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Molecular sexing and sex ratio of the neotropical otter (*Lontra longicaudis annectens)* using non-invasive samples from the Porce III Reservoir, Colombia

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The Neotropical otter is an important mammal in aquatic ecosystems due to its trophic position as a top predator, therefore is a valuable medium and long term bio monitor of the environment. Due to its crepuscular-nocturnal behavior, studies on this species primarily rely on non-invasive methods, such as the analysis of feces and anal-glands jellies, which allow for various ecological and population inferences. With recent advances in molecular techniques, these non-invasive samples have greatly contributed to the understanding of wild populations. Among the relevant population characteristics that might be obtained are individual identification and molecular sexing, which provide key information about demography, kinship relations, permanence, and sex-biased dispersal. This study implemented a molecular sexing methodology using DNA extracted from fresh feces and mucous of Neotropical otters, with the aim of determining the sex ratio in the otter population in a reservoir in northeastern Antioquia, Colombia. The study was conducted in the Porce III reservoir and its main tributaries. Fresh feces and anal-gland jelly from Neotropical otters were collected, DNA was extracted for genotyping. A total of 145 successfully genotyped samples were further amplified using two DNA segments: one homologous to the Y chromosome and another homologous to both sex chromosomes. The amplification products were separated on 3% agarose gels. Sex was successfully determined in 91 samples, corresponding to 40 individuals: 18 males and 22 females, with an average ratio of 2 females for every 1.4 males. A higher presence of females was found in the reservoir, while more males were detected in the tributaries. Females showed a higher recapture rate and lower mobility compared to males. The methodology used proved to be efficient for sex determination without the need to capture individuals. The results are consistent with previous studies suggesting greater female permanence. The information obtained is crucial for the management and conservation of the species, particularly in areas impacted by human activities.

La nutria neotropical es un mamífero importante en los ecosistemas acuáticos por su posición trófica como depredador tope que lo hace un buen biomonitor a mediano y largo plazo del medio ambiente. Debido a su comportamiento crepuscular-nocturno, los estudios sobre esta especie se basan principalmente en métodos no invasivos, como el análisis de heces y mucosidades, que permiten realizar diversas inferencias ecológicas y poblacionales. Con los avances recientes en las técnicas moleculares, estas muestras no invasivas han incrementado su contribución al conocimiento de las poblaciones. Entre los aspectos poblacionales relevantes que se pueden obtener se encuentran la identificación de individuos y el sexado molecular, que proporcionan información clave sobre la demografía, las relaciones de parentesco, la permanencia y la dispersión sesgada por sexo. El presente estudio implementó una metodología de sexado molecular con ADN obtenido a partir de heces y mucosidades de las glándulas anales frescas de nutria neotropical, con el objetivo de establecer la proporción de sexos en la población de nutrias de un embalse del nordeste de Antioquia, Colombia. El estudio se llevó a cabo en el embalse Porce III y sus principales afluentes. Se colectaron heces y mucosidades frescas de las glándulas anales de nutria neotropical, de las cuales se extrajo ADN para su genotipificación. Las muestras efectivas para la genotipificación fueron 145, en las cuales se amplificaron simultáneamente dos secuencias de ADN, una ligada al cromosoma Y, y otra homóloga a ambos cromosomas sexuales. Los productos de amplificación se separaron en geles de agarosa al 3%. Se logró determinar el sexo en 91 muestras, correspondientes a 40 individuos: 18 machos y 22 hembras, con una proporción promedio de 2 hembras por cada 1.4 machos. Se encontró mayor presencia de hembras en el embalse y mayor cantidad de machos en los afluentes. Las hembras presentaron una mayor frecuencia de recapturas y menor movilidad en comparación con los machos. La metodología empleada resultó eficiente para la determinación del sexo sin necesidad de capturar a los individuos. Los resultados coinciden con estudios previos que sugieren una mayor permanencia de las hembras. La información obtenida es fundamental para la gestión y conservación de la especie, especialmente en áreas afectadas por la actividad humana.

