fishes

MDPI

Article

Inadequate Sampling Frequency and Imprecise Taxonomic Identification Mask Results in Studies of Migratory Freshwater Fish Ichthyoplankton

Paulo Santos Pompeu [1,*], Lídia Wouters [1], Heron Oliveira Hilário [2], Raquel Coelho Loures [3], Alexandre Peressin [4], Ivo Gavião Prado [5], Fábio Mineo Suzuki [5] and Daniel Cardoso Carvalho [2]

1 Graduate Program in Applied Ecology, Departament of Ecology and Conservation, Federal University of Lavras, Lavras 37203-202, Brazil; lidia_wouters@hotmail.com
2 Laboratório de Genética da Conservação, Graduate Program in Vertebrate Biology, Pontifícia Universidade Católica de Minas Gerais, Belo Horizonte 30535-610, Brazil; heronoh@gmail.com (H.O.H.); carvalho.lgc@gmail.com (D.C.C.)
3 Cemig Geração e Transmissão SA, Rua Barbacena 1200, Santo Agostinho, Belo Horizonte 30190-131, Brazil; raquel.fontes@cemig.com.br
4 Centro de Ciências da Natureza, Universidade Federal de São Carlos (UFSCar)—Campus Lagoa do Sino, Buri 75775-000, Brazil; alexandre.peressin@gmail.com
5 Pisces—Consultoria e Serviços Ambientais, Lavras 37206-662, Brazil; ivogaviaoprado@gmail.com (I.G.P.); suzuki.fms@gmail.com (F.M.S.)
* Correspondence: pompeu@ufla.br

Abstract: In South America, knowledge of major spawning sites is crucial for maintaining migratory fish populations. In this study, we aimed to understand the spatio-temporal distribution of fish eggs in the upper São Francisco River using high sampling frequency and DNA metabarcoding identification. We evaluated the possible effects of the non-molecular identification of eggs and decreased sampling frequency on the determination of spawning sites and major breeding periods. Collections were carried out every three days from November 2019 to February 2020. We found that, if we had assumed that all of the free and non-adhesive sampled eggs belonged to migratory species, as is usual in the literature, this assumption would have been wrong for both the spawning sites and the breeding periods. Moreover, any decrease in the frequency of sampling could dramatically affect the determination of the major spawning rivers, and the spawning events of some of the migratory species may not have been detected. Therefore, without the proper identification and adequate sampling frequency of eggs, important spawning sites may be overlooked, leading to ineffective or inappropriate conservation measures.

Keywords: DNA metabarcoding; neotropics; South America; São Francisco River; spawning sites

Key Contribution: Studies with ichthyoplankton without molecular identification of eggs and with low sampling frequency may not detect important spawning events. This can lead to the incorrect identification of spawning sites and inadequate conservation strategies.

Citation: Pompeu, P.S.; Wouters, L.; Hilário, H.O.; Loures, R.C.; Peressin, A.; Prado, I.G.; Suzuki, F.M.; Carvalho, D.C. Inadequate Sampling Frequency and Imprecise Taxonomic Identification Mask Results in Studies of Migratory Freshwater Fish Ichthyoplankton. *Fishes* **2023**, *8*, 518. https://doi.org/10.3390/fishes8100518

Academic Editors: Robert L. Vadas, Jr. and Robert M. Hughes

Received: 7 October 2023
Accepted: 17 October 2023
Published: 19 October 2023

1. Introduction

In South America, large migratory fish species are important for commercial and sport fisheries because of their size and abundance [1]. These species are also impaired by river fragmentation by damming because they exhibit complex upstream and downstream movements and require different habitats in order to complete their reproductive cycles [2–4]. Moreover, a lack of connectivity hinders access to spawning sites, and the formation of large reservoirs directly interferes with egg and larvae drift [5], which would normally be carried to floodplains during floods [6].

Considering the high degree of fragmentation in South American basins and the many new hydroelectric projects planned [7], knowledge of the major spawning sites in each basin is crucial for the establishment of appropriate measures for maintaining migratory fish populations [8]. Thus, studies of the early life stages of fish have become increasingly common [9], especially those focusing on the ichthyoplankton of migratory species. These studies have investigated spawning periods and sites, spawning intensity e.g., [10,11], factors related to reproductive success, and the species-specific characteristics of those processes [12].

One of the major methodological challenges in these studies is the correct taxonomic identification of ichthyoplankton. In the case of larvae, despite the high diversity and high rates of endemism [13], taxonomic keys are available for some neotropical river basins [8,9,14], enabling the identification of family or genus. The identification of eggs, on the other hand, has been more limited. Because having free and non-adhesive eggs is a trait associated with migratory species [15], some authors have considered that, in conventional ichthyoplankton sampling, these eggs would predominantly originate from species that exhibit migratory behavior, e.g., [16,17]. Other studies have distinguished between migratory and non-migratory species by using information on the size of the perivitelline space [6,8,9], which is wider in the former. Nonetheless, these traits have been shown to have low accuracy in discriminating between those two species groups [18], which is particularly concerning, given that the presence of eggs is the most accurate indicator of the spawning location.

New genetic tools have shown promise as a means of enabling the improved identification of ichthyoplankton, especially by enhancing the taxonomic resolution of sampled eggs. High-throughput sequencing (HTS) platforms associated with DNA barcoding (i.e., DNA metabarcoding) allow for the identification of multiple species from environmental bulk samples. This methodology has tremendous potential for monitoring and assessing environmental quality [19] has been referred to as DNA-based next-generation biomonitoring, or "Biomonitoring 2.0" [20]. As such, non-invasive methods ensure a rapid and cost-efficient biodiversity assessment of many difficult-to-identify organisms, such as ichthyoplankton samples, which is particularly important when dealing with the megadiverse neotropical ichthyofauna [21].

Another key aspect of ichthyoplankton studies is related to the periodicity of sampling, because neotropical migratory species usually have a single spawning event during the breeding season [22], and the number of spawning events for each species is unknown for most of them. Many neotropical ichthyoplankton studies have been conducted either bi-weekly [23,24] or monthly [25,26]. Studies at a greater sampling frequency are less common but indicate that some spawning events occur over only a few days [4,16].

Therefore, we sought to understand the spatio-temporal distribution of fish eggs in the upper São Francisco River by using a high sampling frequency and DNA metabarcoding identification. We evaluated the possible effects of the non-molecular identification of eggs on the determination of potential spawning sites and on the main breeding periods of migratory species. We also determined how a decreased sampling frequency affected the determination of the major spawning sites in the basin and the identification of the spawning sites of each migratory species.

2. Materials and Methods

2.1. Study Area

Our study comprised the São Francisco River basin upstream of Três Marias Reservoir, Brazil. There, the São Francisco River mainstem has a lotic segment of approximately 400 km and a draining an area of 26,680 km^2. Some local tributaries, such as the Pará River, are recognized feeding sites, whereas the Samburá and Bambuí Rivers and the São Francisco River headwaters are important spawning sites for migratory species [4]. The average annual precipitation in the São Francisco Basin is 1036 mm, and the Köppen climate

is classified as Cwa, which is characterized by an October-to-March rainy season [27]. Fish spawning is concentrated between November and February [4,28].

We selected six sampling sites for ichthyoplankton sampling. Once the ichthyoplankton was transported over a long stretch of river, each point represented its respective upstream river. One site was located in the Samburá River (SAM), one site in the Bambuí River (BAM), and one site in the Pará River (PAR), always near their mouths. Three sites were located along the São Francisco River mainstem (upstream of the Samburá River mouth—SFS; upstream of the Bambuí River mouth—SFB; and upstream of the Pará River mouth—SFP), (Figure 1). The distribution of the sites aimed to encompass all major tributaries of the upper basin, as well as intermediate stretches of the São Francisco River.

