2/3	0/3	0/1

Ch = channel catfish; Hy = hybrid catfish; Bl = blue catfish

*With sample size less than three, fish were either not available for sampling due to mortality or tissue was not captured in histologic section.

Table 2.5. Summary of tissue distribution and other lesions in *E. piscicida* challenges.*

		Day 1			Day 2			Day 3			Day 5			Day 10			Day 14		
	Dose	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**	**Ch**	**Hy**	**Bl**
Inflammatory/necrotizing/hemorrhagic lesions																			
Coelom	Low	0/3	3/3	2/3	3/3	1/3	1/3	3/3	3/3	2/3	2/3	3/3	1/3	0/3	0/3	0/3	1/3	1/3	0/3
	High	1/3	3/3	0/3	3/3	3/3	2/3	2/3	3/3	2/2	1/3	1/1	1/1	1/3	0/1	0/1	0/3	0/0	0/1
Spleen	Low	0/2	0/2	0/3	0/2	1/2	2/2	2/2	3/3	1/3	0/3	0/2	0/3	0/3	0/3	0/3	0/2	0/3	0/3
	High	0/2	0/1	0/3	2/2	2/2	0/3	0/2	1/2	1/2	0/3	0/1	0/1	1/1	0/1	0/1	0/2	0/0	0/1
Liver	Low	0/3	0/3	0/3	0/3	0/3	2/3	1/3	3/3	0/3	0/3	1/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	1/3	1/3	0/3	3/3	3/3	1/2	0/3	0/1	0/1	1/3	0/1	0/1	0/3	0/0	0/1
Brain	Low	0/3	0/3	0/3	0/3	1/3	2/3	1/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/2	0/3	0/1	0/1	1/3	0/1	0/1	0/3	0/0	0/1
Anterior kidney	Low	0/2	0/2	1/2	0/2	0/0	0/0	0/1	1/2	1/3	0/2	0/1	0/2	0/3	0/3	0/3	0/3	0/3	0/2
	High	0/2	0/2	0/1	1/2	0/1	0/1	0/1	0/3	1/1	0/2	0/1	0/0	0/3	0/1	0/1	0/2	0/0	0/1
Posterior kidney	Low	0/3	0/3	0/3	0/3	2/3	2/3	1/3	2/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/2	0/3	3/3	0/3	1/3	1/3	1/2	0/3	0/1	0/1	0/3	0/1	0/1	0/3	0/0	0/1
Spinal cord	Low	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/2	0/3	0/1	0/1	1/3	0/1	0/1	0/3	0/0	0/1
Gill	Low	0/3	0/3	0/3	0/3	1/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	0/3	1/3	0/3	1/3	2/3	1/3	0/3	0/1	0/1	0/3	0/1	0/1	0/3	0/0	0/1
Skin	Low	0/3	0/3	0/3	0/3	1/3	1/3	0/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	1/3	0/3
	High	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/2	0/3	0/1	0/1	1/3	0/1	0/1	0/3	0/0	0/1
Swim bladder	Low	0/3	0/3	0/3	1/3	0/3	0/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/2	2/3	0/3	0/3	1/3	1/3	0/2	0/3	0/1	0/1	0/3	0/1	0/1	0/3	0/0	0/1
Other lesions																			
Gill lamellar hyperplasia	Low	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	0/3	0/3	0/3	1/3	1/3	0/2	0/3	0/1	0/1	0/3	0/1	0/1	0/3	0/0	0/1
Gastric apoptosis	Low	0/3	0/3	0/3	0/3	1/3	0/3	3/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	3/3	1/3	0/2	1/3	2/3	0/2	0/3	0/1	0/1	0/3	0/1	0/1	0/3	0/0	0/1
Gastric edema	Low	0/3	1/3	0/3	0/3	0/3	1/3	3/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	1/3	3/3	0/2	1/3	2/3	0/2	0/3	1/1	0/1	0/3	0/1	0/1	0/3	0/0	0/1
Pancreatic apoptosis	Low	0/3	0/3	0/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	High	0/3	0/3	0/3	0/3	0/3	0/3	1/3	1/3	0/2	0/3	0/1	0/1	1/3	0/1	0/1	0/3	0/0	0/1

Ch = channel catfish; Hy = hybrid catfish; Bl = blue catfish

*With sample size less than three, fish were either not available for sampling due to mortality or tissue was not captured in histologic section.

CHAPTER 3

INTRASPECIFIC VARIATION OF *EDWARDSIELLA ANGUILLARUM* FROM NON-ANGUILLID FISH FROM VARIED GEOGRAPHIC ORIGINS [1]

[1]Armwood, A.R., Rose, D., Waldbieser, G.C., Woodyard, E., Richardson, B.M., Soto, E., Stein, C., Ucko, M., Ware, C., Camus, A.C., and M.J. Griffin. To be submitted to *Journal of Fish Diseases.*

Abstract

Edwardsiella anguillarum is a gram-negative, facultative intracellular bacterium, synonymous with previous descriptions of atypical, fish-pathogenic *Edwardsiella tarda*. Originally described from eels (*Anguilla* spp.) in 2015, *E. anguillarum* has become an increasingly important global fish pathogen, particularly in tilapia aquaculture. This study describes intraspecific phenotypic and genotypic variability among seventeen *E. anguillarum* isolates from non-anguillid fish hosts and varied geographic origins. Isolates demonstrated similar biochemical characteristics, with slight variation in motility and hydrogen sulfide production. Sequenced genomes were analyzed with repetitive extragenic palindromic sequence-based PCR (rep-PCR) and multilocus sequence analysis (MLSA). Rep-PCR with the ERIC II primer set revealed two distinct genetic clusters, while amplification strategies utilizing the GTG_5, BOX, or ERIC I&II primer sets yielded more uniform profiles. While rep-PCR deemed the isolates largely clonal, MLSA schemes using reference genes from published *Edwardsiella* MLSA studies revealed *E. anguillarum* isolates formed five discrete phylogroups. Large, approximately 91.4 kB, plasmids were identified in Costa Rican and Colombian isolates (9/17). This study provides a foundation for delineating drivers of intraspecific variation among *E. anguillarum* isolates from different hosts and geographic regions. Extension of this work is warranted as *E. anguillarum* isolates continue to be reported from new hosts and provenances.

Introduction

The gram-negative bacterium, *Edwardsiella anguillarum,* is an emergent pathogen from the family Hafniaceae associated with disease in diverse fish species (Adeolu et al., 2016; Shao et al., 2015). Previously referred to as "atypical, non-motile, fish-pathogenic *Edwardsiella tarda*", *E. anguillarum* is the latest species to be distinguished from *E. tarda* (Griffin et al., 2013; Matsuyama et al., 2005; Sakai et al., 2007; 2009; Shao et al., 2015; Yamada & Wakabayashi, 1998; 1999). Differentiation from *E. tarda* and the recently identified *Edwardsiella piscicida* relies on species-specific PCR or sequencing of appropriate discriminatory genes (Abayneh et al., 2012; 2013; Griffin et al., 2014; Reichley et al., 2015c; Reichley et al., 2017). Originally described from diseased eels (*Anguilla* spp.) in China in 2015, its presence in archived fish isolates dates back to 1994 (Reichley et al., 2017; Shao et al., 2015).

Despite the species name insinuating the primary hosts are anguillids, *E. anguillarum* also affects non-anguillid hosts including tilapia, blue striped grunt, striped bass, climbing perch, spotted snakehead, silver surfperch, rohu, milkfish, groupers, catfish, and sea bream, from North America, South America, Africa, Europe, and Asia (Abayneh et al., 2013; Armwood et al., 2019; Buján et al., 2018; Dubey et al., 2019; Elgendy et al., 2022; Griffin, 2020b; Griffin et al., 2014; Katharios et al., 2019; López-Porras et al., 2019; Matsuyama et al., 2005; Oguro et al., 2014; Oh et al., 2020; Rahmawaty et al., 2022; Reichley et al., 2017; Shao et al., 2015; Ucko et al., 2016). Matsuyama et al. (2005) investigated the pathogenicity of different "*E. tarda*" strains in yellowtail, flounder, and red sea bream. Based on nucleotide sequences deposited in GenBank, the isolate used in their study (FPC503) possesses genetic elements that share 98–100% sequence identity to multiple *E. anguillarum* genomes (Griffin et al., 2017). Challenges with FPC503 in yellowtail, flounder and red sea bream yielded significantly different mortality trends

than comparable challenges using typical, motile, fish pathogenic *E. tarda* isolate NUF806, which sequence data later identified as *E. piscicida* (Griffin et al. 2017). These early works indicate variable pathogenicity among these two pathogens, which pose different risks for different aquacultured species.

Edwardsiella anguillarum is increasingly important in aquaculture, particularly in grouper, sharpsnout seabream, tilapia, and eel, with outbreaks reported in Costa Rica, Brazil, Korea, China, Israel, Greece, Egypt, and Peru (Armwood et al., 2019; da Costa et al., 2022; Elgendy et al., 2022; Katharios et al., 2015; 2019; López-Porras et al., 2019; Oh et al., 2020; Shao et al., 2015; Sierralta et al., 2020; Ucko et al., 2016). In tilapia, lesions range from multiorgan coagulative necrosis with abundant gram-negative bacterial rods to chronic, discrete granulomas, with sparse bacterial rods (Armwood et al., 2019; Elgendy et al., 2022; Oh et al., 2020). Affected grouper presented with severe suppurative nephritis and large abscesses in adjacent musculature, liver, spleen, and heart (Ucko et al., 2016). Sharpsnout sea bream exhibited grossly visible nodules in the spleen and kidneys (Katharios et al., 2015; 2019). Unlike its counterparts, *Edwardsiella ictaluri* and *E. piscicida*, *E. anguillarum* has minimal significance to US catfish aquaculture (Armwood et al., 2022; Reichley et al., 2018).

Intraspecific variation is described in other significant *Edwardsiella* species affecting fish, including *E piscicida* and *E. ictaluri*, demonstrating bacterial genomic plasticity within the genus (Castro et al., 2011; Griffin et al., 2016; López Porras, 2022; Nguyen et al., 2021; Reichley et al., 2017; Wang et al., 2011). As *E. anguillarum* has only recently been recognized as a separate entity from *E. tarda*, knowledge of diversity between isolates from varying geographic locations and fish host species is limited. Variable motility is reported within the species, with motility in isolates described by Shao et al. (2015), Dubey et al. (2019), and Griffin

et al. (2013) but not in isolates EA011113 and EA181011 described by Katharios et al. (2019) and Ucko et al. (2016), respectively, where the bacteria reportedly lacked flagella.

A French angelfish from a public display aquarium that had died and cultured positive for *E. anguillarum* was submitted postmortem to the Aquatic Pathology Service at the University of Georgia, which prompted this investigation. This study aims to characterize and compare phenotypic, genotypic, and antimicrobial sensitivity differences from a diverse collection of *E. anguillarum* isolates in non-anguillid fish from wide ranging geographic origins. Further, an optimized multilocus sequence analysis (MLSA) scheme is identified for future intraspecific *E. anguillarum* research using the most divergent genes from previously established MLSA schemes (Abayneh et al., 2012; Buján et al., 2018; Griffin et al., 2013).

Methods

Bacterial Isolates and Identification

Seventeen *E. anguillarum* isolates, *E. ictaluri* isolate S97-773, *E. piscicida* isolate S11-285, and *E. tarda* isolate FL95-01 were revived from archived cryopreserved samples at the Thad Cochran National Warmwater Aquaculture Center, Stoneville, MS, USA (Table 3.1). Some isolates have been identified in previous studies (Armwood et al., 2019; Reichley et al., 2017; Ucko et al., 2016), with isolates originating from collections at Louisiana State University, Mississippi State University, the University of California-Davis, the University of Georgia, and the United States Food and Drug Administration Center for Veterinary Medicine. Collection dates range from 1994 to 2019. Cryopreserved isolates were revived by isolation streaking on Mueller-Hinton II Agar (MHB; BBL™, Becton Dickinson and Company) plates supplemented with 5% sheep blood and grown for 24 h at 37ºC. Identities of all study isolates were confirmed

by *Edwardsiella* spp. specific multiplex real-time polymerase chain reaction (mPCR) (Reichley, et al., 2015c; 2017).

Phenotypic Characterization

Stabs of individual colonies from all *E. anguillarum* isolates and the control *E. ictaluri*, *E. piscicida*, and *E. tarda* isolates were inoculated into Remel™ motility test medium with Tetrazolium Chloride indicator (TTC) (Remel Inc., Lenexa, KS) and triple sugar iron (TSI) medium (Oxoid LTD) and incubated at 37ºC (28ºC for *E. ictaluri*) for 48 h. The motility medium was examined for dispersal, and the TSI medium was examined for gas, hydrogen sulfide production, and glucose, sucrose, and lactose fermentation. BBL™ Crystal™ Enteric/Nonfermenter Identification systems (BD, Franklin Lakes, NJ, USA) were inoculated with individual colonies of the bacteria for identification following the manufacturer's protocol. Panels were incubated at 37ºC (28ºC for *E. ictaluri*) for 24 h and corresponding numeric codes used for identification via the BBL CRYSTAL™ ID Systemic Electronic Codebook.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility to Aquaflor® (florfenicol), Romet® (5:1 sulfadimethoxine/ormetoprim), and Terramycin® (oxytetracycline) of all *E. anguillarum* isolates was tested by disk diffusion assays (Hudzicki, 2009) incubated at 37ºC for approximately 40 h. Minimum inhibitory concentrations (MICs) of select antimicrobial agents were also determined using Sensititre™ Vet Avian AVIAN1F plate formats (Thermo Scientific™) following the manufacturer's protocol with *Escherichia coli* ATCC 25922, *E. ictaluri* S97-733, *E. piscicida* S11-285, and *E. tarda* FL9501 used as quality control strains. Three to five colonies of each isolate were suspended in sterile water to match the density of a 0.5 McFarland Standard from which 10 µl were inoculated into 11 ml of cation-adjusted MHB. Fifty µl was added to each

well, and plates were incubated for 24 h at 37ºC, except *E. ictaluri* S97-733 which was incubated at 28ºC. The MIC value for each antibiotic was the lowest concentration with no visible bacterial growth following incubation.

DNA extraction

Individual colonies from revived isolates were expanded overnight in static 9 ml porcine brain-heart infusion broth (BHIb) cultures (Bacto; Becton Dickinson and Company) at 37ºC for 24 h (28ºC for *E. ictaluri*). Cultures were pelleted by centrifugation at 17,000 x *g* for 5 m and genomic DNA (gDNA) isolated from the pellet using the Gentra Puregene Yeast/Bact. Kit (Qiagen, Hilden, Germany). Harvested gDNA was resuspended in 100 µl of EB buffer and quantified spectrophotometrically using a NanoDrop 2000^{TM} (Thermo Fischer Scientific^{TM}). Identities were reconfirmed by mPCR (Reichley et al., 2015c; 2017) and gDNA stored at -80ºC for further analyses.

Repetitive sequence-mediated PCR analysis

Genomic DNA from all *E. anguillarum* and control isolates was analyzed by repetitive sequence-mediated PCR (rep-PCR) using the Enterobacterial Repetitive Intergenic Consensus (ERIC) I and II primers, BOX, and $(GTG)_5$ primers (Table 3.2) following modified protocols from Griffin et al., 2013, Versalovic et al., 1991, and Versalovic et al., 1994. The 30-µl reactions contained 15 µl of IQ^{TM} Supermix (2×) (Bio-Rad Laboratories, Inc.), ~50 ng of gDNA template, nuclease-free water to volume, and either 20 pmol (BOX, ERIC II, GTG_5) or 40 pmol (ERIC I and II). Amplifications were conducted on a C1000 Touch thermal cycler (Bio-Rad Laboratories, Inc.) under the following profiles: a denaturation cycle at 95ºC for 10 m; 5 cycles of 95ºC for 1 m, 40ºC for 1 m, and 72ºC for 5 m; 35 cycles of 95ºC for 1 m, 55ºC for 1 m, and 72ºC for 5 m; and final extension at 72ºC for 5 m. A 1.8% (weight/volume) agarose gel containing 0.3 µg/ml

ethidium bromide was run with 10 µl aliquots of each amplification reaction and the molecular weight standard HyperLadder^{TM} 50bp (Bioline; Meridian Life Sciences). Bands were visualized under ultraviolet light. Final analysis consisted of manual annotation of visibly distinct bands generated from each primer set, and assessment of genetic fingerprints using BioNumerics v7.6 (Applied Maths, Inc.). Dendrograms were generated from Dice coefficient matrices (2% tolerance) based on the unweighted pair-group method using arithmetic averages (UPGMA).

Genome sequencing

Complete genomes were obtained using the Rapid Barcode Sequencing kit (SQK-RBK004; Oxford Nanopore Technologies, Oxford, UK) with v9.4.1 flow cells on a GridION platform (Oxford Nanopore Technologies,) and Illumina paired end reads obtained on a NextSeq platform from the Georgia Genomics and Bioinformatics Core (Athens, GA). Raw nanopore reads were trimmed using NanoFilt to a minimum read quality of 10 and length of 2 kb (Table 3.3), yielding an average 156X genome coverage. Trimmed reads were assembled using Canu v.2.0 (Koren et al., 2017) at an average 42X coverage of canu-corrected reads. Basecall correction on the resulting contigs was initially performed with nanopore sequences using Medaka v1.1.3 (Oxford Nanopore Technologies Ltd.). Indels in the medaka-corrected contigs were subsequently polished using Pilon v1.24 (Walker et al., 2014) at an average 63-fold genome coverage using Illumina reads. These sequence data and annotations are available in GenBank® under accession numbers listed in Table 3.1. Isolates with sequences available in NCBI (EA181011 and LADL05-105) were used as internal sequencing controls, which yielded 100% identity to corresponding NCBI nucleotide sequences for the study reference genes.

Plasmid analysis

Native plasmid DNA harvest was attempted on all *E. anguillarum* isolates using a Qiagen Spin MiniPrep kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. *Edwardsiella ictaluri* isolate S97-773, *E. piscicida* isolate S11-285, and *E. tarda* isolate FL95-01 were included as controls. Harvested plasmids were separated on a 1.5% (weight/volume) agarose gel containing 0.3 µg/ml ethidium bromide and visualized under ultraviolet light. Plasmid sequence data obtained from the previously described Nanopore and Illumina sequencing was submitted to the GenBank® database under accession numbers listed in Table 3.1. Open reading frames (ORFs) were predicated and annotated through the NCBI prokaryotic genome annotation pipeline (PGAP). Circularized plasmids were aligned using the Mauve plugin in Geneious Prime® v11.0.14.1.

Informative Gene Selection

The most informative loci for determining *E. anguillarum* intraspecific variation were identified through estimates of average evolutionary divergence for each gene locus. Among the three evaluated MLSA schemes, the number of base substitutions per site from averaging over all sequence pairs was determined from each prospective MLSA target. Assessments were performed in MEGA11 (Tamura et al., 2013) using the Maximum Composite Likelihood model (Tamura et al., 2004). Codon positions included were 1^{st}, 2^{nd}, 3^{rd}, and noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

Multilocus sequence analysis (MLSA)

All *E. anguillarum* genomes available from the NCBI nr/nt database as of 2021 (n = 8) were downloaded and included in the analysis (Table 3.4). Similarly, reference gene sequences (n = 24) from previous *Edwardsiella* spp. MLSA studies (Abayneh et al., 2012, Buján et al., 2018, and Griffin et al. 2013) were downloaded from NCBI, MAFFT aligned and concatenated

using Geneious Prime® v11.0.14.1. Alignments for individual MLSA loci and subsequent similarity matrices used to identify different allele types. The multiple copy gene *tuf* (Buján et al., 2018) was excluded from analysis. Further, 16S rRNA was also excluded due to high levels of intrageneric homogeneity and low level of intraspecific phylogenetic informativeness (Reichley et al., 2017). The best nucleotide substitution model was determined for each partitioned gene in the concatenated sequence (Table 3.5). Maximum likelihood analysis was performed using IQ-TREE v.1.6.12 with concatenated alignments and partition schemes for each of the previously published MLSA models (Abayneh et al., 2012; Buján et al., 2018; Griffin et al. 2013), as well as the "optimized" scheme employing the appropriate substitution model for each respective gene with 1000 bootstraps (Nguyen et al., 2015). Dendrograms based on Bayesian inference models were produced in FigTree v1.4.4 (Rambaut, 2018).

Histopathology

The French angelfish associated with *E. anguillarum* isolate A20-09557 was examined histologically after postmortem submission from a public aquarium through the Aquatic Pathology Service at the University of Georgia. Representative tissue sections were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (HE). Select sections were additionally examined with Gram and Ziehl-Neelsen acid-fast stains.

Results

Bacterial isolates

The seventeen *E. anguillarum* isolates analyzed in this study originated from six discrete geographic locations, representing four different countries and five different host fish species, including freshwater and marine representatives (Table 3.1). Significant disease was associated

with several isolates, which has been documented in previous work (Armwood et al., 2019; Ucko et al., 2016). Isolate A20-09557, collected from a captive French angelfish (*Pomacanthus paru*), was not associated with acute bacterial-related histologic lesions. The French angelfish had chronic granulomas in the liver, kidney, spleen, and intestine with no discernable bacteria, suggestive of previous parasitic encystment; however, previous or chronic infection with *E. anguillarum* could not be definitively ruled out based on the low sensitivity of histopathology for bacteria present in low numbers.

Biochemical characteristics

A summary of the BBL™ Crystal™ Enteric/Nonfermenter Identification systems results is presented in Table 3.6. All *E. anguillarum* isolates were indole-positive and oxidase-negative, with corresponding enzymatic hydrolysis, deamination, reduction, or utilization of p-nitrophenyl phosphate, proline nitroanilide, p-nitrophenyl-N-acetyl glucosaminide, urea, glycine, citrate, arginine, and lysine. 24% (4/17) produced enzymatic hydrolysis of p-nitrophenyl β-glucuronide and 6% (1/17) tetrazolium. No isolates were positive for enzymatic hydrolysis, deamination, reduction, or utilization of p-nitrophenol α-β-glucoside, p-nitrophenyl β-galactoside, p-nitrophenyl bis-phosphate, p-nitrophenyl xyloside, p-nitrophenyl α-arabinoside, p-nitrophenyl phosphorylcholine, γ-L-glutamyl p-nitoranilide, esculin, p-nitro-DL-phenylalanine, or malonate. Isolates did not use the sugars arabinose, sucrose, melibiose, rhamnose, sorbitol, adonitol, galactose, and inositol. All *E. anguillarum* isolates utilized mannose and 94% (16/17) utilized mannitol, with EA181011 as the only isolate negative for mannitol utilization.

TSI and motility

Results from the TSI and motility mediums are listed in Table 3.7. All *E. anguillarum* isolates were positive for glucose fermentation (K/A) and produced gas. Most isolates produced

scant hydrogen sulfide (14/17; 82.4%). The three isolates that were negative for hydrogen sulfide originated from cultured tilapia isolates from Costa Rica (Armwood et al., 2019). Both *E. piscicida* isolate S11-285 and *E. tarda* isolate FL95-01 exhibited glucose fermentation, gas production, and hydrogen sulfide production. *Edwardsiella ictaluri* isolate S97-773 produced gas and fermented glucose but did not produce hydrogen sulfide. *Edwardsiella anguillarum* isolates varied in motility from none to strong motility, with ten isolates demonstrating no motility and seven isolates demonstrating weak to strong motility. Isolates from Costa Rica were largely non-motile (8/9; 88%) with the exception of isolate R18-14-2. Many isolates from other geographic locations were weakly to strongly motile (6/8; 75%), aside from isolates EA181011 (Israel) and 43472 (Maryland, USA), both of which were non-motile. The *E. piscicida*, *E. ictaluri*, and *E. tarda* control isolates were motile, with weak dispersal for *E. ictaluri* isolate S97-773.

Antimicrobial susceptibility

Antimicrobial susceptibilities measured by Kirby-Bauer disk diffusion assays demonstrated the majority of isolates were responsive to the tested antibiotics, producing inhibition zones ≥ 20 mm for Aquaflor®, Romet®, and Terramycin® (Table 3.8). Isolate R18-14-2 yielded a clearance zone <20 mm for Terramycin®, indicating reduced sensitivity compared to other isolates, but inhibition zones for both Aquaflor® and Romet® were consistent with the rest of the study isolates at >20 mm. MICs varied more widely across isolates (Table 3.9). No antimicrobial was discriminatory between any intraspecific *E. anguillarum* phylogroups. Isolates from Israel, Georgia (USA), and Maryland grew at all concentrations of clindamycin, while isolates from Louisiana, Costa Rica, and Colombia were inhibited at concentrations of 2 mg/liter or lower. Similarly, isolates from Maryland had MICs for penicillin at 4 mg/liter, while those from Israel, Louisiana, Costa Rica, and Colombia had MICs of ≤2

mg/L. The in vivo clinical efficacy of these antibiotics at different concentrations has not been determined for *E. anguillarum*, and, thus, the relevancy of these findings is unknown.

Genomic fingerprinting using rep-PCR

Rep-PCR profiles were generated from BOX, $(GTG)_5$, ERIC II, and ERIC I & II primers (Figures 3.1-4) were consistent with previous work which separated *E. anguillarum* from *E. tarda, E. piscicida,* and *E. ictaluri*. Analysis of the ERIC II primer set revealed two distinct genetic clusters, which loosely correlated with geographic origin. All Maryland, USA isolates segregated into one genetic group. Comparably, results from BOX, $(GTG)_5$, or ERIC I & II primers were mixed, and profiles were largely homogenous without any discrete groupings.

Genome sequencing and plasmid analysis

Genomes ranged from approximately 4.0 to 4.25 Mb, containing approximately 3508 to 3821 coding sequences (CDS) and 3639 to 3957 genes. Initial attempts to identify native plasmids using electrophoresis were unsuccessful, however, based on genome sequencing, the Costa Rican and Colombian isolates (9/17 isolates) carried a single, large, nearly identical plasmid with a molecular weight of approximately 91.49 Kb (range: 91.487 to 91.497 Kb). No plasmids were identified in any other isolates. Predicted coding sequences identified by the NCBI prokaryotic genome annotation pipeline (PGAP) are listed in Table 3.10. The plasmids carried up to 97 coding sequencings, predominantly associated with conjugative and transposable elements, plasmid mobilization (tra operon) and bacterial adhesion (fimbrial elements), with no known antibiotic resistant genes identified. Plasmid sequences were submitted to GenBank® (Table 3.1).

Evolutionary divergence

Average intraspecific evolutionary divergence over sequence pairs for *E. anguillarum* isolates is reported in Table 3.11. Multiple gene targets including *aroA* and *mdh* from Abayneh et al. (2012), *pgi* and *phoU* from Griffin et al. (2013), and *gapA, glnA, groL* from Buján et al. (2018) revealed 100% identity across all *E. anguillarum* isolates. Of the genes demonstrating some divergence, genes with the highest number of base substitutions per site over all sequence pairs were selected for an optimal multilocus sequence assessment, which included *dnaK, gyrB, metG, pyrG* from Abayneh et al. (2012), *gyrA* from Griffin et al. (2013), and *adk, atpD, phoR* from Buján et al. (2018). Of the eight selected loci, *pyrG* from Abayneh et al. (2012) had the greatest number of base substitutions across all sequence pairs.

Multilocus sequence assessment

Bayesian inference and maximum likelihood analysis of concatenated reference genes from Abayneh et al., 2012 (Figure 3.5) and Buján et al., 2018 (Figure 3.6) revealed five discrete phyletic groups. Comparably, the MLSA scheme of Griffin et al., 2013 (Figure 3.7) produced four. Analysis of the concatenation of the 23 genes from all three studies (Figure 3.8) and the optimized eight gene scheme developed in the current study using evolutionary divergence (Figure 3.9) paralleled results from Abayneh et al. (2012) and Buján et al. (2018), with isolates falling into five discrete clades, despite having 2 and 3 non-informative loci, respectively.

In the optimized scheme, clades 2 and 4 were represented by only a single isolate, R17-63 and A20-09557 respectively. Isolates were predominantly divided by geographic origin, with those from Costa Rica and China in Clade 1, Maryland in Clade 3, and the Mediterranean in Clade 5. Tilapia isolates were spread across three different clades (clades 1,2,3). In this analyses, Clade 3 was consistent with grouping from the ERIC II primer set, which also grouped the Maryland isolates together.

Allele types determined by overall mean distances divided isolates into discrete phylogroups which were in complete agreement with their respective MLSA schemes for Abayneh et al. (2012) (Table 3.12), Buján et al. (2018) (Table 3.13), and Griffin et al. (2013) (Table 3.14), as well as the eight genes deemed most informative for assessing *E. anguillarum* intraspecific variation (Table 3.15).

Discussion

Edwardsiella anguillarum is a pathogen of increasing global importance affecting multiple cultured fish species, particularly tilapia (Armwood et al., 2019; Elgendy et al., 2022; Griffin, 2020b; Oh et al., 2020). While the species epithet suggests a predilection for eels, *E. anguillarum* is not limited to anguillid hosts and actually affects a wide range of freshwater, brackish, and marine fish. Despite this, prevalence is likely underestimated as studies conducted prior to its recognition as a separate species in 2015 and even certain contemporary studies misidentify *E. anguillarum* as *E. tarda* (Griffin et al., 2017; Shao et al., 2015). Additionally, while large publicly available databases permit global genomic comparisons, *E. anguillarum* sequences in GenBank continue to be misnamed as *E. tarda*, further confusing the literature and complicating genomic/phylogenetic analyses.

Repetitive sequence mediated PCR has demonstrated intraspecific variation among several other *Edwardsiella* species, though clustering was inconsistent between the amplification sites (Griffin et al., 2013; 2014; 2016; López-Porras et al., 2021; Wang et al., 2011). In contrast to these previous studies, rep-PCR profiles were largely homologous among the *E. anguillarum* isolates evaluated, with only ERIC II primer sets producing two separate genetic clusters. While useful for quick genetic diversity analysis between isolates, the primer sites used here were less informative than the MLSA. Further, unlike rep-PCR, MLSA permitted inclusion of publicly

available *E. anguillarum* genomes and nucleotide sequences from other studies, facilitating analysis of isolates that are not on-hand.

The MLSA schemes of Abayneh et al. (2012) and Griffin et al. (2013) were established either prior to the recognition of *E. piscicida* and *E. anguillarum* and offered substantial supportive evidence that isolates classified as *E. tarda* represented multiple taxa. Likewise, the MLSA of Buján et al. (2018) focused primarily on highlighting interspecific differences, rather than intraspecific diversity. Thus, many of the gene targets included in these MLSA schemes are highly conserved within some taxa and therefore lack informativeness for identifying intraspecific differences. López-Porras et al. (2021) investigated the diversity of *E. piscicida* isolates from catfish culture in the United States, reporting several of the targets established in the three *Edwardsiella* MLSA schemes had limited utility in establishing intraspecific relatedness among *E. piscicida*. Likewise, Divya (2021) found similar redundancy among MLSA targets when investigating two different populations of *E. ictaluri.* This prompted a reassessment of these schemes for their ability to identify variability among *E. anguillarum* variants, as gene loci suitable for one taxon may have limited utility in another.

Edwardsiella anguillarum isolates in this study grouped more by general geographic location than by fish host, with tilapia isolates expanding over three different phylogroups. Griffin et al. (2015) reported three genetically distinct strains among *E. ictaluri* isolates using rep-PCR and *gyrB* sequencing, with catfish isolates, tilapia isolates, and zebrafish isolates each forming a distinct clade; however, *E. ictaluri* isolates from catfish and ornamental aquaculture exhibit minimal genetic variation within their respective industries (Aarattuthodiyil et al., 2020; Divya, 2021; Griffin et al., 2011). López-Porras et al. (2021) described five discrete phylogroups within *E. piscicida* isolates recovered from Mississippi channel and hybrid catfish. Nguyen et al.

(2021) reported the same five clades from ten different fish species, including a sixth clade that was comprised exclusively of isolates from salmonids. The studies of López-Porras et al. (2021) and Nguyen et al. (2021) targeted the reference genes *gyrB, pgi,* and *phoU*. In contrast to these works, *phoU* and *pgi* lack phylogenetic informativeness for *E. anguillarum*. In fact, multiple gene targets, including *aroA* and *mdh* from Abayneh et al. (2012), *pgi* and *phoU* from Griffin et al. (2013) and *gapA, glnA, groL* revealed 100% identity across all *E. anguillarum* isolates examined in the current study and are therefore unsuitable for determining intraspecific variability for *E. anguillarum* due to redundancy.

Isolates genetically consistent with *E. anguillarum* were initially described as atypical, non-motile *E. tarda*, however previous reports describe variable motility among *E. anguillarum* isolates. Both Shao et al. (2015) and Griffin et al. (2013) observed motility, but Katharios et al. (2019) described a non-motile isolate from diseased sharpsnout seabream in Greece, consistent with results observed here indicating inconsistent motility among *E. anguillarum* isolates. Hydrogen sulfide (H_2S) production was present for most groups, except for phylogroup 1 (Figure 1) where a small number of isolates did not produce the black precipitate associated with H_2S production. Phenotypic patterns among BBL crystal codes did not discriminate between phylogroups, which demonstrated only minimal sporadic variation in profiles. These findings are consistent with previous studies comparing characters between *E. tarda, E. anguillarum,* and *E. piscicida*, wherein phenotypic variation failed to reliably discriminate between these taxa (Griffin et al., 2013; Reichley et al., 2017). While there did not appear to be any consistent associations between genetic group and phenotype, discerning relationships between phenotypic characters and *E. anguillarum* genetic groups is precluded by the small number of isolates included in this study.

Plasmids were limited geographically to isolates from Costa Rica and Colombia, where they were universal. The identified plasmids were largely homologous. Plasmids were not obtained from the plasmid DNA harvest, likely due to their large size. While plasmids are described in other *Edwardsiella* spp., they have only been reported in *E. anguillarum* isolate ET080813, which also contained two plasmids, one 2.2 Kb and the second 127.0 Kb (Yang et al., 2012).