Keywords: non-invasive sampling, río Porce, sex ratios, Antioquia, SRY, ZFX/ZFY.

Introduction

Knowing the sex of individuals in wild populations allows for the evaluation of important ecological aspects such as sex ratio, type of reproductive strategy, individual movement or permanence, and home range of the different sexes within the population, which could imply territorial and resource monopolization (Griffiths *et al.* 1996; Gallo-Reynoso et al. 2013; Ferreira et al. 2018). Additionally, it provides a deeper understanding of population dynamics and structure, which is crucial for making management and conservation decisions, especially in isolated or threatened populations (Eggert and Guyétant 2003; Liu *et al.* 2014; Ancona *et al.* 2017), as is the case with some populations of the neotropical otter *Lontra longicaudis*.

This species is a semiaquatic mammal distributed from Mexico to Argentina and Uruguay (Rheingantz *et al.* 2022). Despite its wide distribution, characteristics of its populations, such as sex ratios, are poorly known. This parameter, like other ecological traits, may be especially relevant to support taxonomic differentiation, as there is current discussion regarding whether trans-Andean neotropical otters should be treated as a distinct species, *L. annectens* (de Ferran *et al.* 2024), a specific name we will use onwards.

In Colombia, *L. annectens* is distributed in the northwestern part of the Eastern Cordillera across all biogeographic regions, from lowlands to altitudes of 3,000 meters above sea level (Solari *et al.* 2013; Andrade-Ponce and Angarita-Sierra 2017). The species inhabits freshwater or brackish water bodies and feeds primarily on fish (Rheingantz *et al.* 2022). Its reproduction depends on the availability of food, shelter, and environmental conditions. Females take care of their offspring, extending up to the first year (Gallo-Reynoso 1989). In *Lontra longicaudis*, males have been reported to exhibit greater movement, in contrast to the philopatric behavior of females (Trinca et al. 2013). However, as mentioned earlier, this behavior has not yet been well characterized for trans-Andean otters.

Lontra longicaudis is classified as Near Threatened (NT) globally (Rheingantz *et al.* 2022) and as Vulnerable in Colombia (Ministerio de Medio Ambiente y Desarrollo sostenible MADS, 2024). Given its crepuscular-nocturnal activity patterns (Gallo-Reynoso et al. 2019) and its threatened conservation status, the use of non-invasive genetic methods is especially valuable for studying natural populations. These methods provide critical insights into population characteristics such as size, genetic variability, and sex determination, enabling inferences about the species' biology, behavior, and ecology (Ortega *et al.* 2012; Trinca and Eizirik 2012).

For otters (Lutrinae), sex identification using DNA extracted from feces has been approached mainly through the amplification of sex markers, such as the SRY and ZFX/ZFY genes. The SRY gene is a conserved sequence located on the Y chromosome and is key to sex determination in mammals (Graves 1998). In a PCR, positive amplification of this sequence indicates the presence of a male, while the absence of amplification would suggest a female. However, the lack of SRY amplification can also be due to the degradation or loss of the sequence in the sample, as well as procedural errors (Taberlet et al. 1996), which could lead to overestimating the proportion of females. To control for PCR sex determination failures, some authors have used the SRY marker in conjunction with other nuclear or mitochondrial sequences common to both sexes (Dallas *et al.* 2000, 2003; Huang *et al.* 2005; Hájková *et al.* 2009; Trinca and Eizirik 2012; Quaglietta *et al.* 2013; Lampa *et al.* 2015).

