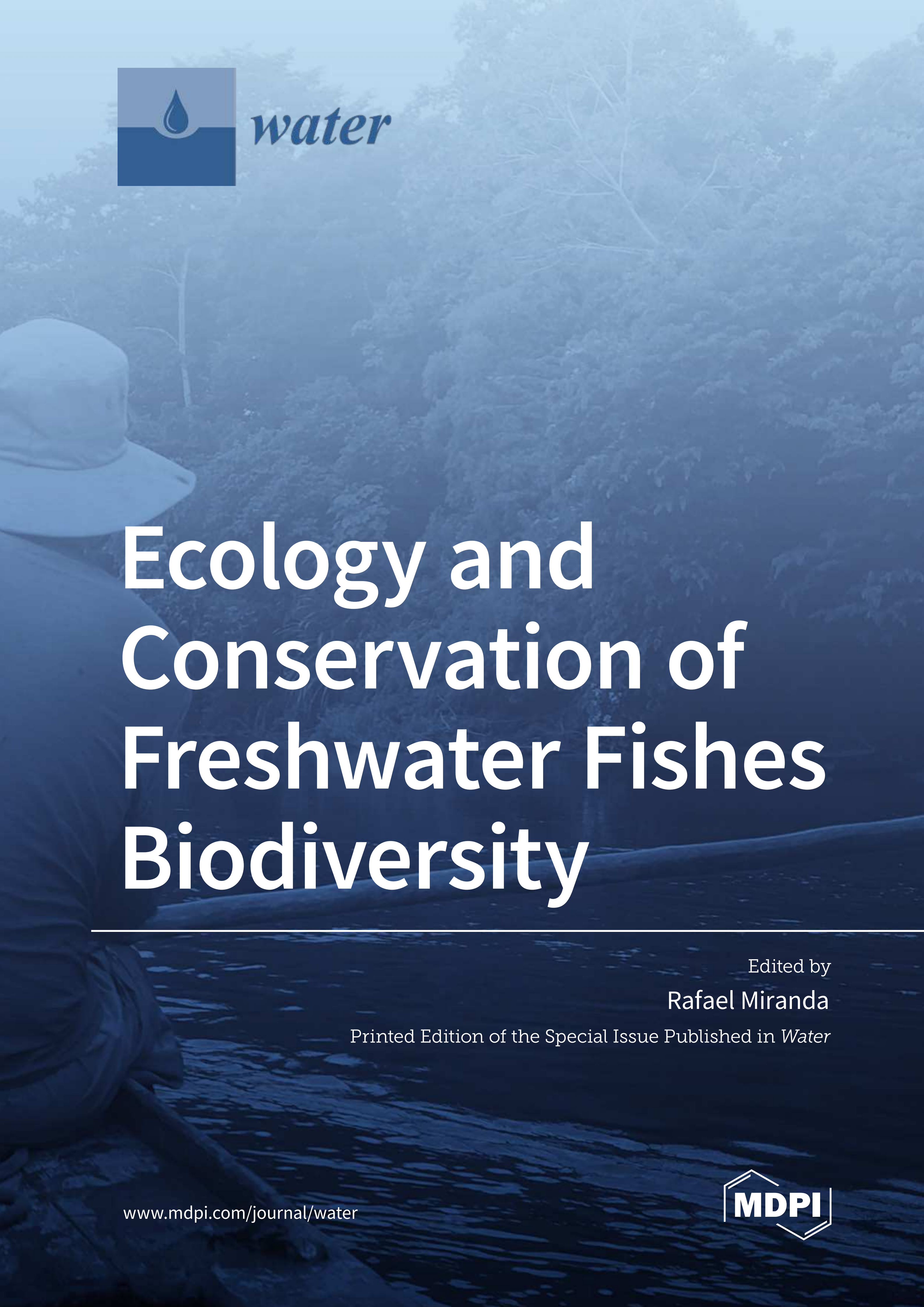

water

Ecology and Conservation of Freshwater Fishes Biodiversity

Edited by

Rafael Miranda

Printed Edition of the Special Issue Published in *Water*

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Editor

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Editor
Rafael Miranda
Institute of Biodiversity and
Environment (BIOMA)
University of Navarra
Pamplona
Spain

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

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Cover image courtesy of Rafael Miranda

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About the Editor

Rafael Miranda

Ph.D. graduated in 1998, and performs his research activity in the field of Hydrobiology and Animal Ecology, especially dealing with Freshwater Fishes and Management and Conservation of Wildlife in the Iberian Peninsula and Latin America. He is a professor and researcher in the Department of Environment Biology at the University of Navarre.

Currently, his research projects aim to analyze the implications of the knowledge of biodiversity for the management and conservation of protected areas, in collaboration with some Latin-American institutions. He is a member of the External Affairs Committee of the American Fisheries Society (AFS) and the treasurer of the Iberian Ichthyologic Society (SIBIC). Dr. Miranda has published more than 90 papers in scientific international journals, related to conservation, hydrobiology, and limnology, especially on the ecological analysis of human impacts.

Preface to "Ecology and Conservation of Freshwater Fishes Biodiversity"

Freshwater fishes are the most diverse vertebrate group, with almost 36,000 species described so far, and more species are being discovered all the time, evenly distributed between marine and freshwater habitats. Freshwater ecosystems serve as a habitat for more than 18,000 fish species, occupying less than 1% of the Earth's surface. Among all ecosystems, inland waters are one of the most affected. Wetlands are disappearing three times faster than forests, and freshwater populations decrease faster than terrestrial biodiversity. Nowadays, freshwater fishes may be considered the most threatened vertebrate group.

Understanding the ecological subjects, environmental necessities, and pressures of freshwater fishes remains a key concern of their conservation biology. This reprint explores the relationships between environmental issues, freshwater fish biodiversity, and human impacts from different perspectives, but always focuses on the conservation biology of species and ecosystems.

A change in mindset is needed to protect biodiversity in the upcoming years. Conservation plans have failed because our current knowledge is deficient and needs to be improved. We need countries to commit to protecting biodiversity and develop realistic targets that can be met while compromising with conflicting needs and interests. The articles included in this reprint emphasize the necessity of having more knowledge to develop conservation strategies. Future conservation targets may be advanced in part based on the knowledge provided by these papers and similar studies to ensure the long-term protection of freshwater fish and other life forms.

Rafael Miranda
Editor

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Editorial

Ecology and Conservation of Freshwater Fishes Biodiversity: We Need More Knowledge to Develop Conservation Strategies

Rafael Miranda [1,2,*] and **Imanol Miqueleiz** [1]

[1] Biodiversity Data Analytics and Environmental Quality Research Group, Department of Environmental Biology, University of Navarra, 31009 Pamplona, Spain; imiqueleiz@alumni.unav.es
[2] Biodiversity and Environment Institute, 31009 Pamplona, Spain
* Correspondence: rmiranda@unav.es

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Freshwater fish represent one-fourth of all vertebrate species, despite freshwater occupying less than 1% of the Earth's surface [1]. They are among the most threatened vertebrates, as they are especially vulnerable to human alterations resulting from species introduction, overexploitation, fragmentation, the degradation of continental watercourses, and climate change [2]. Furthermore, freshwater fish show high levels of endemism because of the particular characteristics of their aquatic ecosystems and their evolutionary isolation; their loss could have irreparable consequences.

Knowledge regarding the conservation status and ecology of freshwater fish is less than that for terrestrial vertebrates due to biases in conservation research and management toward more charismatic species [1]. Research into the ecological subjects, environmental necessities, and pressures of freshwater fishes is considered crucial to develop effective management measures for freshwater ecosystems. Understanding these environmental features remains a key concern of freshwater fish's conservation biology.

This Special Issue of *Water* explores the relationships of environmental issues, freshwater fish biodiversity, and human impacts from different perspectives, but always focused on the conservation biology of species and ecosystems. This Special Issue comprises thirteen papers with contributions from seventy-one authors, including research studies from very diverse ecosystems, places, and countries, from the most extensive basins, such as Middle Rio Grande in New Mexico (USA) [3] or the Yangtze River Basin (China) [4], to remote areas in Andean Amazon piedmont (Peru) [5] or Lake Tana (Ethiopia) [6]. Analyzed ecosystems include basins, rivers and streams, lakes, and ponds from high mountain to estuarine places, from pristine to highly altered environments.

The articles include eleven research articles, one review and a short communication. The nature of studies included in this Special Issue is diverse, considering a variety of scientific approaches in a mixture of field topics. One of the published papers considers methodological approaches to studying freshwater fishes and their conservation status. The authors propose a methodological study and improvement on age estimation for four species of *Labeobarbus* genus using otoliths as a crucial tool for conserving these species. According to the authors, this kind of study is very relevant to Africa, particularly Ethiopia, in order to develop effective management strategies to conserve endemic species [6]. Similarly, Gebremedhin et al. review scientific methods, concepts, and processes related to stock assessment and population dynamics in Africa [7].

Three published papers present results on fish distribution, ecosystem explorations, and ecological features in unique places, using many different techniques. Perivolioti et al. [8] conduct hydroacoustic monitoring of the Lake Trichonis (Greece) fish diversity, with the aim of offering an updated assessment of this unique ecosystem and the associated endemic species, with a focus on management and conservation.

Tobes et al. [5] study the distribution of fish communities related to the environmental variables of the Alto Madre de Dios River, a poorly studied Andean–Amazon watershed of southern Peru. Results show a significant shift in fish diversity regarding altitude, separating headwater and middle-lowland communities. In the light of this scenario where no Andean–Amazon Basin will remain untouched, the studied basin still preserves healthy ecosystems, showing excellent environmental quality overall. This condition makes the basin a perfect candidate for serving as a reference basin for these endangered ecosystems. Schmitter-Soto et al. [9] investigate changes in a fish community during a long-term period, from 1999–2001 to 2015–2018. The results show that changes may be due to morphological changes in the channel. Still, other threats could condition these changes: illegal fishing outside the bay, and erosion in the innermost part, impacting native habitats. Li et al. analyze fish distribution, influencing factors, and habitat requirements in the East Tiaoxi River (a major tributary of the renowned Yangtze River). Results show differences in fish management and several severe threats to their conservation. Complete and continuous scientific research of fish diversity is crucial in order to develop efficient conservation and restoration plans in the Yangtze River Basin [4].

Several papers in this Special Issue explore, in more detail, applications of ecological and biological studies to improve monitoring of environmental changes and impacts on freshwater fishes. Sometimes biological studies permit us to detect reactions to environmental changes. Pastorino et al. [10] describe liver alterations observed in a bullhead population (*Cottus gobio* Linnaeus, 1758) from a mountain lake as an adaptation to extreme ecosystems and adverse conditions. Other studies highlight the interaction between ecological stress factors and biological traits and the use of sentinel species for the long-term monitoring of environmental status. This is the case with the study of Sánchez-Pérez et al. [11], where they analyze biological traits such as the growth, size structure, and somatic condition of the Southern Iberian barbel (*Luciobarbus sclateri* (Günther, 1868)) in a stressed Mediterranean river.

Some studies are focused on severely imperiled species, such as the Rio Grande Silvery Minnow (*Hybognathus amarus* (Girard, 1856)), catalogued as Endangered by the IUCN Red List of threatened species; the Spanish toothcarp (*Aphanius iberus* Valenciennes, 1846), catalogued as Endangered; or the Pyrenean Sculpin (*Cottus hispaniolensis* Bacescu-Master, 1964), included in the Spanish Catalogue of Threatened Species as Endangered [3,12,13]. Archdeacon et al. analyze the inefficiency of a specific conservation tool, the rescue of the Rio Grande Silvery Minnow during streamflow intermittency. Restoring natural flow regimes is the more effective action for species threatened by streamflow intermittency; re-establishment of the biological processes under which fishes evolved advances the conservation of this species [3].