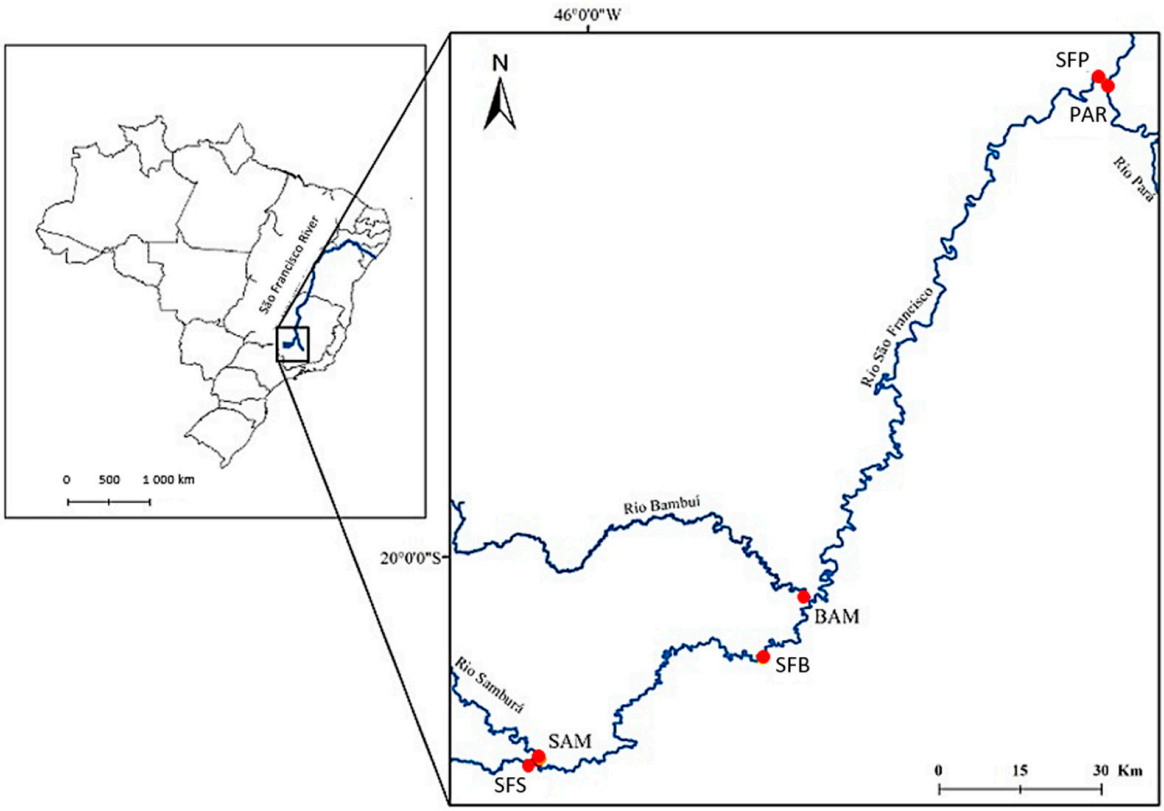

Figure 1. Map of the study area, showing the locations of the sampling sites in the upper São Francisco River, Minas Gerais, Brazil.

2.2. Methods

Migratory fish in the São Francisco and Paraná River basins do not spawn in floodplains. Instead, spawning often occurs in shallower and smaller-sized tributaries, as in the case of our study region [4]. The ichthyoplankton is then passively transported to the floodplains, where they arrive as larvae at the end of yolk sac absorption. Because spawning occurs in such shallow and turbulent rivers, no differences in their distribution in the water column are expected. In fact, even in deep South American rivers, no differences in ichthyoplankton abundance have been observed between samples taken at the surface and those at the river bottom [29]. In the same study, the authors reported differences in ichthyoplankton abundance between sampling hours; however, those also varied with distance from the collection site to the spawning region. Therefore, defining the ideal time is not straightforward, but has been resolved below.

Because the focus of this work was to assess the risks of not considering the genetic identification of eggs for defining spawning sites and times, as well as the effects of reducing the sampling frequency, we took care to replicate the sampling design commonly used in studies of this nature. Therefore, we maximized the sampling frequency (every three

days at all points), a collection effort that is rare in Brazil, but that prevented sampling the same point multiple times per day. That was a choice that had to be made because of our available budget for both field collections and genetic analyses—a budget already much higher than the majority of ichthyoplankton studies in Brazil. Therefore, samples were taken only near the surface and at sunset, because no differences in egg abundance are expected at different depths [29], especially in shallow rivers, and because spawning occurs in the late afternoon and evening, near sunset [30].

The collections were carried out from 1 November 2019 to 29 February 2020, every three days, in the late afternoon, at all six points, resulting in 41 samples per point (246 samples in total). Because the hatching time of migratory fish in the São Francisco basin is always <24 h [31], the 3-day collection interval ensured that each sample represented a potentially independent spawning event. A conical net (40-cm diameter) with a flow meter attached was positioned at the location with the greatest current velocity for 10 min at a depth of 0.5 m. The collected material was placed in 600-mL plastic jars with absolute ethanol and taken to the laboratory for screening via a Bogorov plate and a stereomicroscope.

All samples with fish eggs were analyzed using the DNA metabarcoding method, which allows the determination of multiple species from a single sample through high-performance DNA sequencing (Illumina). DNA was extracted using the salting-out method adapted from [32]. The DNA was quantified on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Cleveland, OH, USA), and then the samples were normalized to 100 ng/µL. Because our focus was to locate spawning rivers, larvae were not considered.

A 655 bp fragment of the 5′ end of the mitochondrial COI gene was amplified via PCR using a combination of different primers (F1 and FishR1), modified from sequences already published in the literature. The original sequences received a tail (Illumina pre-adapter) complementary to the adapter used in a subsequent second PCR. This reaction was performed with a water sample to monitor possible contamination (negative control), as well as a positive control. The PCR products were then purified with magnetic beads (Agencourt AMPure XP®—Beckman Coulter).

After purification, a PCR was performed with Nextera Index kit® adapters (Illumina Inc., San Diego, CA, USA) to amplify the amplicon set from the previous step. In this reaction, adapters compatible with the Illumina next-generation sequencing system (P5 and P7) were used as primers. A single index combination (specific sequences associated with the Illumina adapter) was used for the subsequent identification of each sample, because all points will form a single sequencing set. The amplification product was evaluated on a 1.5% agarose gel.

The samples were successfully amplified in the PCR because they showed the expected band pattern for the COI fragment (655 bp COI + 60 bp adapter + 64 bp index = 780 bp). No amplification was observed for the negative control, indicating no contamination in the reactions. The PCR products were then, again, purified with magnetic beads (Agencourt AMPure XP®—Beckman Coulter), quantified on a Nanodrop, and normalized to 20 ng/µL.

All samples were pooled together, and this pool was purified using a Zymoclean™ Large Fragment DNA Recovery kit (Zymo Research) to remove spurious fragments from the desired-sized fragment (655 bp COI + 60 bp adapter + 64 bp index = 779 bp). Using real-time PCR, performed with a KAPA Biosystems Quantification Kit (Illumina, São Paulo, Brazil) reagent, the pool was quantified, diluted to a concentration of 2 nM, and again quantified to confirm the final concentration. The final solution was denatured and loaded onto the MiSeq® equipment (Illumina) using a Miseq v3 300-cycle sequencing kit (2 × 150 bp), with a final concentration of 16 pM.

