

Threatened fish spawning area revealed by specific metabarcoding identification of eggs and larvae in the Beni River, upper Amazon

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Short title: Threatened fish spawning area in the Upper Amazon

Abstract

Identifying fish spawning areas is of great ecological and conservation importance as fishes are suffering increasing threats levels from anthropogenic activities. However, to date very few studies have done so in the Amazon basin. In the Beni River located in the upper Madeira basin, fishers reported that a particular ecotone near Rurrenabaque city was a fish reproduction area. To test the importance of this zone as a spawning site, we conducted an ichthyoplankton survey during the month when reproduction is most likely to occur. The specific identification of larvae and eggs was made with a metabarcoding analysis. With this approach 13 different fish species of high importance for regional and local fisheries were identified, including the long-distance migratory gilded catfish (*Brachyplatystoma rousseauxii*), considered as endangered in the upper Madeira. Combining the development time of morula-gastrula egg stages with the integrated current velocity of the river, we identified a spawning area that ranges between the last gravel beaches close to Altamarani community and the San Miguel del Bala community on top of Suse strait, the last foothill of the Andes. This spawning area further extends upstream in the Madidi and Pilon Lajas protected areas and Tacana and Tsimane indigenous communities, when considering eggs in final embryo stage. This portion of the Beni River, at the Andean foothills, is heavily impacted by anthropogenic activities, ranging from unmanaged fisheries to contamination coming from the extraction of fluvial aggregates, upstream gold mining, deforestation associated with a sugar cane mill, an existing downstream dam, and projected upstream dam projects. Some

urgent solutions for the conservation of this already impacted area are proposed. Confirming fish spawning zones, although difficult, is crucial to inform the definition of priority areas for conservation and management measures, in particular when these sites host endangered species.

Keywords

ichthyoplankton, Prochilodus, Brachyplatystoma, Zungaro, dams, arid extraction

1. INTRODUCTION

The Amazon basin, which hosts the largest freshwater fish diversity on earth, is facing increasing threats from anthropogenic activities such as overexploitation, habitat degradation, deforestation, hydropower dam construction, invasive species and pollution (Castello *et al.*, 2013; Castello & Macedo, 2016; Hurd *et al.*, 2016). Climate-induced modifications of Amazonian ecosystems are expected to further exacerbate these threats (Xenopoulos *et al.*, 2005; Freitas *et al.*, 2013; Frederico *et al.*, 2016). Beside their exceptional diversity, fish play a major role in the life of Amazonian people (Barthem & Goulding, 2007) with some of the world's highest fish consumption rates per capita: 30-40 kg.year⁻¹ for urban populations and 70-120 kg.year⁻¹ for rural populations (Isaac & Almeida, 2011), with maximum of ~200 kg.year⁻¹ (Batista *et al.*, 1998).

In the Amazon basin, the life cycles of many fishes, including most commercial species, involve a broad range of migrations for feeding and/or reproductive purposes, from a few kilometers to several thousand kilometers (Barthem & Goulding, 1997; Lucas & Baras, 2001; Carolsfeld *et al.*, 2003). As these larger migrations can span over several international boundaries, managing fisheries and conserving migratory species require a good knowledge of their movements and life history strategies. One of the most important issues is to understand

precisely where and when they spawn, so as to take appropriate measures to avoid habitat degradation and overfishing. As reproduction is key to species survival, conserving spawning sites is a priority. Identifying reproductive zones and determining which species use them allows the definition of precise conservation actions based on the specific characteristics of the zone (Castello *et al.*, 2013). Unfortunately, such knowledge is seldom available for Amazonian species.

Many studies have investigated fish reproduction in the Amazon basin using different methodologies, such as the monitoring of reproductive adults (Goulding, 1980, 1981; Vazzoler & Menezes, 1992; Ruffino & Isaac, 1995; Duponchelle *et al.* 2007; García-Vasquez *et al.*, 2009, Agudelo *et al.*, 2013; Bonilla-Castillo *et al.*, 2018), the morphological (Cañas & Pine, 2011; Barthem *et al.*, 2014, 2017; Cella-Ribeiro *et al.*, 2015) or molecular identification of larvae in ichthyoplankton samples (García-Dávila *et al.*, 2015; Maggia *et al.*, 2017), and, more recently, approaches based on citizen science (www.ictio.org). Yet, there is very little information on spawning areas and existing data does not go further than the family level (Oliveira & Ferreira, 2008; Cañas & Pine, 2011).