Other species threatened by water quality have been studied in the Iberian Peninsula. Sgarzi et al. [13] examine abiotic and biotic factors that could influence the size structure and density of Spanish toothcarp in Mediterranean brackish ponds. They suggest that achieving a better pond ecological status may be necessary to conserve this endangered fish. Manubens et al. [12] describe the ex situ conservation plan for the endemic and rare Pyrenean sculpin (*Cottus hispaniolensis* Bacescu-Mester, 1964). The captive breeding process includes six consecutive phases: nesting behavior, courtship, egg fixation, parental care (incubation), hatching, and survival during juvenile development. The management plan implemented for this project has probably allowed the main impediments described in other similar programs to be largely overcome.

Conversely, some studies in this Special Issue are biological and ecological analyses of exotic invasive species, as the Pyrenean Gudgeon (*Gobio lozanoi* Doadrio & Madeira, 2004) in a Mediterranean river or the bleak (*Alburnus alburnus* (Linnaeus, 1758)) in the Iberian Peninsula. Both studies are focused on the significant role of plasticity in the success of these invasive alien species. Latorre et al. [14] assess the variability in dietary traits of the bleak in the Iberian Peninsula and compare the dietary characteristics of this species among the main Iberian rivers and a native bleak population from France. Similarly, Amat-Trigo et al. [15] evaluate other biological traits in addition to the diet. The results of both studies suggest that this wide interpopulation variability will contribute to the species' successful establishment throughout Mediterranean Europe, posing a severe risk to native fish fauna.

All the articles included in this Special Issue point in the same direction. The current status of freshwater fish must be brought to our attention in order to design and implement effective management measures to conserve freshwater fish. While we should maintain our interest in learning and know more about the conservation status of the imperiled freshwater fish, policy and administrations should invert inefficient plans in order to revert, or at least reduce, the current crisis in biodiversity.

A change in mindset is needed to protect biodiversity in the upcoming years. Conservation plans have failed because our current knowledge is deficient and needs to be improved. We need countries to commit to protecting biodiversity and develop realistic targets that can be met while compromising with conflicting needs and interests. The articles included in this Special Issue emphasize the necessity of having more knowledge to develop conservation strategies. Future conservation targets may be advanced in part based on the knowledge provided by these papers and similar studies to ensure the long-term protection of freshwater fish and other life forms [1].

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Article

Fish Ecology of the Alto Madre de Dios River Basin (Peru): Notes on Electrofishing Surveys, Elevation, Palm Swamp and Headwater Fishes

Ibon Tobes [1,2], Adrián Ramos-Merchante [3], Julio Araujo-Flores [4,5,6], Andrea Pino-del-Carpio [2], Hernán Ortega [7] and Rafael Miranda [2,*]

[1] Centro de Investigación en Biodiversidad y Cambio Climático (BioCamb), Facultad de Ciencias del Medio Ambiente, Universidad Tecnológica Indoamérica, Quito EC170103, Ecuador; ibonto-bes@uti.edu.ec
[2] Biodiversity Data Analytics and Environmental Quality Research Group, Department of Environmental Biology, University of Navarra, 31009 Pamplona, Spain; apino@alumni.unav.es
[3] Department of Integrative Science, University of Huelva, Campus Universitario El Carmen, Avda. Andalucí s/n, 21071 Huelva, Spain; ramosadrian24@gmail.com
[4] Centro de Innovación Científica Amazónica, Jr Cajamarca Cdra 1, Puerto Maldonado 17001, Peru; araujojm@wfu.edu
[5] Center for Energy, Environment, and Sustainability, Department of Biology, Wake Forest University, 1834 Wake Forest Rd, Winston-Salem, NC 27109, USA
[6] Colección Científica de Ictiología, Universidad Nacional Amazónica de Madre de Dios, Av. Jorge Chávez 1160, Puerto Maldonado 17001, Peru
[7] Departamento de Ictiología, Museo de Historia Natural de la Universidad Nacional Mayor de San Marcos, Lima 15072, Peru; hortega.musm@gmail.com
* Correspondence: rmiranda@unav.es; Tel.: +3448-425600

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Abstract: Our study analyzes the distribution of fish communities related to the environmental variables of the Alto Madre de Dios River, an Andean-Amazon watershed of southern Peru, between 300 and 2811 m a.s.l. within the Manu Biosphere Reserve. We provide new ecological and diversity data on fishes for these poorly studied rivers and new data for palm swamp habitats. With electric fishing techniques, we collected a total of 1934 fish specimens belonging to 78 species, 42 genera and 15 families. To assess main patterns of diversity we combined SIMPER and ANOSIM with canonical correspondence analysis to obtain an overview of the community structure of fish and their distribution related to aquatic habitats. Our results show an important shift on fish diversity at 700 m a.s.l. separating headwater and middle-lowland communities. Electrofishing was a hindrance due to the depth, flow and low conductivity of the rivers, but also allowed us to capture fish not observed with other techniques. We also compared the use of elevation with slope as an alternative variable for statistical analysis. Our results show that slope offers a solid and equivalent explanation for fish distribution variability, avoids redundance, and instead of giving geographical data offers ecologically solid information.

Keywords: Tropical Andes; Manu Biosphere Reserve; *Astroblepus*; *Trichomycterus*; *Mauritia flexuosa*

1. Introduction

Freshwater ecosystems are often referenced among those that are most altered and threatened by anthropogenic impacts [1] and their fauna is at greater risk than any other animal and plant groups [2]. In this respect, precise knowledge of ecological aspects like species distribution and requirements are a key point for conservation strategies, especially when the focal species are threatened or endangered [3]. Unfortunately, our limited taxonomic knowledge and incomplete information on species distributions for broad territories is very high and represents an insurmountable obstacle for documentation of imperilment and extinction of freshwater biodiversity [4]. In particular, fish assemblage variations in mountain streams of the Andes are poorly understood [5,6].

The Tropical Andes is regarded as the richest of the 25 recognized global biodiversity "hotspots". It includes Andean ecosystems above 500 m a.s.l., extending from Chile and Argentina, through Bolivia, Peru, Ecuador, to Colombia and Venezuela. This hotspot supports an estimated 45,000 plant and 3400 vertebrate species (excluding fishes), which represents about 15% and 12% of all globally known species, respectively, being nearly half of them endemic to the area [7].

Peru is home to 1064 fish species [8], more than the 7% of all the globally known freshwater species. Most of them (more than 800) are found in the Amazonian Basin. Only during the first decade of the XXI century, 155 new species were named in Peru and the experts estimate the total number of Peruvian freshwater fishes at approximately 1200 species [8]. However, as seen before, there have been few ecological surveys focusing on fish and the majority point out the uneven level of knowledge for this group of vertebrates [9,10], with some taxa itemized at the species level, whereas others routinely are catalogued at family level, order or even phylum [4].

Reviewing research on the Department of Madre de Dios where our study was carried out (Figure 1), Pitman et al. [11] reported that all the scientific studies generated for the department found that only 2.8% of the analyzed manuscripts focus on fish or hydrobiology. Only lichens received less attention than fish. Other research works regarding plants or mammals comprise 21.3% and 16.6% of the total scientific literature, respectively.

Streams of the Tropical Andes are also ecologically important as the headwaters of the megadiverse lowland river systems in South America [12]. They are responsible of delivering major loads of inorganic sediment and organic carbon to lowlands [13] and play a key role in the ecological processes along the Andes-to-Amazon fluvial continuum [14]. Furthermore, the Andean flank of the Amazon hosts the highest biodiversity rates and has been least affected by historical climate variability and land use [15].

However, the Andean-Amazonian piedmont is a rapidly changing landscape, part of the "arc of deforestation" [16], caused foremost by the expansion of cattle and soybean production [17]. The recent growth of human populations, the exploitation of natural resources and the proliferation of hydroelectric dams are leading to extensive reductions in habitats and subsequent impacts on rivers [18–20]. Fortunately, many opportunities for protecting these habitats yet exist, particularly in Peru, where entire river systems are still relatively intact and where there are few large dams and other major structural changes to river channels [21].

Our study area, the Alto Madre de Dios River Basin, in the south west of Peru, is an almost pristine basin. It is located in the Andean-Amazonian piedmont, in the transition zone of the Manu Biosphere Reserve, bordered on the northwest by the Manu National Park, and on the southeast by the Amarakaeri Communal Reserve, two important protected areas of the Peruvian Amazon. This region has long been known to tropical biologists as a region high in species diversity, as well as some of the world's largest expanses of pristine tropical forest [22].

Given the pessimistic predictions for the sustainability and conservation of the Andean biodiversity due to environmental alterations [23,24], studies on the relationships among biotic and abiotic factors and the abundance and distribution of Andean fish are critical to document the primary characteristics of the original communities and thereby contribute to the delimitation of appropriate conservation areas and/or to recovery strategies.

One of the most frequent variables used for studying ecology and biodiversity distribution patterns is elevation. Ecologist all around the globe have demonstrated the strong correlation between altitude and changes in community composition, for plants [25], insects [26], amphibians [27], birds [28], and other organisms. Elevation is also commonly used for studying freshwater biodiversity distribution patterns along the watersheds [29], and together with the distance to the mouth or the distance to the source, they are among the most repeated variables for analyzing fish distribution patterns [10,30]. Nevertheless, the use of those geographical variables can be problematic. We aspire to discuss it and evaluate the use of slope as an alternative environmental variable.

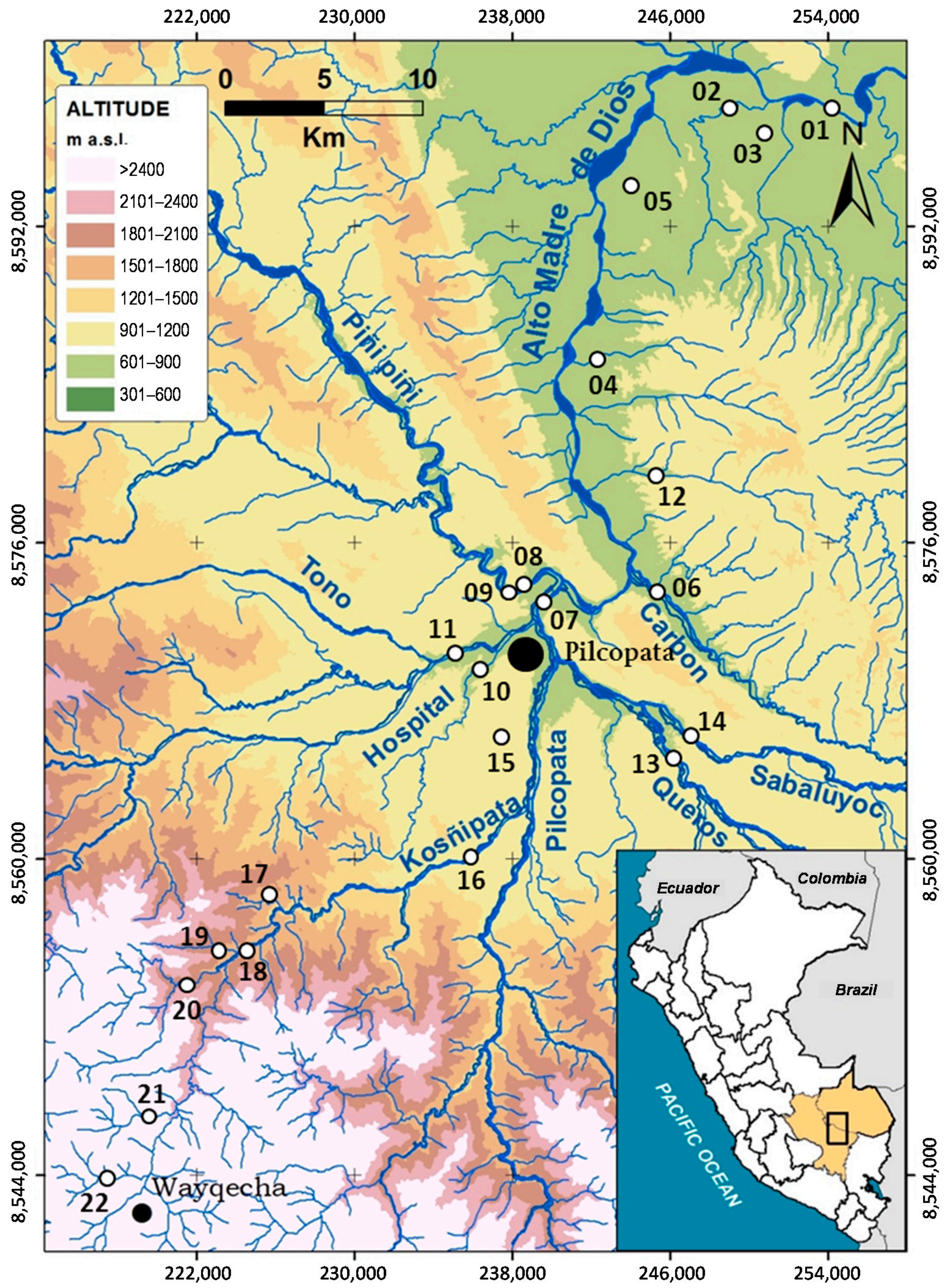

Figure 1. Sampling sites in the Alto Madre de Dios River, Peru (white circles).