The bioinformatic analysis was performed using a custom pipeline written in R (R Core Team, 2022) and DADA2 [33] and Phyloseq [34] packages, as well as the cutadapt program [35]. Briefly, the demultiplexed sequencing reads were downloaded from Basespace (Illumina). An initial quality control step was carried out where reads with undetermined bases (Ns), or a Q-score of <20, were removed. Then, primer sequences were detected and removed. The remaining reads were submitted to dereplication determination of ASVs

(amplicon sequencing variants) and chimera removal, using DADA2 core functions. The R1 and R2 reads were analyzed as complementary datasets and used independently of ASV determination, as the amplicon span hinders read merge by overlap. These ASVs were submitted to a first round of taxonomic classification, performed with a Bayesian classifier integrated into the DADA2 package, using a custom database built from 114,425 vertebrate sequences, available on BOLD (https://www.boldsystems.org/ accessed on 10 August 2022). A second round of taxonomic classification was performed using a similarity search in the NCBInt (nucleotide database) with a local implementation of the BLASTn [36], with thresholds of sequence coverage > 80% and sequence identity > 80%. The taxonomy identification obtained from these complementary classifications was manually curated for each ASV and sample, considering the BLASTn similarity and coverage values and the distribution in the basin of the identified species or close taxa.

Previous work using the DNA metabarcoding approach on ichthyoplankton bulk samples was able to successfully detect alpha diversity at the species level and has provided good estimates of the relative abundance of the larvae [37]. Thus, we used the relative read abundance (RRA) to estimate the species abundance for each bulk sample. The RRA of each ASV on each sample was obtained by dividing the ASV absolute abundance by the total absolute abundance of the sample.

The total density of eggs in each sample was calculated by standardizing the abundance per 10 m^3 of filtered water [6]. The density of eggs from migratory species was also estimated per sample based on their relative read abundance (RRA) in each sample. For both estimates (total and migratory species), periods with abundance peaks were visually compared, and their congruence was tested by Pearson correlation. Species were classified as migratory according to Sato and Godinho [38].

To evaluate the beta diversity patterns of sampling rivers and dates, we performed a non-metric multidimensional scaling (NMDS) analysis. The input data consisted of the taxa abundance of each ichthyoplankton pool as input variables and estimated distances were calculated with Bray–Curtis dissimilarity using the function metaMDS of the R-package vegan [39].

The importance of each river as a spawning ground was evaluated by comparing densities by ANOVA or Kruskal–Wallis, depending on their distribution, considering the total number of eggs, and considering only the estimated number of eggs from migratory species. This evaluation was performed for the entire dataset and for simulations of six- and fifteen-day sampling intervals. In the case of the six-day interval, these were produced considering a start date of November 1st or 4th. For the 15-day interval, scenarios were simulated with collections starting on November 1st, 4th, 7th, 10th, and 13th.

To evaluate the effect of sampling frequency, for the three sampling scenarios (three-, six-, and fifteen-day intervals), the number of migratory species with recorded spawning in each river and the number of spawning rivers for each migratory species were compared by ANOVA.

3. Results

A total of 48,465 fish eggs were collected from the upper São Francisco basin and 71% of the samples contained fish eggs. After DNA metabarcoding analysis, a total of 14,817,732 raw paired DNA reads were obtained for the 149 egg pools, with an average of 49,724 reads per pool. After quality control, error correction, dereplication, and chimera removal, 11,995,008 reads remained, with an average of ~80,500 DNA sequences per pool, corresponding to a total of 507 unique ASVs of 230 bp on average. This enabled the identification of 35 fish taxa, including 7 migratory species (Appendix A). Seven taxa were identified to genus level and three to family.

The majority of the identified ASVs (63.5%) were associated with a single species, mandi *Pimelodus pohli*. The migratory species, *Prochilodus argenteus*, *P. costatus*, *Megaleporinus obtusidens*, and *Leporinus taeniatus*, accounted for 2.92%, 2.88, 2.26%, and 0.9% of the detected DNA sequences, respectively. The other migratory species found (*Pseudoplatystoma*

corruscans, *Brycon* sp., and *Megaleporinus reinhardti*) represented <0.01% each. Despite the dominance of a single species, considerable variation in the ichthyoplankton composition was observed between the sampling points and among the different samples from each river (Figure 2).

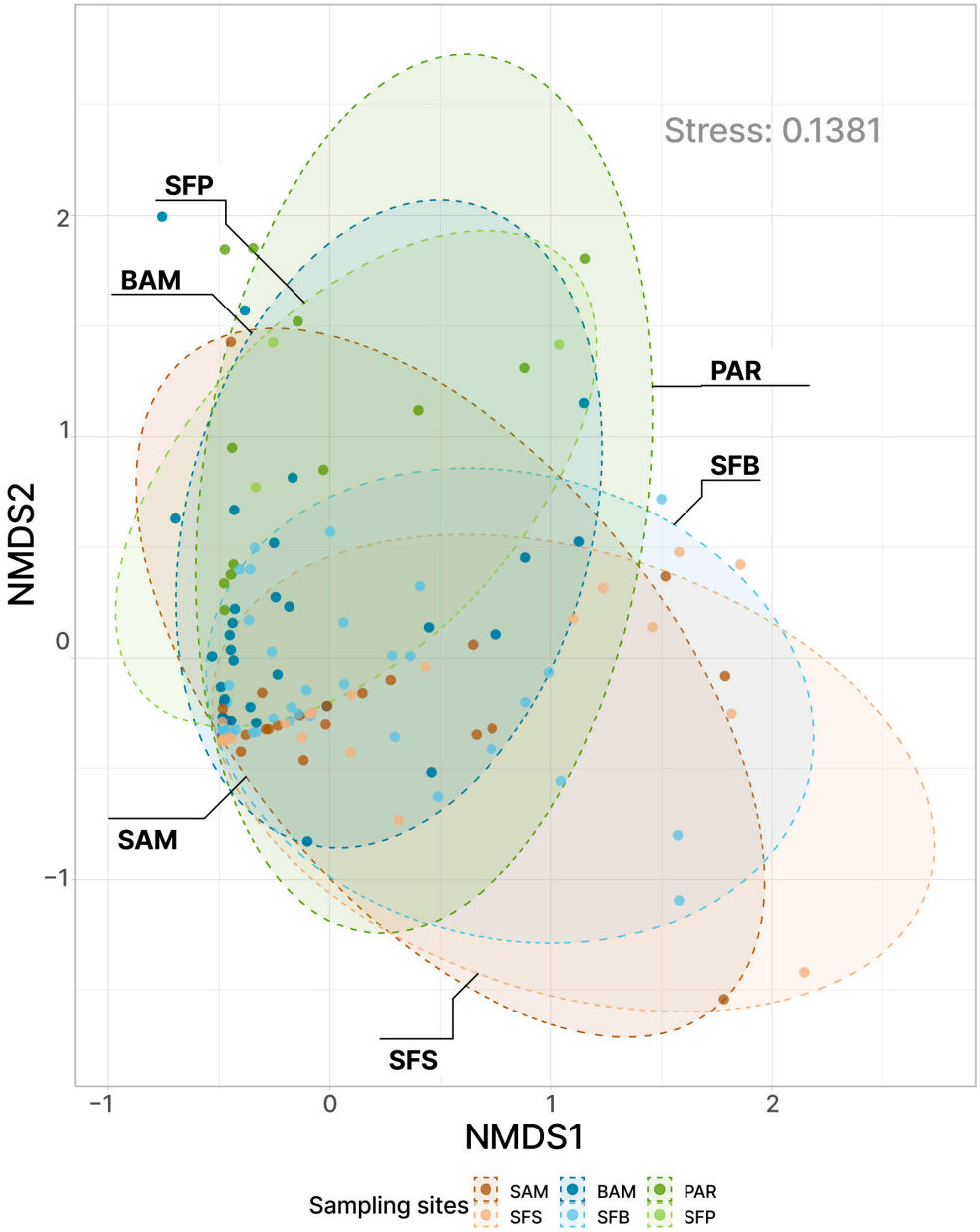

Figure 2. Non−metric multidimensional scaling (NMDS) plot showing ordination based on DNA metabarcoding of taxa abundance, with similarity estimated using Bray–Curtis. Ellipses encompass all samples from the same site. São Francisco River sites are represented by SFB, SFP, and SFS; and tributaries by PAR, BAM, and SAM.

