UNIVERSIDADE FEDERAL DE UBERLÂNDIA

THAÍS CARNEIRO SANTOS RODRIGUES

Leptospira spp., *Brucella* spp. e *Toxoplasma gondii* em peixes-boi amazônicos (*Trichechus inunguis*), botos-cor-de-rosa (*Inia geoffrensis*) e tucuxi (*Sotalia fluviatilis*) na Amazônia brasileira

Uberlândia – MG

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Área de Concentração: Saúde Animal

Orientador: Prof. Dr. André Luiz Quagliatto Santos

Coorientadora: Dra. Miriam Marmontel

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RESUMO

Leptospirose, brucelose e toxoplasmose são zoonoses de grande importância em saúde pública e animal, mundialmente distribuídas. Tais doenças são frequentemente estudadas em mamíferos marinhos, mas pouco se sabe sobre sua ocorrência em mamíferos aquáticos amazônicos. O presente estudo foi realizado com o objetivo de avaliar os níveis de anticorpos contra *Leptospira* spp., *Brucella* spp. e *Toxoplasma gondii* e realizar identificação molecular desses patógenos em peixes-boi amazônicos (*Trichechus inunguis*), botos-cor-de-rosa (*Inia geoffrensis*) e tucuxis (*Sotalia fluviatilis*) de vida livre ou em reabilitação na região do Médio-Solimões, Amazônia brasileira. Amostras de soro de peixes-boi amazônicos foram submetidas aos testes de soroaglutinação microscópica, teste do Antígeno Acidificado-tamponado e 2-Mercaptoetanol, e hemaglutinação indireta para detecção de anticorpos contra *Leptospira* spp., *Brucella* spp. e *T. gondii,* respectivamente. Amostras de tecidos e fluidos corporais desses animais foram testados utilizando dois protocolos de reação em cadeia da polimerase (PCR) para detecção de fragmentos de DNA dos patógenos alvo dessa pesquisa. Tais amostras também foram submetidas à cultura bacteriólogica. Entre as amostras de soro testadas, 63% apresentaram anticorpos contra *Leptospira* spp. e o sorovar Patoc foi considerado o sorovar infectante em todas as amostras positivas. A maior parte dos animais apresentou baixos títulos de anticorpos, sugerindo exposição crônica. Três peixes-boi apresentaram aumento significativo dos títulos de anticorpos durante o tempo em que foram avaliados, o que pode indicar infecção ativa por *Leptospira* spp. Não foram detectados anticorpos contra *Brucella* spp. Anticorpos contra *T. gondii* foram encontardos em 35,3% das amostras e um peixe-boi apresentou título de 1:64 que, em humanos, pode indicar infecção recente. Todas as amostras foram negativas para *Leptospira* spp. e *Brucella* spp. por PCR. O DNA genômico de *T. gondii* foi detectado em amostras de coração e cérebro de botos-cor-de-rosa e de um tucuxi, o que representa o primeiro relato da identificação molecular desse protozoário em golfinhos de rio. As bactérias *Enterococcus faecalis* e *Bacillus* spp. foram isoladas de amostras de tecidos e fluidos corporais. O presente estudo traz informações inéditas sobre a presença *Leptospira* spp., *T. gondii*, *Enterococcus faecalis* e *Bacillus* spp. em mamíferos aquáticos amazônicos. O conhecimento dos patógenos prevalentes em peixes-boi da Amazônia, botos-cor-de-rosa e tucuxis é de grande relevância para a conservação dessas espécies e do bioma amazônico.

Palavras-chave: Cetacea. Isolamento. Leptospirose. PCR. Sorologia. Sirenia. Toxoplasmose. Zoonose.

ABSTRACT

Leptospirosis, brucellosis and toxoplasmosis are common zoonotic diseases with global distribution that represent severe hazard to humans and animals. Although the occurrence of those diseases is broadly studied in marine mammals, very few information is available on their occurrence in Amazonian aquatic mammals. The present study aimed at investigating exposure to and infection by *Leptospira* spp., *Brucella* spp., and *Toxoplasma gondii* in samples from Amazonian manatees (*Trichechus inunguis*), Amazon river dolphins (*Inia geoffrensis*), and tucuxis (*Sotalia fluviatilis*), free-ranging or undergoing *in-situ* rehabilitation in the Mid-Solimões river region, Western Brazilian Amazon. Serum samples from Amazonian manatees were tested by microscopic agglutination test, Rose Bengal test and 2-Mercaptoethanol test, and indirect haemagglutination test for detection of anti-*Leptospira* spp., anti-*Brucella* spp. and anti-*T. gondii* antibody detection, respectively. Tissue and/or body fluid samples from Amazon river dolphins, a tucuxi, and Amazonian manatees, were subjected to a multiplex polymerase chain reaction (PCR) assay for *Leptospira* spp. and *Brucella* spp. and bacterial culture for attempted isolation. Samples of heart and brain from Amazon river dolphins and a tucuxi were tested by PCR for detection *T. gondii* DNA. A total of 63% of Amazonian manatee sera were reactive to *Leptospira* spp. and serovar Patoc was considered the infecting serovar in all positive samples. Titers were generally low, indicating chronic exposure, but active infection was suggested in three manatees with a four-fold in antibody titers. Anti-*Brucella* spp. antibodies were not detected. Anti-*T. gondii* antibodies were present in 35.3% of the samples and one manatee presented a 1:64 titer, considered as indicative of recent infection in humans. All river dolphin samples were negative for *Leptospira* spp. and *Brucella* spp. by PCR. *T. gondii* DNA was detected in heart and brain samples, which represents the first report of molecular identification of the protozoan parasite in river dolphins. *Enterococcus faecalis* and *Bacillus* spp. were isolated from tissue and body fluid samples, although the clinical significance of these opportunistic pathogens is not clear. The present study brings novel information on the occurrence of *Leptospira* spp. and *T. gondii* in Amazonian aquatic mammals in the Western Brazilian Amazon. Knowledge of the pathogens prevalent in Amazonian manatees, Amazon river dolphins, and tucuxis is of great relevance to species conservation and environmental health.

Keywords: Cetacea. Leptospirosis. Microbial isolation. PCR. Serology. Sirenia. Toxoplasmosis. Zoonosis.

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CAPÍTULO 1 - CONSIDERAÇÕES GERAIS

1.1 Instituto de Desenvolvimento Sustentável Mamirauá e área de estudo

A Amazônia representa a maior floresta tropical do mundo, cobrindo uma área de 6.7 milhões de km^2, e um dos maiores reservatórios de diversidade biológica e cultural da Terra (MALHI et al., 2008; PIMM et al., 2014). O bioma amazônico se estende por nove países da América do Sul e 69% de sua área pertence ao Brasil. No país, a Amazônia abrange os estados do Pará, Amazonas, Maranhão, Goiás, Mato Grosso, Acre, Amapá, Rondônia e Roraima, totalizando cerca de 4.871.000 km^2 e uma população em torno de vinte milhões de habitantes (INPE, 2004). Esse bioma é ameaçado principalmente pela exploração e desmatamento desordenados e mudanças climáticas (MALHI et al., 2008; FERREIRA et al., 2005).

Criado em 1999, o Instituto de Desenvolvimento Sustentável Mamirauá (IDSM) tem como missão "promover pesquisa científica sobre a biodiversidade, manejo e conservação dos recursos naturais da Amazônia de forma participativa e sustentável". Trata-se de uma Organização Social fomentada e supervisionada pelo Ministério da Ciência, Tecnologia e Inovação e Comunicações (MCTIC) do Brasil, e atua como uma das unidades de pesquisa do MCTIC. Desde sua criação, o IDSM desenvolve programas de pesquisa, manejo, gestão e assessoria técnica em áreas de proteção da Amazônia brasileira, no Estado do Amazonas (IDSM, 2011).

Buscando a conservação da biodiversidade e melhoria das condições de vida das populações humanas na Amazônia, o IDSM baseia suas atividades no manejo participativo. Dessa forma, as populações locais praticam o uso sustentável dos recursos naturais com assessoria técnica do IDSM, baseada em resultados de estudos científicos. Em Balanço Social do período compreendido entre os anos de 2001 e 2010 (IDSM, 2011), foram apresentados indicadores que sugerem recuperação de populações animais que antes encontravam-se em declínio, assim como melhoria nas condições de vida das populações humanas, em locais de atuação do instituto. Nesse período, a produção científica do IDSM cresceu 212,5%, contribuindo significativamente para o conhecimento geral da Amazônia (IDSM, 2011).

As unidades de conservação, especialmente aquelas de uso sustentável, têm assumido papel fundamental na conservação da biodiversidade na Amazônia (KITAMURA, 2001). Nesse sentido, o IDSM apoia a gestão das Reservas de Desenvolvimento Sustentável Mamirauá (RDSM) e Amanã (RDSA) que, somadas, cobrem uma área de 3.474.000 hectares. A RDSM foi a primeira Reserva de Desenvolvimento Sustentável do país, com área total de 1.124.000 hectares (IDSM, 2010). Sua criação, em 1996, representou importante marco para a conservação no Brasil. É também a maior unidade de conservação em áreas alagadas do Brasil,

e a única do país completamente inserida em área de várzea amazônica. A RDSM é delimitada pelo rio Solimões, rio Japurá e Canal Auati-Paranã e é adjacente à RDSA, situada na margem oposta do rio Japurá, na região do médio curso do rio Solimões (Figura 1). A RDSA foi instituída em 1998 e cobre uma área de cerca de 2.350.000 hectares de florestas de várzea e terra firme, sendo uma das maiores áreas protegidas em floresta tropical na América do Sul (IDSM, 2010; www.mamiraua.org.br).

Figura 1. Mapa do Brasil, com destaque ao estado do Amazonas, mostrando as áreas das Reservas de Desenvolvimento Sustentável Mamirauá e Amanã, rio Negro, rio Amazonas e as cidades de Tefé e Manaus.

Fonte: IDSM, 2011

Adjascente ao Parque Nacional do Jaú e outras unidades de conservação, essas zonas protegidas somam área superior aos territórios de países como Costa Rica e Suíça e representam um dos maiores blocos de área conservada do planeta. Parte desse território é reconhecido pela Convenção de Ramsar das Nações Unidas, que reúne áreas alagadas de importância mundial, compõe a Reserva da Biosfera da Amazônia Central (UNESCO), incluída no Sítio Natural do Patrimônio Mundial (UNESCO), e integra o Corredor Central da Amazônia (UNESCO, 2003; IDSM, 2010; IUCN, 2011).

O acesso às RDSM e RDSA se dá principalmente pela cidade de Tefé, onde se localiza a sede do IDSM. Tefé é o principal centro urbano da região do Médio Solimões, localizada a 516 km de Manaus (IDSM, 2010; RODRIGUES, 2011). A região do Médio Solimões corresponde à região de confluência dos rios Solimões e Japurá, e abrange as áreas dos municípios de Tefé, Alvarães, Uarini, Fonte Boa, Maraã, Juruá e Jutaí, no Estado do Amazonas

(Da COSTA, et al., 2001). É nessa região que o IDSM concentra suas atividades, principalmente nas áreas de várzea, ou floresta inundada, por ser o ecossistema mais pressionado e ameaçado de todo o bioma Amazônia (IDSM, 2017a).

Dois ecossistemas florestais se destacam no bioma amazônico, nos quais as RDSM e RDSA se inserem: a floresta de terra firme e as matas sazonalmente inundadas, que são conhecidas como várzea ou mata de igapó (GAMA et al., 2005) (Figura 2). O ecossistema de várzea amazônica, segundo principal tipo de vegetação na Amazônia, é caracterizado pelo alagamento anual decorrente da variação do nível das águas dos rios e abriga fauna e flora adaptados a essas condições hidrológicas sazonais (HAUGAASEN E PERES, 2005; KALLIOLA et al., 1993). Os alagamentos sazonais dos rios Solimões e Japurá podem causar uma elevação do nível da água de nove a dez metros da estação seca para a cheia, causando a transformação periódica de ambientes terrestres a aquáticos (RAMALHO et al., 2009). Nesse sistema, é possível distinguir as estações do ano baseadas no regime de enchente e cheia, estações com maior média de precipitação mensal, e vazante e seca, caracterizadas por maior amplitude térmica (RAMALHO et al., 2009). Toda a região amazônica é caracterizada por um complexo mosaico de rios, lagos e canais. Durante a cheia, esses corpos d'água se conectam formando grandes espaços abertos em meio à floresta inundada. Já durante o período de seca, quando o nível da água diminui drasticamente, esses lagos se ramificam em canais ou lagos menores e que seguem se ramificando sucessivamente (IDSM, 2010).

Figura 2: Igapó, ou floresta alagada. Foto de Amanda Lelis.

Fonte: Instituto de Desenvolvimento Sustentável Mamirauá.

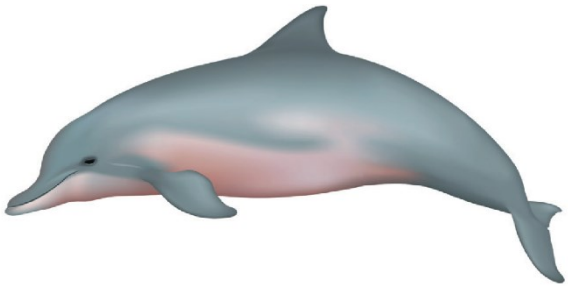
De maneira geral, as florestas de terra firme são áreas de maior biodiversidade em comparação às áreas de várzea, exatamente devido à variação sazonal do nível da água e às alterações ambientais a ela relacionadas (HAUGAASEN E PERES, 2005). Apesar disso, a ocorrência de endemismo na várzea é extremamente alta. As espécies arborícolas e os morcegos constituem maior parte da diversidade, em contraposição aos mamíferos terrestres (IDSM, 2017a). O vasto sistema de rios e corpos d'água oferecem também condições ecológicas para uma fauna aquática extremamente diversa, incluindo mamíferos: peixes-boi amazônicos (*Trichechus inungis*) e botos-cor-de-rosa (*Inia geoffrensis*), espécies endêmicas da bacia amazônica, tucuxis (*Sotalia fluviatilis*), ariranhas (*Pteronura brasiliensis*) e lontras (*Lontra longicaudis*) (BEST, 1984; IDSM, 2010).

1.2 Boto-cor-de-rosa, tucuxi e peixe-boi amazônico

S. fluviatilis (GERVAIS 1853), *I. geoffrensis* (BLAINVILLE, 1817) e *T. inunguis* (NATTERER, 1883) são mamíferos aquáticos amazônicos ainda pouco estudados. *S. fluviatilis*, ou tucuxi, é uma das espécies de cetáceos menos conhecidas (ALVES E ROSA, 2008) e o único delfinídeo exclusivamente fluvial do mundo (SECCHI, 2012). Ocorre nas bacias dos rios Amazonas e Solimões, no Brasil, Peru, Equador e Colômbia, e devido à insuficiência de informações, atualmente está classificada como "Data Deficient", ou "Deficiente em Dados" pela União Internacional para Conservação da Natureza (IUCN) (SECCHI, 2012). A espécie é protegida no Brasil pela Lei Federal de Pesca (Decreto Lei 73.632, de fevereiro de 1967) e pela Lei de Proteção à Fauna (5.197, de março de 1967), sendo classificada como "Quase Ameaçada" na Lista Nacional do Instituto Chico Mendes da Biodiversidade (ICMBio) (BRASIL, 2019).

Os tucuxis têm o dorso de coloração cinza-claro a cinza-azulado e ventre rosado, que são separados por uma linha que vai da abertura da boca até a ponta da cauda. A nadadeira dorsal é triangular e ligeiramente falcada. O rostro é moderadamente fino e possui melão arredondado. O comprimento máximo é de cerca de 152 cm e pode atingir até 53 kg (WÜRSIG, THEWISSEN E KOVACS, 2017; Figura 3).

Figura 3: Ilustração de Uko Gorter representando o tucuxi, *Sotalia fluviatilis*.