In the present study we examine the freshwater ecology for the Alto Madre de Dios River focusing on its ichthyofauna and evaluate the environmental integrity of the studied ecosystems. Our specific objectives are to (1) describe spatial variation of fish assemblage of an unstudied Andean-Amazonian stream, (2) to identify patterns of association between fish assemblages and habitat variables, and to (3) discuss the use of elevation for ecological and fish distribution analysis.

2. Materials and Methods

2.1. Study Area

The Manu National Park has been considered one of the world's most important tropical protected areas since its creation in 1973 (Shepard et al., 2010). Located in the southern Peruvian Amazon rainforest constitutes the core of the Manu Biosphere Reserve and an IUCN World Heritage Site [31]. The southern buffer zone of Manu Biosphere Reserve includes the Alto Madre de Dios basin (11°00′–13°30′ S, 73°30′–68°30′ W) (Figure 1). The river flows, south to north, for 275 km through the rainforest of Cusco and Madre de Dios Departments, draining an area of approximately 1600 km^2. The altitude in the basin varies from up to 3500 to 300 m a.s.l. in the Manu River junction, spanning five Andean vegetation zones: puna (4500–3500 m), upper cloud forest (3500–2500 m), lower cloud forest (2500–1000 m), piedmont (1000–400 m) and lowlands (400–50 m) [32]. Our highest sampling site was located at 2411 m a.s.l. while to lowest was at 398 m a.s.l., 80 kms downstream. Rivers flow through deep and narrow valleys and steep slopes from the source to around 700 m a.s.l., where the valleys open to wide and flat lowland floodplains. Flow regime is highly responsive to rainfall and presents the greatest discharge period from November to April, decreasing progressively in the dry season (from May to October). The rivers remain turbid through the year, with highest sediment loads during the rainy season.

Human population density in the Alto Madre de Dios valley is low, with scattered settlements that are under the administration of the village of Pilcopata in the District of Kosñipata (pop. 4790 in 2007, according to the Statistical National Institute of the Peruvian Government), Department of Cusco.

Nowadays, most of the land is still covered by primary Amazonian forest, although some areas have been deforested and are used for cattle raising. Tourism facilities—lodges and a network of forest trails—are used by a relatively low number of tourists in comparison with other lodges in Madre de Dios area [22].

2.2. Field Sampling and Analysis

Our survey was conducted on twenty-two sampling sites in June 2012, during the dry season (Figure 1). The following environmental variables were recorded at each site using a multiparametric probe (Hanna Instrument, HI 98129 Combo Waterproof, Woonsocket, RI, USA): conductivity (µS/cm), water temperature (°C), and pH. Characterization of habitat structure was done using transects according to Armantrout (1998), and included depth (m), width (m), water velocity ($m \cdot s^{-1}$), percent tree canopy shading and dominant substrate categorized as fines (<2 mm), gravels (~2–64 mm), pebbles (~64–256 mm), boulders (>256 mm), or bedrock and concrete. Additionally, two habitat quality indices were measured: Qualitative Habitat Evaluation Index—QHEI (Rankin, 1989), and Andean adapted version of the riparian forest quality index QBR (Acosta et al., 2009) (Table 1). QHEI jointly considers different habitat parameters, such as bottom substrate and embeddedness, instream structure, velocity and depth regime, canopy cover, channel alteration and pool/riffle or run/bend ratios. QBR index includes aspects of the riparian forest such as total riparian vegetation cover, cover structure, cover quality and channel alterations.

Fish sampling was carried out in wadeable stream stretches (<1 m of height) by electrofishing surveys, using a backpack electrofishing gear (Hans Grassl model IG200/2D, 300–600 V, 0.2–2 A). Surveys was conducted following a single-run depletion methodology and estimating fish abundance based on catch per unit effort (CPUE) (Meador, McIntyre & Pollock, 2003). Collected fish were anesthetized and subsequently counted, measured to the nearest 0.1 cm total length (TL), weighed with a digital scale to an accuracy of 0.05 g and released after the survey, except for some voucher specimens kept to confirm identification.

Voucher specimens were deposited in the fish collection of the Natural History Museum of National University of San Marcos (Lima, Peru). In the laboratory, fish were preserved in alcohol (75%) and identified by Ana María Cortijo, Jessica Espino and Hernán Ortega, members of the Department of Ichthyology of Museum. Scientific names were validated according to W. N. Eschmeyer's Catalog of Fishes [33].

Table 1. Sampling sites and environmental variables in Alto Madre de Dios River, Peru.

Code	River Place	Date	Alt	pH	Ta	Tw	Cd	Wh	Dh	Vc	QBR	QHEI
01R	Aguas Calientes	13 June 2012	398	7.95	24.3	21.7	105	18.2	25.1	0.51	100	72
02R	Shintuya	12 June 2012	424	8.07	24.4	24.4	99	11.2	13.7	0.23	90	72
03A	Anaconda	13 June 2012	428	7.37	26.4	21.4	119	3.45	16.8	0.05	95	61
04Q	Mascuitania	14 June 2012	459	6.82	29.0	25.4	44	7.86	25.6	0.25	85	67
05Q	Puente a km 2,5	14 June 2012	462	8.03	26.1	22.4	169	5.54	18.7	0.15	100	74
06R	Carbón	11 June 2012	499	8.78	26.3	25.3	102	14.4	34.5	0.41	100	85
07R	Pilcopata	10 June 2012	511	6.94	24.8	25.0	119	15.7	15.6	0.04	100	74
08R	Piñi-Piñi	7 June 2012	518	6.86	22.3	22.0	131	6.86	44.1	0.19	100	88
09Q	Villa Carmen	7 June 2012	518	7.20	22.3	20.6	126	4.51	5.11	0.13	100	62
10R	Hospital	8 June 2012	547	7.63	23.1	19.9	26	28.3	36.6	0.74	100	84
11R	Tono	8 June 2012	551	7.70	19.1	18.5	50	6.94	22.1	0.30	100	68
12R	Salvación	11 June 2012	558	7.26	24.0	23.2	33	4.42	30.1	0.39	100	66
13R	Queros	9 June 2012	587	8.00	20.0	18.3	61	10.4	39.5	0.31	80	82
14R	Sabaloyacu	9 June 2012	593	7.70	19.7	21.2	44	22.9	31.0	0.43	100	87
15A	Aguajal	17 June 2012	622	5.77	23.9	22.0	26	2.49	57.4	0.04	50	63
16R	Asunción	10 June 2012	720	7.40	26.4	19.3	65	5.16	19.9	0.32	100	78
17Q	Quitacalzon	16 June 2012	1064	7.83	20.9	18.7	52	4.07	31.4	0.45	100	88
18R	Kosñipata	16 June 2012	1347	7.53	22.2	16.0	89	6.42	24.4	0.15	95	70
19R	San Pedro	16 June 2012	1394	7.81	20.1	18.5	43	7.74	49.4	0.48	95	88
20R	Unión	17 June 2012	1673	7.72	20.2	15.3	64	8.41	32.0	0.72	75	66
21Q	Wayquecha	18 June 2012	2218	7.40	17.3	11.4	51	3.99	30.2	0.83	100	84
22Q	Pacchayoc	18 June 2012	2411	4.41	16.1	11.7	188	6.11	23.9	0.89	100	82

Alt: Altitude (m), Ta: Air temperature (°C), Tw: Water temperature (°C), Cd: Water conductivity ($\mu S \cdot cm^{-1}$), Wh: Mean width (m), Dh: Mean depth (cm), Vc: mean water velocity ($m \cdot s^{-1}$), QBR: riparian forest quality index and QHEI: qualitative habitat evaluation index.

2.3. Data Analysis

From fish community compositional data, richness (Margalef's index $R = (S - 1)/\ln N$), and diversity (Shannon–Wiener index $H' = -\sum pi \log^2 pi$, and Simpson's index $D = \sum pi^2$) measures were calculated, where pi is the proportion of species "i" at a given site, N is the total number of collected specimens, and S is the number of species [34].

First, polynomial regressions were set among the mentioned indices and elevation, distance to the mouth or slope. We wanted to describe and compare patterns of diversity considering these three variables related to location along the basin.

Second, several multiparametric statistical approaches were used to establish the main spatial patterns in the fish community composition [35]. To detect patterns among fish communities, we used a permutation-based test with a nonparametric one-way analysis of similarity (ANOSIM) using a Bray–Curtis similarity index based on abundance data [36,37]. The ANOSIM statistic compares the mean of ranked dissimilarities among groups to the mean of ranked dissimilarities within groups. An R-value close to 1 suggests dissimilarity among groups while an R value close to 0 suggests an even distribution of high and low ranks within and among groups. Post-hoc tests with Bonferroni correction in *p*-values (which were multiplied by the number of comparisons) are done. This correction is very conservative (produces large *p*-values). The sequential Bonferroni option does not output corrected *p*-values, but significance is decided based on step-down sequential Bonferroni, which is slightly more powerful than simple Bonferroni. If ANOSIM revealed a statistically significant result, the relative contribution of each species to group dissimilarities was quantified using a similarity percentage analysis (SIMPER), with a cut-off criterion of 90% (Bray–Curtis similarity index), to identify subsets of the most important species [36]. SIMPER calculates the average Bray–Curtis dissimilarity among all pairs of inter-group samples, expressed in terms of the average contribution from each species. Previously, we created three categorical variables (type of mass of water (river vs. stream), elevation (upstream-downstream 700 m), and stream order). Fish assemblages were compared considering these three categories.

