ARTHUR ROBERTO DA COSTA

TRANSMISSÃO INTERESPÉCIES DE Edwardsiella ictaluri EM TRÊS ESPÉCIES DE PEIXES (Oreochromis niloticus, Pseudoplatystoma corruscans e Clarias gariepinus)

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal da Universidade Estadual de Londrina como requisito parcial para obtenção do título de Mestre.

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Londrina, 26 de fevereiro de 2021.

“O sonho das pessoas não tem fim”

- Marshall D. Teach

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RESUMO

A piscicultura Brasileira alcança novos patamares a cada ano, com a tilápia-do-Nilo (Oreochromis niloticus) como espécie mais produzida, seguida pelos peixes nativos, como o tambaqui (Colossoma macropomum), o pintado (Pseudoplatystoma corruscans) e o pirarucu (Arapaima gigas). A intensificação da produção, a importação e fuga de espécies exóticas, podem contribuir para o estabelecimento de doenças alóctones no país, como os casos recentes de franciselose e Vírus da necrose infecciosa de baço e rim (ISKNV). Edwardsiella ictaluri é a bactéria responsável por ocasionar a septicemia entérica do bagre do canal (ESC), doença que causa prejuízos de milhões de dólares na indústria do bagre do canal (Ictalurus punctatus) nos Estados Unidos e, recentemente, se tornou um risco para a indústria do panga (Pangasianodon hypophthalmus) na Ásia e para a fauna nativa da Austrália, contudo, pouco se sabe sobre este patógeno na piscicultura nacional. No mês de abril de 2020, uma cepa de Edwardsiella sp. foi isolada causando doença em pintados, em uma piscicultura no noroeste do estado do Paraná. Os animais apresentavam ascite e sinais neurológicos, além de taxa de mortalidade maior que 50%. O objetivo deste trabalho foi confirmar se a bactéria se tratava de uma E. ictaluri e verificar se foi a responsável pela mortalidade dos animais; além de, avaliar se espécies exóticas introduzidas na região podem ter atuado como vetores para a disseminação deste patógeno nas pisciculturas locais. Foi conduzido um experimento de infecção por imersão e inoculação intraperitoneal, com pintados, tilápias do Nilo e bagres Africanos. Quarenta e oito animais de cada espécie foram divididos em seis aquários por espécie, contendo oito animais cada. Em paralelo, um experimento de infecção por coabitação com peixes de outras espécies previamente infectados foi realizado em três aquários contendo quatro peixes de cada espécie. Após o sequenciamento do gene 16S, a bactéria foi classificada como E. ictaluri. Todos os pintados desafiados por via intraperitoneal e imersão vieram a óbito em um período de onze e quinze dias, respectivamente. Houve mortalidade também nas coabitações entre todas as espécies. Os sinais clínicos observados foram compatíveis com os relatados na piscicultura e descritos na literatura como ESC. Nas análises histopatológicas foi possível notar a presença de infiltrado inflamatório, principalmente eosinófilos no cérebro de todas as espécies e no olho de tilápias; o fígado, principalmente dos pintados, apresentou alta depleção de glicogênio, congestão e necrose. A bactéria foi reisolada com sucesso de todas as espécies presentes no experimento, mostrando que mesmo os animais que não vieram a óbito se tornaram carreadores do patógeno. Com isto, comprovamos a suscetibilidade do pintado a E. ictaluri, adicionando-o a lista de espécies que podem ser acometidas por esta bactéria. Mais, demonstramos a capacidade de espécies exóticas invasivas, de atuarem como vetores desta bactéria.

Palavras-chave: doenças alóctones; ESC; espécies nativas; infecção por coabitação; pintado; postulado de Koch.

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ABSTRACT

Brazilian aquaculture keeps growing year after year, with Nile tilapia (Oreochromis niloticus) as the most produced species, followed by the native fishes, such as tambaqui (Colossoma macropomum), pintado (Pseudoplatystoma corruscans) and pirarucu (Arapaima gigas). The intensification of production and introduction of exotic species can contribute to the establishment of alien diseases, as seen in the recente cases of francisellosis and Infectious spleen and kidney necrosis virus (ISKNV). Edwardsiella ictaluri is the bacterial pathogen for the enteric septicemia of channel catfish (ESC), a disease responsible for losses of millions of dollars in the channel catfish (Ictalurus punctatus) industry in the United States and, recently became a risk for the panga (Pangasianodon hypophthalmus) production in Asia and for the Australia's native fauna, however, little is known about this pathogen in the national pisciculture. In April 2020, a strain of Edwardsiella sp. was isolated causing disease in pintados, in a fish farm in the northwest of the Parana state. The diseased animals showed ascites and neurological signs, with q mortality rate greater than 50%. The objective of this work was to confirm if the bacterium was an E. ictaluri and to verify if it was the responsible for the fish mortality; in addition, to evaluate whether the exotic species introduced in the region could have acted as vectors for the spread of this pathogen in local fish farms. For this, an experimental infection by immersion and intraperitoneal inoculation was carried with pintado, Nile tilapia and African walking catfish. Forty-eight 48 animals of each specie were divided into six aquariums per species with eight individuals each. In parallel, a cohabitation infection experiment was carried in three aquariums with four fish from each species. After sequencing the 16S gene, the bacterium was identified as E. ictaluri. All pintados challenged by the intraperitoneal and immersion routes died after eleven and fifteen days, respectively. Also, mortality was verified in the cohabitation among all species. Observed clinical signs were compatible with those reported in the farm, and the ones described in the literature as ESC. Histopathology showed the presence of inflammatory infiltrate, mainly eosinophils, in the brain of all species and in the tilapia eye; the liver, especially the for pintado, showed high glycogen depletion, congestion and necrosis. The bacterium has been successfully reisolated from all the fish species, showing that even surviving animals can become carriers of the pathogen. With this, we proved the susceptibility of pintado to E. ictaluri, adding it to species that can be affected by this bacterium. Furthermore, we demonstrate the ability of invasive alien species to act as vectors for this bacterium.

Key-words: alien disease; ESC; native species; cohabitation infection; pintado; Koch postulate.

LISTA DE ILUSTRAÇÕES

REFERENCIAL TEÓRICO

ARTIGO: "Interspecies transmission of Edwardsiella ictaluri in Brazilian catfish (Pseudoplatystoma corruscans) from exotic invasive fish species"

LISTA DE TABELAS

REFERENCIAL TEÓRICO

ARTIGO: "Interspecies transmission of Edwardsiella ictaluri in Brazilian catfish (Pseudoplatystoma corruscans) from exotic invasive fish species"

LISTA DE ABREVIATURAS E SIGLAS

BHI – Brain Heart Infusion

BNP – Bacillar Necrosis of Pangasius

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CEUA/UEL – Comissão de Ética no Uso de Animais / Universidade Estadual de Londrina

CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico

DNOCS – Departamento Nacional de Obras Contra as Secas

EIM – *Edwardsiella ictaluri* medium

ESC – Enteric septicaemia of catfish

EUA – Estados Unidos da América

FAO – Food and Agriculture Organization of the United Nations

FDA – Food and Drug Administration

IBAMA – Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis

IBGE – Instituto Brasileiro de Geografia e Estatística

PEIXE BR – Associação Brasileira da Piscicultura

rRNA – ribossomic RNA

TSI – Triple Sugar Iron

USDA – United States Department of Agriculture

SUMÁRIO

1. INTRODUÇÃO

A aquicultura é uma atividade de produção de alimentos que vem se destacando nos últimos anos como uma alternativa sustentável à captura para a produção de pescados. Apenas no período entre 2015 a 2018, a produção aquícola mundial apresentou um crescimento de 37,5% (FAO, 2020). Atualmente, o Brasil ocupa a décima terceira posição no *ranking* de produtores aquícolas da FAO; o país tem apresentado bons números de produtividade, saltando de 578,8 mil toneladas em 2014 para 758 mil toneladas em 2019, um crescimento de 30,96% (PEIXE BR, 2020). Este intenso crescimento na produção é associado a sistemas intensivos de produção, caracterizados pela alta densidade de animais, fator estressante que pode ocasionar problemas de biosseguridade, como a disseminação de doenças infecciosas e parasitárias (Gabriel & Akinrotimi, 2011).

Uma destas doenças infecciosas é a septicemia entérica do bagre do canal (ESC), causada pela bactéria *Edwardsiella ictaluri* que ocasiona prejuízos estimados em 30 milhões de dólares anualmente na indústria da produção do bagre do canal (*Ictalurus punctatus*) nos Estados Unidos da América (EUA) (Griffin et al., 2020). Após os primeiros relatos da doença (Hawke et al., 1981), diversos grupos de pesquisa isolaram esta bactéria em outras espécies de peixes, totalizando 25 até o presente momento.

Atualmente, além dos graves prejuízos na indústria do bagre do canal, *E. ictaluri* é um dos maiores patógenos para a criação do panga (*Pangasianodon hypophthalmus*) na Ásia, causando a necrose bacilar do Pangasius (BNP) (Phuoc et al., 2020). Na Austrália, país com grande fauna de siluriformes, *E. ictaluri* representa um risco para as espécies nativas de vida livre (Kelly et al., 2018), sendo que a bactéria foi introduzida em seu território através de espécies exóticas carreadoras do patógeno.

Neste trabalho, isolamos *Edwardsiella ictaluri* como agente causador de um surto com alta taxa de mortalidade em pintados (*Pseudoplatystoma corruscans*), um dos representantes da ordem dos Siluriformes na fauna nacional, em uma piscicultura no noroeste do estado do Paraná.

2. REFERENCIAL TEÓRICO

2.1 – AQUICULTURA

A aquicultura se caracteriza como o cultivo de organismos que se desenvolvem (pelo menos em parte da vida) na água, como peixes, moluscos, algas, crustáceos, anfíbios e répteis. Seus primeiros indícios remontam à 2000 a.C. na China com a criação de macroalgas e de carpas. A atividade também é relatada em pinturas egípcias antigas de tanques de peixes ornamentais (Vinatea, 1995).

Atualmente, é uma das atividades que mais cresce no ramo da produção animal; segundo a Organização das Nações Unidas para a Alimentação e a Agricultura, a produção aquícola mundial em 2016, excluindo répteis e plantas aquáticas, foi de aproximadamente 80 milhões de toneladas, com a China, maior produtor aquícola mundial, responsável por 49,2 (61,5%) milhões de toneladas produzidas (FAO, 2020).

No Brasil, a piscicultura é a principal atividade aquícola, com uma produção de 758 mil toneladas em 2019, um crescimento de 4,9% sobre o ano anterior, com um crescimento acumulado de 30,96% desde 2014, com o Paraná apresentando-se como maior produtor aquícola do país, responsável por 146,2 (19,2%) mil toneladas do total do país (PEIXE BR, 2020).