To reduce the risk of errors in sex assignment by tracking only one sex chromosome, several authors have implemented homologous segments from both the X and Y chromosomes in other mammals, such as the "Zinc-finger" marker (ZFX and ZFY) (Mucci and Randi 2007; Mullins *et al.* 2010; Park *et al.* 2011; Brzeski *et al.* 2013; O'Neill *et al.* 2013; Lerone *et al.* 2014; Vergara et al. 2014), a strategy that has also been applied to *Lontra longicaudis* (Ortega *et al.* 2012). This method requires subsequent digestion of the PCR products with a restriction enzyme capable of distinguishing between the amplified fragment from the X chromosome (ZFX) and the amplified fragment from the Y chromosome (ZFY) (Mucci and Randi 2007). Mowry *et al.* (2011) proposed a strategy involving the co-amplification of the ZFX/ZFY segment with the SRY marker, allowing them to identify males and females of *Lontra canadensis* without the need for restriction enzymes.

The otters in the Porce III reservoir, located in Antioquia, Colombia, form a population exposed to various environmental pressures, such as habitat loss and fragmentation, which limit their movement along the Porce River basin and may be affecting population dynamics and gene flow (Mason and Macdonald 1990; Rheingantz *et al.* 2022). This population is also exposed to multiple chemical and organic pollutants that enter the basin from industrial, domestic, and wastewater sources from 10 municipalities in the Valle de Aburrá, as well as 18 others within the Porce River basin, which together host approximately 4 million human inhabitants. Another prevalent contaminant is mercury, derived from gold mining activities around the reservoir (Gómez 2009). The high exposure to this mix of contaminants poses a long-term threat to the population's survival, as these mixtures have been shown to cause DNA mutations, hormonal disruptions, reduced fertility, and changes in sexual behavior in individuals (Storelli *et al.* 2002).

This situation makes it necessary to study the population status of the neotropical otter in the Porce River to establish effective conservation strategies. In this regard, molecular sexing can provide important information about its demographics (sex ratios), reproductive behavior, and whether there is sex-biased dispersal (Tucker et al. 2017; Hrovatin and Kunej 2018; Mengüllüoğlu *et al.* 2019). Therefore, the aim of this research is to shed light on some population aspects of the neotropical otter

present in the area of influence of the Porce III reservoir (Antioquia-Colombia), through a sexing methodology using DNA obtained from feces previously individualized by genotyping with 9 microsatellites, using the SRY and ZFX/ZFY markers.

Methodology

Study Area. The Porce III reservoir is located in the northeastern part of the department of Antioquia, Colombia, approximately 147 km from the city of Medellín. It is part of a hydroelectric generation chain that includes the upstream Porce II reservoir and the Guadalupe-Troneras complex and is primarily fed by the Porce and Guadalupe rivers. This reservoir has been in operation since 2010, has a capacity of 169 million cubic meters, and floods an area of 461 hectares, with a dam height of 151 meters and a maximum flood level of 680 meters above sea level. The Porce River, the main tributary of the reservoir, receives wastewater of industrial and domestic origin from all the municipalities of the Valle de Aburrá, including rural waste from its basin (Hurtado-Alarcón *et al.*, 2007).

Fieldwork. It was conducted between 2012 and 2022, with three field trips per year between 2012 and 2014 and four trips between 2015 and 2022, each trip lasting eight days. Four sectors were selected for sampling, including both the Porce III reservoir and three adjacent lotic systems: the Porce River before and after the reservoir, and the Guadalupe River (Figure 1). Along the banks of each lotic system, two transects of 1 km in length and variable width were traversed. The reservoir was surveyed along both margins using a motorboat at an average speed of 10 km/h along the navigable perimeter, covering approximately 24 km. During each sampling campaign, two surveys per sector were conducted. Fresh otter feces and anal-glands jelly were collected in properly labeled vials with 99% ethanol. The collection sites were georeferenced using GPS, and the samples were kept at -20°C until processing.