For the choice of linear or unimodal analysis, a preliminary DCA (Detrended Correspondence Analysis) was performed [38]. This analysis evaluates the species turnover (the length of gradients) through the first DCA axis. The criterion for choosing linear vs. unimodal ordinations models is to obtain a $SD < 3$ [39]. Our results showed a $SD > 3$

because of the large number of species and their frequency of occurrence. To examine relationships between community composition and several environmental variables a Canonical Correspondence Analysis (CCA, Unimodal response; length of gradient SD > 3) was used through the program CANOCO 4.5 [39,40] based on DCA results. To reduce the number of graphics and considering the similar patterns shown, altitude was selected over distance to mouth ($r = 0.964$, p-value < 0.01) as representant of the geographical variables, to compare it with slope. Two parallel CCA were carried out with altitude or slope as key variables related to location along the headwater-downstream gradient. This direct gradient analysis technique provides ordination axes linearly related to the explanatory variables. The main result is a scatterplot ordination diagram displaying the pattern of fish assemblage variation along the environmental variables shown as vectors. Vector direction and length indicate the relative magnitude and influence of a particular variable on fish assemblages. The significance of the analyses was assessed by a permutation test with 1000 random permutations. Habitat structure and water quality variables were also included in the analysis. To improve linearity, environmental variables were log transformed. Proportional data and abundance fish data were transformed (arcsine and root transformed, respectively). Peebles was removed to avoid a high variance inflation factor (VIF) [41].

All multiparametric analysis were done for species that were captured in more than two sites (i.e., 10% occurrence) and were performed using the R project software version 3.2.3 [42] with the package "vegan" version 2.3–3 [43] except in the case of CCA which had been developed in CANOCO 4.56 [40].

Finally, fish species have been classified according to their trophic specialization [6,44,45], with the aim to analyze the distribution of these trophic groups in the elevation gradient.

3. Results

A total of 1934 specimens were collected, and 78 fish taxa were identified belonging to 43 genera and 14 families (Dataset S1). Twenty-seven species were undetermined, ten were identified as species *affinis*, and 41 species were correctly identified. Characids were the most diverse family with 43% of the species, followed by loricarids (14%), trichomycterids (8%), astroblepids, cichlids and heptapterids (7% each one) and crenuchids (3%). The remaining fish belonged to ten families with only one represented species.

Regarding diversity indices, values presented a marked altitudinal pattern. Shannon–Wiener and Margalef's indices showed negative correlations with altitude, distance to the mouth and slope, whereas Simpson's index showed positive correlation (Figure 2). Site 13 presented the highest diversity and hosted alone 28% of the total fish species (22 species). On the other hand, highest sites, 21 and 20, only had two species (*Astroblepus* sp. and *Trichomycterus* sp.). Finally, site 15 significantly different from the rest of the sampling points. It was a swamp ecosystem isolated from the main river and due to its unique environmental characteristics, it was discarded for subsequent analysis.

Results of one-way ANOSIM showed significant differences in community composition as indicated the high R values (global R: 0.786, $p < 0.001$) with the cut-off point at 700 m a.s.l. This makes fish composition upstream and downstream 700 m a.s.l. well distinguishable. Mass of water and stream order showed not significant differences (global R: 0.04 and R: 0.22, respectively; $p > 0.05$). The SIMPER procedure indicated that using three species was possible to explain more than 38% of variation among elevation categories. *Astroblepus*, *Trichomycterus* and *Knodus* were the most important species explaining the variation in community composition (Table 2).

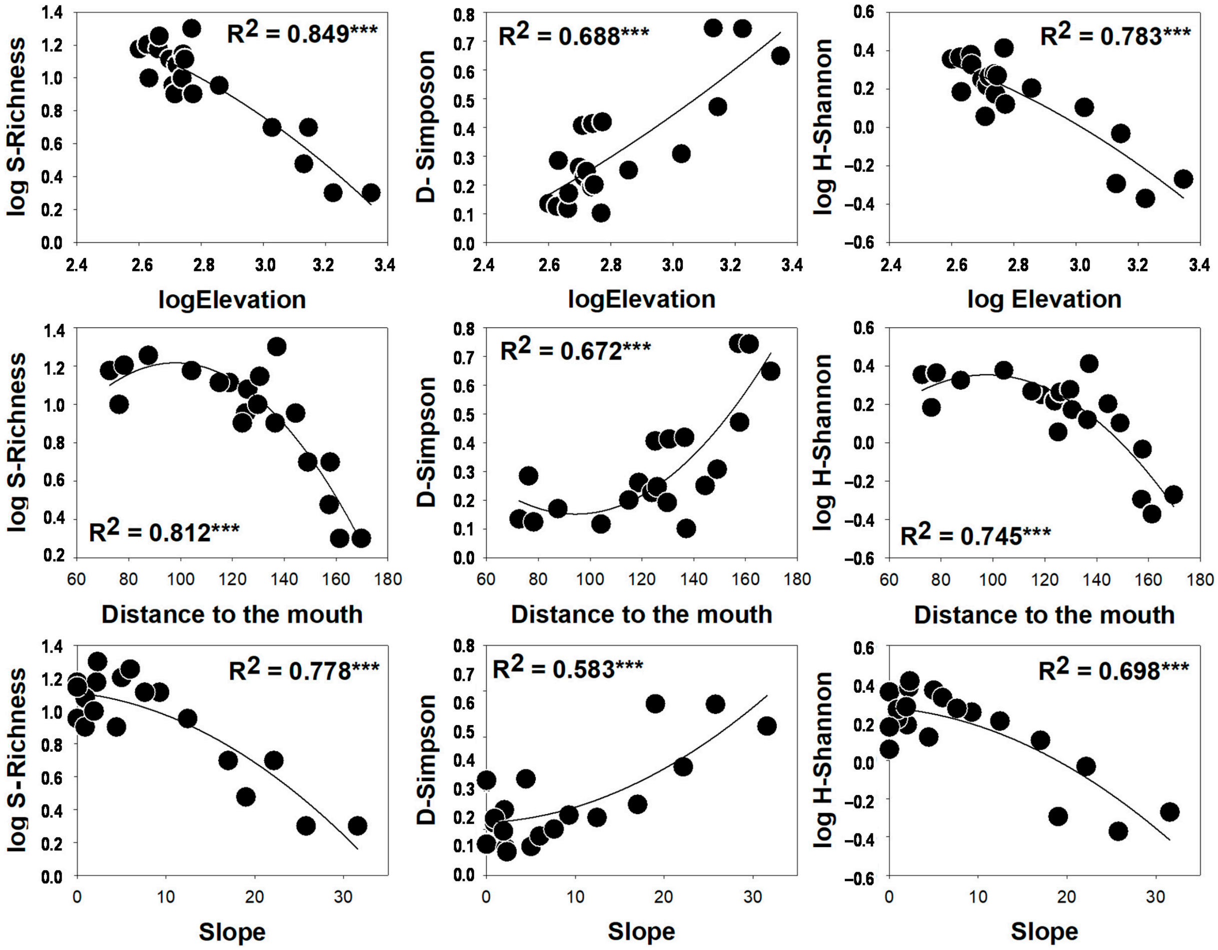

Figure 2. Estimates of the fish community diversity indices along the Alto Madre de Dios River, regarding to elevation, distance to the mouth and slope of sampling points. *** significative coefficient of determination (R^2).

Table 2. Relative abundance of taxa presents under (U) and above (A) 700 m of elevation. Taxa are ordered by their overall weight in the SIMPER analysis (taxa showed with a cut-point of 90% of contribution). Percent of contribution of each taxon accord to SIMPER is show for each group (%contri). In the last row richness of each category of groups are shows.

		Elevation-700 m	
Taxa	**% Contri.**	**U**	**A**
Astroblepus	18.36	1.03	9.00
Trichomycterus	10.76	0.78	5.78
Knodus	8.91	3.92	-
Chaetostoma	7.67	3.54	1.93
Creagrutus	6.35	3.09	-
Bryconamericus	6.01	1.84	2.64
Ceratobranchia	5.88	3.22	-
Astyanax	5.26	2.45	-
Ancistrus	4.57	2.48	0.28
Bujurquina	3.42	1.73	-
Hemibrycon	3.36	1.63	-
Characidium	3.01	1.33	0.28
Aphyocharax	2.67	1.52	-
Rineloricaria	2.36	1.05	-
Hoplias	1.96	0.87	-
Richness		24	6

The relationship between fish and environmental variables are shown in Figure 3 and Tables 3 and 4. The importance of environmental variables is indicated by the marginal effect values (λ_A): for both analysis conductivity (0.22), boulders (0.25), water velocity (0.25) and water temperature (0.38) were most significant. The variables with the highest marginal effect were slope (0.44) for the first CCA (Table 3a) and elevation (0.59) for the second CCA (Table 3b).

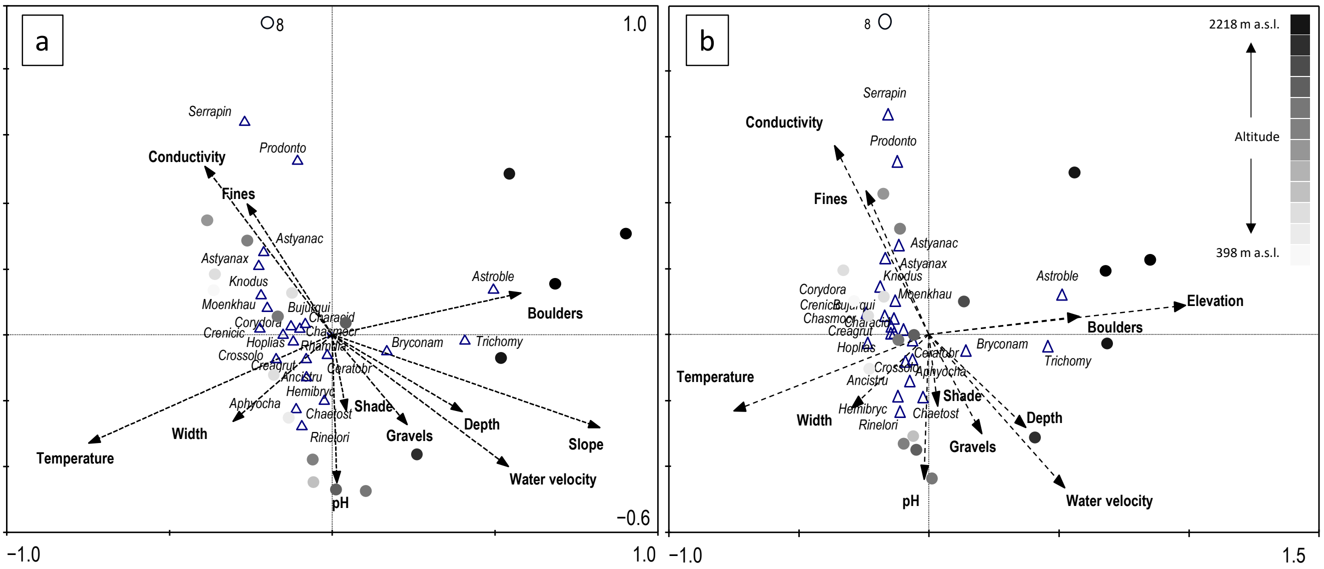

Figure 3. Triplot of results of canonical correspondence analysis carried out with slope (**a**) or elevation (**b**) as key variables related to location, showing site scores (circles), the environmental variables (vectors) and fish abundances (triangles) on the first two canonical axes. The code for site scores and environmental variables refers to sampling sites according to Table 1.

Table 3. Results of canonical correspondence analyses (CCA) carried out with slope (**a**) or elevation (**b**) as key variables related to location along the headwater-downstream gradient, showing canonical coefficients and weighted intraset correlation coefficients of explanatory variables with the first two axes of the CCA. Importance of environmental variables using marginal effects are shown λ_A.