A tilápia-do-Nilo (*Oreochromis niloticus*) é a espécie dominante na aquicultura Brasileira. O país é o quarto maior produtor mundial desta (atrás de China, Indonésia e Egito), com um total bruto de 432.149 toneladas (54,1% da produção do país (PEIXE BR, 2020). A popularização da tilápia se deu graças a sua carne saborosa com baixo teor de gordura, rápido crescimento, rusticidade e resistência a doenças (Raghiante et al., 2017). Com a disseminação da produção da tilápia, os preços de mercado caíram, fazendo com que a produção de peixes nativos surgisse como alternativa em estados de clima mais quente no Centro-Oeste, Norte e Nordeste, já representando 38% da produção Brasileira, com destaques para o tambaqui (*Colossoma macropomum*), pintado (*Pseudoplatystoma corruscans*), pirarucu (*Arapaima gigas*), pacu (*Piaractus mesopotamicus*) e jundiá (*Rhamdia quelen*) (PEIXE BR, 2020).

Estas espécies endêmicas apresentam um potencial único para a aquicultura nacional, não apenas pelo seu sabor diferenciado e potencial de

um produto único para o mercado global da exportação, como pelo menor risco ambiental que a sua produção traz, em contraste com a exótica tilápia (Azevedo-Santos, Rigolin-Sá & Pelicice, 2011).

2.2 – TILÁPIA

A tilápia-do-Nilo (Figura 1) é um peixe da família Cichlidae, introduzida no Brasil no início da década de 70 pelo Departamento Nacional de Obras Contra as Secas (DNOCS) com o intuito de povoar os reservatórios públicos da região Nordeste do país. A espécie, de hábitos onívoros, rapidamente se espalhou em diversas bacias hidrográficas das regiões Nordeste e Sudeste (Schulter & Vieira Filho, 2018). Seu potencial de reprodução acelerado e hábitos alimentares têm comprometido a ictiofauna do país (Bittencourt et al., 2014). Segundo análises de Canonico et al. (2005), a invasão de tilápias em corpos d'água dos EUA, praticamente eliminou espécies com hábitos alimentares semelhantes.

Figura 1: Tilápia do Nilo.

Fonte: The WorldFish Center (disponível em fishbase.org)

Hoje, a maior parte das tilápias produzidas no país é revertida sexualmente (as fêmeas apresentam crescimento reduzido devido ao desvio energético para a maturação sexual), o que diminui os índices de reprodução nas bacias hidrográficas Brasileiras após fuga de animais. Contudo, ainda existe risco devido aos hábitos alimentares vorazes e a disseminação de doenças infecciosas alóctones para as espécies nativas (Meurer et al., 2012).

2.3 – PINTADO

O pintado (Figura 2) é um peixe nativo da ordem dos Siluriformes (bagres/*catfish*) de hábitos carnívoros e noturnos, muito apreciado pela carne macia e sem espinhas. A criação de pintados sofreu muitos empecilhos antes de se estabelecer no país devido aos hábitos carnívoros que resultavam em canibalismo entre as pós-larvas e ao fato de ser um peixe de piracema, que desova nos rios após nadar contra a correnteza. Estas dificuldades foram contornados com a ajuda de estudos e novas tecnologias acostumando os animais com ração extrusada e reprodução induzida por hormônios (Beux & Zaniboni Filho, 2007; Leonardo et al., 2004). Outros siluriformes Brasileiros, como o cachara (*Pseudoplatystoma fasciatum*) e o Jundiá amazônico (*Leiarius marmoratus*) são comumente cruzados com o pintado em busca de híbridos mais resistentes, como o cachapira (♀ *P. fasciatum* x ♂ *P. corruscans*), o pintachara (♀ *P. corruscans* x ♂ *P. fasciatum*) e o pintado da Amazônia (♀ *P. corruscans* x ♂ *L. marmoratus*), que apresentam maior crescimento e comportamento mais dócil (Hashimoto et al., 2016). Para fins de produção a Associação Brasileira da Piscicultura (PEIXE BR) e o Instituto Brasileiro de Geografia e Estatística (IBGE) classificam estes animais como ‘pintados’.

A produção de pintado está concentrada nos estados do Paraná, Goiás, Rondônia e Mato Grosso (55,38% dos 10.094 estabelecimentos produtores do país), com um total estimado de 10.917 toneladas produzidas em 2019 (IBGE, 2020; PEIXE BR, 2020).

Figura 2: Pintado.

Fonte: Timm, Cláudio Dias (disponível em fishbase.org)

Mesmo sendo uma espécie rústica e resistente, em sistemas intensivos de criação, devido à alta densidade populacional e falhas de biosseguridade, pode haver a ocorrência de agentes infecciosos e parasitários, sendo relatadas até o momento: *Lactococcus garvieae* (Fukushima et al., 2017), *Streptococcus spp.* (Tavares et al., 2018), *Flavobacterium columnare* (Barony et al., 2015), *Aeromonas hydrophila* (Silva et al., 2012), *Citrobacter spp.* (Pádua et al., 2014), *Edwardsiella spp.* (Leira et al., 2019) e infestações pelos parasitas *Henneguya sp*., *Epistilys sp*., *Ichthyophthirius multifiliis* e *Trichodina sp*. (Adriano et al., 2012; Pádua et al., 2012).

2.3 – BAGRE AFRICANO

O bagre africano (*Clarias gariepinus*) (Figura 3) é um peixe da ordem dos Siluriformes, assim como o pintado, nativo de maior parte da África e de partes do mediterrâneo (Turquia, Israel e Síria) de hábitos onívoros e noturnos. Este peixe é conhecido como "*African walking catfish*", devido a sua capacidade de sair dos corpos de água e se locomover na terra, com o auxílio das espinhas peitorais, para se alimentar de raízes e insetos durante a noite (Bruton, 1979; Johnels, 1957; Silveira et al., 2018). Esta habilidade advém de um órgão supra branquial especializado, que permite a sobrevivência fora da água por períodos consideráveis.

Figura 3: Bagre Africano.

Fonte: Lovshin, Leonard L. (disponível em fishbase.org)

Seu crescimento rápido, excelente conversão alimentar, resistência à flutuação termal, alta salinidade e capacidade de reproduzir o ano

todo em temperaturas favoráveis a tornaram uma espécie popular para a produção, sendo introduzida em mais de 37 países na Europa, Ásia e América Latina, incluindo o Brasil, em 1986 para fins de produção aquícola (Cambray, 2005; Weyl et al., 2016).

A espécie não teve sucesso no mercado aquícola local, enfrentando competividade com a tilápia e os Siluriformes Brasileiros, ficando limitada a pesque-pague, onde seu crescimento e reprodução rápidos foram bem apreciados (Vitule et al., 2006). A falta de segurança deste tipo de estabelecimento, combinada com a capacidade deste animal de se locomover fora da água, resultou na fuga e invasão de bacias hidrográficas do país, onde seus hábitos alimentares, grande resistência às condições adversas naturais e elevada fertilidade contribuíram para seu estabelecimento como espécie invasora, inicialmente no rio Itajaí-Açu (Santa Catarina - SC) e se disseminando para as bacias dos rios Sorocaba (São Paulo - SP), Doce (Minas Gerais - MG), Grande (MG), São Francisco (MG), Paraná (PR), Ribeira de Iguapé (SP), Almada (Bahia - BA), Iguaçu (PR), Bacia costal do Sul (SC), Laguna dos Patos (Rio Grande do Sul - RS), Paraíba do Sul (MG), Guanabara (Rio de Janeiro - RJ) e D'Una (SC) (Silveira et al., 2018; Weyl et al., 2016).

Vale ressaltar, que a introdução da espécie no país em 1986 foi feita de maneira ilegal, contrariando o disposto no artigo 34 do decreto-lei nº 221 de 1967 (revogado pela lei nº 11.959 de 2009), sobre a proibição da introdução de espécies aquáticas exóticas em águas interiores (Decreto-Lei Nº 221, 1967; Lei nº 11.959, 2009). Para reforçar esta legislação e controlar a invasão por esta espécie, o Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) emitiu a Portaria nº 142 de 22 de Dezembro de 1994, instituindo a proibição do cultivo e comercialização do bagre africano e do bagre do canal nas bacias dos Rios Amazonas e Paraguai (Portaria Nº 142, 1994). Até o momento, não existem relatos destas espécies em rios da bacia Amazônica.

Assim como ocorreu com a tilápia do Nilo, existe a preocupação do povoamento desta espécie espalhar doenças para a fauna Brasileira, principalmente para os Siluriformes locais, como o pintado. Alguns estudos mostram suscetibilidade do bagre africano a patógenos comuns na aquicultura, tais como: trematódeos (Ribeiro et al., 2019), protozoários (Ayawei et al., 2020), *Aeromonas hydrophila*, *Pseudomonas spp*. (Ikpi & Offem, 2011) e *Edwardsiella*

spp. (Hashiem & Abd El-Galil, 2012; Kasornchandra et al., 1987).

2.4 – EDWARDSIELLA

2.4.1 – Classificação

Edwardsiella é um gênero de bactérias, com formato de bastonete, Gram-negativas, intracelulares facultativas, da família Hafniaceae, ordem Enterobacterales (Adeolu et al., 2016). Foram descritas pela primeira vez na década de 60, como “bactérias grupo Asakusa” em enterobactérias com manitol negativo e produção de sulfeto de hidrogênio, isoladas de cobras no Japão (Sakazaki & Murata, 1962) e “bactérias grupo Bartholomew” em isolados em humanos com gastroenterite nos EUA (King & Adler, 1964). O nome *Edwardsiella* foi em homenagem ao microbiologista P. R. Edwards (1901-1966), com o sufixo *tarda* sendo adicionado a espécie devido ao seu lento crescimento (Ewing et al., 1965).

Atualmente, existem cinco espécies no gênero *Edwardsiella*, responsáveis por alterações gastrointestinais e septicêmicas em mamíferos (incluindo humanos), répteis, peixes, aves, sendo: *E. anguillarum*, agente patogênico de animais da ordem Synbranchiformes (Shao et al., 2015); *E. ictaluri*, relacionada a septicemia entérica em Siluriformes, principalmente nos EUA (Griffin et al., 2016; Hawke et al., 1981); *E. piscicida*, que vem ganhando destaque nos últimos anos por infectar múltiplas espécies de peixes (Abayneh et al., 2013; Leung et al., 2019); *E. hoshinae,* bactéria pouco estudada isolada de répteis e aves aquáticas (Grimont *et al.*, 1980; Reichley *et al.*, 2017); e *E. tarda*, principal agente patogênico da edwardsielose, isolado em diversas espécies de peixes, répteis, aves, mamíferos e com potencial zoonótico como patógeno oportunista (Ewing et al., 1965; Michael & Abbott, 1993; Mohanty & Sahoo, 2007). Até 2013, apenas três espécies de *Edwardsiella* eram reconhecidas (*E. hoshinae*, *E. tarda* e *E. ictaluri*), porém, estudos de filogenômica reclassificaram alguns isolados de *E. tarda* em duas novas espécies: *E. piscicida* e *E. anguillarum* (Abayneh et al., 2013; Shao et al., 2015). Esta mudança, somada a uma similaridade de mais de 99% na composição do gene que codifica a subunidade 16S rRNA, faz com que muitos isolados destas bactérias ainda sejam classificados erroneamente (Reichley et al., 2017).