Fonte: WÜRSIG, THEWISSEN E KOVACS, 2017

O tucuxi é uma espécie social, que geralmente ocorre em grupos de mais de dois indivíduos, e de atividade fundamentalmente diurna (MAGNUSSON et al., 1980). Habita os rios, lagos e estuários da bacia Amazônica e geralmente não utiliza áreas de igapó nem águas turbulentas e de alta vazão (SILVA E BEST, 1994). As maiores densidades de tucuxis são observadas nas confluências de rios, com maior profundidade e vegetação reduzida (MARTIN et al., 2004; DOS SANTOS et al., 2012) (Figura 4). Durante a seca, os tucuxis se concentram nos canais principais dos rios, devido à maior disponibilidade de presas (DA SILVA E BEST, 1994).

Figura 4: Fotografia de Pedro Nassar, mostrando um tucucxi (*S. fluviatilis*) em seu ambiente natural.

Fonte: Instituto de Desenvolvimento Sustentável Mamirauá.

Os botos-cor-de-rosa, ou botos-vermelhos, são cetáceos do gênero *Inia*. Até recentemente apenas uma espécie era reconhecida no gênero, *I. geoffrensis*. Porém, devido à sua vasta distribuição geográfica, acredita-se que algumas populações tenham se mantido isoladas por milhares de anos e se diferenciado genética e morfologicamente (HOLLATZ et al., 2011). Atualmente é considerada a existência de mais duas espécies de botos-cor-de-rosa: *I. araguaiaensis,* habitando a bacia do rio Araguaia, e *I. boliviensis*, ao longo do rio Madeira, na Bolívia e no Brasil (BANGUERA-HINESTROZA et al., 2002; HRBEK et al., 2014), além de *I. geoffrensis*, na bacia amazônica (REEVES et al., 2011).

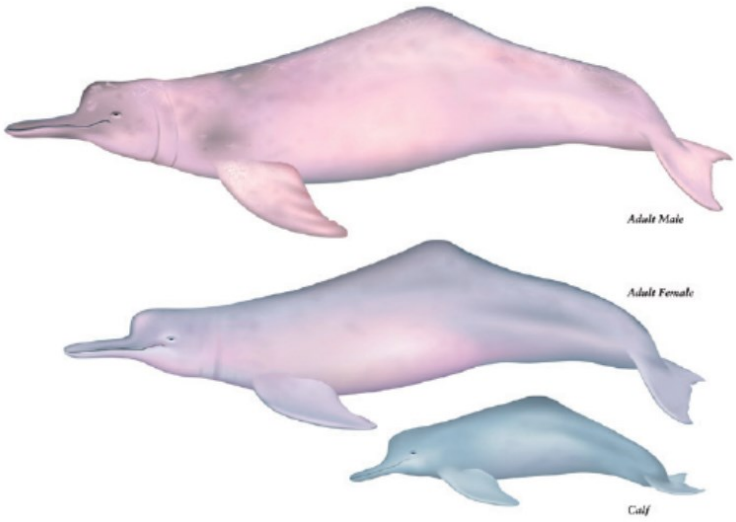
Os botos-cor-de-rosa são os maiores golfinhos de rio existentes, podendo alcançar 255 cm de comprimento e pesar até 153 kg. São sexualmente dimórficos e os machos são maiores e mais pesados que as fêmeas (MARTIN E DA SILVA, 2006). O corpo é extremamente flexível e a cabeça se movimenta em todas as direções devido à ausência de fusão das vértebras cervicais, observada nas demais espécies de golfinhos. As nadadeiras peitorais são amplas e em forma de remo, enquanto a nadadeira dorsal é longa, baixa e em forma de quilha. Essas características anatômicas permitem que os botos naveguem entre os troncos de árvores na floresta alagada, ambiente não utilizado pelos tucuxis. De maneira similar aos tucuxis, os botos-cor-de-rosa preferem águas não turbulentas e de baixa vazão (WÜRSIG, THEWISSEN E KOVACS, 2017; Figura 5).

Figura 5: Fotografia de Marcelo Santana, mostrando um boto-cor-de-rosa (*I. geoffrensis*) em seu ambiente natural.

Fonte: Instituto de Desenvolvimento Sustentável Mamirauá.

Os botos possuem rostro e mandíbula longos e robustos, e olhos pequenos. Possuem dois tipos de dentição, dentes anteriores cônicos e posteriores similares a dentes molares, característica única entre odontocetos. A cor da pele dos botos-cor-de-rosa varia com idade: neonatos geralmente são cinza escuro e a região ventral se torna mais clara e rosada progressivamente. A cor dos adultos varia de cinza a rosa e machos têm coloração mais rosa que as fêmeas, devido à despigmentação da pele por tração durante interações intra-específicas com outros machos (BEST E DA SILVA, 1989; WÜRSIG, THEWISSEN E KOVACS, 2017; Figura 6).

Figura 6: Ilustração de Uko Gorter representando um macho, uma fêmea e um filhote de boto-cor-de-rosa, *Inia geoffrensis*.



Fonte: WÜRSIG, THEWISSEN E KOVACS, 2017.

Botos-cor-de-rosa geralmente ocorrem em grupos de um a sete indivíduos, sendo prevalentemente solitários (DOS SANTOS et al., 2012). As interações sociais de longo prazo se restringem a mães e seus filhotes, enquanto grandes agregações de indivíduos ocorrem durante o forragemento e períodos reprodutivos (WURSIG, THEWISSEN E KOVACS, 2017). Desde junho de 2018 *I. geoffrensis* é classificada como espécie “Ameaçada” na Lista Vermelha da IUCN com base na suspeita de redução de pelo menos 50% do tamanho da população total em três gerações (75 anos) (IUCN, 2018), No Brasil, a espécie é classificada como “Em Perigo” (BRASIL, 2019).

Para ambas as espécies de odontocetos amazônicos, estimativas demográficas e reprodutivas, assim como taxas de mortalidade, são ainda insuficientes (PAVANATO et al., 2016). As principais ameaças à conservação desses cetáceos fluviais são mortalidade acidental

em equipamentos de pesca, como redes e poluição ambiental (SECCHI, 2012). Há ainda caça de botos-vermelhos para uso como isca para pesca (IWC, 2007 em REEVES et al., 2011) e existência de conflitos com humanos por competição por peixes e destruição de material de pesca (REEVES et al., 2011). Os golfinhos de rio são gravemente afetados pela construção de hidrelétricas na Amazônia, que podem provocar isolamento de populações e resultar em redução da variabilidade genética e consequentemente, extinção em níveis locais (PAVANATO et al., 2016).

O peixe-boi amazônico (*T. inunguis*) é o único representante da ordem Sirenia que habita exclusivamente ambientes de água doce e é endêmico à bacia Amazônica. Ocorre desde a Colômbia, Equador e Peru até o Brasil (MARMONTEL, DE SOUZA E KENDALL, 2016). É a menor espécie de peixe-boi, podendo pedir até 3 m de comprimento total e pesar 450 kg. A coloração é cinza escuro a preto e a maior parte dos espécimes possuem mancha ventral branca ou rósea, de tamanho variável, que pode ser utilizada para identificação individual. Diferentemente dos peixes-boi marinhos, a espécie amazônica não possui unhas (ROSAS, 1994; Figura 7).

Figura 7. Ilustração de Uko Gorter representando o peixe-boi amazônico, *Trichechus inunguis*.

Fonte: WURSIG, THEWISSEN E KOVACS, 2017.

Os peixes-boi amazônicos são herbívoros não-ruminantes e alimentam-se várias espécies de plantas aquáticas ou semi-aquáticas. Geralmente ocorrem em temperaturas entre 25 e 30ºC (REYNOLDS E ODELL, 1991) e realizam migrações sazonais de acordo com a mudança no nível da água. Durante a cheia, se deslocam para áreas alagadas e buscam áreas com maior profundidade durante o período de seca, que servem como refúgio. Movimentos migratórios sazonais entre as RDSM e RDSA foram identificados por acompanhamento de peixes-boi amazônicos marcados com cintos rádio-transmissores em frequências únicas de VHF, mostrando que esses animais utilizam as duas reservas e diferentes tipos de ambientes em momentos distintos do ano (MARMONTEL et al., 2002).

A espécie é classificada como “Vulnerável” pela IUCN, devido suspeita de declínio populacional. Apesar de diversos esforços, não há estimativas populacionais, mas acredita-se que número de animais tenha reduzido significativamente devido a centenas de anos de caça descontrolada (MARMONTEL, DE SOUZA E KENDALL, 2016). Peixes-boi amazônicos foram historicamente caçados para consumo de carne, gordura, couro e ossos. Estima-se que a caça comercial tenha sido responsável pelo abate de milhares de animais entre os séculos XVIII e XX, e considera-se esta a principal causa da redução populacional da espécie (DOMMING, 1982; MARMONTEL, DE SOUZA E KENDALL, 2016). Atualmente, apesar de ilegal, a caça de subsistência prevalece em toda a área de distribuição de peixes-boi amazônicos, incluindo áreas de proteção como as RDSM e RDSA, com um pequeno segmento de comércio local (MARMONTEL, DE SOUZA E KENDALL, 2016).

Além da caça, a captura acidental em redes de pesca, aumento da população humana, poluição sonora, degradação ambiental, desmatamento e poluição ambiental decorrente de mineração são importantes ameaças à conservação dos peixes-boi amazônicos (OROZCO, 2001; MARMONTEL, DE SOUZA E KENDALL, 2016). Além disso, sua reprodução é muito lenta, com longos períodos de gestação e lactação. Apenas um filhote é gerado a cada ciclo, com intervalo reprodutivo de dois a três anos (BEST, 1983), o que, somado às ameaças supracitadas, contribui para o risco de extinção da espécie (MARMONTEL, 2008). Estima-se um declínio populacional de pelo menos 30% nas próximas três gerações, considerando gerações em torno de 25 anos (MARMONTEL, DE SOUZA E KENDALL, 2016).

São extremamente discretos e arredios, provavelmente como adapdação à caça. Essas características, somadas às condições ambientais, como a baixa visibilidade das águas que habitam, fazem com que seja muito raro avistar peixes-boi amazônicos e dificultam o estudo da espécie em seu habitat natural (MARMONTEL, DE SOUZA E KENDALL, 2016). Por isso, o conhecimento acerca de populações de vida livre ainda é escasso e geralmente baseado em entrevistas com moradores locais e no uso da radio-telemetria. A maior parte dos estudos fisiológicos, nutricionais, reprodutivos e ecológicos têm sido realizados em animais em cativeiro (COLARES et al., 1992; ROSAS, 1994; ROSAS E PIMENTEL, 2001; SOUSA-LIMA et al., 2002).

Existe grande demanda de investigações acerca da biologia, desde estudos básicos sobre distribuição e abundância, uso de habitat, interações com a pesca a estudos morfológicos, fisiológicos, causas de mortalidade e ocorrência de doenças infeciosas e outras enfermidades, de tucuxis, botos e peixes-boi amazônicos. Essas informações são essenciais para que medidas apropriadas de conservação sejam desenvolvidas e praticadas.

1.3 Grupo de Pesquisa em Mamíferos Aquáticos Amazônicos e Centro de Reabilitação de Peixes-boi Amazônicos de Base Comunitária

O Grupo de Pesquisa em Mamíferos Aquáticos Amazônicos do IDSM (GPMAA/IDSM) se dedica à investigação científica e conservação de mamíferos aquáticos amazônicos nas regiões das Reservas de Desenvolvimento Sustentável Mamirauá e Amanã e nas proximidades da cidade de Tefé (IDSM, 2017a). O grupo realiza, principalmente, estudos acerca de sua ecologia (distribuição, comportamento, contagem e dinâmica populacional, área de vida e rotas de deslocamento, caracterização genética, biologia reprodutiva, alimentação) e medicina da conservação (anatomia e fisiologia, monitoramento de mortalidade, identificação e controle de ameaças à conservação, ocorrência de doenças infecciosas), além de realizar projetos que visam a concientização ambiental da população local (www.mamiraua.org.br). Para isso, o GPMAA/IDSM realiza resgate de carcaças, necropsias, coleta de amostras biológicas e dados epidemiológicos, monitoramento por radiotelemetria de peixes-boi amazônicos e foto-identificação de botos, tucuxis, lontras e ariranhas, e conta com um acervo osteológico das espécies de mais de 20 anos.

O GPMAA/IDSM é também responsável pelo Centro de Reabilitação de Peixes-boi Amazônicos de Base Comunitária (Centrinho), que tem a finalidade de oferecer cuidados clínicos e manejo de filhotes de peixes-boi órfãos, com objetivo de reabilitação e soltura em vida livre. O Centrinho é regularmente cadastrado no Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (Cadastro Técnico Federal/IBAMA n° 561063) e está localizado no Lago Amanã, na RDSA, área de ocorrência de peixes-boi amazônicos.

Em muitos casos, a mortalidade de peixes-boi fêmeas adultas por caça ou emalhe acidental em redes de pesca, ou o emalhe acidental e captura intencional de filhotes, gera peixes-boi órfãos que frequentemente são criados temporariamente em cativeiro ilegal, vendidos para serem mantidos como animais de estimação, ou mortos para consumo. Alguns destes filhotes capturados acidental ou intencionalmente são resgatados pelo Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis – IBAMA e enviados para centros de reabilitação especializados. A equipe técnica do GPMAA/IDSM é responsável pelo resgate, manutenção, reabilitação, soltura e monitoramento de parte desses animais, com o apoio das comunidades ribeirinhas locais, promovendo educação ambiental e engajamento da população na conservação da espécie (IDSM, 2017a; IDSM, 2017b).

Desde sua criação em 2007, 18 filhotes passaram pelo Centrinho. Os animais em reabilitação são alojados inicialmente em piscinas de 4500 l, quando precisam de cuidados

intensivos ou estão em quarentena, e posteriormente transferidos para um tanque flutuante de 12 m de largura, 8 m de comprimento e 2 m de profundidade, contruído em madeira no Lago Amanã de forma que permite o fluxo constante de água do lago (Figura 8). Os filhotes são alimentados com fórmulas de substituição do leite materno até o período de desmame (geralmente por volta dos dois anos de idade), e diversas espécies de plantas macrófitas e capins frescos que fazem parte da alimentação de peixes-boi amazônicos na região, coletadas diariamente no lago. A manutenção dos filhotes em seu habitat e alimentação natural durante a reabilitação reduz a ocorrência de problemas inerentes ao cativeiro e à transloção para áreas mais distantes, e facilita sua readaptação pós-soltura. Além disso, o custo de reabilitação é reduzido, principalmente aquele relacionado à alimentação dos animais, e proporciona a geração de emprego e o engajamento da população local (IDSM, 2017b).

Figura 8: Tanque flutuante localizado no Lago Amanã, onde são mantidos os peixes-boi amazônicos em reabilitação no Centro de Reabilitação de Peixes-boi Amazônicos de Base Comunitária (Centrinho).

Fonte: Instituto de Desenvolvimento Sustentável Mamirauá.

Durante todo o período em que são mantidos no Centrinho, os animais passam por acompanhamento médico-veterinário e realização de exames clínicos, pesagem, biometria, coleta periódica de amostras biológicas e realização de exames complementares, com o objetivo de avaliar seu estado de saúde (Figura 9). Quando indicada, a soltura é realizada em áreas de ocorrência de peixes-boi amazônicos e com abundância de alimentos. Antes da soltura, todos os indivíduos recebem cintos rádio-transmissores em frequências únicas de VHF adaptados a

seus pedúnculos caudais (Figura 10). Os animais são monitorados por radiotelemetria após a soltura, de forma que sua readaptação ao ambiente natural seja acompanhada (IDSM, 2017b).

Figura 9: Tratamento médico-veterinário de peixe-boi amazônico em reabilitação no Centro de Reabilitação de Peixes-boi Amazônicos de Base Comunitária (Centrinho).

