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# Sentinels of the Pantanal: health and territory defense in giant otters

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Ao Gael,  Luana, Torbjörn, Einar e aos que estão por vir,
que vocês possam conhecer e se encantar
pela natureza,ariranhas e o Pantanal.
Que o futuro de vocês seja em um planeta
que ame e respeite a natureza.
A minha Omi, que hoje é floresta.
A minha Xanti, eterna companheira.

*Conselhos de*
*uma Ariranha:*
*Acorde cedo*
*Erga a cabeça*
*Respire fundo e siga em frente*
*Proteja sua casa*
*Proteja sua família*
*Cuide dela e cuide de você*
*Evite conflitos*
*Mas se for preciso grite, brigue*
*Seja forte, mas nunca perca a ternura*
*Deixe sua marca por onde for*
*Ao final do dia vá para casa,*
*Deite sua cabeça e descanse em paz.*

(Foerster, N.
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# Index

# General Abstract

The giant otter (*Pteronura brasiliensis*) is a social and territorial semiaquatic mammal that can be found in the rivers of the Pantanal. Despite of the endangered status, the information regarding the health of the species is scarce. In this thesis we (1) assessed mercury contamination level in giant otters; (2) report a case of myiasis; and (3) evaluate their territorial behavior throughout playback experiments. Our analysis indicated higher mercury levels in connected watercourses near gold mining areas, decreasing downstream. This highlights a gradient of contamination and the far-reaching impact of local polluting activities in the Pantanal. Additionally, herein, we reported the first case of myiasis caused by the fly *Cochliomyia hominivorax* (Calliphoridae) in a giant otter found dead in the Pantanal. The injured otter probably got the larvae after an intraspecific fight and the myiasis probably deteriorated the health of the infested giant otter, which prevented recovery and accelerated its death. For last, in order to evaluate the territorial behavior of the giant otters, we conducted playback experiments, and evaluated their responses to the type of the broadcasted sound (adult-call and snort) and the group-proximity (self, neighbors and non-neighbors), given the presence of cubs and the effective group size. Our results showed that the sociability of giant otters is associated with their defensive capabilities, and that individuals must weigh the costs of exposure and combat. The otters presented difference in responses to the sound types, which may be related to the meaning and type of information conveyed by these sounds and differences in the vocal signature. The groups presented reduced responses when with cubs, suggesting a strategy to minimize the exposition of their cubs to potential threats. Our results did not support the Dear enemy hypothesis, yet our findings support the threat level hypothesis that states that neighbors and non-neighbors can represent different threat levels according to variation in the resources and other environmental variables.

# Resumo Geral

A ariranha (*Pteronura brasiliensis*) é um mamífero semiaquático social e territorial encontrado nos rios do Pantanal. Apesar do seu status de ameaçada, informações sobre a saúde da espécie são escassas. Nesta tese, (1) avaliamos o nível de contaminação por mercúrio nas ariranhas; (2) relatamos um caso de miíase; e (3) avaliamos o comportamento territorial através de experimentos de playback. Nossas análises indicaram níveis mais altos de mercúrio em cursos d'água conectados as áreas de mineração de ouro, diminuindo rio abaixo. Isto destaca um gradiente de contaminação e a abrangência do impacto de atividades locais poluentes no Pantanal. Adicionalmente, relatamos o primeiro caso de miíase causada por moscas *Cochliomyia hominivorax* (Calliphoridae) em uma ariranha encontrada morta no Pantanal. A ariranha provavelmente adquiriu as larvas após um conflito intraespecífico, e a miíase possivelmente deteriorou a saúde da ariranha, o que impediu a recuperação e acelerou sua morte. Por fim, para avaliar o comportamento territorial das ariranhas, realizamos experimentos de playback e avaliamos as respostas ao tipo de som transmitido (chamado de adulto e bufo) e à proximidade do grupo (próprio, vizinho e não-vizinho), considerando a presença de filhotes e o tamanho efetivo do grupo. Nossos resultados mostraram que a sociabilidade das ariranhas está associada à sua capacidade de defesa e que os indivíduos devem avaliar os custos de exposição e combate. As ariranhas apresentaram respostas diferentes aos tipos de sons, o que pode estar relacionado ao significado e ao tipo de informação transmitida por esses sons e às diferenças na assinatura vocal. Os grupos apresentaram respostas reduzidas na presença de filhotes, sugerindo uma estratégia para minimizar sua exposição a ameaças potenciais. Nossos resultados não suportam a hipótese "Querido Inimigo", mas corroboram com a hipótese do nível de ameaça, que afirma que vizinhos e não-vizinhos podem representar diferentes níveis de ameaça de acordo com a variação nos recursos e em outras variáveis ambientais.

# General Introduction

*Giant otters*

Giant otters (*Pteronura brasiliensis*, Zimmermanm 1780) are carnivorous semiaquatic mammals that belong to the Mustelid family, representing the largest species (32 kg; 1.8 m length) in the Lutrinae subfamily, which can reach up to 1.80 m in length and 32 kg (Duplaix 1980; Rosas et al. 2009). Endemic to South America, the giant otter geographic distribution currently includes Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Peru, Suriname, Venezuela, and a small population in Paraguay. The species has been considered extinct in both Argentina and Uruguay (Groenendijk et al. 2021), though individuals were recently re-discovery in Argentina (Leuchtenberger et al. 2023). In Brazil, the species occurs in the Amazon, Cerrado, and Pantanal phytogeographic domains (Leuchtenberger et al. 2018). Giant otters are classified globally as endangered by IUCN (Groenendijk et al. 2022) and as vulnerable in Brazil (Rodrigues et al. 2013). Current threats to the species are mainly habitat loss and degradation, associated with mercury contamination, overfishing, human conflicts, poorly managed tourism, and climate changes (Groenendijk et al. 2022).

Giant otters feed mainly on fishes and live in very cohesive groups ranging from two to 16 individuals (Duplaix 1980; Carter & Rosas 1997). Adults can daily ingest 10% of their body weight, which represents about 3 – 4 kg of fish (Duplaix 1980; Rosas et al. 1999; Staib & Schenck 1994; Cabral et al. 2010; Leuchtenberger et al.2020). Piscivory places the species at the top of the bioaccumulation chain in aquatic systems, therefore is widely considered as an appropriate bioindicator (Utreras & Jorgenson 2003), serving as sentinels of the overall ecosystem.

*Health information in Giant otters*

Despite the status of endangered species, the health of the giant otters remains poorly understand. Currently, the main information is about debilitated individuals found in suboptimal habitats (Ribas et al. 2012), in which dental problems and papillomavirus infection were reported by Leuchtenberger et al. (2015) and Soresini et al. (2022), respectively. There is also scarce information on its parasites (Freitas & Lent 1949, Rosas et al. 2015, Soresini et al. 2023), and two small-scale studies assessed exposure of giant otters to mercury in a small scale (Fonseca et al. 2005, Soresini et al. 2020).

*Mercury in the Pantanal and in giant otters*

Gold mining has been carried out in the northern Pantanal since the last century (Callil & Junk 2011). The process of gold amalgamation uses mercury (Hg), a toxic element in high concentrations that causes neurotoxic and other sub lethal effects in the animals' endocrine systems. Mercury is still being released into the environment through the mining activities, despite regulations and new techniques. This contamination can affect species along the food chain due to its accumulation in tissues, leading to bioaccumulation and biomagnification processes (Brouard et al. 1994).

In the southern Pantanal, mercury concentrations up to 7.15 ± 3.41 $\mu g.g^{-1}$ dry weight were found in fur samples from giant otters, 475 km from the closest gold mining area (Soresini et al. 2020). Other studies reported Hg concentrations in several species along the Pantanal wetland (Del Lama 2011, Vieira et al. 2011, May-Junior et al. 2018, Hylander et al. 2022). In the northern Pantanal, near the gold mining area, May-Junior et al. (2018) found the highest value of Hg concentration (2000 $\mu g.g^{-1}$) ever registered in wild animals in fur samples of a jaguar.

*Selenium and mercury interaction*

Selenium (Se) is an essential element, that can form a compound with Hg (Se:Hg). When in a molar ratio of 1:1, Se seems to mitigate the toxicity effects of Hg (Wren 1984, Palmisano et al. 1995, Dietz et al. 2000, Storelli & Marcotrigiano 2002, Scheuhammer et al. 2008, Nakasawa et al. 2011, Kalinska et al. 2017).When Hg concentration is above the molar ratio of Se, the toxic effects of Hg can develop (Peterson et al. 2009, Zhang 2014).

*Ectoparasites in giant otters*

The knowledge regarding the ectoparasitism in giant otter also is still scarce. Ectoparasite ticks infesting giant otters have been reported only in the Amazon (Rosas et al. 2015) and Pantanal (Soresini et al. 2023), likely because they are a hard to be captured and manipulated, with few sample collections and most information recorded opportunistically from dead individuals. In addition, self-grooming and allogrooming are common comfort activities in giant otters (Duplaix 1980) that may contribute to clean potential ectoparasites, and their semiaquatic habit can reduce ectoparasite infestation as well.

*Territoriality in giant otters*

The giant otter is a territorial species in which the whole group defends their territories through vocalization and scent-marks in the latrines and along the territory (Duplaix 1980, Leuchtenberger & Mourão 2009). In the Pantanal, giant otter territories range from 1.1 to 17.8 km in the dry season along the river channel and expand in the wet season across flooded areas (Leuchtenberger et al. 2015). Agonistic encounters are often observed between individuals from different groups (Ribas & Mourão 2004, Leuchtenberger & Mourão 2009, Ribas et al. 2012), being more common at the borders of the territory and when there is an overlap between groups' areas (Leuchtenberger et al. 2015).

*Giant otter vocal repertoire*

Several researchs described the vocal repertoire of giant otters in different locations, such as in the Guyana (Duplaix 1980), Amazon Basin (Staib 2005, Bezerra et al. 2011, Mumm & Knörschild 2014), and in the Pantanal (Leuchtenberger et al. 2014, Leuchtenberger et al. 2016). Giant otters produce more than 15 different sounds that can be combined increasing the communication of the species (Leuchtenberger et al. 2014, Mumm & Knörschild 2014).The acoustic characteristics of some of those sounds vary between individuals, possibly allowing individual identification (Mumm et al. 2014,Leuchtenberger et al. 2016). However, the ability to recognize and differentiate between neighboring groups or members of distant groups has not yet been evaluated.

*Dear Enemy*

Fisher (1954), suggests suggested that the intensity of territory dispute with known intraspecific neighbors should be lower than when it occurs with unknown ones, a hypothesis named Dear Enemy. Several studies have supported this hypothesis in different groups, such as birds (Brooks & Falls 1975), salamanders (Jaeger 1981), mustelids (Palphramand & White2007), rodents (Rosell et al. 2008), and crustaceans (Booksmythe et al. 2010). The Dear Enemy hypothesis can be explained based on: (1) familiarization, in which repeated interactions between neighbors lead to less intense aggressions, thus conserving energy and time (Wilson 1975, Booksmythe et al. 2010, Zenuto 2010); and (2) the threat level, in a way that passing-by or dispersing strangers without a defined territory can represent a greater risk compared to neighbors with a defined territory (Temeles 1994).

Individual, neighboring, and distant group recognition can demonstrate the strength of relationships and likely contribute to group success. Defensive behavior is especially important for the protection of the offspring. In situ experiments allow us to know how territory defense behaviors occur and to evaluate the recognition capacity among groups of giant otters.

In this thesis, we assess the level of mercury contamination in giant otters across the Pantanal (first chapter), report the occurrence of myiasis in giant otters for the first time (second chapter), and evaluate whether territory defense by giant otters fits the Dear Enemy hypothesis (third chapter).

# <u>Chapter 1:</u> Unveiling the gradient of mercury contamination on a top predator across the Pantanal wetland

## Abstract

Wetlands, such as the Pantanal, are important ecosystems worldwide. Extensive gold exploration in the northern Pantanal resulted in detectable mercury concentration in the endangered giant otters (*Pteronura brasiliensis*) inhabiting distant sites. This study assesses the level of mercury contamination in giant otters in relation to their distance and connectivity to gold mining area in the Pantanal. Ten study sites were selected on a gradient from north to south in the Pantanal. The total mercury (Hg) concentration was determined by ICP-MS. To assess whether the total Hg in giant otters was related to the distance and connection from the gold mining areas, we performed a two-factor generalized additive model with integrated smoothness estimation. Analyses of 20 giant otter groups fur indicated higher mercury levels in watercourses closer and connected to gold mining areas, highlighting a spatial downstream gradient of contamination. This gradient illustrates the far-reaching impact of local polluting activities on complex ecosystems such as wetlands. The Hg concentration found in the Bento Gomes River (located in a gold mining area) was the highest registered for giant otters to date. We found lower Hg concentrations in fur from otters inhabiting unconnected sites, irrespective of their linear proximity to the gold mining sites. Long-term programs for evaluating the concentration of metal contaminants in this species should be conducted to assess the threat status of the species over time. Further studies are necessary to understand how mercury contamination affects the health of giant otters, and of other Pantanal predators as well.

## Resumo

Áreas úmidas, como o Pantanal, são ecossistemas importantes em todo o mundo. A exploração extensiva de ouro no norte do Pantanal resultou em concentrações detectáveis de mercúrio nas ameaçadas ariranhas (*Pteronura brasiliensis*) que habitam locais distantes. Considerando isso, o presente estudo teve como objetivo avaliar o nível de contaminação por mercúrio em ariranhas do Pantanal em relação à sua distância e conectividade com áreas de mineração de ouro. Foram selecionados dez locais de estudo em um gradiente de norte a sul no Pantanal. A concentração total de mercúrio foi determinada por ICP-MS. Para avaliar se o Hg total estava relacionado à distância das áreas de mineração de ouro, realizamos um modelo aditivo generalizado de dois fatores com estimativa de suavização integrada. A análise de 20 grupos de ariranhas indicou níveis mais altos de mercúrio em cursos d'água conectados e próximos a áreas de mineração de ouro, diminuindo com a distância, destacando um gradiente de contaminação. Esse gradiente ilustra o impacto abrangente das atividades poluentes locais em ecossistemas complexos, como áreas úmidas. A concentração de Hg encontrada no Rio Bento Gomes (situado em área de mineração de ouro) foi a mais alta registrada para ariranhas até o momento. Encontramos concentrações mais baixas de Hg nos pelos de ariranhas que habitam locais não conectados, independentemente de sua proximidade linear com os locais de mineração de ouro. Programas de longo prazo para avaliar a concentração de contaminantes metálicos nessa espécie devem ser conduzidos para avaliar o status de ameaça da espécie ao longo do tempo, especialmente devido à expansão das atividades humanas no Pantanal. Além disso, estudos adicionais, como concentrações de metilmercúrio e análises hormonais que podem indicar estresse ou problemas reprodutivos, são necessários para entender como o mercúrio pode afetar a população de ariranhas e outras espécies semiaquáticas no Pantanal.

## Introduction

Wetlands are important ecosystems worldwide, supporting numerous plant and animal species, acting as breeding sites, nurseries, and feeding grounds. They also provide a wide range of ecosystems services, such as carbon sequestration and water quality improvement (Maltby & Acreman 2011). Despite their global importance, wetlands are facing significant threats from industrial and agricultural pollution, drainage, and land conversion (van Asselen et al. 2013). Currently, the world has already lost about half of its wetlands (Davidson 2014). Wetland loss leads to a reduction in biodiversity and disrupts the intricate web of complex ecological interactions (Alho 2008, Nunes et al. 2020).