2.4.2 – *Edwardsiella ictaluri*

Edwardsiella ictaluri é um bastonete gram-negativo, intracelular facultativo, com tamanho de 0.5-2.5 µm e com motilidade abaixo de 30ºC (Hawke et al., 1981; Plumb & Vinitnantharat, 1989), responsável por causar a septicemia entérica do bagre do canal (ESC) e pela necrose bacilar do Pangasius (BNP) (Crumlish et al., 2002; Hawke et al., 1981).

A bactéria apresenta crescimento lento em caldo cérebro coração (BHI) e ágar sangue de ovino 5%, demorando 48h para produzir colônias redondas e esbranquiçadas com aproximadamente 1mm de diâmetro, quando incubada em temperaturas de 25-30ºC (Hawke et al., 1981). Suas características bioquímicas tendem a variar muito pouco, sendo diferenciada de *E. tarda* por não produzir sulfeto de hidrogênio em Triple Sugar Iron - TSI (Tabela 1) (Plumb & Vinitnantharat, 1989; Waltman et al., 1986). Seu crescimento lento faz com que possa ser inibida por outras bactérias, sendo assim, para melhor estudo de isolados de água, Shotts & Waltman em 1990, desenvolveram um meio seletivo (EIM com 0,1% de sais biliares), baseado na resistência de *Edwardsiella spp.* ao antibiótico colistina. O meio foi eficaz em inibir o crescimento de bactérias gram-positivas e, as bactérias gram-negativas com crescimento (algumas *Aeromonas* e *Pseudomonas*) apresentavam características morfológicas e de coloração diferentes de *E. tarda* e *E. ictaluri* devido à produção de metabólitos reagentes ao meio de cultura.

Tabela 1 – Características bioquímicas de *Edwardsiella ictaluri*.

Teste	Resultado
Motilidade 25-30ºC	+
Motilidade > 30ºC	-
Suscetibilidade a NaCl	1,5-2%
Oxidase	-
Catalase	+
Ureia	-
Esculina	-
Citrato	-
Produção de gás (TSI)	+/-
Produção de H_2S (TSI)	-
Vermelho de Metila (VM)	+
Voges-Proskauer (VP)	-

Fonte: Plumb & Vinitnantharat (1989); Waltman et al. (1986).

2.4.3 – Septicemia entérica do bagre do canal

Esta doença foi detectada pela primeira vez em criações de bagre do canal (*Ictalurus punctatus)* em 1976, na região sudeste dos EUA. Os animais acometidos apresentaram sinais clínicos pouco específicos, semelhantes aos sinais clínicos apresentados por outras septicemias causadas por bactérias gram-negativas na aquicultura (*Aeromonas spp., Pseudomonas spp.* e *E tarda*) (Hawke et al., 1981).

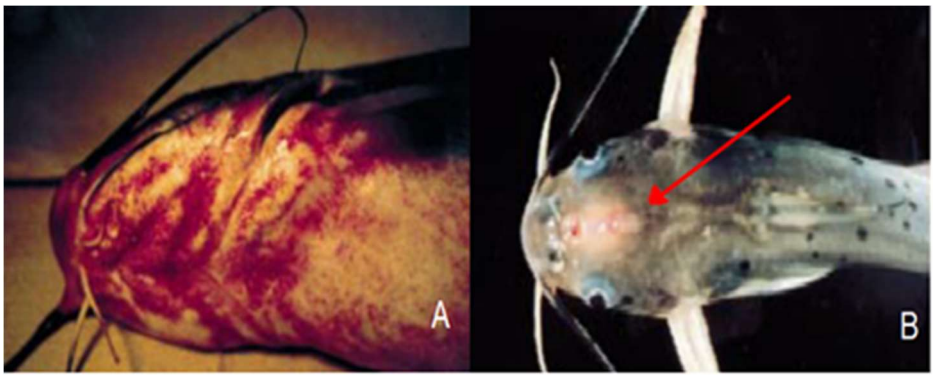
A ESC é uma doença característica do período pós-verão, quando as temperaturas começam a diminuir (entre 20 e 30ºC) afetando principalmente alevinos e animais com imunidade baixa (pós-manejo) (Baxa-Antonio et al., 1992). A transmissão ocorre pela via oral, ingestão da bactéria na água, seja por meio de fezes ou da predação de peixes doentes (o canibalismo é muito comum nos primeiros estágios de vida do bagre do canal) (Xu et al., 2013).

Os sinais clínicos são variáveis: ulcerações brancas e avermelhadas na pele, ascite (Figura 4), petéquias na parte ventral da cabeça (Figura 5-A), exoftalmia, redução total de apetite, natação errática e animais parados na coluna d'água horas antes de irem ao óbito. Em infecções crônicas detecta-se a presença de uma mancha vermelha, semelhante a uma espinha "*pimple-like lession*" (Figura 5-B), na região do forame cranial, por isso o apelido de "*hole-in-head*" (doença do buraco na cabeça) (Hawke et al., 1998). Na necropsia observa-se presença de líquido abdominal, fígado pálido com áreas de necrose, intestino com conteúdo sanguinolento e manchas brancas em fígado, rim e baço, além de inflamação granulomatosa no encéfalo (Booth et al., 2006).

Figura 4: Pintado com dilatação abdominal com suspeita de *E. ictaluri*.

Fonte: Laboratório de Bacteriologia de Peixes (LABBEP-UEL), repositório particular.

Figura 5: Bagre do canal com sinais característicos de Septicemia entérica do bagre do canal: petéquias hemorrágicas na região ventral da cabeça (A) e lesão semelhante a uma espinha próximo ao forame cranial (B).



Fonte: Hawke et al. (1998).

Em análises histopatológicas, observa-se que o acumulo de células inflamatórias no sistema nervoso acarreta necrose de todo o trato olfatório, lobo ocular e meninges. O fígado dos animais afetados apresenta pequenos focos necróticos com atrofia de hepatócitos, e o baço, em alguns casos, apresenta neutrófilos imaturos e disseminação bacteriana com necrose do tecido arterial (Miyazaki & Plumb, 1985). A lesão ulcerativa semelhante a uma espinha na cabeça, exibe necrose de células da pele, com hemorragia e exsudato purulento (Miyazaki & Kaige, 1985).

Mesmo hoje, mais de 40 anos após o surgimento da doença, ela ainda é responsável por grande parte das perdas na indústria dos “catfish” nos EUA, que representa mais de 50% da produção aquícola do país (Akgul et al., 2018; Kelly et al., 2018; USDA, 2018).

O tratamento é feito com o uso de antibióticos, principalmente Terramycin (oxitetraciclina), Romet (sulfadimetoxina-ormetoprim) e Aquaflor (florfenicol) (FDA, 2020). Com o advento de cepas multirresistentes a antibióticos, e a substituição destes medicamentos por alternativas sustentáveis, efeitos de imunoestimulantes (probióticos e fitogênicos) já vem sendo avaliados como alternativas no tratamento e prevenção da ESC (Peterson et al., 2015). Porém, como muitas vezes a doença só é percebida ao já estar instalada, o tratamento é difícil e oneroso. Um dos primeiros sinais clínicos é a inapetência, impedindo a correta ingestão de antibiótico pela via oral. Uma das alternativas, disponível comercialmente, é a vacina viva atenuada contra o patógeno, porém,

a proteção oferecida é limitada a algumas variantes genéticas de *E. ictaluri* (Klesius & Shoemaker, 1999; Kordon et al., 2019).

2.4.4 – *Edwardsiella ictaluri* em outras espécies de peixes

Inicialmente, as infecções por *E. ictaluri* se limitavam a membros da ordem Siluriformes, família Ictaluridae nos EUA: *Ictalurus punctatus*, *Ameirus catus* e *Ameirus nebulosus*. Em 1983, Plumb & Sanchez demonstraram em laboratório que a tilápia azul (*Oreochromis aureus* – ordem Cichliformes) apresentou mortalidade sob altas doses da bactéria ($1,5x10^8$ UFC/0,1mL), enquanto a "*largemouth-bass*" (*Micropterus salmoides* - ordem Perciformes), a carpa dourada (*Notemigonus crysoleucas* - ordem Cypriniformes) e a carpa cabeçuda (*Aristichthys nobilis* - ordem Cypriniformes) não apresentaram nenhuma mortalidade e tiveram baixa taxa de reisolamento.

Atualmente já existem relatos do isolamento de *E. ictaluri* em sete espécies das 33 famílias da ordem Siluriformes (Ictaluridae, Bagridae, Clariidae, Pangasiidae, Siluridae, Plotosidae e Ariidae) e em outras 11 espécies de peixes de diferentes ordens (Tabela 2), com distribuição na América do Norte, Ásia, Turquia e Austrália.

Tabela 2 – Espécies de peixes acometidas por *Edwardsiella ictaluri*.