As *E. anguillarum* was only first recognized as distinct from *E. tarda* in 2015, little has been documented regarding the treatment and prevention of *E. anguillarum*-associated disease (Griffin et al., 2020b; Shao et al., 2015). Concurrent with findings by Armwood et al. (2019) and Reichley et al. (2017), the isolates evaluated were susceptible to a wide variety of antimicrobials *in vitro*, including florfenicol and oxytetracycline, suggesting these compounds may have application during disease outbreaks. Clindamycin and penicillin susceptibilities revealed some differences associated with geographic origin, but while susceptibility profiles varied between isolates and MLSA phylogroups, no antimicrobials were discriminatory between the five clades. Coding sequences associated with plasmid-mediated antibiotic resistance were not identified. Identified plasmid genes were largely associated with adhesion, pilis formation, bacterial conjugation and conjugative transfer, DNA transposition, and plasmid mobility, including genes associated with the transfer (tra) operon indicating a mobilizable plasmid. Vaccine development against *E. anguillarum* is in its early stages and at present no product is commercially available (He et al., 2021; LiHua et al., 2019; Takano et al., 2011). Findings here indicate there is genetic variation among *E. anguillarum* isolates which should be taken into consideration when evaluating potential commrcial vaccines and efficacy against genetically discrete phylogroups should be investigated. While not an important pathogen in US catfish aquaculture, preliminary

studies indicate hybrid catfish exposed to *E. anguillarum* may be protected against *E. piscicida*, suggesting potential for use as a vaccine candidate (Armwood et al., 2022).

This study documents phenotypic and genotypic variation among *E. anguillarum* isolates, establishing an optimized MLSA scheme based on three previously published strategies, which identified five distinct phylogroups. Drivers of this genetic diversity are currently unknown but could be related to fish species, treatment practices, location, culture systems, and/or other factors. This work also expands our understanding of plasmid trafficking among the *Edwardsiella*, documenting the presence of a shared, previously undocumented, mobilizable plasmid among the Central and South American isolates. Admittedly, analyses are limited by the low number of available *E. anguillarum* genomes in comparison to its congeners *E. ictaluri* and *E. piscicida*. Still, this study substantially increases the number of *E. anguillarum* genomes publicly available. As additional isolates continue to emerge in different locations and fish hosts, the MLSA scheme developed in this study will be a useful tool to identify genetic variants, establish intraspecific diversity within *E. anguillarum* and the clinical implications of its genetic plasticity on fish health and disease prevention.

Figures

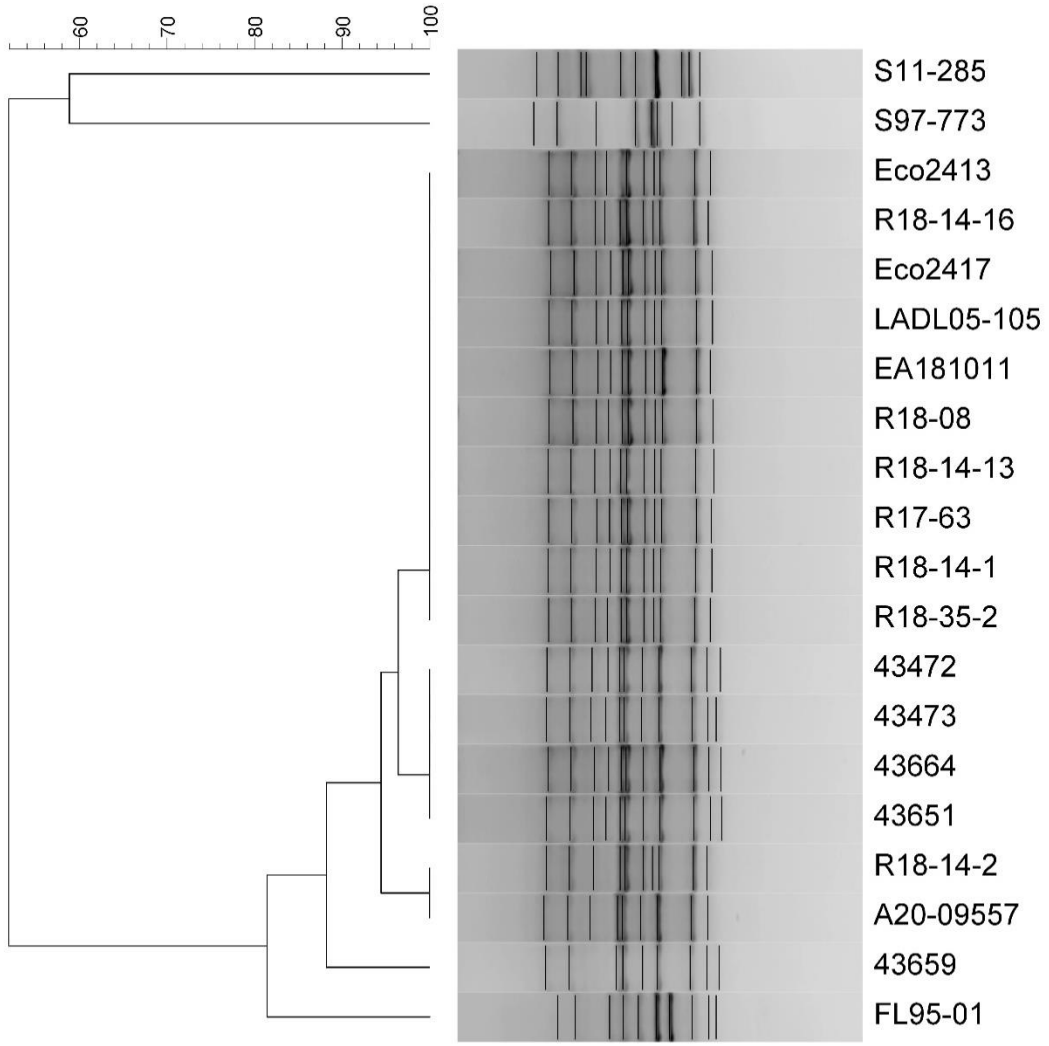

Figure 3.1. Genetic fingerprints created by rep-PCR using BOX primers. Dendrograms are developed from Dice coefficient similarity matrices based on unweighted pair group method with arithmetic mean (UPGMA) at 2% tolerance.

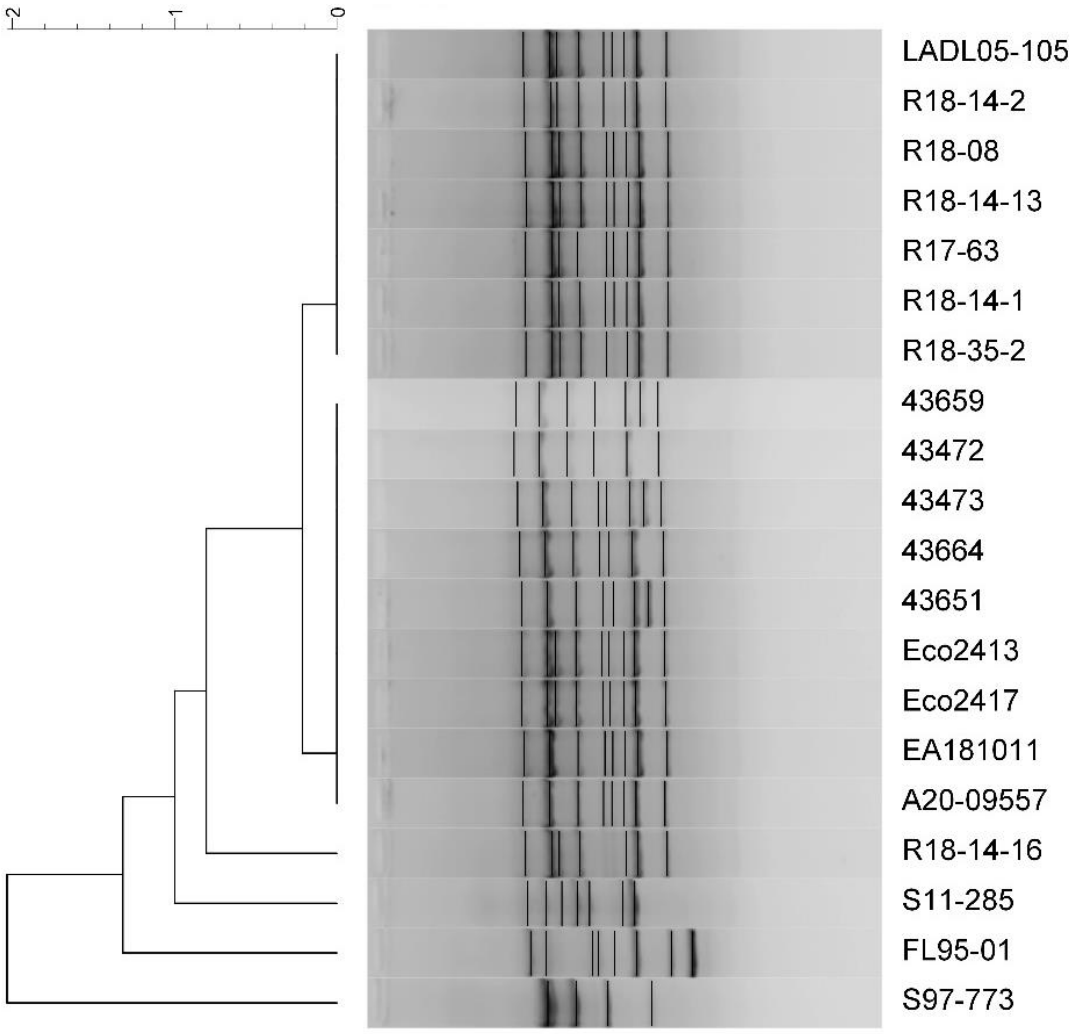

Figure 3.2. Genetic fingerprints created by rep-PCR using $(GTG)_5$ primers. Dendrograms are developed from Dice coefficient similarity matrices based on unweighted pair group method with arithmetic mean (UPGMA) at 2% tolerance.

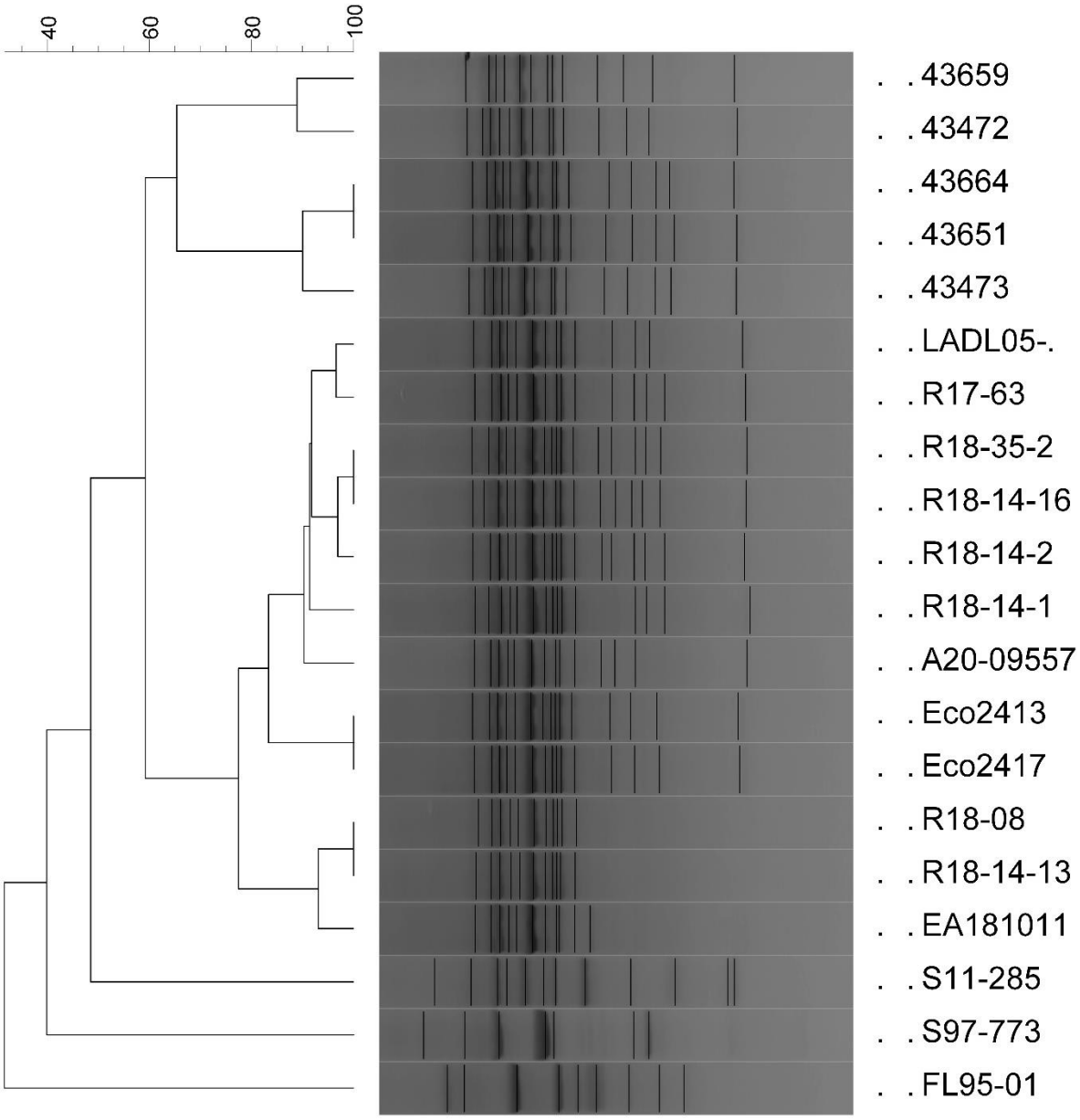

Figure 3.3. Genetic fingerprints created by rep-PCR using ERIC II primers. Dendrograms are developed from Dice coefficient similarity matrices based on unweighted pair group method with arithmetic mean (UPGMA) at 2% tolerance.

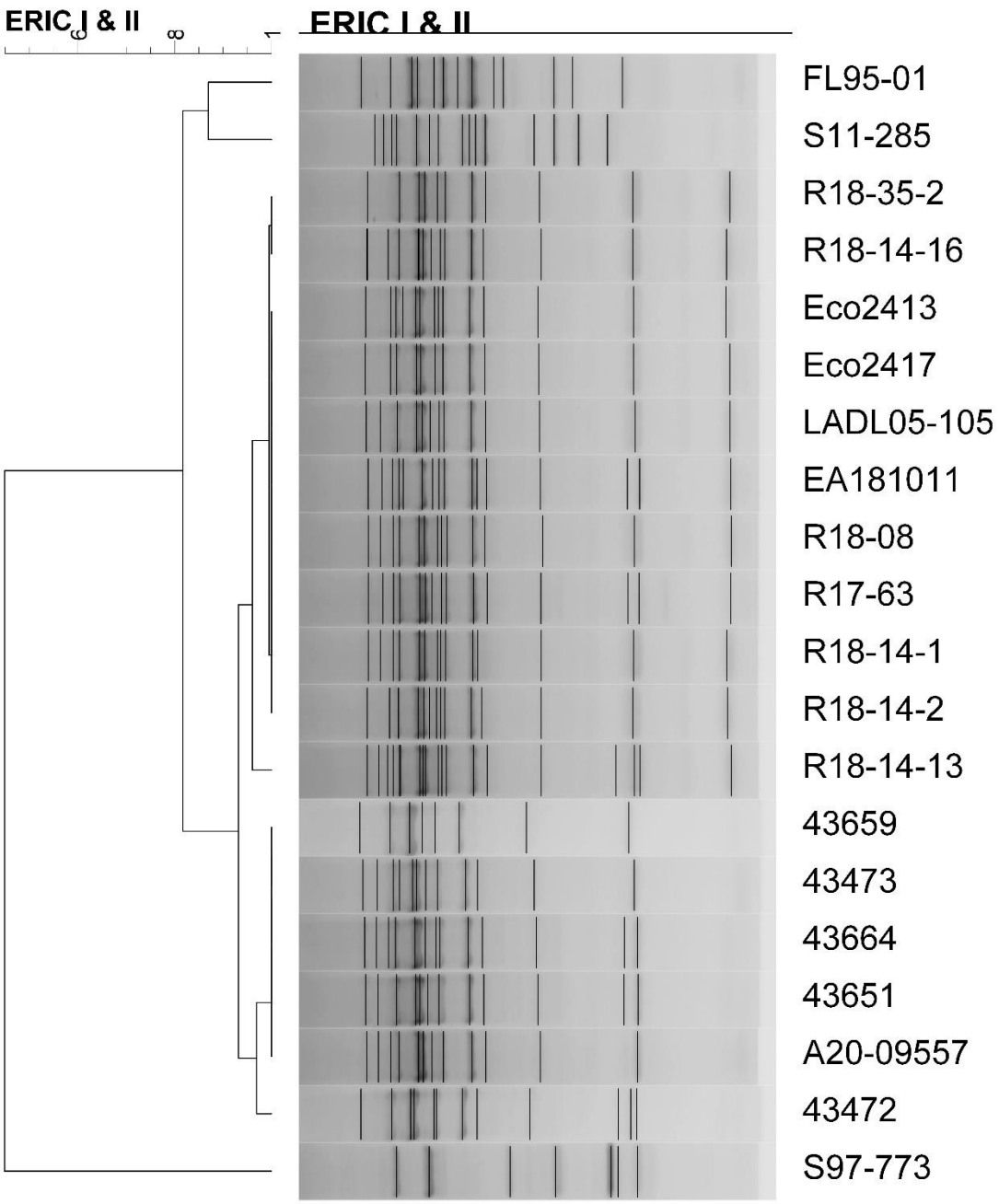

Figure 3.4. Genetic fingerprints created by rep-PCR using ERIC I & II primers. Dendrograms are developed from Dice coefficient similarity matrices based on unweighted pair group method with arithmetic mean (UPGMA) at 2% tolerance.

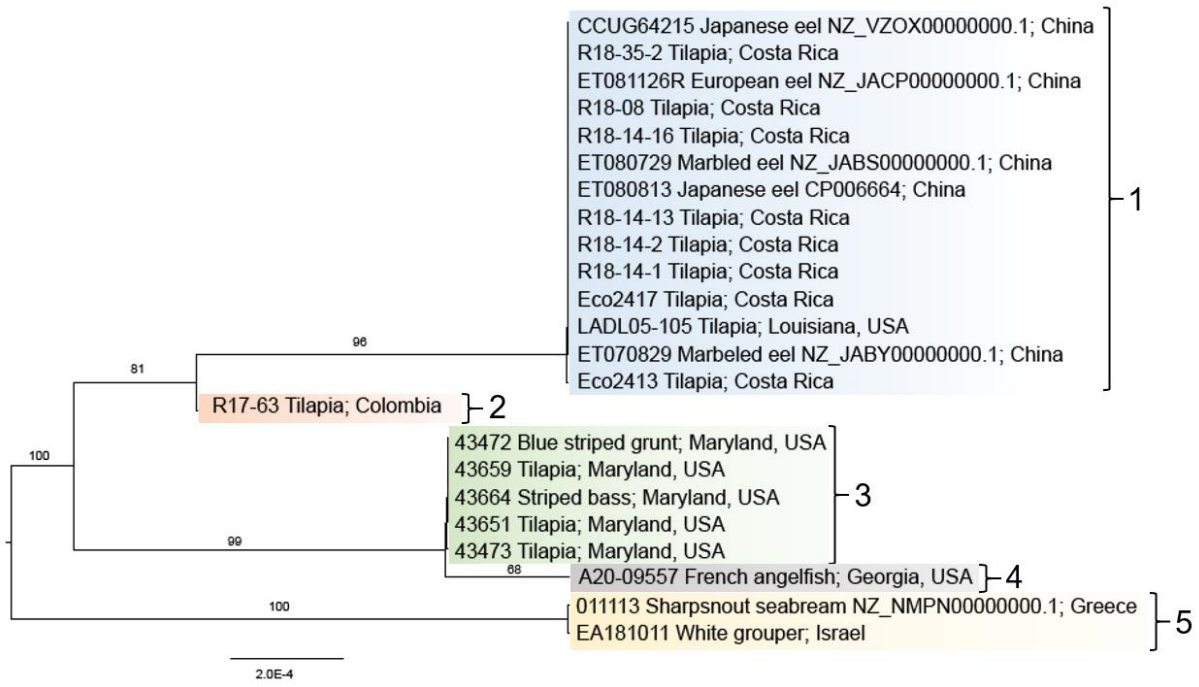

Figure 3.5. Phylogenetic relationships between 23 *E. anguillarum* isolates, including the 17 study isolates and the 6 additional *E. anguillarum* isolates available in the NCBI database, using a concatenation of the *adk, pyrG, aroE, dnaK, phoR, metG, mdh,* and *gyrB* gene sequences from Abayneh et al. 2012. The phylogenetic tree was constructed by Bayesian inference with posterior probabilities calculated by maximum likelihood estimation with bootstrapping (*n* = 1000) in Figtree v1.4.4 and IQTREE v.1.6.12. Numbers above branches represent posterior probabilities (probabilities <50 are excluded). Respective nucleotide substitution models are listed in Table 3.5. Scale bar represents the nucleotide substitution average per site.

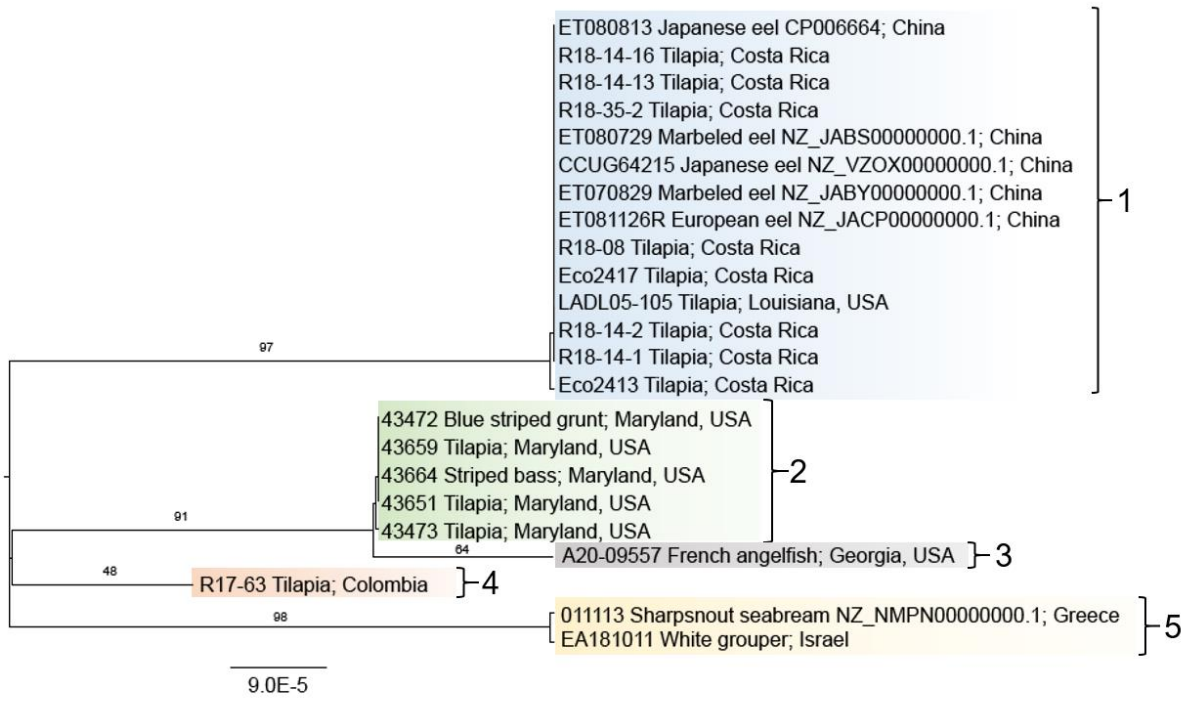

Figure 3.6. Phylogenetic relationships between 23 *E. anguillarum* isolates, including the 17 study isolates and the 6 additional *E. anguillarum* isolates available on the NCBI database, using a concatenation of the *adk, atpD, rpoA, pyrG, gapA, phoR, dnaJ, groL*, and *glnA* genes sequences from Buján et al., 2018. The phylogenetic tree was constructed by bootstrapping (*n* = 1000) following the nucleotide substitution models listed in Table 3.5 in Figtree v1.4.4 and IQTREE v.1.6.12. Scale bar represents the nucleotide substitution average per site.

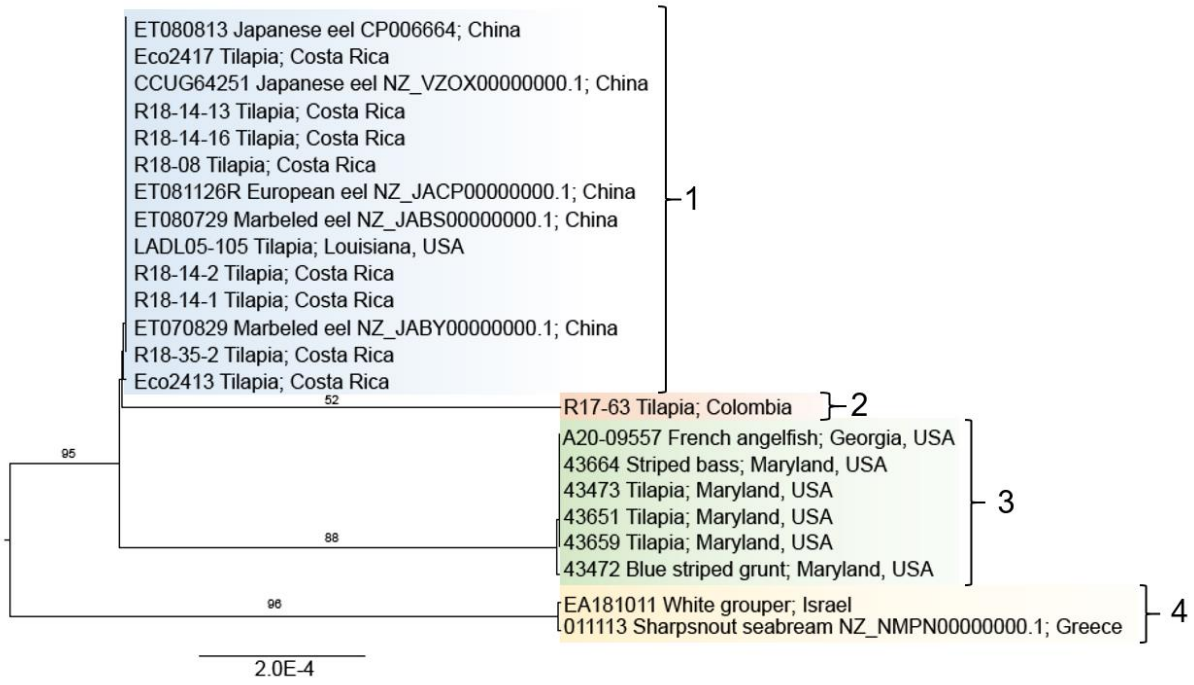

Figure 3.7. Phylogenetic relationships between 23 *E. anguillarum* isolates, including the 17 study isolates and the 6 additional *E. anguillarum* isolates available on the NCBI database, using a concatenation of the *groL*, *gyrA*, *rpoA*, *phoU*, *gyrB*, *pgm*, and *pgi* gene sequence from Griffin et al., 2013. The phylogenetic tree was constructed by bootstrapping (n = 1000) following the nucleotide substitution models listed in Table 3.5 in Figtree v1.4.4 and IQTREE v.1.6.12. Scale bar represents the nucleotide substitution average per site.

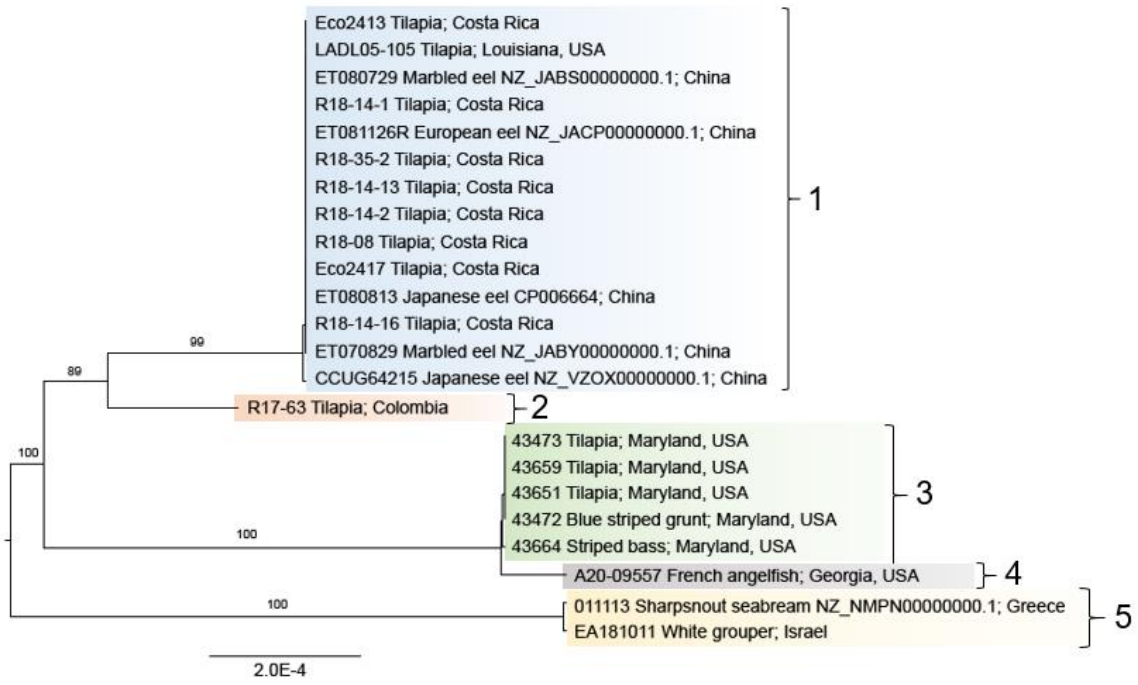

Figure 3.8. Phylogenetic relationships between 23 *E. anguillarum* isolates, including the 17 study isolates and the 6 additional *E. anguillarum* isolates available on the NCBI database, using a concatenation of all genes from Abayneh et al., 2012, Buján et al., 2018, and Griffin et al., 2013 (Table 3.11). The phylogenetic tree was constructed by Bayesian inference with posterior probabilities calculated by maximum likelihood estimation with bootstrapping (n = 1000) in Figtree v1.4.4 and IQTREE v.1.6.12. Numbers above branches represent posterior probabilities (probabilities <50 are excluded). Respective nucleotide substitution models are listed in Table 3.5. Scale bar represents the nucleotide substitution average per site.

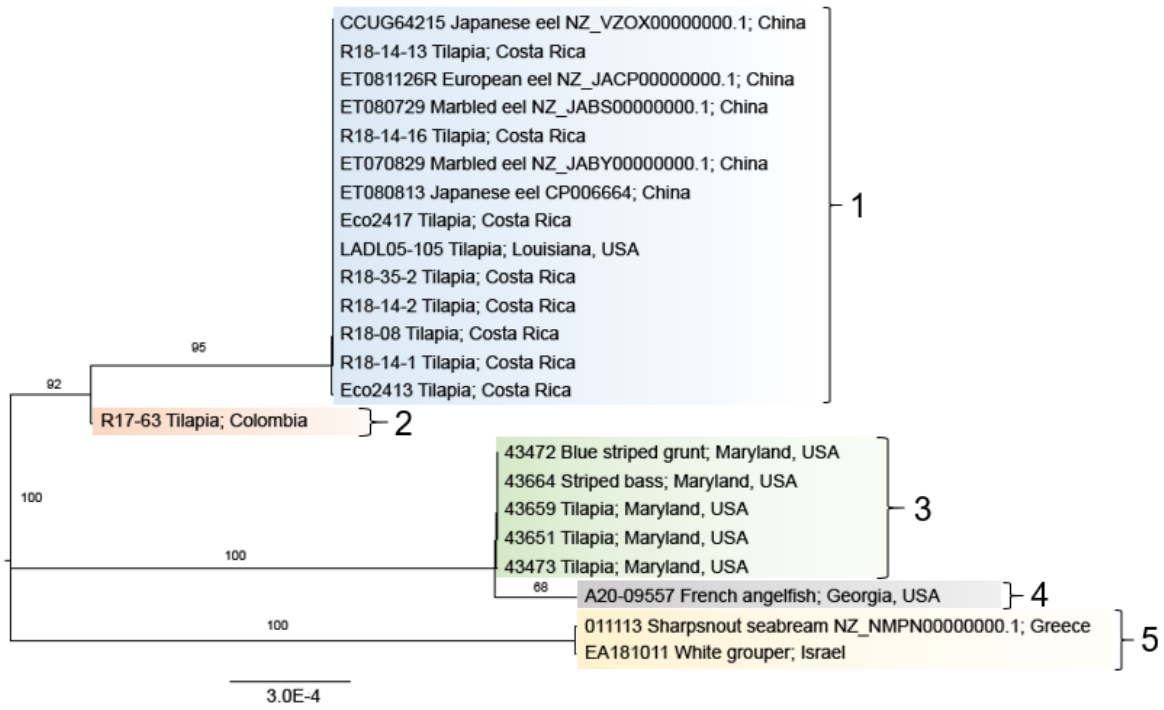

Figure 3.9. Phylogenetic relationships between 23 *E. anguillarum* isolates, including the 17 study isolates and the 6 additional *E. anguillarum* isolates available on the NCBI database, using a concatenation of *dnaK*, *gyrB*, *metG*, *pyrG*, *gyrA*, *adk*, *atpD*, and *phoR* genes deemed most informative by the current study (Table 3.11). The phylogenetic tree was constructed by Bayesian inference with posterior probabilities calculated by maximum likelihood estimation with bootstrapping (n = 1000) in Figtree v1.4.4 and IQTREE v.1.6.12. Numbers above branches represent posterior probabilities (probabilities <50 are excluded). Respective nucleotide substitution models are listed in Table 3.5. Scale bar represents the nucleotide substitution average per site.

Tables

Table 3.1. *Edwardsiella anguillarum* isolates.