The Pantanal, in central South America, is one of the largest tropical wetland in the world. It is a complex ecosystem, composed of several types of water bodies, interspaced with non-flooding grasslands, woody grasslands, savannas, and deciduous to semideciduous forest patches. Numerous water bodies within the floodplains become connected during periods of seasonal flooding. However, over the last few years, rainfall has decreased in the Pantanal and its water levels have gone down. Part of its river systems have remained disconnected, causing many of these connections to cease to exist (Marengo et al. 2021). This scenario has negatively affected aquatic and semiaquatic animals, such as caimans (Campos et al. 2020) as well as many other species, including giant otters (Soresini et al. 2022).

Gold exploration, first through panning and then mining, has been performed in several locations in the northern Pantanal for the last two centuries (Callil& Junk 2001). Mercury (Hg) is a potentially toxic element used in the amalgamation process of gold (Callil&Junk 2001). Despite regulations and new techniques (Callil & Junk 2011, BRASIL 2018), Hg is still being released into the environment through these activities. This contamination can affect species throughout the food chain due to its accumulation in tissues, leading to bioaccumulation and biomagnification (Brouard et al. 1994). At high concentrations, Hg is a toxic metal that can cause neurotoxic and other sub lethal effects in the endocrine system of living organisms.

Giant otter (*Pteronura brasiliensis*) is the largest member of the Lutrinae subfamily. They are among the most social otters in the world and are considered Endangered by the IUCN (Groenendijk et al. 2021) at a global level and Vulnerable in Brazil (Rodrigues et al. 2013). Although giant otters have gone extinct in parts of their original distribution, as is the case in the Atlantic Forest (Garbino et al. 2022), established populations of giant otters can still be found in rivers in the Pantanal. They are top predators in numerous freshwater ecosystems of South America. Their piscivorous habits place them at the top of the bioaccumulation chain in aquatic systems. For this reason, and because of their sensitivity to environmental changes, otters are considered an appropriate bioindicator (Utreras & Jorgenson 2003).

Soresini et al. (2021) found mercury concentrations up to $7.15 \pm 3.41$ $\mu g.g^{-1}$ dry weight in fur samples taken from giant otters inhabiting the southern Pantanal, approximately 475 km linear distance from gold mining areas. Several other studies have reported Hg concentrations in wildlife present in different areas of the Pantanal, including fish (Hylander et al. 2000), caimans (Vieira et al. 2011), wood storks (Del Lama 2011), and jaguars (May-Júnior et al. 2018). May-Júnior et al. (2018) found the highest value (2000 $\mu g.g^{-1}$) ever registered in a wild animal in samples of a jaguar from the northern Pantanal at a site near a gold mining area.

Selenium (Se) is an essential element that can be toxic at high concentrations. Selenium seems to mitigate the toxicity of Hg in several vertebrate species due to the formation of a Se:Hg compound in different tissues when present in a molar ratio of 1:1 (Wren 1987, Palmisano et al. 1995, Dietz et al. 2000, Nakazawa et al. 2011,Kalisinska et al. 2017). When the Se concentration is below the Hg concentration, it may lead to the development of toxic Hg effects (Peterson et al. 2009, Zhang 2014).

The concentration of chemical contaminants in wild animals is related to the time of exposure and concentration in the environment, diet, trophic level, metabolic rate, and tissue type (Hyvärinen et al. 2003, Hosseini et al. 2013, Ackerman et al. 2016, Costa et al. 2023). Keratin-rich structures can serve as a stable long-term record of specific contaminants, namely, potentially toxic elements (Kempson &Lombi 2011, Costa et al. 2023). Once absorbed by the organism, these chemical substances can be stored in the fur as a metabolic elimination pathway (Hyvärinen et al. 2003). Fur samples can be collected using noninvasive techniques (Pedroso et al. 2018) and are a practical solution for hard-to-capture animals such as giant otters.

Therefore, this study assesses whether the level of mercury contamination in giant otters' fur is related to distance and connectivity to gold mining areas in the Pantanal wetland. We also assess selenium concentrations and the Se:Hg molar ratio. We hypothesized that the Hg levels will be higher in otters near gold mining areas and that these levels would decrease according to their distance along rivers and water bodies.

## Methods

*Study site*

Most of the Pantanal wetland is in the Center-West region of Brazil and receives its waters primarily from surrounding uplands. The Paraguay River, which is the main collector of water in the Pantanal, runs from north to south, while most of its tributaries run from east to west (Hamilton et al. 1996). The main gold mining sites are situated in the northern Pantanal (Figure 1).

The 10 study sites selected in the Pantanal wetland were roughly located along a gradient from north to south and included the following rivers and locations: (1) the Bento Gomes River; (2) Corixo Verde, a small seasonal channel; (3) the Claro River; (4) the Pixaim River; (5) the Cuiabá River; and the Paraguay River in two locations: (6) the Amolar region and (7) the city of Corumbá; (8) the Miranda River; (9) the Vermelho River; and (10) the Negro River (Table 1; Figure 1).

Both the site of the Bento Gomes River (1), which is located in a gold mining area, and the city of Corumbá (site 8) are the most disturbed sites, being located close to urban areas along with domestic and industrial waste. The other sites are relatively well preserved, and the main economic activities in these areas are ecotourism, which involves the circulation of boats and cattle farming.

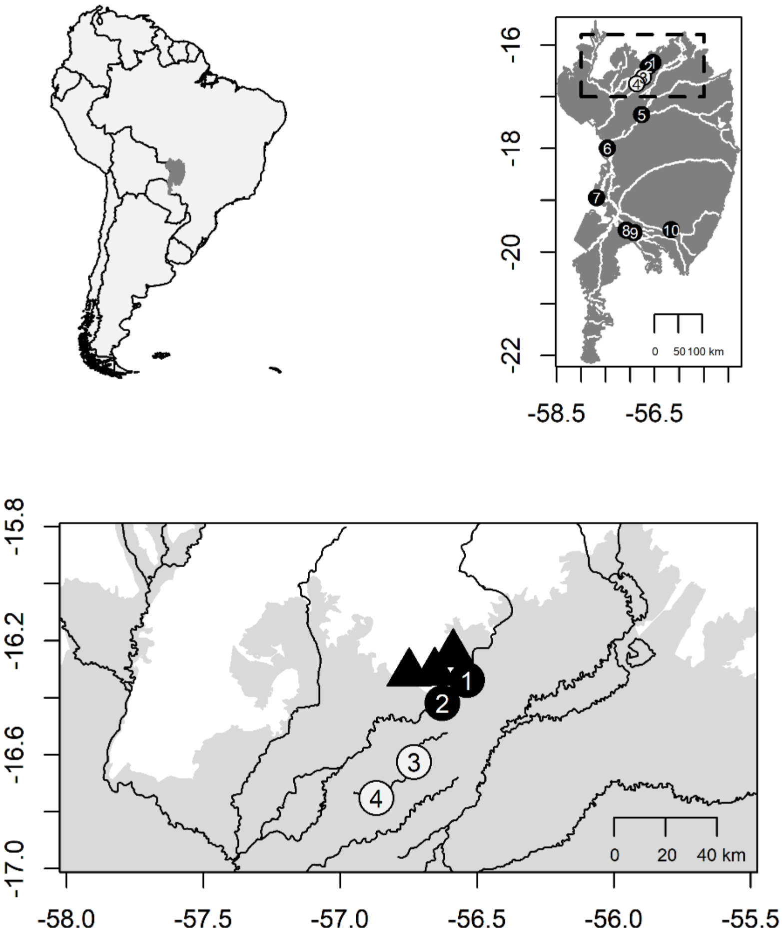


Figure 1. Upper left: a map of South America showing the location of the Pantanal wetland. Upper right: the Pantanal wetland. Here, rivers are represented by white lines. Circles indicate the locations of fur samples collected from groups or individuals of giant otters (*Pteronura brasiliensis*). Black circles indicate that the sampled otters inhabited the river channels or water bodies connected to the river channels. Light gray circles indicate otters from isolated water channels. Bottom: a map detailing the dashed rectangle shown in the previous panel. Black lines represent rivers and isolated water channels. The triangles indicate the location of the gold mining areas, and the numbered circles indicate the sample locations within this area: the connected (1) Bento Gomes River and (2) Corixo Verde; and the unconnected (3) Claro River and (4) Pixaim River.

Table 1. Mercury (Hg) and selenium (Se) concentrations (μg.g $^{-1}$ dry weight) and Se:Hg molar ratios in fur samples from giant otter (*Pteronura brasiliensis*) groups or individuals from 10 sites in the Brazilian Pantanal between September 2020 and October 2021. 'Distance' represents the distance following the river courses and the connecting waters as measured in kilometers from the sampled sites to the gold mining areas.

| Site | | Coordinates | | Distance (km) | Hg | Se | Se:Hg | |
|---|---|---|---|---|---|---|---|---|
| | | Latitude | Longitude | | | | | |
| 1 | Bento Gomes River | 16°19'22.31"S | 56°32'12.67"W | 15.17 | 51.518 | 0.988 | 0.05 | |
| | | 16°44'46.44"S | 56°52'19.76"W | 13.80 | 35.736 | 1.184 | 0.08 | |
| 2 | Corixo Verde | 16°25'10.02"S | 56°37'36.43"W | 32.86 | 4.595 | 0.655 | 0.36 | |
| 3 | Claro River | 16°40'37.86"S | 56°45'27.18"W | - | 4.298 | 1.481 | 0.87 | |
| | | 16°36'20.71"S | 56°43'15,13"W | - | 5.472 | 0.718 | 0.33 | |
| 4 | Pixaim River | 16°44'46.44"S | 56°52'19.76"W | - | 4.515 | 0.844 | 0.47 | |
| 5 | Cuiabá River | 17°19'35.31"S | 56°45'35.12"W | 212.82 | 15.212 | 0.652 | 0.11 | |
| 6 | Amolar - Paraguay River | 18°02'09.00"S | 57°28'07.00"W | 367.46 | 17.008 | 2.290 | 0.34 | |
| | | 18°02'09.23"S | 57°28'07.04"W | 367.46 | 5.606 | 0.016 | 0.01 | |
| | | 19°30'49.95"S | 57°08'28.51"W | 385.11 | 9.276 | 0.589 | 0.16 | |
| | | 18°09'17.11"S | 57°22'39.64"W | 390.46 | 14.995 | 0.047 | 0.01 | |
| | | 17°53'33.64"S | 57°28'38.99"W | 348.82 | 13.462 | 0.522 | 0.10 | |
| | | 18°08'16.14"S | 57°23'16.81"W | 388.30 | 16.490 | 1.265 | 0.19 | |
| | | 18°57'00.70"S | 57°40'57.87"W | 357.16 | 4.998 | 0.139 | 0.07 | |
| | | 17°57'10.23"S | 57°27'36.85"W | 357.53 | 12.163 | 0.658 | 0.14 | |
| 7 | City of Corumbá - Paraguay River | 18° 57'00.70"S | 57°40'57.87"W | 515.46 | 5.547 | 0.435 | 0.20 | |
| 8 | Miranda River | 19°34'36.71"S | 57°02'28.65"W | 709.76 | 8.982 | 1.366 | 0.39 | * |
| 9 | Vermelho River | 19°37'26.78"S | 56°57'50.54"W | 709.72 | 3.551 | 0.533 | 0.38 | |
| 10 | Negro River | 19°34'04.29"S | 56°10'40.14"W | 823.49 | 7.140 | 0.729 | 0.26 | * |
| | | 19°34'41.83"S | 56°09'13.76"W | 821.16 | 7.298 | 0.586 | 0.20 | * |

[a]The'*' indicates samples collected from individuals.

*Data collection*

Fur samples were collected from September 2020 to October 2021 using the fur trap method adapted from Pedroso et al. (2018). However, with the aim of adapting the technique to an even less stressful method, we did not bait the traps with fresh spraints. The traps were placed around active dens and latrines. The number of traps displayed at each site varied according to the number of active latrines and dens found. Camera traps were installed facing the fur traps to allow for the identification of the sampled group. The fur traps remained at least for two days in each site before collection. Fur samples were detached from traps with the aid of tweezers immediately after being removed from the sampling sites, stored in Eppendorf tubes and identified. Due to this collection method, it was not possible to assess which group members were the donors of the fur samples. Therefore, information on individuals such as age, sex and others were missing, but the samples were still representative of giant otters inhabiting those specific locations.

In addition, tissue (liver, kidney, and muscle) and fur samples were obtained from two giant otter carcasses, one found in February 2020 at the Negro River and the other at the Miranda River in September 2021; and only fur was collected from a third carcass in advanced decomposition found in September 2020 at the Negro River. In this site, two scat samples were also collected in the Negro River area. Sample collection were done under licenses SISBIO/ICMBio: 68974, 78746, and 79173.

*Determination of the total concentration of mercury and selenium and quality control*

To measure Hg and Se concentrations, each fur sample was initially washed with acetone P.A., rinsed three times with ultra-purified water (Milli-Q system), and washed again with acetone P.A. to remove external contamination. All samples were oven-dried at 40 °C for 4 hours. Tissues (kidney, liver, and muscle) and scats were not washed nor dried. All the samples were placed in Teflon jars and weighed using an analytical scale. The masses of the samples ranged from 0.7 mg to 0.2 g. Next, 3 ml of distilled nitric acid ($HNO_3$) was added to the samples, which were then digested in a closed system of microwave-assisted digestion. The samples were then diluted with ultra-purified water to 50 ml in centrifuge tubes containing 2.5 ml of 10% (v/v) distilled hydrochloric acid (HCl). Elemental concentrations were determined through inductively coupled plasma-mass spectrometry (ICP-MS, NexION 2000, PerkinElmer), carried out in the Exata Brasil Laboratory in Jataí, Goiás state, Brazil. Standard solutions for Hg and Se tests (NSI Lab Solutions), internal standard solutions (germanium, rhodium, and iridium), and four blank controls were prepared and analyzed through ICP-MS to assess the accuracy of the method. Our results presented good recoveries, ranging from 103.4% to 117% for Hg and 97.8% to 104.8% for Se. In order to present all values in terms of $\mu g.g^{-1}$ dry weight, and compare them to wet weight values, we performed a 3:1 conversion of dry to wet weight (Puls, 1994).

*Connectivity and distance analysis*

We evaluated the gradient of contamination based (1) on the distance from the contaminant sources to the study sites following the river channels and other water bodies and (2) on the presence of connecting waters between the sources and the study sites. Therefore, for this analysis, we only considered the fur samples and sites that presented water coalescence within a period of at least four years. Molting is known to be responsible for eliminating different toxic substances, including Hg. In tropical areas, molting by otter species and other semiaquatic animals usually occurs throughout the year (Kuhn, 2009). Therefore, a four-year period of isolation should be enough to guarantee that the fur collected here represented the current scenario of a given study site within the sampling period.