Fonte: Arquivo pessoal.

Figura 10: Soltura de peixe-boi amazônico após reabilitação, com cinto rádio-transmissor adaptado ao pedúnculo caudal.

Fonte: Instituto de Desenvolvimento Sustentável Mamirauá.

1.4. Leptospirose, brucelose e toxoplasmose em botos-cor-de-rosa, tucuxis e peixes-boi amazônicos

Segundo SIMEONE et al. (2015), o impacto de doenças infecciosas na conservação de mamíferos aquáticos tem sido subestimado. Sabe-se que epidemias virais, bacterianas, parasitárias e fúngicas têm afetado mamíferos marinhos (SIMEONE et al., 2015), mas ainda não se conhece bem a ocorrência dessas doenças em mamíferos fluviais. O aumento da população humana em ambientes compartilhados com essas espécies também gera riscos (SECCHI, 2012). Além da transformação do habitat, a possibilidade de transmissão de agentes zoonóticos entre mamíferos aquáticos e seres humanos, devido ao seu crescente contato, tem sido considerada (WALTZEK et al., 2011).

A ocorrência de leptospirose, brucelose e toxoplasmose tem sido observada em mamíferos aquáticos (BOGOMOLNI et al., 2006) e há indícios da transmissão de leptospirose e brucelose entre mamíferos aquáticos e seres humanos (SMITH et al., 1978 citado por WALTZEK et al., 2012; CAMERON et al., 2008). Estudos relataram a ocorrência de leptospirose em mamíferos marinhos, apontando impactos na saúde desses animais e aumento da mortalidade (HIGGINS, 2000). Na Califórnia, a doença foi estudada em leões marinhos encalhados: 33% desses animais apresentaram sinais de lesão renal e 71% morreram (GULLAND et al., 1996). Outro estudo relatou a presença de *Leptospira* spp. na urina e nos rins de pinípedes, além de encontrar a bactéria na areia contaminada por fezes e urina desses animais, em regiões frequentadas por seres humanos e animais domésticos (CAMERON et al., 2008).

Há raros estudos sobre a presença de anticorpos anti-*Leptospira* spp. em *T. inunguis* e esses indicam a ocorrência do patógeno na espécie e no ambiente amazônico. MATHEWS et al. (2012) relataram que 31% das amostras de soro desses animais foram reagentes no teste de soroaglutinação microscópica (SAM). DELGADO et al. (2012) encontraram 15 amostras reagentes no teste sorológico entre as 19 utilizadas. Não foram realizados estudos sobre a doença em *I. geoffrensis* e *S. fluviatilis*.

Sabe-se muito pouco sobre a epidemiologia da brucelose no ambiente aquático, mas estudos sorológicos sugerem que cetáceos e pinípedes têm sido infectados por *Brucella* spp. (NIELSEN et al., 2001) e existem evidências de transmissão horizontal e vertical da doença nesses animais (NYMO, TRYLAND E GODFROID, 2011; WALTZEK et al., 2012). Há também relatos de anticorpos anti-*Brucella* spp. em peixes-boi marinhos (HERNÁNDEZ-MORA, PALACIOS-ALFARO E GONZÁLEZ-BARRIENTOS, 2013), inclusive no Brasil

(ATTADEMO, 2014). Em estudo realizado com 161 botos-vermelhos da Reserva de Desenvolvimento Sustentável Mamirauá e de outras localidades, a ocorrência de infecção não foi evidenciada (ROCCA, 2014).

Acredita-se que uma cepa nova ou atípica de *Brucella* spp. tenha sido isolada de um feto de golfinho-nariz-de-garrafa abortado nos Estados Unidos (EWALT et al., 1994). Em animais de ciclo reprodutivo lento, como no caso do peixe-boi amazônico, doenças que afetem a capacidade reprodutiva, como brucelose e leptospirose, podem causar impactos significativos na conservação da espécie em longo prazo. GONZÁLEZ-BARRIENTOS et al. (2010) identificaram *Brucella* spp. causando meningoencefalite em golfinhos-riscados (*Stenella coeruleoalba*) que apresentavam dificuldade de natação.

Infecções por *Toxoplasma gondii* têm sido identificadas como causadoras de aumento da mortalidade em mamíferos marinhos (DUBEY et al., 2003) e queda na eficiência reprodutiva de lontras-marinhas (CONRAD et al., 2005). Em estudo realizado no Brasil, 8,3% dos peixes-boi marinhos do Centro Nacional de Pesquisa, Conservação e Manejo de Mamíferos Aquáticos/IBAMA (CMA) apresentaram anticorpos anti-*T. gondii* (SILVA et al., 2001). Acredita-se que tanto os felídeos domésticos como selvagens atuem na transmissão da toxoplasmose no ambiente aquático. Esses animais eliminariam oocistos nas fezes em cursos de água doce, ou próximo a eles, e assim haveria consequente contaminação do ambiente marinho (CONRAD et al., 2005).

Em estudo sobre a ocorrência de anticorpos anti-*T. gondii* em peixes-boi amazônicos utilizou-se 74 animais do Instituto Nacional de Pesquisas da Amazônia (INPA) e do Centro de Pesquisas e Conservação de Mamíferos Aquáticos (CPPMA). Desses, 39,2% foram positivos (MATHEWS et al., 2012). Outro trabalho mostrou 63% (12/19) dos *T. inunguis* positivos para o teste sorológico na Amazônia peruana (DELGADO et al., 2013). SANTOS et al. (2011) sugeriram alto nível de contaminação ambiental com *T. gondii* e relataram a presença de antiporcos contra o patógeno em 86% dos botos-vermelhos utilizados no estudo.

Em peixe-boi *Trichechus manatus manatus* na Guiana, taquizoítos de *T. gondii* foram identificados em cortes de tecido cardíaco, causando miocardite não supurativa e necrose multifocal (DUBEY et al., 2003). Um boto cinza (*S. guianensis*) fêmea, no Paraná, apresentou pneumonia intersticial e hepatite e diagnosticou-se toxoplasmose. Através de imunohistoquímica, observou-se a presença de taquizoítos e cistos de *T. gondii* ao redor de áreas de necrose moderada a severa e infiltração de células mononucleares. Nesse caso, a ocorrência da doença no boto cinza foi atribuída principalmente a alterações ambientais, como

a poluição da Baía de Paranaguá com esgoto de áreas urbanas e presença de felinos domésticos e selvagens (GONZALES-VIEIRA et al., 2013).

Mamíferos aquáticos podem atuar como importantes indicadores da saúde do ecossistema que habitam, sinalizando previamente a ocorrência de alterações no ambiente e a presença de agentes infecciosos (BOSSART, 2007). Dessa forma, o estudo de leptospirose, brucelose e toxoplasmose em tucuxis, botos-vermelhos e peixes-boi amazônicos, além de permitir maior conhecimento das doenças nessas populações, pode gerar informações importantes sobre a ocorrência de patógenos de importância em saúde pública no ambiente amazônico.

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CAPÍTULO 2 - Serological and molecular investigation of *Leptospira* spp. and *Brucella* spp., and bacteriological culture in samples from Amazonian manatees (*Trichechus inunguis*), Amazon river dolphins (*Inia geoffrensis*), and tucuxi (*Sotalia fluviatilis*)

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*"Anti-*Leptospira *antibodies and isolated bacteria in Amazonian aquatic mammals"*

Serological and molecular investigation of *Leptospira* spp. and *Brucella* spp., and bacteriological culture in samples from Amazonian manatees (*Trichechus inunguis*), Amazon river dolphins (*Inia geoffrensis*), and tucuxi (*Sotalia fluviatilis*)

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Abstract: Leptospirosis and brucellosis are common zoonotic diseases with global distribution that represent severe hazard to humans and animals. The present study aimed at investigating exposure to and infection by *Leptospira* spp. and *Brucella* spp. in samples from Amazonian manatees (*Trichechus inunguis*), Amazon river dolphins (*Inia geoffrensis*), and tucuxi (*Sotalia fluviatilis*), free-ranging or undergoing *in-situ* rehabilitation in the mid-Solimões river region, Brazilian Amazon. Serum samples from Amazonian manatees were tested by microscopic agglutination test, Rose Bengal test and 2-Mercaptoethanol test. Tissue and/or body fluid samples from Amazon river dolphins, a tucuxi, and Amazonian manatees, were subjected to a multiplex PCR for *Leptospira* spp. and *Brucella* spp. and bacterial culture for attempted

isolation. A total of 63% of sera were reactive to *Leptospira* spp. and serovar Patoc was considered the infecting serovar in all positive samples. Titers were generally low, indicating chronic exposure, but active infection was suggested in three Amazonian manatees. Anti-*Brucella* spp. antibodies were not detected. All samples were negative by PCR and *Leptospira* spp. and *Brucella* spp. were not isolated. Instead, *Enterococcus faecalis* (from uterine fluid, lymph node, and lung) and *Bacillus* spp. (from milk and synovial fluid) were isolated. Knowledge of the pathogens prevalent in Amazonian manatees, Amazon river dolphins, and tucuxis is of great relevance to species conservation and environmental health. Further research is needed to elucidate the clinical relevance of infection by *Leptospira* sp. serovar Patoc, *E. faecalis*, and *Bacillus* spp. in Amazonian aquatic mammals.

Keywords: Cetacea, Microbial isolation, Serovar Patoc, Serology, Sirenia, Zoonoses

1. INTRODUCTION

Leptospirosis and brucellosis are important zoonotic diseases with global distribution. Leptospirosis was considered the most widespread and prevalent zoonotic disease in the world (WHO 1999). It represents a re-emerging public health problem, with markedly increasing incidence rates and multiple outbreaks reported in all continents (Hartskeerl et al. 2011). It is estimated that around half a million severe human cases occur annually, while rates of the disease in animals are unknown (WHO 1999, Hartskeerl et al. 2011). Leptospirosis is caused by gram-negative, aerobic spirochetes of the *Leptospira* genus that includes several pathogenic and saprophytic species. Such species are further classified into serogroups that can include several genetic varieties, called serovars (Adler & Moctezuma 2010). Most mammalian species are natural carriers of *Leptospira* spp. and the risk of acquiring leptospirosis is associated with contact with animals (WHO 2003, Barthi et al. 2003). It is expected that leptospirosis will remain a significant veterinary and public health threat for the coming years (Hartskeerl et al. 2011).

Brucellosis is a communicable and One Health disease of public health significance that presents a severe hazard to humans and domestic animals (Suluku et al. 2019). It is caused by gram-negative, facultative anaerobic, intracellular bacteria of genus *Brucella*. In domestic animals, *Brucella* spp. infection may cause abortion and other reproductive issues resulting in huge economic losses. Human infection is frequently associated to direct or indirect contact with infected animals or their products (Corbel 2006). Very few countries in the world are officially considered free of brucellosis and the disease is endemic and widely prevalent in many developing countries (Corbel 2006, Rubach et al. 2013, Machavarapu et al. 2019). In India, the disease is estimated to cause economic losses up to 3.4 billion US dollars per year

(Singh et al. 2015). Brucellosis is endemic in Brazil and human outbreaks have been reported (Lemos et al. 2018).

The Amazon is the largest rainforest in the world and in the past decades human population has exponentially increased in this region, enhancing environmental degradation and contact between humans, wildlife, and domestic animals. High prevalence of leptospirosis was reported for humans, domestic animals, and wildlife in the Amazon (Carvalho et al. 2000, Homem et al. 2001, Jori et al. 2009, Jesus et al. 2012, Paz et al. 2015, Chiebao et al. 2015, Albuquerque et al. 2017, Ribeiro et al. 2018). Exposure to *Brucella* spp. was reported for humans and animals in the Amazon (Mayor et al. 2006, Aguiar et al. 2007). It was proposed that the incidence of these diseases in the region could be consequence of social and natural conditions such as: the climate conditions favoring the survival and spread of the bacteria in the environment; close contact between urban, rural, and wild areas, enhancing livestock/wildlife interface and potential of pathogen transmission; scattered veterinary service in remote areas; and lack of basic sanitation and infectious disease prevention measures (Donaires et al. 2012, Chiebao et al. 2015).

Despite the diversity and abundance of the Amazonian wild fauna, little is known about the role of wild species in the epidemiology of leptospirosis and brucellosis, and the impacts of those diseases in Amazonian wildlife health. Capybaras (*Hydrochoerus hydrochaeris*) in the western Brazilian Amazon were massively infected by *Leptospira* spp. and may act as resivors for the spirochetes in the environment (Albuquerque et al. 2017). A serological investigation showed that 4.9% collared peccaries (*Tayassu tajacu*) sampled in the eastern Amazon had antibodies against *Brucella* spp. and 9.8% animals had antibodies to *Leptospira* spp. (Mayor et al. 2006). High seroprevalence of anti-*Leptospira* spp. antibodies was reported in captive collared peccaries in the Peruvian Amazon and such herds may play a role in the maintenance and amplification of the pathogen in the environment (Jori et al. 2009).

Aquatic mammals can act as sentinels of environmental health by indicating the presence of pathogens in the environment over space and time (Bossart 2007). Although leptospirosis and brucellosis have been described in marine mammals (Zuerner et al. 2009, Sulzner et al. 2012, Guzmán-Verri et al. 2012, Aragón-Martínez et al. 2014, Sánchez-Sarmiento et al. 2019), few studies have been conducted on those infectious diseases in Amazonian aquatic mammals (Mathews et al. 2012, Delgado et al., 2015; Sánchez-Sarmiento et al. 2018). The aim of this study was to evaluate the exposure to and infection by *Leptospira* spp. and *Brucella* spp. in Amazonian manatees (*Trichechus inunguis*), Amazon river dolphins (*Inia geoffrensis*), and tucuxis (*Sotalia fluviatilis*), free-ranging or undergoing *in-situ* rehabilitation in the mid-Solimões river region, Brazilian Amazon, using serological, molecular, and culture based diagnostic techniques.

2. MATERIALS & METHODS

2.1 Samples and study area

This study was conducted in the areas of Amanã Sustainable Development Reserve (ASDR) and Mamirauá Sustainable Development Reserve (MSDR) and Lake Tefé, Amazonas state, Brazil, located in the mid-Solimões river region, between the Negro, Japurá and Solimões river basins. The study area is included in the Amazonian várzea ecosystem, an annually inundated seasonal floodplain forest located in the Amazon biome. Both riverine dolphin species occur extensively throughout the studied area, and Amazonian manatees are known to seasonally migrate between the ASDR and MSDR (Arraut et al. 2010). The Community-based Amazonian Manatee Rehabilitation Center (Centrinho), maintained by the Mamirauá Sustainable Development Institute (IDSM), is located at Lake Amanã, within the ASDR, an area historically known to concentrate Amazonian manatees. The Centrinho performs orphaned

Amazonian manatee rescue, rehabilitation and release within the ASDR and MSDR. During rehabilitation the animals are kept in a wooden floating pen within Lake Amanã, with water flow-through from the lake, and are fed with replacement formula and vegetation collected locally (Figure 1). The IDSM also opportunistically collects carcasses of free-living Amazon river dolphins, tucuxis, and Amazonian manatees found within the study area for necropsies and collection of biological samples that are archived for future studies.

A total of 50 serum samples were collected from Amazonian manatees for detection of antibodies against *Leptospira* spp. and *Brucella* spp. (Table 1). Of these, five serum samples were collected from five free-ranging Amazonian manatees captured at the MSDR during a capture-release health assessment study conducted in 2005. The remaining 45 serum samples were collected from 14 Amazonian manatees in rehabilitation at Centrinho between 2009 and 2014. Up to seven serum samples were opportunistically collected from each animal throughout their rehabilitation period, when physical restrain and blood sampling were performed for clinical evaluation. All blood samples were collected from the vascular bundle of the pectoral limb, accessed through its plantar surface (Fawcett 1942, Bossart 2001). After refrigerated and allowed to clot, samples were centrifuged, the serum was separated and kept frozen until analyzed.