Isolate	GenBank Accession Number	Host species	Year collected	Geographic location	Reference
43472	Chromosome: CP094323	Blue striped grunt	2003	Maryland, USA	Reichley et al., 2017
43664	Chromosome: CP094322	Striped bass	1994	Maryland, USA	Reichley et al., 2017
43473	Chromosome: CP094321	Tilapia	1997	Maryland, USA	Reichley et al., 2017
43659	Chromosome: CP094320	Tilapia	1998	Maryland, USA	Reichley et al., 2017
43651	Chromosome: CP094319	Tilapia	1999	Maryland, USA	Reichley et al., 2017
LADL05-105	Chromosome: CP011516	Tilapia		Louisiana, USA	Reichley et al., 2015
EA181011	Chromosome: CP011364	White grouper	2011-2012	Israel	Reichley et al., 2015; Ucko et al., 2016
R17-63	Chromosome: CP094317 Plasmid: CP094318	Tilapia	2017	Colombia	Armwood et al., 2019
Eco2413	Chromosome: CP094324 Plasmid: CP094325	Tilapia†	2017	Costa Rica	Armwood et al., 2019
Eco2417	Chromosome: CP094315 Plasmid: CP094316	Tilapia†	2017	Costa Rica	Armwood et al., 2019
R18-14-16	Chromosome: CP094313 Plasmid: CP094314	Tilapia†	2018	Costa Rica	Armwood et al., 2019
R18-14-2	Chromosome: CP094311 Plasmid: CP094312	Tilapia†	2018	Costa Rica	Armwood et al., 2019
R18-14-1	Chromosome: CP094308	Tilapia†	2018	Costa Rica	Armwood et al., 2019

	Plasmid: CP094309				
R18-14-13	Chromosome: CP094306 Plasmid: CP094307	Tilapia†	2018	Costa Rica	Armwood et al., 2019
R18-08	Chromosome: CP094304 Plasmid: CP094305	Tilapia†	2018	Costa Rica	Armwood et al., 2019
R18-35-2	Chromosome: CP094302 Plasmid: CP094303	Tilapia†	2018	Costa Rica	Armwood et al., 2019
A20-09557	Chromosome: CP094301	French Angelfish	2019	Georgia, USA	Present study

† Isolates from the same farm.

Table 3.2. Primers used for repetitive sequence mediated PCR (Versalovic et al., 1994).

Primer	**Sequence (5'-3')**	**Temperature (C)**
BOX	CTACGGCAAGGCGACGCTGACG	52
ERIC I	ATGTAAGCTCCTGGGGATTCAC	52
ERIC II	AAGTAAGTGACTGGGGTGAGCG	52
$(GTG)_5$	GTGGTGGTGGTGGTG	40

Table 3.3. Raw sequence processing details.

Isolate	**Nanopore Read Depth**	**Assembly Depth**	**Illumina Read Depth**
A20-09557	200	44.06	63
43472	116.34	40.16	52
43473	162.75	40.61	58
43651	200	43.95	53
43659	185.76	42.51	53
43664	200	44.51	67
EA181011	45.93	40.27	60
Eco2413	135.65	40.27	48
Eco2417	94.74	40.26	60
LADL05-105	122.38	40.15	64
R18-14-1	200	43.4	79
R18-14-13	200	42.7	72

R18-14-16	200	43.7	49
R18-14-2	200	42.7	90
R17-63	124.68	40.3	46
R18-08	200	43.3	79
R18-35-2	66.81	40.25	81

Table 3.4. *Edwardsiella anguillarum* isolates from NCBI used in multilocus sequence analysis.

Isolate	**GenBank Accession Number**	**Host Species**	**Reference**
ET080813	CP006664	*Anguilla japonica*	Shao et al., 2015
01113	NMPN00000000	*Diplodus puntazzo*	Katharios et al., 2019
CCUG 64215	VZOX00000000	*Anguilla japonica*	Tunovic et al., unpublished
ET070829	JABY00000000	*Anguilla marmorata*	Shao et al., 2015
ET080813	JABS00000000	*Anguilla marmorata*	Shao et al., 2015
ET081126R	JACP00000000	*Anguilla*	Shao et al., 2015
LADL05-105	CP011516	*Oreochromis* sp.	Reichley et al., 2015a
EA181011	CP011364	*Epinephelus aeneus*	Reichley et al., 2015a; Ucko et al., 2016

Table 3.5. Nucleotide substitution models selected for the partitioned genes for phylogenetic analysis in IQ-TREE v.1.6.12 using Bayesian inference and maximum likelihood estimation.

Gene	Originating study	Nucleotide substitution model for gene partition
adk	Abayneh et al., 2012; Buján et al., 2018	F81 + F, JC
aroE	Abayneh et al., 2012	F81 + F, JC
dnaK	Abayneh et al., 2012	F81+F
gyrB	Abayneh et al., 2012	F81+F
mdh	Abayneh et al., 2012	F81 + F, JC
metG	Abayneh et al., 2012	F81 + F, JC
phoR	Abayneh et al., 2012; Buján et al., 2018	F81 + F, JC
pyrG	Abayneh et al., 2012; Buján et al., 2018	F81+F
atpD	Buján et al., 2018	F81 + F, JC
dnaJ	Buján et al., 2018	F81 + F, JC
gapA	Buján et al., 2018	F81 + F, JC
glnA	Buján et al., 2018	F81 + F, JC
groL	Buján et al., 2018	F81+F
groL	Griffin et al., 2013	F81 + F, JC
gyrA	Griffin et al., 2013	F81 + F, JC, HKY + F
gyrB	Griffin et al., 2013	F81+F
pgi	Griffin et al., 2013	F81 + F, JC
pgm	Griffin et al., 2013	F81 + F, JC
phoU	Griffin et al., 2013	F81+F
rpoA	Buján et al., 2018; Griffin et al., 2013	F81 + F, JC

F81 + F = Felsenstein + Empirical Base Frequencies
HKY + F = Hasegawa-Kishino-Yano
JC = Jukes-Cantor

Table 3.6. BBL™ Crystal™ Enteric/Nonfermenter Identification system results.

Isolate	Code	Indole	Oxidase	Identification	CL (%)†
E. anguillarum					
43472	2403114013	+	-	*E. tarda*	99.99
43664	2403114033	+	-	*E. tarda*	99.99
43473	2403114013	+	-	*E. tarda*	99.99
43659	2403114013	+	-	*E. tarda*	99.99
43651	2403114133	+	-	*E. tarda*	99.97
LADL05-105	2403114013	+	-	*E. tarda*	99.99
EA181011	2403110013	+	-	*E. tarda*	99.99
R17-63	2403114013	+	-	*E. tarda*	99.99
Eco2413	2403114013	+	-	*E. tarda*	99.99
Eco2417	2403114013	+	-	*E. tarda*	99.99
R18-14-16	2403114013	+	-	*E. tarda*	99.99
R18-14-2	2403114013	+	-	*E. tarda*	99.95
R18-14-1	2403114013	+	-	*E. tarda*	99.99
R18-14-13	2403114013	+	-	*E. tarda*	99.99
R18-08	2403114013	+	-	*E. tarda*	99.99
R18-35-2	2403114033	+	-	*E. tarda*	99.99
A20-09557	2403114033	+	-	*E. tarda*	99.99
E. ictaluri					
S97-773	2403110003	-	-	*E. tarda*	99.97
E. piscicida					
S11-285	2403110013	+	-	*E. tarda*	99.99
E. tarda					
FL95-01	2403110013	+	-	*E. tarda*	99.99

†CL, Confidence level

Table 3.7. *Edwardsiella anguillarum* TSI and motility medium results.

Isolate	TSI†	Motility ‡
E. anguillarum		
43472	K/A + gas + H_2S (scant)	-
43664	K/A + gas + H_2S (scant)	+ (weak)
43473	K/A + gas + H_2S (scant)	+
43659	K/A + gas + H_2S (scant)	+
43651	K/A + gas + H_2S (scant)	+
LADL05-105	K/A + gas + H_2S (scant)	+
EA181011	K/A + gas + H_2S (scant)	-
R17-63	K/A + gas + H_2S (scant)	-
Eco2413	K/A + gas + H_2S (scant)	-
Eco2417	K/A + gas + H_2S (scant)	-
R18-14-16	K/A + gas; no H_2S	-
R18-14-2	K/A + gas; no H_2S	+

R18-14-1	K/A + gas + H_2S (scant)	-
R18-14-13	K/A + gas + H_2S (scant)	-
R18-08	K/A + gas + H_2S (scant)	-
R18-35-2	K/A + gas; no H_2S	-
A20-09557	K/A + gas + H_2S (scant)	+ (weak)
E. ictaluri		
S97-773	K/A + gas; no H_2S	+ (weak)
E. piscicida		
S11-285	K/A + gas + H_2S	+
E. tarda		
FL95-01	K/A + gas + H_2S	+

†K/A = glucose fermentation only
‡+ = motile, - = non-motile, weak = less than other samples

Table 3.8. Disk diffusion assay antimicrobial sensitivity patterns of *Edwardsiella anguillarum* isolates.

Diffusion zone diameters	Antibiotic	Number of isolates
≥ 20 mm		
	Aquaflor®	17/17
	Romet®	17/17
	Terramycin®	16/17
11-19 mm		
	Aquaflor®	0/17
	Romet®	0/17
	Terramycin®	1/17†
≤ 10 mm		
	Aquaflor®	0/17
	Romet®	0/17
	Terramycin®	0/17

†*E. anguillarum* isolate R18-14-2.

Table 3.9. Minimal inhibitory concentrations (MICs) of antimicrobials for *Edwardsiella anguillarum* isolates. Values in the lowest concentration of the antibiotic represent the growth inhibition at the lowest available concentration. MICs not achieved at the highest concentration are cited at the next concentration above their range. Growth was inhibited for all isolates at combinatory concentrations of 2/38 mg/liter for trimethoprim-sulfamethoxazole (data not shown).

	Isolates	**Number of isolates with MIC (mg/liter)**									
Antibiotic (range: mg/liter)		≤0.5	1	2	4	8	16	32	64	128	≥256
Aminoglycosides											
Gentomycin (0.5-8)	All	17									
Neomycin (2-32)	All			17							
Spectinomycin (8-64)	All					16		1			
Streptomycin (8-1024)	All					16					1
Macrolides											
Erythromycin (0.12-4)	All				1	16					
Tylosin tartrate (2.5-20)	All							17			
Pencillins											
Amoxicillin (0.25-16)	All	9	8								
Penicillin (0.06-8)		6	2	4	5						
Sulfonamides											
Sulphadimethoxine (32-256)	All							7	1		9
Sulfathiazole (32-256)	All							8	3	1	5
Tetracyclines											
Oxytetracycline (0.25-8)	All	8	9								
Tetracycline (0.25-8)	All	5	12								
Others											
Ceftiofur (0.25-4)	All	16				1					
Clindamycin (0.5-4)		7	1	1		8					
Enrofloxacin (0.12-2)	All	16	1								
Florfenicol (1-8)	All		16	1							
Novobiocin (0.5-4)	All		1	2	3	11					

Table 3.10. Summary of coding sequences (CDS) annotated by the NCBI prokaryotic genome annotation pipeline (excluding hypothetical proteins) from *Edwardsiella anguillarum* plasmids (n = 9) in tilapia with Costa Rican and Colombian origins (GenBank accession numbers: CP094303, CP094305, CP094307, CP094309, CP094312, CP094314, CP094316, CP094318, CP094325).

Name	Number of isolates with CDS
AAA family ATPase CDS	9
AAA family ATPase CDS	9
AAA family ATPase CDS	9
adhesin biosynthesis transcription regulatory family protein CDS	9
antibiotic biosynthesis monooxygenase CDS	9
antirestriction protein CDS	9
ATP-binding protein CDS	1
caspase family protein CDS	9
chromosome partitioning protein ParB CDS	1
class I SAM-dependent methyltransferase CDS	9
conjugal transfer pilus assembly protein TraU CDS	9
conjugal transfer protein TraB CDS	9
conjugal transfer protein TraH CDS	9
CRISPR-associated helicase/endonuclease Cas3 CDS	9
DJ-1/PfpI family protein CDS	1
DNA helicase UvrD CDS	9
DUF2726 domain-containing protein CDS	9
DUF2913 family protein CDS	9
DUF3560 domain-containing protein CDS	9
DUF4496 domain-containing protein CDS	9
fimbria/pilus outer membrane usher protein CDS	9
fimbria/pilus periplasmic chaperone CDS	9
fimbrial protein CDS	9
fimbrial protein CDS	9
finO CDS	9
gloB CDS	9
integrase core domain-containing protein CDS	9
IS1 family transposase CDS	9
IS1 family transposase CDS	9
IS110 family transposase CDS	9
IS3 family transposase CDS	9
IS3 family transposase CDS	9
IS3 family transposase CDS	9
IS3 family transposase CDS	9

IS3 family transposase CDS	9
IS5 family transposase CDS	9
IS5 family transposase CDS	9
IS5 family transposase CDS	9
IS5/IS1182 family transposase CDS	9
IS5/IS1182 family transposase CDS	9
istA CDS	9
istB CDS	9
nuclease PIN CDS	8
ParB/RepB/Spo0J family partition protein CDS	9
ParB/RepB/Spo0J family partition protein CDS	9
plasmid SOS inhibition protein A CDS	9
psiB CDS	9
relaxosome protein TraM CDS	9
replication initiation protein CDS	9
Replication protein CDS	9
single-stranded DNA-binding protein CDS	9
site-specific integrase CDS	9
site-specific integrase CDS	9
ssb CDS	9
traA CDS	9
traC CDS	9
traD CDS	9
traE CDS	9
traF CDS	9
traG CDS	9
traI CDS	9
traK CDS	9
traL CDS	9
traN CDS	9
transcriptional regulator CDS	9
transglycosylase SLT domain-containing protein CDS	9
transposase CDS	9
traQ CDS	9
traT CDS	9
traV CDS	9
traW CDS	9
traX CDS	9
traY domain-containing protein CDS	9
trbB CDS	9
trbC CDS	9
trbI CDS	9
type 1 fimbrial protein CDS	9

type 1 fimbrial protein CDS	9
type 1 fimbrial protein CDS	9
type 1 glutamine amidotransferase CDS	9
type II toxin-antitoxin system VapC family toxin CDS	9
vapB CDS	9
vapB CDS	9
vapC CDS	9
zincin-like metallopeptidase domain-containing protein CDS	9

Table 3.11. Estimates of average evolutionary divergence within *Edwardsiella anguillarum* isolates. The number of base substitutions per site from averaging over all sequence pairs within each group are shown. Genes selected for the current study optimal MLSA scheme are highlighted in grey. Analyses were conducted using the Maximum Composite Likelihood model.

Previous study	**Gene**	**Number of base substitutions per site over all sequence pairs**
Abayneh et al., 2012		
	adk	0.001283111
	aroE	0
	dnaK	0.001680572
	gyrB	0.000459206
	mdh	0
	metG	0.000652491
	phoR	0.000334208
	pyrG	0.001684575
Griffin et al., 2013		
	groL	0.000116072
	gyrA	0.001476075
	gyrB	0.000240281
	pgi	0
	pgm	0.000188976
	phoU	0
	rpoA	0.000119221
Buján et al., 2018		
	adk	0.001411176
	atpD	0.001023644
	dnaJ	0.000228419
	gapA	0
	glnA	0
	phoR	0.000540355
	groL	0
	pyrG	0.001588272
	rpoA	0.000109662

Table 3.12. Allele types using genes from Abayneh et al. 2012 on the 17 study isolates and 6 additional *Edwardsiella anguillarum* isolates from NCBI database.

Isolate	***adk***	***pyrG***	***aroE***	***dnaK***	***phoR***	***metG***	***mdh***	***gyrB***	**Allele type**	**Sequence type**
A20-09557	1	1	1	1	1	1	1	1	11111111	1
43472	1	2	1	1	1	1	1	1	12111111	2
43473	1	2	1	1	1	1	1	1	12111111	2
43651	1	2	1	1	1	1	1	1	12111111	2
43659	1	2	1	1	1	1	1	1	12111111	2
43664	1	2	1	1	1	1	1	1	12111111	2
ET080813	3	3	1	3	1	2	1	2	33131212	3
Eco2413	3	3	1	3	1	2	1	2	33131212	3
Eco2417	3	3	1	3	1	2	1	2	33131212	3
ET080729	3	3	1	3	1	2	1	2	33131212	3
ET070829	3	3	1	3	1	2	1	2	33131212	3
ET081126R	3	3	1	3	1	2	1	2	33131212	3
LADL05-105	3	3	1	3	1	2	1	2	33131212	3
R18-14-1	3	3	1	3	1	2	1	2	33131212	3
R18-14-2	3	3	1	3	1	2	1	2	33131212	3
R18-14-13	3	3	1	3	1	2	1	2	33131212	3
R18-14-16	3	3	1	3	1	2	1	2	33131212	3
R18-08	3	3	1	3	1	2	1	2	33131212	3
R18-35	3	3	1	3	1	2	1	2	33131212	3
CCUG64215	3	3	1	3	1	2	1	2	33131212	3
R17-63	1	3	1	3	1	2	1	2	13131212	4
EA181011	2	3	1	2	2	2	1	3	23122213	5
011113	2	3	1	2	2	2	1	3	23122213	5
Numbers of Alleles	3	3	1	3	2	2	1	3		

Table 3.13. Allele types using genes from Buján et al. 2018 on the 17 study isolates and 6 additional *Edwardsiella anguillarum* isolates from NCBI database.

Isolate	***adk***	***atpD***	***rpoA***	***pyrG***	***gapA***	***phoR***	***dnaJ***	***groL***	***glnA***	**Allele type**	**Sequence type**
A20-09557	1	1	1	1	1	1	1	1	1	111111111	1
43472	1	1	1	2	1	1	1	1	1	111211111	2
43473	1	1	1	2	1	1	1	1	1	111211111	2
43651	1	1	1	2	1	1	1	1	1	111211111	2
43659	1	1	1	2	1	1	1	1	1	111211111	2
43664	1	1	1	2	1	1	1	1	1	111211111	2
EA181011	2	2	1	2	1	2	2	1	1	221212211	3
011113	2	2	1	2	1	2	2	1	1	221212211	3
ET080813	3	2	1	3	1	1	1	1	1	321311111	4
Eco2413	3	2	1	3	1	1	1	1	1	321311111	4
Eco2417	3	2	1	3	1	1	1	1	1	321311111	4
ET080729	3	2	1	3	1	1	1	1	1	321311111	4
ET070829	3	2	1	3	1	1	1	1	1	321311111	4
ET081126R	3	2	1	3	1	1	1	1	1	321311111	4
LADL05-105	3	2	1	3	1	1	1	1	1	321311111	4
R18-14-1	3	2	1	3	1	1	1	1	1	321311111	4
R18-14-2	3	2	1	3	1	1	1	1	1	321311111	4
R18-14-13	3	2	1	3	1	1	1	1	1	321311111	4
R18-14-16	3	2	1	3	1	1	1	1	1	321311111	4
R18-08	3	2	1	3	1	1	1	1	1	321311111	4
R18-35-2	3	2	1	3	1	1	1	1	1	321311111	4
CCUG64215	3	2	1	3	1	1	1	1	1	321311111	4
R17-63	1	2	2	2	1	1	1	1	1	122211111	5
Numbers of Alleles	3	2	2	3	1	2	2	1	1		

Table 3.14. Allele types using genes from Griffin et al. 2013 on the 17 study isolates and 6 additional *Edwardsiella anguillarum* isolates from NCBI database.

Isolate	*groL*	*gyrA*	*rpoA*	*phoU*	*gyrB*	*pgm*	*pgi*	Allele type	Sequence type
A20-09557	1	1	1	1	1	1	1	1111111	1
43472	1	1	1	1	1	1	1	1111111	1
43473	1	1	1	1	1	1	1	1111111	1
43651	1	1	1	1	1	1	1	1111111	1
43659	1	1	1	1	1	1	1	1111111	1
43664	1	1	1	1	1	1	1	1111111	1
ET080813	2	2	1	1	1	1	1	2211111	2
Eco2413	2	2	1	1	1	1	1	2211111	2
Eco2417	2	2	1	1	1	1	1	2211111	2
ET080729	2	2	1	1	1	1	1	2211111	2
ET070829	2	2	1	1	1	1	1	2211111	2
ET081126R	2	2	1	1	1	1	1	2211111	2
LADL05-105	2	2	1	1	1	1	1	2211111	2
R18-14-1	2	2	1	1	1	1	1	2211111	2
R18-14-2	2	2	1	1	1	1	1	2211111	2
R18-14-13	2	2	1	1	1	1	1	2211111	2
R18-14-16	2	2	1	1	1	1	1	2211111	2
R18-08	2	2	1	1	1	1	1	2211111	2
R18-35-2	2	2	1	1	1	1	1	2211111	2
CCUG64215	2	2	1	1	1	1	1	2211111	2
EA181011	2	3	1	1	2	1	1	2311211	3
011113	2	3	1	1	2	1	1	2311211	3
R17-63	2	2	2	1	1	2	1	2221121	4
Numbers of Alleles	2	3	2	1	2	2	1		

Table 3.15. Allele types using the 8 genes from Abayneh et al. 2012, Buján et al. 2018, or Griffin et al. 2013 deemed most informative for intraspecific variation within *Edwardsiella anguillarum* in the current on the 17 study isolates and 6 additional *E. anguillarum* isolates from NCBI database.

	Abayneh	**Abayneh**	**Abayneh**	**Abayneh**	**Buján**	**Buján**	**Buján**	**Griffin**		
Isolate	***dnaK***	***gyrB***	***metG***	***pyrG***	***adk***	***phoR***	***atpD***	***gyrA***	**Allele type**	**Sequence type**
A20-09557	1	1	1	1	1	1	1	1	11111111	1
43472	1	1	1	2	1	1	1	1	11121111	2
43473	1	1	1	2	1	1	1	1	11121111	2
43651	1	1	1	2	1	1	1	1	11121111	2
43659	1	1	1	2	1	1	1	1	11121111	2
43664	1	1	1	2	1	1	1	1	11121111	2
ET080813	3	2	2	3	2	1	2	2	32232122	3
Eco2413	3	2	2	3	2	1	2	2	32232122	3
Eco2417	3	2	2	3	2	1	2	2	32232122	3
ET080729	3	2	2	3	2	1	2	2	32232122	3
ET070829	3	2	2	3	2	1	2	2	32232122	3
ET081126R	3	2	2	3	2	1	2	2	32232122	3
LADL05-105	3	2	2	3	2	1	2	2	32232122	3
R18-14-1	3	2	2	3	2	1	2	2	32232122	3
R18-14-2	3	2	2	3	2	1	2	2	32232122	3
R18-14-13	3	2	2	3	2	1	2	2	32232122	3
R18-14-16	3	2	2	3	2	1	2	2	32232122	3
R18-08	3	2	2	3	2	1	2	2	32232122	3
R18-35	3	2	2	3	2	1	2	2	32232122	3
CCUG64215	3	2	2	3	2	1	2	2	32232122	3
R17-63	3	2	2	3	1	1	2	2	32231122	4
EA181011	2	3	2	3	3	2	2	3	23233223	5
011113	2	3	2	3	3	2	2	3	23233223	5
Numbers of Alleles	3	3	2	3	3	2	2	3		

CHAPTER 4

COMPARATIVE IMMUNE RESPONSES TO EXPERIMENTAL *EDWARDSIELLA ICTALURI* CHALLENGES IN VACCINATED AND NON-VACCINATED CHANNEL AND CHANNEL ♀ X BLUE ♂ HYBRID CATFISH [1]

[1]Armwood, A.R., Cai, W., Leary, J., López-Porras, A., Richardson, B.M., Ware, C., Purcell, S.L., Wise, D., Fast, M.D., Griffin, M.J., and A.C. Camus. To be submitted to *Journal of Fish Diseases.*

Abstract

Edwardsiella ictaluri is an important bacterial pathogen of farm-raised catfish in the southeastern United States, exhibiting varied virulence in channel (*Ictalurus punctatus*) and channel x blue *(Ictalurus furcatus*) hybrid catfish fingerlings. Research has demonstrated that channel catfish are more susceptible to the agent, which is supported by diagnostic submissions to the Aquatic Research and Diagnostic Laboratory in Stoneville, MS, where channels account for nearly 70% of *E. ictaluri* diagnoses. In Mississippi, and to some extent Arkansas, a live-attenuated *E. ictaluri* vaccine (LAV) is available, which results in robust protection against subsequent *E. ictaluri* exposure and has significantly improved on-farm survival. Further, there is strong laboratory evidence indicating a cross-protective effect of the LAV against the closely related *E. piscicida.* To better understand the immune mechanisms underlying this protective effect, the susceptibility and immune responses to *E. ictaluri* challenge in vaccinated and non-vaccinated channel and hybrid catfish fingerlings was assessed. Catfish were immunized via immersion using the LAV and challenged by intraperitoneal injection with *E. ictaluri* 33 days post-vaccination (dpv). Serum IgM ELISAs were performed against *E. ictaluri* and *E. piscicida* antigen at 28 dpv and 28 days post challenge (dpc). Expression of immune genes was quantified with RNA-seq analysis and verified by qPCR with RNA harvested from posterior kidneys (5-6 fish/treatment group) at 5 dpc. Vaccinated catfish were significantly ($p < 0.001$) more likely to survive the challenge than their unvaccinated counterparts. Serum IgM against *E. ictaluri* varied with vaccination and challenge status at 28 dpv and 28 dpc in channel catfish, however, differences in serum IgM activity in vaccinated and naïve fish were only present in hybrids at 28 dpc. Interestingly, only vaccinated, subsequently challenged channels produced a significant antibody response to *E. piscicida* antigen ($p <0.05$). Differentially expressed genes (DEGs) were

identified between fish types and across treatments, although no DEGs were attributed to vaccination alone. Notable variably regulated genes were largely related to innate immune responses, complement, and coagulation. These data illustrate how bacterial infection, vaccination, and fish type influence the host response to *E. ictaluri* infection in channel and hybrid catfish.

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Introduction

Globally, aquaculture produced 85.3 million tonnes of fish, crustaceans, and mollusks in 2019, comprising 48% of the total harvest (FAO, 2021). Catfish are the top food fish produced in the United States, with sales of $398 million in 2021 (USDA/NASS 2022). Despite its status, the catfish industry has undergone significant contraction since its peak in the early 2000s. Contributing factors include competition from foreign imports, high feed and energy costs, more profitable land use alternatives, and losses from infectious disease. Infectious diseases impact producer profitability through direct mortalities, reduced growth performance, and treatment expenditures (Hawke & Khoo, 2004).

While commercial production in the southeastern United States developed around the channel catfish (*Ictalurus puncatus*), channel ♀ x blue (*Ictalurus furcatus*) ♂ hybrid catfish now represent greater than 60% of commercial harvests (Hegde et al., 2022). Hybrid catfish are increasingly favored by producers for their improved feed conversion, superior growth, crowding tolerance, and increased disease resistance (Dunham et al., 2014; Griffin et al., 2010; Hegde et al., 2022; Wolters et al., 1996). In relation to disease, channel and hybrid catfish notably demonstrate interspecific variation in susceptibility to commercially important pathogens, with channel catfish showing susceptibility to *E. ictaluri*, *Henneguya ictaluri* and, to some extent, Ictalurid herpesvirus 1 (IcHV1), while hybrid catfish are more vulnerable to *Edwardsiella piscicida* (Bosworth et al., 2003; Griffin et al., 2010; Plumb et al., 1975; Wolters et al., 1996).

Edwardsiella ictaluri, the causative agent of enteric septicemia of catfish (ESC), is primarily a pathogen of channel catfish. In the southeastern US, outbreaks occur in late summer or early fall at water temperatures of 22 to 28°C (Francis-Floyd et al., 1987; Wise et al., 2004). Transmission occurs horizontally through water-borne exposure via the intestinal mucosa

following ingestion or by direct contact with the olfactory epithelium (Thune et al., 1993). Infection typically results in mortality of naïve first-year fingerlings. Surviving fish can clear the bacteria and become resistant, develop chronic disease, and/or become asymptomatic carriers (Hawke & Khoo, 2004; Klesius, 1992; Mqolomba & Plumb, 1992; Thune et al., 1993). In hybrid catfish, *E. piscicida* has emerged as a major pathogen concurrent with the rise in their increased use as a production variety. Although disease induced by *E. piscicida* is similar in many respects to *E. ictaluri*, converse to *E. ictaluri*, *E. piscicida* is not considered a problem for fingerling operations and is more commonly recovered from stocker and market-sized fish (Griffin et al., 2019).

Management and prevention of *Edwardsiella* spp. outbreaks involves feed restriction, antibiotic administration, and vaccination (Wise et al., 2004; 2015). Despite substantial research investment and an abundance of available vaccine candidates, commercially available *E. ictaluri* vaccines have failed to gain widespread industry adoption due to minimal investment returns, logistical challenges associated with delivery, reluctance to change hatchery management practices, and intrinsic factors related to age and immunocompetency in fry (Bebak & Wagner, 2012; Lawrence et al., 1997; Petrie-Hanson & Ainsworth, 1999; Thune et al., 1994; 1999). Recently, an orally delivered, live attenuated *E. ictaluri* vaccine (U.S. Patent No. 8,999,319) developed by sequential passage of isolate S97-773-340X2 on Rifamycin has provided significant protection against *E. ictaluri* in channel and hybrid catfish fingerlings (Aarattuthodiyil et al., 2020; Chatakondi et al., 2018; Greenway et al., 2017; Peterson et al., 2016; Wise et al., 2015; 2020). Over 1.5 billion catfish have been vaccinated since 2013 and over 80% of producers now utilize vaccination in fingerling production, achieving net economic

benefits to channel and hybrid producers of $3,199 and $6,145 per hectare, respectively (Hegde et al., 2022; Kumar et al., 2019).

In addition to *E. ictaluri*, the live attenuated vaccine can also protect channel and hybrid catfish against *E. piscicida* infection (Griffin et al., 2020; López-Porras et al. 2022). This varied response between the two fish hosts, and the mechanisms driving this cross-protective effect congeners have not been fully investigated, but genetically determined immune responses specific to the two catfish varieties are suspected to play a major role. At present, information on immune responses to *Edwardsiella* spp. infections and vaccination in catfish has concentrated primarily on individual immune gene expression and serum antibody quantification (Bilodeau & Waldbieser, 2005; Chen et al., 2020; Elibol-Flemming et al., 2009; Pridgeon et al., 2010; Yao et al., 2014).

Transcriptomics, including RNA-seq, allows for quantification of gene expression levels unveiling host responses to pathogens or vaccination. Several studies have employed detailed RNA-seq analysis in catfish, one revealing 1633 differentially expressed genes in *E. ictaluri* challenged catfish intestine 3 h, 24 h, and 3 days post exposure including genes associated with cytoskeletal dynamics, junctional modification, lysosome/phagosome, immune activation/inflammation, pathogen recognition, and endocrine/growth disruption (Li et al., 2012). In this study, susceptibility and immune responses to *E. ictaluri* challenge in vaccinated and non-vaccinated channel and hybrid catfish fingerlings is compared using ELISAs, qPCR, and RNA-seq to illustrate how bacterial infection, vaccination, and fish type influence the host response to *E. ictaluri.* This work will lay the foundation for future research optimizing *Edwardsiella* vaccine efficacy in channel and hybrid catfish.

Methods

Fish

Channel and channel (♀) x blue (♂) hybrid catfish fingerlings were obtained as fry from a local hatchery and reared indoors at the National Warmwater Aquaculture Center (NWAC) at Mississippi State University Delta Research and Extension Center in Stoneville, Mississippi. At the initiation of the study, channel and hybrid catfish averaged 5.1 g and 5.4 g, respectively. Fish were maintained in 55 L glass aquaria containing 22 L of aerated, flow-through (2 L/m) well water. Throughout the challenges, the mean water temperature was 24.7°C ± 1.8°C (range: 21.6 - 27.6°C). Fish were acclimated for 48 h and fasted for 24 h prior to vaccination. All animal handling procedures were conducted in compliance with the Mississippi State University Institutional Animal Care and Use Committee.

Fish vaccination

Fish were arbitrarily stocked (55 fish/aquarium) into 24 aquaria (12 channel, 12 hybrid) for sample collection. An additional 24 aquaria (12 channel, 12 hybrid) were stocked (20 fish/aquarium) to estimate mortality of the delivered challenge doses without sampling bias. Aquaria were divided into two treatments, vaccinated and sham-vaccinated (naïve). Vaccinated fish were immunized by immersion using an attenuated *E. ictaluri* isolate (S97-773-340X2) following previously established procedures (Wise et al., 2015) using a frozen vaccine serial (V19-BHP 050219). The frozen vaccine was thawed, diluted 1:100 with deionized water, and expanded at 27°C for 18 h in 6 L porcine brain-heart infusion broth (BHIb) (Bacto; Becton Dickinson and Company) with shaking (250 RPM). The estimated quantity of viable bacterial cells in the vaccine broth was 2.3 x 10^9 CFU/ml, determined by standard plate count procedures performed on Mueller-Hinton II Agar plates with 5% sheep blood (MHBA) (BBL™; Becton and

Dickinson Company, Franklin Lakes, New Jersey, USA). The immersion bath was administered by ceasing water flow and adding 100 ml of culture to the tank water, resulting in an immunizing dose of ~$1x10^7$ CFU/ml. After 30 m, water flow was restored. Sham-vaccinated tanks received 100 ml of sterile BHIb under similar conditions.

Fish were monitored for adverse reactions twice daily for 33 days post vaccination (dpv). Moribund fish were euthanized by overdose in 300 mg/L of tricaine methanesulfonate (MS-222; Western Chemical, Ferndale, WA). Posterior kidneys from select moribund and fresh dead fish were cultured on trypticase soy agar (TSA) plates supplemented with 5% sheep blood with and without Rifamycin SV sodium salt (340 µg/ml; Sigma-Aldrich, St. Louis, Missouri) to confirm the isolated bacteria were the attenuated vaccine strain rather than wild-type (Wise et al., 2015). Bacterial identities were confirmed by multiplex real-time polymerase chain reaction (mPCR) (Reichley et al., 2015c; 2017).