Subsequently, to evaluate the connectivity between the sampling sites and gold mining areas, we used Sentinel-2 satellite images of the study region from 2018 to 2021 retrieved from the USGS Earth Explorer data portal (USGS, 2022). The images were processed with QGIS software (QGIS.org, 2022), and the normalized difference water index (NDWI) was calculated using the formula proposed by Gao (1996):

$$NDWI = \frac{pNIR - pMIR}{pNIR + pMIR}$$

where pNIR refers to near infrared reflectance and pMIR to mid-infrared reflectance. The NDWI enables the highlighting and delineation of water bodies (Mcfeeters, 1996).

We measured the distance between sample sites using the ‘measuring tool’ in the QGIS software, by following the water courses and other water bodies connecting the sites.

*Statistical analysis*

To evaluate whether the total Hg measured in the fur of the giant otters was related to distance from gold mining areas upstream, we performed a two-factor generalized additive model with integrated smoothness estimation (GAM), available in the mgcv package (Wood, 2017), and running within the R statistical environment (R development Core Team, 2022). To determine the feasibility of merging the dataset from the present study and that from a previous study, which was performed in the Pantanal from 2016-2017 (Soresini et al. 2021), we included the datasets as a factor. A non-significant result indicates the feasibility of merging the data. The other variable in the model was the distance from the sampling locations to the gold mining area following the rivers and connected water body channels. For this analysis, we excluded sites 3 and 4, which were not connected to the other sites during the selected period. On the other hand, we included site 2 though it was also not connected during the collection years (2020 and 2021) because it was connected to the Bento Gomes and to Paraguay rivers during the floods of 2018 and 2019.

To calculate the Se:Hg molar ratio, we converted the dry weight measurements to molar concentrations by dividing the concentration (µg.g$^{-1}$) by the molecular weight. The molecular weight of Se is 78.96 g.mol$^{-1,}$ and that of Hg is 200.59 g.mol$^{-1}$.

## Results

In total, we analyzed fur samples from 20 giant otter groups, samples from two giant otter carcasses (kidney, muscle, liver, and fur), and scat samples collected from the latrines of two different groups. We detected mercury (Hg) and selenium (Se) in all the fur samples (Table 1), scats, and in all the tissues (Table 2).

Table 2. Mercury (Hg) and selenium (Se) concentrations (µg.g$^{-1}$ dry weight) and Se:Hg molar ratios detected in the kidney, liver, muscle, and fur from two carcasses of giant otters (*Pteronura brasiliensis*), one from Negro River in February 2020 and another from Miranda River in September 2021, in the southern Pantanal, Brazil.

| Location | Tissue | Hg | Se | Se:Hg |
|---|---|---|---|---|
| Miranda River | Kidney | 4.104 | 3.922 | 0.97 |
| | Liver | 19.314 | 5.592 | 0.29 |
| | Muscle | 0.378 | 0.429 | 1.14 |
| | Fur | 8.982 | 1.366 | 0.15 |
| Negro River | Kidney | 3.338 | 3.541 | 1.06 |
| | Liver | 26.570 | 8.251 | 0.31 |
| | Muscle | 0.676 | 0.620 | 0.92 |
| | Fur | 7.298 | 0.586 | 0.08 |

The total mercury concentration found in the fur samples ranged from 3.55 $\mu g.g^{-1}$ to 51.51 $\mu g.g^{-1}$ (mean 12.4 $\mu g.g^{-1}$ ± 11.85 $\mu g.g^{-1}$). The highest values were found in two fur samples from Bento Gomes River (51.51 $\mu g.g^{-1}$ and 35.73 $\mu g.g^{-1}$), followed by the Amolar region (12.62 $\mu g.g^{-1}$ ± 4.28 $\mu g.g^{-1}$) and one sample from Cuiabá River in Porto Jofre (16.49 $\mu g.g^{-1}$). Despite the geographic proximity to gold mining areas, samples from the unconnected sites of the Claro and Pixaim rivers, (30 to 50 km of linear distance, respectively),showed low values (4.29 $\mu g.g^{-1}$ and 4.51 $\mu g.g^{-1}$, respectively). The lowest value of Hg concentration was found in a sample from the Vermelho River (3.55 $\mu g.g^{-1}$), far from the gold mining area (705 km following the courses of the river).

From the two giant otter carcasses, we obtained the highest Hg values in the liver (26.57 $\mu g.g^{-1}$ and 19.31 $\mu g.g^{-1}$), followed by the fur (8.98 $\mu g.g^{-1}$ and 7.29 $\mu g.g^{-1}$), the kidney (4.10 $\mu g.g^{-1}$ and 3.33 $\mu g.g^{-1}$), and lastly, the muscle (0.67 $\mu g.g^{-1}$ and 0.37 $\mu g.g^{-1}$). The mercury concentration in the scat samples varied from 0.157 $\mu g.g^{-1}$ to 0.23 $\mu g.g^{-1}$.

The two-factor GAM analysis indicated that the fur samples collected from 2016-2017 by Soresini et al. (2021) and from the present study (2020-2021) can be merged ($t = -0.123$, $p = 0.903$, $n = 36$) and resulted in a significant smooth term (reference equivalent degrees of freedom or edf = 2.402, $p < 0.001$). Overall, the model explained approximately 51.2% of the deviance. Hence, the total Hg measured in the fur of the giant otters was related to distance when following the river channels and other water bodies to the gold mining areas downstream. On the other hand, the fur of giant otters from unconnected water bodies had the lowest Hg concentrations, irrespective of their proximity to the gold mining areas (Figure 2).

The selenium concentration found in the giant otter fur ranged from 0.016 $\mu g.g^{-1}$ to 2.290 $\mu g.g^{-1}$ (mean 0.78 $\mu g.g^{-1}$ ± 0.54 $\mu g.g^{-1}$). From the two giant otter carcasses, the concentrations of Se were 3.922 $\mu g.g^{-1}$ and 3.541 $\mu g.g^{-1}$ in the kidneys, 5.592 $\mu g.g^{-1}$ and 8.251 $\mu g.g^{-1}$ in the liver, and 0.429 $\mu g.g^{-1}$ and 0.620 $\mu g.g^{-1}$ for the Miranda and Negro rives, respectively.

The Se:Hg molar ratio ranged from 0.01 $\mu g.g^{-1}$ to 0.87 $\mu g.g^{-1}$ in the fur samples (Table 1). In the carcasses, the molar ratio was smaller in the fur (0.08 $\mu g.g^{-1}$ and 0.15 $\mu g.g^{-1}$), followed by the liver (0.29 $\mu g.g^{-1}$ and 0.31 $\mu g.g^{-1}$), muscle (0.92 $\mu g.g^{-1}$ and 1.14 $\mu g.g^{-1}$) and kidneys (0.97 $\mu g.g^{-1}$ and 1.06 $\mu g.g^{-1}$) (Table 2).

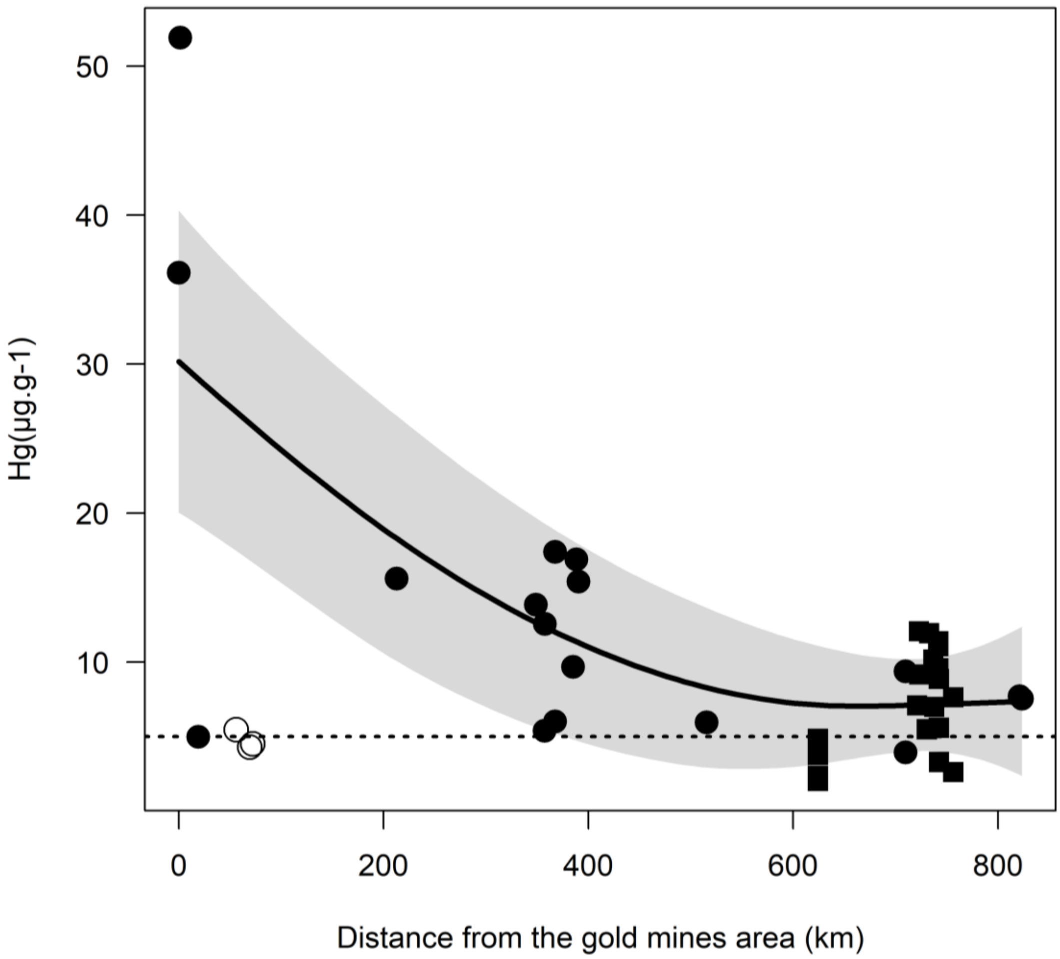


Figure 2. Generalized additive model (GAM) plot to evaluate the total mercury (Hg) concentration ($\mu g.g^{-1}$ dry weight) in giant otters' fur as a function of distance (km) from the gold mining area in the Pantanal. Black dots are sites included in the analysis; circles indicate sample sites of the present study and squares indicate data from Soresini et al. (2021) in the southern Pantanal. White dots indicate sites unconnected with gold mining, and excluded before GAM analysis. Shaded area indicates the 95% confidence interval.

## Discussion

This study was the first to evaluate the concentrations of Hg and Se in giant otters on a broad scale. Previous studies evaluated the mercury concentrations over a comparatively small area of the south of the Brazilian Pantanal (Fonseca et al., 2005; Soresini et al., 2021), which is approximately 475 km (or 750 km following the river channels) from any gold mining sites. The Hg concentration that we obtained from giant otter fur samples from the Negro River in the southern Pantanal was higher than those found by Fonseca et al. (2005) (2.94 $\mu g.g^{-1}$ and 3.68 $\mu g.g^{-1}$) and similar to the values found by Soresini et al. (2021) (2.62 $\mu g.g^{-1}$ to 12.06 $\mu g.g^{-1}$) in the Vermelho and Miranda rivers. Most of the Hg concentrations we found in fur from the giant otters in this region are higher than the 5 $\mu g.g^{-1}$ limit found in a previous study with other otters species in areas free from known contamination sources (Sheffy & St. Amant 1982). According to the U.S. Fish and Wildlife Service, results above that threshold should be considered evidence of an environmental problem related to mercury (Eisler 2000).

The gradient of contamination is evident as the total mercury concentration in fur samples of giant otters exhibited higher levels in connected watercourses near the gold mining areas. This indicates that the concentration of mercury in giant otters decreases downstream as their distance from the mining areas increases. However, it is important to note that the contamination persists in distant areas, affecting the entire aquatic ecosystem. This gradient illustrates the far-reaching impact of local polluting activities on complex ecosystems such as wetlands. A study conducted in Madre de Dios, Peru, elucidates the pervasive presence of mercury in sediments, suspended solids, and fish, near gold mining sites and in areas located hundreds of kilometers away (Diringer et al. 2015). The authors highlight the concerning consequences of this contamination on human communities residing far from the original sources of pollution (Diringer et al. 2015). In the same region of Peru, Barrocas et al. (2023) evaluated the relationship between mercury concentrations in fish and the presence of giant otters. However, the authors did not measure the mercury in the otters and did not find a significant correlation between mercury concentration in fishes and occurrence of giant otters. Even though, they suggest that habitat destruction resulting from gold mining activities may negatively affect the otter population (Barrocas et al. 2023).

The Hg concentration found in the Bento Gomes River (51.51 $\mu g.g^{-1}$) was the highest value registered for giant otters to date. A study on jaguars (*Panthera onca*) in the Pantanal found a considerable difference in Hg concentration in this species between the northern and southern Pantanal (May-Júnior et al. 2018). These authors reported the highest Hg value ever recorded in a wild animal (2000 $\mu g.g^{-1}$) in an area near one of our sampling sites in the Cuiabá River, approximately 200 km from the Bento Gomes River (May-Júnior et al. 2018). We found the lowest Hg concentrations in fur from otters inhabiting unconnected sites, irrespective of their linear proximity to gold mining sites.

Soresini et al. (2021) also found a lower Hg concentration in the fur of giant otters living in relatively isolated water bodies along a road in the southern Pantanal than in that of the fur taken from otters inhabiting rivers. A study on piscivorous wood storks (*Mycteria americana*) found smaller contamination values in birds from seasonally flooded areas than in birds from permanently flooded areas or mainstream rivers, regardless of the distance from gold mining areas (Del Lama et al. 2011). Therefore, we expect that both the distance and connectivity between the Hg source and the giant otter habitats affect the Hg concentration in otters.

The distribution of the concentration of elements may vary according to element and tissue specificities. Differences between tissues occur because of variations in their absorption and the excretion of Hg (Eisler 2006). Hyvärinen et al. (2003) found that an adult male Eurasian otter (*Lutralutra*) could eliminate 7.3 mg of mercury during a complete molt, showing that Hg elimination through fur is an important mechanism in otters. Considering the results of Hg concentration in the fur from Fonseca et al. (2005) of 2.94 $\mu g.g^{-1}$ and 3.68 $\mu g.g^{-1}$and the present study, giant otters appear to present 2 to 3.6 times more Hg in their livers than in their fur, differing from other otter species from temperate climates that usually have similar Hg concentrations in their fur and liver (Evans 2000, Hyvärinen et al. 2003, Sleeman et al. 2010). Otter species from temperate regions present a definite molt season (Kuhn, 2009), which likely causes accumulation of more mercury in their fur until the beginning of the molting. On the other hand, a molt season tends to be absent among tropical semiaquatic animals, including otters (Kuhn 2009), with a continuous fur renewal throughout the year that may act as an important route of mercury elimination in giant otters. Other routes can include Hg elimination through feces, as found in the Baltic gray seals *Halichoerus grypus*) (Grajewska et al. 2020). The concentration of Hg was small in the two scat samples analyzed in our study, similarly to that found in feces from giant otters close to gold mining sites in Peru (Gutleb et al. 1998). Although considering small Hg concentration in feces, giant otters defecate several times throughout the day due to a very fast digestion (Duplaix 1980, Carter & Rosas 1997), thus a high frequency of defecation may turn it an important route of Hg elimination in this species. However, more studies are necessary to evaluate the effectiveness of these routes as a mercury elimination pathway.