Recent conservation scenarii have evaluated the vulnerability of the main river basins of the Amazon and their evolution until 2050 under current and planned threats (Jézéquel *et al.*, 2020a). The Beni River basin is currently ranked within the second most vulnerable watershed category in the Amazon, and is predicted to negatively evolve into the most vulnerable category by 2050. Another recent study highlighted the knowledge gap in fish diversity for the upper Beni watershed (Jézéquel *et al.*, 2020b).

In spite of this lack of information about ichthyofauna, two of the most important national protected areas of the tropical Andes are located in this part of the Beni watershed, just above

Rurrenabaque: the Madidi National Park and Natural Area of Integrated Management and the Pilon Lajas Biosphere Reserve and Indigenous Territory (Figure 1). This area also hosts Tacana, T'simane-Mosetene and Uchupiamonas indigenous territories. These protected areas and indigenous territories play a critical role in the conservation of ichthyofauna and Bolivian biodiversity in general (SERNAP & CRTM 2009; SERNAP & Uchupiamonas 2009; CIPTA & CIMTA 2014; Identidad Madidi & SERNAP 2018). The natural boundary between the Madidi and Pilon Lajas protected areas is the Beni River, which crosses the core of the conservation area that these national parks represent. This portion of the Beni River is the main aquatic corridor linking both protected areas to the rest of the watershed, and hence the main migration route for the fish, but also for the indigenous communities that inhabit them, such as Asuncion del Quiquibey (Pilon Lajas) and San Jose de Uchupiamonas (Madidi). This route also crosses a series of marked ecological transitions including the Chepete, Beu, Bala and Suse mountain ranges (200-300 m a.s.l.) and the valleys in between (Figure 1). Another important transition is downstream of Rurrenabaque at Altamarani, a Tacana indigenous community, where a marked ecotone between the Andean foothills and the Amazonian alluvial plain is characterized by a change from a stone and gravel substrate upstream towards fine sand downstream (Figures 1 & 2). Local fishermen have identified this transition zone as a breeding site for many species of fish. Currently, this zone is highly impacted by several anthropogenic activities, including arid extraction, overfishing, and contamination by human and industrial waste, and could be impacted by dam projects in the near future.

The aim of this research was to confirm if this threatened ecotone is important for fish reproduction. A metabarcoding approach was implemented for taxonomic identification of fish species and resulting frequency estimation in plankton samples (Mariat *et al.*, 2018).

2. MATERIAL AND METHODS

2.1 Study area

The specific study site is located in the Beni basin, near the Altamarani village (67.555 W, 14.333 S, altitude 185 m a.s.l.), between the last Andean foothills and the beginning of the Beni floodplain, and 22.8 km downstream from the nearest town: Rurrenabaque (Figure 1). The Beni River is one of the largest rivers in Bolivia, at nearly 1000 km in length with a mean discharge of $2050 \text{ m}^3\text{s}^{-1}$ at the Bala strait (Gautier *et al.*, 2006, 2010). At the sampling site, the Beni River has a mean width of 269 m. The average depth is 7 m, reaching 45 m at the Suse strait (Figure 1). Sampling was conducted at the lowest point of this sand-gravel ecotone, thereby ensuring that egg and larvae drift from the majority of the watershed upstream was included in the sample (Lechner *et al.*, 2016). The hydrological regime registered at this location shows high amplitude variations related to rainfall (Molina *et al.*, 2020). Owing to ongoing human activities that threaten its integrity, this site was prioritized for the collection of ichthyoplankton (eggs and larvae). It was then sampled in February 2017, during the breeding season of most fish species in the upper Madeira (Cañas & Pine, 2011; Van Damme *et al.*, 2011; Barthem *et al.*, 2014; Cella Ribeiro *et al.*, 2015).