Bacterial culture and challenges

Thirty-three dpv, vaccinated and sham-vaccinated channel and hybrid catfish in sampling tanks (55 fish/sampling aquarium; 3 aquaria/treatment group; 8 treatment groups) and non-sampling tanks (20 fish/sampling aquarium; 3 aquaria/treatment group; 8 treatment groups) received 100 µl intracoelomic injections of either *E. ictaluri* isolate S97-773 or sterile BHIb. The archived *E. ictaluri* isolate S97-773 was revived from cryogenic storage on MHBA and incubated for 48 h at 28°C. Individual colonies were expanded overnight in static 9 ml cultures of BHIb at 28°C from which a 1 ml aliquot was used to seed a 1 L culture that was incubated for 18 h with shaking (200 rpm) at 28°C. Expanded cultures were diluted 1:100,000, resulting in a delivered dose of 3.5 x 10^3 CFU/fish, which was determined using standard plate count techniques. Again, bacterial identity was confirmed via mPCR (Reichley et al., 2015c; 2017).

Challenge fish were anesthetized via immersion in 100 mg/L of MS-222 and given an intracoelomic (IC) injection of 100 µl of the bacterial broth or sterile BHIb. The IC challenge route was selected over an immersion route because the study was originally designed to directly compare immune responses to *E. ictaluri* against responses to *E. piscicida,* which requires the IC injection route for consistent challenge results (Reichley, 2017). Fish were monitored for morbidity and mortality for 28 days post challenge (dpc). Posterior kidneys of select moribund fish and fresh dead fish considered suitable for necropsy were cultured on MHB and trypticase soy agar (TSA) plates supplemented with 5% sheep blood. Identities of recovered bacterial isolates were confirmed by mPCR (Reichley et al., 2015c; 2017).

Sampling

Two fish were opportunistically collected from each triplicate sampling aquarium (n = 6 fish per treatment group per sampling time) at four different time points: 28 dpv, 37 dpv (4 dpc), 38 dpv (5 dpc), and 61 dpv (28 dpc) and euthanized with 300 mg/L MS-222 (Figure 4.1). Sampling days post-challenge were established from the current study's mortality peak. In fish sampled 28 dpv and 61 dpv (28 dpc), blood was collected from the caudal vein using a hematocrit tube and transferred to a 1.5 ml microcentrifuge tube. The blood-filled tubes were stored for 24 h at 4°C before concentration at 4,000 x *g* for 10 m. Serum was removed from the cell pellet and frozen at -20°C until further analysis. In fish sampled 37 dpv (4 dpc) and 38 dpv (5 dpc), portions of the posterior kidneys were aseptically removed, placed in RNAlater for 24 h at 4°C, and then frozen at -80°C until RNA isolation. The bodies of fish with kidneys removed were fixed whole in 10% neutral buffered formalin (NBF).

Enzyme linked immunosorbent assay

Serum antibodies directed against *E. ictaluri* were quantified using an enzyme-linked immunosorbent assay (ELISA) from individual fish sampled 28 dpc and 28 dpv (n = 6 total fish/treatment/timepoint). Archived *E. ictaluri* isolate S97-773 and *E. piscicida* isolate S11-285 (Reichley et al., 2016) were revived from cryogenic storage by isolation streaking on MHBA, grown for 48 h at 28ºC, and identities confirmed by mPCR (Reichley et al., 2015c; 2017). Individual colonies were inoculated into 200 ml of BHIb and expanded for 48 h at 27ºC with shaking (200 rpm). For preparation of crude whole cell lysates, the bacterial cells were centrifuged at 2700 x *g* for 20 m at 10ºC. The resulting pellets were flash frozen at -80ºC and stored until subsequent analysis. Frozen pellets were thawed, resuspended in borate buffered saline (BBS), and sonicated using a Sonic Dismembranator Model 100 (Fisher Scientific) on ice (20 s pulses, 10-15 watts, repeated seven times). The crude antigenic preparations were centrifuged at 10,000 rpm for 10 m to pellet cellular debris, and the supernatant collected and stored at -20ºC.

Protein concentrations of the bacterial lysates were measured by Bradford assay (Coomassie Protein Assay Reagent; ThermoFisher #1856209) and lysate concentrations diluted to 5 µg/ml with BBS. ELISA strips (Immunlon 2; ThermoFisher #6302) arranged in 96-well strip holders were coated with 100 µl of the diluted lysate and incubated overnight at 4°C to permit antigen binding. Plates were washed twice with phosphate buffered saline containing Tween 20® (PBST), the wells incubated in 250 µl blocking buffer (ELISA Blocker, ThermoFisher, #N502) at room temperature (RT) for 2 h, and then sealed and temporarily stored at 4°C. For storage >1 week, blocking buffer was removed and the plates air-dried prior to sealing and storage at 4°C.

For the ELISA, serum samples from each treatment group were first pooled and serially diluted to determine the ideal concentration for quantification. Serum diluted 1:320 in 1% fetal

bovine serum (FBS)-PBST was added to wells and further serially diluted to 1:2560 in triplicates for each sample. The plate was incubated overnight at 4ºC (~18 h) then washed 3 times with PBST. Wells were incubated in 50 µl of 1% FBS-PBST diluted (1:200) mouse immunoglobulin G1 (IgG1) anti-channel catfish IgM monoclonal antibody (9E1; University of Mississippi Medical Center, Jackson, Mississippi) for 2 h at RT, followed by 3 washes with PBST. Plates were incubated with 50 µl of 1% FBS-PBST diluted (1:5000) goat anti-mouse IgG1 antibody (Thermo Fischer Scientific) for 2 h at RT then washed again 3 times with PBST. Samples were incubated in 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate in the dark for 25 m at RT. Finally, 100 µl of 2N H_2SO_4 stop solution was added to the wells. Optical densities were measured at 450 nm on a BioTek ELX808 plate reader using GEN5 3.10 software. Optical densities of the 1:320 dilution were used for further analysis. Samples were individually normalized using an internal positive control of pooled serum from *E. ictaluri* challenged channel catfish. Analysis included a two-way analysis of variance (ANOVA), performed with GraphPad v9.2.0, followed by Tukey's *post hoc* test ($\alpha = 0.05$).

RNA Isolation and Sequencing

RNA was isolated from posterior kidneys collected during the two days of peak mortality 36 dpv (4 dpc) and 37 dpv (5 dpc) for challenged fish and control fish (vaccinated and sham-vaccinated) for qPCR and/or RNA-seq. Posterior kidney total RNA was isolated using Qiagen RNeasy Mini Kits (Hilden, Germany) following the manufacturers' protocol. Tissues were homogenized using a needle and syringe and lysed in RLT buffer. Genomic DNA contamination was removed from the RNA preparation using a Qiagen RNase-free DNase Set. The RNA quantity and quality was measured using spectrophotometry (Nanodrop 2000, Thermo Fischer Scientific), and RNA samples with an A260/280 from 1.8 to 2.0 utilized for future analyses.

Between each step, samples were stored at -80°C until further processing. The final sample was re-suspended in RNase-free water and stored at -20°C until analysis. Samples with a minimum RIN of 5 were included in library construction; 27 or 40 samples had a RIN over 6. Total RNA was submitted to Genewiz (Azenta Life Sciences) for library construction and standard RNA-seq.

RNA-seq Data Analysis

Illumina-specific adaptor sequences and low-quality reads were trimmed/eliminated from the raw reads using Trimmomatic v0.39 (Bolger et al., 2014) and the remaining trimmed reads mapped to the channel catfish reference genome (GenBank Accession No. GCF_001660625.2) using STAR v2.7 (Spliced Transcripts Alignment to a Reference; Dobin, 2022). FeatureCounts (Liao et al., 2013) was utilized to count features at the gene level. Differentially expressed genes (DEGs) were identified in R Studio v2022.22.1 using the package edgeR v3.38.1. DEGs were defined as genes with an adjusted p value < 0.05 and a log2 |fold-change| of >1. Annotations for the DEGs were conducted using R-based AnnotationHub v3.4.0 for the *I. punctatus* database (database number: AH101427). Data visualization and gene ontology (GO) term enrichment analysis was conducted using the R package clusterProfiler v4.4.2. Reported GO terms and pathways have an adjusted p value (false discovery rate [FDR] ≤ 0.05).

Quantitative PCR

For quantitative PCR, first strands of cDNA were synthesized using 2 µg of total RNA, SuperScript® IV reverse transcriptase, and Oligo-dT primer from Invitrogen life technologies. The previously developed immune gene-specific primers (4 primer sets) and newly designed primers (6 primer sets) utilized are listed in Table 4.1 (Xu et al., 2017). The new primers were designed using Geneious Prime and contig sequences from significantly expressed transcripts.

qPCR was conducted in triplicate reactions, with amplification reactions consisting of 10 µl of SsoAdvanced Universal SYBR Green Supermix (BioRad), 10 µm of the forward and reverse primers, 2 µg of template cDNA, and nuclease-free water to volume, for a total reaction volume of 12 µl. Transcripts were amplified on a CFX96 Thermal Cycler (BioRad) using the following settings: 95°C for 3 m followed by 35 cycles at 95°C for 10 s and 54°C for 30 s. Conventional PCR performed on control posterior kidney cDNA was used to generate amplicons for standard curve generation. Products were gel purified (Qiagen Gel Extraction Kit) and quantitated by Qubit fluorometry (Invitrogen). DNA (1 ng/µl) was tenfold serially diluted in distilled water and dilutions of 10^{-2} thru 10^{-8} evaluated by qPCR. Efficiencies and linearity evaluation are listed in Table 4.1. To confirm gDNA contamination absence, no reverse transcriptase controls were performed. Immune gene expression was normalized to three stable reference genes, *elongation factor 1-alpha* (*ef1α*), *glyceraldehyde-3-phosphate dehydrogenase* (*gadph*), and *beta-actin* (*β-actin*) (Table 4.1). Calibrated normalized relative quantities (CNRQ) were calculated in Bio-Rad CFX Manger v3.1 using primer-specific amplification efficiencies and sample-specific normalization factors. Two-way or three-way analysis of variance (ANOVA) was performed with GraphPad v9.2.0, followed by Tukey's *post hoc* test ($\alpha = 0.05$).

Data analysis

Relative percent survival (RPS) was calculated using the average of the three replicate non-sampling tanks per treatment group and the formula, RPS = [1-(cumulative mortalities in vaccinates / cumulative mortalities in non-vaccinates) x 100%] (Amend, 1981). Cumulative mortality among vaccinated and sham-vaccinated, channel and hybrid catfish was analyzed by two-way ANOVA, followed by Tukey's *post hoc* test ($\alpha = 0.05$). Analyses were performed in GraphPad 9.2.0. Kaplan–Meier survival curves were produced and analyzed using the survminer

(v0.4.9; Kassambara et al., 2021), survival (v3.4-0; Therneau, 2022; Therneau & Grambsch, 2000) and dplyr (v1.0.8; Wickham et al., 2022) packages in R statistical software (v4.0.2; R Core Team, 2021).

Detection of Edwardsiella ictaluri

Edwardsiella species specific multiplex PCR as previously described and published (Reichley et al., 2015c; 2017) was performed retrospectively on the cDNA from the posterior kidneys of fish in duplicates to determine bacterial presence or absence.

Histopathology

Individual fish submitted for RNA-seq and select moribund fish deemed adequate for analysis were examined histologically. Fish were decalcified in Kristensen's solution for 24-48 h after a minimum of 24 h fixation in 10% NBF. Serial transverse sections through the head and coelomic cavity were placed into tissue cassettes, processed routinely, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (HE). Select sections were stained with a modified Brown and Hopps Gram stain.

Results

Vaccination and bacterial challenge

Some post-vaccination, pre-challenge mortality occurred within the first 28 dpv in non-sampling tanks within vaccinated channel (3.3%; 4/120 fish) and hybrid catfish (7.5% mortality; 9/120 fish), indicating an extreme immunizing dose. No mortality occurred in sham-vaccinated and control fish. Mortality in vaccinated hybrid catfish within the first 28 dpv was significantly higher ($p < 0.01$) than in non-vaccinated hybrids. All isolates recovered from dead and moribund fish prior to challenge grew on rifamycin and were identified as *E. ictaluri* by mPCR, confirming the presence of the vaccine strain. Tukey's *post hoc* multiple comparisons revealed vaccinated

channel and hybrid catfish were significantly ($p < 0.0001$) more likely to survive through 28 dpc than unvaccinated fish exposed to wild-type *E. ictaluri* (Figure 4.2). Immersion baths with the live attenuated *E. ictaluri* vaccine resulted in relative percent survival (RPS) at 28 dpc of 87.7% in channel catfish and 93.2% RPS in hybrid catfish (Table 4.2). Kaplan Meier Survival curves are presented in Figure 4.3A, B. Intracoelomic *E. ictaluri* challenge produced high mortality in sham-vaccinated channel and hybrid catfish by 5 dpc, with no losses after 16 dpc (Figure 4.3A). Mortality in vaccinated fish did occur but was substantially lower and delayed until 7 dpc, persisting out to 16 dpc. There were no significant differences in cumulative mortality and survival probability in channel and hybrid catfish (Figure 4.3B). Bacteria isolated from moribund and fresh dead posterior kidneys from challenged fish were all identified as *E. ictaluri* by mPCR.

ELISA data

Serum IgM levels against *E. ictaluri* 28 dpv were significantly elevated in vaccinated compared to non-vaccinated channel catfish ($p < 0.001$) (Figure 4.4A). In contrast, serum IgM levels 28 dpv in vaccinated hybrids were not significantly different from non-vaccinated hybrid cohorts ($p = 0.13$) (Figure 4.4B). At 28 dpc, serum IgM against *E. ictaluri* was significantly increased in vaccinated, challenged channel ($p < 0.001$) and hybrid catfish ($p < 0.05$) compared to non-vaccinated, non-challenged controls (Figure 4.5A). Only vaccinated, challenged channels produced a significant antibody response to *E. piscicida* ($p <0.05$) compared to their non-challenged, non-vaccinated channel counterparts (Figure 4.5B). Channel catfish produced significantly ($p < 0.05$) more serum IgM antibodies against *E. piscicida* than hybrid catfish at both 28 dpv and 28 dpc. Individual antibody levels often varied considerably between individual fish in the same treatment group when the median was elevated.

RNA-sequencing data

Global posterior kidney transcriptional profiles between vaccinated and unvaccinated, *E. ictaluri* challenged and non-challenged, channel and hybrid catfish 4 or 5 dpc are listed in Table 4.3 and illustrated in Venn diagrams in Figure 4.6.

Transcriptomic response of comparing vaccinated and unvaccinated, hybrid and channel catfish

Transcriptomic profiles of vaccinated, non-challenged channel and hybrid catfish showed no significant differences compared to unvaccinated (sham) channel and hybrid catfish, respectively, 37 and 38 dpv. Comparisons between vaccinated channel and hybrid catfish, however, displayed varying global transcriptomic profiles with 631 DEGs, characterized mainly by transcript overexpression (571 transcripts) in the vaccinated hybrid catfish compared to the vaccinated channel catfish. GO pathway enrichment analysis revealed over-expressed genes primarily involved in coagulation and wound healing (e.g., *coagulation factor II [thrombin], coagulation factor VII, coagulation factor IXb, coagulation factor X, plasma kallikrein, fibrinogen gamma chain, plasminogen, vitamin K-dependent protein C*), complement (e.g., *complement 3, complement factor H, complement component 8, complement factor B, complement C1r subcomponent*), and metabolic pathways (e.g., *glucokinase, phosphoglycerate mutase 2, urate oxidase, fatty acid desaturase 2*) (Figure 4.7). No GO pathways were enriched with the 60 under-expressed genes from this comparison.

Unvaccinated channel and hybrid catfish showed similar differences in transcriptomic profiles (Figure 4.8), with 480 DEGs and upregulation of 462 transcripts in hybrid catfish compared to channel catfish and only 19 downregulated transcripts. Upregulated genes revealed enrichment of pathways involved in hemostasis, wound healing, complement activation, and metabolic processes (small molecule biosynthetic process, oxoacid metabolic process, etc.), similar to the enriched pathways between vaccinated hybrids and channel catfish. When

comparing the overexpressed genes in hybrids, 352 (51.8%) were overexpressed in vaccinated and sham-vaccinated hybrids, when compared to their respective channel catfish groups. Vaccinated hybrids upregulated an additional 219 (32.3%) genes compared to vaccinated channels, and sham-vaccinated hybrids an additional 108 (15.9%) genes compared to sham-vaccinated channels. With GO enrichment analysis, the 19 under expressed genes were enriched in "chemokine activity" (*C-C motif chemokine 3-like 1, C-C motif chemokine 4-like, C-C motif chemokine ligand 25b*).

Transcriptomic response during infection with E. ictaluri in non-vaccinated channel and hybrid catfish

Non-vaccinated, challenged channel and hybrid catfish had 4976 and 6029 DEGs, respectively, in comparison to their non-challenged counterparts. Channel catfish had 2722 over-expressed transcripts and 2246 under-expressed transcripts, while challenged hybrid catfish had 2456 over-expressed and 3573 under-expressed transcripts. Over-expressed genes in channel catfish involved the innate immune response (e.g., *interferon gamma 1, tumor necrosis factor a, toll-like receptor 1, toll-like receptor 2, toll-like receptor 5, toll-like receptor 13, interleukin 1 beta, chemokine (C-X-C motif) ligand 14*) complement system (e.g., *complement components C1, C1r, C1q, C3, C4b C6, C7b, C8a/b, C9, B, H, D*), coagulation system (e.g., *plasma kallikrein, fibrinogen alpha/beta/gamma chains, protein C, protein Z, plasminogen, tissue factor pathway inhibitor a,* and *factors 2, 7, 9b,* and *10*), ribosome biosynthesis (e.g., *BMS1 ribosome biogenesis factor, EMG1 N1-specific pseudouridine methyltransferase*), and amino acid activation (e.g., *alanyl-tRNA synthetase 1, tryptophanyl-tRNA synthetase 1*) (Figure 4.9). Significantly downregulated transcripts in challenged catfish involved pathways including blood vessel morphogenesis, cell adhesion, transporter activity, and the extracellular matrix.

Transcripts upregulated in challenged hybrid catfish (2456 DEGs) included pathway enrichment in the immune response (e.g., *nucleotide-binding oligomerization domain containing 1, interleukin 8, interleukin 1 beta, C-X-C motif chemokine 11-6, C-X-C motif chemokine 9, C-C motif chemokine 3, nucleotide-binding oligomerization domain containing 1, toll-like receptor 1, toll-like receptor 2, toll-like receptor 4, toll-like receptor 5, tumor necrosis factor a*), apoptosis, ribosome biogenesis, amino acid activation, and antigen processing and presentation (e.g., *endoplasmic reticulum aminopeptidase 1a, proteasome activator subunit 1, tapasin*) (Figure 4.10). In contrast to channel catfish, complement (e.g., *complement component 1q subcomponent, complement C1r subcomponent, complement factor 7-like, complement factor 8 alpha/beta peptides, complement factor 9, mannose-binding protein C)* and coagulation were among down-regulated transcript pathways. Other pathways underexpressed in challenged hybrids included metabolic processes, such as organic acid catabolic processes, responses to hormones, and glycogen metabolic processes.

Transcriptomic response during infection with E. ictaluri in vaccinated channel and hybrid catfish

Global transcript profiles contained 3495 DEGs between vaccinated, challenged and sham-vaccinated, challenged channel catfish, with 2060 upregulated transcripts in sham-vaccinated fish and 1435 downregulated. Upregulated transcripts characterized by enrichment in GO terms including immune response, blood coagulation, complement activation, and wound healing (Figure 4.11). Upregulated complement components included *complement factor B, complement factor H, complement factor 1, complement c3, complement component c3b, complement 4b, complement component 5, complement component 7b, complement component 8, complement component 9,* and *complement factor D.* Downregulated gene transcripts contained

enrichment in GO terms such as pigment biosynthetic process, cell adhesion, blood vessel morphogenesis, extracellular matrix, leukocyte mediated immunity, and response to cytokine. Leukocyte mediated immunity contained only a few complement components such as *complement component C7-like* and *complement 1 q subcomponents.*

In contrast to the channel catfish, only 362 DEGs were identified between the vaccinated, challenged hybrid fish and the sham-vaccinated, challenged hybrid catfish, characterized by 319 upregulated genes in sham-vaccinated fish and 7 downregulated genes. Significantly enriched GO pathways included coagulation, complement activation, wound healing, and regulation of body fluids (Figure 4.12). Upregulated complement and coagulation are similar to those listed in the previous section and include the chains of complement component 1q subcomponent. The downregulated genes include *DEAD (Asp-Glu-Ala-Asp) Box polypeptide 58* (*ddx58*) and *radical S-adenosyl methionine domain containing 2 (RSAD2).*

When comparing vaccinated, non-challenged channel catfish to vaccinated, challenged channel catfish, 506 DEGs were identified, with 216 upregulated and 290 downregulated transcripts in the challenged fish. Overexpressed gene transcripts were concentrated in the immune response, specifically of cytokines such as *tnf, ifng1,* and *ccl25b.* Coagulation and complement pathways were not enriched or differentially regulated. Underexpressed genes were predominated by catfish reproductive genes. Regarding hybrids, vaccinated, challenged hybrids downregulated 481 genes and upregulated 1008 genes, totaling 1489 DEGs, compared to vaccinated, non-challenged hybrids. Upregulated genes involved GO pathways involving the immune response, programmed cell death, cytokine signaling, and antigen processing and presentation, while downregulated pathways involved metabolic processes. Immune genes

largely revolved around cytokine signaling and pathogen recognition receptors. Hybrids similarly did not differentially regulate complement or coagulation pathways.

Transcriptomic responses comparing challenged channel and hybrid catfish

Comparisons of vaccinated, challenged hybrid and channel catfish revealed only 48 DEGs, with 2 upregulated and 46 downregulated in channels. Downregulated genes formed pathways with 1-5 genes, enriching GTPase activity and cytokine activity (*C-X-C motif chemokine 9, C-C motif ligand 19a tandem duplicate 2*). Upregulated genes were *urokinase plasminogen activator surface receptor* and *glycine N-acyltransferase-like protein 3.*

Sham-vaccinated, challenged channel catfish had higher numbers of DEGs compared to the hybrid counterparts, with 446 DEGs characterized by underexpression in 17 and overexpression in 429 genes. Pathways with increased expression in channels compared to hybrid catfish included coagulation, complement activation, and response to wounding (Figure 4.13); humoral pathways were composed of complement components. The downregulated genes enriched no pathways.

RNA-seq validation and gene expression with qPCR

Nine immune genes of interest were selected for qPCR validation of the RNA-seq, including *tnfα, ifnγ, il1β, c9, c3, cfX, nod1, tlr5,* and *csf1* (Figure 4.14). A subset of genes were quantified on both 4 and 5 dpc (*tnfα, ifnγ, il1β, nod1, C9*). No significant differences existed between the same treatment groups in the 4 and 5 dpc samples (data not shown), so qPCR was conducted on samples from 5 dpc for the remaining genes (*c3, cfX, tlr5, csf1*). Genes were selected due to their immunologic relevance or variable regulation identified with RNA-seq analysis. No significant differences were identified in *c3, c9,* and *cfX.* Transcript expression of these genes correlated between qPCR and RNA-seq.

Bacterial presence

The presence or absence of bacteria was determined using multiplex qPCR on synthesized cDNA, with a cut-off cycle threshold value of 33; detection was predominantly between 28 and 32. *Edwardsiella ictaluri* DNA was detected in all non-vaccinated, challenged samples 4 dpc (n = 6 fish/treatment group/day). In contrast, bacterial presence was identified in only 16.7% (1/6) of vaccinated, challenged hybrids and in 0% (0/6) of vaccinated, challenged channels 4 dpc.

Histopathology

Histopathologic examination conducted on individual fish submitted for RNA-seq (4 or 5 dpc) revealed inflammatory responses parallel with immune response and morbidity trends (Table 4.4). Lesions were typical of gram-negative bacterial sepsis, with multifocal necrotizing lesions containing abundant bacteria (Figure 4.15a) ranging to granulomatous inflammation with rare bacteria. Necrotizing lesions with abundant bacteria predominated and were more severe in the non-vaccinated, challenged catfish compared to their vaccinated, challenged counterparts, where granulomatous lesions with few bacteria predominated. Necrotizing lesions varied with the tissue, but all included gram-negative bacteria, cellular debris, loss of tissue architecture, and fibrin. Additional changes in the liver, included peripheral hypereosinophilic hepatocytes with pyknotic nuclei. Vaccinated, challenged catfish often had mild granulomatous inflammation, characterized by small aggregates of macrophages and lymphocytes (Figure 4.15b), with minimal histologically evident bacteria. The most frequently affected tissues included gill, spleen, liver, anterior kidney, and posterior kidney. Microscopic examination of affected gills often revealed moderate, multifocal lamellar aneurysms, occasionally associated with fibrin, few macrophages, and abundant bacteria (Figure 4.15c,d). Mild granulomatous coelomitis commonly

occurred in both vaccinated and non-vaccinated, challenged fish. Multifocal hemorrhages were present occasionally in the central nervous system (brain and spinal cord) and gills of non-vaccinated, challenged fish.

Discussion

Understanding basic host immune mechanisms in response to *E. ictaluri* vaccination and infectious challenge builds a foundation for improved vaccine development and disease prevention in the US catfish industry. Serum antibody levels provide a direct measure of host responsiveness to the bacterium, while transcriptome analyses permit quantification of gene expression across the many pathways related to innate and adaptive immunity. The current study compares serum antibody levels and transcriptome responses in two fish with differing susceptibilities to *E. ictaluri* infection, channel and channel x blue hybrid catfish, in relation to their immunization status and subsequent bacterial challenge, providing insight into their respective immune responses to this important pathogen.

Contrary to reports of greater susceptibility to *E. ictaluri* infection in channel catfish (Armwood et al., 2022; Griffin et al., 2019), non-vaccinated channel and hybrid catfish both experienced high losses with no significant differences between catfish type in survival and cumulative mortality in the current study. The high mortality is attributable to the large bacterial challenge dose and IC delivery route. However, immersion vaccination provided significant protection against *E. ictaluri* IC challenge in both catfish types, again with no significant differences between the two. Consistent with previous studies, findings support the live-attenuated vaccine provides robust protection against *E. ictaluri* challenge (Wise et al., 2015; 2020). The vaccine caused low levels of mortality, indicative of an extreme immunizing dose which was administered to ensure an adequate response from hybrids (López-Porras et al., 2022).

In experimental pond trials, the orally administered live attenuated vaccine produced 18-fold increases in antibody levels in channel catfish fingerlings at 20 dpv, with values reported as the endpoint titer representing a serum dilution having antibody activity (Wise et al., 2015). Wolters et al. (1996) also described higher antibody levels in channels than hybrids following *E. ictaluri* challenge. Antibody levels in this study generally increased following vaccination and subsequent challenge in both catfish types but varied dramatically between individual fish, as seen in other studies (Wise et al., 2015). Despite this, vaccination afforded a high level of protection to both channel and hybrid catfish, although serum IgM was only significantly elevated in channel catfish 28 dpv when compared to non-vaccinated controls. These results suggest *E. ictaluri*-specific antibodies circulating prior to challenge may not play a significant role in the protective immunity in hybrid catfish and that *E. ictaluri* may be less immunogenic in hybrid catfish. At 28 dpc *E. ictaluri*-specific IgM antibodies were significantly elevated in vaccinated channel and hybrid catfish compared to non-vaccinated controls, demonstrating that vaccinated hybrid catfish have elevated antibody levels in response to challenge. Antibodies play important roles in pathogen neutralization, opsonization, antibody-dependent cellular cytotoxicity, and complement activation. Based on the elevated antibody levels in vaccinated, challenged fish surviving 28 dpc, antibodies may play a role in an effective immune response. Serum IgM comparisons from non-vaccinated, challenged catfish were limited by high mortality in non-vaccinated fish, with bacterial doses used to ensure significant disease and immunostimulation for RNA-seq analysis.

Based on evidence of cross-protection between *Edwardsiella* congeners *E. ictaluri* and *E. piscicida*, ELISAs were run against *E. piscicida* on serum from fish 28 dpv and vaccinated fish 28 dpc (Armwood et al., 2022; Griffin et al., 2020a; López-Porras et al., 2022). Incongruously,

E. ictaluri vaccinated channel catfish had significantly elevated levels of cross-reactive antibodies against *E. piscicida* compared to their non-vaccinated counterparts or to vaccinated hybrid catfish 28 dpv and 28 dpc. The lack of significant antibody production in vaccinated, challenged hybrids further supports the assertion that circulating anti-*Edwardsiella* antibodies may play a negligible role in protecting hybrid catfish against *Edwardsiella* spp. infections. In contrast, levels of circulating antibodies in channel catfish suggest they may play a greater protective role against *E. piscicida* and *E. ictaluri* in this species and that vaccination boosts this immune response. However, the immune correlates of protection are not well understood, and antibody avidity requires further investigation.

Mucosal immunity also plays a likely role in natural *E. ictaluri* infection, based on *E. ictaluri* pathogenesis and use of the gastrointestinal and olfactory epithelia as infection routes (Thune et al., 1993). However, IC injection was chosen over an immersion route because the initial study design aimed to compare immune responses elicited by *E. ictaluri* and *E. piscicida*, and IC injection is necessary to produce consistent infection results for *E. piscicida* (Reichley, 2017). Unfortunately, IC injection bypasses the mucosa, limiting elicitation of a mucosal immune response. Mucosal immune responses to *E. ictaluri*, natural and vaccine induced, are poorly characterized in both catfish types, providing a notable area for future work in examining the role of adaptive humoral immunity in protection. The IC challenge route may have also biased the host response transcriptionally with underrepresentation of genes associated with mucosal immunity, although mortality trends, histologic lesions, and vaccine-induced protection mimic those of natural infection.

Transcriptome analysis revealed extensive variation in gene expression between treatment groups. DEGs were not identified in comparisons of vaccinated channel or hybrid

catfish to their non-vaccinated counterparts pre-challenge. Changes in immune gene expression are expected during the immediate post-vaccination period, including those associated with interferon signaling, antigen processing, antigen presentation, complement, coagulation, and potentially humoral immunity. However, sampling occurred 37 to 38 dpv, potentially too late to observe these early patterns of gene transcription. In other fish species such as Arctic charr, transcriptomic responses to vaccination were observed after 517 degree-days post-vaccination with little change observed after (Braden et al., 2018). Mortality from wild-type *E. ictaluri* immersion challenge typically peaks at 8 to 10 days, suggesting peak immune stimulation from the live-attenuated vaccine isolate may occur on a similar timeline. Future research comparing immune responses to vaccination with *E. ictaluri* at earlier timepoints is needed to explore differences in the magnitude and quality of the immune response in channels and hybrids.

The most robust expression increases occurred in non-vaccinated fish following *E. ictaluri* challenge. Complement and coagulation pathways upregulated in channel catfish while they were downregulated in hybrids compared to their non-challenged counterparts. Interestingly, vaccinated and non-vaccinated, non-challenged hybrids had significantly greater expression of complement components compared to channels. When comparing challenged fish, non-vaccinated hybrid and channel catfish had upregulated expression of complement compared to vaccinated fish, which could represent detrimental activation or that the complement system had already been primed by vaccination. However, global transcriptomic profiles comparing non-challenged channel to hybrid catfish revealed significant upregulation of coagulation, complement, and metabolic process pathways in hybrids in both vaccinated and non-vaccinated fish comparisons, suggesting expression of coagulation and complement pathways is at higher baseline levels in hybrids than channels. Counterparts to many mammalian components of

complement exist in teleosts where they likely play similar pro-inflammatory roles, such as chemoattraction and opsonization (Grayfer et al., 2018; Sunyer et al., 2005). Coagulation is also important in combating infection by entrapping bacteria and preventing systemic spread. However, both systems can produce detrimental effects, such as intravascular invasion and thrombosis and overwhelming systemic inflammation if overly activated, and in channel catfish could potentially lead to higher mortality (Berends et al., 2014). The significant upregulation of coagulation and complement pathways in non-vaccinated over vaccinated fish suggests similar processes could contribute to mortalities. Regulation of coagulation relies on inhibitors (e.g., α-2-plasmin, antithrombin), which, in this study, were upregulated in challenged channel catfish compared to unchallenged fish, but interestingly downregulated in hybrid catfish. These inhibitors may play a role in protecting the channels from uncontrolled coagulopathy. The consequences to hybrids are unclear, as they appear to have higher baseline expression of the coagulative cascade prior to challenge.

Protection against *E. ictaluri*, a facultative intracellular pathogen, is more likely the result of M1 macrophage polarization and cell-mediated immune processes (Grayfer et al., 2018). Macrophages play a large role in the immune response to *E. ictaluri*, linking innate immunity to the adaptive response through upregulated macrophage-associated genes such as colony stimulating factor 1 and interferon gamma. Macrophages phagocytize and kill pathogens, generate reactive oxygen and nitrogen intermediates, restrict available nutrients, and induce abundant inflammatory mediators (Grayfer et al., 2018). Pathogen recognition receptors (PRRs) of teleost macrophages recognize different components of extracellular and intracellular pathogens (Grayfer et al., 2018). NOD-like receptors and toll-like receptors (TLRs) 1, 2, and 5 were variably upregulated in certain groups in response to challenge. TLR 5 recognizes bacterial

flagellin, which is upregulated to recognize the peritrichous flagella of *E. ictaluri* (Panangala et al., 2009). TLRs 1 and 2 recognize various lipopeptides. Activated PRRs stimulate various transcription factors and modulate the downstream innate immune response (Palti, 2011; Zhang et al., 2014). IFNγ and TNFα, both upregulated in response to *E. ictaluri*, play vital roles against obligate and facultative intracellular pathogens (Grayfer et al., 2018). Release of IFNγ by Th1 helper lymphocytes and natural killer cells corresponds with classical/M1 macrophage activation, which then skews cytokine production toward a Th1 response (Grayfer et al., 2018). Th1 responses are effective against intracellular pathogens, such as *E. ictaluri.* Classically activated macrophages upregulate various inflammatory and antigen presentation effectors, such as tapasin, which was upregulated in response to bacterial challenge (Landis et al., 2006).