Selenium seems to mitigate the toxic effects of Hg in several species through the immobilization of mercury due to the formation of a Se:Hg compound in different tissues of vertebrate species (Nakazawa et al. 2011). In many of these species, the molar ratio between Hg and Se is 1:1 (Palmisano et al. 1995, Dietz et al. 2000). When the Se concentration is below the Hg concentration, it can lead to a Se availability deficiency and the development of Hg toxicity (Peterson et al. 2009, Zhang 2014). The results presented here show very small ratios between Hg and Se in liver samples, which may also indicate possible mercury poisoning in this organ.

Even with a moderate degree of contamination, subclinical effects may significantly impact giant otter populations (Fewtrell et al. 2004), primarily due to reproductive impairment (Scheuhammer 1991). Intrauterine accumulation of Hg has been observed in the North American river otter (*Lontra canadensis*) (Beck et al. 1977), and environmental factors, such as droughts, can aggravate Hg contamination in fish (Hylander et al. 2000), which in turn increases contamination in otters. Recently, the Pantanal suffered from severe droughts, and in the context of climate change, these droughts will likely become more frequent (Marengo et al. 2021), thereby worsening Hg contamination through food chains.

Moreover, considering that the liver of Hg-contaminated giant otters present Hg concentrations 2 to 3.6 times higher than the Hg concentration observed in their fur, the giant otters inhabiting Bento Gomes River, the site closest to the mining area, would present liver Hg concentrations of greater than 100 $\mu g.g^{-1}$. These values are above the critical Hg-concentration threshold of 90 $\mu g.g^{-1}$ in the livers of experimentally dosed *Lontra canadensis* determined by O'Connor& Neilsen (1981), which was associated with increasing otter mortality. In addition, although we did not measure methylmercury, this methylated form of mercury is easier absorbed by the organism than its inorganic form (Wolfe et al. 1998). Methylmercury can cross the blood-brain barrier and cause neurotoxicity and death (Kerper et al. 1992, Wolfe et al. 1998).

Because giant otters are aquatic top predators, they are likely to reach higher levels of Hg and other contaminants than most aquatic or semiaquatic animals (Basu 2012). We found Hg concentrations above the limit were considered normal for uncontaminated otters at sites approximately 400 to 750 km away from gold mining area, supposed to be a major source of Hg in this region. Although other sources, such as fertilizers, pesticides, or even microbial-mediated methylmercury could be locally available (Soresini et al. 2021), gold mines continue to be the main putative Hg source in contaminated giant otter fur. The presence of a mercury concentration gradient in giant otters related to the distance and connectivity of water bodies with gold mining areas reinforces the idea that gold mines are the main source of Hg contamination. However, regardless of whether the Hg sources are local or situated hundreds of kilometers away, the high Hg concentrations in the giant otters reported here indicate that many other organisms in the Pantanal are also exposed to this contaminant.

Considering that giant otters are classified as endangered (Groenendijk et al. 2021) the increase in human activity, including mining and other activities that lead to environmental contamination and/or promote climate change, accompanied by extreme droughts, may jeopardize this species even further, aggravating the threats to its survival.

## Conclusion

The data obtained in this study highlight the existence of a gradient of mercury contamination in giant otters across their habitat, extending from the northern Pantanal to distant sites downstream. Giant otters are at the top-predator of the freshwater food chain, and the concentration of potentially toxic elements and other contaminants in this species reflects the preservation status of the environment and can be an indicator of human health, especially for riverine communities (Basu 2012). The use of fur traps, as described in Pedroso et al. (2018) and modified in this study, enables the establishment of long-term monitoring programs to assess inorganic contaminant concentrations in this species. These programs would facilitate the assessment of the threatened status of the species over time, especially due to the expansion of human activity in the Pantanal. Moreover, further studies, including methylmercury concentrations and hormonal analyses that can reveal potential stress or reproductive problems, are necessary to understand how mercury may affect the giant otter populations and other semiaquatic species in the Pantanal.

# Chapter 2: First report of myiasis caused by *Cochliomyia hominivorax* in free-ranging giant otter (*Pteronura brasiliensis*)


## Abstract

Giant otters are territorial semi-aquatic mammals. It is common to find several individuals exhibiting wounds and scars due to intraspecific conflicts. Myiasis is a parasitic infestation on living tissues of vertebrates caused by dipterous larvae that usually develops in freshly open wounds and can seriously threaten the host's health. Ectoparasites seem to be rare among giant otters and myiasis had not been recorded in this species until now. Here, is presented one record of myiasis in a free-ranging giant otter found dead in the Pantanal, Brazil. An ulcerative lesion was found in the frontoparietal region, from which 22 larvae were recovered and identified as *Cochliomyiahominivorax.* The low occurrence of ectoparasites in giant otters might reflect their semi-aquatic habits and their grooming behavior, which makes it difficult for parasites to remain on the skin. The injured otter probably got the larvae after an intraspecific fight. Agonistic encounters between groups of giant otters have been reported before and these fights can result in serious wounds or even death. It was hypothesized that the myiasis caused by *C. hominivorax*deteriorated the health of the infested giant otter, which prevented recovery and accelerated its death.


*Chapter published in *Brazilian Journal of Veterinary Parasitology* - see Appendix

## Resumo

As ariranhas são mamíferos semiaquáticos. É comum encontrar vários indivíduos apresentando feridas e cicatrizes devido a conflitos intraespecíficos. A miíase é uma infestação parasitária em tecidos vivos de vertebrados, causada por larvas de dípteros que, geralmente, se desenvolvem em feridas recém-abertas, podendo ameaçar seriamente a saúde do hospedeiro. Ectoparasitos parecem ser raros em ariranhas e, até o presente, não há relatos de miíase nesta espécie. Aqui, é apresentado um registro de miíase em uma ariranha de vida livre, encontrada morta no Pantanal, Brasil. Foi encontrada uma lesão ulcerativa na região frontoparietal, na qual foram recuperadas 22 larvas identificadas como *Cochliomyia hominivorax*. A baixa ocorrência de ectoparasitos em ariranhas pode refletir seus hábitos semiaquáticos e seu comportamento de limpeza, o que dificulta a permanência dos parasitos na pele. A ariranha ferida, provavelmente, se infestou com as larvas após uma briga intraespecífica. Encontros agonísticos entre grupos de ariranhas já foram relatados antes, e essas lutas podem resultar em ferimentos graves ou até mesmo em morte. Hipotetizamos que a miíase causada por *C. hominivorax* deteriorou a saúde da ariranha infestada, o que impediu a recuperação e acelerou sua morte.

## Introduction

Giant otters (*Pteronura brasiliensis*) are semi-aquatic mammals and the largest members of the family Mustelidae. They are social and territorial. Giant otters scent-mark and vocalize a wide repertoire to mark their territories and to avoid agonistic encounters with other groups (Leuchtenberger & Mourão 2009). However, during the dry season their territories shrink, and the conflicts tend to increase (Ribas & Mourão 2004, Leuchtenberger & Mourão 2009), and it is common to find several individuals exhibiting wounds and scars (Rosas & Mattos 2003).

Myiasis is a parasitic infestation on living or necrotic tissues of vertebrates caused by dipterous larvae (Hope 1837, Zumpt 1965). Myiasis can involve heavy infestation of freshly open wounds causing swelling, inflammation, pain, and thus seriously threat the host's health (Hall 1997, Yan et al. 2019). The North American river otter (*Lontra canadensis*) is susceptible to myiasis (Kimber & Kollias 2000). However, it was unknown if the giant otter was also susceptible to myiasis.

There are a few records of myiasis in free-ranging mammals in Brazil, including in maned wolf (*Chrysocyon brachyurus*) (Cansi et al. 2011), porcupine (*Coendou prehensilis*) (Lacey & George 1981), opossum (*Didelphis marsupialis*) (Reis et al. 2008) and gracile mouse opossum (*Gracilinanus* sp.) (Reis et al. 2008). Recently, May-Júnior et al. (2021) captured 13 jaguars in the Pantanal presenting subcutaneous nodules due to parasitism by *Dermatobia hominis* larvae. In some of these jaguars, myiasis caused by *Cochliomyia hominivorax* was also found. In mustelids, only two records of myiasis infestation have been reported in Brazil. One occurred in a captive lesser grison (*Galictiscuja*) (Figueiredo et al. 2010), and the other in a neotropical otter (*Lontralongicaudis*) that was rescued exhibiting health problems on the banks of a lake in southern Brazil (Michelazzo et al. 2022). A report of myiasis in the North American river otter resulted in death three days after it was captured due to extensive damage caused by the dipteran larvae, which were not specifically identified (Serfass et al. 1993).

Here, is presented one record of myiasis caused by the larval stage of *Cochliomyia hominivorax*, in a free-ranging giant otter in the Pantanal, a large wetland located near the center of South America. To the author's knowledge, this is the first report of myiasis in giant otters.

## Methods

On the morning of September 4, 2021, a dead giant otter was found floating at the side of the Miranda River (19° 31' 13.95" S 57° 7' 12.96" W), in the state of Mato Grosso do Sul, Brazil. The animal was near the entrance of a former den. The carcass was collected and taken straight to a field lab (license SISBIO/ICMBio 79173-1). Judging from the fresh condition of the carcass, the time of death was estimated as only a few hours before the animal was found.

It was a young male, in poor body condition, measuring 116 cm in total length and weighing 18.77 kg. This individual had several injuries along its body, most of them probably due to bites and other wounds from a possible fight (Figure 1A and 1B). At the necropsy, it was observed that the animal no longer had any fat tissue remaining and the internal organs had normal macroscopic appearance. In a skin lesion measuring 8.5 x 6 cm, in the frontoparietal region with many cavitations on the edges, dipteran larvae were found. All the larvae were collected and stored in 70° GL ethyl alcohol for subsequent identification.

The larvae were cleaned with the aid of a brush and then were examined for their taxonomic characteristics by means of light microscopy, under a Leica M205 C™ stereomicroscope or a Leica DM5500 B™ microscope, both equipped with Leica cameras, models DFC 420 and 490, respectively (Leica Microsystems™, Wetzlar and Mannheim, Germany). Images were registered in the Leica Application Suite image analysis system (LAS™ 3.8; Leica Microsystems™, Wetzlar and Mannheim, Germany). Two specimens were randomly selected and passed through a clarification process, using a solution of potassium hydroxide (KOH) (10% w/v) (Zumpt, 1965), and were placed in an oven at 46°C until translucent (approximately five hours). The specimens were dehydrated in a progressive ethyl alcohol series, from 70 to 99° GL, at one-hour intervals between each dilution (70, 80, 90 and 99° GL). Then they were immersed in hexamethyldisilazane (cat. number 440191; Sigma-Aldrich™) for 10 minutes, followed by deposition onto carbon conductive tabs (12 mm OD, Pelco Tabs™; Ted Pella®, Inc., USA) attached to Pelco® Q pin stubs of dimensions 12.7 × 12.7 mm (Ted Pella®, Inc., USA). The images were documented using a Hitachi® model TM3000™ scanning electron microscope (Hitachi, Tokyo, Japan) in the analysis mode. The specimens were deposited in the Zoological Reference Collection of the Federal University of Mato Grosso do Sul (ZUFMS-DIP01276).

The taxonomic characteristics considered were those previously described by Laake et al. (1936), Knipling (1939), James (1947), Zumpt (1965) and Shewell (1981).

## Results and Discussion

During the necropsy examination of the giant otter, 22 live larvae were recovered. Macroscopically, the larvae were whitish, cylindrical, and tapered anteriorly, with 12 visible segments surrounded by band spines (Figure 1C). The lesion was characterized as typical primary ulcerative myiasis (Laake et al. 1936,Knipling 1939, James 1947).

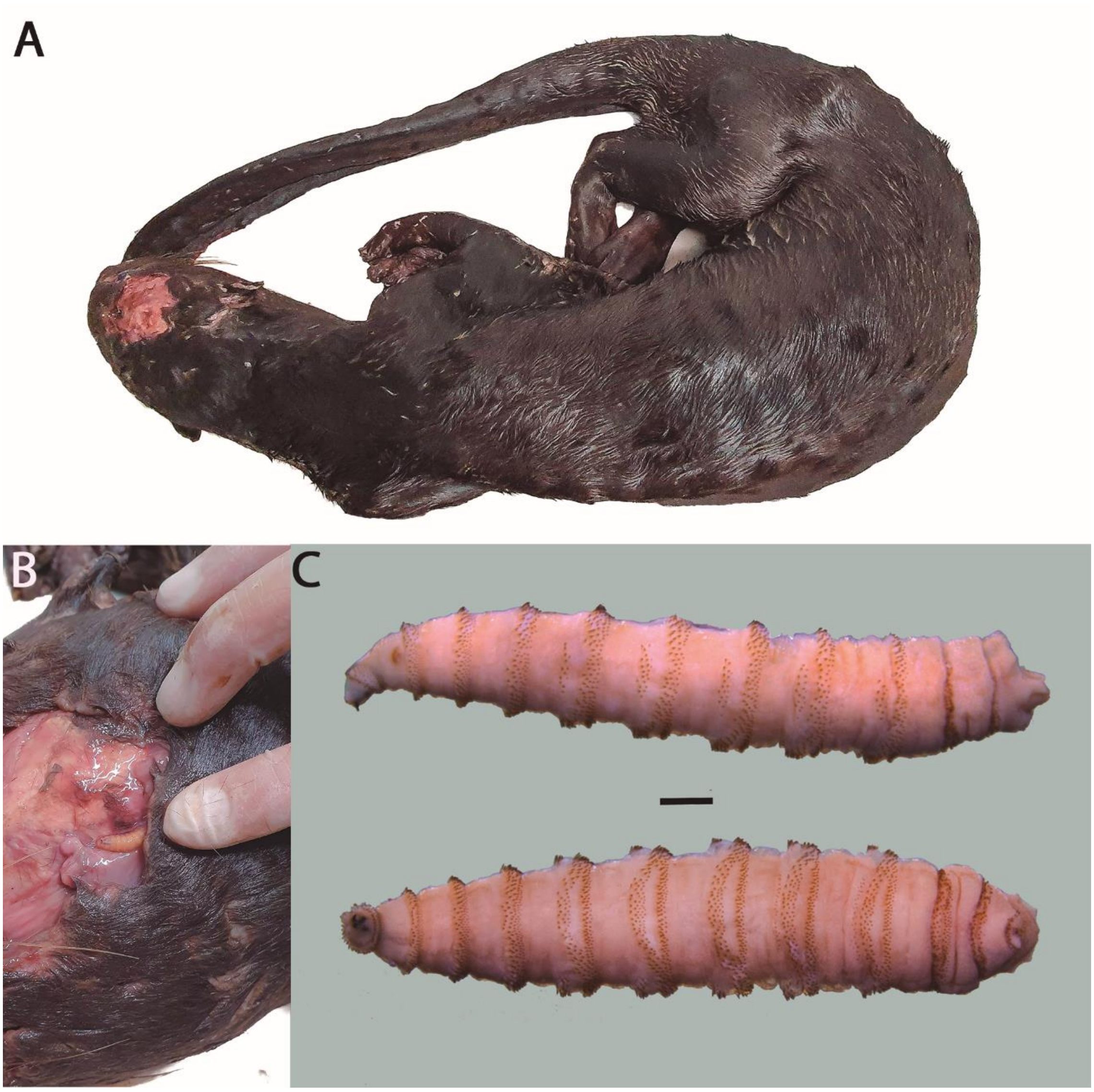


Figure 1**.** Carcass of a giant otter (*Pteronura brasiliensis*) found in the wild with myiasis lesion. A – General view of the animal; B – Details of the lesion, with

third-instar larva; C – Third-instar larva of *Cochliomyia hominivorax* (Coquerel, 1858) recovered from skin; lateral view and ventral view (bar = 1 mm).