From IDSM's archive, 21 frozen samples of lymph nodes (n=2), ovaries (n=2), uterus/uterine fluid (n=2), spleen (n=3), liver (n=2), lungs (n=2), abscesses (n=4), synovial articular fluid (n=1), and milk (n=3), collected from 12 Amazon river dolphins, one tucuxi, and two Amazonian manatees were selected for molecular identification and attempted isolation of *Leptospira* spp. and *Brucella* spp., and opportunistic bacterial culture (Table 2).

2.2 Detection of antibodies against *Leptospira* spp. and *Brucella* spp.

All serum samples were examined for the presence of *Leptospira* serovar antibodies by the microscopic agglutination test (MAT). The MAT was performed at the Laboratory of Infectious Diseases, Federal University of Uberlandia (UFU), using a panel of 22 reference serovars classified within the species *L. interrogans*, *L. biflexa*, *L. kirschneri*, *L. borgpetersenii*, and *L. noguchii* (Table 3), following standard methodology (Brasil 1995, Cumberland et al. 1999). The MAT titer ≥1:100 was used as cut-off to determine seropositivity, as previously used by Aragón-Martinez et al. (2014). Samples considered positive in the screening test were subjected to antibody titration in two-fold serial dilutions (1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200). Positive reaction to more than one serovar is possible due to cross reactivity or exposure to multiple serovars (Brasi 1995, Haake & Levett 2015). The presumptive infecting serovar was based on highest titer. Infection by multiple serovars was assumed in case of equally highest MAT titers against two or more serogroups (Chadsuthi et al. 2017).

The presence of *Brucella* spp. antibodies in the serum samples was screened using Rose Bengal Test (RBT) and 2-Mercaptoethanol Test (2-ME) following previously described methodology (OIE 2012, Brasil 2006). RBT was conducted using 8% buffered *Brucella* antigen mixed to the serum in equal volumes, and samples were considered positive by RBT when visible agglutination was noted. For 2-ME, samples were subjected to serial dilutions (1:25, 1:50, 1:100, and 1:200). A sample was considered positive for *Brucella* spp. antibodies only when positive by both RBT and 2-ME tests (Brasil, 2006).

2.3 Multiplex polymerase chain reaction (mPCR) to *Leptospira* spp. and *Brucella* spp.

At the Biological Institute of São Paulo, samples from lymph nodes, ovaries, uterus/uterine fluid, spleen, liver, lungs, abscesses, synovial articular fluid, and milk (Table 2) were individually ground in sterile mortar and pestle, and resuspended as 10% suspension (w/v)

in ultrapure water (Cortez et al. 2001). DNA was extracted from the sample suspensions using the Invitrogen DNAzol Reagent (Genomic DNA Isolation Reagent), according to manufacturer's instructions (Chomczynski et al. 1997).

The multiplex polymerase chain reaction (mPCR) to *Leptospira* spp. and *Brucella* spp. assay was carried out in 500 µl microtubes with 50 µl cocktails consisting of 15 µl of Milli-Q water, 5 µl of reaction buffer 10x (500 mM KCl, 100 mM Tris–HCl, pH 9.0), 8 µl of dNTPmix (200 µm of each dCTP, dTAP, dGTP, and dTTP), 1.5 µl of MgCl250 mM, 2.5 µl of each primer B4, B5, Lep1, and Lep2 at 10 pM/µl, 0.5 µl of Taq DNA polymerase (5 U/ml), and 10 µl of extracted DNA (Richtzenhain et al. 2002). The primer set B4 (TGGCTCGGTTGCCAATATCAA) and B5 (CGCGCTTGCCTTTCAAGGTCTG) were used to amplify a 223 bp sequence of *Brucella* spp. DNA (Baily et al. 1992). Primers Lep1 (GGCGGCGCGTCTTAAACATG) and Lep2 (TTAGAACGAAGTTACCCCCCTT) were used to amplify a 330 bp sequence of *Leptospira* spp. DNA (Merien et al. 1992). Ultrapure water was used as negative control. *Brucella abortus* strain ATCC 544 bacterial suspension (2.8×10^9) and *Leptospira borgpetersenii* serovar Hardjo strain Hardjoprajitino were used as positive controls (Richtzenhain et al. 2002).

The initial denaturation was 3 min at 94°C followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 60°C for 60 seconds, and extension at 72° C for 90 seconds, and a final elongation step at 72° C for 10 min. PCR products were subjected to electrophoresis on a 2% agarose gel stained with ethidium bromide, using a molecular weight marker with 100 bp increments (100 bp ladder Gibco-BRL) for size standards. Gel images were acquired and analyzed using Kodak DC120 Digital Camera and Kodak 1D Image Analysis Software (Richtzenhain et al. 2002).

2.4 Isolation of *Leptospira* spp. and *Brucella* spp. and microbiological culture

For microbiological culture, samples from lymph nodes, ovaries, uterus/uterine fluid, spleen, liver, lungs, abscesses, synovial articular fluid, and milk (Table 2) were prepared into 20% tissue suspensions in sterile saline, at the Biological Institute of São Paulo. *Brucella* spp. isolation was performed by adding 0.1 ml of tissue suspensions in culture plates of *Brucella* agar (Difco®) with 5% of defibrinated sheep blood incubated for as long as 10 days at 37ºC, in duplicates: one plate was incubated in aerobic conditions, and the other was incubated in microaerophilic jars under a 10% CO2 atmosphere (OIE 2012). Aerobic and microaerophilic colonies were isolated and identified using Gram staining and biochemical tests, using standard procedure (Kirkbride 1990, Holt et al. 1994, Scarcelli et al. 2004, OIE 2012). Attempted *Leptospira* spp. isolation was performed by adding 0.1 ml of tissue suspensions in 5 ml of Fletcher medium supplemented with 10% rabbit serum in aerobic conditions at 28ºC. Resulting cultures and subcultures were observed weekly in dark-field microscopy for 6 weeks (Faine 1994).

3 RESULTS

Seropositivity to *Leptospira* spp. was detected in 22 (44%) of 50 samples from 12 (63.1%) of 19 Amazonian manatees. *L. biflexa* serovar Patoc was considered the presumptive infecting serovar in all positive samples, from both free-ranging and in-rehabilitation animals, although reaction to more than one serovar was observed in five samples (22.7%). Titers varied from 1:100 to 1:800, and titers of 100 (59%) were most frequent. While most animals were sampled only once (11/19; 57.8%), it was possible to study the curve of anti-*Leptospira* spp. titers through time on four Amazonian manatees undergoing rehabilitation in Centrinho, which were identified as Piti, Japurá, Helena, and Luna (Table 1, Figure 2).

All serum samples were considered negative to antibodies against *Brucella* spp. by RBT and 2-ME (Table 1). All samples were negative for *Brucella* spp. and *Leptospira* spp. DNA by m-PCR and attempted isolation (Table 2). *Enterococcus faecalis* was isolated from the uterine fluid (case 2), lymph node (case 5), and lung (case 12) samples from three Amazon river dolphins. *Bacillus* spp. was isolated from milk (cases 3 and 15) and synovial fluid (case 9) from two Amazon river dolphins and from the milk sample from an Amazonian manatee (Table 2).

4 DISCUSSION

Our results suggest the exposure of free-ranging and in-rehabilitation Amazonian manatees to *Leptospira biflexa* serovar Patoc. This represents only the third report of anti-*Leptospira* spp. antibodies in Amazonian manatees and the first report in free-ranging animals (Mathews et al. 2012, Delgado et al. 2015). In our study, serovar Patoc was considered the infecting serovar in all positive animals by MAT. MAT is the most widely used method for diagnosing leptospirosis and the gold-standard serological test suggested by the World Organization for Animal Health (OIE 2012). Although the specificity of the test is good, significant serological cross-reactivity between serovars and serogroups of *Leptospira* spp. occurs and therefore, serology does not definitively identify infecting serovars (OIE 2012). As a complementary diagnostic measure, the isolation of the bacteria followed by its molecular characterization is recommended. When serological diagnostic tests are used, specific knowledge of circulating serovars is important to yield useful results and improve sensitivity (OIE 2012, Agampodi et al. 2016). An incomplete panel of serovars can induce false negative results (Musso & La Scola 2013).

Similarly to our study, the serovar Patoc was also reported as the most frequent *Leptospira* spp. serovar in Amazonian manatees maintained in captivity at two rehabilitation

units in the Lower-Solimões river region (Mathews et al. 2012) and the third most frequent in Amazonian manatees in captivity in the Peruvian Amazon (Delgado et al. 2015). Patoc was also the most prevalent *Leptospira* spp. serovar reported in humans living in rural areas of the western Amazon, Brazil (46.7%) (Aguiar et al. 2007), and in ovine (29,7%) from 15 farms in the same region (Aguiar et al. 2010). Serovar Patoc was the second most frequent (20%) serovar in domestic dogs in another region of the Brazilian Amazon (Paz et al. 2015). Serological response to this serovar was also reported in cattle (7.9%) and equines (26,6%) in the Brazilian Amazon (Aguiar et al. 2006, Aguiar et al. 2008). Our results, in addition to those previous reports, suggest the occurrence of serovar Patoc in the Amazonian environment, with serological exposure of humans and domestic and wild animals, for at least 10 years.

Serovar Patoc belongs to the *L. biflexa* species, considered a free-living, saprophytic, aquatic spirochete. *L. biflexa* serovar Patoc strain Patoc1, was initially isolated from stream water (Picardeau et al. 2008). The significance of the high prevalence of sero-reaction to Patoc in animals and humans in the Amazon is unknown. Serovar Patoc is known to cross-react with pathogenic *Leptospira* (Correa et al. 1970). Because of that, most of the commercially available enzyme-linked immunosorbent assay (ELISA) kits use serovar Patoc as a genus-specific antigen for serological investigations of *Leptospira* spp. (Musso & La Scola 2013). In marine mammals, severe clinical leptospirosis was reported in pinniped species (Waltzek et al. 2012). Periodic large-scale stranding and mortality events of California sea lions were attributed to *L. interrogans* serovar Pomona (Gulland et al. 1996, Cameron et al. 2008, Norman et al. 2008, Zuerner et al. 2009). Transmission to humans was reported following contact with fluids from infected sea lions and with tissues during necropsy of a sea lion that died from leptospirosis (Smith et al. 1978).

The seroprevalence of anti-*Leptospira* spp. antibodies in Amazonian manatees appears to be high: 63% in our study, 78.9% as reported by Delgado et al. (2015), and 31.1% as reported

by Mathews et al. (2012). Amazonian environmental conditions are highly favorable for the maintenance and transmission of *Leptospira* spp., and therefore, wild mammals would probably be exposed to the spirochetes (Barthi et al. 2003, Jori et al. 2009). Mathews et al. (2012) suggested that the most likely source of *Leptospira* spp. infection for Amazonian manatees would be the exposure to water contaminated with urine from infected terrestrial, domestic, and wild animals. In pinnipeds, leptospirosis is believed to be directly spread among individuals via infected urine at rookeries (Cameron et al. 2008, Norman et al. 2008, Zuerneret al. 2009).

MAT detects IgM and IgG antibodies, and does not differentiate between current, recent, or past infections (Musso & La Scola 2013). In our study, serum titers were mainly low, indicating chronic infection or past exposure to *Leptospira* spp. (OIE 2012). In areas with frequent exposure, as in most tropical countries, a higher cut-off titer is necessary to indicate active infection. In very high endemic areas, a single titer of 1:800 in symptomatic patients is generally indicative of disease (Musso & La Scola 2013). According to the Center for Disease Control (CDC 1997), for humans, the laboratory criteria for diagnosis of leptospirosis should consist of a fourfold or greater increase in the agglutination titer between serum specimens obtained $\geq$ 2 weeks apart and studied at the same laboratory, along with clinical disease. That increase in titers in paired sera confirms the diagnosis regardless of the interval between samples (Levett 2001). Although no clinical signs compatible with leptospirosis were observed in the sampled Amazonian manatees in this study, the individual Piti showed greater than a fourfold increase of titers to serovar Patoc in seven months and maintained the higher titers in the following sampling, three months later. Japurá and Helena also showed a fourfold increase in titers in samples collected around 10 months apart (Table 1; Figure 2). Therefore, the Amazonian manatees Piti, Helena, and Japurá may have had active lesptospiral infections. The same pattern of antibody increase was reported for Antillean manatees (*Trichechus manatus*

manatus) from a landlocked lake in Mexico, and active infections were suggested (Aragón-Martinéz et al. 2014).

All three Amazonian manatees had increased serum titers in samples collected between Nov 2012 and Feb 2013, in the dry season, indicating active infection or re-infection in the rehabilitation pen in that period (Table 1; Figure 2). During the dry season, when the level of water lowers considerably and food availability decreases, Amazonian manatees tend to concentrate in terra-firme lakes or main river channels (Calvimontes 2009). Arraut et al. (2010) demonstrated that during lowwater season (October–November), Amazonian manatees migrate to Lake Amanã, where Centrinho is located. Concentration of animals can influence the exposure and transmission of *Leptospira* spp. (Hashimoto et al. 2015). Differently, in Manaus, capital of Amazonas state, located around 500 km from Lake Amanã, the largest number of confirmed human leptospirosis cases from 2000 to 2010 occurred in May, followed by March and April, a period of intense rainfall (Jesus et al. 2012). Higher seroprevalence and titers to *L. interrogans* were also reported to occur during the rainy season in Antillean manatees in Mexico (Aragón-Martinéz et al. 2014).

In our study, *Leptospira* spp. was not identified in tissue samples of dolphins by PCR or bacterial culture. *Leptospira* spp. has never been isolated or molecularly identified in Amazonian river dolphins (Higgins 2000, Sánchez-Sarmiento et al. 2018). The first isolation of *Leptospira* sp. from a cetacean, a southern right whale (*Eubalaena australis*) calf stranded in Argentina, occurred in 2015 (Loffler et al., 2015). On that study, a novel species of the genus *Leptospira*, likely to be pathogenic, was isolated from the kidney tissue (Loffler et al. 2015).

In marine cetaceans, infection by *Brucella* spp. has been reported in a broad range of species with widespread distribution (Higgins 2000, Guzmán-Verri et al. 2012, Hernández-Mora et al 2013, Sánchez-Sarmiento et al. 2018). The infection can be asymptomatic or cause severe pathology in dolphins, such as meningoencephalitis and neurological disorders,

endocarditis, placentitis, lung lesions, and death (Hernández-Mora et al. 2008, Guzmán-Verri et al. 2012, Sánchez-Sarmiento et al. 2018). *Brucella* sp. has been isolated from multiple organs in cetaceans, including subcutaneous lesions, liver, spleen, blood, lymph nodes, uterus, mammary glands and several tissues from an aborted fetus (Ross et al. 1996, Higgins 2000, McAloose et al. 2016). *B. ceti* was recently detected in the brain of a dolphin stranded in northeastern Brazil, using PCR (Attademo et al. 2018). The zoonotic potential of marine mammal *Brucella* strains is recognized (McDonald et al. 2006). Two cases of neurobrucellosis and intracerebral granulomas in humans believed to be infected in Peru, were confirmed to be caused by marine mammal *Brucella* sp. (Sohn et al. 2003). In agreement with our results, infection by or exposure to *Brucella* spp. has never been reported in riverine dolphins or manatees (Guzmán-Verri et al. 2012, Hernández-Mora et al. 2013, Sánchez-Sarmiento et al. 2018). Despite of that, continued surveillance is recommended due to the potential conservation and public health impacts of brucellosis in the Amazonian environment.