2.2 Sampling

Ichthyoplankton samples were collected (eggs and larvae) using ichthyoplankton nets, with a length of 2.5 m x 0.5 m in diameter and 250 μm mesh. Three nets were used simultaneously, tied to a string to sample at 1, 2 and 3 m depths. The nets were horizontally towed seven times for 15 minutes each during daylight. The bottom of the three nets were

weighted down and were held against the current from a boat running approximately at the same speed as the water flow (Barthem *et al.*, 2014). The larvae and eggs collected were macroscopically separated from the organic matter retained in the nets (by adding alcohol in the water to turn them a whitish color easier to identify) directly in the field, preserved in ethanol 95% and stored at 4°C for later determination of developmental stages and metabarcoding analysis.

2.3 Estimation of the upstream limit of the spawning area

The upstream limit for spawning was estimated using the relation between the current velocity and the timing of specific embryonic stages. Water flow was estimated using 2015 and 2016 hydrological data from the HYBAM project station at Rurrenabaque. Owing to the construction of the Rurrenabaque – San Buenventura bridge (Figure 1), the historical HYBAM sampling station had to be moved to another location, which explains the unavailability of data for 2017 (Timouk, 2017). Hence, the average current velocity was calculated from the 11 days preceding the collection date (25/02/2017) taken over the years 2015 and 2016. From the average of these 22 velocity measurements (1.74 m/sec), the range of values at 95% CI (i.e. average speed ± 1.96 SE) was calculated: 1.21 to 2.28 ms^{-1} . These two range values were then used to define the lower and upper distance of a first part of the spawning area using the age, in hours, of the morula-gastrula developmental times. The second part of the spawning area corresponds to the ranges calculated from the development time of the final embryo stage (15h).

For that, it was assumed that eggs can be assimilated to inactive particles, that they drift downstream as soon as they are spawned, and that they do so at the same speed as the water flow in the river (Barros & Rosman, 2018). Eggs, but not larval development stages, were used for this

calculation because the length of egg developmental stages is more constant between species (Nakatani *et al.*, 2001; Andrade *et al.*, 2016). As several species were involved, we used the approximate mean development times provided in the literature for some of these taxa (at the genus level at least) and some close relatives (Table 1). Water temperature is generally warmer in controlled conditions (Table 1) than in the river (in our case $\sim 24^{\circ}\text{C}$), which might also delay the development time, albeit more for developed embryo and larvae than for cleavage stages (morula and gastrula). For this reason, the development time selected has been rounded up to 2 hours for the morula stage, 4 hours for the gastrula stage and 15 hours for the final embryonic stage (means provided in Table 1).

In order to delimit a spawning area as conservatively (extensively) as possible, the lower limit of the spawning area was approximated using the shortest development time for the morula-gastrula stages (2h) and the slowest current velocity (1.21 m/s). We then defined two upper limits, the first using the longest development time (4h) of the morula-gastrula stages and the second using the development time of the final embryo stage (15h), both with the fastest current velocity (2.28 m/s).

2.4 Sequencing and taxonomic assignation

2.4.1 DNA extractions and NGS libraries preparation

Two DNA bulk extractions were performed, one each for the larvae and egg samples, following the rapid isolation of mammalian DNA procedures (Sambrook *et al.*, 1989). The single COI (cytochrome c oxidase subunit I) probe production, as well as all steps and conditions for enriched libraries by capture preparations (DNA shearing, DNA end repair, adapters ligation, Bst Polymerase treatment, hybridizations and real time PCR) followed published protocols (Mariac *et*

al., 2018). One negative (blank) and two positive controls (mock community samples with known species composition) were included during NGS (Next Generation Sequencing) libraries preparation. Indexed libraries were pooled at an equimolar ratio for the capture enrichment step, followed by paired-end sequencing using MiSeq v2 reagents and 2×150 bp. Sequencing was carried out at the CIRAD facilities (Montpellier, France).

2.4.2 Data cleaning and taxonomic assignation

Demultiplexing based on the 6-bp internal index was performed using the PYTHON script DEMULADAPT (<https://github.com/Maillol/demultadapt>). Adapters were removed using CUTADAPT 1.2.1 (Martin, 2011). The NGS sequences have been aligned to a COI database (command lines in S1) with the MALT version 0.3.8 program (Herbig, 2016) to generate a file in blast format. The reference database used contains 160,387 Actinopterygii COI sequences extracted from Genbank and Bold (Ratnasingham & Hebert, 2007) on 21st February 2017 (reference database S2). Among the 16,270 species or subspecies it contains, at least 445 are species described in the Amazon region.