Nome Popular	Nome Científico	Ordem	Família	Referência
Bagre do canal "*Channel catfish*"	*Ictalurus punctatus*	Siluriformes	Ictaluridae	(Hawke et al., 1981)
Peixe gato branco "*White bullhead*"	*Ameirus catus*	Siluriformes	Ictaluridae	(Hawke et al., 1981)
Peixe gato castanho "*Brown bullhead*"	*Ameirus nebulosus*	Siluriformes	Ictaluridae	(Hawke et al., 1981)
Peixe gato preto "*Black bullhead*"	*Ameirus melas*	Siluriformes	Ictaluridae	(Shotts & Plumb, 2003)
Peixe gato azul "*Blue catfish*"	*Ictalurus furcatus*	Siluriformes	Ictaluridae	(Newton et al., 1988)

"Tadpole madtom"	*Noturus gyrinus*	Siluriformes	Ictaluridae	(Klesius et al., 2003)
Peixe gato coreano "*Yellow catfish*"	*Pelteobagrus fulvidraco*	Siluriformes	Bagridae	(Ye et al., 2009)
Bagre andador "*Walking catfish*"	*Clarias batrachus*	Siluriformes	Clariidae	(Kasornchandra et al., 1987)
Hybrid catfish	*C. macrocephalus x C. gariepinus*	Siluriformes	Clariidae	(Suanyuk et al., 2014)
Panga "*Striped catfish*"	*Pangasianodon hypophthalmus*	Siluriformes	Pangasiidae	(Crumlish et al., 2002)
Peixe gato chinês "*Southern catfish*"	*Silurus meridionalis*	Siluriformes	Siluridae	(Geng et al., 2013)
"Toothless catfish"	*Anodontiglanis dahli*	Siluriformes	Plotosidae	(Kelly et al., 2018)
Butter jew	*Neosilurus ater*	Siluriformes	Plotosidae	(Kelly et al., 2018)
"*Wet tropics Tandan*"	*Tandanus tropicanus*	Siluriformes	Plotosidae	(Kelly et al., 2018)
Bagre tubarão de Berney "Highfin catfish"	*Neoarius berneyi*	Siluriformes	Ariidae	(Kelly et al., 2018)
Tilápia-do-Nilo "*Nile tilapia*"	*Oreochromis niloticus*	Cichliformes	Cichlidae	(Soto et al., 2012)
Tilápia azul "*Blue tilapia*"*	*Oreochromis aureus*	Cichliformes	Cichlidae	(Plumb & Sanchez, 1983)
Tilápia vermelha "*Hybrid red tilapia*"	*O. mossambicus x O. niloticus*	Cichliformes	Cichlidae	(Dong et al., 2019)
Peixe-zebra "*Zebrafish*"	*Danio rerio*	Cypriniformes	Cyprinidae	(Hawke et al., 2013)
Danio	*Devario devario*	Cypriniformes	Cyprinidae	(Waltman et al., 1985)
Barbo rosado "*Rosy barb*"	*Pethia conchonius*	Cypriniformes	Cyprinidae	(Humphrey et al., 1986)

Salmão-Rei "*Chinook salmon*"*	*Oncorhynchus tshawytscha*	Salmoniformes	Salmonidae	(Baxa et al., 1990)
Truta arco-íris "*Rainbow trout*"	*Oncorhynchus mykiss*	Salmoniformes	Salmonidae	(Keskin et al., 2004)
Perca-gigante "*Asian seabass*"	*Lates calcarifer*	Perciformes	Latidae	(Gibson-Kueh et al., 2004)
Ituí transparente "*Glass knifefish*"	*Eigenmannia virescens*	Gymnotiformes	Sternopygidae	(Kent & Lyons, 1982)
Ayu	*Plecoglossus altivelis*	Osmeriformes	Plecoglossidae	(Nagai et al., 2008)

***** Apenas infecção experimental.

Com este estudo, adicionaremos a esta lista o primeiro bagre nativo sul-americano, o pintado, relatando a ocorrência de um surto causado por *E. ictaluri* em uma piscicultura no estado do Paraná.

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669

4. HIPÓTESE

O peixe brasileiro, pintado, é susceptível a *Edwardsiella ictaluri,* e desenvolve a septicemia entérica do bagre do canal quando exposto a esta bactéria.

Espécies alóctones, susceptíveis a edwardsielose causada por *Edwardsiella ictaluri* podem atuar como reservatórios e contaminar peixes nativos sensíveis, como o Pintado.

5. OBJETIVOS

5.1 – OBJETIVO GERAL

Confirmar que o peixe nativo Pintado (*Pseudoplatystoma corruscans)* é suscetível a *Edwardsiella ictaluri*.

5.2 – OBJETIVOS ESPECÍFICOS

- Demonstrar que peixes da família Pimelodidae (bagres da América Latina) podem ser acometidos pela Septicemia entérica do bagre do canal;
- Identificar a *Edwardsiella* isolada de uma piscicultura no noroeste do estado do Paraná como pertencente a espécie *E. ictaluri*;
- Avaliar diferentes vias de infecção / transmissão e a taxa mortalidade de *Edwardsiella ictaluri* em pintados (*Pseudoplatystoma corruscans*);
- Estudar a transmissão de *Edwardsiella ictaluri* entre a espécie invasora tilápia do Nilo (*Oreochromis niloticus*) e a espécie nativa pintado (*Pseudoplatystoma corruscans*);
- Estudar a transmissão de *Edwardsiella ictaluri* entre a espécie invasora bagre africano (*Clarias gariepinus*) e a espécie nativa pintado (*Pseudoplatystoma corruscans*).

6. ARTIGO ACEITO NO PERIÓDICO DISEASES OF AQUATIC ORGANISMS

Interspecies transmission of *Edwardsiella ictaluri* in Brazilian catfish (*Pseudoplatystoma corruscans*) from exotic invasive fish species

Enteric septicemia of catfish in pintado

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ABSTRACT

Infections caused by *Edwardsiella ictaluri* are one of the biggest problems for the catfish industry in North America and was reported in other fishes around the world. An occurrence of *E. ictaluri* was detected in farmed pintado juveniles (*Pseudoplatystoma corruscans*), a Brazilian catfish, in Paraná state. The diseased animals showed ascites and neurological signs, with more than 50% mortality. Some species susceptible to this bacterium are reported as invasive in the area. Also, we assessed the susceptibility of pintado against *E. ictaluri* in an experimental infection via intraperitoneal and immersion, as well as a cohabitation experiment with Nile tilapia (*Oreochromis niloticus)* and African walking catfish (*Clarias gariepinus*), two exotic invasive species. All pintados challenged by intraperitoneal and immersion routes and cohabiting with infected *C. gariepinus* died within 17 days of the challenge. Nile tilapia had 71.42% mortality in the intraperitoneal challenge and 35.71% in immersion within 28 days, whereas African walking catfish

showed zero mortality. Observed clinical signs were compatible with those in the farm, and the described in the literature as Enteric Septicemia of Catfish. With this, we demonstrated the susceptibility of *P. corruscans* to *E. ictaluri*, as well as interspecies transmission of this bacterium.

Keywords: ESC, alien disease, cohabitation infection, Koch postulate.

1. INTRODUCTION

Edwardsiella ictaluri is a Gram-negative rod, facultative intracellular bacteria, responsible for the enteric septicemia of catfish (ESC), an important disease responsible for loses of more than U$ 30 million in the *Ictalurus punctatus* industry in United States (Griffin et al., 2020). The disease characteristically occurs in the period after summer, when temperature starts to drop (20-30ºC), affecting mainly fingerlings, juveniles, and animals with low immunity (after handling) (Baxa-Antonio et al., 1992). Clinical signs range from ulcerations, petechiae in the ventral part of the body, ascites, anorexia, erratic swimming, and animals immobile in the water column, hours before dying, while in chronical infections the animals also show a pimple-like lesion in the cranial foramen region, giving it the “hole-in-the-head disease” nickname (Booth et al., 2006, Hawke et al., 1998).

After its first isolation in channel catfish in 1976 (Hawke et al., 1981), the bacterium was isolated in other fish species and regions, not being limited to catfishes (Crumlish et al., 2002, Dong et al., 2019, Geng et al., 2013, Gibson-Kueh et al., 2004, Keskin et al., 2004, Newton et al., 1988, Soto et al., 2012, Suanyuk et al., 2014, Ye et al., 2009), attaining the status of an important pathogen for *Pangasianodon hypophthalmus* aquaculture in Asia (responsible for the bacillar necrosis of Pangasius – BNP) (Phuoc et al., 2020) and to native catfish in Australia (Kelly et al., 2018), place where it established after being introduced from imported animals (Humphrey et al., 1986).

762 Brazil has one of the biggest fish fauna in the world, with many important catfish
763 species, such as pintado (*Pseudoplatystoma corruscans*), cachara (*P. fasciatum*), surubim
764 (*P. reticulatum*), and jundiá Amazônico (*Leiarius marmoratus*). These species and its
765 hybrids are a growing part of the native fish aquaculture, especially in the northern and
766 southwestern regions of the country. To date, to the best of our knowledge, there is no
767 published literature describing the isolation or identification of *E. ictaluri* in those species,
768 and there is limited knowledge about the interspecies transmission of this pathogen.
769 In the present study, we described a disease outbreak in a pintado farm in Paraná
770 State, where a Gram-negative bacterium, identified as *Edwardsiella sp.* was isolated.
771 Thus, our objectives were to identify the isolated *Edwardsiella*, fulfill the Koch postulate
772 to determine if it was responsible for the described outbreak, and inquire if exotic fish
773 species introduced in the region for aquaculture purposes (African walking catfish –
774 *Clarias gariepinus* and Nile tilapia – *Oreochromis niloticus*) could act as vectors of the
775 pathogen for native catfish.
776

777 ## 2. MATERIALS AND METHODS

778 The present experiment was conducted according to the norms of wellbeing and
779 ethics in animal experimentation approved by the Ethics Committee on the Use of
780 Animals of State University of Londrina (CEUA/UEL) with protocol number 030.2020.
781

782 ### 2.1. Case report

783 A pintado (*P. corruscans)* farm located in the Northwest region of the Paraná State
784 (Figure S1) related high mortality levels (> 50%) during April 2020 (Brazil mid-autumn,
785 water temperature ranged from 23 to 26ºC) in juveniles with approximately 30 to 50 cm.
786 Affected fish presented ascites, swollen gills, erratic swimming, and immobility in the

787 water column. Parasites (monogenetic and trichodine) were identified, but considered
788 insignificant. The farmer also related a similar case at 2019 mid-December (late spring/
789 early summer, water temperature between 25 and 28ºC), but with less mortality, in bigger
790 fish (approximately 1m). In both cases the fish were treated with oxytetracycline (100
791 $mg \cdot kg^{-1}$) for 14 days until the mortality stopped.
792
793 **2.2. Culture and bacterial identification**
794 Microbiological diagnostic procedures were carried out on six fish in the
795 laboratory. Animals showed signals of edwardsiellosis, liver congestion and ascites. The
796 eye, brain, liver, kidney, and spleen of all the fishes were inoculated in blood agar with
797 5% sheep blood and incubated aerobically at 28ºC for 48h.
798 After incubation, bacterial colonies were analyzed using Gram stain, Oxidase test,
799 and the Bactray I commercial identification kit (Laborclin®, Pinhais, PR, Brazil), as well
800 as a conventional biochemical identification test composed of TSI, indole, urea, citrate,
801 Voges-Proskauer, methyl red, fermentation of lactose, sorbitol, trehalose, and maltose,
802 motility at 25º and 30ºC. The antibiotic susceptibility test as conducted with the Kirby
803 and Bauer methodology, according to the standardization of the Clinical and Laboratory
804 Standards Institute (CLSI, 2019), with the antibiotics penicillin, amoxicillin, florfenicol,
805 gentamicin, enrofloxacin, tetracycline, and streptomycin.
806 Additionally, the isolated bacterium had its DNA extracted with the Purelink™
807 Genomic DNA Mini Kit (Invitrogen™, USA) with PCR amplification for the 16S rRNA
808 using the methodology and primers described by Weisburg et al. (1991). For the
809 sequencing of the purified DNA an ABI3500 Genetic Analyzer (Applied Biosystems,
810 Foster City, CA, USA) was used. The sequencing result was analyzed with the MEGA X
811 software (Kumar et al., 2018), using representatives of all *Edwardsiella* species and