E. faecalis is an opportunistic pathogen considered as normal flora in skin and gastrointestinal and genital tracts in humans, many animals and insects (Facklam et al. 1999, Kau et al. 2005, Higuita & Huycke 2014). It is also frequently reported as the causative agent of infections in humans, including urinary tract infections, endocarditis, bacteremia, and wound infections (Kau et al. 2005). *E. faecalis* was the most common *Enterococcus* spp. identified in fecal samples from humpback whale (*Megaptera novaeangliae*) and Risso's dolphin (*Grampus griseus*) (Prichula et al. 2016). The bacteria were also isolated from the anus of a necropsied bottlenose dolphin (*Tursiops truncatus*) (Buck et al. 1987), and from blowhole and gastric fluid samples of wild bottlenose dolphins (Morris et al. 2011). *E. faecalis* was previously associated with a multisystemic infection in a neonate dugong (*Dugong dugon*) in Australia, including meningoencephalitis. In that case, *E. faecalis* was cultured from the cerebrospinal fluid, brain tissue, and urine (Nielsen et al. 2013). *Enterococcus* spp. were commonly isolated from

inflammatory lesions in pinnipeds stranded alive in California. In those animals, *Enterococcus* spp. were cultured from superficial abscesses, wounds, ocular and urethral discharges, livers from septicemic animals, brains with encephalitis or meningitis, and pneumonia affected lungs (Thornton et al. 1998). Herein, *E. faecalis* was isolated from an Amazon river dolphin's verminous pneumonia affected lung (case 12), associated to hydrothorax, focal parasitic granuloma, and multifocal intrabronchial nematodes. The dolphin from case 5, from which *E. faecalis* was isolated from the lymph node, was presented with lymphadenitis, bronchopneumonia, and suspected osteomyelitis. No lesions were reported on case 2.

We isolated *Bacillus* spp. from milk samples of an Amazonian manatee and an Amazon river dolphin. *Bacillus* spp. are saprophytes widely distributed in the environment, and commonly isolated from soil, air, water, plants and animals. These bacteria are frequently reported in milk and dairy industry as contaminating organisms (IDF 2016). *Bacillus* sp. was reported to be present in 5% of donor human milk pools before pasteurization, and the most predominant contaminant cultured after pasteurization in a human milk bank (Landers & Updegrove et al. 2010). Potential clinical implications of *Bacillus* sp. contamination in human milk are still unclear (Landers & Updegrove et al. 2010). No other reports of *Bacillus* spp. in aquatic mammal's milk were found in the literature and the normal milk microbiota for Amazon river dolphins and Amazonian manatees is unknown. *Bacillus* spp. were one of the most common gram-positive bacteria cultured from blowhole and gastric fluid samples of wild bottlenose dolphins (Morris et al. 2011). In correlation with the isolation of *Bacillus* spp. from an Amazon river dolphin's synovial fluid in this study (case 9), the bacteria were between the array of microorganisms isolated from blood and/or joint tissues of wild dolphins presenting joint disease (Turnbull and Cowan 1999). Gross lesions of chronic joint disease, such as roughening or erosion of the articular cartilage were not observed during case 9 necropsy, but there was soft tissue edema associated with the left humeroscapular joint and the synovial fluid

was abnormal, displaying slightly red coloration. Whether *Bacillus* spp. isolated from the joint was associated to the observed lesions is not clear.

Although commonly reported in other animals, this study represents the first report of *E. faecalis* and *Bacillus* spp. isolation in tissues and/or body fluids from Amazon river dolphins and Amazonian manatees. Very few information is available on the clinical significance of the isolation of opportunistic pathogens in aquatic mammals and the role of these bacteria in disease, if any, has not been established. Knowledge of infectious agents prevalent in Amazonian manatees, Amazon river dolphins, and tucuxis is of relevance to species conservation. Furthermore, aquatic mammals can act as sentinels of environmental health by indicating the presence of pathogens, including zoonotic agents, in the environment (Bossart 2007). Our results reinforce the importance of ongoing studies for the detection of infectious agents that can affect the health of aquatic mammals, humans, and the Amazonian environment. Further research is needed for molecular confirmation of serovar Patoc in the Amazonian manatees, and better understanding of the clinical relevance of infection by *Leptospira* spp., *E. faecalis*, and *Bacillus* spp. in Amazonian aquatic mammal's health.

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Table 1. Amazonian manatee (*Thichechus inunguis*) serum samples subjected to microscopic agglutination test (MAT), Rose Bengal Test (RBT), and 2-Mercaptoethanol Test (2-ME). Animal identification, sex, age class, date of sampling, and interval between blood samples collection for each animal, and serological tests result are reported. [F]: free-ranging; [R]: in rehabilitation; M: male; F: female; ND: not determined; CA: calf; JU: juvenile; AD: adult; —: negative.

Animal ID	Sex	Age class	Sampling date	Sampling interval (days)	MAT (Serovar and titer)	RBT	2-ME
A2005-02 [F]	M	AD	Nov-2005	Single sample	Patoc 1:400	—	—
A2005-03 [F]	F	JU	Nov-2005	Single sample	—	—	—
A2005-04 [F]	F	JU	Nov-2005	Single sample	Patoc 1:100	—	—
A2005-004 [F]	M	AD	Nov-2005	Single sample	Patoc 1:100	—	—
A2005-6 [F]	M	AD	26/11/2005	Single sample	Patoc 1:200, Sentot 1:100	—	—
Piti [R]	M	JU	16-May-2009	0	Patoc 1:100	—	—
		JU	07-Apr-2012	1057	Patoc 1:100	—	—
		AD	15-Nov-2012	1279	Patoc 1:800, Sentot 1:100	—	—
		AD	17-Feb-2013	1373	Patoc 1:800, Sentot 1:100	—	—
		AD	07-Feb-2014	1728	Canicola 1:100, Icterohaemorragiae 1:100, Djasiman 1:100, Panama 1:100, Patoc 1:200	—	—
		AD	16-Jul-2014	1887	Patoc 1:100	—	—
Japurá [R]	M	CA	04-Jun-2010	0	—	—	—
		JU	07-Apr-2012	673	—	—	—
		JU	25-Feb-2013	997	Patoc 1:400	—	—
		JU	08-Feb-2014	1345	Patoc 1:100	—	—
		JU	15-Jul-2014	1502	—	—	—
Jovenal [R]	M	CA	07-Apr-2012	Single sample	Patoc 1:200	—	—
Benguela [R]	M	CA	07-Apr-2012	Single sample	Patoc 1:100	—	—
Alagoilton [R]	M	JU	07-Apr-2012	Single sample	Patoc 1:100	—	—
Negão [R]	M	JU	07-Apr-2012	Single sample	Patoc 1:100	—	—
Helena [R]	F	JU	07-Apr-2012	0	Patoc 1:100	—	—
		JU	25-Feb-2013	324	Patoc 1:400	—	—
		JU	07-Feb-2014	671	Patoc 1:100	—	—
		JU	15-Jul-2014	829	—	—	—
		JU	24-Oct-2014	930	Canicola 1:100, Tarassovi 1:100, Patoc 1:200	—	—
		JU	21-Nov-2014	958	—	—	—
		JU	15-Dez-2014	982	Patoc 1:100	—	—
Vilemar [R]	M	CA	25-Feb-2013	Single sample	—	—	—
Luna [R]	F	CA	15-Jul-2013	0	—	—	—
		CA	22-Jul-2013	7	—	—	—
		CA	07-Feb-2014	207	—	—	—
		CA	27-Aug-2014	408	Patoc 1:100	—	—
		CA	21-Nov-2014	494	—	—	—

Jerusa [R]	F	CA	25-Feb-2013	0	—	—	—
		CA	07-Feb-2014	319	—	—	—
		CA	27-Aug-2014	520	—	—	—
Jurema [R]	F	CA	25-Feb-2013	0	—	—	—
		CA	09-Apr-2013	43	—	—	—
		JU	07-Feb-2014	347	—	—	—
		JU	15-Jul-2014	505	—	—	—
		JU	21-Nov-2014	634	—	—	—
Castanha [R]	F	CA	25-Feb-2013	0	—	—	—
		CA	09-Apr-2013	43	—	—	—
		CA	07-Feb-2014	347	—	—	—
		JU	15-Jul-2014	505	—	—	—
Valentina [R]	F	CA	27-Jan-2014	Single sample	—	—	—
Cassí [R]	F	CA	27-Aug-2014	0	—	—	—
		CA	20-Oct2014	40	—	—	—
		CA	21-Nov-2014	54	—	—	—
		CA	06-Dez-2014	86	—	—	—

1

Table 2. Samples from Amazon river dolphins (*Inia geoffrensis*), tucuxi (*Sotalia fluviatilis*), and Amazonian manatees (*Trichechus inunguis*) subjected to multiplex polymerase chain reaction (mPCR) to *Leptospira* spp. and *Brucella* spp., and microbiological culture. Animal identification, species, sex, age class, samples tested, and diagnostic tests results are reported. M: male; F: female; ND: not determined; JU: juvenile; AD: adult; —:negative.

Case #	Animal ID	Sex	Age class	Sample	mPCR	Bacterial culture
Inia geoffrensis						
1	WY404 Aranapu V. Iriarte	F	AD	Milk	—	—
2	RDSM Boa União V.Iriarte	F	ND	Uterine fluid	—	*Enterococcus faecalis*
3	EEM Ig 9601 200cm	F	AD	Milk	—	*Bacillus* spp.
4	Abial Lago Tefé	ND	ND	Liver	—	—
5	IDSM Ig 29/11/13	F	JU	Lymph node	—	*Enterococcus faecalis*
				Ovaries	—	—
6	Ig 30/01/2014	F	AD	Lymph node	—	—
7	Ig 17/01/2014	M	AD	Abscess	—	—
8	Ig 3/2014	M	AD	Spleen	—	—
				Liver	—	—
9	Ig 27/09/2014	M	JU	Synovial fluid	—	*Bacillus* spp.
				Abscess	—	—
10	Ig2015/03	ND	ND	Spleen	—	—
				Uterus	—	—
11	Ig Lago Tefé 01/06/2016	F	JU	Ovaries	—	—
				Spleen	—	—
12	Ig Muralha 08/07/2016	ND	AD	Lung	—	*Enterococcus faecalis*
Sotalia fluviatilis						
13	IDSM Sf 02-2014 22/05/2014	ND	ND	Lung	—	—
				Abscess	—	—
Thichechus inunguis						
14	Maria Coló	F	JU	Abscess	—	—
15	A2005-6 IDSM	F	AD	Milk	—	*Bacillus* spp.

Table 3: Species, serogroups, and serovars of *Leptospira* spp. used as antigens for the microscopic agglutination test (MAT).

Species	Serogroup	Serovar
L. biflexa	Andamana	Andamana
	Semarang	Patoc
L. borgpetersenii	Javanica	Javanica
	Tarassovi	Tarassovi
	Celledoni	Whitcombi
L. interrogans	Autumnalis	Autumnalis
	Australis	Australis
	Bataviae	Bataviae
	Australis	Bratislava
	Canicola	Canicola
	Icterohaemorrhagiae	Copenhageni
	Djasiman	Djasiman
	Sejroe	Hardjo
	Hebdomadis	Hebdomadis
	Icterohaemorrhagiae	Icterohaemorrhagiae
	Pomona	Pomona
	Pyrogenes	Pyrogenes
	Djasiman	Sentot
	Sejroe	Wolffi
L. kirschneri	Cynopteri	Cynopteri
	Grippotyphosa	Grippotyphosa
L. noguchii	Panama	Panama

Figure 1. Map of the study area in the mid-Solimões River, showing the Amanã and Mamirauá Sustainable Development Reserves (ASDR and MSDR), Lake Tefé, and the Community-based Amazonian Manatee Rehabilitation Center (Centrinho), Amazonas state, Brazil.

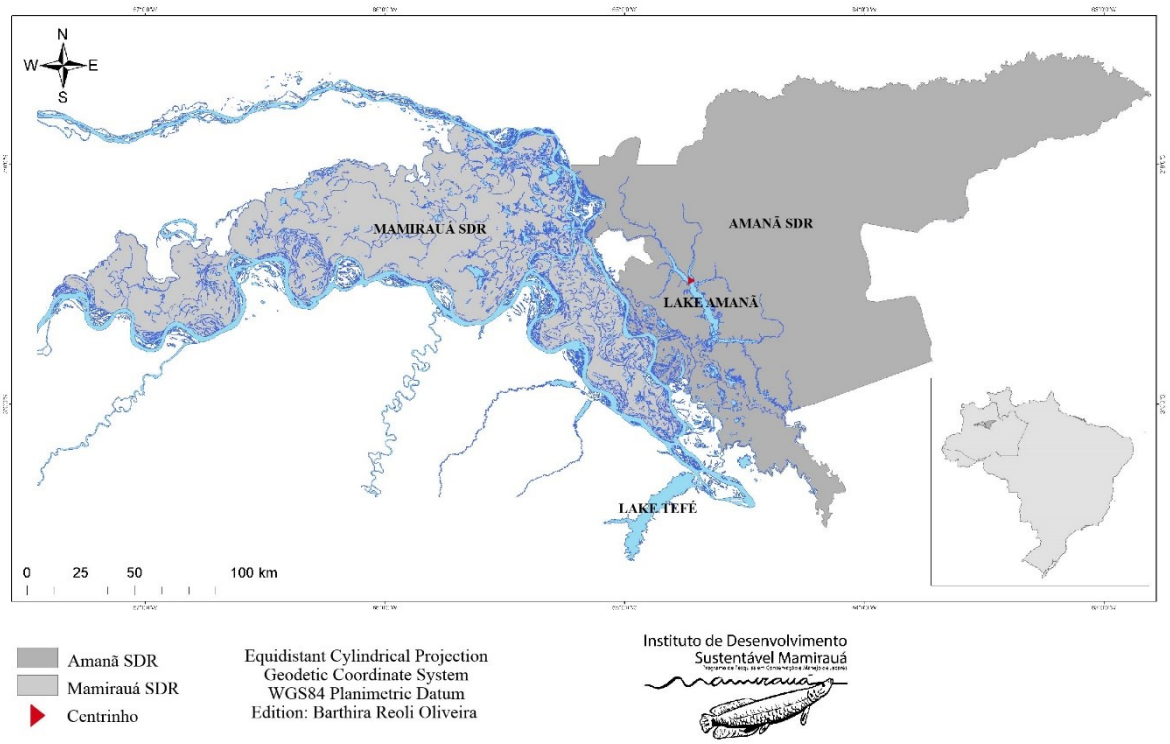
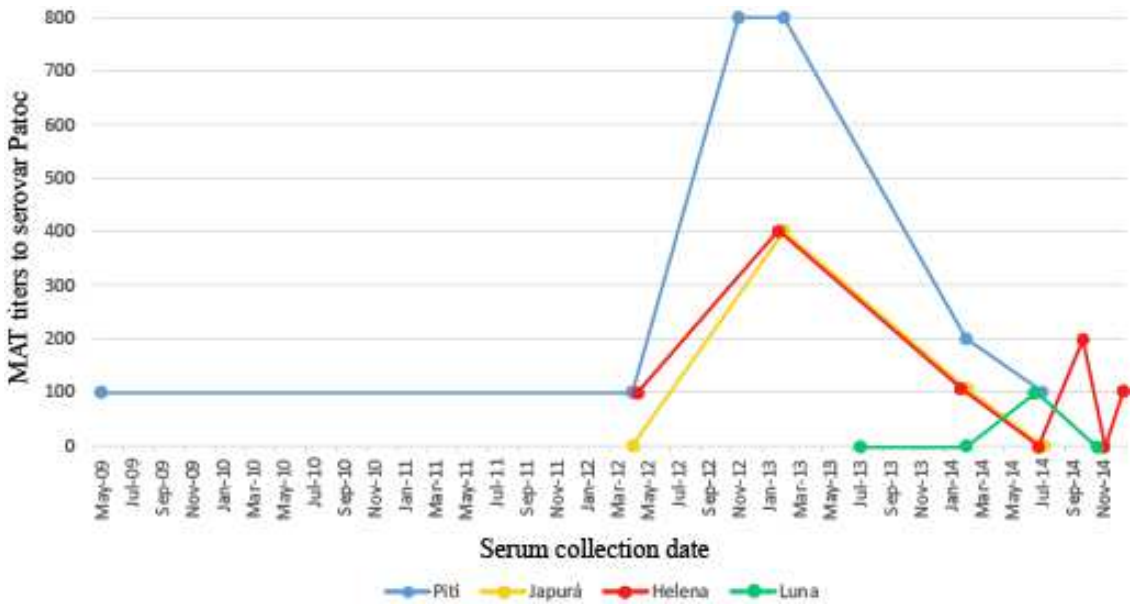


Figure 2. Microscopic agglutination titers anti-*Leptospira* sp. serovar Patoc in serum of four Amazonian manatees (*Thichechus inunguis*) in rehabilitation at the Community-based Amazonian Manatee Rehabilitation Center (Centrinho), Amazonas state, Brazil.