When comparing the total egg densities, the SFB site stood out, having both the greatest averages and the greatest peaks of egg densities (Figure 3A) (KW = 2.47; $p = 0.01$). However, when considering only migratory-fish-egg abundances, the Bambuí (BAM) and Samburá (SAM) River sites indicated the greatest reproductive activity (Figure 3B) (F = 4.76; $p < 0.001$).

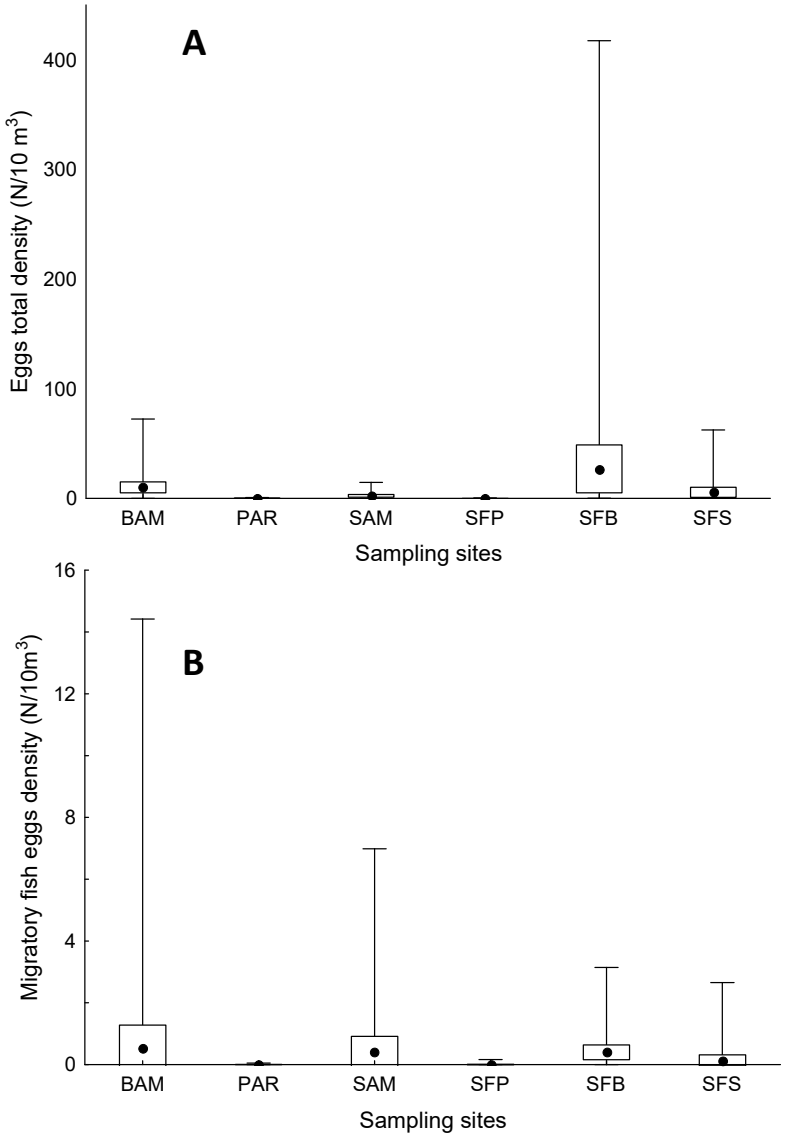

Figure 3. Estimated egg density of all species (**A**) and migratory species (**B**) per sampling point in the upper São Francisco River from samplings every three days (dot = mean; box = standard error; whisker = range).

When considering the total egg densities, the largest spawning events occurred between the second half of November and the first week of January. However, when considering only the abundance of the migratory fish eggs, the largest reproductive events occurred in the first half of November and from the second half of January to the first half of February (Figure 4), and both of these evaluations were not congruent (r = 0.05; $p = 0.47$).

For the total egg density, both of the six-day sampling simulations pointed to significant differences among the sites (F = 4.74; $p < 0.001$) but produced different results regarding the importance of each river for fish spawning. Whereas, in one simulation, the SFB site remained the most important breeding site, in the other, the BAM and SFS sites had similar migratory-fish-egg densities (Figure 5A). The two six-day interval simulations for estimating migratory-egg densities were not able to capture differences among the sites (F = 1.34; $p = 0.24$). In addition, in one simulation, the Bambuí River (BAM) stood out, whereas, in the other, Samburá River (SAM) was the most important breeding location (Figure 5B).

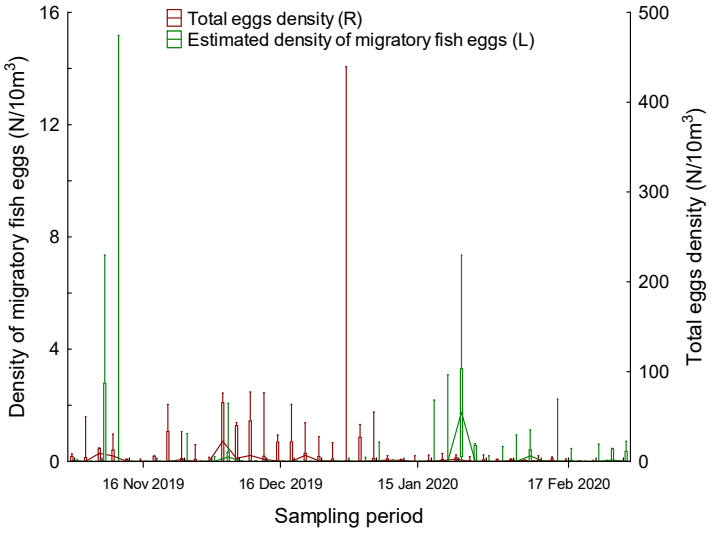

Figure 4. Total egg density (red) and estimated density of migratory species eggs (green) during the sampling period in the upper São Francisco River from sampling every three days (dot = mean; box = standard error; whisker = range).

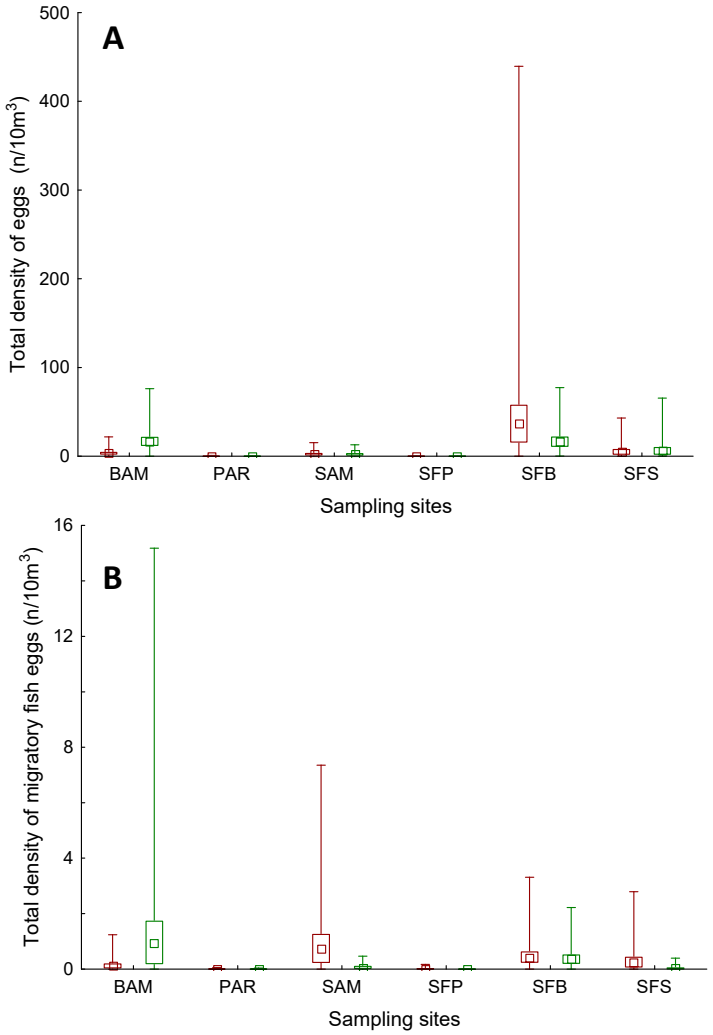

Figure 5. Total egg density (**A**) and estimated egg density of migratory species (**B**) by collection point in the upper São Francisco River, considering samplings every six days. Different colors represent each of the two simulations (dot = mean; box = standard error; whisker = range).