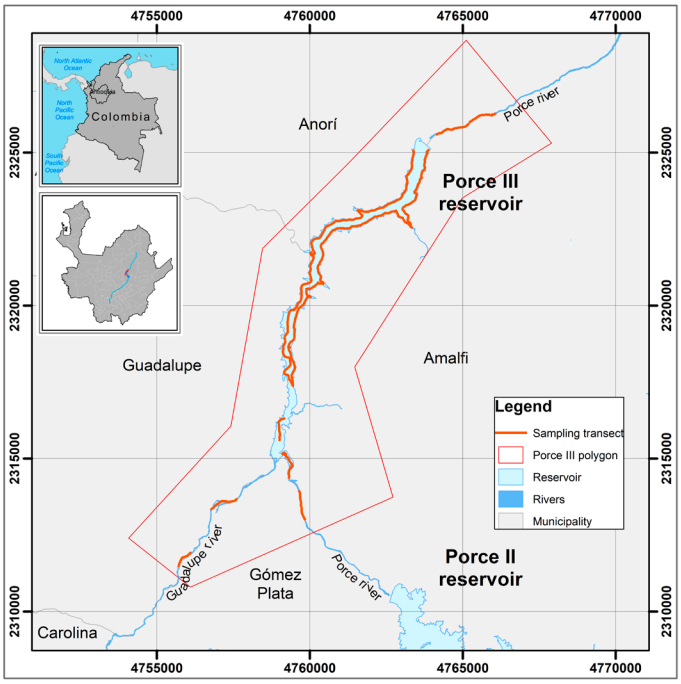

Figure 1. Study area and transects: Guadalupe River, Porce River before the Porce III reservoir and Porce River, past the reservoir.

Laboratory Work. Given that the samples were from impure sources such as feces and anal-gland jelly, a validation of the sexing method was performed using a tissue sample from a male otter and a blood sample from a female otter, whose DNA was extracted using the ZR Genomic DNA Tissue Miniprep Kit - Cat #D3051 (Zymo Research, CA, US), a specific extraction kit for tissues. Subsequently, for the extraction of total DNA from fecal samples, was used the ZymoBIOMICS DNA Miniprep Kit - Cat #D4300 (Zymo Research, CA, US), a specific extraction kit for feces. In both cases, the manufacturer's established protocol was followed.

For the total DNA extracted from otter feces, the mitochondrial DNA (mtDNA) control region was first amplified and sequenced, and polymorphisms were assigned. Then, it was determined whether the samples corresponded to the same individual through genotyping with nine microsatellite markers (Aristizábal-Duque et al. 2025, unpublished data): Lut435, Lut453, Lut715, Rio11, Lut701, 04OT17-1, RIO19, Lolo13, and Lolo29 (Dallas *et al.* 1999; Huang *et al.* 2005; Latorre-Cardenas *et al.* 2020). Both methodologies were validated with otter tissue and blood samples.

To identify the sex of the otters from fecal samples, 145 samples were selected, where the Y chromosome-specific SRY marker and the ZFX/ZFY markers present on both sex chromosomes were used. Initially, a multiplex PCR was performed where the SRY gene (Dallas *et al.* 2000) and the ZFX/ZFY gene (Mucci and Randi 2007) were simultaneously amplified (Table 1). The PCR mix included 5 µL of Phusion Flash High-Fidelity PCR Master Mix (2X) (Phusion Flash II DNA polymerase - Thermo Scientific™ CA, US), 2 µL of DNA template from fecal samples or 1 µL in the case of tissue samples, 0.5 µL of MgCl2 (0.25 mM), 0.5 µL of each primer (10 µM), 0.039 µL of BSA (20 mg/mL), and mQ H2O to adjust the final volume to 10 µL. Amplification was carried out in a MultiGene™ OptiMax thermal cycler (Labnet™) under the following conditions: one cycle at 98°C for 10 seconds, followed by 30 cycles at 98°C for 1 second, 60°C for 5 seconds, 72°C for 10 seconds, and finally, one cycle at 72°C for 1 minute for the final extension. Each amplification included a negative PCR control consisting of the reaction mix without a DNA template. PCR products were verified

Table 1. Molecular markers used for the molecular sexing of the neotropical otter.