All the larvae were in the third stage and were identified as *Cochliomyia hominivorax* (Coquerel 1858). They showed the following characteristics: anterior portion armed with a pair of strong mouth hooks (Figure 2A and 2B); peritremes of posterior spiracle incomplete and not defined enclosing the button poorly; encircling three straight sub parallel opening lined up diagonally (Figure 2C and 2D); cephalopharyngeal apparatus well developed and highly sclerotized, with conspicuous paired of mandibular hooks (Figure 3A and 3B); tracheal trunks lightly blackened, pigmented from the posterior spiracle (Figure 3C); dorsal region of the cornua not incised (Figure 3D); and anterior spiracle with short flattened stalk, with 10 nodular branches arranged fanwise (Figure 3E) (Knipling 1939, James 1947, Shewell 1981). The length of the larvae ranged from 10 to 15 mm.

*Cochliomyia hominivorax*, commonly known as the New World screwworm (NWS), is a dipteran species of the family Calliphoridae. The NWS is one of the main causes of myiasis in livestock, wildlife, and humans in tropical and subtropical parts of the Americas where it has not been eradicated, including Brazil (Wyss 2000,Zumpt 1965). Gravid adult female *C. hominivorax* lay their eggs in open wounds on the host. Upon hatching, the fly larvae, or maggots, also known as screwworms, feed on living dermal or sub dermal tissues of the parasitized host (Knipling 1939).

Myiasis is very rare in aquatic vertebrates, with only a few records in fish (Bristow et al. 1990,Öktener& Alas 2009, Zumpt 1965). The low occurrence of ectoparasites in giant otters, especially dipterans, might reflect their semi-aquatic habits, but also their grooming and allogrooming behavior, which makes it difficult for parasites to remain on the skin.

The NSW cannot develop in carrion; it feeds only on living tissues. The time taken for the larvae to reach the third stage is 5 to 7 days (Hall 1997). Thus, the otter probably acquired the NSW after being injured either by an accident or even intraspecific fights. Agonistic encounters between groups of giant otters have been reported before (Ribas& Mourão 2004). These fights can result in serious wounds or even death (Rosas & Mattos 2003, Leuchtenberger et al. 2015), even though many individuals can show rapid recovery from injuries in the wild (Foerster, person. obs.). It was hypothesized that the myiasis caused by *C. hominivorax* deteriorated the health ofthe infested giant otter, which prevented recovery and accelerated its death.

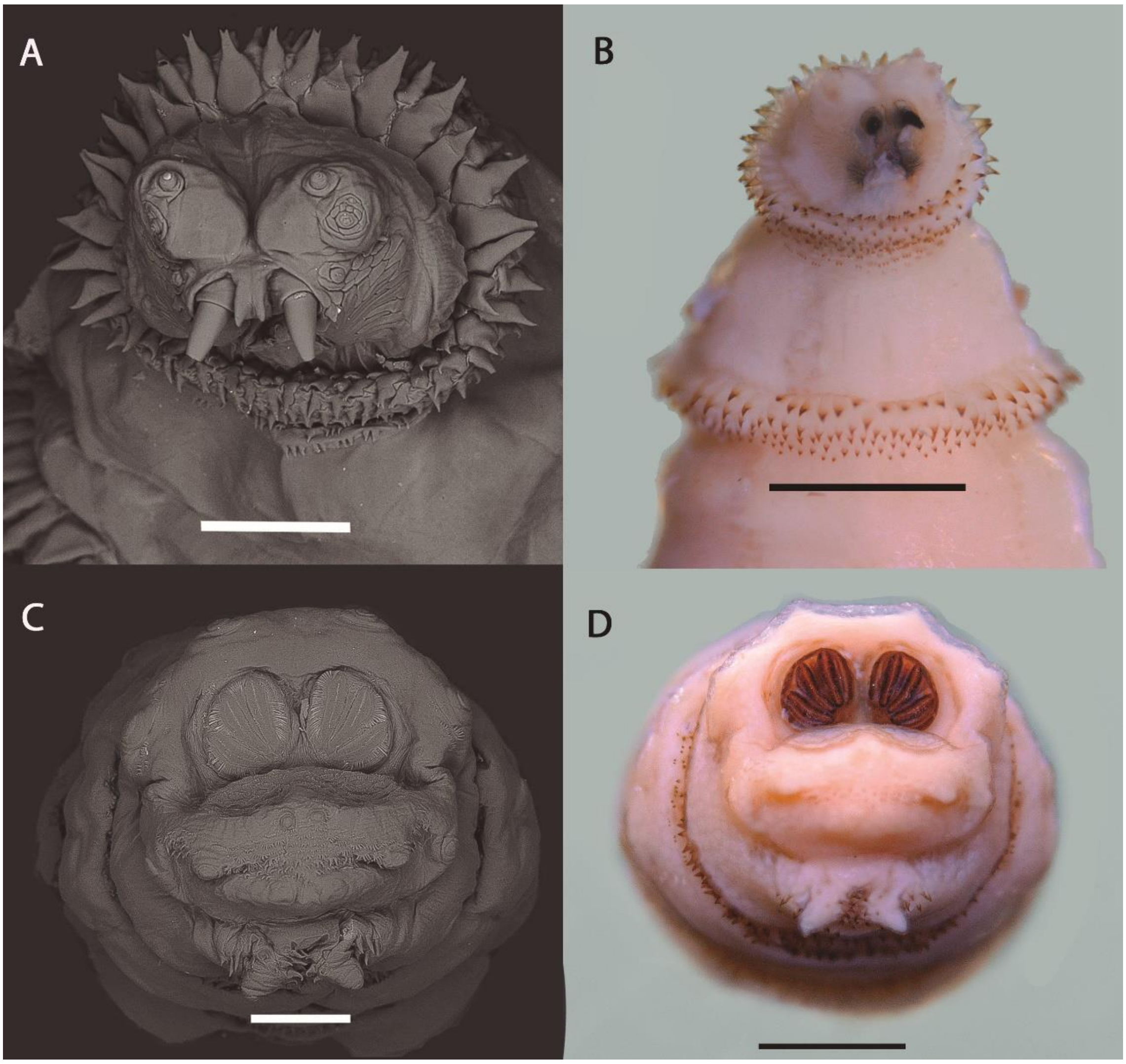


Figure 2.Third-instar larva of *Cochliomyiahominivorax*(Coquerel, 1858) recovered from skin lesion in giant otter (*Pteronura brasiliensis*) in the Pantanal, Brazil. A and B – Details of the anterior portion; C and D – View of the posterior portion. Scale bars: A and C = 250 μm; B and D = 1 mm.

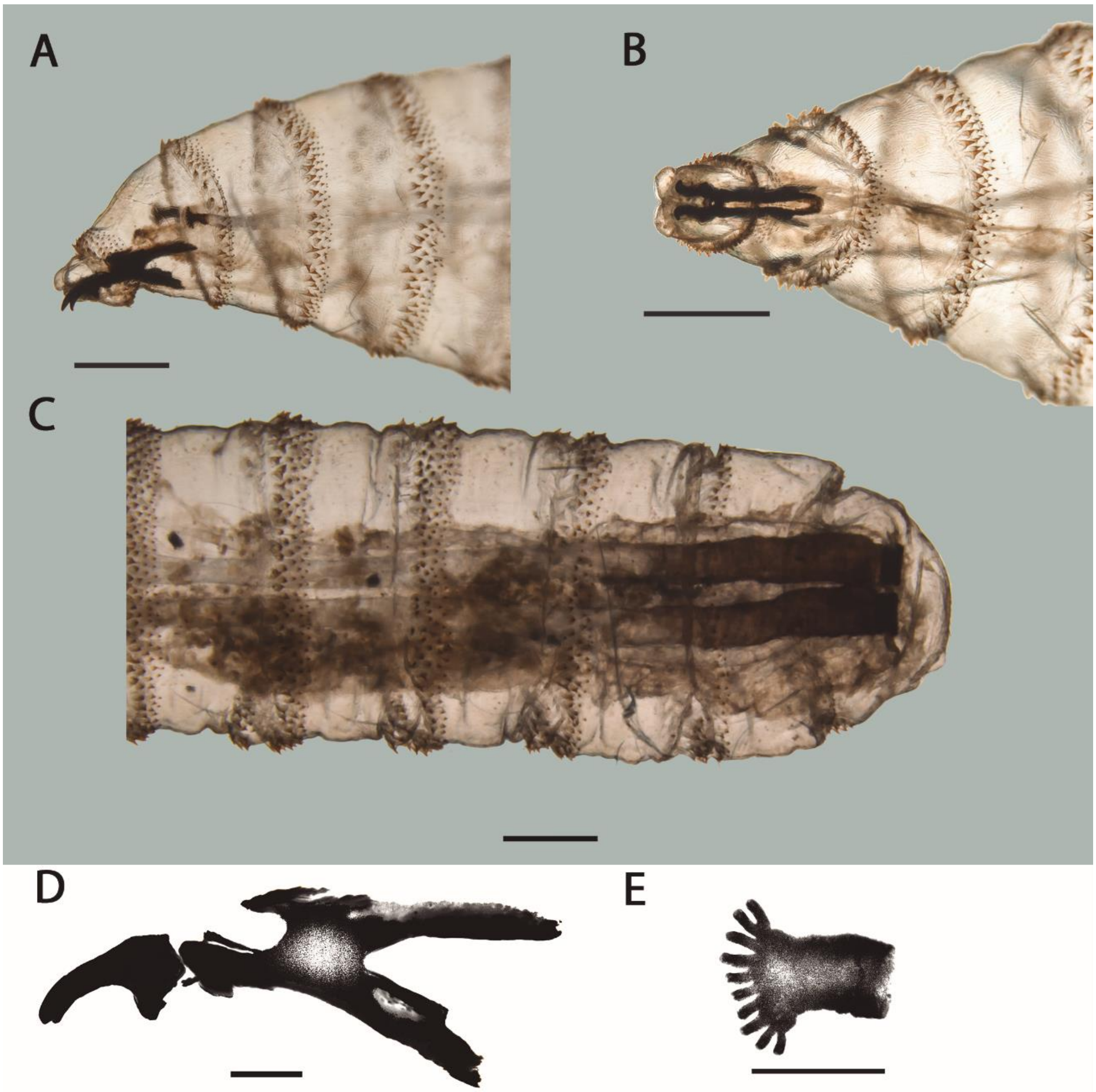


Figure 3.Third-instar larva of *Cochliomyia hominivorax* (Coquerel, 1858) recovered from skin lesion in giant otter (*Pteronura brasiliensis*) in the Pantanal, Brazil. A, B and C – specimen cleared with 10% solution of potassium hydroxide (KOH) (bar = 1 mm); A – Anterior portion in lateral view; B – Anterior portion in ventral view; C – Posterior portion in ventral view, highlighting the pigmentation of the tracheal trunks. D – Diagrammatic drawing of cephalopharyngeal apparatus in the lateral view (bar = 200 μm); and E – Diagrammatic drawing of anterior spiracle (bar = 250 μm).

# Chapter 3: Balancing Conflict: behavioral strategies of territory defense in a social top predator

## Abstract

The intraspecific interactions related with dispute and defense of territory can vary according to the purpose of how each species uses the territory. The Dear Enemy hypothesis states that the intensity of territory defense against nearby neighboring individuals should be lower than non-neighboring ones. In this study, we evaluate this hypothesis for giant otters (*Pteronura brasiliensis*) in the Pantanal wetland, Brazil, through behavioral responses to playback experiments, according to the type of the broadcasted sounds (adult-call and snort) and to the group-proximity the sound emitter (self, neighbor, and non-neighbor), given the presence of cubs and the effective group size. Our results showed that lonely individuals presented lower arousal responses than larger groups. The sociability of giant otters is associated with their defensive abilities, whereby larger groups have significant competitive advantages over smaller groups in such situations. Therefore, individuals must weigh the costs of exposure and combat, especially when they are outnumbered. The otters presented differences in responses to snort and adult-call sounds, which may be related to the meaning and information conveyed by them and differences in the vocal traits. The groups presented reduced responses when accompanied by cubs, suggesting being a strategy to minimize the exposition of their cubs to potential threats. In two occasions, the groups responded with the agonistic chorus to the self adult-call sound, indicating limitations on individual recognition. Our results did not support the Dear Enemy hypothesis for giant otters in the Pantanal, instead they apparently conform to the threat level hypothesis. Considering the constant scent marking conducted by giant otters, future studies should assess whether these markings contain individual and group identity information and evaluate how groups respond to distinct evidence of neighboring and non-neighboring intruders.

## Resumo

As relações intraespecíficas de disputa e defesa de território podem variar de acordo com o propósito de como cada espécie utiliza o território. A hipótese "Querido Inimigo" sugere que a intensidade da defesa do território contra vizinhos da mesma espécie deve ser menor do que quando ocorre com não-vizinhos. Neste estudo, avaliamos essa hipótese para ariranhas (*Pteronura brasiliensis*) no Pantanal, Brasil. em resposta a experimentos de playback, de acordo com o tipo de som reproduzido (chamado de adulto e bufo) e a proximidade do grupo com o emissor do som (próprio, vizinhos e não-vizinhos), considerando a presença de filhotes e o tamanho efetivo do grupo. Nossos resultados mostraram que indivíduos solitários apresentaram respostas de excitação mais baixas, enquanto grupos maiores responderam com maior excitação. A sociabilidade das ariranhas está associada à sua capacidade defensiva; grupos maiores têm vantagens competitivas significativas sobre grupos menores em tais situações. Portanto, os indivíduos devem avaliar os custos da exposição e do combate, especialmente quando estão em menor número. As ariranhas apresentaram diferenças nas respostas aos sons chamado de adulto e bufo, o que pode estar relacionado ao significado e ao tipo de informações transmitidas por esses sons e às diferenças na assinatura vocal. Os grupos apresentaram respostas reduzidas quando acompanhados por filhotes, sugerindo ser uma estratégia para minimizar a exposição da prole a ameaças potenciais. Em duas ocasiões, os grupos responderam com o coro agonístico ao som de chamado de adulto próprio, indicando limitações no reconhecimento individual. Nossos resultados não apoiaram a hispótese "Querido Inimigo" para ariranhas, em vez disso, aparentemente estão em conformidade com a hipótese do nível de ameaça. Considerando a marcação constante de odor realizada pelas ariranhas, estudos futuros devem avaliar se essas marcações contêm informações de identidade individual e de grupo e avaliar como os grupos respondem a diferentes evidências de intrusos vizinhos e não-vizinhos.