Even more divergent results were observed for the 15-day sampling intervals. For the total egg density, in only two simulations did the SFB site remain the most important, whereas, in the other three, the densities were more similar between the Bambuí (BAM), Samburá (SAM), and upper São Francisco River (SFS) sites (Figure 6A). For migratory fish, the importance of the Bambuí River (BAM) was shown in only one simulation, and in only two out of five simulations for the Samburá River (SAM) (Figure 6B). In all of these cases, the differences between the points were not statistically significant.

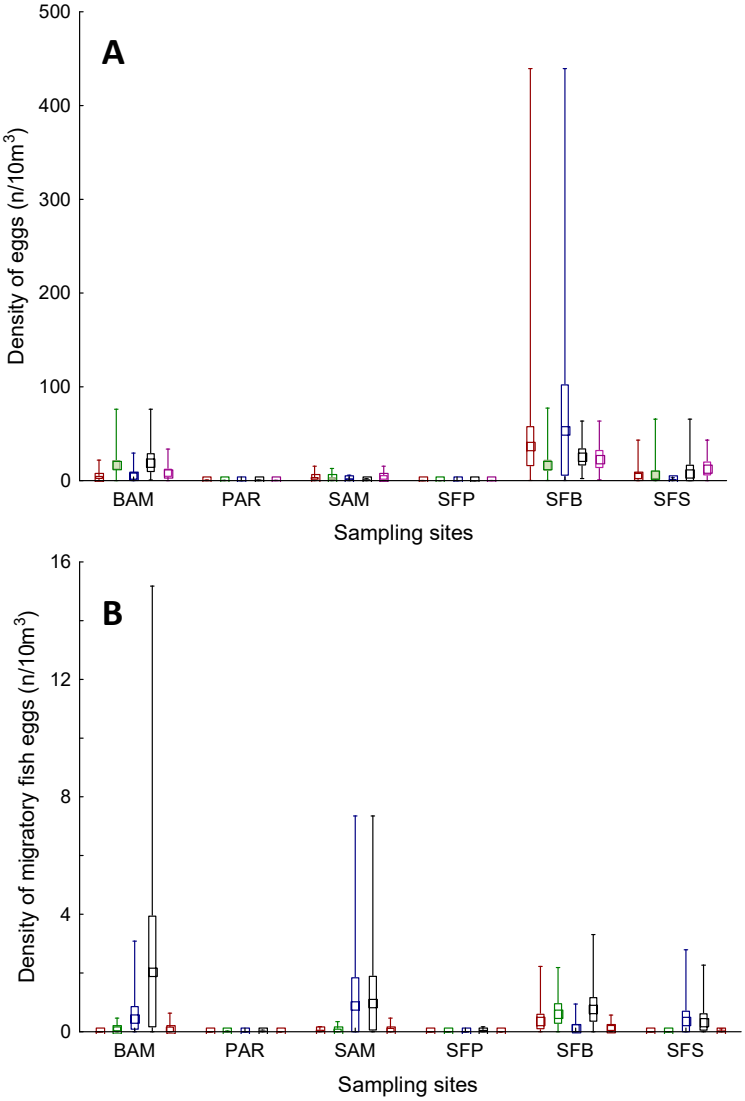

Figure 6. Total egg density (**A**) and estimated density of eggs from migratory species (**B**) per sampling site in the upper São Francisco River, considering sampling every 15 days. Different colors represent each of the five simulations (dot = mean; box = standard error; whisker = range).

Regarding the spawning grounds for migratory species, an increase in the sampling interval directly interfered with the quality of the information about the importance of the sampled sites ($F = 8.88$; $p < 0.001$). For all of the sites, increasing the sampling interval from three to six days prevented the identification of some species, and, for four sites, a fifteen-day interval resulted in a total non-recording of migratory species (Figure 7). Similarly, a progressive increase in the sampling interval decreased the number of spawning rivers identified for each migratory species ($F = 14.26$; $p < 0.001$), and four of them (*Brycon* sp., *P. corruscans*, *P. costatus*, and *M. reinhardti*) may not have their reproductive event recorded in the basin if sampled only every 15 days (Figure 8).

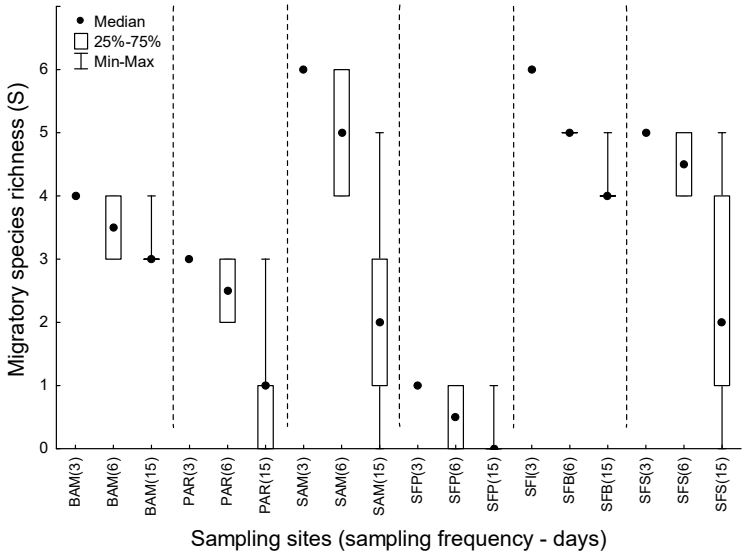

Figure 7. Number of migratory species with spawning recorded for each sampling point, considering intervals of 3, 6, or 15 days between collections.

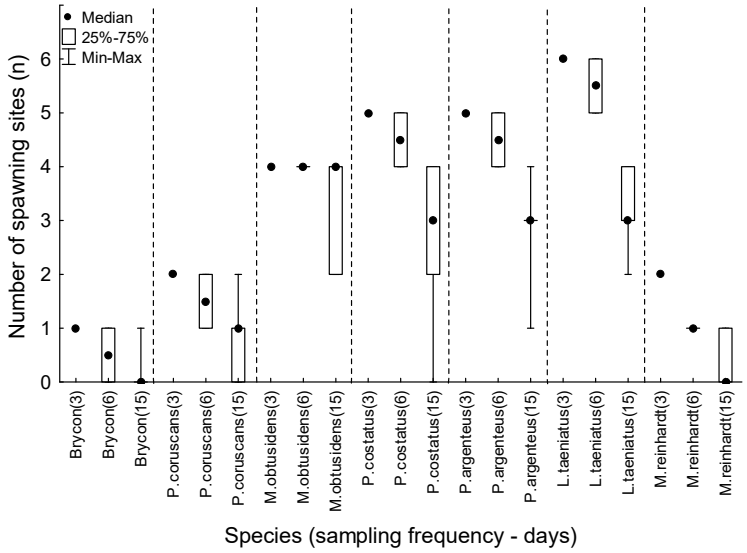

Figure 8. Number of spawning sites inferred for each migratory species, considering sampling intervals of 3, 6, or 15 days.

4. Discussion

We determined the spatio-temporal distribution of fish eggs in the upper São Francisco River using high frequency sampling and DNA metabarcoding identification. The molecular identification of eggs and high frequency sampling was necessary to obtain reliable data. For instance, our results showed that, if we had assumed that all of the sampled eggs belonged to migratory species, this assumption would have been wrong for both the spawning sites and the breeding periods. Moreover, the six- and fifteen-day sampling intervals dramatically affected the determination of the major spawning rivers in the upper basin, and the spawning events of some migratory species were not detected.