Gene	Primer	Sequence (5′-3′)
SRY	Lut-SRY (Forward)	GAATCCCCAAATGCAAAACTC
	Lut-SRY R (Reverse)	GGCTTCTGTAAGCATTTTCCAC
ZFX/ZFY	P1-5EZ (Forward)	ATAATCACATGGAGAGCCACAAGCT
	Zfxyrb (Reverse)	TTGTTCAGCTGTCTCATATTCACA

with electrophoresis on 3% agarose gels stained with Gel Red (Biotium, Hayward, CA, USA), and a DNA size marker ranging from 50 bp to 1000 bp (GeneRuler 50 bp DNA Ladder - Cat #SM0371 - Thermo Scientific™ CA, US) was used. Males were distinguished by the presence of two bands of 70 and 180 bp, corresponding to SRY and ZFX/ZFY, respectively, while females showed only a 180 bp band from the ZFX gene. Because DNA was obtained from fecal samples, three to four independent amplifications were performed (depending on the sample volume available) to validate the consistency of the PCR products (Taberlet et al. 1996). To prevent the presence of human-associated Y chromosome sequences, samples were not collected or processed by human males. Extractions, amplifications, and genotyping were performed at the laboratory GAIA at the Universidad de Antioquia.

Due to the extended sampling period, sex ratios were calculated annually using data from the years 2014, 2015, 2018, 2019, and 2022, which had the highest number of samples and individuals. The ratio was determined as the relationship between the number of individuals of each sex (females and males).

Results

The tissue and blood samples from a female and a male otter were successfully sexed using the methodology implemented in this research. As expected, since females have two X chromosomes, only a 180 bp segment corresponding to the ZFX gene was amplified. Males, having one X and one Y chromosome, produced two bands: a 70 bp band derived from the SRY gene, and a 180 bp band derived from the ZFX/ZFY segment, which is present in both the Y and X chromosomes (Figure 2). These results were consistent across all rounds of amplification evaluated.

Of the 145 samples selected for analysis, 91 were successfully sexed with consistent results in at least three independent rounds of amplification, corresponding to 40 different individuals. This means that 51 samples were recaptures, and the sex assigned to the same individual was consistent across all recaptures, further validating the effectiveness of the method.

Of the 40 individuals sexed in the study area over the 10 years of sampling, 22 were females and 18 were males. By sector, the records are as follows: at the Porce III reservoir, 14 females and 9 males were registered, with one individual of each sex recaptured in the Porce River upstream of the reservoir. In the Porce River upstream of the reservoir, 4 females and 5 males were recorded, including those shared with the reservoir. In the Porce river downstream of the reservoir, there were 4 females and 2 males, and in the Guadalupe River, there were 2 females and 2 males.

The average female-to-male ratio over the analyzed years was 2:1.4 (Table 2). However, when analyzed by system, the rivers had a lower average female-to-male ratio of 1.7:2.4, whereas the Porce III reservoir had a higher female-to-male ratio of 2.2:1. Regarding the years, the data show that while in 2014 and 2015 the proportion of males was higher compared to females, in 2018, 2019, and 2022 the proportion of females was greater. This pattern was consistent across the lotic systems, while in the reservoir, the proportion of females was always higher compared to males (Table 3).