812 *Hafnia alvei* as an out-group of the *Hafniaceae* family (Adeolu et al., 2016). The tree was
813 inferred using the Maximum Likelihood method and Kimura-2 model (Kimura, 1980),
814 with a bootstrap value of 1,000 replicates (Felsenstein, 1985)
815
816 **2.3. Fish and experimental design**
817 To fulfill the Koch Postulate, 60 pintado juveniles with approximately 10g were
818 acquired from a commercial hatchery in Paraná State, Brazil. Also, to test if invasive fish
819 species in the area could be the transmitter agent of edwardsiellosis, 60 Nile tilapia
820 (*Oreochromis niloticus*) juveniles and 60 African walking catfish (*Clarias gariepinus*),
821 both with approximately 5g, were acquired from the same hatchery. Those species were
822 introduced in the area for aquaculture purposes and escaped to the natural basins of the
823 area, affecting the ecosystem of the region (Birck et al., 2019, Vitule et al., 2006).
824 The fish were distributed and kept in 40L aquariums during a 15-day
825 acclimatization period before the experimental challenge. Temperature was kept at 26ºC,
826 and oxygen > 5 $mg \cdot mL^{-1}$, measured using an AK87 multiparameter instrument (Akso
827 Measuring Instruments, São Leopoldo, RS, Brazil); whereas pH was kept at 7.2 and total
828 ammonia <0.4 $mg \cdot L^{-1}$ which were evaluated using commercial aquarium kits (Alcon Pet,
829 Camboriú, SC, Brazil), with a 12h day-night photoperiod. The animals were fed four
830 times a day until apparent satiety with commercial extruded feed proper for each of the
831 species.
832 To evaluate the potential of the isolated bacteria to reproduce the disease in the
833 pintados, an experimental challenge was designed with infections via intraperitoneal
834 injection, an immersion bath with a bacterial solution, and cohabitation with infected fish
835 from other species. The intraperitoneal, immersion and negative control groups where
836 executed in duplicate, whereas the cohabitation challenge had no replicates. Therefore,

the experimental groups were stated here, and in Table 1 for better visualization, as follows:

$IP_{Pintado}$: 8 pintados experimentally challenged via intraperitoneal injection;
$IM_{Pintado}$: 8 pintados experimentally challenged via immersion bath;
$N_{Pintado}$: 8 pintados not challenged (negative control);
$IP_{African}$: 8 African walking catfish challenged via intraperitoneal injection;
$IM_{African}$: 8 African walking catfish challenged via immersion bath;
$N_{African}$: 8 African walking catfish not challenged (negative control);
$IP_{Tilapia}$: 8 tilapias experimentally challenged via intraperitoneal injection;
$IM_{Tilapia}$: 8 tilapias experimentally challenged via immersion bath;
$N_{Tilapia}$: 8 tilapias not challenged (negative control);
$CH_{Pintado}$: 4 pintados experimentally challenged via intraperitoneal injection in the same aquarium as 4 tilapias and 4 African walking catfish not challenged;
$CH_{African}$: 4 African walking catfish experimentally challenged via intraperitoneal injection in the same aquarium as 4 tilapias and 4 pintados not challenged;
$CH_{Tilapia}$: 4 tilapias experimentally challenged via intraperitoneal injection in the same aquarium as 4 African walking catfish and 4 pintados not challenged;

The fishes in the cohabitation aquariums ($CH_{Pintado}$, $CH_{African}$, and $CH_{Tilapia}$) were kept together from the beginning of the acclimation to verify possible cannibalism or behavioral changes, which were not detected.

2.4. Experimental challenge

To observe the mortality rate, clinical signs, and bacterial re-isolation, the outbreak-isolated bacterium, named BEP194 (Genbank access number MW667584), was cultivated in sheep blood agar (5%) for 48h and diluted in saline solution until an apparent 0.5 Mcfarland scale (a 1.93e8 $CFU \cdot mL^{-1}$ dose, value obtained with posterior bacterial count), resulting in the D0 inoculum.

The water in the aquariums of the immersion bath challenge was lowered to 10L (1/4 of its total capacity), and 10 mL of the D0 inoculum were added to them (resulting in a 1.93e5 $CFU \cdot mL^{-1}$ dose). The aquariums were kept at this water level for three hours, followed by a 100% water change.

For the intraperitoneal infection, the D0 inoculum has diluted, with saline solution, till a 1.93e5 $CFU \cdot mL^{-1}$ inoculum was obtained. The fish were anesthetized in a bath of 0.03$mg \cdot mL^{-1}$ benzocaine solution until apparent loss of consciousness, and then 0.1mL of the intraperitoneal solution was injected (resulting in 1.93e4 CFU·fish dose). Negative control groups were inoculated with sterile saline solution to simulate the stress of an infection.

The fish separated for the cohabitation test (4 pintados for the CHP group, 4 African walking catfish for the CHA group, and 4 tilapias for the CHT group) were submitted to the same procedure as the intraperitoneal infection group, but after being inoculated with the pathogen, were kept for 30 min in three separate aquariums before being placed in their respective aquarium, to avoid inoculum droplets to contaminate the other fishes in the cohabitation aquariums.

2.5. Histopathological sampling

Five and 10 days after the experimental challenge, one fish of each species from the intraperitoneal infection was euthanized with a benzocaine overdose, and the brain

and liver were streaked in sheep blood agar (5%). These organs, along with the eyes, were also collected for histopathological analysis. Fish from the immersion infection were sampled at the days seven and 14 and submitted to identical procedure. Also, four weeks after the start of the experimental challenge, all the surviving fish were euthanized and had their brain and eye collected. The fixed tissue samples were embedded in paraffin at 60 °C to 5µm thick cross-sections and stained with hematoxylin-eosin. The slides (Entellan, Darmstadt, Germany) were mounted for microscopic evaluation.

2.6. Histopathological analysis

For the histopathology analysis a scale was used to classify each analyzed organ: eye scores used a range to 0 (insignificant inflammatory lesions/ non-significant findings) to 3 (prominent inflammatory lesions/ involving iris and/or choroidal gland and/or periocular/retrobulbar tissues)**,** the liver score was based on the glycogen accumulation in the hepatocytes, adapted from Favero et al. (2021), with + being accentuated glycogen accumulation, ++ moderate glycogen accumulation and +++ mild glycogen accumulation concomitant or not with lipid vacuoles. For the analysis of the brain, a more complex injury score was created to translate the tissue damage as a whole, considering circulatory lesions (0 to 3 points, according to the alteration intensity) and inflammatory lesions (attributing 2 points to discrete lesions, 4 to moderate lesions and 6 to accentuated lesions) that combined, resulted in a definitive 0 to 3 score according to the attained points (0-2 points received a 0 score, 3-4 a 1, 5-6 a 2, and 7-9 the 3 points score).

3. RESULTS

A Gram-negative, oxidase negative and long-rod was isolated from the brain, kidney, and liver of all the six fishes. The Bactray I commercial test showed presence of

911 Lysine decarboxylase and Ornithine decarboxylase, resulting in the 4100 code
912 (*Edwardsiella hoshinae*) with 62.68% probability. All the conventional biochemical
913 analysis, except maltose fermentation, were negative and an A/A TSI with gas production
914 (Table 2); the bacteria showed sensitivity to all the antibiotics tested. The DNA
915 sequencing analyzed in the MEGA X software identified our strain in the *E. ictaluri* clade
916 (Figure 1)

917 Erratic swimming, anorexia, discrete ascites, and hemorrhagic petechial were
918 displayed by infected pintados (three days after infection for the intraperitoneal challenge,
919 five days for the immersion challenge, 10 days for the cohabitation with infected African
920 walking catfish, and 12 days for the cohabitation with infected Nile tilapia). The pintados
921 also showed the characteristic behavior of standing still in the water column, hours before
922 death and, nine days after the infection, the pintados challenged via immersion presented
923 a "pimple-like lesion", as the one described by Hawke et al. (1998) for ESC. Nile tilapia
924 infected intraperitoneally showed ascites (25%). None of the African walking catfish
925 showed visible clinical and/or behavior alterations.

926 Mortality was 100% for pintados challenged intraperitoneally (15 days after the
927 infection) and via immersion (12 days after the infection). Nile tilapia showed 71.42%
928 mortality in the intraperitoneal challenge and 35.71% in the immersion challenge (during
929 the four weeks of the trial). African walking catfishes in both, immersion and
930 intraperitoneal groups, showed no mortality during the trial.

931 In the cohabitation aquariums, half of the pintados died in the $CH_{Pintado}$ group and
932 $CH_{Tilapia}$ groups, and all of them in the $CH_{African}$ group. The $CH_{African}$ group also showed
933 100% mortality for the African walking catfish, whereas in the $CH_{Tilapia}$ group three of
934 the four Nile tilapia infected died, more, one tilapia within the $CH_{Pintado}$ died 25 days after

935 the infection. The mortality data can be seen in more detail in Table 3 (experimental
936 infection) and in Table 4 (cohabitation infection).

937 Brain culture of all dead fishes resulted in a Gram negative, oxidase negative rod,
938 similar to the one isolated. For the sampled fishes, all collected pintados produced the
939 same bacteria for the brain and liver. Nile tilapia collected from the intraperitoneal
940 challenged had growth from the brain in the five days' sample and from the brain and
941 liver in the 10 days' sample. The African walking catfish showed similar colonies only
942 in the brain of the 10 days' sample. All the sampled pintados and the intraperitoneally
943 challenged tilapias exhibited liver congestion at macroscopic analysis.

944 At the end of the experiment, the surviving Nile tilapia from the CHT group, two
945 from the 11 survivors of the immersion challenge, and one from the five survivors of the
946 intraperitoneal challenge presented the bacteria in brain colonies, as well as the surviving
947 pintados from the CHP group. No bacteria were recovered from the surviving African
948 Walking Catfish brains.