The DNA metabarcoding identification was able to determine the presence of eggs from seven migratory fish species. Of those known to occur in the basin [40], only the eggs of dourado (*Salminus franciscanus*) were undetected. Most of the eggs were identified to species level; however, the absence of complete genetic databases limited some identifications to genus or family levels only [21]. As expected, eggs from species with parental care, such as Loricariidae and Cichliformes [41], or with internal fertilization,

such as Auchenipteridae [42] and Poeciliidae [43], were not recorded. However, eggs from rheophilic species that do not undertake long migrations predominated in the samples, especially *Pimelodus pohli*. This species performs fractional spawning and reproduces throughout the year, with spawning peaks occurring between November and February [44].

The identification of ichthyoplankton through morphological characteristics observation is highly complex, especially during the embryonic phase, when there is great morphological similarity among species [6,45]. Even considering the differences in the perivitelline space of the eggs, which tends to be larger in migratory species than in sedentary species [6], the distinction between the groups is imprecise [18]. Given this challenge, it is common for eggs to only be quantified and evaluated collectively. Although these analyses can contribute to a general understanding of reproductive dynamics, the use of such methods to evaluate the reproductive patterns of a specific group is inadequate. In this study, higher egg densities from migratory species were recorded in the Samburá and Bambuí Rivers, two recognized spawning rivers based on previous telemetry studies [4]. The importance of these two São Francisco River tributaries for migratory species would be greatly underestimated if all of the sampled eggs were presumed to be from migratory species. Similarly, non-molecular identification would point to spawning periods that would lead to different interpretations regarding the main environmental factors that trigger the reproductive process.

In addition to the method of identification, sampling frequency is another key factor that directly affects the detection of reproductive patterns. This factor was particularly evident in the significant temporal and spatial variation observed in ichthyoplankton composition. Ichthyoplankton studies conducted in neotropical rivers have commonly adopted biweekly or longer sampling intervals, e.g., [24,25,46–48]. Incomplete identification becomes even more critical when we consider the fish monitoring programs developed by the hydroelectric sector. In most cases, these studies are conducted with quarterly or biannual frequency [49], resulting in sporadic collections that may or may not be outside of the reproductive period of migratory species. The minimum ichthyoplankton sampling protocol [50], a reference used by environmental agencies to guide the environmental regulation of hydropower plants, recommends monthly collections over a period of one year for surveys and for monthly collection to be carried out for at least four months in the reproductive season for monitoring. Our results indicate that the greater the sampling interval, the lower the chances of recording important reproductive events. Migratory species exhibit spawning that is highly synchronized with environmental variables [4,11,24], and they exhibit reproductive homing [51], with fidelity to a single spawning site. Therefore, an inadequate sample frequency leads to the erroneous conclusion that a river or region is not important as a spawning site for some migratory species. An error such as this would result in inadequate management and conservation measures, because having quality data is essential for us to understand the impact of existing or new dams on migratory fish populations [8]. In the upper São Francisco River, the spawning of four out of the seven migratory species would have gone undetected if the sampling had been conducted biweekly or monthly. Among those species is Surubim *Pseudoplatystoma corruscans*, the largest-sized species in the basin, which is currently listed as endangered [52], and *Prochilodus argenteus*, which comprises half of the fisheries in some stretches of the São Francisco River basin [38,53].

5. Conclusions

Most South American river basins, which harbor an enormous biodiversity of fish, are already highly fragmented [54]. In these systems, free-flowing river segments are refuges for migratory species, some of which are rare and/or threatened [11,24,47,48,55]. In this context, the identification of critical habitats, especially spawning sites, is essential for taking appropriate conservation measures. We have found that, without the proper identification and adequate sampling frequency of eggs, important spawning sites will be overlooked, leading to ineffective or inappropriate conservation measures. Therefore, it is

necessary to improve the ichthyoplankton surveys and monitoring programs conducted in environmental impact studies related to aquatic ecosystem projects. Given the importance of molecular techniques to improve species identification accuracy, it is crucial to provide incentives and support in order to make them more financially accessible.

Author Contributions: P.S.P., R.C.L., A.P. and L.W. conceived and designed the investigation. L.W., A.P., I.G.P. and F.M.S. performed the fieldwork. P.S.P., I.G.P., A.P., F.M.S. and L.W. analyzed the data. D.C.C. and H.O.H. performed the genetic analyses of eggs. P.S.P. wrote the paper. All co-authors revised the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Companhia Energética de Minas Gerais (CEMIG) P&D Agência Nacional de Energia Elétrica (ANEEL) GT612. P.S.P. and D.C.C were supported by the CNPq productivity grants (302328/2022-0; 312102/2022-4, respectively).

Institutional Review Board Statement: All proceedings were carried out under capture permission (SISBIO 10327) and the approval of the ethics committee of the Universidade Federal de Lavras (CEUA UFLA 003/2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available upon request from the corresponding author (P.S.P.).

Acknowledgments: We thank the local residents who volunteered to collect ichthyoplankton samples, as well as the collectors from the Fish Ecology Laboratory who assisted with sample screening. We also thank the anonymous reviewers and special-issue editors for their comments on the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Relative abundance (ASV %) of fish species identified by DNA metabarcoding in ichthyoplankton samples from the upper São Francisco River. Migratory species and parental care are also indicated [22,31,36,56,57].

Fish Species	ASV %	Migratory (Y/N)	Parental Care (Y/N)
ORDER CLUPEIFORMES			
Family Engraulidae			
Anchoviella vaillanti (Steindachner, 1908)	0.06	N	N
ORDER CHARACIFORMES			
Family Crenuchidae			
Characidium fasciatum Reinhardt, 1867	<0.01	N	N
Characidium zebra Eigenmann, 1909	0.07	N	N
Family Parodontidae			
Parodon hilarii Reinhardt, 1867	<0.01	N	N
Family Serrasalmidae			
Myleus micans (Lütken, 1875)	0.01	N	N
Myleus sp.	<0.01	N	N
Myloplus sp.	<0.01	N	N
Serrasalmus sp.	<0.01	N	Y
Family Anostomidae			
Leporellus vittatus (Valenciennes, 1850)	0.86	N	N
Leporinus piau Fowler, 1941	0.04	N	N
Leporinus taeniatus Lütken, 1875	2.26	Y	N
Megaleporinus obtusidens (Valenciennes, 1837)	0.90	Y	N
Megaleporinus reinhardti (Lütken, 1875)	<0.01	Y	N
Schizodon knerii (Steindachner, 1875)	1.94	N	N

Table A1. *Cont.*

Fish Species	ASV %	Migratory (Y/N)	Parental Care (Y/N)
Family Curimatidae			
Curimatidae sp.	<0.01	N	N
Family Prochilodontidae			
Prochilodus argenteus Spix and Agassiz, 1829+	2.92	Y	N
Prochilodus costatus Valenciennes, 1850 +	2.88	Y	N
Family Bryconidae			
Brycon sp.	<0.01	?	N
Family Characidae			
Astyanax scabripinnis (Jenyns, 1842)	0.02	N	N
Bryconamericus sp.	0.26	N	N
Piabina sp.	0.11	N	N
Psalidodon fasciatus (Cuvier, 1819)	0.08	N	N
Tetragonopterus chalceus Spix and Agassiz, 1829	<0.01	N	N
ORDER SILURIFORMES			
Family Cetopsidae			
Cetopsidae sp.	0.05	N	N
Family Doradidae			
Doradidae sp.	<0.01	N	N
Family Heptapteridae			
Cetopsorhamdia iheringi Schubart and Gomes, 1959	0.57	N	N
Imparfinis sp.	<0.01	N	N
Family Pimelodidae			
Bergiaria westermanni (Lütken, 1874)	4.84	N	N
Pimelodus fur (Lütken, 1874)	1.29	N	N
Pimelodus maculatus Lacepède, 1803	11.54	N	N
Pimelodus pohli Ribeiro and Lucena, 2006	63.54	N	N
Pseudoplatystoma corruscans (Spix and Agassiz, 1829)	0.01	Y	N
ORDER GYMNOTIFORMES			
Family Sternopygidae			
Eigenmannia sp.	<0.01	N	N
ORDER PERCIFORMES			
Family Sciaenidae			
Pachyurus sp.	3.89	N	N
Plagioscion squamosissimus (Heckel, 1840)	<0.01	N	N