(a) Slope	Marginal Effects	Intraset Correlations		Regression/Canonical Coefficients	
Groups	λ_A	**CCA1**	**CCA2**	**CCA1**	**CCA2**
Slope	0.44	0.786	−0.261	0.755	−0.114
Water temperature	0.38	−0.715	−0.304	−0.418	−0.433
Water velocity	0.25	0.517	−0.370	0.047	0.002
Boulders	0.25	0.554	0.118	0.006	0.111
Conductivity	0.22	−0.374	0.472	0.007	0.987
Depth	0.19	0.382	−0.216	−0.076	0.377
Fines	0.15	−0.250	0.368	−0.149	0.282
Width	0.14	−0.291	−0.243	−0.132	0.064
pH	0.13	0.014	−0.415	−0.090	−0.662
Gravels	0.07	0.220	−0.252	0.018	0.236
Shade	0.07	0.041	−0.214	−0.266	−0.289
(b) Elevation	**Marginal Effects**	**Intraset Correlations**		**Regression/Canonical Coefficients**	
Groups	λ_A	**CCA1**	**CCA2**	**CCA1**	**CCA2**
Elevation	0.59	0.963	0.081	1.069	0.077
Water temperature	0.38	−0.730	−0.213	0.189	−0.330
Water velocity	0.25	0.510	−0.427	0.074	−0.032
Boulders	0.25	0.563	0.051	0.091	−0.010
Conductivity	0.22	−0.354	0.526	−0.068	1.064
Depth	0.19	0.364	−0.259	−0.080	0.402
Fines	0.15	−0.236	0.400	0.045	0.287
Width	0.14	−0.289	−0.205	−0.084	0.184
pH	0.13	−0.018	−0.403	0.164	−0.787
Gravels	0.07	0.198	−0.276	−0.035	0.261
Shade	0.07	0.034	−0.199	−0.083	−0.250

Table 4. Summary statistics table for CCA ordination presented, with slope (**a**) or elevation (**b**) as key variables.

(a) Slope	**CCA Axis**	**1**	**2**	**3**	**4**
Eingenvalues		0.579	0.264	0.167	0.126
Species-environmental correlations		0.956	0.924	0.884	0.950
Cumulative percentage variance					
of species data		27.9	40.6	48.7	54.7
of species-environmental relation		38.4	55.9	67.0	75.4
(b) Elevation	**CCA Axis**	**1**	**2**	**3**	**4**
Eingenvalues		0.597	0.263	0.170	0.132
Species-environmental correlations		0.976	0.918	0.903	0.931
Cumulative percentage variance					
of species data		28.8	41.5	49.7	56.0
of species-environmental relation		38.8	55.9	67.0	75.5

For (**a**) Significance of the axis by the Monte Carlo permutation test is given by F = 3.10 ($p < 0.05$). All canonical axes were significant. Values in bold indicate significant difference at $p < 0.05$. For (**b**) Significance of the axis by the Monte Carlo permutation test is given by F = 3.23 ($p < 0.05$). All canonical axes were significant. Values in bold indicate significant difference at $p < 0.05$.

Outcomes were equivalent using slope and elevation as main variables, therefore, only results for CCA with slope are described next. The inertia in the assemblage composition in the studied sites was 0.579 for axis 1, 0.264 for axis 2, 0.167 for axis 3 and 0.126 for axis 4. The CCA ordination revealed strong relationships between species abundances and measured environmental variables explaining 72.6% of species distribution. The first canonical axis (axis 1) accounted for 38.4% of the variation in the data set, the second axis (axis 2) accounted for 17.5% of the variation in the data set. An unrestricted Monte Carlo permutation test indicated that all canonical axes were significant ($p < 0.05$).

First Canonical Correspondence Analysis CCA1 eigenvalue accounts 0.579 and it is composed by slope, boulders and water velocity on the positive side and water temperature on the opposite side (Figure 3a). First Canonical Correspondence Analysis was interpreted as a longitudinal gradient from high to lowlands with sites above 700 m a.s.l. being distributed on the right area of the graph (except for site 12, slightly right from the axis) and sites below that altitude spread in the left area. Second Canonical Correspondence Analysis CCA2 (eigenvalue = 0.264) is dominated by conductivity on the positive side and pH and water temperature on the negative side. Second Canonical Correspondence Analysis was interpreted as hydrochemical parameters (Table 3a; Figure 3a).

On the right area of the graph (Figure 3a) a first species group composed by *Trichomycterus* (Figure 4) and *Astroblepus* genera (Figure 5) was separated from the remaining and it was positively related to headwaters showing distinctive features: high velocities of water, big boulders and lower temperatures. *Bryconamericus* genera species were also related to these headwater streams, although they do not appear so high in the altitudinal gradient. A second group of fish represented by *Serrapinnus*, *Prodontocharax*, *Astyanax*, *Astyanacinus* and *Knodus* genera, located on the top-left area of the graph, was positively related to still waters with low velocities, higher conductivities and fine substrates (mainly sands). A third cluster, located on the top-left area of the graph, included *Rineloricaria*, *Chaetostoma*, *Aphyocharax*, *Hemibrycon*, *Ancistrus*, *Creagrutus* and *Crossoloricaria*. They were related to open places with highest water temperatures and wide riverbeds. The rest of the genera were distributed between these two groups along the first axis on the left of the graph, including *Hoplias*, *Rhamdia*, *Chasmocranus* and *Characidium*.

Attending to fish distribution and trophic specialization [6] along the altitudinal gradient (Figure 6), no fish were found above 2200 m a.s.l. *Astroblepus* and *Trichomycterus* were the only genus found on highest reaches (above 1400 m a.s.l.), feeding exclusively on invertebrates. Even though they appear tightly related to headwaters, they were found all along the basin with occasional and scarce occurrence also in lowland streams. At 1400 m a.s.l. three new genera appeared: one invertivorous *Bryconamericus*, and the first two herbivore-detritivores *Chaetostoma* and *Ancistrus* genera. Next invertivorous *Characidium* genus was found below 1100 m. Above the 700 m other three invertivorous genera were found: *Rhamdia*, *Creagrutus* and *Chasmocranus*. The rest of the fish taxa were found below 622 m, representing most of the diversity. The first piscivorous species, *Hoplias malabaricus* and *Crenicichla semicincta* were found at this altitude.

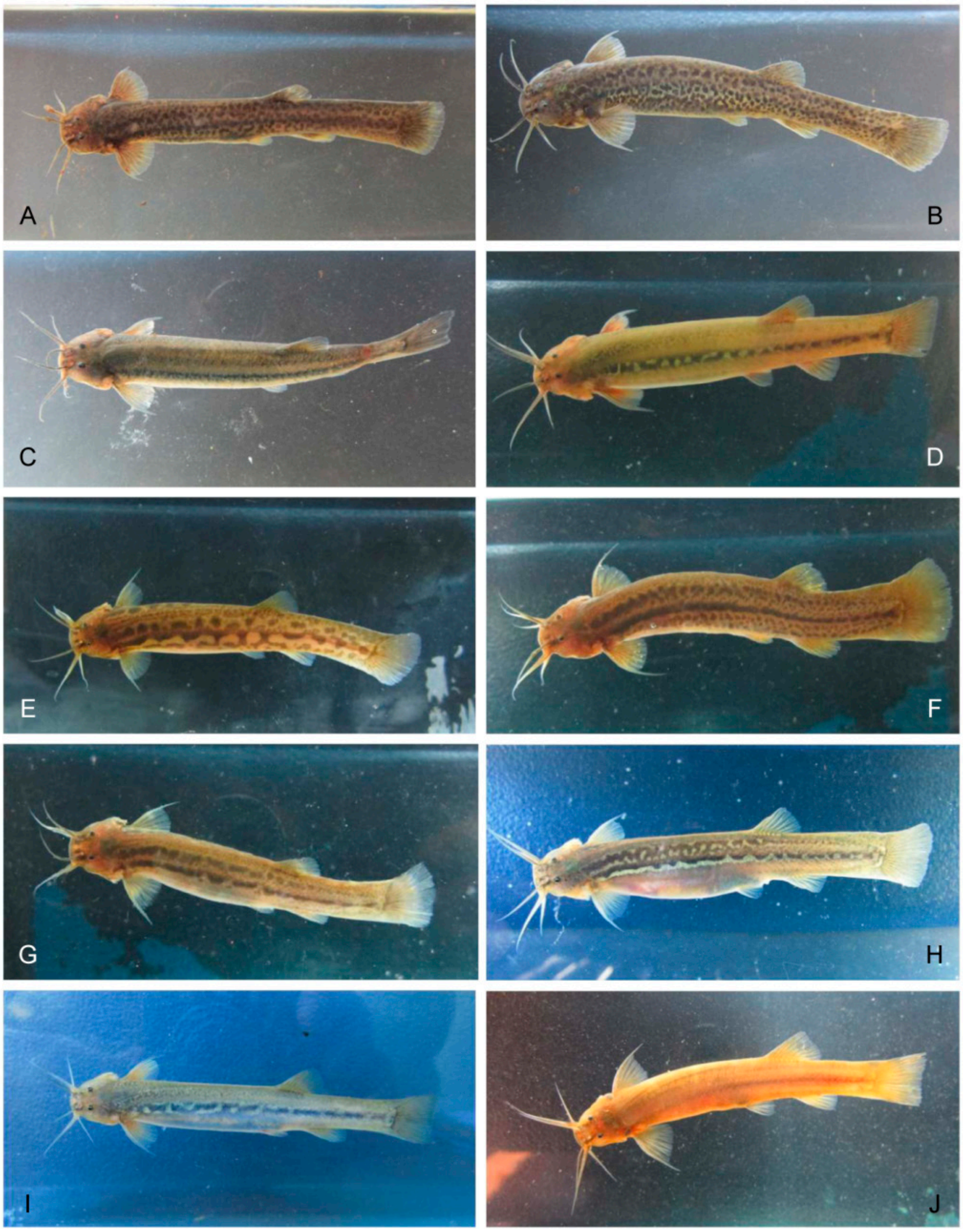

Figure 4. Species and morphotypes of genus *Trichomycterus* collected in the upper Madre de Dios River. (**A**,**B**) *Trichomycterus* sp.3 collected on Américo stream (21Q), (**C**) *Trichomycterus* sp.2 collected on Unión River (20R), (**D**) *Trichomycterus* sp.2 collected on San Pedro River (19R), (**E**) *Trichomycterus* sp.3 collected on Quitacalzon stream (17Q), (**F**,**G**) *Trichomycterus* sp.5 collected on Quitacalzon stream (17Q), (**H**) *Trichomycterus* sp.4 collected on Salvación River (12R), (**I**) *Trichomycterus* sp.6 collected on Kosñipata River (16R), and (**J**) *Trichomycterus* sp.1 collected on Queros River (13R).