Taxonomic assignation of the blast results was performed with MEGAN software version 6.12.3 (Huson *et al.*, 2016) using the paired reads option and the weighted LCA method. Reads mapping COI were assigned to a species if a minimum alignment score value of 150 and 98% identity was reached with a reference sequence in the database. If a read mapped with the same score to more than one species it was assigned to their lowest common ancestor. Additional files containing synonymous and disabled taxa (S3) were included for assignment under MEGAN software. These files were used in order to control mis-assignments related to typographical errors and taxonomic revisions. The percentage of reads assigned, not assigned (reads with a hit

but failing to meet the score and identity requirements) and reads with no hits to the COI database are reported (S4). A minimum frequency threshold was defined using the MaxSSS method (Manel *et al.*, 2001; Liu *et al.*, 2013) with the two mock control samples. The value of this threshold, set at the maximum sum of specificity and sensitivity, was determined by comparing the frequencies estimated by NGS in mock samples to their known frequencies (established by an individual Sanger sequencing). As previously described in Mariac *et al.* (2018), species identified in egg and larvae samples whose frequencies were below this threshold were discarded.

3. RESULTS

The number of larvae and eggs collected at the sampling point in February 2017 was 404 and 49, respectively. Among the eggs, 8.7% were in the morula stage (~ 2 h post-fertilization), 87% in the gastrula stage (~ 4 h post-fertilization), and 4.3% in the final embryo stage (~ 15 h post fertilization) (Table 2), which implies that the corresponding species spawned very close to the sampling point. Among the larvae, the highest proportion of individuals were found in early stages of development with 60% in the *early flexion* stage, 37.5%, in the *pre-flexion* stage and 2.4% were yolk-sac larva (newly hatched individuals); or equivalent to ~ 62 h, 26 h and 14 h of total life, respectively. The larvae of more advanced stages were found in a lower proportion: most of them were more than 48 h old, with a variation depending on the species.

3.1 Sequencing results and taxonomic assignation

The total number of raw reads was 1,230,896 from which 1,089,701 (88.5%) were successfully paired and then processed for the taxonomic identification of eggs and larvae. The number of reads per library ranged between 86,040 and 227,368 (see S3 for details) with a mean

percentage of reads assigned against the COI reference database of 66.6% (SE= 4.7). Only 176 reads were retrieved in the negative control and none of them were assigned. Taxa with a frequency of less than 0.6% were discarded. This minimum frequency threshold was determined by the maxSSS method using mock control samples (supplementary data S5 and S6). Overall fifteen different taxa were identified, 13 at the species level and 2 at the genus level. These taxa belong to eight genera and two orders (Table 3). Eleven and five fish species were identified in eggs and larvae samples, respectively. The percentage of assigned reads was highest for the genus *Prochilodus*, with 33.1% in the egg sample and 62.35 % in the larva sample. *Prochilodus nigricans* is the most represented species in both eggs and larvae with 18.15 and 26.1% respectively (Table 3). *Brachyplatystoma rousseauxii* and *Zungaro zungaro* were identified only in the egg samples and represent 17% and 8% of the assigned reads, respectively. The remaining reads were assigned to the genus *Pseudopimelodus* and to ten other species, including five species of *Prochilodus* in both eggs and larvae stages (Table 3). Of the 15 taxa recorded, 8 were observed only in egg samples (*Brachyplatystoma rousseauxii*, *Zungaro zungaro*, *Potamorhina altamazonica*, *Psectogaster rutiloides*, *Pimelodus blochii*, *Mylossoma albiscopum*, *Prochilodus aff. argenteus*, *Rhinodoras boehlkei*), 3 by both eggs and larvae (*P. nigricans*, *P. aff. lineatus*, *P. aff. rubrotaeniatus*) and 2 only in larvae samples (*P. aff. costatus*, *Pseudopimelodus* sp. LBP2397) (Table 3). It can therefore be assumed that at least the 11 species whose eggs were sampled reproduce in the spawning area closest to the sampling point (red stretch in Figure 1).