References

1. Agostinho, A.A.; Pelicice, F.M.; Gomes, L.C. Dams and the fish fauna of the Neotropical region: Impacts and management related to diversity and fisheries. *Braz. J. Biol.* **2008**, *68*, 1119–1132. [CrossRef] [PubMed]
2. Agostinho, A.A.; Thomaz, S.M.; Gomes, L.C. Conservação da biodiversidade em águas continentais do Brasil. *Megadiversidade* **2005**, *1*, 70–78.
3. Godinho, A.L.; Pompeu, P.D.S. A importância dos ribeirões para os peixes de piracema. In *Águas, Peixes e Pescadores do São Francisco da Minas Gerais*; PUC Minas: Belo Horizonte, Brazil, 2003; pp. 361–372.
4. Lopes, J.D.M.; Pompeu, P.S.; Alves, C.B.M.; Peressin, A.; Prado, I.G.; Suzuki, F.M.; Facchin, S.; Kalapothakis, E. The critical importance of an undammed river segment to the reproductive cycle of a migratory Neotropical fish. *Ecol. Freshw. Fish* **2019**, *28*, 302–316. [CrossRef]
5. Pelicice, F.M.; Pompeu, P.S.; Agostinho, A.A. Large reservoirs as ecological barriers to downstream movements of Neotropical migratory fish. *Fish Fishes* **2015**, *16*, 697–715. [CrossRef]
6. Nakatani, K.; Agostinho, A.A.; Baumgartner, G.; Bialetzki, A.; Sanches, P.V.; Makrakis, M.C.; Pavanelli, C.S. *Ovos e Larvas de Peixes de Água Doce: Desenvolvimento e Manual de Identificação*; EDUEM: Maringá, Brazil, 2001.
7. Brasil, Ministério de Minas e Energia, Empresa de Pesquisa Energética. *Plano Nacional de Energia 2050/Ministério de Minas e Energia*; Empresa de Pesquisa Energética, MME/EPE: Brasília, Brazil, 2020.
8. Pompeu, P.S.; Agostinho, A.A.; Pelicice, F.M. Existing and future challenges: The concept of successful fish passage in South America. *River Res. Appl.* **2012**, *28*, 504–512. [CrossRef]
9. Orsi, M.L.; Almeida, F.S.; Swarça, A.C.; Claro-García, A.; Vianna, N.C.; Garcia, D.A.Z.; Bialetzki, A. *Ovos, Larvas e Juvenis dos Peixes da Bacia do Rio Paranapanema uma Avaliação para a Conservação*; Triunfal Gráfica e Editora: Assis, Brazil, 2016; 136p.

10. Reynalte-Tataje, D.A.; Nakatani, K.; Fernandes, R.; Agostinho, A.A.; Bialetzki, A. Temporal distribution of ichthyoplankton in the Ivinhema River (Mato Grosso do Sul State/Brazil): Influence of environmental variables. *Neotrop. Ichthyol.* **2011**, *9*, 427–436. [CrossRef]
11. Suzuki, F.M.; Pompeu, P.S. Influence of abiotic factors on ichthyoplankton occurrence in stretches with and without dams in the upper Grande River basin, south-eastern Brazil. *Fish. Manag. Ecol.* **2016**, *23*, 99–108. [CrossRef]
12. Baumgartner, G.; Nakatani, K.; Gomes, L.C.; Bialetzki, A.; Sanches, P.V.; Makrakis, M.C. Fish larvae from the upper Paraná River: Do abiotic factors affect larval density? *Neotrop. Ichthyol.* **2008**, *6*, 551–558. [CrossRef]
13. Reis, R.E.; Albert, J.S.; Di Dario, F.; Mincarone, M.M.; Petry, P.; Rocha, L.A. Fish biodiversity and conservation in South America. *J. Fish Biol.* **2016**, *89*, 12–47. [CrossRef]
14. Nascimento, F.L.; Lima, C.A.R.M.A. *Descrição das Larvas das Principais Espécies de Peixes Utilizadas pela Pesca, no Pantanal*; Embrapa: Brasília, Brazil, 2000; 25p.
15. Rizzo, E.; Sato, Y.; Barreto, B.P.; Godinho, H.P. Adhesiveness and surface patterns of eggs in neotropical freshwater teleosts. *J. Fish Biol.* **2002**, *61*, 615–632. [CrossRef]
16. Pompeu, P.S.; Nogueira, L.B.; Godinho, H.P.; Martinez, C.B. Downstream passage of fish larvae and eggs through a small-sized reservoir, Mucuri River, Brazil. *Zoology* **2011**, *28*, 739–746. [CrossRef]
17. Reynalte-Tataje, D.A.; Agostinho, A.A.; Bialetzki, A.; Hermes-Silva, S.; Fernandes, R.; Zaniboni-Filho, E. Spatial and temporal variation of the ichthyoplankton in a subtropical river in Brazil. *Environ. Biol. Fishes* **2012**, *94*, 403–419. [CrossRef]
18. Becker, R.A.; Sales, N.G.; Santos, G.M.; Santos, G.B.; Carvalho, D.C. DNA barcoding and morphological identification of neotropical ichthyoplankton from the Upper Paraná and São Francisco. *J. Fish Biol.* **2015**, *87*, 159–168. [CrossRef]
19. Leese, F.; Bouchez, A.; Abarenkov, K.; Altermatt, F.; Borja, Á.; Bruce, K.; Ekrem, T.; Čiampor, F., Jr.; Čiamporová-Zaťovičová, Z.; Costa, F.O.; et al. Why we need sustainable networks bridging countries, disciplines, cultures and generations for aquatic biomonitoring 2.0: A perspective derived from the DNAqua-Net COST action. *Adv. Ecol. Res.* **2018**, *58*, 63–99.
20. Baird, D.J.; Hajibabaei, M. Biomonitoring 2.0: A new paradigm in ecosystem assessment made possible by next-generation DNA sequencing. *Mol. Ecol.* **2012**, *21*, 2039–2044. [CrossRef]
21. Carvalho, D.C. Ichthyoplankton DNA metabarcoding: Challenges and perspectives. *Mol. Ecol.* **2022**, *31*, 1612–1614. [CrossRef]
22. Godinho, A.L.; Lamas, I.R.; Godinho, H.P. Reproductive ecology of Brazilian freshwater fishes. *Environ. Biol. Fishes* **2010**, *87*, 143–162. [CrossRef]
23. Cheshire, K.J.M.; Ye, Q.; Gillanders, B.M.; King, A. Annual variation in larval fish assemblages in a heavily regulated river during differing hydrological conditions. *River Res. Appl.* **2015**, *32*, 1207–1219. [CrossRef]
24. Rosa, G.R.; Salvador, G.N.; Bialetzki, A.; Santos, G.B. Spatial and temporal distribution of ichthyoplankton during an unusual period of low flow in a tributary of the São Francisco River, Brazil. *River Res. Appl.* **2018**, *34*, 69–82. [CrossRef]
25. Soares, M.d.L.; Massaro, M.V.; Hartmann, P.B.; Siveris, S.E.; Pelicice, F.M.; Reynalte-Tataje, D.A. The main channel and river confluences as spawning sites for migratory fishes in the middle Uruguay River. *Neotrop. Ichthyol.* **2022**, *20*, e210094. [CrossRef]
26. Melo-Silva, M.; da Silva, J.C.; Bialetzki, A. Community structure of fish larvae in different biotopes of a neotropical river. *Community Ecol.* **2022**, *23*, 1–12. [CrossRef]
27. CBHSF (Comitê da Bacia Hidrográfica do Rio São Francisco). *Diagnóstico da Dimensão Técnica e Institucional Volume 2: Caracterização da Bacia*; CBHSF: Belo Horizonte, Brazil, 2016; 292p.
28. Bazzoli, N. Parâmetros reprodutivos de peixes de interesse comercial na região de Pirapora. In *Águas, Peixes e Pescadores do São Francisco das Minas Gerais*; PUC Minas: Belo Horizonte, Brazil, 2003; pp. 291–306.
29. Hermes-Silva, S.; Reynalte-Tataje, D.; Zaniboni-Filho, E. Spatial and temporal distribution of ichthyoplankton in the upper Uruguay River, Brazil. *Braz. Arch. Biol. Tech.* **2009**, *52*, 933–944. [CrossRef]
30. Baumgartner, G.; Nakatani, K.; Gomes, L.; Bialetzki, A.; Sanches, P. Identification of spawning sites and natural nurseries of fishes in the upper Paraná River, Brazil. *Environ. Biol. Fishes* **2004**, *71*, 115–125. [CrossRef]
31. Sato, Y.; Fenerich-Verani, N.; Nuñer, A.P.O.; Godinho, H.P.; Verani, J.R. Padrões reprodutivos de peixes da bacia do São Francisco. In *Águas, Peixes e Pescadores do São Francisco das Minas Gerais*; PUC Minas: Belo Horizonte, Brazil, 2003; pp. 229–274.
32. Aljanabi, S.M.; Martinez, I. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Res.* **1997**, *25*, 4692–4693. [CrossRef]
33. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef]
34. McMurdie, P.J.; Holmes, S. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **2013**, *8*, e61217. [CrossRef]
35. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **2011**, *17*, 10–12. [CrossRef]
36. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]
37. Nobile, A.B.; Freitas-Souza, D.; Ruiz-Ruano, F.J.; Nobile, M.L.M.; Costa, G.O.; De Lima, F.P.; Camacho, J.P.M.; Foresti, F.; Oliveira, C. DNA metabarcoding of Neotropical ichthyoplankton: Enabling high accuracy with lower cost. *MBMG* **2019**, *3*, e35060. [CrossRef]
38. Sato, Y.; Godinho, H.P. Migratory Fishes of the São Francisco River. In *Migratory Fishes of South America: Biology, Fisheries and Conservation Status*; Carolsfeld, J., Harvey, B., Ross, C., Baer, A., Eds.; IDRC-World Bank: Ottawa, ON, Canada, 2003; pp. 195–232.