CAPÍTULO 3- Molecular detection of *Toxoplama gondii* in Amazon river dolphins (*Inia geoffrensis*) and tucuxi (*Sotalia fluviatilis*), and anti-*T. gondii* antibody survey in Amazonian manatees (*Trichechus inunguis*), Western Brazilian Amazon

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Short Communication

Molecular detection of *Toxoplama gondii* in Amazon river dolphins (*Inia geoffrensis*) and tucuxi (*Sotalia fluviatilis*), and anti-*T. gondii* antibody survey in Amazonian manatees (*Trichechus inunguis*), Western Brazilian Amazon

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Abstract: Amazonian manatees (*Trichechus inunguis*), Amazon river dolphins (*Inia geoffrensis*), and tucuxis (*Sotalia fluviatilis*) are aquatic mammals endemic to the Amazon Basin, classified as Vulnerable, Endangered, and Data Deficient, respectively. Although *Toxoplasma gondii* is an important pathogen in marine mammals and highly prevalent in the Amazonian environment, little is known about infection in Amazonian aquatic mammals. The present study was designed to investigate the presence of *T. gondii* in tissue samples of Amazonian cetaceans by polymerase chain reaction assay, and seroprevalence of anti-*T. gondii* antibodies in Amazonian manatees by indirect haemagglutination test. We provide de first reports of molecular identification of *T. gondii* in heart samples of Amazon river dolphins and in the heart and brain samples of a tucuxi, in addition to significant titer of anti-*T. gondii* antibodies in a wild Amazonian manatee. Our results highlight the importance of zoonotic disease surveillance in Amazonian aquatic mammals as sentinels of environmental health. Further research to access the clinical significance of *T. gondii* infection in Amazonian aquatic mammals and its potential impacts on population conservation is warranted.

Keywords: Zoonosis, toxoplasmosis, Cetacea, Sirenia, aquatic mammal, wildlife, heart, brain,

Amazonian manatees (*Trichechus inunguis*), Amazon river dolphins (*Inia geoffrensis*), and tucuxis (*Sotalia fluviatilis*) are aquatic mammals endemic to the Amazon Basin. *I. geoffrensis* is an Endangered species (Da Silva et al., 2018a) with estimated population decline of 70.4% in a period of one generation in the Mamirauá Sustainable Development Reserve (MSDR), Brazilian Amazon (Da Silva et al., 2018b). Due to limited knowledge on ecology, population, and threats, *S. fluviatilis* is classified as Data Deficient by the International Union for Conservation of Nature (IUCN) (Secchi 2012), while considered Near Threatened in Brazil (ICMBio 2011). High population size reduction was also reported for

tucuxis in the MSDR (Da Silva et al., 2018b). The Amazonian manatee is listed as Vulnerable by the IUCN based on suspected population decline of at least 30% within the next three generations (Marmontel et al., 2016). The three species are regarded as indicators of the Amazon's aquatic ecosystem quality and may indicate the presence of pathogens in the environment (Bonde et al., 2004; ICMBio 2011). Although it is suggested that Amazonian aquatic mammals are susceptible to several infectious pathologies that could contribute to population decline, there is insufficiency of data on the diseases that affect such species (Rodrigues et al. 2018).

Toxoplasma gondii is a worldwide spread zoonotic protozoan parasite reported to play a role in morbidity and mortality in manatees, cetaceans, and pinniped species (Dubey et al. 2003; Bossart et al. 2012; Roe et al. 2017). Although a few studies indicate exposure to *T. gondii* Amazonian aquatic mammals (Santos et al. 2011; Mathews et al. 2012; Delgado et al. 2013), little is known about infection among these species. *T. gondii* is highly prevalent in humans in Brazil and very common in the Amazon (Pappas et al. 2009). One of the highest *T. gondii* prevalence rates worldwide was reported in an indigenous population of the Amazon River basin (Bóia et al. 2008). Amazonian environmental changes due to deforestation may facilitate genetic flow between wild and domestic strains of *T. gondii* generating more virulent strains that may impact animal and public health (Mercier et al. 2011). Therefore, extensive interdisciplinary surveillance of the pathogen is warranted (Confalonieri et al. 2014). Herein, we report molecular and serological investigation of *T. gondii* in Amazonian aquatic mammals in Western Brazilian Amazon.

Fragments of heart (n=9) and brain (n=1) were collected from carcasses of eight free-ranging Amazon river dolphins and a tucuxi (Table 1), opportunistically encountered in the areas of Amanã and Mamirauá SDRs (ASDR and MSDR) and Lake Tefé, Amazonas state, Brazil. Genomic DNA was extracted from tissue samples using the Illustra Tissue and Cells

GenomicPrep Mini Spin Kit according to manufacturer's instructions (GE Healthcare) and quantified in spectrophotometer (Thermo Scientific NanoDrop 1000). *T. gondii* DNA in tissue samples was assessed by a polymerase chain reaction assay (PCR) described previously (Wahab et al., 2010).

Serum samples were collected from 12 Amazonian manatees undergoing rehabilitation at the Community-based Amazonian Manatee Rehabilitation Center (Centrinho) and kept in a wooden floating pen within Lake Amanã, in the ASDR, with water flow-through from the lake and locally collected vegetation. Additional serum samples were collected from five free-living adult Amazonian manatees captured at the ASDR during a capture-release tagging study. The collected sera (Table 1) were subjected to an indirect haemagglutination test assay (IHA) for detection of total anti-*T. gondii* antibodies using a Toxotest HAI commercial kit (Wiener lab.), following manufacturer's instructions. Samples were tested in two-fold serial dilutions up to 1:64.

T. gondii DNA was identified in the heart samples of four Amazon river dolphins (4/8, 50%) and in the brain and heart tissues of the tucuxi (Table 1). Although high seroprevalence of antibodies against *T. gondii* was reported on free-living Amazon river dolphins in MSDR (82/95, 86.3%) in a previous study (Santos et al. 2011), the present study represents the first report of molecular identification of the parasite in river dolphins. Evidence of *T. gondii* infection in *S. fluviatilis* had never been reported. *T. gondii* was identified by PCR in multiple tissues, including heart and brain, of Hector's dolphins (*Cephalorhynchus hectori*), an endangered coastal species, that died due to disseminated toxoplasmosis (Roe et al. 2013). Cerebral toxoplasmosis was reported in striped dolphins (*Stenella coeruleoalba*) that stranded in Italy presenting nonsuppurative meningoencephalitis, high *T. gondii* antibodies serum titers, and immunohistochemical evidence of cysts and tachyzoites in the brains (Di Guardo et al. 2010). Bossart et al. (2012) reported necrotizing myocarditis in cases of disseminated

toxoplasmosis in Antillean manatees (*Trichechus manatus manatus*). Toxoplasmosis is also frequently considered a secondary disease in cetaceans, becoming symptomatic only when hosts are immunosuppressed (Roe et al., 2013). High loads of environmental pollutants reported in Amazonian dolphins, especially high levels of mercury (Lailson-Brito Jr et al. 2008), are known to induce immunosuppression in marine mammals (Desforges et al. 2016).

Anti-*T. gondii* antibodies were found in 6/17 (35.3%) Amazonian manatees in the present study. Most samples presented low titers (1:8 and 1:16) and one wild captured manatee presented a 1:64 titer (Table 1). The only previous study conducted in Amazonian manatees in Brazil reported similar seroprevalence: 39.2% (Mathews et al. 2012). In the Peruvian Amazon the seroprevalence in Amazonian manatees in rehabilitation was higher (63.2 %) (Delgado et al. 2013). Both previous studies reported only low titers, indicating exposure to *T. gondii*. Titers ≥1:64 are the cut off parameter for serological survey of toxoplasma infection in domestic animals (Figueiredo et al. 2001). In humans, titers ≥1:64 are suggestive of recent *T. gondii* infection (Karim & Ludlam 1975).

Our study provides novel evidence of infection of river dolphins by *T. gondii* and significant seroconversion in a manatee. By detecting *T. gondii* in dolphin's tissues, we also demonstrate presence of the protozoan in the Amazonian aquatic ecosystem and highlight the importance of zoonotic disease surveillance in Amazonian aquatic mammals as sentinels of environmental health. Further research is needed to determine if *T. gondii* infection in Amazonian aquatic mammals is associated with clinical disease and its potential impacts on population dynamics and conservation.

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Table 1: Samples from Amazon river dolphins (*Inia geoffrensis*), tucuxi (*Sotalia fluviatilis*), and Amazonian manatees (*Trichechus inunguis*) subjected to polymerase chain reaction (PCR) to *Toxoplasma gondii* and indirect haemagglutination test (IHA) for detection of total antibodies against *T. gondii*, Brazilian Amazon. Animal species, case number, date of sample collection, sex, age class, and results for PCR and IHA (serum titers) are reported. M: male; F: female; nd: not determined; J: juvenile; A: adult; +: positive; —: negative; nt: not tested.

Case #	Collection date	Sex	Age class	Sample	PCR	IHA
Inia geoffrensis						
1	29-Nov-2013	F	J	Heart	+	nt
2	27-Sep-2014	M	J	Heart	+	nt
3	25-Nov-2014	M	A	Heart	—	nt
4	28-Jan-2015	nd	nd	Heart	—	nt
5	29-Jan-2015	nd	nd	Heart	—	nt
6	04-Feb-2015	nd	nd	Heart	—	nt
7	01-Jun-2016	nd	nd	Heart	+	nt
8	08-Jul-2016	nd	nd	Heart	+	nt
Sotalia fluviatilis						
9	17-Jan-2014	nd	nd	Heart	+	nt
				Brain	+	nt
Trichechus inunguis **(free-living)**						
10	2005	F	J	Serum	nt	—
11	2005	M	A	Serum	nt	—
12	2005	M	A	Serum	nt	—
13	2005	F	J	Serum	nt	—

14	2005	nd	nd	Serum	nt	1:64
***Trichechus inunguis* (in rehabilitation)**						
15	16-May-2009	M	J	Serum	nt	1:8
16	07-Apr-2012	M	J	Serum	nt	1:8
17	07-Apr-2012	F	J	Serum	nt	1:8
18	07-Apr-2012	M	J	Serum	nt	—
19	27-Jan-2014	F	J	Serum	nt	—
20	07-Feb-2014	F	J	Serum	nt	1:16
21	07-Feb-2014	F	J	Serum	nt	1:16
22	08-Feb-2014	M	J	Serum	nt	—
23	07-Feb-2014	F	J	Serum	nt	—
24	07-Feb-2014	F	J	Serum	nt	—
25	07-Feb-2014	F	J	Serum	nt	—
26	06-Oct-2014	F	J	Serum	nt	—

ANEXO A

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 55144-2	Data da Emissão: 08/12/2016 23:48	Data para Revalidação*: 07/01/2018

* De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Thaís Carneiro Santos Rodrigues	CPF: 016.100.276-55
Título do Projeto: Avaliação do perfil sanitário de mamíferos aquáticos	
Nome da Instituição : INSTITUTO DE DESENVOLVIMENTO SUSTENTAVEL MAMIRAUA	CNPJ: 03.119.820/0001-95

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta oportunítica de carcaças	08/2016	08/2018
2	Coleta oportunítica de carcaças	08/2016	08/2018
3	Elaboração de artigos e comunicações científicas	08/2016	08/2018
4	Análises laboratoriais de amostras mamíferos aquáticos amazônicos	08/2016	08/2018
5	Coleta de amostras de mamíferos aquáticos amazônicos	08/2016	08/2018

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
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5	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
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8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	ANNA MONTEIRO CORREIA LIMA RIBEIRO	Executora	966.309.496-68	5420105 SSP-MG	Brasileira
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7	Camila Carvalho de Carvalho	Auxiliar	396.826.068-61	35045008-0 SSP-SP	Brasileira
8	Eliana Scarcelli Pinheiro	Executora	130.177.378-61	13261822-9 SSP-SP	Brasileira
9	DANIELLE DOS SANTOS LIMA	Executora	008.744.446-16	7386876 SSP-MG	Brasileira

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Dados do titular

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Nome da Instituição : INSTITUTO DE DESENVOLVIMENTO SUSTENTAVEL MAMIRAUA	CNPJ: 03.119.820/0001-95

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1	TEFE	AM	Instituto de Desenvolvimentos Sustentável Mamirauá	Fora de UC Federal

1	Amostras biológicas	Regurgitação/conteúdo estomacal, Urina, Sangue, Animal encontrado morto ou partes (carcaça)/osso/pele, Outras amostras biológicas, Fezes, Fragmento de tecido/órgão
2	Método de captura/coleta	Captura manual

Atividades X Táxons

#	Atividade	Táxons
1	Coleta/transporte de amostras biológicas ex situ	Sotalia fluviatilis, Trichechus, Inia geoffrensis
2	Coleta/transporte de amostras biológicas in situ	Sotalia fluviatilis, Trichechus, Inia geoffrensis

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	INSTITUTO DE DESENVOLVIMENTO SUSTENTAVEL MAMIRAUA	
2	INSTITUTO BIOLOGICO	
3	Universidade Federal de Uberlândia	

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Nome da Instituição : INSTITUTO DE DESENVOLVIMENTO SUSTENTAVEL MAMIRAUA	CNPJ: 03.119.820/0001-95

Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº 03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

* Identificar o espécime no nível taxonômico possível.

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ANEXO B

Inter-Research Science Publisher

Inter-Research > Journals > Guidelines_Authors

Diseases of Aquatic Organisms

1. Manuscript length and structure

The target lengths for the different manuscript types (Research Article, Note, Review, Comment, Reply Comment and Opinion Piece) are listed in 'Author guidelines'.

Manuscripts should be structured as follows:

- Title page (title, author list, author affiliations, corresponding author email, running page head, abstract, key words)
- Main text (see below)
- Acknowledgements
- Literature cited
- Tables (with legends)
- Figures (with legends)
- Appendices (length: up to 2 printed pages; longer materials should be submitted as online supplementary materials in a separate file)

Main text:

- **Research Articles** and **Notes** should follow the IMRAD format (Introduction, Materials & Methods, Results, Discussion and an optional Conclusions section). Note that separate Results and Discussion sections are strongly preferred, but exceptions are possible if the manuscript's content warrants a combined Results & Discussion section.
- **Reviews**, **Comments**, **Reply Comments** and **Opinion Pieces** may deviate from the IMRAD format as necessary.

2. Title page

Title: The title should be concise and informative, i.e. summarizing either the subject or the most important findings of the study rather than merely the hypothesis addressed. It should

have around 100 characters (ca. 15 words), and 150 characters at most (including spaces). Avoid 'A', 'An', 'The', 'On', etc. at the beginning. Avoid questions in the title.

Provide a **running page head** with 3 to 6 words; e.g. 'Detection of shrimp WSSV'.

Authors and addresses: If a manuscript has several authors from different institutions:

- use superscript numerals for identification;
- provide the address of each author's institution, identifying any present address(es) if applicable. Include zip or postal code but not street address or box number;
- use an asterisk (*) to refer to a footnote that identifies the single corresponding author and provide her/his e-mail.

Abstract: Limit length to 250 words. Provide concise information on your work, its significance and its principal results. Avoid literature cites, series of data, or meaningless clauses such as 'the results are discussed'.