Histopathologic findings were consistent with lesions induced by other *Edwardsiella* species in channel and hybrid catfish fingerlings, particularly *E. piscicida* (Armwood et al., 2022; López-Porras et al., 2021). Subjectively, changes were more common, necrotizing, and involved higher numbers of bacteria in non-vaccinated than vaccinated fish. Efficacy of the vaccine at stimulating bacterial clearance is further supported by qPCR results that demonstrated an absence of bacteria in the kidneys of 11/12 vaccinated fish, compared to their presence in all kidneys of non-vaccinated fish, indicating enhanced bacterial clearance in immunized cohorts. Moreover, foci of macrophages and lymphocytes with few associated bacteria indicates an immune response primed for efficient pathogen clearance. The mild coelomitis observed in nearly all challenged fish was mild and consistent with the intracoelomic challenge route.

Findings in this study illustrate how bacterial infection, vaccination, and fish type influence the host response to *E. ictaluri* infection in channel and hybrid catfish. Results suggest the differing susceptibilities of the two catfish types and protection conferred by vaccination to

E. ictaluri challenge integrate multiple components of the host immune response with variable antibody production and notable differential regulation of genes related to innate immunity, complement, and coagulation. DEGs were identified between fish types and across treatments, although no DEGs were attributed to vaccination alone. However, the live-attenuated vaccine conferred significant protection against wild-type bacterial challenge in both channel and hybrid catfish, supported by less frequent detection of *E. ictaluri* in tissues by PCR and fewer necrotizing lesions with histopathology. The expression patterns of immune related genes determined by transcriptomic analysis were limited to the posterior kidney, and likely vary in different tissues. Immersion challenge and investigation of DEGS in the immediate post-challenge period with exploration of mucosal immunity is needed to further elucidate the host response to *E. ictaluri* infection and vaccination.

Figures

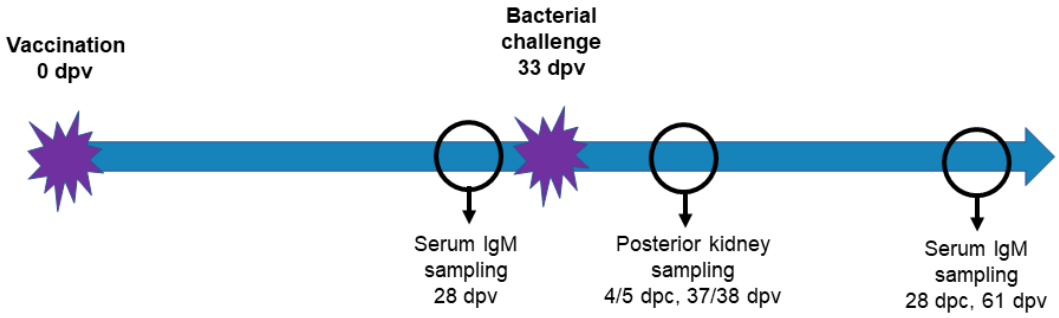

Figure 4.1. Experimental design timeline. Fish were vaccinated or sham-vaccinated 33 days prior to bacteria challenge. Serum was sampled for relative IgM quantification 28 dpv (days post vaccination) and 28 dpc (days post challenge). Posterior kidneys were collected at 4/5 dpc for RNA-sequencing and qPCR validation, and then the whole fish were preserved in formalin for histopathologic examination.

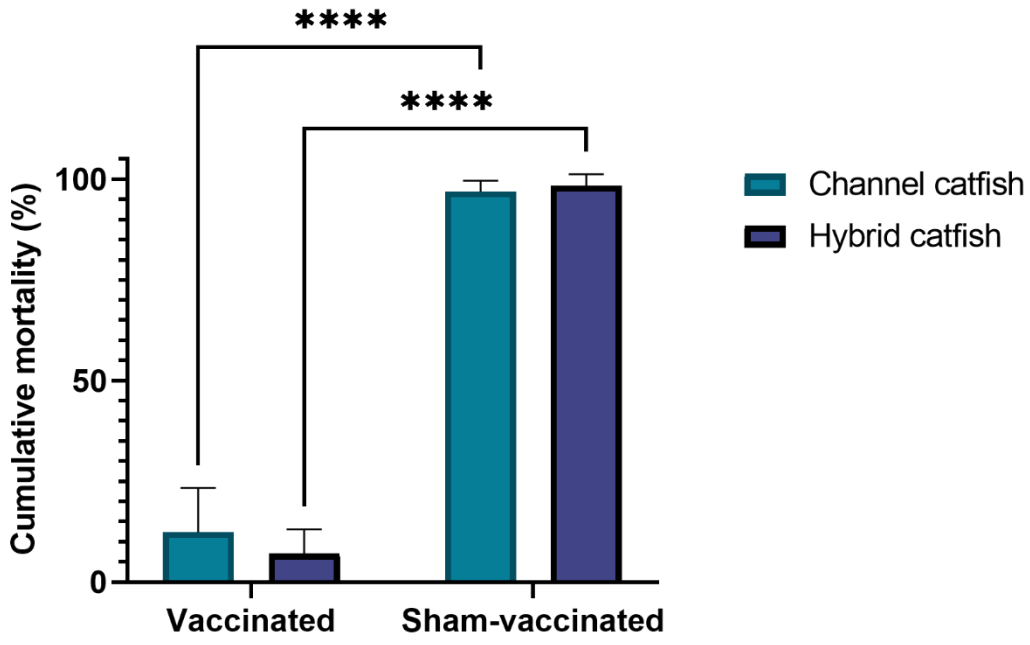

Figure 4.2. Cumulative mortality of vaccinated and sham-vaccinated, channel and hybrid catfish 28 days after exposure to *Edwardsiella ictaluri*. Vaccinated and sham-vaccinated controls had 0% cumulative mortality and were not significantly different than vaccinated fish. **** = $p <$ 0.0001.

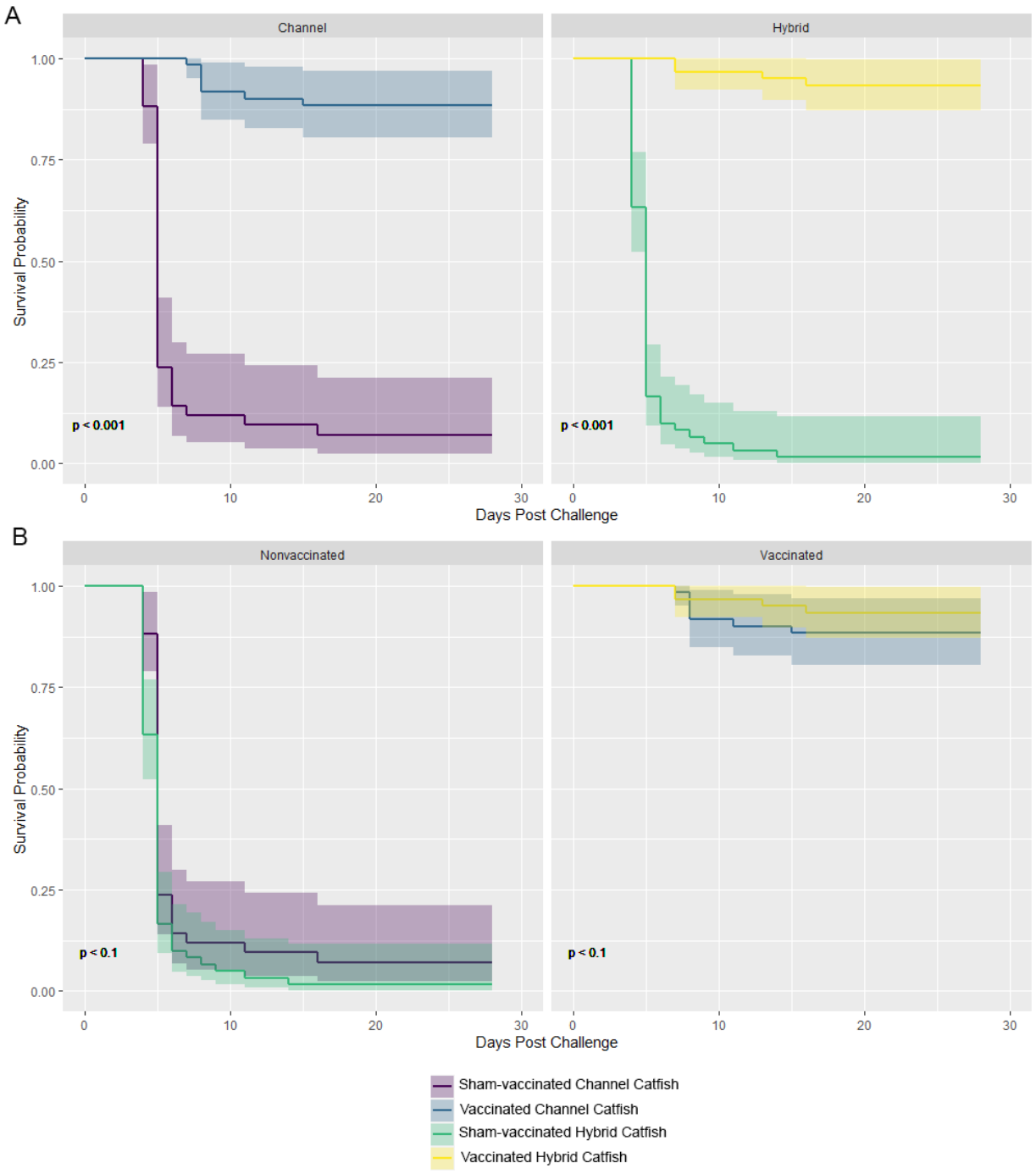

Figure 4.3. Kaplan-Meier survival curves comparing survival probability after *Edwardsiella ictaluri* intracelomic challenge in vaccinated and non-vaccinated channel and hybrid catfish from non-sampling tanks (n = 20 fish per tank; 3 tanks per treatment). Fish were vaccinated with a

live-attenuated *E. ictaluri* isolate (S97-773-340x2; Wise et al., 2015) and, at 33 dpv, challenged with an intracoelomic injection of *E. ictaluri* isolate S97-773. A) Survival probability was significantly higher in vaccinated hybrid and channel catfish ($p < 0.001$). B) No significant difference in survival probability was present comparing vaccinated channel catfish to vaccinated hybrid catfish or non-vaccinated channel catfish to non-vaccinated hybrid catfish after *E. ictaluri* challenge. Data presented include the mean survival curves and the 95% confidence intervals.

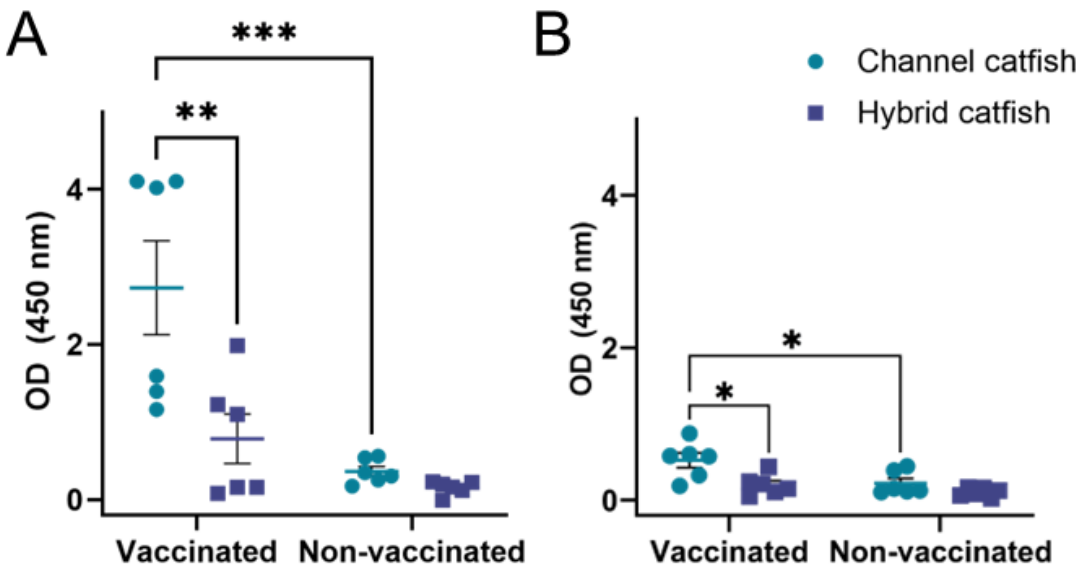

Figure 4.4. Relative IgM antibody concentrations (OD = optical density) were measured by ELISA at 28 days post vaccination from 1:320 dilutions of serum from vaccinated and non-vaccinated, channel and hybrid catfish (n = 6). Statistical significance was established using a two-way ANOVA followed by Tukey *post-hoc* HSD ($p < 0.05$). A) Antibodies against *Edwardsiella ictaluri* were significantly elevated in vaccinated channel catfish when compared to non-vaccinated channel catfish and vaccinated hybrid catfish, with no significant difference between vaccinated and non-vaccinated hybrid catfish. B) Similarly, antibodies against *E. piscicida* antigen were significantly elevated in vaccinated channel catfish compared to non-vaccinated channel catfish and vaccinated hybrid catfish, with no significant differences between the vaccinated and non-vaccinated hybrid catfish. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

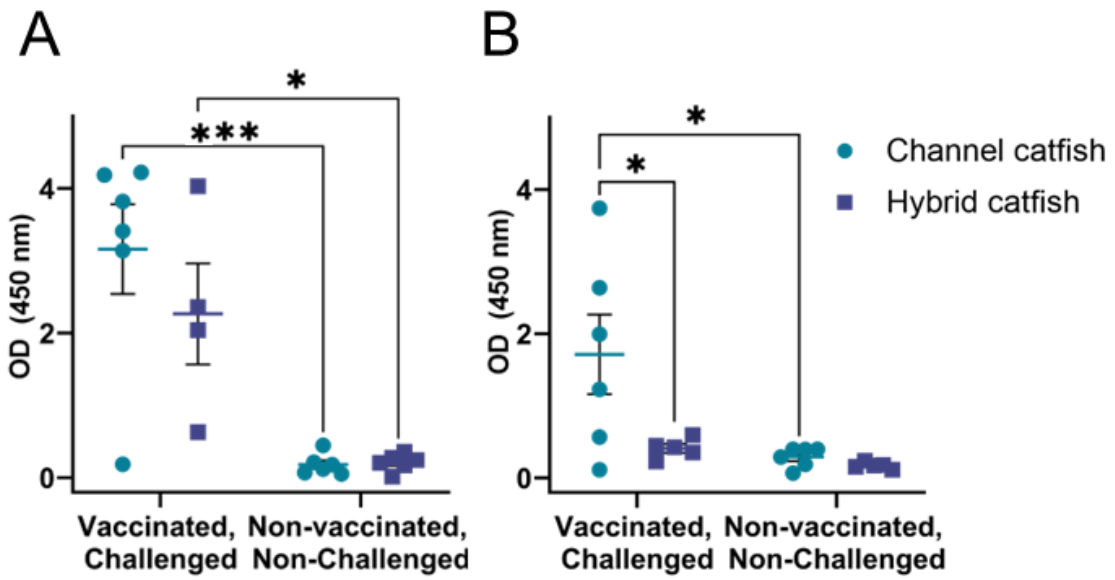

Figure 4.5. Relative serum IgM antibody concentrations (OD = optical density) were measured by ELISA at 28 days post challenge from 1:320 dilutions of serum from vaccinated and non-vaccinated, channel and hybrid catfish (n = 6). Statistical significance was established using a two-way ANOVA followed by Tukey *post-hoc* HSD ($p < 0.05$). A) Vaccinated, challenged catfish had significantly elevated serum IgM antibody levels against *Edwardsiella ictaluri* in comparison to their non-vaccinated, non-challenged counterparts. B) Vaccinated, challenged channel catfish had significantly higher serum IgM levels against *Edwardsiella piscicida* than vaccinated, challenged hybrid catfish and non-vaccinated, non-challenged channel counterparts. No significant differences existed between vaccinated, challenged hybrid catfish and non-vaccinated, non-challenged hybrid catfish. * = $p < 0.05$; *** = $p < 0.001$.

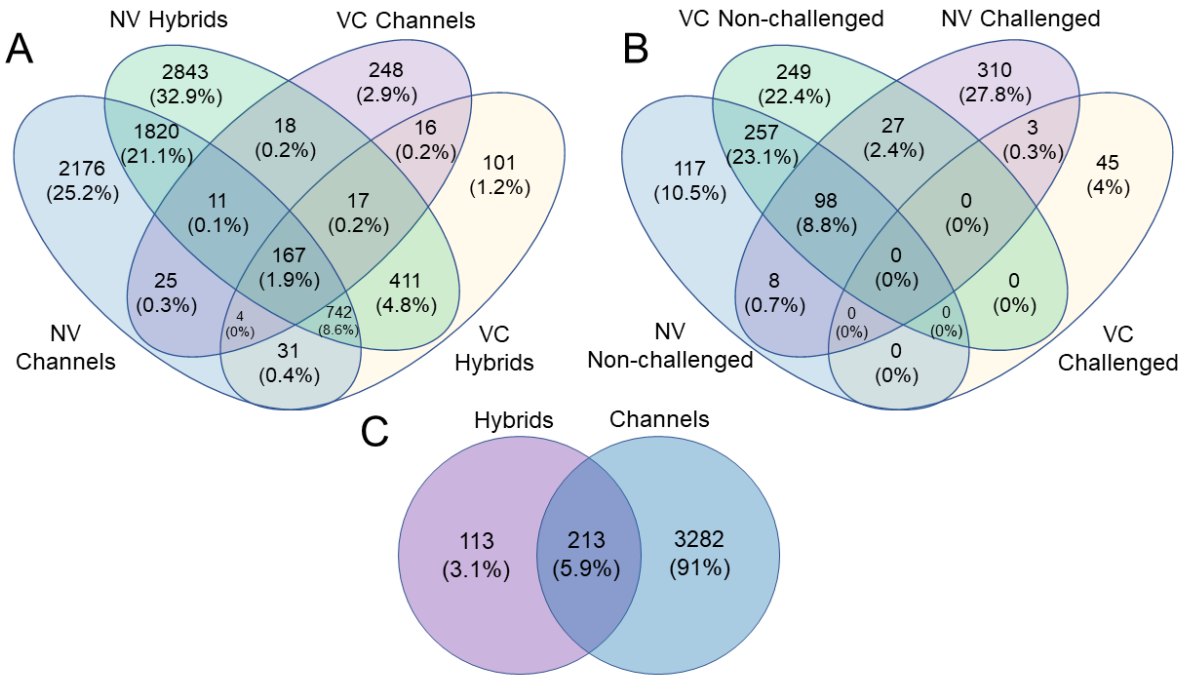

Figure 4.6. Venn diagrams illustrating global transcript expression profiles showing: A) distribution of DEGs between challenge status among fish variety and vaccine status, B) distribution of DEGs between fish variety among vaccine and challenge statuses, C) distribution of DEGs between vaccinated and sham-vaccinated challenged fish, comparing catfish varieties. NV = non-vaccinated; VC = vaccinated, DEG= differentially expressed gene.

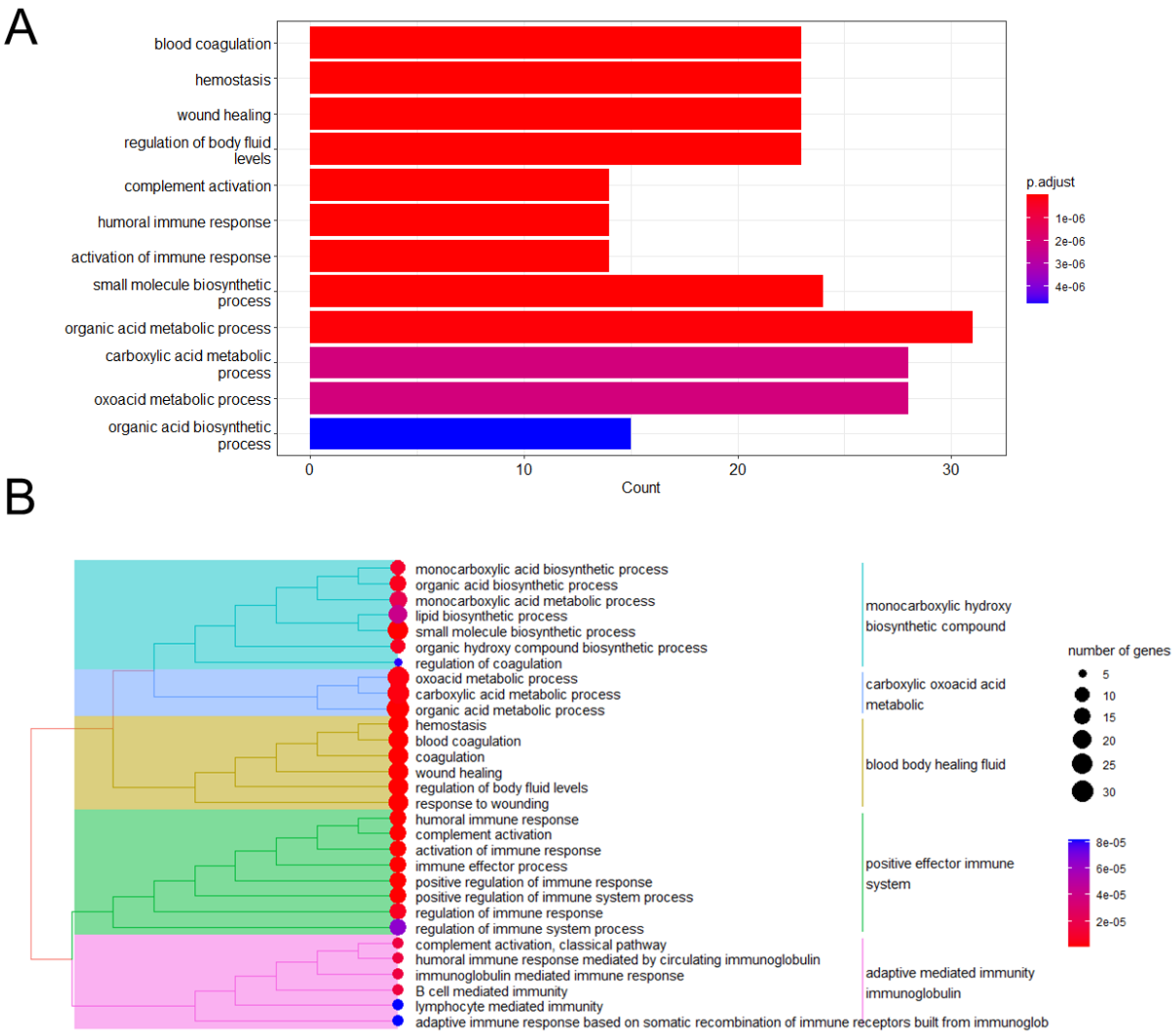

Figure 4.7. Gene Ontology (GO) pathway enrichment analysis showing: A) enriched pathways among genes upregulated in vaccinated hybrid catfish compared to vaccinated channel catfish, B) a heatmap of enriched terms in vaccinated hybrid catfish compared to vaccinated channel catfish.

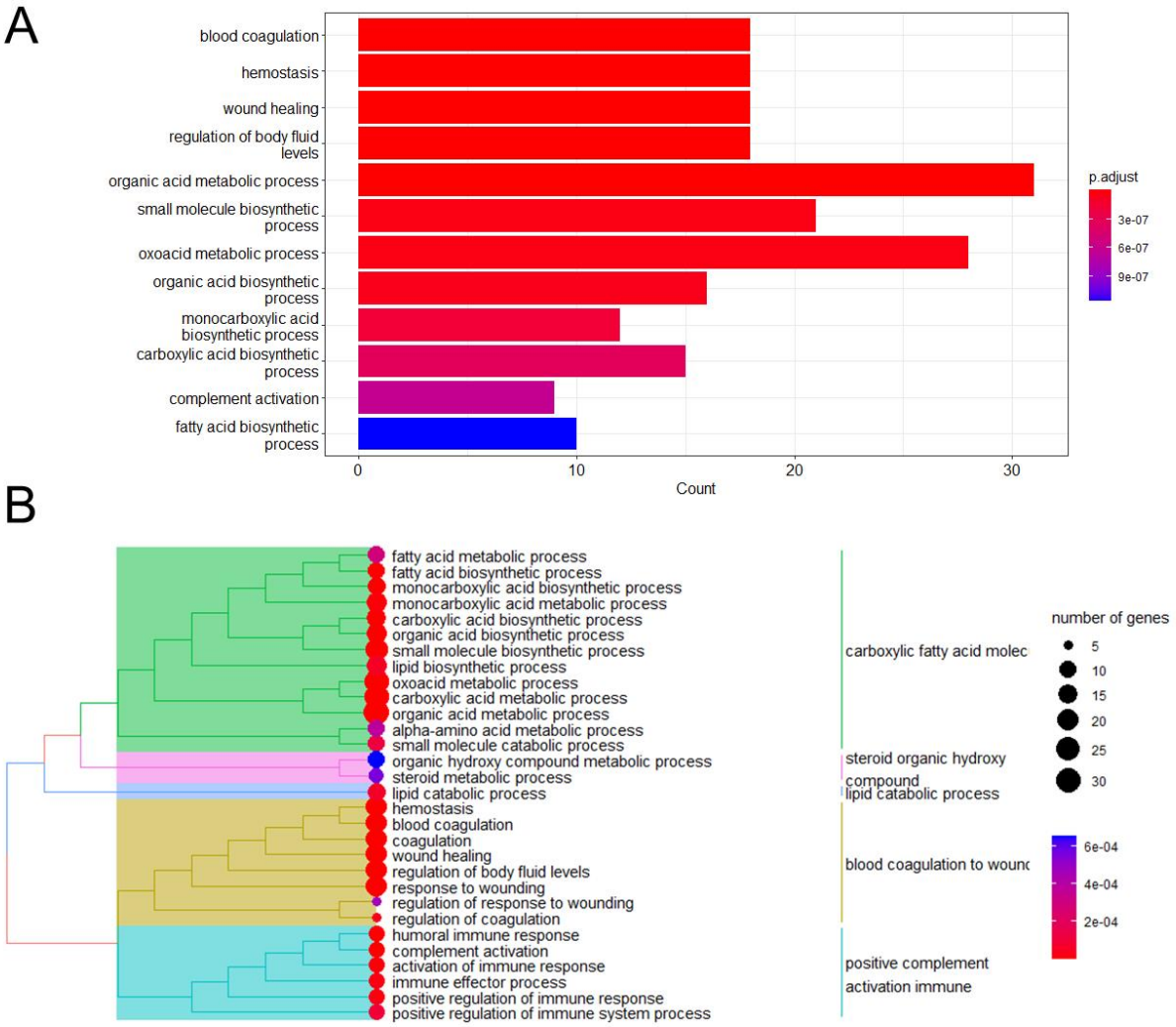

Figure 4.8. Gene Ontology (GO) pathway enrichment analysis showing: A) enriched pathways among genes upregulated in sham-vaccinated hybrid catfish compared to sham-vaccinated channel catfish, B) a heatmap of enriched terms in sham-vaccinated hybrid catfish compared to sham-vaccinated channel catfish.

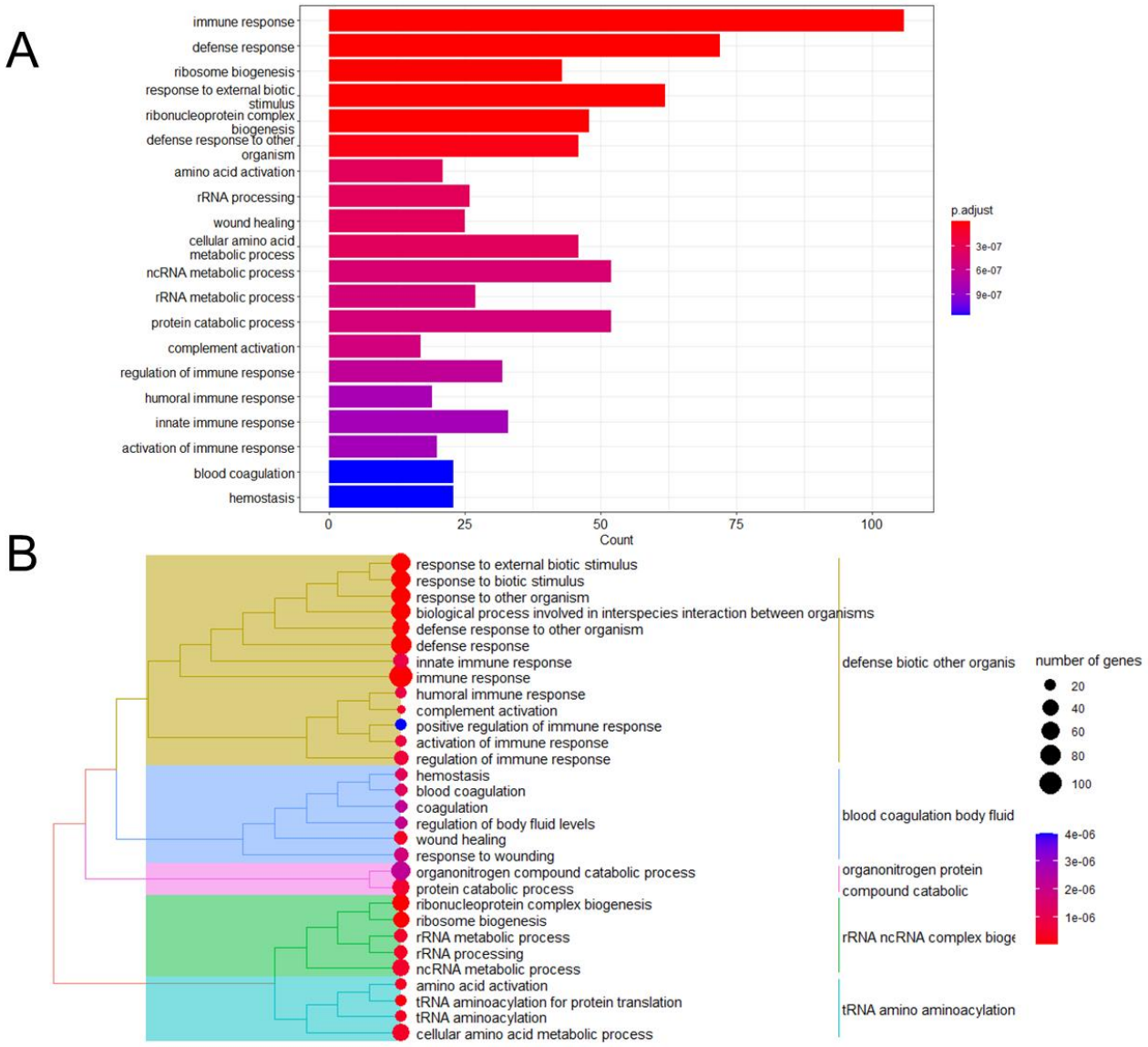

Figure 4.9. Gene Ontology (GO) pathway enrichment analysis showing: A) enriched pathways among genes upregulated in challenged, sham-vaccinated channel catfish compared to non-challenged, sham-vaccinated channel catfish, B) a heatmap of enriched terms in challenged, sham-vaccinated channel catfish compared to non-challenged, sham-vaccinated channel catfish.

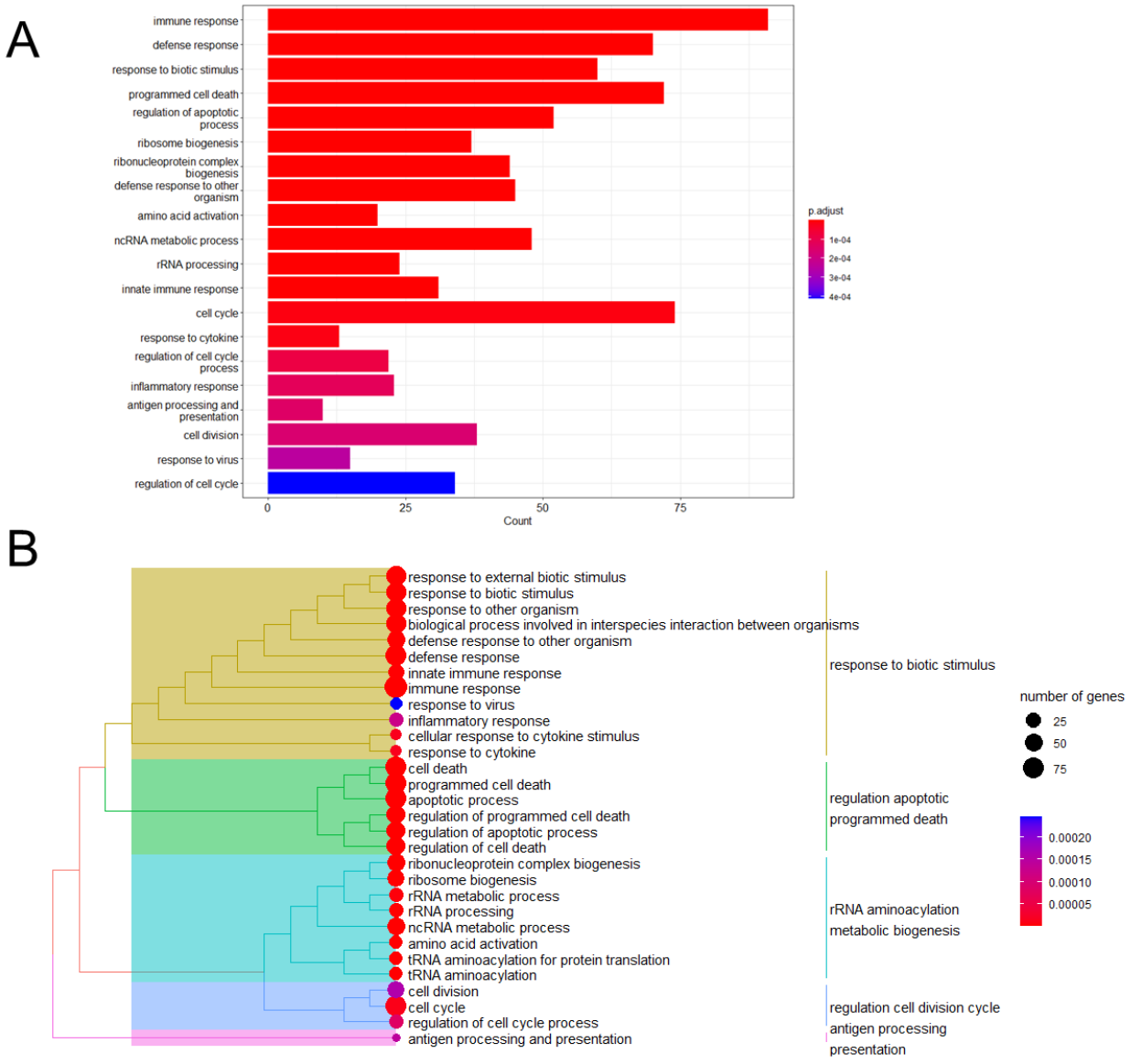

Figure 4.10. Gene Ontology (GO) pathway enrichment analysis showing: A) enriched pathways among genes upregulated in challenged, sham-vaccinated hybrid catfish compared to sham-vaccinated, non-challenged hybrid catfish, B) a heatmap of enriched terms in challenged, sham-vaccinated hybrid catfish compared to sham-vaccinated, non-challenged hybrid catfish.