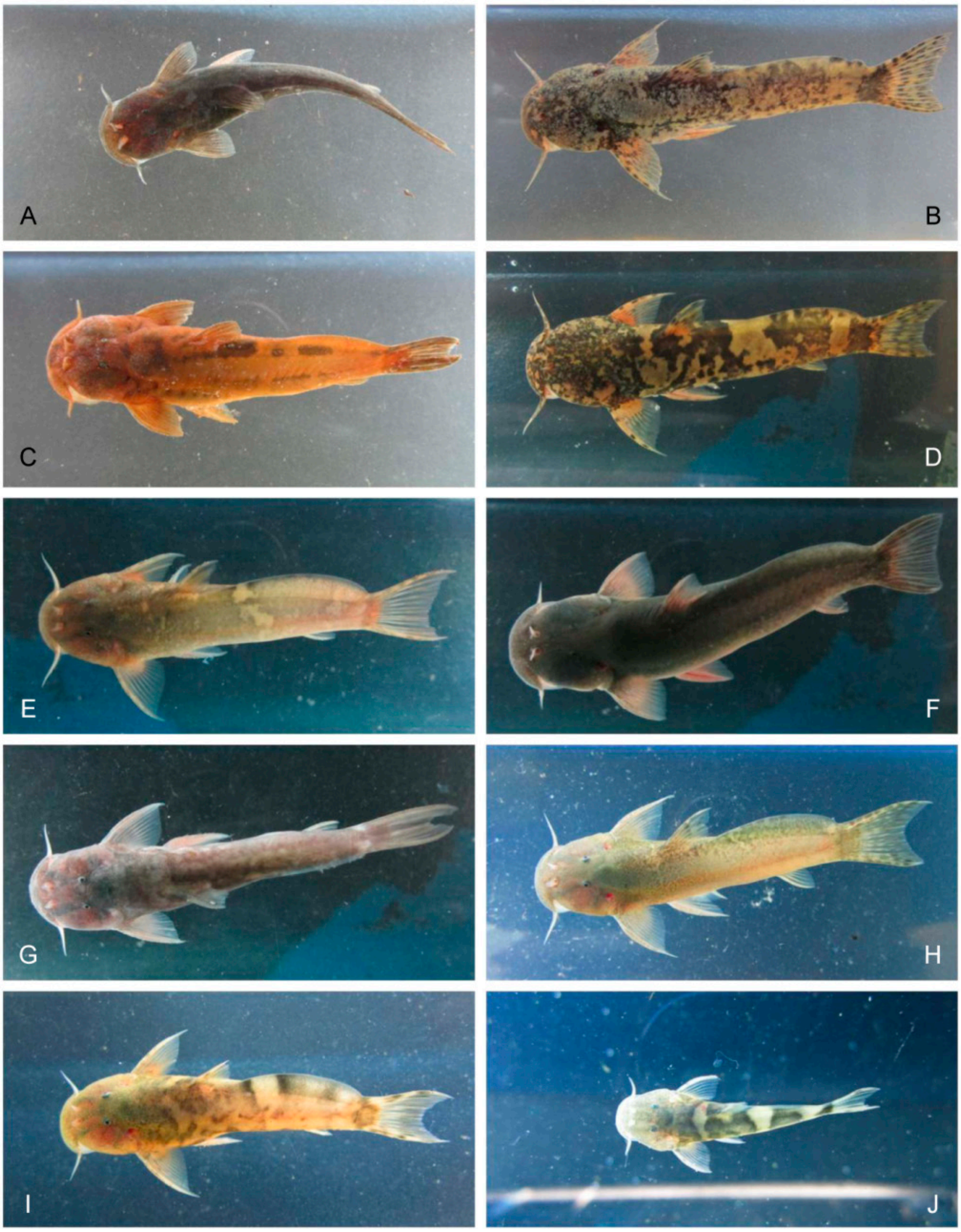

Figure 5. Species and morphotypes of genus *Astroblepus* collected in the upper Madre de Dios River. (**A**) *Astroblepus* sp.2 collected on Américo stream (21Q), (**B**) *Astroblepus* sp.1 collected on Unión River (20R), (**C**) Reddish specimen of *Astroblepus* sp.1 collected on Unión River (20R), (**D**) *Astroblepus* sp.1 collected on San Pedro River (19R), (**E**) *Astroblepus mancoi* collected on San Pedro River (19R), (**F**) *Astroblepus* sp.2 collected on San Pedro River (19R), (**G**) *Astroblepus* sp.1 collected on Quitacalzon stream (17Q), (**H**) *Astroblepus aff. longifilis* collected on Kosñipata River (16R), (**I**) *Astroblepus mancoi* collected on Queros River (13R), and (**J**) *Astroblepus aff. trifasciatus* collected on Salvación River (12R).

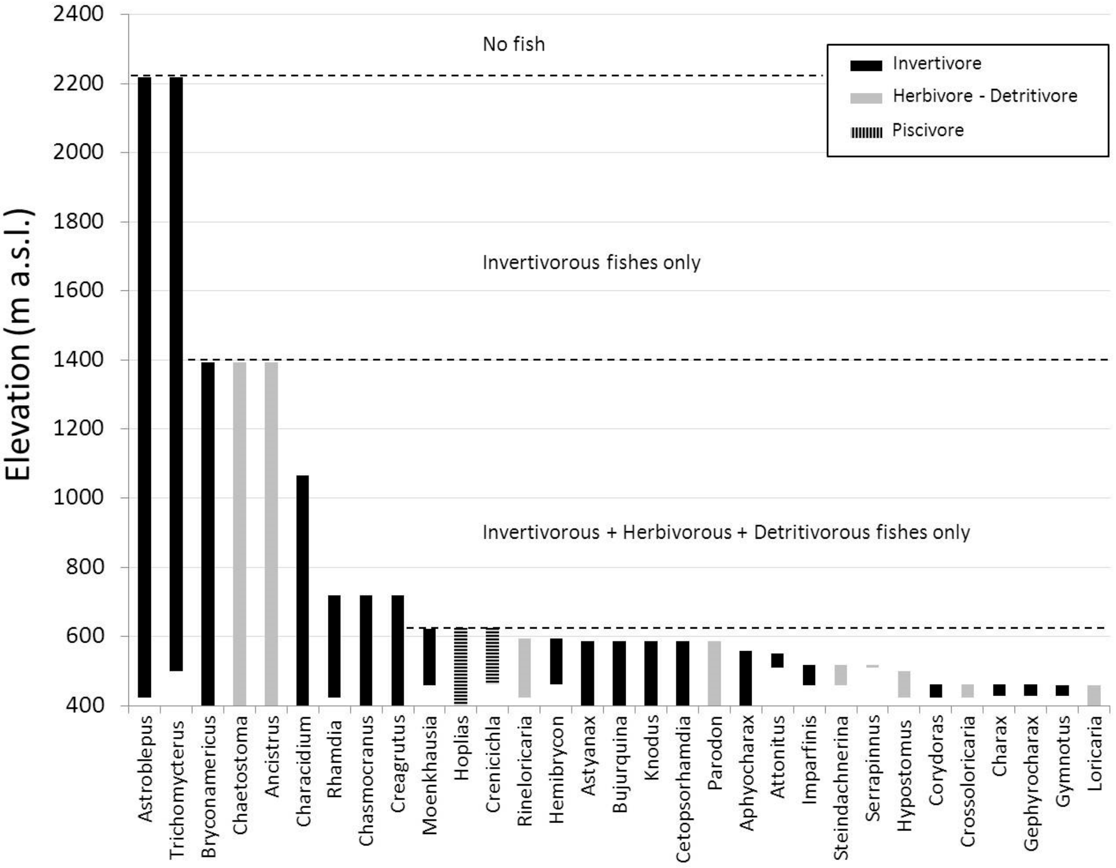

Figure 6. Elevational ranges and trophic specializations of fish species occurring at elevations >400 m a.s.l. in tributary and main-channel sites within the Alto Madre de Dios watershed, south-eastern Peru.

4. Discussion

A gradual increase in species richness and diversity is expected along longitudinal gradients in lotic systems, associated with changes in resource availability, channel morphology, flow regime and substrate type [46,47]. Addition of species is usually related to an increase of habitats motivated for the structural diversification of the environment downstream [48]. Our study confirms this pattern (Figure 2), with the only exception of one sampling point 15, that does not fit the trend due to its low diversity values, explained by its peculiar features as discussed below.

All the studied environmental variables were correspondent with the values reported by other works for natural and unaltered streams of this territory [6,30,49]. Quality indices of riparian and fluvial habitat (QBR and QHEI) got very high scores in all the sampling points, highlighting the good environmental quality, with some remarkable exceptions (Table 1). Riparian forest (and adjacent jungle) on the right bank of the sampling point 13, in the Queros River, was lacking due to deforestation for extensive cattle raising.

Besides, sampling point 15, located at 622 m a.s.l., showed significant differences both in environmental and diversity features, as already mentioned. This site belongs to a special type of aquatic ecosystem of Amazon forest known as *aguajal*. They are back swamp forests, defined as forest on low-lying areas outside of streams courses, only connected to them during high flood season. They are usually dominated by palms (*Mauritia flexuosa*), although other tree species like figs (*Ficus* species) can be abundant as well [50]. They also tend to be clear and acidic, in contrast to the higher turbidity and circumneutral pH of the streams around [51,52]. This *aguajal* was confined by extensive cattle pastures surrounding it and was used as drinking reservoir for cattle, showing a remarkable eutrophication. Sampling point 15 presented the described characteristics and showed low habitat quality indices (Table 1) and low diversity of fishes (Figure 2), with only three extant species: *Moenkhausia oligolepis* (70 specimens), *Crenicichla semicincta* (three specimens) and *Hoplias malabaricus* (one specimen). However, diversity might increase in the rainy season, when this habitat is connected to the river and other species present in the river could occupy this place. Species of genera *Astroblepus*, *Trichomycterus*, *Bryconamericus*, *Ceratobranchia*, *Creagutus* and *Rhamdia* are present in the nearest sampling points (Sites 14 and 16).

Because of its special and no-comparable characteristics, this peculiar sampling point has been excluded for the general comparative analysis. Although the importance of the *aguajales* has been widely recognized because of their ecological and social relevance [53,54], no fish species records are available on the literature. Our fish and ecological data throw some light on these poorly studied freshwater habitats.

When analyzing richness, diversity and dominance in relation with position from headwaters-downstream indicated by elevation, distance to mouth and slope, results were solid and similar for the three variables (Figure 2). The Canonical Correspondence Analyses through the marginal effect values (λ_A) highlighted principal role of elevation (0.59) or slope (0.44) for explaining fish distribution along the basin (Table 3). The resulting graphics were also analogous when using both variables (Figure 3). Elevation and distance to mouth are widely used for multivariate analysis [55,56]. Nevertheless, they do not give environmental information, they are geographical variables and indicate spatial position. Although they show strong correlation with ecologic changes, their use for ecological analysis together with environmental variables may be problematic. Instead, slope is a purely environmental variable that measures a geophysical characteristic of the site. Furthermore, it is strongly related to position along the basin but is independent from other variables, something that avoids "noise" on multivariate analyses. After validating its robustness for explaining fish distribution as well as elevation, we recommend the use of slope as alternative variable to elevation or distances to mouth/source.

Fish community composition along the basin presented two distinct groups with a clear cut-off point occurring at 700 m a.s.l as indicated by the ANOSIM, separating mountainous fish from piedmont communities. The boundary between both zones is marked by geomorphological changes on the basin, when it shifts from the steep mountainous streams flowing through narrow valleys, to flat and wide floodplains where river channels widen. As pointed out by SIMPER procedure *Astroblepus* and *Trichomycterus* are the most distinctive species from the mountainous streams, while *Knodus* was the most important species from lowlands explaining the variation in community composition (Table 2).

Fish abundance and distribution in response to environmental variables was represented using the CCA analysis and showed similar results using slope or elevation (Figure 3). The observed distribution of species along the longitudinal gradient of the basin was comparable to other fish assemblages found in rivers with similar characteristics in adjacent areas [10,30,49]. This biotic zonation corresponds to discontinuities in river geomorphology or abiotic conditions and are usually related to smooth transitions of abiotic factors contributing to nested patterns of assemblage composition along the altitudinal gradient [57]. The graph highlights this zonation, distributing headwater sites and their fish assemblages on the right of the diagram, related to fast running waters, steep slope, boulders and low water temperatures, opposed to middle and lowland streams on the left of the graph related to higher water temperatures, wider streams, low velocities, higher conductivity and fine substrates.

Genera *Astroblepus* and *Trichomycterus* co-occur in high reaches, being the unique species present on the sites above 1400 m a.s.l. These genera are adapted to headwaters, related to rocky substrates and cold, clean and well oxygenated water (Figure 3). They are benthonic species without scales and a powerful sucker mouth or opercular odontodes [58]. Nevertheless, they occasionally occur in lower areas, although their distribution is usually limited to elevations greater than 400 m.a.s.l. (Lujan et al., 2013). *Bryconamericus*, a rheophile genus, is the next genus joining the headwater assemblage at 1400 m a.s.l. (Figure 6).