## Introduction

Territory is a defined space defended by one individual or group against conspecifics (Davies & Houston 1984). The intraspecific interactions of dispute and defense of territory have been studied in different animal groups (e.g. Boydston et al. 2001, Cavalcanti & Gese 2009, Leuchtenberger & Mourão 2008, Zenuto 2010). These relationships vary according to the purpose of how each species uses the territory: if only for reproduction (Hyman 2005), or for multiple purposes (Temeles 1994). The defense of the territory is important to guarantee access to resources, and thus the ecological success of the owner (individual or group) in each environment. However, the energy expenditure for maintaining the territory can be high (Jaeger 1981).

For many species, once a territory is established and after several encounter, neighbors become a known threat, and often the energy and time spent defending territory against those neighbors tends to diminish (e.g. Eason & Hannon 1994,Temeles 1994), resulting in a beneficial relationship for both (Jaeger 1981, Gosling & McKay 1990).Fisher (1954) named this hypothesis as Dear Enemy and suggested that the intensity of territory defense against known neighbors should be lower than against non-neighbors. Several studies have supported this hypothesis for different groups, such as crustaceans, salamanders, birds, rodents, and mustelids (Brooks & Falls 1975, Jaeger 1981, Palphramand & White 2007, Rosell et al. 2008, Booksmythe et al. 2010).

One of the explanations for the Dear Enemy hypothesis is based on the "expected familiarity" hypothesis, in which repeated interactions between neighbors eventually lead to less intense aggressions, resulting in less expenditure of energy and time (Booksmythe et al. 2010, Zenuto 2010). However, social species might present several different behavioral responses (e.g.Christensen & Radford 2018, Kranstauber & Manser 2018, McGregor &Bee 2018, Radford & Christensen 2018, Ridley & Mirville 2018, Stamps 2018, Thompson &Cant 2018). Alternatively, the "relative threat" hypothesis (Temeles 1994) states that neighbors and non-neighbors can represent different threat levels according to the resources they compete for, which can vary in availability and may depend on other environmental variables. The recognition and discrimination of neighbor and non-neighbor groups can demonstrate the strength of relationships and contribute to the ability of a group to maintain its territory and, therefore, its success. *In situ* experiments allow us to understand how territory-defense behaviors occur and to assess the capacity of individual recognition among social species.

In this study, we evaluate the behavioral response of free-ranging giant otters (*Pteronura brasiliensis*) to different simulated intruders in their territories. Giant otters are a reliable model to understand more about the territorial behavior of highly social species. They live in very cohesive groups, ranging from two to 16 individuals, formed by a breeding couple and non-breeding individuals that help with the care of the cubs (Duplaix 1980, Leuchtenberger & Mourão 2008). The groups perform almost all activities together and show social interactions like grooming, allogrooming, and rubbing (Duplaix 1980, Carter & Rosas 1997). Giant otters have a vocal repertoire composed of more than 15 different sounds for different contexts, and different combinations of sounds enlarges the communication possibilities among individuals (Leuchtenberger et al. 2014a, Mumm & Knörnschild 2014).

Giant otter groups are territorial along river stretches, and linear territories range from 2 to 20 km. They often patrol such territories and spend near 10% of their active time scent-marking the banks along the water bodies (Leuchtenberger et al. 2014b). Defense capacity usually improves as the number of adults in the group increases (Leuchtenberger et al. 2015). Scent marks combined with vocalizations (Leuchtenberger et al. 2015) have been considered as defense signs leading to discourage intruders. Despite this, agonistic encounters often occur among giant otters (Ribas& Mourão 2004, Ribas et al.2012, Leuchtenberger et al. 2015), conducting to high-energy expenditure and causing severe injuries to individuals (Leuchtenberger et al. 2015). Therefore, the ability to recognize members of their own group and members of neighboring groups, and to differentiate them from possible passers-by or invaders from non-neighboring groups, can represent an adaptive advantage.

Some types of sounds emitted by giant otters, such as calls and snorts, carry acoustic signatures or acoustic traits that may allow individuals to distinguish each other, as well as other groups and potential rivals(Mumm et al. 2014, Leuchtenberger et al. 2016a). However, the ability to recognize and differentiate between neighboring groups and individuals passing by or members of non-neighboring groups has not yet been evaluated in giant otters.

Here, we evaluate whether the behavioral and vocal responses of giant otters differ according to the type (adult-call and snort) of broadcasted sounds and to the group-proximity with the sound emitter (self, neighbor, and non-neighbor), given the presence/absence of cubs and the effective group size. Considering the acoustic traits of the snorts and adult-calls, we hypothesized that giant otters could discriminate between those sounds emitted by different individuals according to the group-proximity. In that case, the group members should recognize and differentiate these sounds emitted by individuals of their own groups from those emitted by neighbors or members of non-neighboring groups. Due to the territorial nature of giant otters (Leuchtenberger et al. 2015), the responses to these broadcasted sounds would be different if they were recorded from the self-group or from an intruder giant otter. In addition, otter responses could differ if the broadcasted sound is from a known neighboring or with a non-neighboring intruder giant otter. Based on this, we examined whether giant otters fit the Dear Enemy hypothesis, i.e., whether they respond less aggressively to playbacks from neighboring groups than to those from non-neighbor groups. In addition, we hypothesized that responses by giant otters will differ depending on their group size and on the presence or absence of cubs.

## Methods

*Study site*

This study was carried out in the southern region of the Pantanal floodplain (approximately 160.000 $km^2$), in the center of South America, in the Mato Grosso do Sul State, Brazil (Figure 1). The Pantanal is characterized by wet and dry seasons. Data were collected in two sites. One was a stretch of 60 km long of the Miranda River (19°34'36.71” S, 57°02'28.65” W) and 20 km of its tributary, the Vermelho River (19°37'26.78” S, 56°57'50.54” W). The site 2 was a stretch of 30 km long of the Negro River (19°34'42" S, 56°09'11" W).

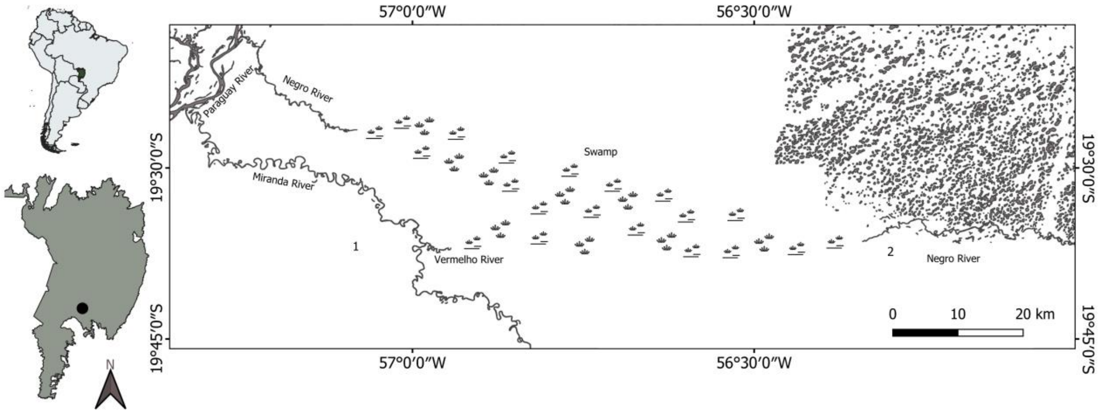


Figure 1. Location of the Pantanal in South America, (top-left), the study site in the Pantanal (bottom-left), and the rivers (right) where playback experiments with giant otter (*Pteronura brasiliensis)* groups were carried out between August 2019 and November 2021: (1) Miranda and Vermelho rivers where nine groups were sampled; (2) Negro River, where six groups were sampled, surrounded by lakes (dark spots) characteristic of the area. Vermelho and Negro rives are connected by a large swamp.

*Monitoring* of *giant otter groups*

We monitored nine groups of giant otters at the Miranda and Vermelho rivers monthly from August to November 2019 and from June to November 2021. Additionally, we monitored six groups at the Negro River monthly from March to September 2020. The field trips varied from seven to 30 days. The surveys were conducted by boat or canoe. Since giant otters are mainly diurnal (Leuchtenberger et al., 2014b), the groups were monitored from sunrise to sunset to record group size, presence/absence of cubs, territory boundaries, and their vocalizations.

*Playback experiment*

We conducted a playback experiment to evaluate, throughout their responses to those sounds, whether giant otters can recognize and differentiate their own calls and to those from neighbors and non-neighbors conspecifics, thus assuming that individuals can recognize each other through acoustic signatures. We selected two sound types, the adult-call and snort, which may present acoustical traits that might indicated acoustical vocal signature (Mumm et al. 2014, Leuchtenberger et al. 2016a). For each sound types (i.e., adult-call and snort), we established three treatments: (S) Self-group sounds (self-group, i.e., sounds produced by one individual of the focal group); (A) adjacent-neighbor sounds (i.e. sounds produced by individuals from a group-territory adjacent to the focal group); (D) non-neighboring sounds (i.e., a sound produced by individuals from a distant, non-adjacent territory).

Sounds used during the playback experiments were recorded from individuals from the monitored groups, with a directional microphone (Sennheiser, ME-66) connected to a digital audio recorder (Zoom H4N; 16-bit resolution and 44.1 kHz sampling rate) and saved as wav files. For playbacks, we edited and selected only high-quality sounds on the Raven Pro 1.6 software (Cornell Lab of Ornithology, Ithaca, NY, U.S.A.). Playback stimuli were edited also on the Raven Pro 1.6 software in a way that each consisted of three call sequences of the same sound type, broadcasted three times in each experiment with an interval of 5 seconds between each sequence.

In each experiment, we set a loudspeaker (JBL charge 4) hidden on the bankside in areas of habitual use of the groups, in which likely the groups would pass (Figure 2). After setting up the equipment, we waited for the group to approach the location and then we broadcasted the playback. We stood at a minimum distance of 20 mfrom the loudspeaker. We installed the loudspeaker when the groups were not in the area and we avoided being perceived by the groups during the experiment, making sure that our presence would not affect their behavior.

Figure 2. Giant otters (*Pteronura brasiliensis*) approaching the loudspeaker during a playback experiment at the Negro River in the Southern Pantanal, Brazil. The white circle indicates where the loudspeaker is hidden on the bankside.

We conducted a single experiment for each group per day, wherein the sequence of treatments and sound types were randomly selected. Therefore, most groups were submitted to each combination of treatment and sounds, with at least a 24-hour interval between consecutive playbacks for the same group. We recorded the behavior and sound responses of these groups for up to 15 min.

We evaluated if the group size and the presence of cubs could be affecting the responses of the playbacks. Cubs were considered those individuals up to six months old (Groenendijk et al. 2005).

All the data collection and the playback experiment were authorized under environmental license from the Federal Environmental Agency of Brazil (SISBIO/ICMBio 68974/2019) and from the Animal Use Ethics Committee of Embrapa Pantanal (protocol 004/2019).

*Response Measurements*

In our analysis, the input data was the group collective response, since individual responses recognition to the playback was not always possible. We recorded five explanatory variables and nine response variables (Table 1).

Table 1. Variables recorded during playback experiments with giant otters (*Pteronura brasiliensis*), from August 2019 to November 2021, in two sites in the Southern Pantanal of Brazil.

| **Explanatory variables** | **Type** | **Description** | **Levels** |
|---|---|---|---|
| Playback | Binomial | Sound played | snort, adultcall |
| Group-proximity | Category (three levels) | Classification of the broadcasted sound according to the origin of the caller | neighbor, non-neighbor, self |
| Cub | Binomial | Cubs | Present, absent |
| Effective group size | Count | Number of individuals of a given group present during the experiment (except for cubs) | 1-8 |
| **Response variables** | **Type** | **Description** | **Levels** |
| Approach | Binomial | Did the otters approach towards the sound source? | Yes, no |
| Attack | Binomial | Did the otters move fast and escalate the riverbank towards the sound source? | Yes, no |
| Hide | Binomial | Did the otters hide after the playback? | Yes, no |
| Latency | Continuous | Time latency between the start of the playback and the first movement in relation to the sound source | Up to 300 sec |
| Flee | Binomial | Did the otters fled from the sound source? | Yes, no |
| Alarm calls | Count | Number of alarm calls emitted after the playback | 0-300 |
| Close-contact calls | Count | Number of close-contact calls emitted after the playback | 0-300 |
| Arousal Rank | Rank | Arousal rank ranging from close contact calls to agonistic chorus (see text) | 0-10 |
| Vocalization | Binomial | Did the otters vocalize after the playback? | Yes, no |

In response to the playbacks, we recorded seven types of sounds, previously described by Duplaix (1980), Ribas & Mourão 2004, Leuchtenberger et al. (2014a) and Mumm &Knörnschild (2014): close-contact sounds, HAH, adult call, snorts, begging, scream, and agonistic chorus. Most sounds present physical structures that enable us to recognize their arousal state, which can range from lower to higher arousal, depending on the situation (Morton 1977). Therefore, based on the social context in which each sound is used, from close-contact and coordination to more alarmed and threatening situations (Leuchtenberger et al. 2014a), and the detection of acoustical arousal traces on the spectrograms, we ranked the arousal state based on vocal response of the groups to the playbacks. This resulted in 11 categories of arousal rank (Figure 3, Table 2), ranging from no vocalization to close contact calls and agonistic chorus, as follows: (0) no vocalization, (1) close-contact low, (2) close-contact high, (3) HAH low, (4) HAH high, (5) adult call low, (6) adult call high, (7) snort high, (8) begging high, (9) scream, and the (10) agonistic chorus. Close-contact calls were classified as high arousal when presented at lower frequencies. The HAH sound was classified as high arousal when it was produced in sequences of more than one in intervals shorter than two seconds. Snorts were considered in high arousal when presented harsh and more pulses ends. The sounds snort low and begging low were absent responses or associated with other sounds of higher arousal, therefore, not considered on the arousal rank analyses. Adult-calls and begging were considered in high arousal when became harsher at the end and/or transitioned to scream-like sounds. Scream and agonistic chorus are sounds emitted in higher arousal states. The sounds snort, begging-scream, scream and agonistic chorus were always vocalized in high arousal in response to the playbacks. Other sounds such as growl, high-scream, and double whistle were emitted in some responses; however, they were excluded from the scale because other sounds with higher score were also emitted in the same response.