3.2 Estimation of the spawning area

Linking the development time for the most abundant gastrula-morula stages (2h-4h) with the range of current velocity of the Beni river during the sampling period (1.21 to 2.28 m/sec:

Table 1), we estimate that the collected eggs were drifting from a zone located between 8.71 km ($2\text{h} * 1.21 \text{ m/s} * 3.6$) and 32.83 km ($4\text{h} * 2.28\text{m/s} * 3.6$) upstream from the sampling point, illustrated as a red stretch in Figure 1. The final embryo stages (15h) would be drifting from a zone between 65.3 to 123.1 km above the sampling point through the Chepete and Beu Straits, illustrated as an orange stretch in Figure 1. These two stretches delimit the whole spawning area (SPA) based on egg samples collected in the present study. This SPA integrates parts of the Madidi and Pilon Lajas protected areas and indigenous territories of the Tacana, T'simane Mosetene and Uchupiamonas people. Regarding the larvae, most were more than 48 h old, which also suggests spawning areas further upstream inside the Madidi and Pilon Lajas protected areas and indigenous territories.

4. DISCUSSION

Our study is based on a single sampling date and locality, which constrains the extent of its conclusions. It is impossible, for instance, to state how many species exactly spawn in the identified spawning area, or to precisely determine the extent of the spawning area. We cannot assess either the relative importance of this spawning area for the different species, as they were sampled in very different proportions, probably because of the sampling scheme. Answering these questions would require a broader temporal and spatial sampling coverage. But in spite of these inherent limitations, our study nevertheless provides clear evidence that our sampling area is used by several fish species, including species of major commercial and cultural importance, as a spawning ground, and that it is under immediate threat by anthropogenic activities. Our results also allow, for the first time in the Bolivian Amazon, and one of the first in the Amazon basin,

the delimitation of a spawning area for at least 15 taxa around Rurrenabaque in the Beni River, including 13 species, with 11 sampled at the egg stage. It is likely indeed, that further sampling at different times of the hydrological cycle would increase the number of species using the identified spawning area. Contrary to the few studies that have identified reproduction areas in the Amazon basin (e.g. Oliveira & Ferreira, 2008; Cañas & Pine, 2011), the delimitation of the spawning area in the present study was based on the specific molecular identification of eggs in early development stages and not of larvae, which allows for a more precise estimation of the spawning area. Approximations relative to current velocity and water temperature have to be made in both cases, as eggs and even more so larvae that can have swimming abilities, are not homogeneously distributed in the water column or in the width of the rivers (Oliveira & Araujo-Lima, 1998; Barthem *et al.*, 2014; Cella-Ribeiro *et al.*, 2015), and current velocity strongly varies in the river section. Current velocity can also vary among stretches of the river, especially when the slope or the width of the riverbed varies. However, early egg developments are counted in a few hours, which limits potential approximation errors to a few kilometers, as opposed to tens of km with larvae that develop over a few days (Nakatani *et al.*, 2001; Nogueira *et al.*, 2012; Andrade *et al.*, 2016; Nakauth *et al.*, 2016; Souza *et al.*, 2016).

The identified spawning area extends between Altamarani below Rurrenabaque and well over 100 km upstream. Previous studies had suggested spawning area for migratory catfishes between 200 and 400 masl (Van Damme *et al.*, 2011; Barthem *et al.*, 2017). Although the spawning area identified in the present study is extensive, our results also indicate that at least 11 species reproduce in an area close to Rurrenabaque, between the Altamarani and San Miguel indigenous Tacana communities (~ 40 km) (the red stretch in Figure 1). This relatively restricted portion of the spawning area is exposed to high anthropogenic threats. Indeed, the portion of the

Beni River just downstream from Rurrenabaque is used intensively to extract gravel and stone for local infrastructure like roads and bridges (Figure 1). This activity was already identified as a threat to the migration of the small and culturally important *Trichomycterus barbouri* catfishes, which migrate along the water edge (Miranda-Chumacero *et al.*, 2015). It is also likely to alter the nature of the substrate, as well as physico-chemical conditions, in this particular ecotone, threatening its suitability as a spawning ground for fishes. Additionally, waste from a sugar mill 2 km from the sampling site, gold mining extractions and deforestation upstream of the spawning area, represent supplementary water pollution risks.