Regarding recaptures and individual mobility, 16 individuals (10 females and 6 males) were recorded only once during the 10 years of monitoring. The remaining 24 individuals (12 females and 11 males) had between 1 and 7 recaptures during the monitoring period, with females having a higher frequency of recapture. Considering the time during which the same individual was recorded, which varied between 1 and 6 years for females and between 1 and 2 years for males, the average displacement for females in the reservoir was 3.2 km and for males, 4.4 km. In the rivers, the displacements were shorter, with an average of 0.6 km for females and 0.4 km for males, and a much lower recapture rate compared to the reservoir (Figure 3). Since the Porce River upstream of the reservoir and the Porce III reservoir are connected at their closest point, 2 individuals, 1 male and 1 female, used both sectors for more than one year, including them in their movements. Additionally, 4

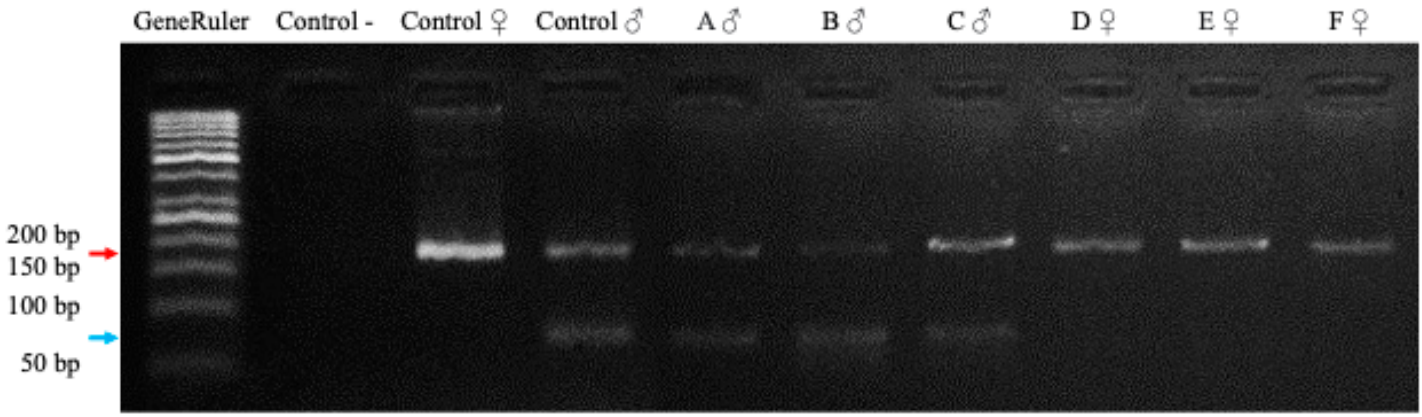

Figure 2. Verification of PCR products by 3% agarose gel electrophoresis. The bands are amplified from the co-amplification of the SRY and ZFX/ZFY genes for tissue samples (♀ and ♂ Controls) and for fecal samples (A-F) of *Lontra longicaudis* in the study area. The blue arrow indicates the size (bp) of the SRY fragment, and the red arrow indicates the size (bp) of ZFX/ZFY.

Table 2. Number of female and male otters by year

Year	Females	Males	Ratio
2014	3	7	1 : 2.3
2015	3	5	1 : 1.7
2018	13	5	2.6: 1
2019	6	3	2 : 1
2022	7	2	3.5 : 1
Average			2 : 1.4

Table 3. Number of female and male otters by year and water system

Year	Lotic (Rivers)			Lentic (Reservoir)		
	Females	Males	Ratio	Females	Males	Ratio
2014	1	6	1 : 6	2	1	2 : 1
2015	1	4	1 : 4	2	1	2 : 1
2018	5	2	2.5: 1	8	3	2.7 : 1
2019	2	0	2 : 0	5	3	1.7 : 1
2022	2	1	2 : 1	5	2	2.5 : 1
	Average		1.7 : 2.4	Average		2.2 : 1

individuals with extensive movements within the reservoir (over 7 km) were found, including 1 female and 3 males.