Key words: Supply 3 to 8 key words, listed in order of importance.

3. Text

Please number all pages and restart line numbering on each page (important: do not use ‘continuous’ line numbering), 12 point font, double spacing and numbered sections. Manuscripts that do not use correct English grammar, spelling and punctuation will be returned to authors without review; if you are not a native English speaker, you should have the text edited by someone who is, before submitting your manuscript. You may also wish to consult a 'How to' book such as Day & Gastel (2011; How to write and publish a scientific paper, 7th edn. Greenwood Press, Santa Barbara, CA).

Section headings: Main sections (IMRAD) should be numbered '1. INTRODUCTION’, ‘2. MATERIALS & METHODS’, etc. Subsections should be numbered as e.g. '2.1. Study site', ‘2.2. Sample collection’, etc. Avoid going beyond third-level subsections (e.g. 3.1.1.).

Verbosity: Please eliminate verbiage; example:

Verbose – 'The speed was chosen because past studies by Miller (1995) and Smith (1998) have shown this to be slightly greater than the maximum sustained swimming speed.'

Not verbose – 'The speed is slightly greater than the maximum sustained swimming speed (Miller 1995, Smith 1998).'

Verbose – 'It has been shown that boat noise affects whale behaviour (Smith 1994).' (and similar phrases such as 'it has been reported/found that', 'it is possible/suspected that', 'results show that')
Not verbose – 'Boat noise influences whale behaviour (Smith 1994).'

Genus and species names must be in italics; write the genus name in full at first mention in each section (Abstract, Introduction, Materials and Methods, Results, Discussion) and abbreviate whenever mentioned again in the same section. When referring to a species, do not use the genus name alone, unless you have previously defined it that way; be precise when using 'sp.' (singular) and 'spp.' (plural).

At first mention in a section – 'The filter feeding of blue mussels Mytilus edulis was examined'.
After first mention in a section – 'Filter feeding rates of M. edulis increased with increasing temperature.'

Sequence data: Full sequence information is required when molecular methods are used. The sequences of novel primers must be given. Novel nucleotide or protein sequences must be deposited in the GenBank, EMBL or DDBJ databases and an accession number obtained.

Conservation Evidence: If your study is testing for an intervention, please check the existing evidence for your intervention at www.conservationevidence.com. State whether or not relevant evidence is available there, and if so, briefly summarise it in your Introduction. Otherwise, search for individual studies testing the action, and summarise any relevant evidence. If Conservation Evidence does not yet cover the topic and no individual studies exist, please state this; you may briefly reference other relevant literature, but this is not essential.

Ocean acidification data reporting: When presenting methods and results reporting ocean acidification the 'Guide to best practices for ocean acidification research and data reporting' must be followed. Specifics for reporting ocean acidification data in scientific journals are outlined in Annex 1 of the 2015 addendum.

Abbreviations: Define abbreviations and acronyms in the Abstract and at first mention in the main text, and thereafter use only the abbreviation / acronym.

Equations and units: Use standard SI units. Relations or concentrations (e.g. mg per l) must be given as 'mg l^{-1}' (not mg/l). Variables are usually italicised (except for Greek letters). Italicisation should be consistent in normal, superscript and subscripted text. Example of proper spacing: '$p < 0.05$, $r^2 = 0.879$' (not '$p<0.05$, $r^2=0.879$'); but: 'we studied organisms of size <0.5 μm'

Figures and tables

Please consult 'Figures' for details.
Figures, tables, and their captions should be self-explanatory; e.g. abbreviations and acronyms must be defined here. For table footnotes, use superscripted lower case letters; asterisks can be used to indicate statistical significance (must be defined in the legend).

4. Acknowledgement section

Declare all sources of funding of the study. In addition, you may wish to acknowledge any assistance you received from anyone not listed as author. Include this section before your Literature Cited.

5. References

All references cited in the text must be listed in the Literature Cited section, and all listed literature must appear in the text, using Harvard (Name-Year) referencing style. Citing references as 'in press' implies that the article has been accepted for publication; if pagination information is not available yet, the DOI should be included in the citation in the Literature Cited section. Unpublished results and submitted articles should be cited as: author's name

unpub. data (e.g. N. Smith unpubl. data) in the text only.

a. Within the text

2 authors: use ‘&' between last names
3 or more authors: use the first author’s last name followed by ‘et al.’ (not italicized and not separated by a comma)
If the same first author published multiple papers in the same year and the within-text citations would look identical, distinguish these citations with a lower case letter (a, b, c, etc.) after the year.
Multiple citations within a single bracket: separate cites with a comma (not a semi-colon). Sort multiple cites chronologically from oldest to newest and if several cites are from the same year, sort them alphabetically.
Examples:
(Smith & Miller 1965, Ahmed 1968, Miller et al. 2000a)
(Burns 2000, Miller et al. 2000a,b, Quinn 2000, Barni in press)

b. Literature Cited section

Format required for citing literature (examples):

Periodicals: Use standard abbreviations according to 'BIOSIS Serial Sources' or use the style for your selected journal in a reference managing software. A list of over 30000 journal names and BIOSIS abbreviations can be found here. In addition, Endnote users may download styles for IR journals in this zip file for import into reference managing software.

- Dempster T, Holmer M (2009) Introductory editorial. Aquacult Environ Interact 1:1–5

Books: Write the title of the book in lower case, and give the publisher and place of publication. In the case of book series, give the series editor as well. Example:

- Hanski I (2005) The shrinking world: ecological consequences of habitat loss. In: Kinne O (ed) Excellence in ecology, Book 14. International Ecology Institute, Oldendorf/Luhe

Papers from books, conference reports, symposium proceedings, etc.: Give the title of the chapter, the editor(s) and title of the volume, the publisher and place of the publisher (not the location where the conference was held), and the pages of the chapter. The date cited must be the year of publication (not the year in which the conference was held). Example:

- West TL, Amrose WG (1992) Abiotic and biotic effects on population dynamics of oligohaline benthic invertebrates. In: Colombo G, Ferrari I, Ceccherelli VU, Rossi R (eds) Marine eutrophication and population dynamics. Proc 25th Eur Mar Biol Symp. Olsen & Olsen, Fredensburg, p 189–194

Dissertations: Write the title in lower case, the type of thesis / dissertation (e.g. MS / MSc / PhD), and give the university and its location. Example:

- Eve TM (2001) Chemistry and chemical ecology of Indo-Pacific gorgonians. PhD dissertation, University of California, San Diego, CA

Websites: Permanent databases such as FishBase, GenBank, or climatological sources may be included in the Literature Cited list; the access date must be given. URLs for printed publications also available online may be included with their citations. Example:

- Froese F, Pauly D (2009) FishBase. www.fishbase.org (accessed 13 Jan 2013)

Other website references should only be cited in the body text.

6. Final checklist

Please cross-check your manuscript using this list. Consult recent IR publications as a general guide for formatting:

Include page numbers and line numbers (restart on each page)

- Ensure that abbreviations are defined at first mention in the abstract, main manuscript Included text and figure/table legends, and that the legends are informative
- Use periods instead of commas as decimal signs
- Correctly present your statistical results (e.g. include two sets of degrees of freedom for ANOVA results and significance/p-values of regressions)
- Cite all of the references in the text and vice versa
- Correctly label your figure axes with a title and a unit where applicable

- Create legible figures: i.e. large enough font size (at least 10 pt) with sufficient resolution for pdf viewing
- Prepare the manuscript (text, figures and tables) as a single file

ANEXO C

JOURNAL OF
WILDLIFE DISEASES

INFORMATION FOR AUTHORS AND REVIEWERS

GENERAL INFORMATION

The Journal of Wildlife Diseases (JWD) is published quarterly by the Wildlife Disease Association (WDA). The WDA is an international organization of scientists, academicians, wildlife and fisheries specialists, and others whose mission is to acquire, disseminate, and apply knowledge of the health and diseases of wild animals in relation to their biology, conservation, and interactions with humans and domestic animals. For a listing of the JWD Editor, Assistant Editors, and Editorial Board see:
http://www.jwildlifedis.org/site/misc/edboard.xhtml
The JWD publishes reports of wildlife disease investigations, research papers, brief research notes, case and epizootic reports, review articles, and book reviews. The JWD publishes the results of original research and observations dealing with all aspects of infectious, parasitic, toxic, nutritional, physiologic, developmental and neoplastic diseases, environmental contamination, and other factors impinging on the health and survival of free-living or occasionally captive populations of wild animals, including fish, amphibians, reptiles, birds, and mammals. Papers on zoonoses involving wildlife and on chemical immobilization of wild animals are also published. Manuscripts dealing with surveys and case reports may be published in the Journal provided that they contain significant new information or have significance for better understanding health and disease in wild populations. Authors are encouraged to address Conservation, wildlife management, or One Health implications of their studies, when appropriate.
Manuscripts should be submitted with the understanding that the information and ideas are original (with the exception of reviews), have not been published previously, and are not being considered for publication elsewhere. Authors also affirm that, to the best of their knowledge, they have complied with all applicable treaties, laws, and regulations in the collection, maintenance, and disposal of animals used in their study. Authors of manuscripts

that are not in the public domain must transfer full copyright interests to the WDA. Forms for this transfer are provided when proofs are sent to authors.

EDITORIAL AND ETHICAL POLICIES

The WDA serves the wildlife disease profession and society at large in several ways, including publication of the results of scientific research in JWD. The Editor of the Journal has the responsibility to establish and maintain guidelines for selecting and accepting papers submitted to the Journal. These guidelines derive from the WDA's definition of the scope of the Journal and from the Editor's perception of standards of quality for scientific work and its presentation.

An essential feature of a profession is the acceptance by its members of a code that outlines professional behavior and specifies obligations of members to each other and to the public. This code stems from a desire to maximize the benefits to the wildlife disease profession and society at large, and to limit actions that serve self interests of individuals.

This set of ethical guidelines is presented for those involved in the publication of research in the Journal of Wildlife Diseases. These guidelines are offered from a conviction that the observance of high ethical standards is so vital to the scientific enterprise that a definition of those standards should be brought to the attention of all concerned. Also, reaching a common understanding on the responsibilities of editors, authors, assistant editors, and manuscript reviewers, and on their expectations of each other, can help all parties work together more effectively in fulfilling the Journal's mission to the wildlife disease profession and the public.

Endangered Species and Humane Treatment of Animals

The JWD, as the primary publication of the WDA, subscribes to the rules, regulations, and laws as established by national and international agencies of all countries represented by WDA membership.

ETHICAL RESPONSIBILITIES OF AUTHORS

The responsibilities outlined for authors and reviewers are reprinted in large part with permission from "Ethical Guidelines to Publication of Chemical Research," Chemical Reviews 95: pp. 11A-13A (1995). Copyright 1985, 1989, 1995 American Chemical Society.

1. The ultimate responsibility for all material published in the manuscript lies with the authors. An author is obligated to present an accurate account of the research performed and an objective discussion of its significance. All work must be free of plagiarism, falsification, fabrications, or omission of significant material.

2. Because Journal space is limited and costly, an author has an obligation to use it wisely and economically.

3. A primary research report should contain sufficient detail and reference to public sources of information to permit the evaluation and repetition of the study by skilled workers.

4. An author should cite those publications that have been influential in determining the nature of the reported work and that will guide the reader quickly to the earlier work that is essential for understanding the present investigation. Conflicting evidence from the work of others should be included to help readers judge the soundness of the conclusions presented in the manuscript. Except in a review, citation of work that is not essential to building a foundation or interpreting the reported research should be avoided.

5. Any previously unrecognized and unusual hazards identified in an investigation should be clearly noted in a manuscript in which that work is reported.

6. Authors are responsible to be aware of, and adhere to, all laws, treaties, and regulations currently applying to their work. This includes the review and approval of the research protocol by an institutional animal care and use committee, where applicable, and the acquisition of all appropriate permits.

7. Fragmentation of research reports should be avoided. A scientist who has done extensive work on a system or group of related systems should organize the publications so that each report gives a well-rounded account of a particular aspect of the general study. Fragmentation excessively consumes Journal space and unduly complicates literature searches. The convenience of readers is served if reports on related studies are published in the same journal, or a small number of journals.

8. Research findings should not be presented as original material in more than one scientific publication. It is inappropriate for an author to submit manuscripts describing essentially the same research to more than one journal, except for the resubmission of a manuscript rejected by, or withdrawn from, another journal.

9. In submitting a manuscript for publication, an author should inform the editor of related manuscripts that the author has under editorial consideration or in press. The relationships of

such manuscripts to the one submitted should be clarified, and copies of the related manuscripts should be included with the manuscript submission.

10. An author should identify the source of all information quoted or offered, except that which is common knowledge. Information obtained privately, as in conversation, correspondence, or discussion with third parties, should not be used or reported in the author's work without explicit permission from the investigator with whom the information originated, usually by a personal communication. Information obtained in the course of professional services, such as reviewing manuscripts or grant applications, should also be treated as confidential.

11. Strong criticism of the work of another scientist may be given. However, in no case is sarcasm or criticism of a personal nature appropriate. Authors of a criticized work will have the opportunity to respond.

12. Coauthors of a paper should be only those persons who have made significant scientific contributions to the work reported and who share responsibility and accountability for the results. Other contributions should be indicated in the Acknowledgments section. Deceased persons who meet the criterion for inclusion as coauthors should be so included, with a footnote indicating their death. No fictitious name should be included as an author or coauthor. The author who submits a manuscript for publication accepts the responsibility of having included as coauthors all persons appropriate and none inappropriate. Prior to submission of the manuscript, the corresponding author should have sent each coauthor a draft copy of the manuscript and obtained assent to coauthorship from each coauthor. Manuscripts submitted to JWD that have not been approved by all authors may be rejected without review.

13. All funding sources should be identified in the manuscript. Authors should disclose to the Editor any potential conflict of interests, such as consulting or financial interest in a company that might be affected by publication of the results contained in a manuscript. Authors should ensure that no contractual relations or proprietary considerations exist that would affect the publication of information in a submitted manuscript.

14. When appropriate, representative biological material should be deposited in a nationally or internationally recognized professional museum. Accession numbers should be reported in the manuscript.

ETHICAL RESPONSIBILITIES OF REVIEWERS

1. A Reviewer should submit a report in a timely manner. If circumstances preclude prompt attention to a manuscript, the Reviewer should decline the request to review. Alternatively, the Reviewer should notify the Assistant Editor (AE) or Editor of probable delays and propose a revised completion date for the review.
2. A Reviewer who feels inadequately qualified to judge the research reported in a manuscript should promptly notify the AE (or Editor) with a brief explanation.
3. A Reviewer should judge objectively the quality of the manuscript, its experimental and theoretical work, and its interpretations and its exposition, with regard to the maintenance of high scientific and literary standards. A Reviewer should respect the intellectual independence of the Authors.
4. A Reviewer should be sensitive to the potential for a conflict of interest when the manuscript under review is closely related to the Reviewer's work in progress or published. If in doubt, the Reviewer should promptly advise the AE (or Editor). Alternatively, the Reviewer may furnish a review stating the Reviewer's interest in the work, with the understanding that it may, at the Editor's discretion, be transmitted to the Author.
5. A Reviewer should decline to evaluate a manuscript authored or coauthored by a person with whom the reviewer has a personal or professional connection if the relationship would bias judgment of the manuscript.
6. A Reviewer should decline to evaluate a manuscript if the Reviewer may experience a possible financial gain or loss with publication of that manuscript if this financial connection would bias judgment of the manuscript.
7. A Reviewer should treat each manuscript received as a confidential document. It should neither be shown to nor discussed with others except, in special cases, persons from whom specific advice is being sought; in that event, the identities of those consulted should be disclosed to the AE or Editor.
8. Reviewers should explain and support their judgments adequately so that AE, the Editor, and Authors may understand the reasons for their comments. Any statement that an observation, derivation, or argument in the manuscript was reported previously should be accompanied by the relevant citation. Unsupported assertions by Reviewers (or by Authors in rebuttal) should be avoided.
9. A Reviewer should comment if the authors fail to cite relevant work by other scientists.