Another major threat is the accelerated construction of hydroelectric dams in the Amazon basin (Finer & Jenkins 2012; Lees *et al.*, 2016; Winemiller *et al.*, 2016; Latrubesse *et al.*, 2017; Anderson *et al.*, 2018). The dams planned between the protected areas of the Madidi and Pilon Lajas at the Bala, Chepete and Beu straits, would result in important alterations in the volume and flow of sediments, homogenizing downstream habitats (Forsberg *et al.*, 2017), including the newly identified spawning area. There are also plans to build other dams in Bolivian territory, such as that on the border with Brazil in Nova Mamoré-Guayaramirim. Downstream, two large hydroelectric dams, Santo Antonio and Jirau, were recently (2011-2012) completed on the Brazilian portion of the river, between Porto Velho and the Bolivian border (Fearnside, 2014). Although located well downstream from the spawning area, these two major hydroelectric dams strongly disrupt the migration patterns of fish species, in particular upstream movements towards reproduction zones, including for several Pimelodidae like goliath catfishes (*Brachyplatystoma* spp.: Duponchelle *et al.*, 2016; Hauser, 2018; Hauser *et al.*, 2019).

One particularly interesting fact about the newly identified spawning area is that it also hosts the reproduction of *Brachyplatystoma rousseauxii*, one of the most emblematic and

commercially important fishes for the food security and economic sustainability of riverine human populations in the Amazon basin (Barthem & Goulding, 2007), and the Madeira in particular (Doria *et al.*, 2018; Santos *et al.*, 2018; Lima *et al.*, 2020). As a migratory top predator (Barthem & Goulding, 1997), *B. rousseauxii* plays a crucial role for the stability of food webs (Angelini *et al.*, 2006; Estes *et al.*, 2011; Bauer *et al.*, 2014). This species also performs the longest migration known in fresh waters: a round trip of up to 12,000 km (7,500 km for this specific spawning area) between its breeding areas in the Andean foothills and its nursery in the Amazon estuary (Barthem & Goulding 1997, 2017; Duponchelle *et al.*, 2016). Moreover, this exceptional migration involves a natal homing behavior (Batista & Alves-Gomes, 2006), in which most fish return to spawn in the specific geographic area where they were hatched in the upper Amazon (Hauser *et al.*, 2020), including the upper Madeira (Duponchelle *et al.*, 2016). Recent analyzes of otolith microchemistry have shown that the construction of Santo Antônio and Jirau dams on the Brazilian portion of the Madeira have profoundly altered its migration pattern, preventing pre-adults to home back to their breeding areas in the upper Madeira and reducing the downstream drift of larvae to the estuary (Hauser, 2018; Hauser *et al.* 2018). The populations of *B. rousseauxii* are now totally isolated in the upper Madeira basin: all the individuals caught in the upper Madeira after dam construction were hatched and bred within the Upper Madeira, without ever reaching their nursery area in the lower Amazon-estuary, a behavior rarely observed before the dams (Duponchelle *et al.*, 2016; Hauser, 2018). This situation has already led to a drastic post-dam reduction in the catches of *B. rousseauxii* in Puerto Villarroel, located in another major river basin of the Bolivian Amazon (Van Damme *et al.*, 2019). This species is the most vulnerable *Brachyplatystoma* species, classified as Vulnerable (VU) (Van Damme *et al.*, 2009),

and with a proposal to categorize it as Critically Endangered (CR) in the Madeira basin (Van Damme, *in prep*).