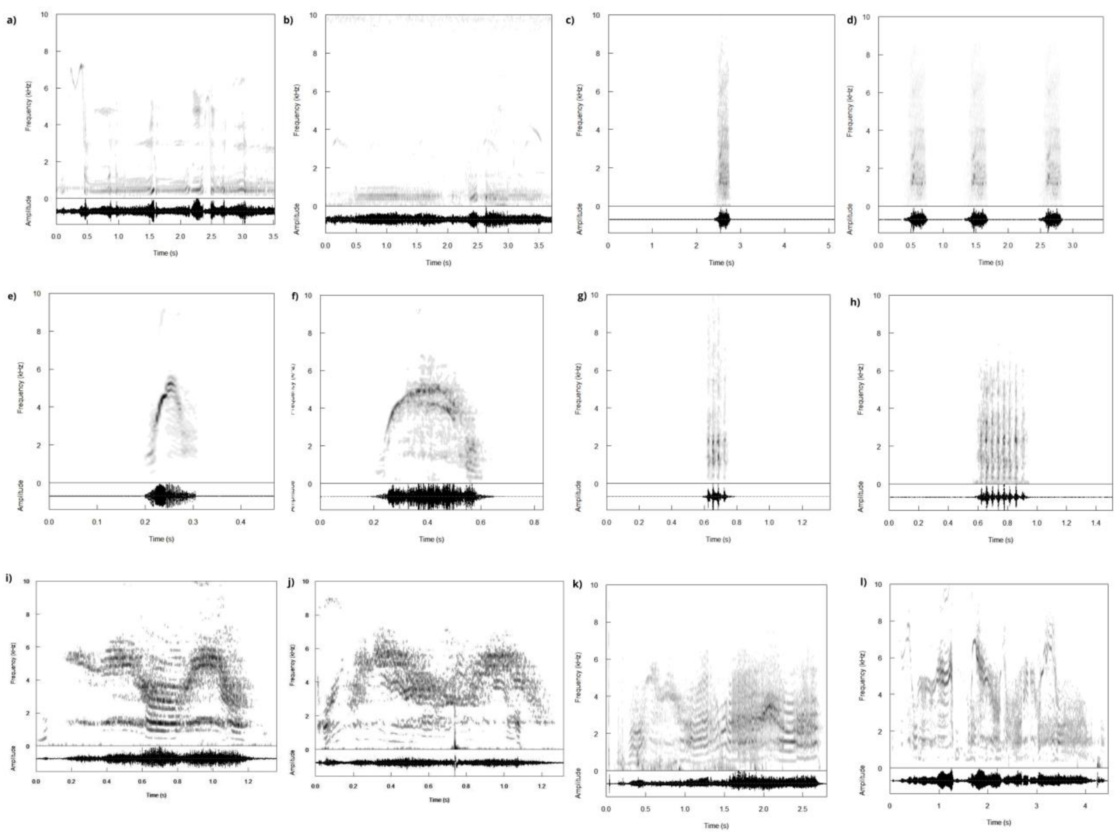


Figure 3. Spectrograms and oscillograms of vocalizations emitted by free-ranging giant otters in the Pantanal, characterized according to their arousal state (low or high) ranging from close-contact calls to agonistic chorus, as follow: a) close-contact low; b) close-contact high; c) HAH low; d)HAH high; e) adult-call low; f) adult-call high; g)snort low*; h) snort high; i) begging low*; j) begging high; k) scream; l) agonistic chorus. * The sounds snort low and begging low were not present on the responses or were always associated with another sound of higher arousal, therefore, not considered on the arousal rank analyses.

Table 2. Categories of arousal rank and the number o times each category showed according to the playback sound (adult-call or snort) and the treatment (self, neighbor and non-neighbor) during playback experiments with free-ranging giant otters (*Pteronura brasiliensis*) in the Brazilian Pantanal.

| Sound | Arousal state | Arousal rank | **Treatment** | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Self | | Neighbor | | Non-neighbor | |
| | | | Adult-call | Snort | Adult-call | Snort | Adult-call | Snort |
| No vocalization | -- | 0 | 2 | 7 | 3 | 4 | 2 | 4 |
| Close-contact | low | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| Close-contact | high | 2 | 0 | 0 | 0 | 0 | 2 | 0 |
| HAH | low | 3 | 0 | 2 | 1 | 2 | 2 | 1 |
| HAH | high | 4 | 0 | 0 | 3 | 4 | 2 | 4 |
| Adult-call | low | 5 | 0 | 0 | 0 | 0 | 0 | 1 |
| Adult-call | high | 6 | 0 | 0 | 0 | 0 | 0 | 1 |
| Snort | high | 7 | 0 | 0 | 2 | 0 | 0 | 1 |
| Begging | high | 8 | 2 | 0 | 0 | 0 | 0 | 0 |
| Scream | -- | 9 | 5 | 0 | 2 | 0 | 0 | 0 |
| Agonistic chorus | -- | 10 | 2 | 1 | 5 | 1 | 6 | 0 |

*Statistical analysis*

All analyses were performed in the R (version 4.2.2.) environment for statistical analyses (R Core Team 2022). We fitted general linear models (GLM) using binomial distribution to estimate whether the effective group size, the group-proximity with the sound emitter, the type of the broadcasted sound, and the presence/absence of cubs in the group affected the probability of the groups adopt one of the four non-vocal behavioral response to the playbacks. The four examined non-vocal behaviors were (1) approached or not (2) fled or not, (3) to hide or not, or (4) attacked or not the point of origin of the broadcasted sound. We estimate the McFadden's $R^2$ for each of theseanalyses as 1 – residual deviance / null deviance. To plot the results of these regression models, we used the visreg package (Breheny & Burchett 2017).

We fitted a linear mixed-effects model using the function lmer() of the library lme4 (Bates et al. 2015), associated with the library lmerTest (Kuznetsova et al. 2017), to model the arousal rank produced by the groups in response to the interaction of the playback types (adult-call or snort) with the treatment (group-proximity). We included the presence/absence of cubs in the group and the identity of the groups (id) as random variables, as we did not have full control over them. The lmerTest provides t-test using the Satterthwaite's method to the lmer outputs, and then allowing the computation of probability values (Kuznetsova et al. 2017). Also, we used the function r.squaredGLMM() of the MuMIn package (Bartoń 2009) to estimate the coefficient of determination of the full model (conditional $R^2$) and of the fixed effects (marginal $R^2$) of the lmer model (Nakagawa et al. 2017). The agonistic chorus is the unequivocal vocal sign of the otters' willingness to fight for territory. Therefore, we performed a chi-squared test to assess the significance of the differences in response frequencies between agonistic chorus response and the group-proximity during adult-call experiments.

To exam whether the overall behavioral and vocal responses of the giant otters respond to the type of the broadcasted sound and the group-proximity between the tested groups with the group of the sound emitter, we performed a permutational multivariate analysis of variance using distance matrices (PERMANOVA). For this analysis, we used the function adonis2() of the vegan package (Oksanen et al. 2022) and included the presence/absence of cubs and the effective group size as explanative variables. Finally, we run a non-metric multidimensional scaling (nMDS) ordination that allowed producing a biplot, to provide a graphic evaluation of our data.

## Results

The total sampling effort was of 1440 hours distributed in 120 days. We conducted the experiment with 15 different free-ranging giant otter groups in the Pantanal, six at the Negro River and nine at the Miranda and Vermelho rivers. Group size varied from two to eight, but during some experiments, the number of individuals present varied from one to eight. In total, we executed 76 playback experiments: 17 neighbor adult-call, 14 non-neighbor adult-call, and 11 self adult-call; 12 neighbor snort, 12 non-neighbor snort, and 10 self snort.

Giant otters responded to all experiments, displaying a range of different vocal and non-vocal responses, and their first movement always was to turn the head or to periscope towards the loudspeaker. They vocalized 71% (n=54) of the times in response to the playbacks.

Examining the behavioral non-vocal responses, we found that the giant otters approached the sound source occurred in 74% (n=56), nevertheless, none of the four studied explanative variables (playback sound type, group-proximity, presence of cubs, and effective group size) were significant ($|z| \leq 1.190$, $p_{partial} > 0.234$) and the model explained only 0.05% of the variability of the dependent variable. The otters fled the area following the playbacks in 20% of the times (n=15), however, none of the explanative variables (effective group size, group-proximity, playback type, presence of cubs) were significant ($|z| \leq 0.782$, $p_{partial} > 0.142$) and only about 0.07% of the variability could be explained for the full model. In about 13% (n=10) of the times, the otters hid in response to the playbacks and the hid behavior was inversely related with the number of individuals present during the playbacks (-1.148, $z = -2.759$, $p_{partial} = 0.006$, Figure 4). However, neither group-proximity with the sound emitter ($z = 0.596$, $p = 0.551$ for neighbor and $z = 0.509$, $p = 0.611$ for non-neighbor originated sound), nor the playback sound ($z = -0.236$, $p = 0.813$ for the snort sound in comparison to the adult call) were significant. The presence of cubs was also not significant ($z = 1.482$, $p = 0.138$). The full model explained almost one third of the deviance (McFadden's $R^2$ approximation = 0.322).Finally, the giant otters attempt to attack the sound source of the playback occurred in 14% (n=10) of the times. In none of these cases of attack, there were cubs with the experimental group. In addition, the response attack always occurred in response to the adult call, with one exception ($z = -1.999$, $p_{partial} = 0.046$, Figure 5). The only case when a group responded to a snort playback with an attack occurred when the specific sound broadcasted had more pulses than usual and was recorded from an ex-member of the group that formed a new neighboring group. All the attack responses occurred when playbacks were from an adjacent group that overlapped their territories and it coincided with areas where we registered more often intraspecific agonistic encounters during our monitoring (nine of 14 registers).

In relation to the vocal responses, we found that the snort sound led to lower arousal ranks responses from the experimental groups($t = -4.334$, $df = 62.856$, $p_{partial} < 0.001$) than the adult call, irrespective of the group-proximity to the original caller (Figure 6A). Nor the caller group-proximity, nor its interaction with the playback sounds significantly affected the arousal ranks, although the sound snort, recorded from the own group, tended to lead to lower arousal ranks in the vocal responses of the groups ($t = 1.766$, $df = 63.425$, $p_{partial} = 0.082$) (Figure 6B). The larger the effective group size, higher the arousal ranks ($t = 2.523$, $df = 23.938$, $p_{partial} = 0.019$) (Figure 6C), and the presence of cubs in the experimental group lead to lower arousal ranks (t = -3.141, df = 12.027, $p_{partial}$= 0.009) (Fig. 6d).The full model explained about 44% of the variability ($R^2_{conditional}$ = 0.444), while the fixed factors accounted for about 40% of the model variability ($R^2_{conditional}$ = 0.403). In two occasions, the focal group responded with agonistic chorus to self-adult-call playbacks. Although it was less frequent than the times that the focal group responded with this chorus to the adult-call sounds recorded from neighboring and non-neighboring groups ($\chi^2$ = 27.073, df = 16, p = 0.041, Table 2).

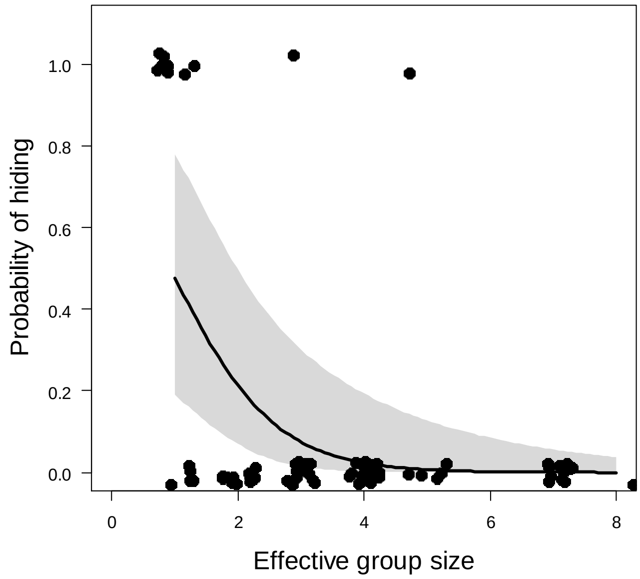


Figure 4. Partial probability of giant otters (*Pteronura brasiliensis*) hiding as a function of the effective group size during playbacks experiments (see methods). The full model also included playback sound type (adult call or snort) and group-proximity with the sound emitter (self, neighbor, non-neighbor), which resulted in non-significant partial terms (see text).

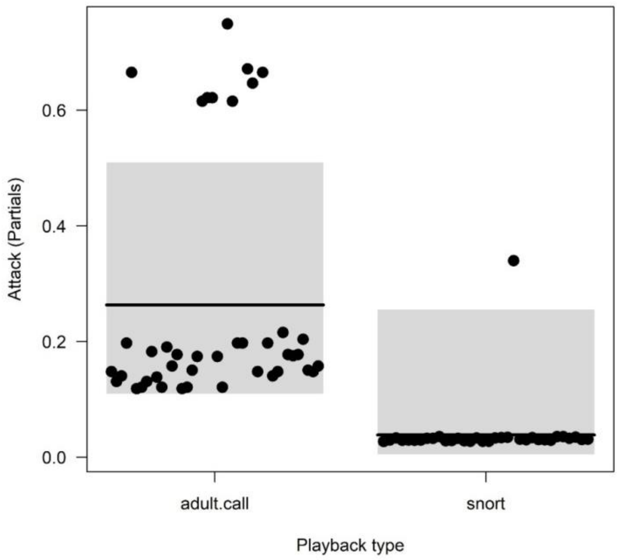


Figure 5. Partial residuals plot resulted from a mixed effect model relating the attack response towards of groups of giant otter (*Pteronura brasiliensis*) to the playback sound (adult call or snort)

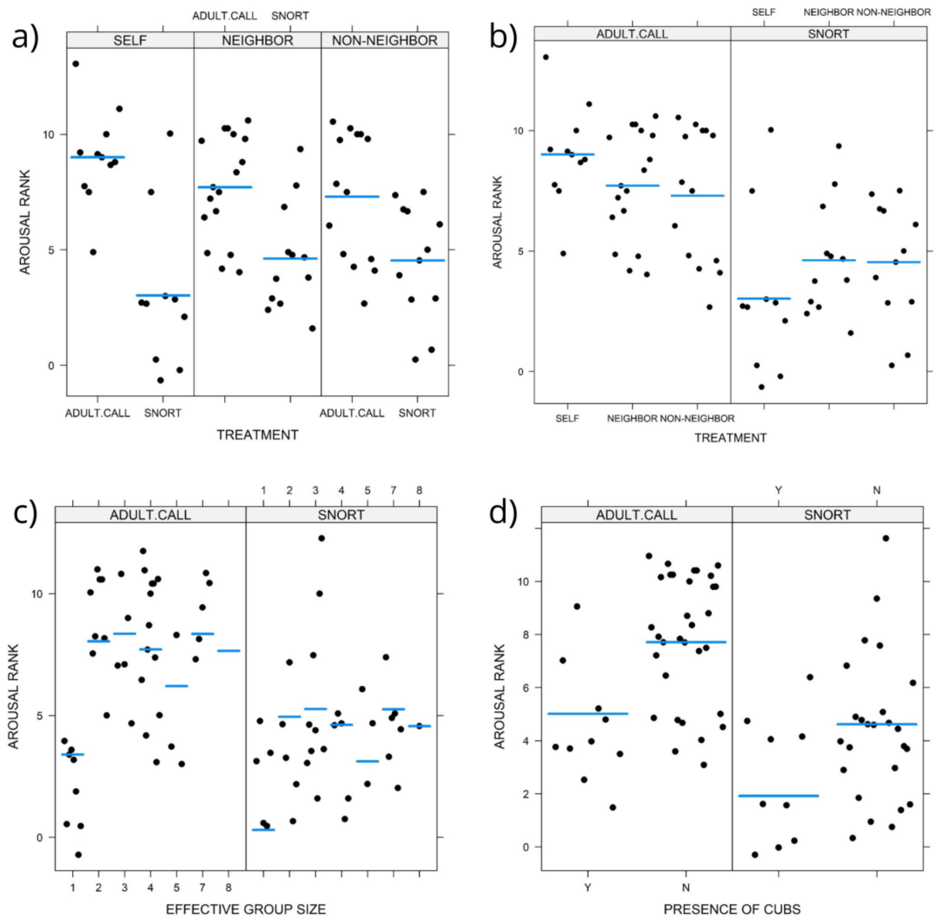


Figure 6.Partial residuals plot resulted from a mixed effect model relating to the arousal rank that indexed the degree of awareness of vocal response of groups

of giant otter (*Pteronura brasiliensis*) to:(a) the playback sound (adult-call or snort) given the group-proximity with the sound emitter (self, neighbor, non-neighbor); (b) to the group-proximity given playback sound; (c) to the effective size of the group given the playback sound; and (d) to the presence of cubs in groups (yes or no) given the playback sound. Groups were included in the analysis as a random variable (see text for details).