10. A Reviewer should call to the AE's attention any substantial similarity between the manuscript under consideration and any published paper or any manuscript submitted concurrently to another journal.
11. Although not specifically encouraged, Reviewers may divulge their identity to the authors.
12. Reviewers should not use or disclose unpublished information, arguments, or interpretations contained in a manuscript under consideration. However, if this information provides evidence that some of the Reviewer's work is unlikely to be productive; the Reviewer ethically could discontinue the work.
13. Reviewers should contact the Editor, JWD if they have questions concerning manuscripts that may report dual use research of concern (http://oba.od.nih.gov/biosecurity/biosecurity.html).
14. Flaws in a study sometime may justify strong criticism of the work of an Author. However, in no case is sarcasm or criticism of a personal nature appropriate.

PEER REVIEW

Acceptance and publication of a manuscript is based on scientific merit as determined by stringent peer review. The criteria and standards for publication are outlined in Instructions to Reviewers, below.
Manuscripts submitted to JWD generally receive two or more evaluations from external reviewers and an Assistant Editor (AE). Final selection of reviewers will be determined by the editorial staff; however, authors should provide the editorial staff with a list of four to five potential unbiased reviewers, who are experts in the subject area. Include their affiliations, and email addresses. Although the review process for most manuscripts is handled through AEs, all correspondence is through the office of the Editor. If you have questions during the preparation of a manuscript please contact the Editorial office at jwdwda@gmail.com.

Prior or Duplicate Publication

In the cover letter accompanying the manuscript, the author should make a full statement to the Editor about all submissions and previous reports that might be regarded as prior or duplicate publication of the same or very similar work. Copies of such material should be included with the submitted paper as Word or PDF files uploaded to AllenTrack as

supplemental information files. We do not consider deposition of raw data in online data repositories 'previous publication' of data, and doing so does not preclude publication in the Journal. However, analyses and interpretation of data presented elsewhere could preclude comparable presentation in JWD.

Transfer of Copyright

Following acceptance of a manuscript, we will require a signed Copyright Transfer Agreement (CTA) from one author (usually the corresponding author) with the understanding that all authors have seen and agree to the contents of the manuscript. The CTA will be sent to the corresponding author with the proofs. Return the signed CTA to the Editor's Office, preferably by email (WildlifeDisease@gmail.com), otherwise by FAX (404-378-7895) or mail (James N. Mills, Editor, Journal of Wildlife Diseases, 1335 Springdale Road, NE, Atlanta, GA 30306, USA).

CATEGORIES OF PAPERS PUBLISHED

Full-Length Manuscripts

Manuscripts that report novel results from completed quantitative or qualitative research studies and illustrate important advances in the field of wildlife diseases are published as Full-length manuscripts. Full-length manuscripts are ≤4000 words of text (Introduction through Discussion). The first page should be a title page containing a Running Head (<75 characters including spaces) consisting of author's last name (or first author plus "et al.") and an abbreviated title; the full title; the authors' names; each author's affiliation with **complete** mailing address; and the name, address, phone and fax numbers, and email address of the corresponding author (see a recent issue for format). Provide the word count at the bottom of the title page. The next pages should contain (in the following order): The Abstract (≤300 words), four to eight Key Words (or phrases) in alphabetical order, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Literature Cited, tables (each table on a separate page), and Figure Legends (also on one [or more if needed] separate page). Figures and images should be submitted as separate files (see below).

Short Communications

Shorter manuscripts that report completed studies that are narrower in scope than those described in Full-length manuscripts are published as Short Communications. Methodological

or descriptive studies that represent significant contributions to the literature may also be considered as Short Communications. These are ≤2000 words, *including the Literature Cited*; not including the title page, abstract, or acknowledgements. The first (title) page should have a Running Head entitled "Short Communications," the full title, the authors' names, affiliations, and complete mailing addresses, and the name, address, phone and fax numbers, and email address of the corresponding author. Provide the word count at the bottom of the title page. The next page should contain: an Abstract (≤225 words), 4-8 Key Words (or phrases) in alphabetical order, and then continue right on with the main body of the paper without subheadings, Literature Cited, Tables (each table on a separate page) and Figure Legends. Figures and images should be submitted as separate files (see below).

Letters

Two categories of letters are published in JWD:

1. *Long letters* are brief notes and case reports that describe novel findings that contribute significantly to understanding of wildlife diseases within scope for the Journal: Limited to 1000 words of text plus a 50-word abstract, 15 references, and 2 tables or figures or 1 of each. Title and authors follow Short Communication style.
2. *Short letters* provide commentary on other articles in JWD: Restricted to 500 words and 7 references and have no abstract.

Key words are not provided in Letters. The first (title) page should have a Running Heading entitled "Letters," the full title, the authors' names, affiliations, and complete mailing addresses, and the name, address, phone and fax numbers, and email address of the corresponding author following Short Communication format. Provide the word count at the bottom of the title page.

Reviews

Reviews are on current topics relevant to JWD readership that provide a critical review and synthesis of the literature (NOT simply a restatement of what is available in the literature), and include discussion of important gaps in knowledge in the subject area. Although reviews are generally solicited by the Editor, authors interested in publishing a review are encouraged to contact the Editor, JWD (email: WildlifeDisease@gmail.com). Reviews may be longer than a regular article (>4000 words).

Book Reviews

Book reviews, limited to approximately 1200 words, are managed by the JWD Book Review Editor. To request that a book be reviewed or to offer to review a book, contact the Book Review Editor, Richard Botzler (botzlerr@sbcglobal.net).

Normal Hematology and Biochemistry

Articles presenting normal hematology and biochemistry values will be considered as letters (routine normal values) or short communications (studies involving multiple species, examining temporal or spatial trends, or comparing between normal and diseased populations). In addition to adhering to word limits of letters and short communications (see above), these papers should adhere to the following guidelines (headings in italics are provided for organization and should NOT be added to manuscript).

Introduction: Not to exceed 200 words. State what is known about normal values on the species being examined or on closely related species, and the purpose of the study.

Methods: Provide the following concerning sampling and collection: Health status of animals; month, year, and time of day of sampling; age, sex, and location of animals; number of animals immobilized; drugs used for sedation; time from immobilization to sample collection; parameters monitored (e.g., body temperature, respiration rate); how blood was collected and from what anatomic location. For biochemistries, provide make and model of biochemistry analyzer; whether plasma (citrate, EDTA, heparin) or serum was used; how and for how long samples were stored prior to centrifugation; how and for how long serum was stored before analysis. For hematology, state whether differentials were done manually or automatically (if latter, indicate make and model of machine). For total cell counts, indicate method (e.g., unopette, Natt & Herricks) and number of samples stored prior to processing and for how long.

Results: Should consist of one table for hematology and one table for biochemistry values. Values for differentials should be expressed as 103/μL in cases where total white cell count is available or % when not. Values for biochemistry should be expressed in SI units. All values in table should have mean, range, SE, and n. If applicable, add a column of other published values from same or closely related (same genus) species.

Discussion: Not to exceed 200 words and should state: These values can be compared with the following species or added to table (with appropriate citations) or differences found between or among populations and differences in spatial/temporal trends. If appropriate, provide statement regarding how findings might impact investigations of wild populations.

NATIONAL INSTITUTES OF HEALTH COMPLIANCE GUIDELINES

The Journal of Wildlife Diseases supports the National Institutes of Health (NIH) Public Access policy. Authors should visit the NIH website to determine if their manuscripts meet the NIH requirements. Using the NIH Manuscript Submission System, the accepted manuscript should be submitted by the lead author to PubMed Central. The accepted manuscript is the version of the manuscript that has been accepted prior to editing, image quality control, and production and should include the published paper's full reference citation (including DOI). Public release of NIH-funded research will be allowed by the Journal of Wildlife Diseases 12 months after the manuscript is accepted for publication, and as such, during the process of submission, the author should designate 12 months as the release date to the public.

ENGLISH LANGUAGE EDITING

Prior to submission, authors who believe their manuscripts would benefit from professional editing are encouraged to use a language-editing service. Some services available are listed here. The Journal of Wildlife Diseases does not take responsibility for or endorse these services; use of an English-language editing service is not mandatory and will not guarantee acceptance or preference for publication, however it may minimize delays in the editing process by increasing the effectiveness of the manuscript. The authors assume financial responsibilities of utilizing professional editing services.

SUBMISSION OF MANUSCRIPTS

How to submit your manuscript to JWD for consideration for publication

AllenTrack is the online manuscript tracking system provided by our publisher, Allen Press. Using this system, all aspects of the manuscript review process are carried out online. Online submission is required, except in special circumstances. To submit an article, please go to the Journal’s AllenTrack website at http://jwd.allentrack.net.

File Formats

Electronic text files should be submitted as Microsoft (MS) Word files. Tables should be prepared using the Table function of MS Word and included in the manuscript text file. Do

not upload text files in PDF format. Each manuscript should have >25 mm margins all around and be typed in 12-point font (Times New Roman, Courier, or Arial preferred). Lines of type should be justified left with ragged right margins and contain no end-of-line hyphens. Double space all parts of the manuscript, including the title page, Literature Cited, and Tables. Use the American form of English for spelling. Number all pages in the upper right corner, starting with the title page. It is not necessary to insert line numbers in the MS Word files; these are automatically inserted when the manuscript is converted to PDF in AllenTrack.

Author Charges and Payment for Publication

See: Author Charges

Open Access

Online access to manuscripts published in the Journal of Wildlife Diseases is available only to members of the Wildlife Disease Association for the first 18 months following publication. Authors may choose to avoid this embargo period for their manuscripts by choosing the Open Access option when they submit their manuscript and specifying the same in their cover letter to the Editor. Authors who choose the Open Access option will be billed an additional $1,000 (for WDA members) and $1,500 (for nonmembers) when the manuscript is accepted. The manuscript will appear as open access at the time it is posted on the JWD website.

GENERAL INSTRUCTIONS FOR MANUSCRIPT PREPARATION

In order to minimize delays, authors are urged to carefully read these instructions. Manuscripts will be returned if authors do not follow instructions for manuscript preparation. Only original papers written in English will be accepted. All manuscripts must be free of plagiarism. The Journal of Wildlife Diseases is a member of CrossCheck and may screen manuscripts for potential plagiarism.

Cover letter

A cover letter to the Journal Editor should include the following:

 A declarative statement that the manuscript represents new information that has not been previously submitted or published elsewhere; or an explanation of any previous publication or presentation of all or parts of the manuscript.

 A declarative statement that all authors of the paper have read and approved the final version of the manuscript submitted and that all have made substantive contributions to the work.

 Specification of the type of manuscript that is being submitted.

 A description of how the information provided in the manuscript is original, new, timely, significant, and relevant to the readers of JWD.

Manuscript Components

Manuscripts should be composed of the following elements in the below order. Figures or images should be submitted as separate files.

Title Page

The first page should be a title page containing a Running Head. See description above in Categories of Papers Published for a detailed description of title pages by manuscript type (see a recent issue for format). To avoid delays, authors should ensure that the word count is within the limits specified for the manuscript type.

Abstract

Abstracts are unstructured. References are not cited and figure or table callouts not allowed. Provide inclusive dates of the study in the Abstract and main body of the text. The abstract and body of the text should provide a clear statement of the objective(s), such as the hypothesis tested or the question addressed. Abstracts should highlight new information made available as a result of the work being described. Provide the genus and species of each organism the first time it is given in the Abstract, and again in the text.

Keywords

Key words are included in Review, Full-length, and Short Communication articles. Four to eight key words should appear in alphabetical order, separated by commas.

Headers

Headers for Full-length articles include: Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Literature Cited. There are no textual heads in Short Communications or Letters except for Literature Cited.

Acknowledgments

For Reviews, Full-length manuscripts, and Short communications, acknowledgments are placed at the end of the text before the Literature Cited in one indented paragraph. For Short Communications there is no "Acknowledgments" header. Grant and funding information

appears in acknowledgments, omitting the number sign and abbreviation (“No.”). Honorific titles such as Dr or Ms, and degrees (MD, PhD, etc.) are not included.

Literature Cited

The Literature Cited section of the manuscript should be prepared in appropriate Journal style. References in the body of the text follow the author–year style, with parenthetical entries in chronological, then alphabetical, order. Use BIOSIS journal abbreviations. Many university libraries provide online lists by discipline. In addition, authors may check the Serials Source List for Biological Sciences from Cambridge Scientific Abstracts.

 The authors should carefully check that all literature cited appears in the text, and vice versa.

 As a rule, use only one literature citation to make each point in the text; omit redundant citations; if multiple citations are required in the text, list them in chronological order, from oldest to most recent separated by semicolons.

 Meeting abstracts, unpublished materials, and non-peer reviewed materials generally are not acceptable as citable materials; exceptions must be justified by the authors.

 Theses and dissertations, state and federal documents intended for professional distribution, and *peer-reviewed* proceedings of meetings generally are acceptable citations.

Article in a journal:

Smith AB, Jones CD. 1994. Hepatitis of viral origin in Canidae: An etiologic hypotheses. *J Wildl Dis* 76:371–380

Smith AB, Jones CD, Garwin EF. 1995. An outbreak of cowpox in captive cheetahs: Virologic and epidemiologic studies. *J Hygiene* 89:72–79.

Chapter in a book or an edited book:

Smith AB. 1998. *The insects of Australia*, 2nd Ed. Commonwealth Scientific and Industrial Research Organisation, Division of Entomology, Melbourne University Press, Melbourne, Victoria, Australia, 542 pp.

Jones CD. 1997. *Biostatistical analysis*. Prentice-Hall, Inc. Englewood Cliffs, New Jersey, 153 pp.

Jordan FT. 1996. Avian mycoplasmosis. In: *Poultry diseases*, Jordan FT, Pattison M, editors. W. B. Saunders Company Ltd., Philadelphia, Pennsylvania, pp. 81–93.

Cheville NF, editor. 1994. *An introduction to interpretation*. Iowa State University Press, Ames, Iowa, 502 pp.

Proceedings:

Dickinson VM, Jarchow JL, Trueblood MH. 2002. Hematology and plasma biochemistry reference range value for free-ranging desert tortoises in Arizona. In: *Proceedings of the 54th annual meeting on pathology and medicine of reptiles and amphibians*, American Veterinary Association, Phoenix, Arizona, 17–21 January, pp. 129–134. (if published: ... 17–21 January; W. B. Saunders, Philadelphia, Pennsylvania, pp. 129–134.)

Dissertation/Thesis:

Hathaway SC. 1978. *Leptospirosis in free living animals in New Zealand, with particular reference to the possum (*Trichosurus vulpecula*)*. Ph.D. Thesis, Veterinary Pathology and Public Health, Massey University, Palmerston North, New Zealand, 434 pp.

URLs:

United States Department of Agriculture. 2001. *The Federal Agriculture Improvement and Reform Act of 1996*, www.usda.gov/farmbill/title0.htm. Accessed April 2002.

Tables

Tables should be prepared using the Table function in MS Word. Omit all vertical lines. Do not enclose tables with borders. Footnotes in the table should be identified by superscript lower-case letters. The table caption should appear above the table and should be complete enough to "stand alone" without reference to the text.

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Figure captions are included on a separate page following the Tables. Figure captions must "stand alone." Include "what, when, and where." Provide scientific names for all species mentioned and explain all abbreviations.

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Journal style follows Council of Science Editors, 7th Edition. Nomenclature for mammals (both common and scientific names) follows Wilson and Reeder, Mammal Species of the World, 3rd Edition, 2005 (http://www.bucknell.edu/msw3/); for birds, use the latest Clements checklist (http://www.birds.cornell.edu/clementschecklist/downloadable-clements-checklist). Capitalize standard common names of birds. GenBank accession numbers should be provided for nucleotide sequences.

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