Interestingly, we identified 6 different genetic entities of *Prochilodus* in the geographic area, with a genetic differentiation at a specific level according to the identification criterion in our methodology: *P. nigricans* already known in the Madeira basin (Castro & Vari, 2004), but also *P. aff. rubrotaeniatus*, *P. aff. argenteus*, *P. aff. costatus* and *P. aff. lineatus*, as well as a sixth entity *Prochilodus* (without valide assignation at the species level). Only *P. nigricans* and *P. rubrotaeniatus* are supposed to be present in the Amazon basin (Castro & Vari, 2004; Melo *et al.*, 2016, 2018). These results suggest cryptic diversity in the Upper Madeira with more naturally occurring species. Although these cryptic taxa are most likely not *P. argenteus*, *P. lineatus* and *P. costatus*, which belong to other river basin of South America (Castro & Vari, 2004; Melo *et al.*, 2016, 2018), these newly identified taxa are nevertheless genetically close to these species, hence our use of *affinis* in their names (Table 3). This genetic proximity is not surprising given the very low divergence in the COI barcode among lineages of the genus *Prochilodus* (Melo *et al.*, 2018). The existence of several putative species of *Prochilodus* in Bolivia was reported by Loubens *et al.* (1991), in a review in which 6 species of *Prochilodus* were identified in Bolivia, including a new species: *P. labeo*. However, this diversity was neglected by a posterior revision of the Prochilodontidae in which only one fish from Bolivia was analyzed (Castro & Vari, 2004). Nevertheless, the existence of greater species diversity in the genus *Prochilodus*, including possible endemism in the Beni watershed, is also supported by the local knowledge of the T'simane indigenous people (Daillant, 1999, Sarmiento J., pers. comm.). These results emphasize the need for molecular studies on *Prochilodus* in the upper Madeira to shed light on these taxonomic issues.

Among the identified species, many are of major importance for local and regional fisheries (Van Damme *et al.*, 2011; Doria *et al.*, 2018), e.g. *Brachyplatystoma rousseauxii*, *Prochilodus nigricans* and *Zungaro zungaro*. These species are also critical for the livelihoods of the indigenous Tacana communities, whose catches are dominated (85%) by migratory catfishes (CIPTA & WCS, 2010). These species also constitute a large part of the annual income of Tacana communities. In these communities ~ 65% of the fish are sold whereas the other 35% are for communal consumption (Miranda-Chumacero *et al.*, 2011). Alterations in the reproductive cycles of these species could affect the food security and sovereignty of hundreds of indigenous families living in this spawning area. Furthermore, a large part of the fish traded in the principal Bolivian cities come from the Beni watershed, and therefore underpin the livelihoods of hundreds of commercial fisher people in Rurrenabaque and San Buenaventura (Carvajal-Vallejos *et al.*, 2011).

Preserving the integrity and function of the endangered spawning area around Rurrenabaque is paramount for several reasons: it is used by many important species for local and regional fisheries; it is used as a corridor by migratory species, including the culturally important *T. barboursi* (Miranda-Chumacero *et al.*, 2015); it is located within two regional protected areas and indigenous territories; and it is the first precisely identified spawning area of *B. rousseauxii* in the Amazon basin, and likely one of the last remaining spawning areas for the already strongly diminished and isolated population of *B. rousseauxii* in the upper Madeira (Van Damme *et al.*, 2019). Indeed, the breeding areas above Puerto Maldonado in the Peruvian portion of the Madre de Dios (Cañas & Pine, 2011) are now severely compromised by the illegal mining activities that have drastically altered the river's geomorphological and chemical properties (Diringer *et al.*, 2015; Martinez *et al.*, 2018).

Fish life cycles face several natural and anthropogenic threats (dams, pollution, fishing) and the destruction of their spawning sites magnifies their vulnerability. To guarantee the permanence of the species that use this spawning area, it will be important to: 1) maintain the connectivity within and between the still free-flowing Beni, Madre de Dios and Mamoré sub-basins, avoiding the construction of dams with large reservoirs in the lowlands, such as the binational dam planned at Nova Mamore-Guayaramerim, or the Bala, Chepete, Beu dams projected upstream of Rurrenabaque (Anderson *et al.*, 2018), 2) declare a general fishing ban in the upper Madeira for the most threatened fish species, e.g. the goliath catfishes (Ruffino *et al.*, 2018), to allow the remaining individuals to recruit (Hauser *et al.*, 2020), and accompany this with a fishing monitoring program in the Rurrenabaque area, 3) regulate and zone the extraction of gravel and stone excluding it from the breeding area to avoid alterations to the hydro-geomorphological characteristics that migratory species seek for their reproduction, 4) conduct studies to determine the impact of agro-industrial waste and gold extraction on the Beni River, 5) initiate a system for monitoring the density of larvae and their identification using NGS metabarcoding, 6) establish information campaigns with a citizen science approach, like the Ciencia Ciudadana para la Amazonia project (<https://www.amazoniacienciaciudadana.org>) for local residents and fishermen to raise awareness of the importance of this spawning area for local fisheries and, finally, 7) strengthen territorial management in both protected areas and indigenous lands by increasing their role in fish and aquatic biodiversity protection, highlighting the importance of rivers and their connectivity for conservation.