The PERMANCOVA indicated a significant interaction between the playback sound and the treatment related to the group-proximity with the original transmitter of the broadcasted individual ($F_{5,75}$= 3.510, $p < 0.002$, $R^2_{partial} = 0.177$). The number of individuals at the time of the experiment ($F_{1,75} = 6.630$, $p < 0.001$, $R^2_{partial} = 0.067$) and the presence of cubs in groups ($F_{1,75} = 7.004$, $p = 0.001$, $R^2_{partial} = 0.071$) also affected the otters response. Despite the relatively low proportion of the model variability explained by the presence of cubs in groups, when they were present, the focal groups tended to respond less aggressively to the playbacks and did not vocalize at all in most of the times (58%).

The nMDS analysis resulted in a smoothed diagram of Sheppard (linear fir $R^2$ = 0.97) with a relatively small stress (0.091), indicating that the analysis captured most of the variability of the model. The inspection of the biplot resulted from the nMDS analysis (Figure 7) suggests that the ordination captured the main strategies utilized by wild giant otters when confronting intruders within their territory.

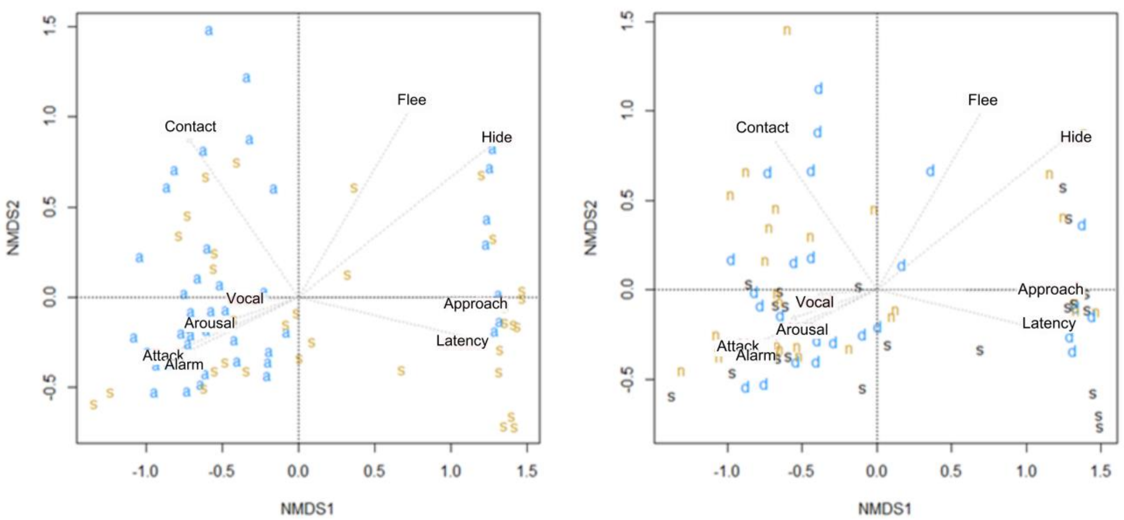


Figure 7. Bivariate plots resulting from the NMDS ordination analysis conducted to reduce the dimensionality of up to nine vocal or behavioral responses of giant

otter groups (see table 1). These responses followed the playback of either an adult-call (a) or a snort (s) (left panel), combined with the degree of group-proximity (neighboring or non-neighboring) between the focal group and the giant otter individual which had originally produced the sound: s = sound of a giant otter of the self-group, n = sound of a giant otter of a neighboring group; and d = sound of a giant otter of a distant non-neighboring group (right panel).

## Discussion

The giant otters presented a wide range of different vocal and non-vocal responses to the experiments. They often approached the playback source and rarely fled the area, regardless the stimuli type. This investigative behavior is typical of the giant otters as they are constantly inspecting new things and scents along their territories. Nevertheless, this behavior is generally believed to serve for gathering information rather than for territory defense (Stamps 2018). Besides, the responses also varied according to the presence of cubs and group size, what may have masked patterns. Moreover, variations in group living species can occur due to individual variations between group members, such as sex and hierarchy (Christensen & Radford 2018).

The otters vocalized in most of the playback experiments, although lonely individuals did not vocalize and used to hide, whereas larger groups responded with higher arousal ranks. The sociability of giant otters seems to be associated with their protective and defensive capabilities (Leuchtenberger et al. 2016b), contributing to the overall success of the group (Groenendijk et al. 2015). In intergroup conflicts, group size can be a positive competitive advantage. Generally, larger groups have significant competitive advantages over smaller groups in such situations (McComb et al. 1994, Cant et al. 2002). Therefore, the results support that individuals likely weigh the costs of exposure and combat, hiding when in numeric disadvantage as a strategy to avoid agonistic encounters and escalated injuries. Leuchtenberger et al. (2015) observed that small groups of giant otter often remained hidden in marginal swamps or lakes, while large groups were invading their territories. Hiding seems also a strategy of lonely giant otters to scape potential predators like jaguars (*Panthera onca*), while groups often engage in mobbing the predator to force it to leave their territory (Leuchtenberger et al. 2016b).

The otters vocalized much less in response to self-snort playback. In addition, it was possible to observe during most of the experiments of self-adult-call that one individual responded first, spent more time observing and looking at the sound source, and usually this one vocalized first (mainly begging scream) and then the rest of the group. This behavior was not observed in cases of adult-call playbacks of other groups. These subtle observations indicate that giant otters recognize individual or group signatures of adult-call and snort, as proposed by Mumm et al. (2014) and Leuchtenberger et al. (2016a).

The observed difference in responses between the giant otter groups to snort and adult-call sounds may be related to the meaning and type of information conveyed by these sounds. Snorts are typically vocalized in alarm contexts, serving as an alert to other group members (Leuchtenberger et al. 2014a). Snort can become louder or be emitted in double bursts when animals are startled (Leuchtenberger et al. 2014a), potentially encoding the level and type of threat, such as observed in alarm calls of other social mammals (Manser et al. 2001, Digweed et al. 2005). The otters seem to pay less attention when another member of the group vocalizes regular snorts in comparison to the harsh and pulsed ones. In general, broadcasted snorts resulted in lower arousal ranks responses than broadcasted adult-calls. However, when a more pulsed snort sound (higher arousal) was used in two experiments (one self and one neighbor), giant otters exhibited responses at higher arousal levels, including vocalizing in agonistic chorus, and attacking. This suggests that individuals might perceive acoustic variations in the snort sound and attribute significance to it based on the conveyed information. This differentiation also occurs in suricates (*Suricata suricatta*), that respond differently to playbacks that indicate the urgency of the situation highlighting the ability of individuals to perceive differences in the structure of the calls (Manser et al. 2001), . Yet, more experiments are necessary to understand more how giantotters respond to variations in the acoustic traits of snort sounds.

Adult-calls appear to have an intragroup function and are emitted when animals are searching for other group members (Leuchtenberger et al. 2014a). Group members responded to adult-calls with sounds of high arousal rank, and engaged into agonistic chorus especially when the playback call was from an intruder. The reason why the adult-call sound always elicited responses in higher arousal could be attributed to the more accentuated vocal signature in comparison to snort (Mumm et al. 2014,Leuchtenberger et al. 2016a). In addition, this sound may carry more important information about location than the snort, and increase the group cohesion compared to the regular snort. Due to the significance of the adult-call, individuals are more attuned and responsive to this sound as they provide valuable information for survival and social cohesion. Additionally, less common sounds that are infrequently heard, like the adult-call, may grab more attention and elicit stronger responses. This natural response ensures that individuals remain attentive to potentially novel or significant information in their environment. Therefore, adult-calls represent a less common vocalization in comparison with snort, and it is likely to attract more attention and generate stronger responses from individuals.

Groups with presence of cubs exhibited a reduced response to the playbacks than those with cubs absent, suggesting that the adults intended to minimize the exposure of cubs to potential threats. As k-strategists, giant otters invest significantly in reproduction, with the breeding couple and non-reproductive members cooperating in cub rearing (Staib 2005, Rosas et al. 2009). Cubs represent a costly investment for the entire group. In the Pantanal, female giant otters reproduce only once a year, producing two newborns in average. Newborns remain nestled in dens until the third month of life, when they start following their parents on brief excursions, but still are prone to be killed by carnivores, caiman or infanticidal giant otters (Schweizer 1992, Mourão & Carvalho 2001). Therefore, the reduced responsiveness to playbacks exhibited by groups containing cubs is likely a strategy to safeguard them from detection and potential involvement in intraspecific conflicts.

Most of the times that the giant otters responded the playbacks with the agonistic chorus occurred in response to non-self sounds, demonstrating their ability to recognize sounds emitted by otters outside their groups. However, in two occasions, the groups responded with the agonistic chorus to the self-adult-call sound, indicating that the discrimination process is not always accurate. Captive giant otters also presented individual recognition during playback experiments, but some individuals did not recognize their own sound (Mumm et al. 2014). Several other studies have shown that species with large vocal repertoires might present limitations on individual recognition (Goddard 1993, Botero et al. 2007).

Overall, the responses observed in this study represent the main array of strategies employed by wild giant otters when encountering intruders in their territories. On the one hand, these otters may respond aggressively to the playback, vocalizing intensively alarming sounds and ultimately attacking towards the speaker. On the other hand, the approach involves a more measured reaction, with the otters taking a longer time to respond, quietly advancing to investigate the playback's source, and eventually leaving the area or hiding. Our observations at this study evidence how the behavior of giant otters is diverse and can be influenced by a range of factors (e.g. Potts et al. 2019).

Despite the significant interaction between the playback type and the group-proximity, our results did not support the Dear Enemy hypothesis. Several studies have shown that social species present broader behavioral responses than those behaviors that leaded to the proposition of the Dear enemy hypothesis (Christensen & Radford 2018, Kranstauber& Manser 2018, McGregor & Bee 2018, Radford & Christensen 2018, Ridley & Mirville 2018, Stamps 2018,Thompson &Cant 2018). Similar responses to neighbors and non-neighbors were previously recorded for other social species (e.g. Battiston et al. 2015, Christensen et al. 2016).The threat level hypothesis states that the perceived threat posed by neighbors and non-neighbors depends on factors such as resource availability, group sizes, and social context (Christensen & Radford 2018, Decellieres et al. 2021). In certain situations, neighbors and non-neighbors may represent similar threat levels, resulting in comparable responses (Tumulty et al. 2018).For instance, in wild Diana monkeys (*Cercopithecus diana*) the territorial threat level varies according to the group density, predation pressure, and food availability (Decellieres et al. 2021).

Giant otter groups are territorial, and territory defense is important to avoid agonistic encounters (Rosas & Mattos 2003, Ribas& Mourão 2004, Leuchtenberger& Mourão 2009). Groups invest a substantial amount of daily time in scent-marking (Leuchtenberger et al. 2014b) and often patrol the area. Despite all this effort in signaling territory boundaries, territory overlap can occur (Leuchtenberger et al. 2015). Territory size is associated with the availability of resources, which allows an increase in the number of individuals in the group, reproductive success and consequently an improvement in fitness (Groenendijk et al. 2015). However, marginal environments can be quite harmful to the species, leading them to feed on unusual preys (Ribas et al. 2012, Leuchtenberger et al. 2020), present health problems (Leuchtenberger et al. 2020), and even the dissolution of groups and death of individuals (Leuchtenberger et al. 2015). Shared territories incur an energetic cost for the otters, mainly due to heterogeneous distribution of the resources (Groenendijk et al. 2015). The territory boundaries of giant otters can change with seasons, expanding or shrinking according to resources availability and disputes (Leuchtenberger et al. 2015). Therefore, even a single individual or a small group of intruders can potentially pose a significant threat to the territory holder (Ferrandiz-Rovira et al. 2020). Hence, the dispute for optimal territories seems to be constant. Consequently, regardless of group-proximity, all intruders could be similarly perceived as a similar threat by the territory owners. As a result, the response to both neighbors and non-neighbors tends to be similar. Thus, given the constant nature of territorial disputes and the potential dangers posed by intruders, our findings support the threat level hypothesis.

Scent marking is widespread among mustelids and can carry diverse information like sex, age, reproductive status, and resource usage (Hutchings & White 2000, Buesching et al. 2002, Zhang et al. 2003). In the case of giant otters, scent markings predominantly occur at communal latrines, where allo-marking takes place. Allo-marking involves all individuals depositing their scents, ultimately mixed by the dominant male (Leuchtenberger & Mourão 2009), potentially creating a group identity. These markings are not solely conducted by territory owners within their respective territories, but also by intruders when exploring the territory (Potts et al. 2019). It is common to observe giant otters sniffing these markings both within their own territory and even when encroaching foreign areas. Frequently, intruders leave these markings without encountering the group that holds the given territory. However, through these markings, the territory owners can detect the presence of intruders (Christensen & Radford 2018). Leuchtenberger & Mourão (2009) observed that following an agonistic encounter,giant otters invested significantly more time in scent marking latrines than usual, highlighting the role of scent marking in territorial defense. Future studies should assess whether these markings contain individual and group identity information and evaluate how groups respond to distinct evidence of neighboring and non-neighboring intruders.

## General Conclusion

In summary, our study yielded several significant findings for giant otters (*Pteronura brasiliensis*):

(1) There is a mercury contamination gradient in giant otters across the Pantanal.

(2) Mercury concentrations are higher in giant otters inhabiting sites near gold mining areas.

(3) Mercury concentrations are lower in giant otters inhabiting unconnected sites.

(4) To the best of our knowledge we reported the first case of myiasis in giant otters.

(5) Playback experiments revealed that lonely individuals display reduced arousal responses, while larger groups exhibit heightened arousal. This indicates that sociability of giant otters is associated with their defensive capabilities.

(6) Variations in responses to snort and adult-call sounds are related to the conveyed information type and meaning, as well as vocal traits of these sounds.

(7) Giant otters appear to reduce conflicts when accompanied by cubs, probably as strategy to minimize exposing their offspring to potential threats.

(8) Although giant otters exhibit some signs of individual recognition, it appears to have limitations.

(9) Giant otters do not fit the Dear Enemy hypothesis. Our findings support the "threat level hypothesis," indicating that giant otters perceive all conspecific intruders as potential threats.

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# Appendix

**Chapter 2 published**

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Short Communication

## First report of myiasis caused by *Cochliomyia hominivorax* in free-ranging giant otter (*Pteronura brasiliensis*)

Primeiro relato de miíase causada por *Cochliomyia hominivorax* em ariranha (*Pteronura brasiliensis*) de vida livre

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