Discussion

The effectiveness of the sexing method in this study was 62.8%, a value higher than that obtained in similar research (Dallas *et al.* 2000, 2003; Hájková *et al.* 2009; Trinca and Eizirik 2012; Ortega *et al.* 2012; Brzeski *et al.* 2013; Lerone *et al.* 2014; Vergara *et al.* 2014; Lampa *et al.* 2015; Biffi and Williams 2017). This can be attributed to the exclusive collection of fresh samples (feces and anal gland jelly), the preservation method employed, and the use of feces-specific DNA extraction kits. However, the most determining factor of success was the selection of samples previously characterized for the control region of mtDNA, genotyped and individualized with microsatellites, which ensured high-quality DNA for sexing. Amplification was conducted using Phusion Flash II polymerase (Thermo Scientific™, CA, US), which is known for its high specificity and reduced formation of non-specific products, potentially contributing to the success rate.

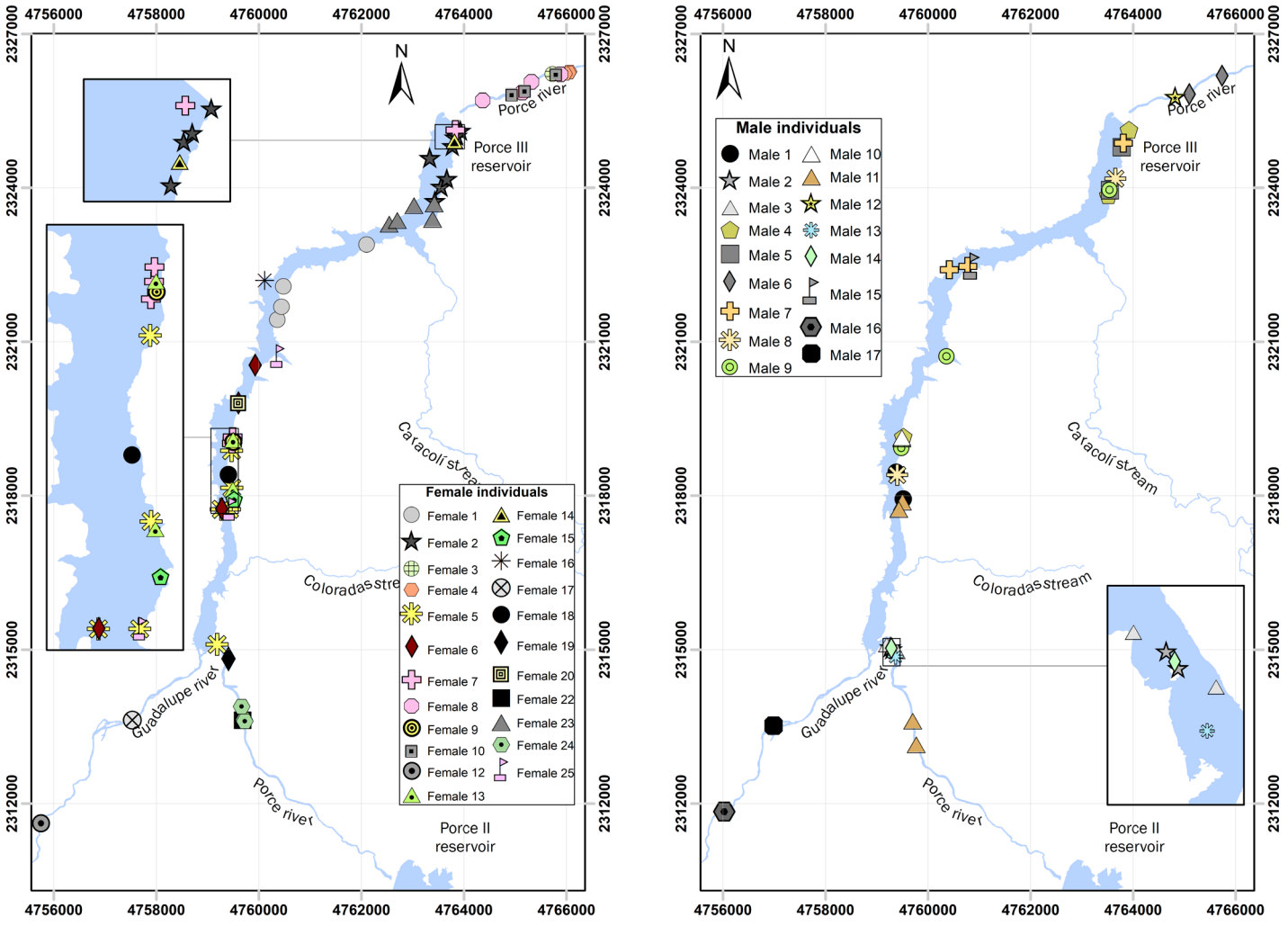

Figure 3 Distribution of individuals in Porce III reservoir and river with assigned sex. A) females B) males

The implemented methodology was effective for sexing individuals in the Porce III reservoir otter population with no need to capture and handling individuals. It is also a more economical and efficient method than others that have been used for non-invasive samples of the neotropical otter (Ortega *et al.* 2012; Trinca and Eizirik 2012). Using the ZFX/ZFY gene as a control for SRY gene amplification allowed for the identification of both males and females in a single co-amplification without the need for additional steps, such as the use of restriction enzymes. Furthermore, sex was confirmed by performing four independent PCRs for each sample, which reduced the possibility of genotyping errors due to sequence loss or amplification of non-specific products (Taberlet and Luikart 1999). In those cases where consistent results were not achieved across repetitions, this was likely due to low DNA quality, or the presence of PCR inhibitors commonly found in otter's feces.

The average sex ratios over the evaluated years in the study area, show a slight predominance of females over males. This result is strongly influenced by the otters inhabiting the reservoir area, as the proportion of males was higher in the rivers. These findings are consistent with reports in the literature for *Lontra longicaudis* (Trinca and Eizirik 2012) and *Lutra lutra* (Lampa *et al.