39. Oksanen, J.; Blanchet, F.G.; Kindt, R.; Legendre, P.; Minchin, P.R.; O'Hara, R.B.; Simpson, G.; Solymos, P.; Stevens, M.; Wagner, H. Vegan: Community Ecology Package. R Package, Version 2.5.6.1-29; 2015. Available online: https://CRAN.R-project.org/package=vegan (accessed on 1 August 2022).
40. Sato, Y.; Cardoso, E.L.; Amorim, J.C.C. *Peixes das Lagoas Marginais do Rio São Francisco a Montante da Represa de Três Marias (Minas Gerais)*, 3rd ed.; CODEVASF: Brasília, Brazil, 1988.
41. Blumer, L.S. A bibliography and categorization of bony fishes exhibiting parental care. *Zool. J. Linn.* **1982**, *75*, 1–22. [CrossRef]
42. Calegari, B.B.; Vari, R.P.; Reis, R.E. Phylogenetic systematics of the driftwood catfishes (Siluriformes: Auchenipteridae): A combined morphological and molecular analysis. *Zool. J. Linn.* **2019**, *187*, 661–773. [CrossRef]
43. Thibault, R.E.; Schultz, R.J. Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). *Evolution* **1978**, *32*, 320–333. [CrossRef]
44. Veloso-Junior, V.C.; Arantes, F.P.; dos Santos, J.E. Short communication Population structure and reproduction of the Neotropical catfish *Pimelodus pohli*, Ribeiro and Lucena, 2006 (Teleostei: Pimelodidae). *J. Appl. Ichthyol.* **2014**, *30*, 399–402. [CrossRef]
45. Reynalte-Tataje, D.A.; Lopes, C.A.; Massaro, M.V.; Hartmann, P.B.; Sulzbacher, R.; Santos, J.A.; Bialetzki, A. State of the art of identification of eggs and larvae of freshwater fish in Brazil. *Acta Limnol. Bras.* **2020**, *32*, e6. [CrossRef]
46. Suzuki, F.M.; Pires, L.V.; Pompeu, P.S. Passage of fish larvae and eggs through the Funil, Itutinga and Camargos Reservoirs on the upper Rio Grande (Minas Gerais, Brazil). *Neotrop. Ichthyol.* **2011**, *9*, 617–622. [CrossRef]
47. Lopes, C.A.; Zaniboni-Filho, E. Mosaic environments shape the distribution of Neotropical freshwater ichthyoplankton. *Ecol. Freshw. Fish* **2019**, *28*, 544–553. [CrossRef]
48. Azevedo-Santos, V.M.; Daga, V.S.; Pelicice, F.M.; Henry, R. Drifting in a free-flowing river: Distribution of fish eggs and larvae in a small tributary of a Neotropical reservoir. *Biota Neotrop.* **2021**, *21*, e20211227. [CrossRef]
49. Loures, R.C. Effectiveness of Fish Monitoring Programs in Hydropower Plant Reservoirs, Lavras. Ph.D. Thesis, Federal University of Lavras, Lavras, Brazil, 2019.
50. Bialetzki, A.; Tataje, D.; Oliveira, E.; Zaniboni-Filho, E.; Baumgartner, G.; Makrakis, M.; Leite, R. Protocolo mínimo de amostragem do ictioplâncton de água doce para estudos de levantamento, inventário e monitoramento ambiental para implantação de empreendimentos hidrelétricos. *Bol. Soc. Bras. Ictiol.* **2015**, *113*, 32–34.
51. Godinho, A.L.; Kynard, B. Migration and spawning of radio-tagged zulega *Prochilodus argenteus* in a dammed Brazilian river. *Trans. Am. Fish. Soc.* **2006**, *135*, 811–824. [CrossRef]
52. MMA (Ministério meio Ambiente). *Portaria no 148 de 7 de Junho de*; Diario Oficial da União: Brasília, Brazil, 2022.
53. Sato, Y.; Cardoso, E.L.; Godinho, A.L.; Godinho, H.P. Hypophysation parameters of the fish *Prochilodus marggravii* obtained in routine hatchery station conditions. *Rev. Bras. Biol.* **1996**, *56*, 59–64.
54. Nilsson, C.; Reidy, C.A.; Dynesius, M.; Revenga, C. Fragmentation and flow regulation of the world's large river systems. *Science* **2005**, *308*, 405–408. [CrossRef]
55. Silva, J.C.; Rosa, R.R.; Galdioli, E.M.; Soares, C.M.; Domingues, W.M.; Veríssimo, S.; Bialetzki, A. Importance of dam-free stretches for fish reproduction: The last remnant in the upper Paraná River. *Acta Limnol. Bras.* **2017**, *29*, e106. [CrossRef]
56. Graça, W.J.; Pavanelli, G.C. *Peixes da Planície de Inundação do Alto Rio Paraná e Áreas Adjacentes*; Eduem: Maringá, Brazil, 2007; p. 241.
57. Suzuki, H.I.; Pelicice, F.M.; Luiz, E.A.; Latini, J.D.; Agostinho, A.A. Estratégias reprodutivas da assembléia de peixes da planície de inundação do alto rio Paraná. Pesquisas Ecológicas de Longa Duração–PELD.(Org.). In *A Planície Alagável do Rio Paraná: Estrutura e Processos Ambientais*; PELD: Maringá, Brazil, 2002.