949 Nile tilapias exhibited a large number of inflammatory infiltrate cells (especially
950 eosinophils) in the eye slides, on iris, choroidal gland, posterior chamber, periocular
951 connective tissue, and retrobulbar adipose tissue (Figure 2); whereas other species
952 showed no significant alterations in the eye. Brain of all species displayed varying degrees
953 of congestion (diffuse in Nile tilapia and pintado, multifocal for African walking catfish)
954 and edema perivascular, perineuronal and periventricular, the alterations were mostly
955 insignificant in the initial sampling during the trial but, for the surviving animals
956 (especially Nile tilapias), were more prominent (Figure 3). The pintados liver presented
957 necrosis and congestion, with low glycogen accumulation and some mononuclear cells
958 after seven days of infection (Figure 4), while, in the African walking catfish no
959 difference was observed and intraperitoneally infected Nile tilapia showed a decrease in

960 the glycogen deposits (Figure 5), with no other alteration. All the histopathology analysis
961 results are displayed in Tables 5 and 6.
962
963 **4. DISCUSSION**
964 Although it has initially considered a disease of the Ictaluridae fish family in the
965 USA (Hawke et al., 1981), *E. ictaluri* showed its potential to infect and cause disease in
966 other Siluriformes families (Crumlish et al., 2002, Geng et al., 2013, Kasornchandra et
967 al., 1987, Kelly et al., 2018, Ye et al., 2009), and more, in other fish orders such as
968 Cichliformes and Cypriniformes (Dong et al., 2019, Hawke et al., 2013, Soto et al., 2012).
969 In the present study, we showed that the family Pimelodidae of Latin American catfish,
970 represented here by the *P. corruscans*, can be included in the list of species naturally
971 affected by *E. ictaluri*. Other data from our laboratory, characterizes *Edwardsiella*
972 isolates, without H_2S production, in pintado farms in Paraná State since 2016; these cases
973 were not considered owing to low mortality and limited clinical signals reported, but
974 could show that *E. ictaluri* has been circulating in the region for longer than we recognize.
975 There are some limitations of using only one gene as sequencing target for the
976 *Edwardsiella* genus, due to its high resemblance between species (Reichley et al., 2017).
977 Therefore, we designed primers to elaborate a species-specific PCR for *Edwardsiella*,
978 where our strain attested as *E. ictaluri* (unpublished data). The result of the 16S
979 sequencing, together with the microbiological identification, similar to the one described
980 by Plumb & Vinitnantharat, 1989, and our in-development specific-PCR is enough to
981 correctly classify this bacterium.
982 The high mortality and clinical signs resemble those of the ESC (Hawke et al.,
983 1998), in both, the acute and chronic forms. The 100% mortality with a high-dose
984 infection also is related in other catfish species, such as *I. punctatus* (Plumb & Sanchez,

1983) and *Pelteobagrus fulvidraco* (Ye et al., 2009), in laboratory infection. Unexpectedly, two of the infected pintados survived (in the $CH_{Pintado}$ aquarium), but it's unclear why, since the bacterium has been isolated from their brain after four weeks of the intraperitoneal infection and moderate diffuse congestion was observed in the histopathology; this can be part of an individual resistance of those fish.

Infected Nile tilapia showed mortality similar to that demonstrated in other experimental infections (Plumb & Sanchez, 1983, Soto et al., 2012), however, African walking catfish showed only casualties in the $CH_{African}$ group, this could be due to the stress generated for the competition with the other fish species for the feed, since it has observed that Nile tilapia often ate the feed designated for other species in the cohabitation aquariums or the presence of the highly susceptible pintado, acting as a multiplier for the bacteria. Suanyuk et al. (2014), reported intense mortality in *C. macrocephalus x C. gariepinus* hybrids, both in laboratory and naturally infected, in contrast, here we show a high resistance of *C. gariepinus* front an experimental challenge with *E. ictaluri*, even though the pathogen was isolated from the brain of an intraperitoneally challenged fish ten days after the infection, and minor alterations were detected in their nervous system histopathology analysis of some surviving fish. This can be explicated with the theorized by Griffin et al. (2020), that hybrid fish are more susceptible to edwardsiellosis.

Histopathology analysis of eye and nervous system shown varying levels of inflammatory cells, with Nile tilapia presenting the most congested ones. Only tilapia presented inflammatory infiltrate in the eye; it's important to note that the pintados received for diagnosis had their eyes stroked in blood agar, with no resulting bacterium growth, this may indicate a lack of tropism in *E. ictaluri* for the ocular tissue of pintados also, no exophthalmia was noted in them during the experimental challenge, as opposed

to other diagnosed species such as *I. punctatus* (Hawke et al., 1998) and *P. fulvidraco* (Ye et al., 2009). Nervous system alterations were more present on the surviving animals, suggesting that the bacteria take more time to install itself in the brain, whereas in the liver, necrosis and glycogen depletion were already present five days after the infection. The cells and alterations found were similar as those described in the previous studies with *I. punctatus* (Miyazaki & Kaige, 1985, Miyazaki & Plumb, 1985).

Although Suanyuk et al. (2014) reported several findings on *Clarias* hybrid histopathology after two weeks of infection, our samples of *C. gariepinus* showed only minor multifocal congestion of the nervous system (after 28 days on some of the fish from all the infections types) and a moderate depletion of glycogen on the intraperitoneally infected, at the 10 days sampling, in contrast with the vacuolation and necrosis showed by the authors. Again, resistance differed from the pure species to the hybrid to *E. ictaluri* infections. A comparison of the hybrid, *C. gariepinus* and *C. macrocephalus* infected by the same bacterial strain can help us understand better this relation.

The cohabitation challenge demonstrated that pintados could acquire the disease from both, infected Nile tilapia and African walking catfish. All the pintados living with infected African Walking Catfish and half of the ones living with infected tilapias died, whereas the other half showed moderate diffuse congestion in the brain. Considerable escapement from farms occurs in Brazilian aquaculture, resulting in the introduction of exotic fish species in natural basins (Azevedo-Santos et al., 2011, Weyl et al., 2016). Cage aquaculture farms located in rivers, often see several native fish in their vicinity attracted by the leftover food, possible providing hotspots for the transmission of infectious diseases between endemic and cultured fish. Escaping Nile tilapia, are also prey for some indigenous *P. corruscans* (Birck et al., 2019); thus, establishing another possible infection

route of *E. ictaluri* in environmental conditions, since the cannibalism is related as one of the most prominent causes of ESC transmission in channel catfish (Xu et al., 2013).

In summary, this study concluded that *E. ictaluri* can cause ESC in the native fish *P. corruscans*, with high-mortalities outbreaks in seasons similar to the observed in *I. punctatus* farms (late summer to autumn). Invasive species such as Nile tilapia, and possibly, African walking catfish, can act as reservoirs for the bacterium, and transmit the disease to native Siluriformes. Furthermore, this is the first study that describes the presence of *E. ictaluri* in Brazilian waters, creating the need to monitor its presence and risk in other native species.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHOR CONTRIBUTION

ARC, DDG and UPP designed the study; DCA isolated and characterized the bacteria, ARC and RTC conducted the *in-vivo* experiment; KMSES and GWS realized the

histopathological analysis; GWS, DDG and UPP read and approved the final manuscript; all authors helped analyzing and interpreting the results.

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Table 1 – Design of the experimental challenge with BEP 194.

Group Name	Fish Species	Number of Animals	Number of repetitions	Infection Route
$IP_{Pintado}$	Pintado	8	2	Intraperitoneal
$IM_{Pintado}$	Pintado	8	2	Immersion
$N_{Pintado}$	Pintado	8	2	No Infection[a]
$IP_{African}$	African catfish	8	2	Intraperitoneal
$IM_{African}$	African catfish	8	2	Immersion
$N_{African}$	African catfish	8	2	No Infection[a]
$IP_{Tilapia}$	Nile tilapia	8	2	Intraperitoneal
$IM_{Tilapia}$	Nile tilapia	8	2	Immersion
$N_{Tilapia}$	Nile tilapia	8	2	No Infection[a]
$CH_{Pintado}$	All three	12 (4 of each species)	1	Cohabitation with infected pintados
$CH_{African}$	All three	12 (4 of each species)	1	Cohabitation with infected African catfish
$CH_{Tilapia}$	All three	12 (4 of each species)	1	Cohabitation with infected tilapia

[a] Intraperitoneally inoculated with sterile saline solution

Table 2 – Characterization of the bacterium BEP194 – *Edwardsiella ictaluri* isolated from the brain of a *Pseudoplatystoma corruscans.*

Test	Result
Gram stain	Gram Negative
Morphology	Long Rods
Presence of catalase	Positive
Presence of oxidase	Negative
Motility at 25ºC	Positive
Motility at 30ºC	Negative
Triple sugar iron	A/A with gas
Production of H_2S	Negative
Urease	Negative
Esculin	Negative
Citrate	Negative
Indole	Negative
Methyl red	Negative
Voges-Proskauer	Negative
Fermentation of lactose	Negative
Fermentation of sorbitol	Negative
Fermentation of trehalose	Negative
Fermentation of maltose	Positive

Table 3 – Absolute cumulative mortality of fish experimentally challenged with BEP194.

Days after infection	IPP (*n=16*)	IMP (*n=16*)	IPA (*n=16*)	IMA (*n=16*)	IPT (*n=16*)	IMT (*n=16*)
0	0	0	0	0	0	0
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	2 (14.28%) + 1 HPT[a]	0	0 + 1 HPT[a]	0	0 + 1 HPT[a]	0
6	3 (21.23%)	0	0	0	0	0
7	6 (42.85%)	0 + 1 HPT[a]	0	0 + 1 HPT[a]	0 + 1 HPT[a]	0
8	8 (57.14%)	0	0	0	0	0
9	11 (78.57%)	1 (7.14%)	0	0	3 (21.23%)	0
10	12 (85.71%) + 1 HPT[a]	10 (71.43%)	0 + 1 HPT[a]	0	3 + 1 HPT[a]	0
11	12	14 (100%)	0	0	3	0
12	12	-	0	0	3	0
13	12	-	0	0	3	0
14	12	-	0	0 + 1 HPT[a]	4 (28.57%)	0 + 1 HPT[a]
15	14 (100%)	-	0	0	5 (35.71%)	0
16	-	-	0	0	5	1 (7.14%)
17	-	-	0	0	5	1
18	-	-	0	0	8 (57.14%)	1
19	-	-	0	0	8	1
20	-	-	0	0	8	2 (14.29%)
21	-	-	0	0	8	2
22	-	-	0	0	10 (71.43%)	2
23	-	-	0	0	10	2
24	-	-	0	0	10	3 (21.43%)
25	-	-	0	0	10	5 (35.71%)
26	-	-	0	0	10	5
27	-	-	0	0	10	5
28	-	-	0	0	10	5
TOTAL	**100%**	**100%**	**0**	**0**	**71.43%**	**35.71%**

IPP: *P. corruscans* challenged intraperitoneally; IMP: *P. corruscans* challenged via immersion bath; IPA: *C. gariepinus* challenged intraperitoneally; IMA: *C. gariepinus* challenged via immersion bath; IPT: *O. niloticus* challenged intraperitoneally; IMT: *O. niloticus* challenged via immersion bath.

[a] At two predetermined moments, one fish of each experimental group was euthanized for histopathological samples. Those fish were not counted for the absolute percentage.

Table 4 – Absolute cumulative mortality of fish living in aquariums with fish experimentally challenged with BEP194 (cohabitation infection).