* 2015). A greater movement of males compared to females was also observed, primarily in the reservoir, meanwhile in the rivers, movements were short and similar between sexes. These results suggest a higher dispersion of males and a philopatric tendency in females, at least in the reservoir, as reported in other studies (Ortega *et al.* 2012; Quiagletta *et al.* 2013; Trinca et al. 2013; Biffi and Williams 2017; Michalski *et al.* 2021). Considering that the availability of food and shelter are key factors influencing habitat use by otters, the movement of individuals to the lotic systems adjacent to the reservoir may reflect a search for areas with reduced competition for these resources (Zapata-Escobar et al. 2015). The higher aggregation in the reservoir area could be attributed to the sufficient availability of both food and shelter for females and their offspring. Males observed with the group of females in the reservoir area could be transient individuals or temporary residents contributing to gene flow, or juveniles remaining with their mother until the onset of the next breeding season (Michalski *et al.* 2021), a hypothesis that requires confirmation through kinship analysis.

It is important to consider that due to the high exposure to contaminants that may affect the survival and reproduction of otter individuals, it is relevant to complement this study with analyses of sex hormones to determine the levels of estrogens and testosterone in the already sexed individuals, as well as the detection of endocrine disruptors that may be affecting reproduction. Even though otters are frequently observed, the sight of neonates is low. Furthermore, it is crucial to implement effect-based methodologies to analyze whether exposure to mutagenic or endocrine-disrupting contaminants varies by sex, using fecal samples. This is essential because otters play a key role in the conservation of the aquatic and riparian ecosystems they inhabit.

In conclusion, the species *Lontra annectens* remains poorly understood compared to other otters, such as *Lutra lutra*. This lack of information hampers conservation efforts. However, this project represents a significant advancement as it is the first study in Colombia to apply molecular sexing techniques to this species. In addition to providing valuable data on sex ratios, this study also presents initial findings on individual mobility. This innovative approach not only provides valuable data for conservation but also sets a precedent for future studies on the biology and ecology of *Lontra longicaudis* and *L. annectens*.

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