They prefer areas of moderate to strong current and present even in the torrential flows, occupying intermediate places between mountain upstream and jungle downstream [30,59]. We also found some *Chaetostoma* and *Ancistrus* fishes at these reaches, although they appeared closely related with lower middle section streams (Figure 3).

We found most of the species related to these middle-low reaches, distributed along the left area of the CCA (Figure 3): habitats with fine substrata, very slow water velocities, mild slope and more conductivity, related with *Serrapinus*, *Prodontocharax*, *Astyanancistrus*, *Astyanas*, *Knodus* and *Moenkhausia*, on the top-left area of the diagram (Figure 3b) [60]; other lotic sites presenting the widest habitats with highest temperature and higher pH, were distributed on the bottom left of the diagram, related with *Aphyocharax* and *Hemibrycon* genera characids and loricarids like *Rineloricaria*, *Chaetostoma* and *Ancistrus* [47,59,61].

We also observed a remarkable zonation related to trophic niches (Figure 6). Headwaters were dominated exclusively by invertivorous *Astroblepus* (Figure 5) and *Trichomycterus* (Figure 4) fishes due to the scarcity of other food resources in these clean waters [9,58]. Although the first herbivore-detritivore *Chaetostoma* and *Ancistrus* individuals were found starting at 1400 m a.s.l., they were more abundant below 1000 m a.s.l., once the river carries enough organic matter coming from the surrounding forest offering a new trophic niche [9,49]. The first piscivorous species, *Hoplias malabaricus* and *Crenicichla semicincta*, were distributed around 600 m a.s.l. and below, first found at site 15, the *aguajal*, where the *Moenkhausia oligolepis* community was very abundant providing enough biomass to feed the predators. This distribution pattern is according with the observations of Lujan et al. [6].

According to some ecological studies and inventories carried out in this area [8,62], more than 130 species of freshwater fishes have been reported for the Alto Madre de Dios Basin. In the present study, only 78 species were collected, due to two main reasons: (1) the highest biodiversity is found on the lowest reaches of the basin and our lowest sampling was at 398 m a.s.l.; (2) electrofishing technique has some limitations on these ecosystems.

Regarding electrofishing, only wadeable stretches were sampled, whereas larger courses or deep stretches were avoided. This limitation directed our sampling efforts to smaller rivers and tributaries where the electrofisher gained efficiency [63]. Although this represents a significant impediment for carrying out a complete biological survey, also brings up new opportunities. Most of the ichthyofaunal studies undertaken in Amazonia have focused on the large rivers and commercially valuable species, therefore small and noncommercial fishes and secondary streams have been usually overlooked [64]. Guided by our limitations, part of our surveying efforts was concentrated on this poorly studied ecosystems and species.

Besides the restrictions for choosing samplings sites, fish resistance to electrofishing was significant. This resistance was favored by low water conductivity and temperature, high velocities, shelter's abundance or low visibility [65]. Furthermore, capture efficiency depended on the mobility of species: little benthic fish were collected easily, in contrast to larger, strong-swimming species that escaped the electrofishers range. However, according to other authors [65,66] electrofishing is the best sampling method when your objectives are to estimate and quantify freshwater fish populations in streams and wadable rivers and to correlate these abundances with environmental features, habitat characteristics, hydrochemical parameters or other ecological measures. Besides, although electrofishing surveys have been widely achieved on the world, they have been scarce in remote places like the Alto Madre de Dios Basin due to the more complex logistics.

If we match our results with the survey carried out with seine nets by Araújo-Flores in 2013 [62] we find remarkable differences. Comparing the total 44 species reported by Araújo-Flores [62] with our 78 species only 17 of them were captured by both studies. Nevertheless, survey of Araújo-Flores focused on two rivers and its tributaries (Pilcopata and Piñi-Piñi Rivers), distributing ten sampling sites in a smaller area with a limited altitudinal range (500–600 m a.s.l.). If we compare our data for the three sites coinciding with the survey of Araújo-Flores (sites 7, 8 and 9), although our sampling effort was lower (3 vs. 10 sites) and our captured diversity smaller (26 vs. 44 species), we collected 15 species missing in the survey of Araújo-Flores [62]. Therefore, although electrofishing is not as effective in tropical streams as it is for temperate streams, it may be more effective for capturing some species that seem to be underestimated by other techniques. Consequently, as we focused on understudied small rivers and streams, catching poorly known small fish, using an uncommon technique for tropical freshwaters, our study presents new and relevant taxonomic and ecological information, providing data for some fish never captured before by previous surveys in the area.

Although our results highlight the good conservation status of the Alto Madre de Dios Basin, the territory endures severe threats: logging [22,31], gold mining [67], climate change [15,23], fossil fuel extraction [68], and hydropower projects [20,23]. The development and implementation of management plans is crucial for anticipating and mitigating future impacts. Nevertheless, the principal threat for this peculiar area, with high endemic biodiversity and very good conservation status, is the lack of knowledge regarding its fauna and flora. Studies of the fish fauna are critical to document the primary characteristics of the original communities and thereby contribute to the delimitation of appropriate conservation areas and/or to recovery strategies for degraded streams [69]. Therefore, Peruvian freshwater fish faunal inventory (including the Alto Madre de Dios River Basin) is a priority [8,69]. There are many remote areas, with poor accessibility, where knowledge of freshwater fish fauna is negligible and for most fish taxa basic taxonomic work is still required, not only for this area, but for all the Amazon and adjacent basins [8]. Besides, there is even less information, in some cases null, on the ecology of many species. This knowledge gap highlights the necessity of basic taxonomic works and the creation of field guides providing new research projects an essential tool [70]. Fortunately, there are some remarkable guides for the Madre de Dios Basin [52,61] and adjacent areas [71,72] that offer inestimable material about freshwater fishes in this region, although, the information in these guides is incomplete for many species. In this sense, the lack of basic knowledge may hamper the development of community-level analyses [3,73]. Although fish diversity for the hotspot is unclear yet, some papers estimate fish richness for the Andean region more than 600 species [69], with more species found at low elevations compared to higher reaches [5]. Around 2700 species have been recognized for the hotspot inside the Amazon Basin territory [74]. In contrast to richness, endemism tends to increase at higher elevations and particularly concentrates in isolated patches of habitat such as valleys and mountain tops [7]. An IUCN report evaluated fish fauna conservation status for the Tropical Andes [75] considering 666 endemic species: 13 spp. critically endangered; 33 spp. vulnerable; 36 spp. near threatened; 341 spp. least concern; 215 spp. data deficient. One of the main conclusions points out the significant information gap: 32% of the evaluated fish were poorly known.

Concerning this taxonomic constraint, freshwater fishes of high headwaters demand special attention. All of them are Andean species and live on high altitudes, areas where human settlements and their subsequent impact has a longer and more intense presence, compared with Amazonian streams [23]. We found five different species of *Astroblepus* (Figure 5), two of them undetermined, five species of *Bryconamericus*, and six species of *Trichomycterus* all undetermined (Figure 4). Astroblepids and trichomycterids are typically restricted in their geographical distribution and endemic to single or adjacent river systems of the Andes, and their taxonomy is poorly known and in constant revision [76–78]. Advances on the taxonomic and ecological knowledge of these high mountain species, related to tropical montane cloud forests, are mandatory for the design of conservation and management plans in the area.

According to Ortega et al. [8], there is not an official national Peruvian red list of freshwater fishes because of this lack of knowledge (despite several attempts and proposals). Although conservation lists have their limitations and critics, they represent an essential tool required for protecting biodiversity [79]. Therefore, it is imperative to continue with the study of freshwater fish species in Peru with the aim of improving management actions and conservation plans.

In the light of this scenario where no Andean-Amazon Basin will remain untouched, the Alto Madre de Dios River Basin still preserves healthy ecosystems, with mild human impacts affecting some stretches, but showing a good environmental quality overall. Therefore, this makes the basin a perfect candidate for being preserved and considered as a reference basin for these seriously endangered ecosystems.

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Variability in Population Traits of a Sentinel Iberian Fish in a Highly Modified Mediterranean-Type River

Ana Sánchez-Pérez [1,*], Francisco J. Oliva-Paterna [1], Fátima Amat-Trigo [2] and Mar Torralva [1]

[1] Departamento de Zoología y Antropología Física, Facultad de Biología, Campus de Espinardo, Universidad de Murcia, 30100 Murcia, Spain; fjoliva@um.es (F.J.O.-P.); torralva@um.es (M.T.)
[2] Department of Life and Environmental Sciences, Faculty of Science and Technology, Bournemouth University, Poole, Dorset BH12 5BB, UK; fatima.amat@um.es
* Correspondence: ana.sanchez15@um.es

Abstract: Human pressures on water resources have been suggested as a driver of biological traits that induce changes in native fish populations. This study highlighted the interplay between environmental stress factors, mostly related to flow regulation, and the longitudinal river gradient in biological traits such as the growth, size structure and somatic condition of a sentinel fish, *Luciobarbus sclateri*. We found an increase in size-related metrics and somatic condition at population levels associated with downstream reaches, although fragmentation and habitat alteration, flow regime alteration and the abundance of non-native fish were also significantly involved in their variability. Age-related parameters and growth were only explained by flow regime alterations and the abundance of non-native fish species. The high plasticity observed in *L. sclateri* population traits suggests that this is a key factor in the species adaptability to resist in a strongly altered Mediterranean river basin. However, the interplay of multiple stressors plays an important role in fish population dynamics and could induce complex responses that may be essential for long-term monitoring in sentinel species.

Keywords: sentinel species; longitudinal gradient; human impacts; flow regime alteration; non-native fish; fragmentation; habitat alteration; Mediterranean rivers; Segura River basin

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1. Introduction

Freshwater ecosystems are considered among the most altered as a consequence of the historical pressure of human activities [1,2]. Hydraulic management to take advantage of water resources and the effects of climate change are inducing quantitative and qualitative changes in river systems [3,4]. These changes imply hydro-morphological, chemical and biological alterations which affect the freshwater fauna [2,5]. Hydraulic management is especially intense in Mediterranean regions where water resources are scarce [6]. Rivers in semi-arid regions, such as the Iberian Peninsula, are heavily impacted by the construction of a large number of dams and weirs [7,8].

Mediterranean regions are characterized by marked seasonality and inter-annual variability with severe periods of floods and droughts [9,10]. The native freshwater fauna is adapted to such natural variability and displays great resistance and resilience [11–13]; however, it is considered especially sensitive to human impacts [14,15]. Human pressures are particularly severe in Mediterranean regions and they coincide with high natural variability, causing severe alterations to fluvial ecosystems [8,16,17]. Despite the high adaptability of freshwater fauna, the expected increase in human impacts under future scenarios of global climate change could increase its vulnerability to such pressure, especially in Mediterranean regions [11,18,19].

The flow regime is considered one of the main driving forces of freshwater ecosystems, determining the structure and ecological dynamic of rivers [19,20]. In the Iberian Peninsula, the alteration of the natural flow regime by dam regulation is one of the most important stress factors that negatively affects the native fish fauna [21–23]. The effects of flow regime alteration have been widely documented in Mediterranean fish populations [24–27]. In addition, flow management infrastructures cause loss of connectivity and habitat alterations related to the fragmentation process (e.g., increase in lentic habitats, changes in water quality), which could facilitate the establishment and spread of non-native fish species, altering the composition of the community [28,29]. However, the effects of these stress factors have been poorly studied in Mediterranean rivers [30–32].