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TABLES

Table 1. Development times of ontogenetic stages in some Amazonian taxa. Reference 1:

Nogueira *et al.* (2012); 2: Souza *et al.* (2016); 3: Nakauth *et al.* (2016); 4: Andrade *et al.* (2016).

Taxa	Temperature	Developmental time			Ref
		Morula (M)	Gastrula (G)	Final embryo (FE)	
<i>Zungaro</i>	26.7	2h10	4h40	13h30	1
<i>Prochilodus</i>	26 (FE)-28 (M-G)	1h	4h	22h	2
<i>Brycon</i>	30	1h10	1h50	9h30	3
<i>Pseudoplatystoma</i>	28.2	2h	4h	12h	4
Mean (h)		1.6	3.6	14.25	

Table 2. Stages and reference sizes of all eggs registered at the spawning area in February 2017.

Stage	Life Stage (h) ^a	N	(%)	Diameter (mm)
Morula	2	5	(8.7%)	1.24
Gastrula	4	41	(87.0%)	1.75
Final embryo	15	3	(4.3%)	2.16
Total		49	100	1.73

^a adapted from Andrade *et al.*, 2016

Table 3. Taxa identified in egg and larvae samples at the spawning area, and their percentage.

Only the taxa with a percentage above the threshold value of 0.6% are reported.

Order	Family	Genus	Species	Eggs	Larvae	
Characiformes	Curimatidae		<i>Potamorhina altamazonica</i>	6.39	0.00	
			<i>Psectrogaster rutiloides</i>	6.02	0.00	
	Prochilodontidae	<i>Prochilodus</i>			33.15	62.35
				<i>Prochilodus</i> aff. <i>argenteus</i>	1.16	0.00
				<i>Prochilodus</i> aff. <i>costatus</i>	0.00	0.88
				<i>Prochilodus</i> aff. <i>lineatus</i>	0.92	2.95
				<i>Prochilodus</i> aff. <i>rubrotaeniatus</i>	0.62	0.76
				<i>Prochilodus nigricans</i>	18.15	26.10
	Serrasalminidae		<i>Mylossoma albiscopum</i>	1.39	0.00	
Siluriformes	Doradidae		<i>Rhinodoras boehlkei</i>	0.68	0.00	
	Pimelodidae		<i>Brachyplatystoma rousseauxii</i>	17.61	0.00	
			<i>Pimelodus blochii</i>	2.94	0.00	
			<i>Zungaro zungaro</i>	8.81	0.00	
	Pseudopimelodidae	<i>Pseudopimelodus</i>			0.00	4.41
				<i>Pseudopimelodus</i> sp. LBP2397	0.00	0.70
Taxa below threshold				2.16	1.86	
Total (%)				100	100	

FIGURE LEGENDS



Figure 1. Upstream delimitation of the spawning area from the sampling point (yellow point), estimated from eggs at the morula-gastrula stages (red line), and from eggs at the final embryo stage (orange line). The map also indicates existing and potential threats in the area, as well as the Madidi and Pilon Lajas protected area limits and the location of indigenous territories.



Figure 2. Ecological transition close to the sampling point (67.5555 W, 14.3329 S) between sand beaches (a), and stone beaches (b). Extraction activity 10 km upstream from the sampling point, at the spawning area, showing the machinery (c) used for extracting gravel for construction materials. Photos by Pascal Arditi.

APPENDICES

Data accessibility

DNA sequences in.fastq file format and MEGAN project file (rma) used in this study are available from NCBI's Sequence Read Archive (SRA). Bioproject number PRJNA507878. Biosample accession numbers SAMN10511183-SAMN10511185.

Supporting information

S1 Text. Command lines and software used.

S2 Text. COI reference database used in this study for taxonomic assignment.

S3 Text. Synonym-and-disable-taxa.txt

S4 Table. Number of reads and assignment rates in libraries.

S5 Table. Species frequencies calculated for each library.

S6 Table. maxSSS value calculated from mock control samples.