Days after infection	$CH_{Pintados}$ (Infected pintados)			$CH_{African}$ (Infected African catfish)			$CH_{Tilapia}$ (Infected Nile tilapia)		
	Pintados (*n=4*)	African catfish (*n=4*)	Nile tilapia (*n=4*)	Pintados (*n=4*)	African catfish (*n=4*)	Nile tilapia (*n=4*)	Pintados (*n=4*)	African catfish (*n=4*)	Nile tilapia (*n=4*)
0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	1 (25%)
6	0	0	0	0	0	0	0	0	1
7	0	0	0	0	0	0	0	0	1
8	1 (25%)	0	0	0	0	0	0	0	1
9	1	0	0	0	0	0	0	0	1
10	1	0	0	0	0	0	0	0	3 (75%)
11	1	0	0	0	0	0	0	0	3
12	1	0	0	0	0	0	0	0	3
13	1	0	0	0	2 (50%)	0	0	0	3
14	2 (50%)	0	0	0	2	0	0	0	3
15	2	0	0	2 (50%)	4 (100%)	0	1 (25%)	0	3
16	2	0	0	2	-	0	1	0	3
17	2	0	0	4 (100%)	-	0	1	0	3
18	2	0	0	-	-	0	1	0	3
19	2	0	0	-	-	0	1	0	3
20	2	0	0	-	-	0	1	0	3
21	2	0	0	-	-	0	1	0	3
22	2	0	0	-	-	0	2 (50%)	0	3
23	2	0	0	-	-	0	2	0	3
24	2	0	0	-	-	0	2	0	3
25	2	0	0	-	-	0	2	0	3
26	2	0	1 (25%)	-	-	0	2	0	3
27	2	0	1	-	-	0	2	0	3
28	2	0	1	-	-	0	2	0	3
TOTAL	**2 (50%)**	**0**	**1 (25%)**	**4 (100%)**	**4 (100%)**	**0**	**2 (50%)**	**0**	**3 (75%)**

1214 **Table 5** – Histopathological results of fish sampled during the experimental challenge with BEP 194

Fish	Infection	Time after infection (days)	Eye Score (inflammatory infiltrate) 0 to 3	Nervous System Score (inflammation and circulatory damage) 0 to 3	Liver Score (glycogen depletion) + / ++/ +++
Nile tilapia	Intraperitoneal	5	0	0	++ with congestion
Nile tilapia	Immersion	7	0	0	+
Nile tilapia	Intraperitoneal	10	1 (moderate presence of eosinophils in the choroidal gland and periocular connective tissue)	0 (A few multifocal congestions and eosinophil presence)	++ with congestion
Nile tilapia	Immersion	14	1 (multifocal eosinophil presence in the choroidal gland)	0	+
African Catfish	Intraperitoneal	5	0	0	+
African Catfish	Immersion	7	0	0	+
African Catfish	Intraperitoneal	10	0	0	+++
African Catfish	Immersion	14	0	0	+

Pintado	Intraperitoneal	5	0	0	+++ with multifocal hepatocytes necrosis
Pintado	Immersion	7	0	0 (Starting of diffuse congestion and edema)	++ with focal necrosis
Pintado	Intraperitoneal	10	0	0 (Starting of diffuse congestion and edema)	++ with moderate congestion and presence of mononuclear cells
Pintado	Immersion	14[a]	Not applicable	Not applicable	Not applicable

[a] There was no pintado alive for this sampling.

1217 **Table 6** – Histopathological results of fish that survived 28 days in the experimental challenge with BEP 194

Experimental Group	Fish Species	Infection type	Eye Score (Inflammatory infiltrate) 0 to 3	Nervous System Score (Inflammation and circulatory damage) 0 to 3
$IP_{Tilapia}1$	Nile tilapia	Intraperitoneal	1 (Moderate presence of eosinophils, lymphocytes and melanomacrophages in the choroidal gland and periocular connective tissue)	2 (Accented diffuse congestion and perivascular and perineuronal edema, mild presence of eosinophils periventricular)
$IP_{Tilapia}2$	Nile tilapia	Intraperitoneal	2 (Moderate presence of eosinophils, lymphocytes and melanomacrophages in the choroidal gland and periocular connective tissue)	2 (Moderate diffuse congestion and perivascular and perineuronal edema, eosinophilic meningitis and mild presence in the superficial cortex)
$IM_{Tilapia}1$	Nile tilapia	Immersion	2 (Moderate presence of eosinophils, lymphocytes and melanomacrophages in the choroidal gland, mild eosinophils in the retrobulbar adipose tissue)	1 (Congestion in the leptomeninges and perivascular and perineuronal edema)
$IM_{Tilapia}2$	Nile tilapia	Immersion	1 (Mild presence of eosinophils, lymphocytes and melanomacrophages in the choroidal gland)	0 (Moderate diffuse congestion and perivascular and perineuronal edema)
$IP_{African}1$	African Catfish	Intraperitoneal	0	0
$IP_{African}2$	African Catfish	Intraperitoneal	0	0 (Mild multifocal congestion and perivascular and perineuronal edema)
$IM_{African}1$	African Catfish	Immersion	0	0

$IM_{African}2$	African Catfish	Immersion	0	0 (Mild multifocal congestion and perivascular and perineuronal edema)
$CH_{Pintado}$	Pintado	Intraperitoneal	0	0 (Moderate diffuse congestion and perivascular and perineuronal edema)
$CH_{Pintado}$	Nile tilapia	Cohabitation	2 (Moderate presence of eosinophils, and melanomacrophages in the choroidal gland and periocular connective tissue, accented eosinophils in the retrobulbar adipose tissue)	2 (Moderate diffuse congestion and perivascular and perineuronal edema, eosinophilic meningitis and mild periventricular presence)
$CH_{Pintado}$	African Catfish	Cohabitation	0	0 (Mild multifocal congestion and perivascular and perineuronal edema)
$CH_{Tilapia}$	Pintado	Cohabitation	0	0 (Moderate diffuse congestion and perivascular and perineuronal edema)
$CH_{Tilapia}$	Nile tilapia	Intraperitoneal	2 (Accented presence of eosinophils with mild presence of melanomacrophages and lymphocytes in the choroidal gland and retrobulbar adipose tissue)	1 (Moderate diffuse congestion and perivascular and perineuronal edema, mild eosinophilic meningitis)
$CH_{Tilapia}$	African Catfish	Cohabitation	0	0
$CH_{African}$	Nile tilapia	Cohabitation	2 (Moderate presence of eosinophils, and melanomacrophages in the choroidal gland and periocular connective tissue, mild eosinophils in the retrobulbar adipose tissue)	1 (Mild diffuse congestion and moderate perivascular and perineuronal edema, eosinophilic meningitis and discrete periventricular presence)

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Figure 1: Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Kimura 2-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0,1102)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 *Edwardsiella* nucleotide sequences of the 16S rRNA gene, and one *Hafnia alvei* as outgroup. There were a total of 1024 positions in the final dataset. Our strain, BEP 194 was classified as *E. ictaluri*.

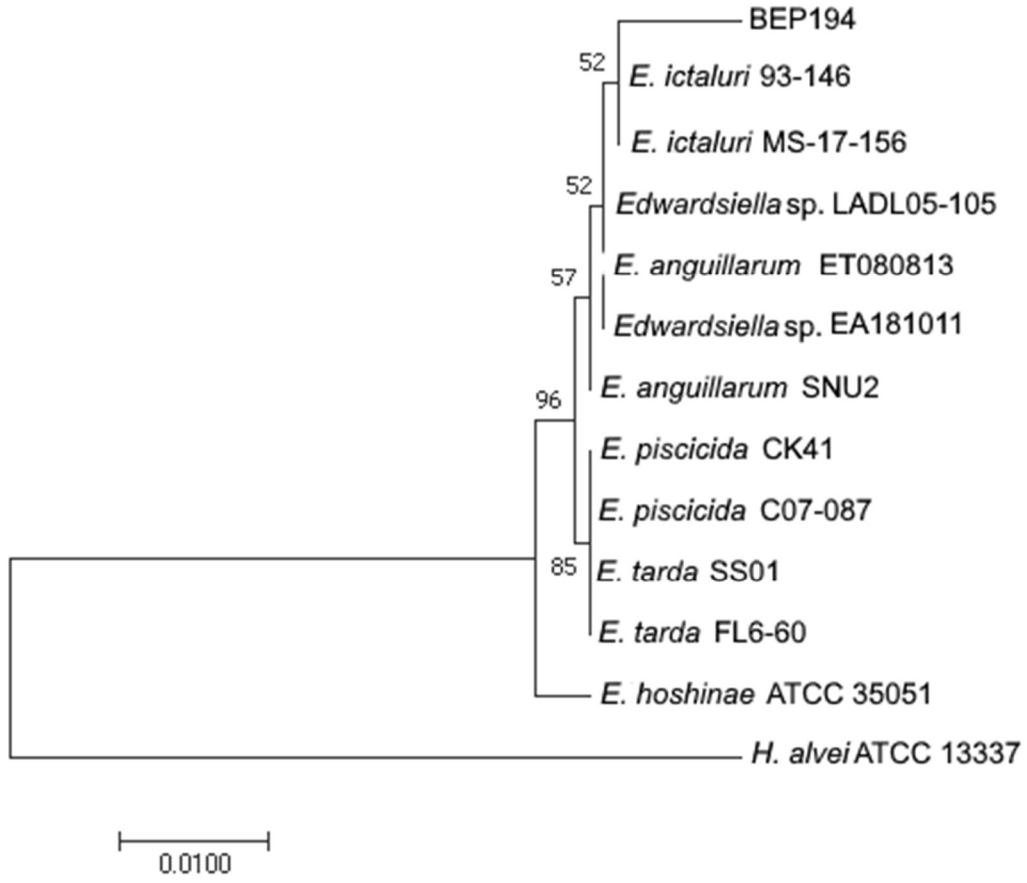


Figure 2: Digital microscopy of the eye of a Nile tilapia that survived a cohabitation challenge with pintados infected with BEP 194 showing inflammatory cells. A: Choroidal gland. Engorgement of blood vessels with erythrocytes characterizing vascular congestion (open arrows) and groups of eosinophils scattered throughout the tissue (arrows); B: Retrobulbar connective tissue. Eosinophils (arrows) spread out in the connective tissue, mainly from the perivascular region (open arrow).