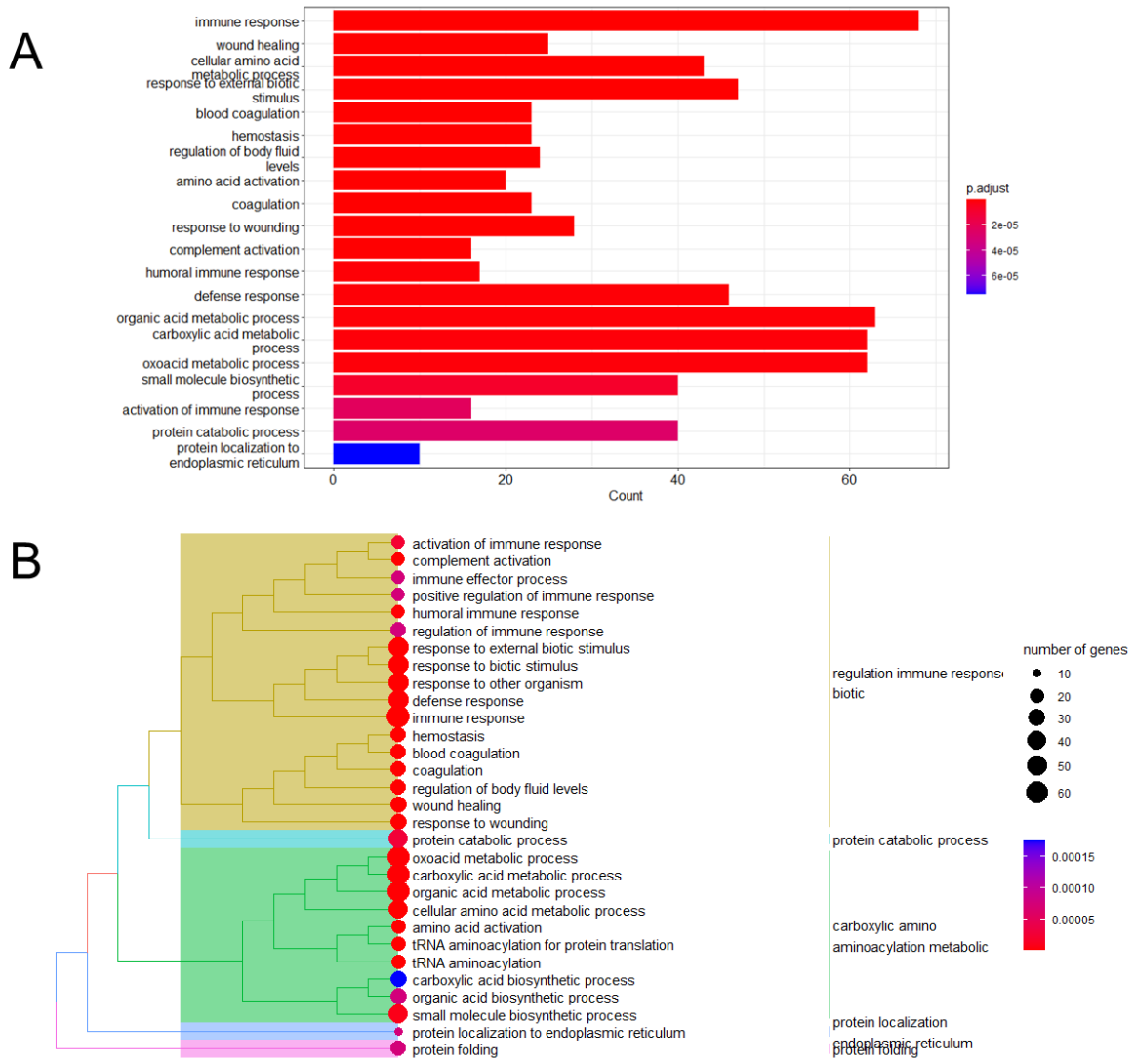

Figure 4.11. Gene Ontology (GO) pathway enrichment analysis showing: A) enriched pathways among genes upregulated in challenged, sham-vaccinated channel catfish compared to challenged, vaccinated channel catfish, B) a heatmap of enriched terms in challenged, sham-vaccinated channel catfish compared to challenged, vaccinated channel catfish.

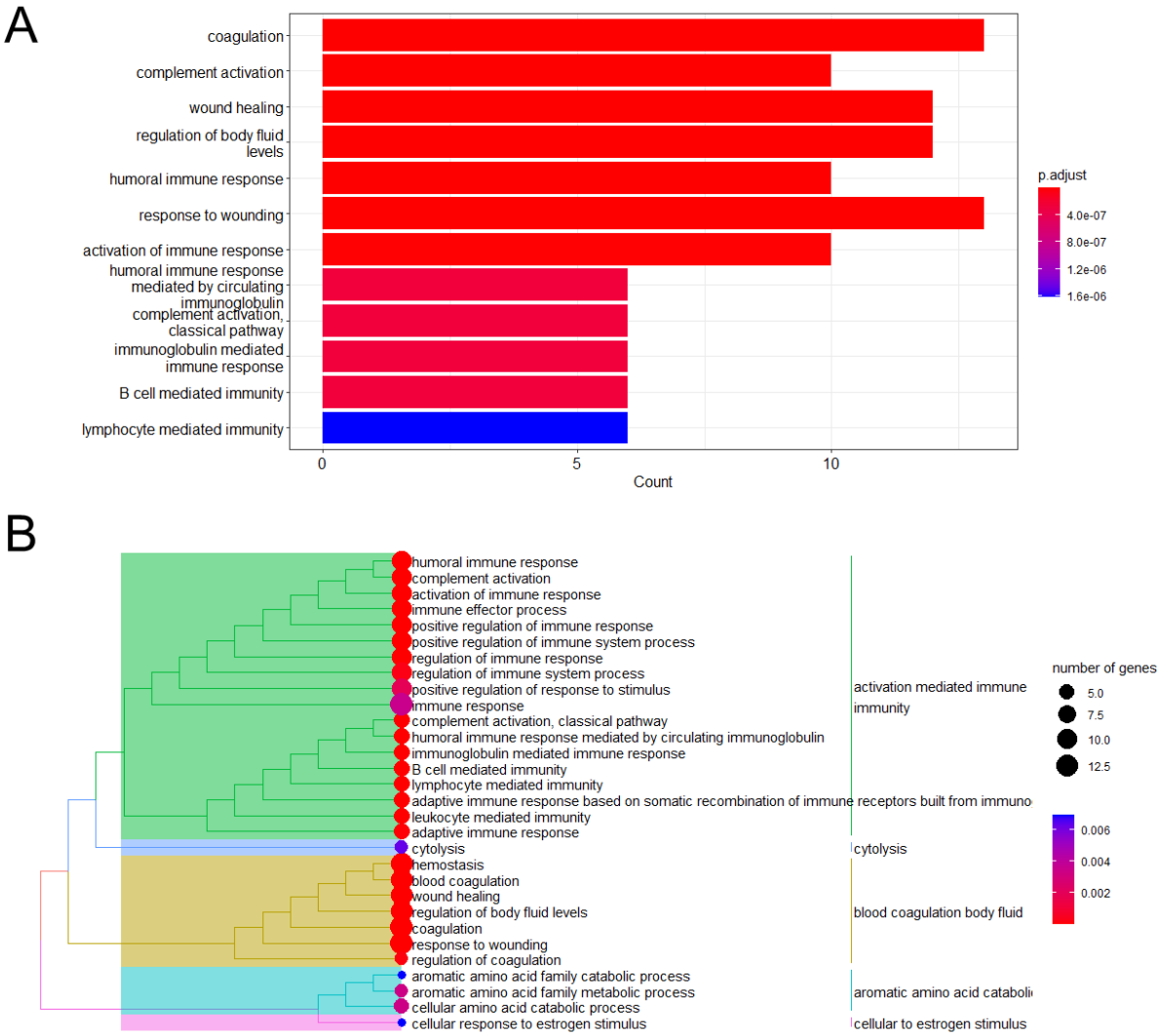

Figure 4.12. Gene Ontology (GO) pathway enrichment analysis showing: A) enriched pathways among genes upregulated in challenged, sham-vaccinated hybrid catfish compared to challenged, vaccinated hybrid catfish, B) a heatmap of enriched terms in challenged, sham-vaccinated hybrid catfish compared to challenged, vaccinated hybrid catfish.

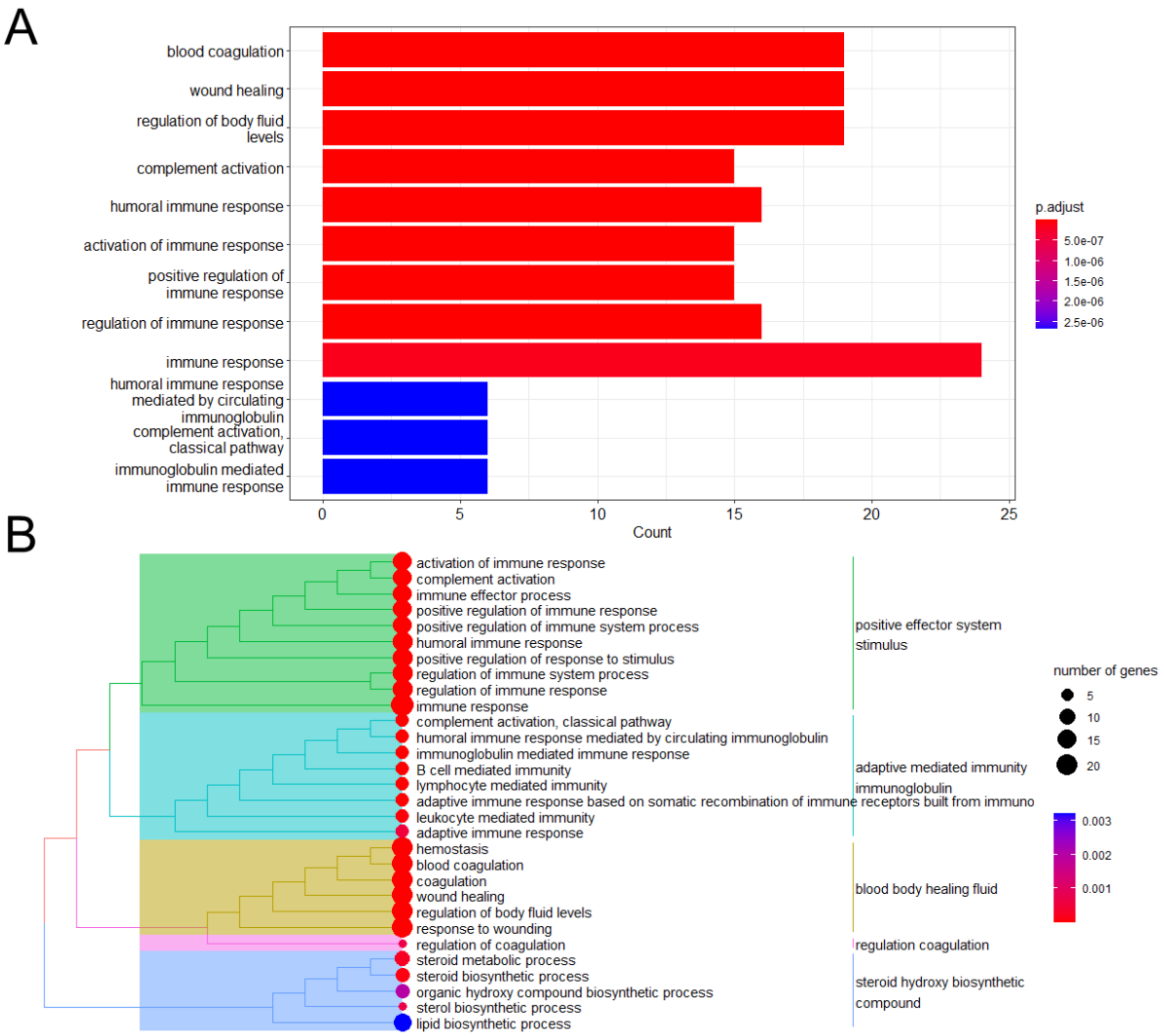

Figure 4.13. Gene Ontology (GO) pathway enrichment analysis showing: A) enriched pathways among genes upregulated in challenged, sham-vaccinated channel catfish compared to challenged, sham-vaccinated hybrid catfish, B) a heatmap of enriched terms in challenged, sham-vaccinated channel catfish compared to challenged, sham-vaccinated hybrid catfish.

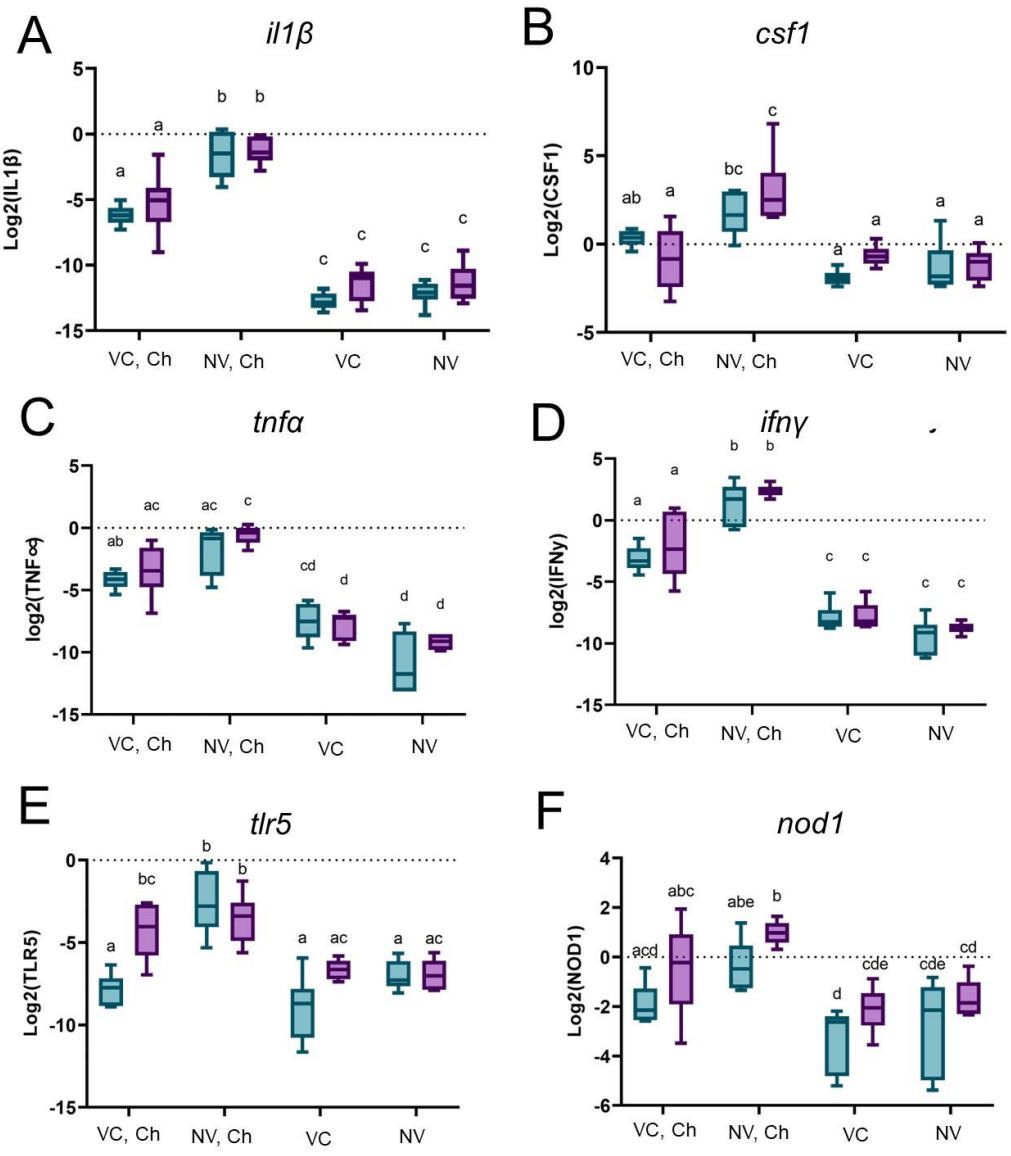

Figure 4.14. qPCR validation of RNA-seq data with select immune genes. The box plots represent the median log2CNRQ with 95% confidence intervals. Significance was determined using two-way or three-way ANOVA followed by *post-hoc* Tukey HSD. Significance is indicated with letters ($p < 0.05$). VC = vaccinated; Ch = challenged; NV = non-vaccinated.

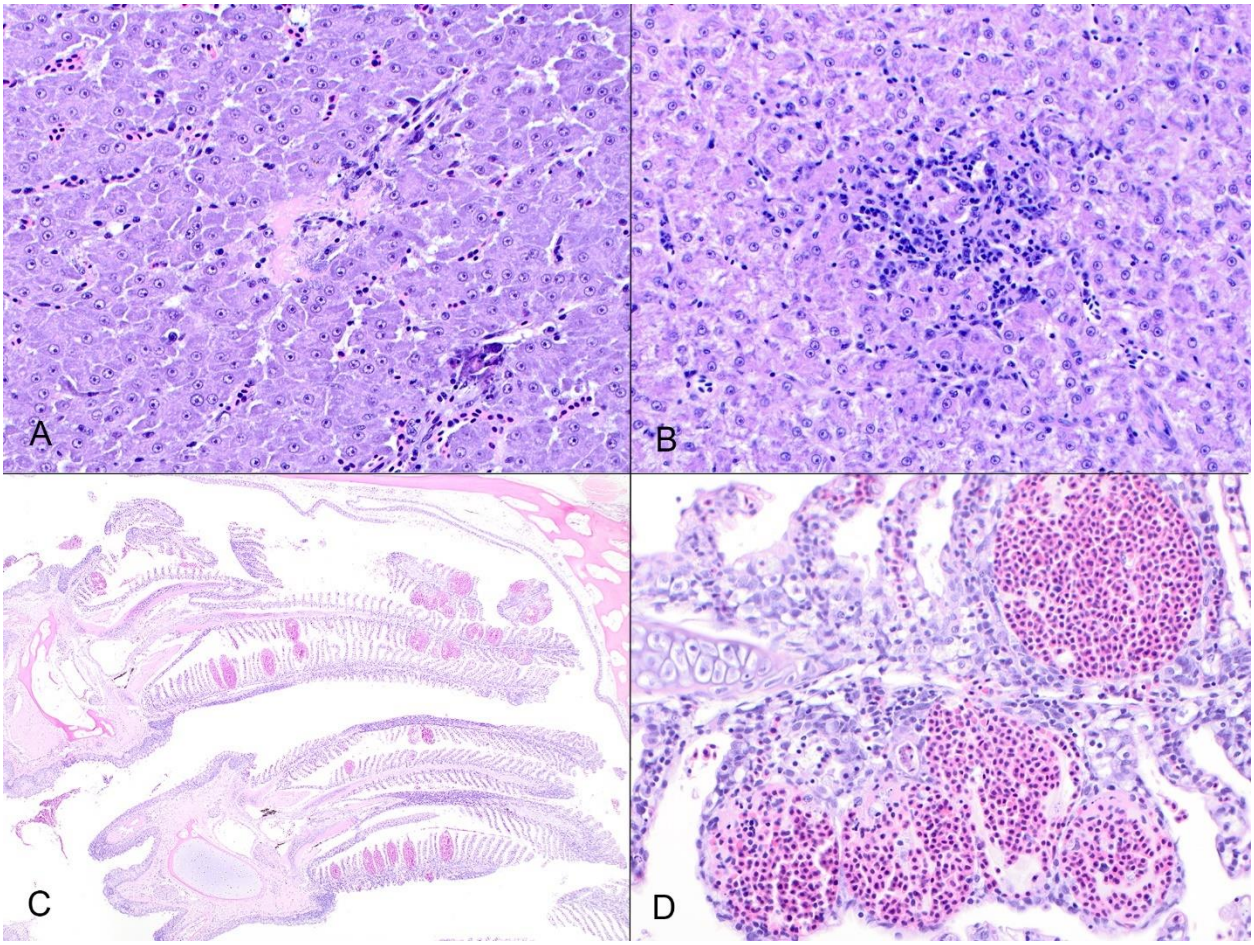

Figure 4.15. Histopathologic findings in catfish fingerlings challenged intracoelomically with *Edwardsiella ictaluri.* A) Liver. Necrotizing lesions, characterized by hypereosinophilic hepatocytes with fragmented nuclei, cellular debris, and abundant bacterial rods, predominated in non-vaccinated, challenged fish. B) Liver. Vaccinated, challenged catfish often had small aggregates of lymphocytes and fewer macrophages, with minimal histologically evident bacteria. C) Gills. The gills occasionally had moderate, multifocal lamellar aneurysms. D) Gills. Lamellar aneurysms were often associated with fibrin, few macrophages, and abundant bacterial rods.

Tables

Table 4.1. Primer sets for study reference genes and genes of interest for qPCR.

Gene	Name	Forward (5'-3')	Reverse (5'-3')	Amplicon size (bp)	Efficiency (%)	R^2
Reference genes						
tubulin	*TUB*	AGCCATACAATTCCATCCTGACC	GCGGCAGATGTCGTAGATGG	95	95	0.987
beta-2-microglobulin	*B_2M*	GCCTTTGCTAAGGAGTCTCCACC	GACCTGCTGAAGAATGGCGAGGT	143	92	0.978
beta-actin	*β-actin*	CCCATCTATGAGGGTTATGCTCTG	GCTCGGTCAGGATCTTCATCAG	98	101	0.950
glyceraldehyde-3-phosphate dehydrogenase isoform 1	*GADPH*	TCTTATGAGCACTGTCCATGCC	TAATGTTCTGGCTGGCACCAC	101	98	0.988
elongation factor 1-alpha	*EF1α*	TCAGTGAAGGACATCCGTCG	AGCGTAGCCCTGAGAGATC	129	92	0.987
Immune genes of interest						
interleukin 1 beta	*IL1β*	TTTGGCCATGAGCGGCAG	GTTTCAGCCTCTGCAAAGCG	116	97	0.988
interferon gamma	*IFNγ*	GGACCTTAAGGAAAATGACCCACGG	TTAGCTTGACGTCGTCTCCG	124	101	0.974
tumor necrosis factor alpha	*TNFα*	TCGTGGTCTTCTTCAGGAG	GTTCTGCGTCTTGTTTTGAG	102	100	0.989
toll-like receptor 5	*TLR5*	GTCTCAAAACGGCTTCAGCG	ACTCCCTTTCCCATGCTGCC	111	96	0.994
nucleotide-binding oligomerization domain-containing protein 1	*NOD1*	AGCAGTGTGGAAGTTTTGGC	CAGTTTGGCTCCAACATCCG	99	96	0.969
complement component 3	*C3*	TCAATAAGGACTCGCTGGCG	TATCCCATTGTGAGCTGCGG	123	97	0.996
complement component 9	*C9*	ACAGCTTGGACTATGTGGGC	TGAGTCGTTGGTTTGCAGGG	143	97	0.996
coagulation factor X	*CfX*	CATGAGGGTGGTCTACAGGC	GCATCCTTTTCCTCCTTGGC	140	91	0.991

colony stimulating factor 1	*csf1*	GAACACACACACAACAGCCC	ACACATGGACCAGGTACAGC	102	93	0.990

Table 4.2. Relative percent survival (RPS) for channel and hybrid catfish challenged with *Edwardsiella ictaluri* at 28 dpc. Values are averages of three replicate tanks per treatment ± standard deviation of the mean.

Fish group	**Relative Percent Survival (RPS)**
Channel catfish	87.7%
Hybrid catfish	93.2%

Table 4.3. Global transcript expression profiles.

Comparison category	**Treatment comparison**	**Number of differentially expressed genes (DEGs)**		
		Upregulation	**Downregulation**	**Total**
Vaccine status				
	Challenged sham-vaccinated channels vs. challenged vaccinated channels	2060	1435	3495

	Non-challenged vaccinated channels vs. non-challenged sham-vaccinated channels	0	0	0
	Challenged sham-vaccinated hybrids vs. challenged vaccinated hybrids	7	319	326
	Non-challenged vaccinated hybrids vs. non-challenged sham-vaccinated hybrids	0	0	0
Bacterial challenge				
	Non-challenged sham-vaccinated channels vs. challenged sham-vaccinated channels	2250	2726	4976
	Non-challenged vaccinated channels vs. challenged vaccinated channels	290	216	506

	Non-challenged sham-vaccinated hybrids vs. challenged sham-vaccinated hybrids	3573	2546	6029
	Non-challenged vaccinated hybrids vs. challenged vaccinated hybrids	481	1009	1489
Fish types				
	Challenged vaccinated channels vs. challenged vaccinated hybrids	2	46	48
	Challenged sham-vaccinated channels vs. challenged sham-vaccinated hybrids	429	17	446
	Non-challenged vaccinated channels vs. non-challenged vaccinated hybrids	60	571	631
	Non-challenged sham-vaccinated channels vs. non-challenged sham-vaccinated hybrids	18	462	480

False discovery rate (FDR) < 0.05

Table 4.4. Histopathologic analysis summary of fish submitted for RNA-seq analysis (n = 5 fish/treatment group), indicating fish with hemorrhage, necrosis, and/or granulomatous inflammation.

Treatment group	***Affected tissues***						
	Liver	Anterior kidney	Posterior kidney	Gill	Spleen	Coelom	Central nervous tissue
Hybrid catfish							
Vaccinated, challenged	2/4	1/2	0/4	1/5	0/3	5/5	0/5
Non-vaccinated, challenged	5/5	4/4	4/5	3/5	2/2	5/5	3/5
Vaccinated, non-challenged	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Non-vaccinated, non-challenged	0/5	0/3	0/5	0/5	0/2	0/5	0/5
Channel catfish							
Vaccinated, challenged	*3/5*	1/5	0/5	1/5	2/5	4/5	0/5

Non-vaccinated, challenged	2/3	2/3	3/5	1/5	3/4	5/5	2/5
Vaccinated, non-challenged	0/5	0/4	0/4	0/5	0/4	0/5	0/5
Non-vaccinated, non-challenged	0/5	0/2	0/5	0/5	0/2	0/5	0/5

If number per examined tissue is less than 5, the tissue was not captured in histologic section in all fish.

CHAPTER 5

IMMUNOLOGIC ANALYSIS OF *EDWARDSIELLA PISCICIDA* CHALLENGE IN VACCINATED AND NON-VACCINATED CHANNEL AND CHANNEL ♀ X BLUE ♂ HYBRID CATFISH [1]

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[1]Armwood, A.R., Cai, W., Leary, J., López-Porras, A., Richardson, B.M., Ware, C., Purcell, S.L., Wise, D., Fast, M.D., Griffin, M.J., and A.C. Camus. To be submitted to *Journal of Fish Diseases.*

Abstract

Edwardsiella piscicida is an emerging pathogen in United States catfish aquaculture, causing disease in predominantly market-sized or stocker-sized channel (*Ictalurus punctatus*) x blue *(I. furcatus*) hybrid catfish. Recently, a live-attenuated *Edwardsiella ictaluri* vaccine has been shown to induce cross-protective immunity against *E. piscicida* in channel and hybrid catfish. Herein, the immune response to *E. piscicida* challenge in vaccinated and non-vaccinated channel and hybrid catfish was assessed. Catfish fingerlings were immunized via immersion with a live-attenuated *E. ictaluri* vaccine, and a subset challenged 33 days later with an intracoelomic injection of *E. piscicida*. Relative percent survival (RPS) was 84.0% in channels and 38.1% in hybrids, with vaccinated fish more likely to survive challenge than non-vaccinated fish. At 28 dpc, serum IgM levels were significantly increased in non-vaccinated, challenged channel catfish. Comparably, no significant increases in serum IgM levels were observed in vaccinated, challenged channels, control channels, or any of the hybrid catfish treatments. Channel catfish developed a more robust humoral and Th1 response to *E. piscicida* challenge than hybrid catfish. Transcription of complement and coagulation genes were upregulated in challenged, vaccinated channel catfish compared to non-challenged counterparts, suggesting the *E. ictaluri* vaccine may confer protection to channels through rapid induction of the complement system and coagulation cascade. This study lays the foundation for understanding catfish host responses to *E. piscicida* challenge.

Introduction

Edwardsiella piscicida is an emerging gram-negative, facultative intracellular bacterial fish pathogen synonymous with isolates historically designated "typical, motile, fish pathogenic *Edwardsiella tarda*" (Abayneh et al., 2013; Griffin et al., 2017; Matsuyama et al., 2005; Yamada and Wakabayashi, 1998; 1999). Improved molecular techniques, with species-specific PCR and discriminatory genetic markers, revealed *E. tarda* contained three genetically distinct species, including *Edwardsiella anguillarum*, *E. tarda,* and *E. piscicida* (Abayneh et al., 2013; Shao et al., 2015). Since its description, *E. piscicida* associated disease has been diagnosed in an extensive and growing list of fish across multiple continents, becoming a notable pathogen of southeastern United States catfish aquaculture (Armwood et al., 2022; Griffin et al., 2019; 2020b; Reichley et al., 2018).

Channel (*Ictalurus punctatus*) and channel ♀ x blue (*I. furcatus*) ♂ hybrid catfish comprise the largest aquaculture industry in the United States, with approximately 51,800 water surfaces acres dedicated to commercial production in 2022 (USDA/NASS, 2022). Hybrid catfish are becoming increasingly popular over channel catfish due to their superior feed conversion, improved growth, crowding tolerance, and reduced disease susceptibility (Arias et al., 2012; Dunham et al., 2008; 2014; Griffin et al., 2010; Hegde et al., 2022; Kumar & Engle, 2010; Wolters et al., 1996). Concurrent with the recent industry pivot toward hybrid catfish production, *E. piscicida* has emerged as the most important bacterial pathogen to affect hybrid culture (Griffin et al., 2019). Records from the Aquatic Research and Diagnostic Laboratory at the Thad Cochran National Warmwater Aquaculture Center (NWAC) in Stoneville, Mississippi indicate hybrids accounted for 89.1% of *E. piscicida* cases from 2013 to 2017 (Griffin et al., 2019). This trend has continued, with hybrids accounting for 91.5% of *E. piscicida* diagnoses in 2020 and

95.0% in 2021 (M.J. Griffin, unpublished data). Furthermore, experimental challenges corroborate the increased susceptibility of hybrids over channel catfish (Armwood et al., 2022; López-Porras et al., 2021; 2022; Reichley et al., 2018).

The closely related *Edwardsiella ictaluri* is another significant pathogen in the US catfish industry, predominantly affecting the fingerling stage of production (Wise et al., 2004). Comparably, *E. piscicida* primarily affects stocker or market-sized catfish. Mortality in these larger fish, which frequently occurs in their second growing season as they approach harvest size, incurs significant costs to the producers as substantial investment has already been made in the crop that cannot be recovered if fish die before harvest (Griffin et al., 2019). Natural infections result in generalized sepsis, with gross lesions including dermal ulcerations, exophthalmia, abdominal distension, and splenomegaly (Griffin et al., 2019). Histologic lesions include mononuclear meningoencephalitis, hemorrhagic branchitis, splenitis, ulcerative dermatitis, granulomatous interstitial nephritis, hepatitis, and/or hemorrhagic enteritis (Griffin et al., 2019). Lesions induced by intracoelomic challenges mimic the natural disease, involving the spleen, kidneys, brain, gill, and central nervous tissue, with changes ranging from acute and necrotizing with abundant bacteria to chronic and granulomatous with few bacteria, as described in Chapter 2 (Armwood et al., 2022; López-Porras et al., 2021).

Management of *E. piscicida* outbreaks includes treatment with antimicrobials, with reported susceptibility to florfenicol, oxytetracycline, and a 5:1 combination of sulfadimethoxine and ormetoprim (Griffin et al., 2019; Reichley et al., 2017). However, preventive strategies such as vaccination are highly desired by the industry. Currently, an orally delivered, live attenuated *E. ictaluri* vaccine (U.S. Patent No. 8,999,319) is used extensively in the southeast United States for control of Enteric Septicemia of Catfish (ESC) (Aarattuthodiyil et al., 2020; Chatakondi et

al., 2018; Greenway et al., 2017; Hegde et al., 2022; Peterson et al., 2016; Wise et al., 2015; 2020). This attenuated strain, derived from a wild-type *E. ictaluri* isolate originating from a disease outbreak on a commercial farm near Stoneville, MS, has also shown cross-protective effects against *E. piscicida* (Griffin et al., 2020a).

Bath exposure of channel catfish to *E. piscicida* protected them against immersion challenge with wild-type *E. ictaluri* (Griffin et al., 2020a). Likewise, López-Porras et al. (2022) described relative percent survivals (RPS) of 54.7 to 77.8% in hybrid catfish challenged with multiple *E. piscicida* variants 30 days after vaccination with the attenuated *E. ictaluri* vaccine isolate and 80.5% to 100% RPS in channels. Similarly, bath immersion in *E. piscicida* provided protection 30 days later against immersion challenge with wild-type *E. ictaluri* (López-Porras et al., 2022). Chapter 2 (Armwood et al., 2022) revealed channel catfish surviving *E. piscicida* challenge had a greater chance of surviving *E. ictaluri* challenge 100 days later than naïve fish, and that hybrid catfish surviving *E. piscicida* or *E. anguillarum* challenge exhibited greater survival against *E. piscicida* challenge than naïve controls. Taken collectively, these findings provide evidence for cross-protective effects between *Edwardsiella* congeners and a potential for polyvalent or cross-protective vaccines for industry use.

Due to the recent recognition of *E. piscicida* as a distinct taxon, little is known concerning immune responses of catfish to infection or why hybrid catfish are more susceptible than channels. In Chapter 4, immune responses to the live attenuated *E. ictaluri* vaccine and to challenge with wild-type *E. ictaluri* were examined using enzyme-linked immunosorbent assay, quantitative PCR, and RNA-seq analysis. Channel catfish produced a significantly elevated serum IgM response against *E. ictaluri* in response to vaccination, while hybrids did not. This study builds on previous work reported in Chapter 4, applying a similar study model and

methods to vaccinated and non-vaccinated, channel and hybrid catfish, challenged instead with *E. piscicida.* Differentially expressed genes (DEGs) between treatment groups included predominantly genes involved in the innate immune system, coagulation cascade, and complement system. This work investigates hybrid and channel catfish susceptibility and immune responses to *E. piscicida* bacterial challenges using similar methods and analyses.

Methods

Methods parallel those completed in chapter 4, with some exceptions detailed in the following paragraphs.

Fish Husbandry and Vaccination

All animal work was conducted in compliance with the Mississippi State University Institutional Animal Use and Care Committee (IACUC). Channel and hybrid catfish were obtained from a local hatchery and reared indoors at the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS. Channel (average 5.1 g) and hybrid (average 5.4 g) catfish fingerlings were arbitrarily assigned to 55 L glass aquaria holding 22 L of aerated, flow through well-water. The mean water temperature was 24.7°C ± 1.8°C (range: 21.6 - 27.6°C) throughout the bacterial challenges. Fish were fed once daily at approximately 1-2% body weight with a commercial floating catfish feed containing 40% crude protein (Rangen, Buhl, Idaho).

The channel and hybrid catfish were divided arbitrarily into vaccinated and non-vaccinated treatment groups. Fish were divided into 6 aquaria per catfish variety and vaccine treatment (24 total aquaria; 6 aquaria/treatment group) for both sampling tanks (55 fish/aquarium) and non-sampling tanks (20 fish/aquarium) used to determine mortalities without sampling bias. Fish were acclimated for 48 h and fasted for 24 h before vaccination. A live-attenuated *E. ictaluri* isolate (S97-773-340X2; vaccine serial V19-BHP 050219) was thawed,

diluted 1:100 in deionized water, and expanded for 18 h at 27°C in 6 L porcine brain-heart infusion broth (BHIb; Bacto; Becton Dickinson and Company) (Greenway et al., 2017). Standard plate counts, conducted on Mueller-Hinton II Agar plates with 5% sheep blood (MHBA; BBL™; Becton and Dickinson Company, Franklin Lakes, New Jersey, USA) after incubation at 28°C for 48 h, determined an estimated bacterial concentration of 2.3 x 10^9 CFU/ml in the vaccine broth.

For the immersion vaccination, flow-through water to aquaria was stopped and 100 ml of vaccine broth at an estimated final concentration of 2.3 x 10^9 CFU/ml was added. After 30 m, flow-through conditions were resumed, and fish were fed daily to near satiation. Non-vaccinated fish were sham-vaccinated by adding 100 ml of sterile BHIb under similar conditions. Fish were observed for morbidity and mortality twice daily for 33 days post vaccination (dpv) during which moribund fish were euthanized by overdose (300 mg/L) in tricaine methanesulfonate (MS-222; Western Chemical, Ferndale, WA). Following procedures outlined in Chapter 4, select posterior kidneys from euthanized moribund and fresh dead fish were cultured on trypticase soy agar (TSA) plates supplemented with 5% sheep blood, with and without Rifamycin SV sodium salt (340 µg/ml; Sigma-Aldrich, St. Louis, Missouri). If bacteria grew in the presence of antibiotic, they were considered to be the attenuated *E. ictaluri* vaccine isolate (Wise et al., 2015). Bacterial identities were confirmed by *Edwardsiella* species specific multiplex real-time polymerase chain (mPCR) (Reichley et al., 2015c; 2017).

Experimental Bacterial Challenges

Cryopreserved *E. piscicida* isolate S11-285 (host *I. punctatus*; Mississippi, USA; Griffin et al., 2013; Reichley et al., 2016) was revived by isolation streaking on MHBA and incubating for 24 h at 37°C. Identification was confirmed using mPCR (Reichley et al., 2015c; 2017). Individual bacterial colonies were expanded for approximately 18 h in 9 ml of porcine brain

heart infusion broth (BHIb) from which a 1 ml aliquot was seeded into 1 L BHIb and grown for 18 h at 200 rpm and 28°C. The expanded bacterial cultures were diluted to concentrations approximating previously established hybrid median lethal doses of 2.22 x 10^4 CFU/g of fish (Reichley et al., 2018). Standard plate counts, performed as described for the vaccine broth, were used to determine the actual dose of approximately 3.9 x 10^4 CFU/fish.

In both the sampling and non-sampling aquaria subsets, experimental groups for bacterial challenges and non-challenged controls were replicated (3 aquaria/treatment group; 8 treatment groups). Food was restricted for 24 h prior to bacterial challenge. Thirty-three dpv, fish were anesthetized in MS-222 (100 mg/L bath) and administered an intracoelomic (IC) injection with 0.1 ml of the dilute bacterial broth. Non-challenged groups were injected with an equivalent volume of sterile BHIb. The IC challenge route was selected based on previous studies demonstrating it provides consistent infection results for *E. piscicida*, while immersion and oral routes do not (Reichley, 2017). Fish were monitored twice daily for 28 days, and moribund fish euthanized in 300 mg/L MS-222. Posterior kidneys of select moribund fish and fresh dead fish were cultured on MHBA, and isolate identity confirmed by mPCR (Reichley et al., 2015c; 2017).

Sampling Design

Two fish were sampled from each challenged sampling tank (n = 6 fish/treatment group) 35 dpv (2 dpc), 36 dpv (3dpc), and 61 dpv (28 dpc) (Figure 5.1). Vaccinated and non-vaccinated, non-challenged control fish were sampled 37 dpv (4 dpc). Fish were euthanized by overdose of MS-222 (300 mg/L). At 28 dpc, serum was collected in hematocrit tubes from the caudal vein following transection of the caudle peduncle. Blood was transferred to 1.5 ml microcentrifuge tubes and cells allowed to settle for 24 h at 4°C before centrifuging at 4,000 x g for 10 m. Serum was removed from tubes and stored at -20°C for future use. Posterior kidneys were collected

from euthanized fish 2 and 3 dpc for challenged fish and 4 dpc for non-challenged control fish. Kidneys were aseptically collected, placed in RNAlater at 4°C for 24 h, then frozen at -80°C for molecular analysis. Remaining tissues from the sampled fish were preserved in 10% neutral buffered formalin (NBF). The study was concluded 28 dpc.

Histopathology

The bodies of whole fish were preserved in 10% NBF for a minimum of 24 h, decalcified in Kristensen's solution for 24-48 h, and serial transverse sections through the head and coelomic cavities processed routinely for histopathology. Tissues were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (HE). Additional select sections were stained with modified Brown and Hopps Gram stain.

Enzyme-Linked Immunosorbent Assay

An enzyme-linked immunosorbent assay was used to evaluate serum IgM levels (n = 6 fish/treatment) against *E. piscicida* antigen 28 dpc. Antigens were prepared from cryopreserved *E. piscicida* isolate S11-285. The isolate was revived by isolation streaking on MHBA, grown for 24 h at 37ºC, and its identity confirmed by mPCR (Reichley et al., 2015c; 2017). Isolated colonies were expanded in 200 ml of BHI for 48 h at 27ºC with shaking (200 rpm). The expanded broth culture was centrifuged at 2700 x *g* for 20 m at 10°C and pelleted bacteria frozen at -80°C until analysis. Thawed bacterial pellets were resuspended in borate buffered saline (BBS) were sonicated on ice using a Sonic Dismembranator Model 100 (Fisher Scientific) (20 s pulses, 10-15 watts, repeated seven times). The crude antigenic preparations were centrifuged at 10,000 rpm for 10 m to pellet cellular debris, and the supernatant collected and stored at -20ºC.

Bradford assays (Coomassie Protein Assay Reagent; ThemoFisher #1856209) were used to quantify bacterial lysate protein concentrations and the lysates diluted to 5 µg/ml with BBS.

ELISAs were conducted using Immulon 2 strips (ThermoFisher #3602) organized in 96-well strip holders. Antigen was bound to wells by coating wells with 100 µl of the diluted lysate and incubating for approximately 18 h at 4°C. Phosphate buffered saline with Tween 20® (PBST) was used to wash plates twice before 250 µl of blocking buffer (ELISA Blocker, ThermoFisher, #N502) was added to wells then incubated at room temperature (RT) for 2 h. Prepared plates were either sealed and stored temporarily at 4°C or, for storage over one week, were emptied, air dried, sealed, and then stored at 4°C. Initially, ideal concentrations for relative IgM quantification were determined by serially diluting pooled serum from treatment groups. Triplicates of serum were diluted 1:320 in 1% fetal bovine serum (FBS)-PBST and serially diluted to 1:2560 for each individual sample. Sequential plate processing steps included: incubation at 4°C for approximately 18 h, washing 3 times with PBST, incubation for 2 h at RT in 50 µl of 1% FBS-PBST diluted (1:200) mouse immunoglobulin G1 (IgG1) anti-Channel Catfish IgM monoclonal antibody (9E1; University of Mississippi Medical Center, Jackson, Mississippi), washing 3 times with PBST, incubation at RT for 25 m in 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate in the dark, and addition of 100 µl of 2N H_2SO_4 stop solution to the wells. A BioTek ELX808 plate reader using GEN5 3.10 software measured optical densities of each well at 450 nm. Interplate calibration was performed using a pooled serum internal positive control from fish challenged with *E. piscicida.* Optical densities at 1:320 dilutions were analyzed via a two-way analysis of variance (ANOVA) performed with GraphPad v9.2.0 and followed by Tukey's *post hoc* test ($\alpha = 0.05$).

Nucleic Acid Isolation

Posterior kidney samples from 36 fish sampled from 2, 3, and/or 4 dpc ($n = 6$ fish/treatment group), as previously described, were chosen for qPCR analysis and/or RNA-seq.

Days 2 and 3 were selected for challenged fish based on peak mortality in this study. The posterior kidneys were homogenized mechanically with a syringe and needle, and the homogenized tissues lysed in RLT buffer. Samples were maintained at -80°C until a Qiagen RNeasy Mini Kit (Hilden, Germany) was used to extract RNA and a Qiagen RNase-free DNase Set used to remove genomic DNA contamination, both according to manufacturer's directions. RNA concentrations and purity were estimated with a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific). Isolated RNA with an A260/289 ranging from 1.8 to 2.0 was incorporated into the study. Final RNA samples were re-suspended in RNase-free water and maintained at -20°C until qPCR and/or RNA-seq analysis.

RNA-Sequencing and Analysis

Forty samples (n = 5/treatment group; 8 treatment groups) were submitted for RNA sequencing. Genewiz from Aventa Life Sciences determined the sample RIN, constructed a library with samples having a minimum RIN of 5 (26/40 samples with an RIN over 6), and performed standard RNA-seq. Trimmomatic v0.39 (Bolger et al., 2014) was used to trim Illumina-specific adaptor sequences and low-quality reads from the raw reads. STAR v2.7 (Spliced Transcripts Alignment to a Reference; Dobin, 2022) was utilized to map the trimmed reads to the channel catfish reference genome (GenBank Accession No. GCF_001660625.2). Features at the gene level were counted using featureCounts (Liao et al., 2014). R Studio V2022.22.1 utilizing the package edgeR v3.38.1 was used to identify differentially expressed genes (DEGs), specified as genes with a log2 |fold-change| of >1 and an adjusted p value < 0.05. The R package clusterProfiler v4.4.2 was utilized for data visualization and gene ontology (GO) term enrichment analysis, with presented GO terms and pathways having an adjusted p value (False discovery rate [FDR] < 0.05).

Quantitative Polymerase Chain Reaction Validation

Quantitative PCR was conducted on 9 immune genes of interest on cDNA synthesized from posterior kidneys of fish sampled on 2 and/or 3 dpc. The cDNA was synthesized from 2 µg of total RNA with SuperScript® IV reverse transcriptase and Oligo-dT primer (Invitrogen Life Technologies). Primers, listed in Table 5.1, were designed in Geneious Prime from contig sequences of significantly expressed transcripts from the RNA-seq or from previously published work (Xu et al., 2017). Quantitative PCR was performed in triplicates using 10 µl of SsoAdvanced Universal SYBR Green Supermix (BioRad), 10 µm of the forward and reverse primers, 2 µg of the template cDNA, and nuclease-free water to volume, for a total reaction volume of 12 µl. Amplification was conducted using the following settings on a CFX96 Thermal Cycler (BioRad): 95°C for 3 m followed by 35 cycles at 95°C for 10 s and 54°C for 30 s. Standard curves were generated using amplicons developed from conventional PCR of control posterior kidney cDNA. The products were gel purified using a Qiagen Gel Extraction Kit and quantified using Qubit fluorometry (Invitrogen). Diluted DNA (1 ng/µl) was used for tenfold serial dilutions in dH2O. Dilutions were evaluated by qPCR to establish efficiencies and linearity (Table 5.1). No reverse transcriptase controls were used to confirm genomic DNA contamination absence. Three stable reference genes, *elongation factor 1-alpha* (*ef1α*), *glyceraldehyde-3-phosphate dehydrogenase* (*gadph*), and *beta-actin* (*β-actin*), were used to normalize immune gene expression. The calibrated normalized relative quantities (CNRQ) were determined in Bio-Rad CFX Manger v3.1 with primer-specific amplification efficiencies and sample-specific normalization factors. Gene expression was analyzed using two-way analysis of variance (ANOVA) in GraphPad v9.2.0, followed by Tukey's *post hoc* test ($\alpha = 0.05$).

Data Analysis

Kaplan-Meier survival analyses were conducted in R statistical software (v4.0.2; R Core Team, 2021) using the following packages: survminer (v0.4.9; Kassambara et al., 2021), survival (v3.4-0; Therneau, 2022; Therneau & Grambsch, 2000), and dplyr (v1.0.8; Wickham et al., 2022). Post-challenge, non-sampling tank cumulative mortality was evaluated via two-way ANOVA followed by Tukey's *post hoc* test ($\alpha = 0.05$) in GraphPad 9.2.0. Relative percent survival (RPS) from non-sampling tanks was calculated according to Amend 1981 with the following equation: RPS = [1-(cumulative mortalities in vaccinates / cumulative mortalities in non-vaccinates) x 100%].

Results

Bacterial challenge

Post-vaccination and pre-challenge mortality are described in Chapter 4. In non-sampling tanks, vaccinated, *E. piscicida* challenged channel and hybrid catfish had significantly lower cumulative morality than their non-vaccinated counterparts ($p < 0.001$) (Figure 5.2). Vaccinated channel catfish were more likely to survive *E. piscicida* bacterial challenge than vaccinated hybrid catfish ($p < 0.01$), but no significant difference existed in cumulative mortality between non-vaccinated channel and hybrid catfish. No non-vaccinated or vaccinated control fish died after sterile BHIb injection. The relative percent survival (RPS) was 84.0% and 38.1% in channel and hybrid catfish, respectively (Table 5.2). Kaplan-Meier Survival analyses are shown in Figure 5.3 A and B. In non-vaccinated fish, mortality peaked 2 dpc, with no additional mortalities 6 dpc. In vaccinated fish, mortality was steady, with no distinct peak, from 2 to 8 dpc. Survival probability at any given time point was significantly different between vaccinated and non-vaccinated fish as well as between vaccinated channel and hybrid catfish, similar to patterns in

cumulative mortality. Post-challenge bacterial cultures and mPCR from all moribund fish were consistent with *E. piscicida.*

Histopathology

Histopathologic examination of fish processed for RNA-seq 2 or 3 dpc (n = 5 / treatment group) revealed sporadic lesions consistent with gram-negative bacterial sepsis in both vaccinated and non-vaccinated, challenged fish (Table 5.3). Visceral lesions were frequently characterized by multifocal, random necrosis with gram-negative bacterial rods, predominantly in the liver, but also in the anterior kidney, posterior kidney, and rarely spleen. Splenic capsules were often surrounded by moderate numbers of macrophages, which occasionally infiltrated the parenchyma. Stomach lesions consisted of multifocal necrosis in the mucosal and lamina propria surrounded by rims of macrophages. Gill lamellae were multifocally fused, with moderate numbers of intravascular bacterial rods and infiltrating macrophages. Coelomic inflammation was typically fibrinous, with moderate numbers of macrophages and lymphocytes. Interestingly, while both channel and hybrid catfish had lesions, channel catfish tissues were more commonly affected than hybrid tissues.

Relative antibody quantification

At 28 dpc, non-vaccinated, *E. piscicida* challenged channel catfish had significantly elevated IgM levels against *E. piscicida* compared to controls, non-vaccinated challenged hybrid catfish, and vaccinated hybrid catfish (Figure 5.4). Hybrid catfish did not develop a significant antibody response following bacterial challenge or vaccination and bacterial challenge combined. Channel catfish antibody levels varied widely across individual fish.

RNA-Seq Analysis

Global transcript expression profiles are listed in Table 5.4.

Transcription Following Bacterial Challenge in Unvaccinated Fish

Comparisons of transcriptomic profiles between non-vaccinated, non-challenged and challenged channel catfish revealed 716 DEGs. Of these, 623 genes were overexpressed. The upregulated transcripts included enrichment in terms such as immune response, response to cytokine, programmed cell death, and toll-like receptor signaling pathway (Figure 5.5). Specific select upregulated genes included *cd28, toll-like receptor (tlr) 5, tlr4, tlr1, tlr13, interferon gamma, heat shock protein 5,* and *interleukins 1β, 6, 8, 11, 12, 17,* and *22.* Ninety-three underexpressed genes were identified, enriching telomeric DNA binding, carbohydrate phosphatase activity, and cytidylyltransferase activity.

Between non-challenged and challenged non-vaccinated hybrid catfish, 218 genes were differentially regulated, with 187 overexpressed and 31 underexpressed transcripts. Enrichment of GO terms included transcripts for processes such as response to bacterium, chemokine activity, inflammatory response, programed cell death, and proton-transporting V-type ATPase complex (Figure 5.6). Immune response upregulation included genes such as *ccl25b, cscl71, il6,* and *tlr5*. There was no enrichment of GO terms in the downregulated transcripts.

Transcription Following Bacterial Challenge in Non-Vaccinated versus Vaccinated Fish

When comparing non-vaccinated to vaccinated, challenged channel catfish, 479 DEGs were identified, with 33 upregulated transcripts and 446 downregulated transcripts in the non-vaccinated fish. Downregulated GO enriched pathways included autophagy and various enzyme activities (e.g., phosphoric ester hydrolase, phosphatase, CoA-ligase). Upregulated genes did not enrich any pathways. No DEGs were identified between non-vaccinated, challenged hybrid and vaccinated, challenged hybrid catfish.

Transcription in Vaccinated Fish With and Without Bacterial Challenge

The response in channel catfish vaccinates to bacterial challenge revealed 2799 DEGs, with 1998 overexpressed and 801 underexpressed in challenged vaccinates compared to non-challenged vaccinates. GO analysis of the upregulated transcripts in the challenged fish revealed enrichment in wound healing, the immune response, blood coagulation, and complement activation (Figure 5.7). While many procoagulant components of the coagulation cascade were overexpressed (e.g., *f2, f7, f9a, f9b, f10*), other anticoagulant components were also upregulated (e.g., *antithrombin, plasminogen activator inhibitor-1*). Complement activation included upregulation of 17 genes, including *c1r subcomponent, c3, c3b.2, c6, c7b, c8a, c8b, c9,* and *cfd.* Immune response upregulation was characterized by increased expression of components such as *interferon gamma 1, interleukin 1β, toll-like receptor 3, toll-like receptor 5, toll-like receptor 13, tumor necrosis factor α,* and various chemokines. Downregulated transcripts primarily enriched pathways involved in fish reproduction. In hybrids, only 33 genes were differentially regulated between challenged and non-challenged vaccinates. The 15 overexpressed genes in challenged fish include *NK-lysin type 1, NK-lysin type 2,* and *lysozyme C.* Downregulated genes did not enrich any GO pathways.

Comparisons Between Channel and Hybrid Catfish

When comparing vaccinated, challenged hybrid catfish to channel catfish, 1137 transcripts were differentially regulated, characterized by 819 upregulated and 318 downregulated transcripts. Overexpressed transcripts included genes involved in the inflammatory response and signaling receptor activator activity. Underexpressed transcripts in channels were in the pathways, triglyceride lipase activity and carboxylic ester hydrolase activity. Non-vaccinated, challenged channel catfish upregulated 142 genes and downregulated 734 genes compared to non-vaccinated, challenged hybrids. The overexpressed transcripts

contained 4-6 genes enriching chemokine activity, cytokine activity, and signaling receptor regulator activity. Hybrid catfish transcript overexpression included enrichment of wound healing, immune effector processes, coagulation, and complement activation.

Quantitative PCR

Select immune genes of interest were quantified from posterior kidneys using qPCR, including *tnfα, ifnγ, il1β, c9, c3, cfX, nod1, tlr5*, and *csf1* at 3 dpc and *tnfα, ifnγ, il1β, nod1* and *c9*, at 2 dpc. Gene expression did not significantly vary between 2 or 3 dpc with *tnfα, ifnγ, il1β, nod1* or *c9* (data not shown), and therefore only expression of genes at 3 dpc are compared here. Significant differences were detected between treatment groups with *tnfα, ifnγ, il1β,* and *tlr5* expression (Figure 5.8). No significant differences in transcript expression were identified in *c3, c9, crfX, csf1,* or *nod1*. Transcript expression of these genes correlated between qPCR and RNA-seq data.

Discussion

Edwardsiella piscicida, which was only recently identified as a species separate from *E. tarda,* has quickly emerged as an important pathogen in US catfish aquaculture. Its growing significance highlights the need for efficacious preventative measures, such as vaccination (Griffin et al., 2019). A live attenuated *E. ictaluri* vaccine is currently in use in Mississippi catfish aquaculture, which confers significant protection against *E. ictaluri* outbreaks. Further, research indicates the *E. ictaluri* vaccine also offers protection against *E. piscicida* (Griffin et al., 2020a; Hegde et al., 2022; López-Porras et al., 2022; Wise et al., 2015; 2020). However, the historical literature is convoluted in regard to *E. piscicida,* given it was only recognized as a discrete taxon in 2013. Previous work involving "typical, fish pathogenic *E. tarda*" is likely attributable to *E. piscicida,* however historical reports are muddled due to inconsistent reporting

of isolate identities and limited molecular data available for comparison. As a result, little is known regarding the immune response of channel and hybrid catfish to *E. piscicida* infection or the mechanisms responsible for their varied susceptibilities. ELISAs permit relative quantification of antibodies produced against *E. piscicida*, while transcriptomics offers detailed insight into immune mechanisms and the degree of responses against the bacteria. The present study used ELISAs, RNA-seq, and qPCR to quantify and characterize the response of vaccinated and non-vaccinated, channel and hybrid catfish with and without *E. piscicida* bacterial challenge. Additionally, data presented here corroborates previous studies detailing the efficacy of the live attenuated *E. ictaluri* vaccine against IC *E. piscicida* challenge. Collectively, these works provide insight of how bacterial infection, vaccination, and fish type influence channel and hybrid catfish immune responses to *E. piscicida*, which will facilitate optimization of vaccination protocols and preventive strategies to reduce *Edwardsiella* related losses in these two important commercial catfish varieties.

The live attenuated *E. ictaluri* vaccine conferred protection 33 dpv against *E. piscicida* IC challenge, with significantly decreased mortality in channel and hybrid catfish. Vaccinated channel catfish were more likely to survive than vaccinated hybrid catfish, which agrees with previous studies suggesting hybrid catfish have increased susceptibility to *E. piscicida* and that the protective effect is more pronounced in channels (Armwood et al., 2022; Griffin et al., 2020a; Reichley et al., 2018). In this study, survival was not significantly different between non-vaccinated channel and hybrid catfish. This is likely multifactorial, with influences including small fish size, low sample size in replicate tanks, and the relatively high IC bacterial dose, which was selected to stimulate immune responses and clearly distinguish the effects of vaccination on survivability.

Histopathologic evidence of systemic disease predominated in vaccinated, challenged channel catfish and non-vaccinated, challenged channel and hybrid catfish. Mortality peaked and resolved earlier in vaccinated, challenged catfish, suggesting a higher percentage of living but severely affected fish available for histopathologic sampling may have contributed to lesion quantities in the vaccinated channel catfish. Necrotizing lesions with an abundance of bacteria outweighed other inflammatory patterns, such as granulomatous, likely based on the sampling time frame of 2 to 3 dpc, supporting previous histopathologic studies including work from Chapter 2 (Armwood et al., 2022; López-Porras et al., 2021). Coelomitis was the most common lesion, likely resulting from the IC challenge route. Histologic lesion distribution and severity likely does not mimic natural infection, which follows mucosal surface entry routes. However, this challenge route was selected based on previous studies determining it ensured the most consistent initiation of bacterial infection (Reichley, 2017).

In this study, only non-vaccinated, challenged channel catfish produced significant serum IgM levels against *E. piscicida.* Interestingly, in Chapter 4, vaccinated channels challenged with *E. ictaluri* also produced significant IgM levels against *E. piscicida*. In this study, vaccinated channels challenged with *E. piscicida* did not. Because *E. ictaluri* is more pathogenic in channel catfish, it is possible that wild-type *E. ictaluri* is more immunogenic in the channel catfish host, stimulating a more robust humoral response, giving rise to cross-reactive IgM antibodies. Surprisingly, hybrid catfish did not mount a significant antibody response against *E. piscicida* in either vaccinated or non-vaccinated catfish following *E. piscicida* injections. The lack of significant anti-*E. piscicida* antibody production following vaccination in both channels and hybrids, and the negligible antibody response in naïve hybrids following challenge suggests circulating antibodies do not play a significant role in the immune response against *E. piscicida*

in channel and hybrid catfish. Again, this could be related to the IC challenge route and circumvention of natural mucosal infection routes by *E. piscicida*. Mucosal immune responses, including mucosal antibody production, were not considered in this study but could play an important role in protection. Future work should assess mucosal immunity and the effects of various vaccine delivery methods (oral, bath, injection) on mucosal antibody levels.

Bacterial challenges induced immune responses in both channel and hybrid fish, affecting pathogen recognition receptor signaling, cytokines, and chemokines. Channel catfish upregulated three-fold the number of genes as hybrids, with a more diverse subset of overexpressed toll-like receptors (TLRs) and interleukins. Additionally, channels upregulated *cd28*, which encodes a protein on T cells that provides costimulatory signals needed for T cell activation (Bernard et al., 2006). *Il12*, also upregulated in channels, enhances expression of IFNγ during T cell activation and stimulates NK cells, promoting cell-mediated immunity and the Th1 response (Wang et al., 2014). Among these genes in hybrids, only *il12* was upregulated, with no increases in cd28 or IFNγ, findings further corroborated by qPCR findings. *Il10*, a suppressor of the Th1 response, was not differentially regulated in channels or hybrids. IFNγ and TNFα prime M1 macrophages, also known as classically activated macrophages (Grayfer et al., 2018). M1 macrophages participate in inflammatory and microbicidal responses, killing pathogens through reactive oxygen and nitrogen species production, phagocytosis, and nutrient restriction. Channels may respond to *E. piscicida* challenge with a more rapid and/or robust Th1 response, making them less susceptible to terminal infection. Additionally, channels upregulate more TLRs than hybrid catfish, including *tlr1, 2,* and *13*. TLRs recognize various pathogen-associated molecular patterns (PAMPs) on bacteria, leading to activation of various transcription factors that dictate the innate immune response (Palti, 2011; Zhang et al., 2014).

When comparing responses to bacterial challenge in vaccinated and non-vaccinated fish, only channel catfish had differential gene regulation; however, these were predominantly genes related to autophagy and enzyme activity. The increased expression of autophagy in the vaccinated challenged fish potentially represents increased antigen presentation. During self-digestion, antigens can be processed and presented by the major histocompatibility complex (Johnstone & Chaves-Pozo, 2022). The lack of expression variation in challenged vaccinated and non-vaccinated hybrid catfish suggests the protection conferred by the vaccine does not rely on gene expression.

Interestingly, *E. piscicida* challenge induced less DEGs in the posterior kidney at peak mortality in both non-vaccinated hybrid and channel catfish than *E. ictaluri* in Chapter 4, especially in hybrid catfish where only 218 genes were differentially expressed. While the days post challenge likely played a role in this large difference (3 dpc in *E. piscicida* and 5 dpc in *E. ictaluri* challenges), sampling occurred at peak mortality for the respective bacteria, supporting the conclusion that the observed differences in transcription are comparable and possibly contribute to the differences in susceptibility. Coagulation and complement activation were not among the enriched pathways following *E. piscicida* challenge in channel catfish compared to the *E. ictaluri* challenge.

Similar to the non-vaccinated fish, when comparing vaccinated challenged and non-challenged catfish, challenged channels produced a much more robust response than hybrids, compared to their respective counterparts. While similar immune components were upregulated in the channels, other GO pathways were enriched including wound healing, coagulation, and complement. Complement plays an integral role in chemoattraction and opsonization and can be activated by pathogens or antibodies (Grayfer et al., 2018). Coagulation contributes to

containment of the bacteria, preventing systemic spread (Berends et al., 2014). Potentially, early activation of coagulation and complement contribute to the improved survival seen in vaccinated channel catfish compared to non-vaccinated fish and vaccinated hybrids.

The current work suggests channel catfish develop a more robust humoral and Th1 response to *E. piscicida* challenge than hybrid catfish, potentially contributing to the increased susceptibility of hybrids to infection. Complement and coagulation seem to play less of a role in *E. piscicida* challenge than seen in the *E. ictaluri* challenges described in Chapter 4 but are significantly upregulated in challenged vaccinated channel catfish compared to their non-challenged counterparts. These findings suggest the live attenuated *E. ictaluri* vaccine confers protection to channel catfish through rapid induction of the complement system and coagulation cascade. Hybrids do not develop a significant serum IgM response. However, the responses identified by transcriptomics were limited to the posterior kidney 3 days after IC challenge and may not represent the full extent of the immune reaction. The impacts of the protection conferred against *E. piscicida* from the live attenuated *E. ictaluri* vaccination in hybrid production systems remains unclear and warrants additional study.

Figures

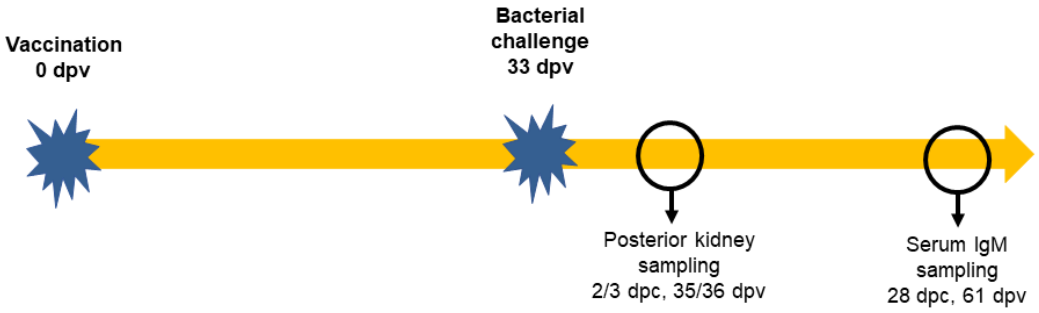

Figure 5.1. Study design timeline. Fish were vaccinated via immersion with a live attenuated *E. ictaluri* vaccine and challenged after 33 days by intracoelomic injection with *E. piscicida.* Serum was sampled 28 dpc (61 dpv) for relative IgM quantification. Poster kidneys were sampled 2 and 3 dpc (35 and 36 dpv) for evaluation of immune gene expression and the sampled fish preserved for histopathologic examination. dpv = days post vaccination; dpc = days post challenge.

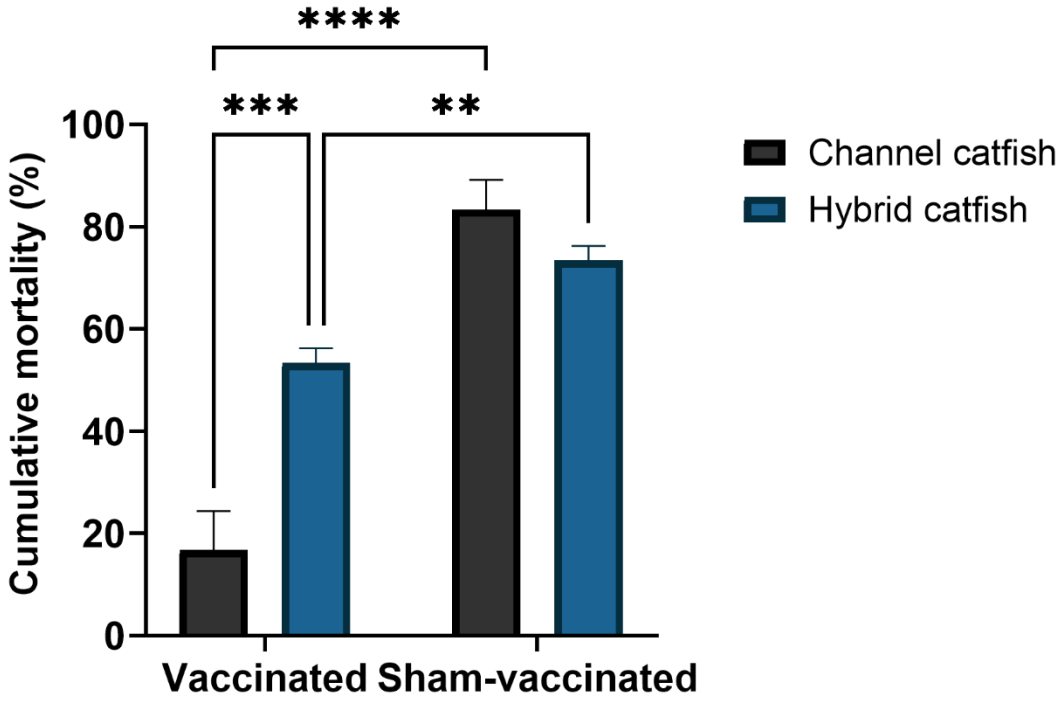

Figure 5.2. Cumulative mortality in channel and hybrid catfish after *Edwardsiella piscicida* intracoelomic challenge with and without previous vaccination. Vaccinated and non-vaccinated control fish (not picture) had 0% post-challenge cumulative mortality, which was significantly ($p < 0.001$) lower than their vaccinated, challenged channel catfish counterparts ($p < 0.001$) and their vaccinated, challenged hybrid counterparts ($p < 0.0001$). Graph includes the mean and standard deviation between cumulative mortality in non-sampling tanks. ** = $p < 0.01$; *** = $p < 0.001$; **** = p 0.0001.