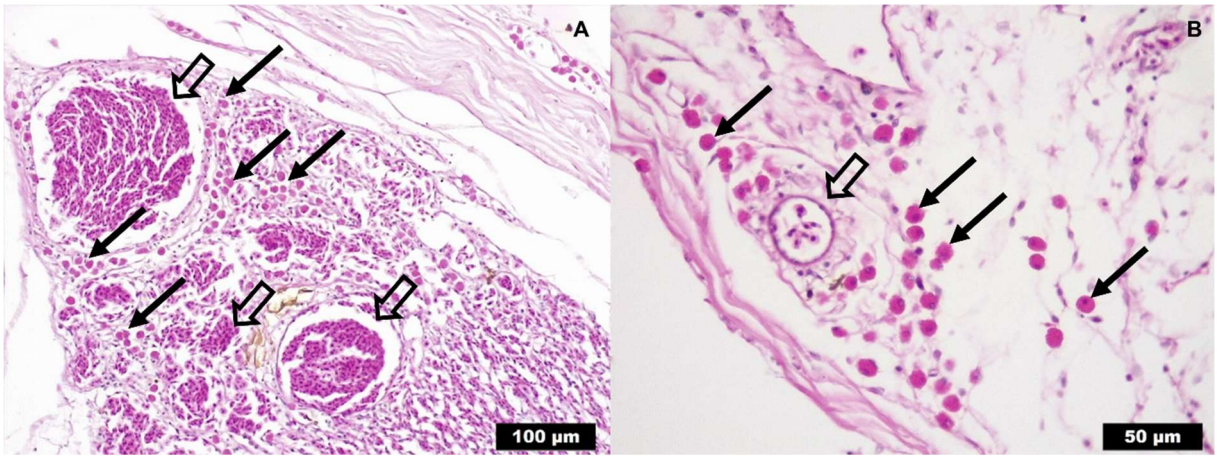


Figure 3: Digital microscopy of the brain of a Nile tilapia that survived for 28 days after an intraperitoneal experimental infection with BEP 194. A: Few eosinophils lining the periventricular region (arrows) and a mild perivascular edema (open arrow). B: Moderated leptomeningeal thickening by eosinophils (arrows).

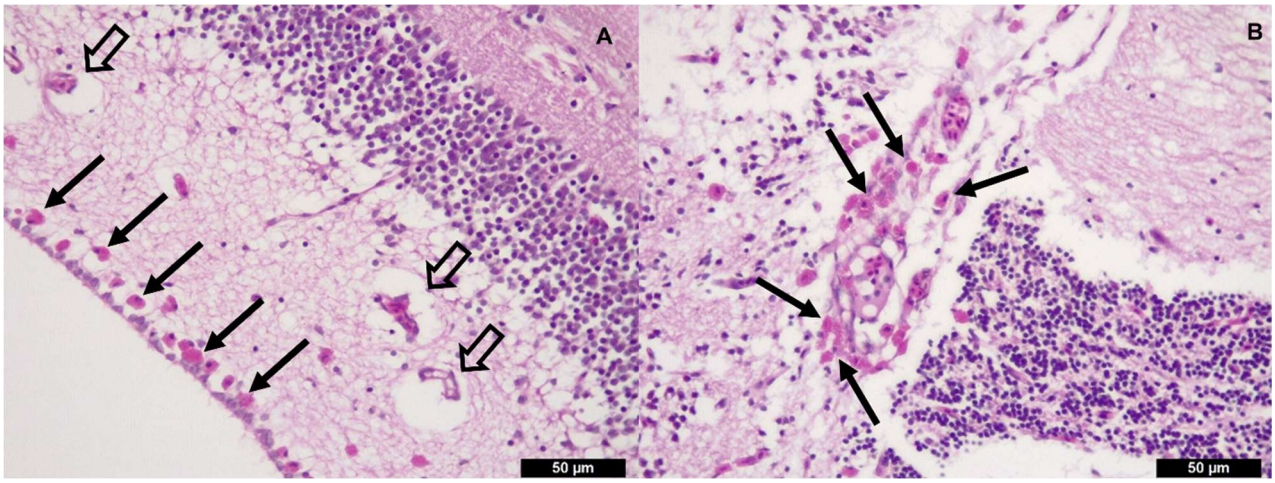


Figure 4: Digital microscopy of liver of a euthanized pintado. A: In the left side of the picture there is a small necrosis area where there are many hepatocytes with pyknotic (hyperchromatic and shrunken) nuclei (circles), while in the right side there are mainly normal nuclei in the hepatocytes (arrows). B: Moderate perivascular mononuclear inflammatory infiltrate (arrow).

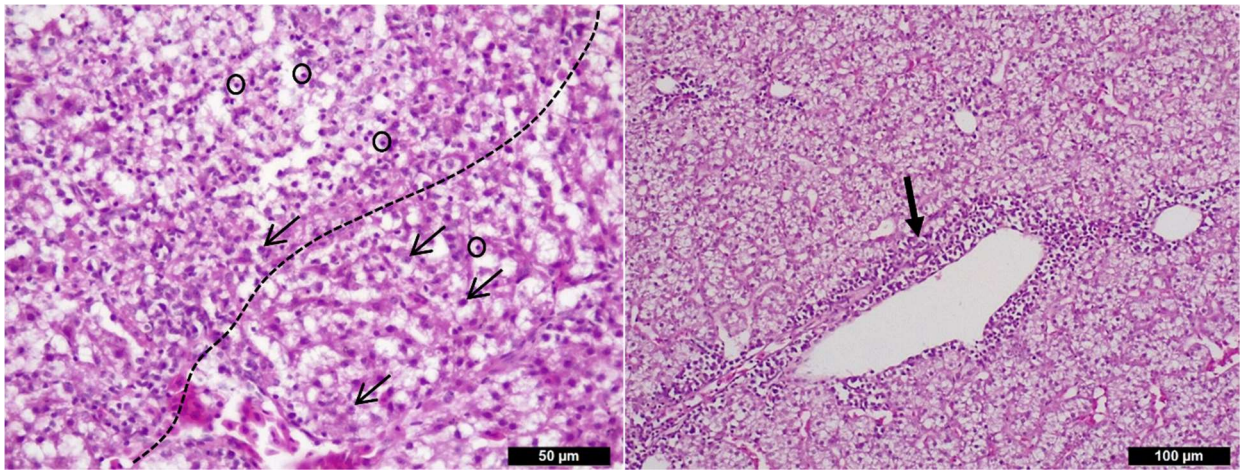


Figure 5: Digital microscopy of liver of fishes infected with BEP 194 (E. ictaluri). A: Score (+) accentuated and diffuse glycogen accumulation in an African Walking Catfish, 14d after an immersion challenge; B: Score (++) moderate and diffuse glycogen accumulation in a Nile tilapia, 5d after an intraperitoneal challenge; C: Score (+++) Mild and diffuse glycogen accumulation (arrows) and rare hepatocytes containing lipids (open arrow) in a pintado, 5d after an intraperitoneal challenge.

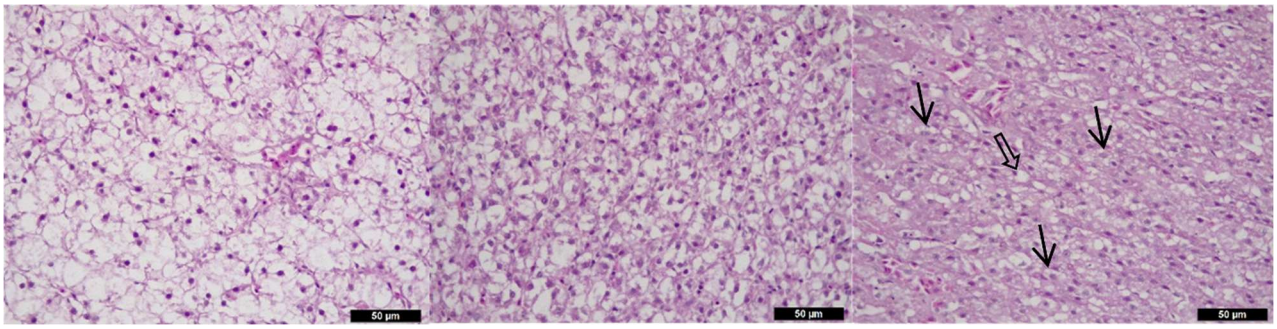
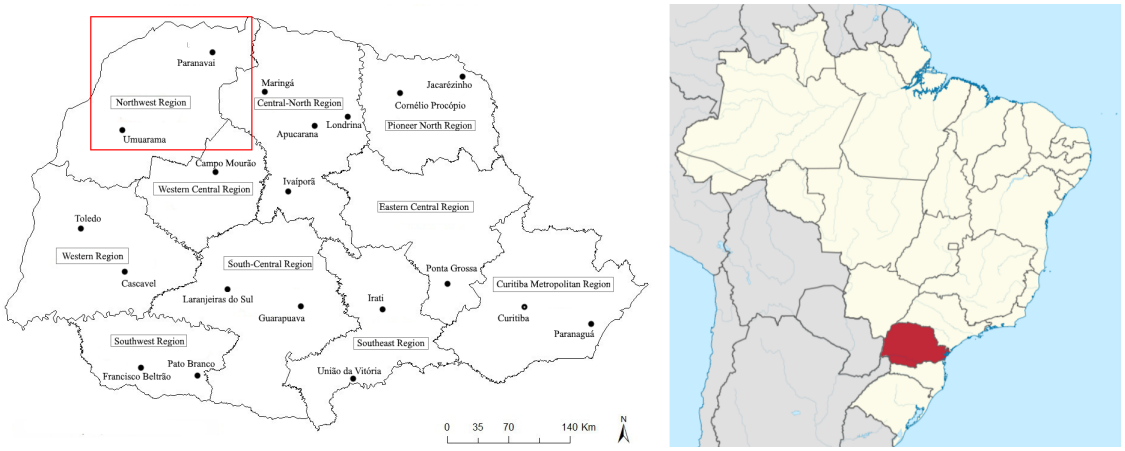


Figure S1: Paraná State and Brazil map, outlining the region where an outbreak of *Edwardsiella ictaluri* was detected.



7. CONSIDERAÇÕES FINAIS

Com este trabalho, conseguimos através do experimento proposto, confirmar que o pintado pode ser acometido por doença causada por *E. ictaluri*, adicionando esta espécie Brasileira na lista de espécies globalmente afetadas por esta bactéria. Foi possível confirmar que a bactéria isolada na piscicultura no noroeste do estado do Paraná se tratava de uma *E. ictaluri*, indicando que está bactéria alóctone já circula na região.

A partir do experimento realizado, observamos que os sinais clínicos apresentados pelos pintados são parecidos com os apresentados pelo bagre do canal acometido por ESC. Os animais apresentaram taxa de mortalidade elevada em ambas as vias de infecção, imersão e inoculação intraperitoneal, indicando que ambas as vias podem ser utilizadas em projetos futuros. A coabitação indicou que houve transmissão entre as três espécies (tilápia-do-Nilo, bagre Africano e pintado), com os animais apresentando sinais clínicos, e alterações histopatológicas.

Mais estudos se fazem necessário quanto ao potencial patogênico de *E. ictaluri* em outras espécies brasileiras, especialmente membros da ordem Siluriforme importantes para a piscicultura nacional, visto a sensibilidade do pintado e a comprovação do patógeno estar circulante no estado do Paraná.