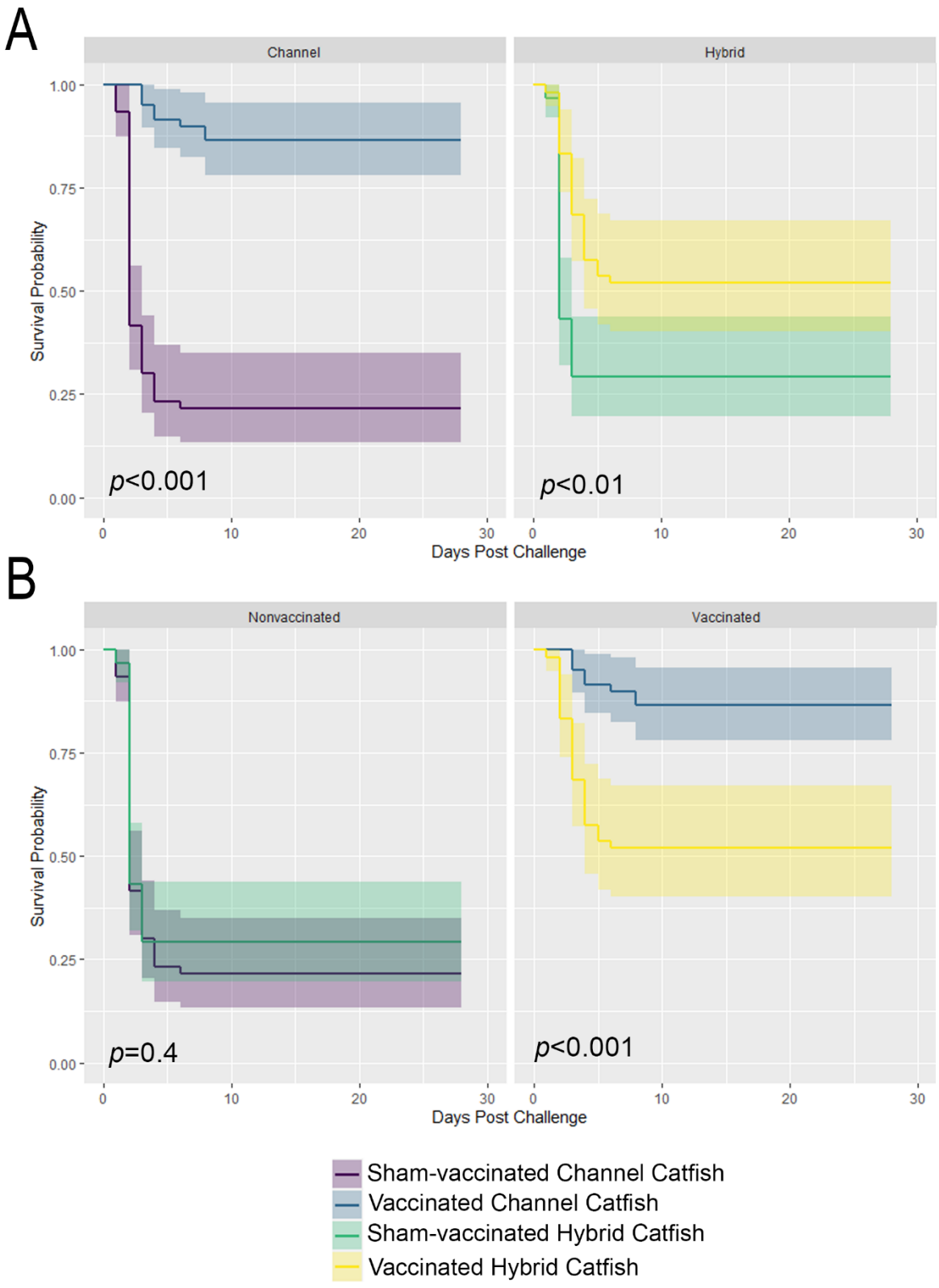
A
Channel
Hybrid
Survival Probability
1.00
0.75
0.50
0.25
0.00
p<0.001
p<0.01
0
10
20
30
Days Post Challenge
B
Nonvaccinated
Vaccinated
p=0.4
p<0.001
Sham-vaccinated Channel Catfish
Vaccinated Channel Catfish
Sham-vaccinated Hybrid Catfish
Vaccinated Hybrid Catfish

Figure 5.3. Kaplan-Meier survival curves assessing survival probability in non-sampling tanks after intracoelomic challenge with *E. piscicida* in vaccinated and non-vaccinated, channel and hybrid catfish (n = 20 fish per aquaria; 3 aquaria per treatment group). Fish were vaccinated by immersion in a live-attenuated *E. ictaluri* isolate (Wise et al., 2015) or non-vaccinated and challenged 33 days later with an intracoelomic injection of *E. piscicida* isolate S11-285. A) Survival probability was significantly higher in vaccinated channel ($p < 0.001$) and hybrid ($p < 0.01$) catfish compared to their non-vaccinated counterparts. B) Vaccinated channel catfish had higher survival probability ($p < 0.001$) than vaccinated hybrid catfish. There was no significant difference between survival probability in non-vaccinated hybrid and channel catfish. Data presented include the mean survival curves and the 95% confidence intervals.

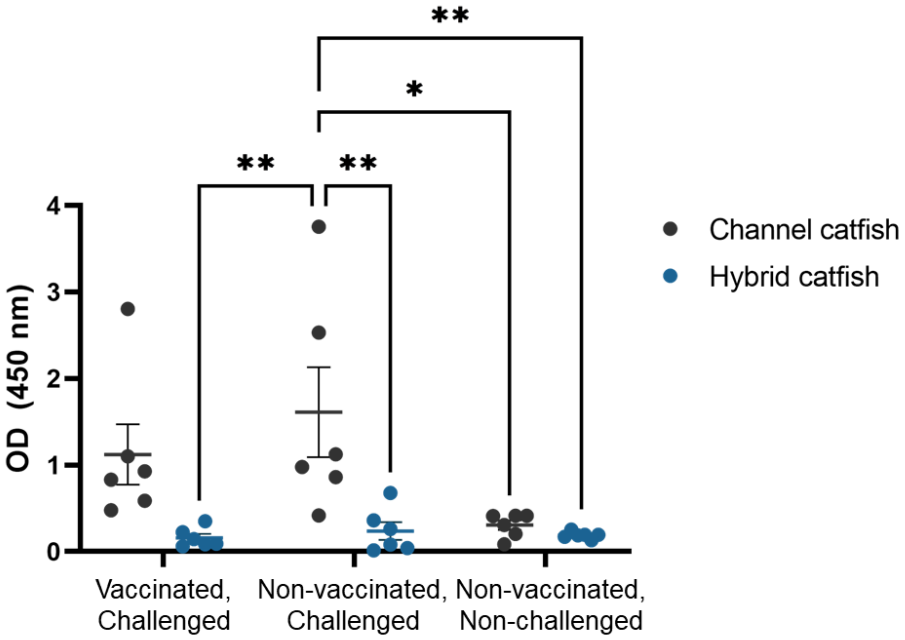

Figure 5.4. Relative serum IgM levels against *E. piscicida* 28 days post challenge were quantified using ELISAs on 1:320 serum dilutions from vaccinated challenged, non-vaccinated challenged, and non-vaccinated non-challenged hybrid and channel catfish (n = 6 fish/treatment group). Statistical significance was determined by two-way ANOVA followed by Tukey *post-hoc* HSD ($p < 0.05$). Non-vaccinated, *E. piscicida* challenged channel catfish had significantly elevated IgM compared to controls, non-vaccinated challenged hybrid catfish, and vaccinated hybrid catfish. * = $p < 0.05$; ** = $p < 0.01$; OD = optical density.

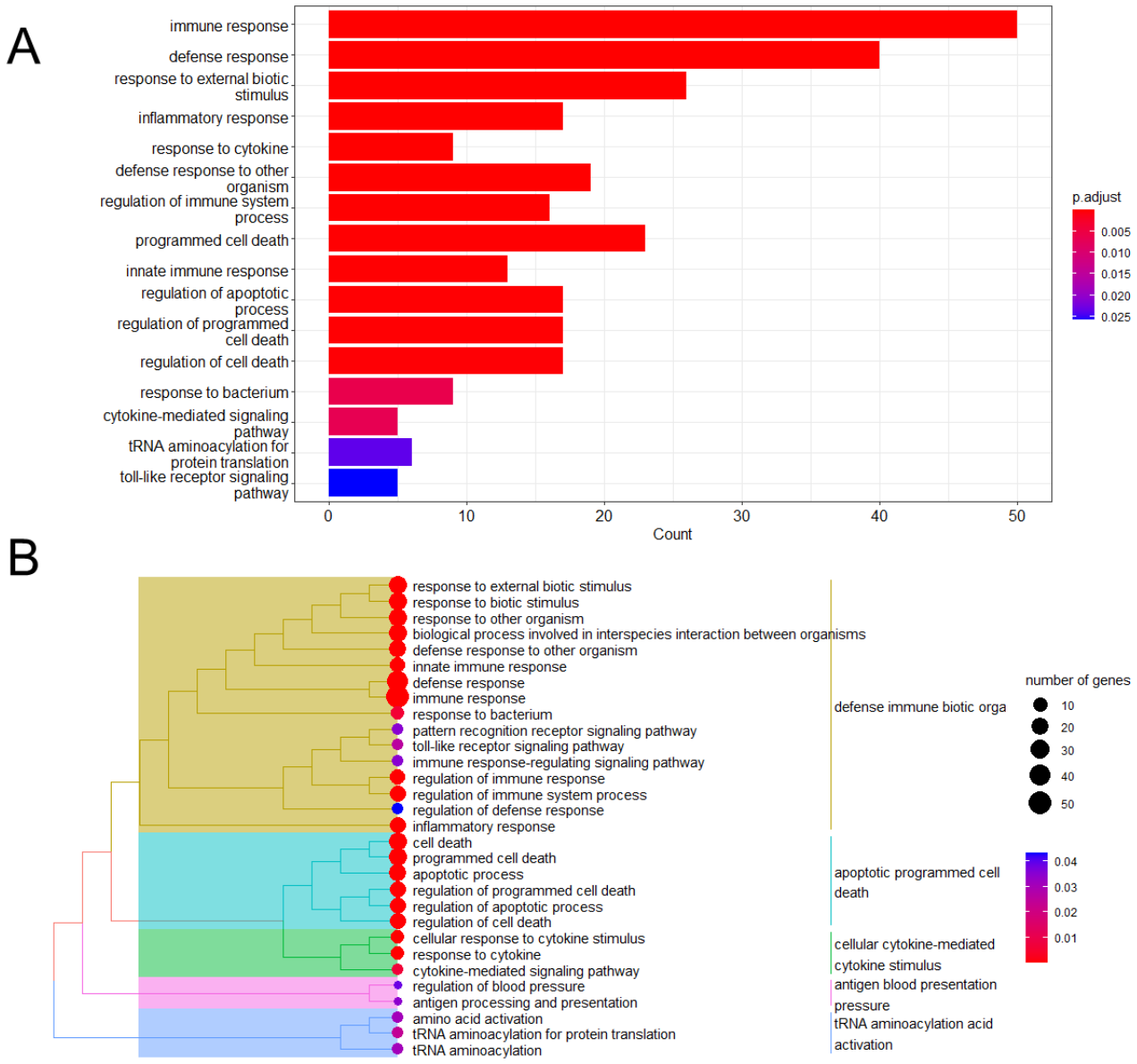

Figure 5.5. Gene Ontology (GO) pathway enrichment analysis in non-vaccinated channel catfish revealing: A) enriched pathways among genes upregulated in challenged fish and B) a heatmap of enriched terms in challenged fish.

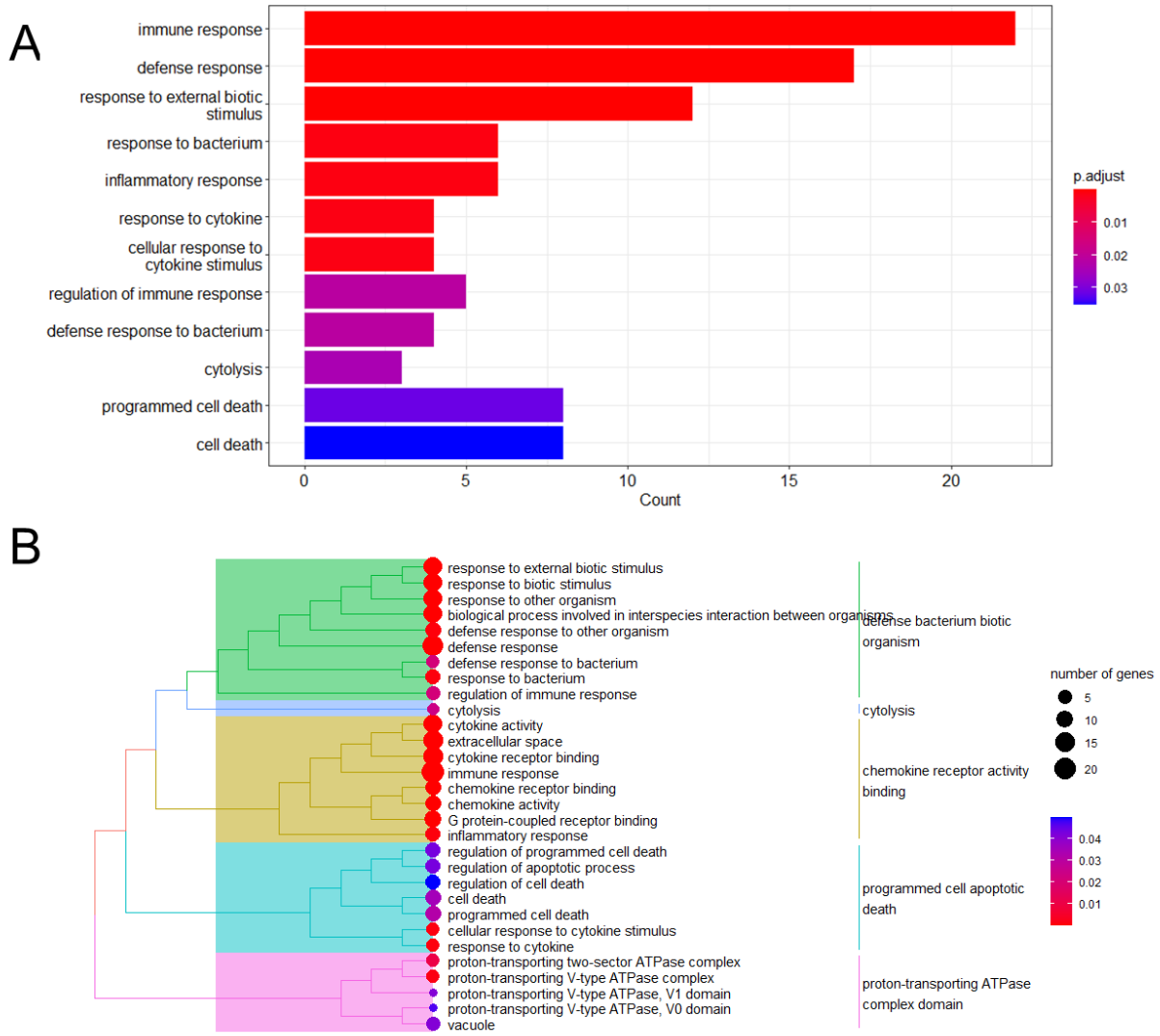

Figure 5.6. Gene Ontology (GO) pathway enrichment analysis in non-vaccinated hybrid catfish revealing: A) enriched pathways among genes upregulated in challenged fish and B) a heatmap of enriched terms in challenged fish.

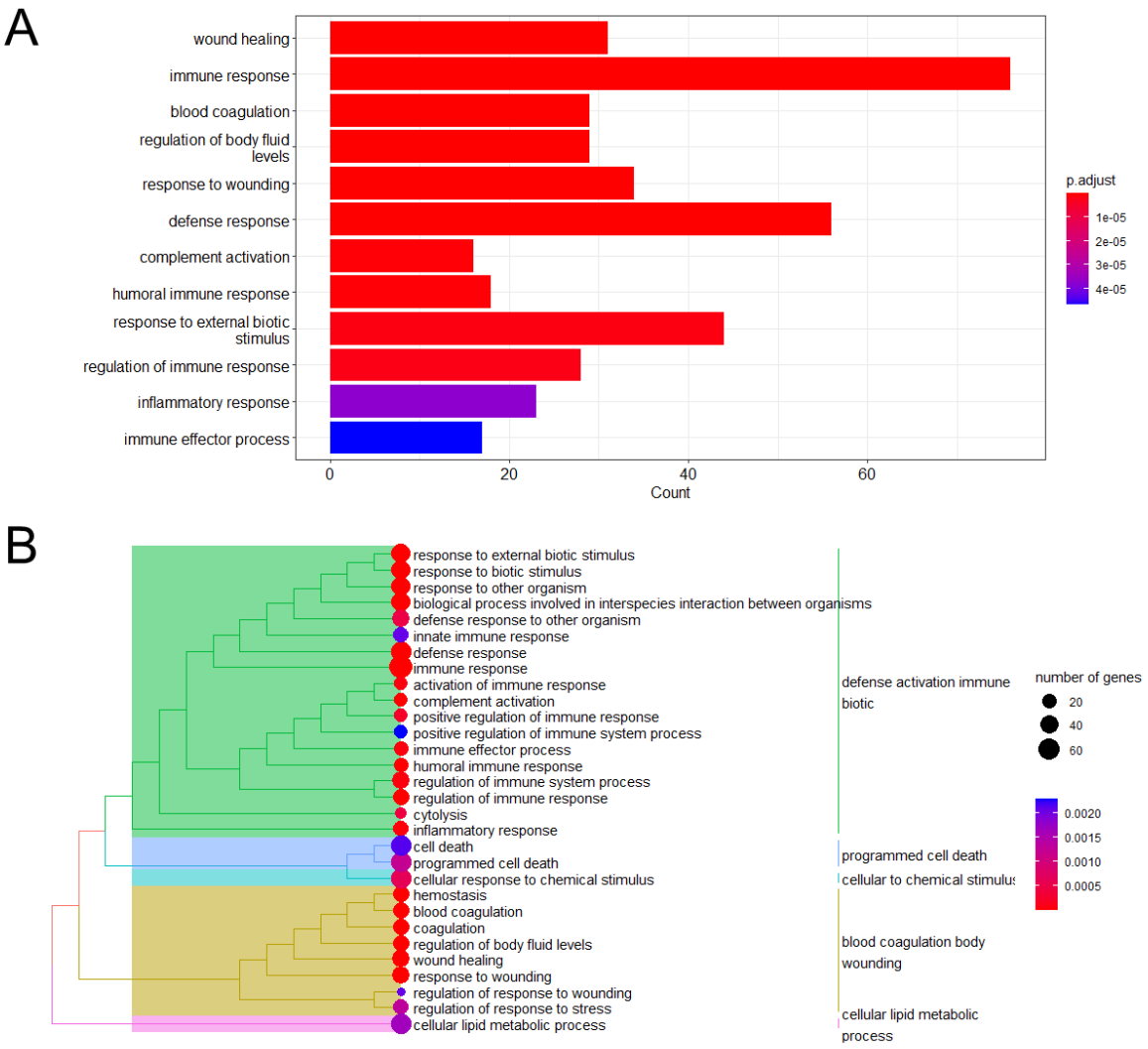

Figure 5.7. Gene Ontology (GO) pathway enrichment analysis in vaccinated channel catfish revealing: A) enriched pathways among genes upregulated in challenged fish and B) a heatmap of enriched terms in challenged fish.

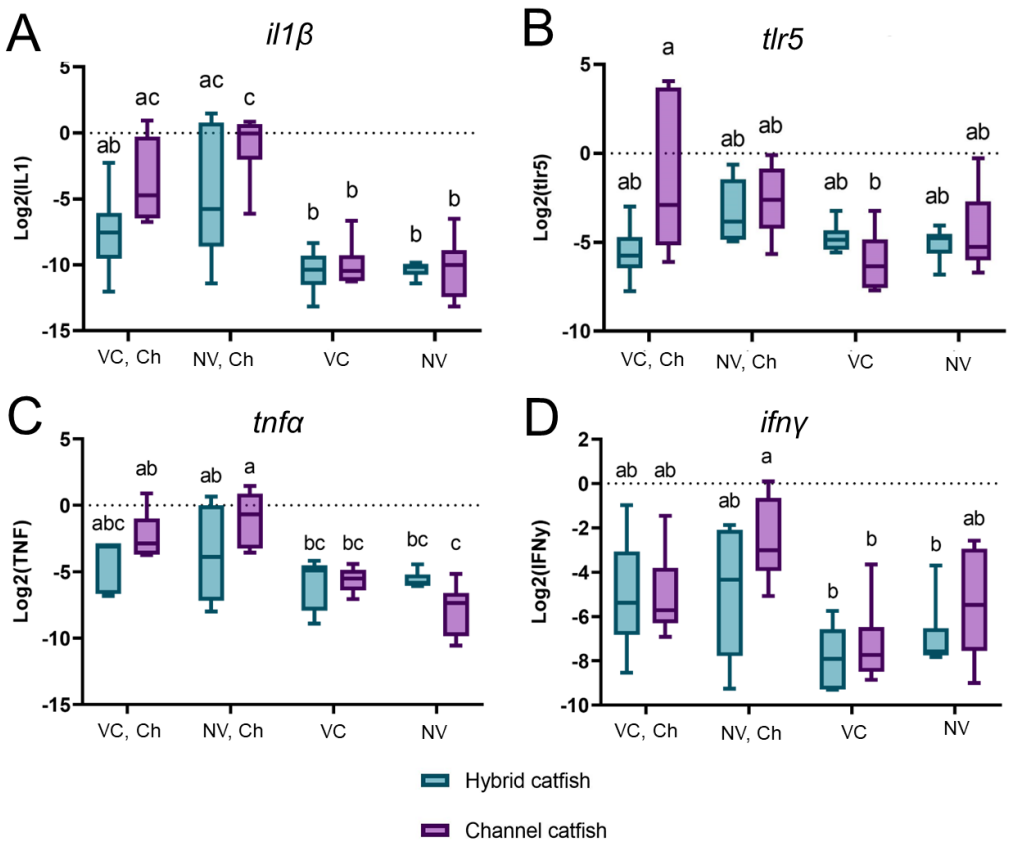

Figure 5.8. Quantitative PCR of select immune genes including: A) *il1β,* B) *tlr5*, C) *tnfα,* and D) *ifnγ*. The box plots represent the median log2CNRQ with 95% confidence intervals. A two-way ANOVA followed by post-hoc Tukey HSD determined significance. Significance is indicated with letters ($p < 0.05$). VC = vaccinated; Ch = challenged; NV = non-vaccinated.

Tables

Table 5.1. Primer sets for study reference genes and genes of interest for qPCR (see Chapter 4).

Gene	Name	Forward (5'-3')	Reverse (5'-3')	Amplicon size (bp)	Efficiency (%)	R^2
Reference genes						
tubulin	*TUB*	AGCCATACAATTCCATCCTGACC	GCGGCAGATGTCGTAGATGG	95	95	0.987
beta-2-microglobulin	*B_2M*	GCCTTTGCTAAGGAGTCTCCACC	GACCTGCTGAAGAATGGCGAGGT	143	92	0.978
beta-actin	*β-actin*	CCCATCTATGAGGGTTATGCTCTG	GCTCGGTCAGGATCTTCATCAG	98	101	0.950
glyceraldehyde-3-phosphate dehydrogenase isoform 1	*GADPH*	TCTTATGAGCACTGTCCATGCC	TAATGTTCTGGCTGGCACCAC	101	98	0.988
elongation factor 1-alpha	*EF1α*	TCAGTGAAGGACATCCGTCG	AGCGTAGCCCTGAGAGATC	129	92	0.987
Immune genes of interest						
interleukin 1 beta	*IL1β*	TTTGGCCATGAGCGGCAG	GTTTCAGCCTCTGCAAAGCG	116	97	0.988
interferon gamma	*IFNγ*	GGACCTTAAGGAAAATGACCCACGG	TTAGCTTGACGTCGTCTCCG	124	101	0.974
tumor necrosis factor alpha	*TNFα*	TCGTGGTCTTCTTCAGGAG	GTTCTGCGTCTTGTTTTGAG	102	100	0.989
toll-like receptor 5	*TLR5*	GTCTCAAAACGGCTTCAGCG	ACTCCCTTTCCCATGCTGCC	111	96	0.994
nucleotide-binding oligomerization domain-containing protein 1	*NOD1*	AGCAGTGTGGAAGTTTTGGC	CAGTTTGGCTCCAACATCCG	99	96	0.969
complement component 3	*C3*	TCAATAAGGACTCGCTGGCG	TATCCCATTGTGAGCTGCGG	123	97	0.996
complement component 9	*C9*	ACAGCTTGGACTATGTGGGC	TGAGTCGTTGGTTTGCAGGG	143	97	0.996
coagulation factor X	*CfX*	CATGAGGGTGGTCTACAGGC	GCATCCTTTTCCTCCTTGGC	140	91	0.991

colony stimulating factor 1	*csf1*	GAACACACACACAACAGCCC	ACACATGGACCAGGTACAGC	102	93	0.990

Table 5.2. Relative percent survival 28 dpc against *Edwardsiella piscicida* IC challenge in fish vaccinated with a live-attenuated *E. ictaluri* immersion vaccine

Fish group	Relative Percent Survival (RPS)
Channel catfish	84.0%
Hybrid catfish	38.1%

Table 5.3. Summary of histopathologically affected tissues from fish submitted for RNA-seq analysis (n = 5 fish/treatment group). Lesions were primarily characterized by hemorrhage, necrosis, and/or granulomatous inflammation

Treatment group	***Affected tissues***						
	Liver	Anterior kidney	Posterior kidney	Gill	Spleen	Coelom	Stomach
Hybrid catfish							
Vaccinated, challenged	0/5	0/4	0/4	0/5	0/5	5/5	0/5
Non-vaccinated, challenged	2/4	0/3	1/5	0/5	1/5	4/5	0/5
Vaccinated, non-challenged	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Non-vaccinated, non-challenged	0/5	0/3	0/5	0/5	0/2	0/5	0/5
Channel catfish							
Vaccinated, challenged	3/5	1/2	0/5	2/5	2/5	5/5	2/5
Non-vaccinated, challenged	2/5	0/5	1/3	1/5	2/3	5/5	1/5
Vaccinated, non-challenged	0/5	0/4	0/4	0/5	0/4	0/5	0/5
Non-vaccinated, non-challenged	0/5	0/2	0/5	0/5	0/2	0/5	0/5

If the total tissue number is < 5, the tissue was not available for histologic examination in every fish.

Table 5.4. Global transcript expression profiles.

Comparison category	Treatment comparison	Number of differentially expressed genes (DEGs)		
		Upregulation	**Downregulation**	**Total**
Vaccine status				
	Challenged non-vaccinated channels vs. challenged vaccinated channels	33	446	479
	Challenged non-vaccinated hybrids vs. challenged vaccinated hybrids	0	0	0
Bacterial challenge				
	Non-challenged non-vaccinated channels vs. challenged non-vaccinated channels	93	623	716
	Non-challenged vaccinated channels vs. challenged vaccinated channels	801	1998	2799
	Non-challenged non-vaccinated hybrids vs. challenged non-vaccinated hybrids	31	187	218
	Non-challenged vaccinated hybrids vs. challenged vaccinated hybrids	18	15	33
Fish types				
	Challenged vaccinated channels vs. challenged vaccinated hybrids	819	318	1137
	Challenged non-vaccinated channels vs. challenged non-vaccinated hybrids	142	734	876

False discovery rate (FDR) < 0.05

CHAPTER 6

CONCLUSIONS

The genus *Edwardsiella* contains several significant pathogens of teleost fish, especially within catfish aquaculture. The recent reorganization of the genus *Edwardsiella* into five distinct species has obscured previous descriptions of the pathogenicity and pathology associated with each bacterium. Additionally, little has been documented concerning intraspecific genotypic and phenotypic characteristics among isolates of one of the newly designated species, *Edwardsiella anguillarum.* Moreover, another newly designated species, *Edwardsiella piscicida*, has emerged as an increasingly significant pathogen in hybrid catfish aquaculture, highlighting the need for further information on preventative strategies and host responses.

This work investigated variation in susceptibility and disease in blue, channel, and channel x blue hybrid catfish to challenges with the three taxa recently differentiated from *Edwardsiella tarda*: *E. anguillarum, E. piscicida,* and *E. tarda.* The most severe disease and mortality occurred in fish challenged with *E. piscicida*. These findings support earlier reports of increased virulence by *E. piscicida* in the commercially important ictalurids, and that *E. anguillarum* and *E. tarda* merit only minimal concern to the industry. Acute pathologic lesions elicited by all three bacterial species were predominantly necrotizing and characteristic of gram-negative sepsis but became progressively granulomatous over time. The 100-day survivors exposed to approximate median lethal doses of *E. piscicida* and *E. ictaluri* had variable, but increased survivability, suggesting potential cross-protective effects among *E. piscicida*, *E. anguillarum,* and *E. ictaluri*. *Edwardsiella tarda* did not confer any protection against

subsequent *E. piscicida* or *E. ictaluri* challenge. This highlights the potential cross-protective affects among fish associated *Edwardsiella* spp.

As *E. anguillarum* was only recently recognized a species separate from *E. tarda*, this study described the intraspecific phenotypic and genotypic variability among *E. anguillarum* isolates from non-anguillid fish hosts and differing geographic origins. Isolates exhibited comparable biochemical characteristics, with slight variation in motility and hydrogen sulfide production. Prior to its renaming, *E. anguillarum* was synonymous with "atypical, non-motile, fish-pathogenic *E. tarda"* but previously described isolates were reported to be non-motile and motile. This work clarifies that motility varies within this bacterial species. Sequenced genomes were evaluated with repetitive extragenic palindromic sequence-based PCR (rep-PCR) and multilocus sequence analysis (MLSA). Rep-PCR with the ERIC II primer set revealed two distinct genetic clusters. No discrete clusters were formed utilizing the $(GTG)_5$, BOX or ERIC I&II amplification strategies. Though rep-PCR categorized the isolates as mostly clonal, MLSA schemes using reference genes from published *Edwardsiella* MLSA schemes showed *E. anguillarum* isolates formed five discrete phylogroups. The established MLSA scheme in this study can be used in future work regarding *E. anguillarum* genetic diversity as additional isolates from new hosts and provenances are identified. Large plasmids were identified in Costa Rican and Colombian isolates, but additional characterization is needed to better understand their function, such as virulence or antibiotic resistance. This study provides a foundation for defining drivers of *E. anguillarum* intraspecific variation among different hosts and geographic regions.

This research also investigated the varied virulence of *E. ictaluri* in farm-raised channel and channel x blue hybrid catfish fingerlings as well as the immune response and protective effects of a live-attenuated *E. ictaluri* vaccine. To do this, the immune responses to *E. ictaluri*

challenge in vaccinated and non-vaccinated, channel and hybrid catfish fingerlings were studied using ELISAs, RNA-sequencing, and qPCR. The vaccine conferred protection against intracoelomic *E. ictaluri* challenge. In channel catfish, serum IgM against *E. ictaluri* was elevated by the vaccine and challenge 28 dpv and 28 dpc. In contrast, hybrid serum IgM levels were only elevated 28 dpc, indicating the vaccine did not induce substantial IgM production in hybrids. Additionally, only vaccinated, challenged channels produced a significant antibody response to *E. piscicida*. Differentially expressed genes (DEGs) were identified between fish types and across treatments, but no DEGs were credited to vaccination alone. The differentially regulated transcripts were commonly related to innate immune responses, complement, and coagulation. The data presented in Chapter 4 illustrate how bacterial infection, vaccination, and fish type influence the host response to *E. ictaluri* infection in channel and hybrid catfish.

Chapter 5 built on Chapter 4 by investigating the pathogenicity of and immune responses to *E. piscicida* bacterial challenge in *E. ictaluri* vaccinated and non-vaccinated channel and hybrid catfish with similar methods. The vaccine conferred protection in both channel and hybrid catfish, with a lower relative percent survival in hybrid catfish and higher survival probability in vaccinated channels than hybrids. Only non-vaccinated, challenged channel catfish produced robust serum IgM levels. Channel catfish had a greater humoral and Th1 response to *E. piscicida* challenge than hybrid catfish. Complement and coagulation gene transcripts were upregulated in challenged, vaccinated channel catfish compared to non-challenged counterparts, indicating the *E. ictaluri* vaccine may confer protection to channels through rapid complement system and coagulation cascade induction. While this study provides significant data for understanding catfish host responses to *E. piscicida* challenge, additional work is warranted to further

characterize host-pathogen interactions and preventative strategies for *E. piscicida* infections, particularly in hybrid catfish.

These chapters build a foundation for research on intraspecific variation in *E. anguillarum* and build on previous research on the pathogenicity, immune responses, and cross-protective effects induced by *Edwardsiella* species in commercially important ictalurid species. The data and techniques used here can be applied to further develop disease prevention methods specifically regarding potential cross-protective vaccines.

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