The Segura River basin is located in the southeast of the Iberian Peninsula and is characterized by a marked environmental variability and displays a wide variety of human impacts along its longitudinal gradient [33,34]. Furthermore, this basin shows a well-documented range of impacts on the natural flow regime [35,36] and is considered one of the most regulated Mediterranean river systems [37]. Therefore, the Segura River offers an opportunity to study the effect of multiple human-induced stressors along an intensively altered basin. The fish assemblage in the Segura River basin is characterized by a low number of species where *Luciobarbus sclateri* (Günther, 1868) appears to be dominant [38]. This native species is an endemic potamodromous fish considered a sentinel species in the southern Iberian Peninsula [12], and its biology and ecology have been well documented [39–41], being the unique native fish widely distributed in the Segura River basin [42]. Therefore, *L. sclateri* populations in the Segura River could be considered a useful tool through which to assess the intra-specific variability along a wide longitudinal gradient strongly affected by environmental alterations mainly related to flow regulation. However, few authors have described the effects of multiple stressors on its populations, and nothing is known about its intra-specific variability along longitudinal gradients [43–45].

The main objective of the present study was to assess the variability of *L. sclateri* population traits in relation to different environmental stressors along the longitudinal gradient of a highly regulated river system. Our hypothesis was that *L. sclateri* would exhibit high phenotypic plasticity that enables populations to survive along the longitudinal gradient of a highly impacted river basin. *L. sclateri* has been evaluated as near threatened (NT) on a regional scale due to the intensification of human pressures which caused a decrease in habitat quality (mainly pollution and flow regime alteration) and an increase in the establishment and spread of several non-native fish species [46]. Furthermore, in recent years, a severe decline in *L. sclateri* populations has been documented in the Segura River basin [38,47]. The use of different biological traits provides ecological insights into how populations respond to multiple stress factors, allowing us to understand how species' traits could predispose species to local extinctions [48] and to establish more successful management and recovery programs [8,49].

2. Materials and Methods

2.1. Study Area and Sampling Design

This study was conducted in the Segura River basin, situated in the semi-arid Mediterranean climatic zone in the southeast of the Iberian Peninsula (Figure 1). The river basin drains an area of 18,870 km^2 and is characterized by a strong climatic and altitudinal gradient, and significant annual and inter-annual natural variation in the flow regime [6]. Multiple human activities—primarily related to agriculture, but also to electricity generation and human supply—have developed in the study area [33,34,37]. Irrigation accounts for 90% of the water demand and is considered the main pressure on the water resources. A total of 33 dams (>10 m height and >1 hm^3 of reservoir) and 170 smaller obstacles exist along the longitudinal gradient of the river with a capacity of regulation of approximately 1200 hm^3. Since 1979, this basin has received an external water transfer from the Tajo River with an average of 350 hm^{3}year^{-1}, so the storage capacity increased by around 140% of the natural input (871 hm^{3}year^{-1}) [37,50]. Furthermore, local agricultural practices add an artificial source of pollutant discharge (mainly phosphates and nitrates) into the river [51,52]. As a result, the fluvial and riparian habitats of the Segura River basin have been severely altered [33,53], in addition to strong modifications in the natural flow regime [54].

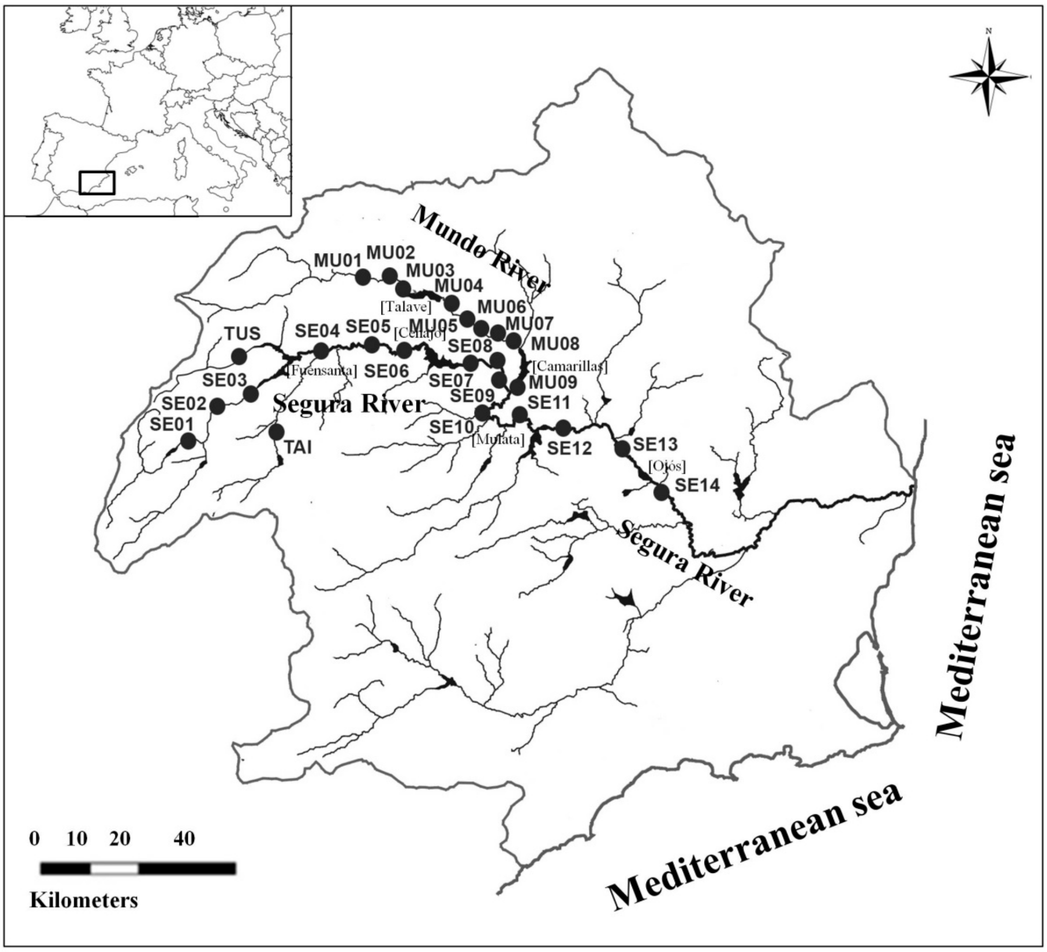

Figure 1. Study area. Location of the Segura River basin in the southeast of the Iberian Peninsula. Sampling sites are marked (●): TAI (Taibilla River), TUS (Tus River), MU (Mundo River) and SE (Segura River). Names of large reservoirs are between parentheses [].

We sampled a total of 25 sites in fluvial reaches distributed along upstream–downstream gradients from the upper Segura main stem (SE) to the middle Segura (195 km), from the upper Mundo River (MU) to the Mundo–Segura confluence (51 km) and in two upper tributaries (Tus and Taibilla) (Figure 1). The range in altitude is 112–809 m.a.s.l. in the study area; the water conductivity ranged from 316 to 1303 µs cm^{-1} and the range of the mean annual water temperature was 13.9–16.9 °C during the study period. The distribution of sampling sites reflects the different hydrological flow regimes present in the study area. Sampling sites placed in areas with the most natural flow regime (e.g., MU01 and SE01; Figure 1) were characterized by a strong seasonal variation, alternating summer droughts and spring/autumn short-time flow peaks (Figure 2a). An impact gradient on the natural flow regime in the study area was described by Amat-Trigo [35] and Amat-Trigo et al. [36]. Impacted areas exhibit a seasonal inversion of the natural flow pattern (high flow levels in spring and summer, low flow levels in autumn and winter) due to the water demand for agricultural practices. Furthermore, impacted areas can be characterized by two extreme flow impacts: reaches downstream of the Cenajo reservoir (sampling sites SE06, SE07 and SE09; Figure 1) showed a high level of contingency and low variability, but also low predictability (Impact 1, Figure 2b), while other reaches (e.g., sampling sites MU09, SE12 and SE13; Figure 1) were characterized by more stable and high levels of base flow throughout the year, high values of temperature and spell peaks, in addition to the inversion in flow seasonality (Impact 2, Figure 2c).

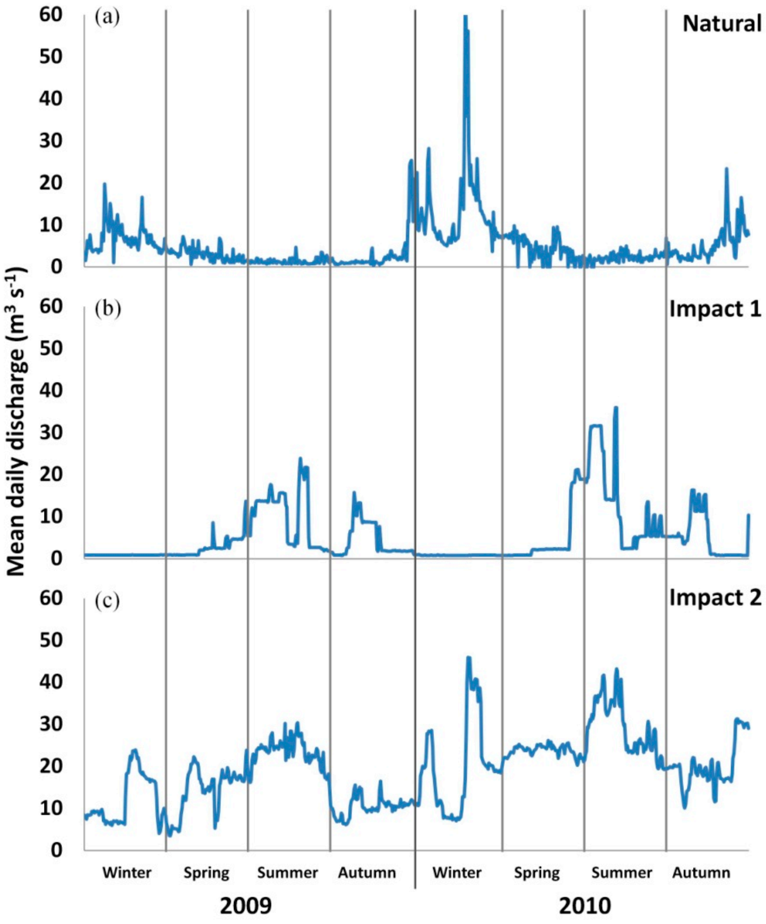

Figure 2. Flow variation (mean daily discharge; m^3 s^{-1}) at three representative fluvial sectors in the study area. Repre-sentative flow regimes were measured at three gauging stations: (**a**) Natural flow regime at the upper part of the Mundo River, (**b**) Impact 1 just downstream of the Cenajo reservoir, and (**c**) Impact 2 downstream of the La Mulata dam (see location in Figure 1). Flow discharge data were obtained from the Segura Hydrographic Confederation.

The fish assemblage in the study area is composed of both native and non-native species [38]. Non-native species are dominant (90% of total species richness) and *L. sclateri* is the only widely distributed native species in the study area. The most abundant species are cyprinids: the native *L. sclateri* and the non-native species *Pseudochondrostoma polylepis* (Steindachner, 1864), *Gobio lozanoi* (Doadrio and Madeira, 2004) and *Alburnus alburnus* (Linnaeus, 1758). The non-natives *Cyprinus carpio* (Linnaeus, 1758) and *Lepomis gibbosus* (Linnaeus, 1758) are locally abundant. Furthermore, the natives *Squalius pyrenaicus* (Günther, 1868) and *Salmo trutta* (Linnaeus, 1758), as well as the non-natives *Gambusia holbrooki* Girard 1859, *Oncorhynchus mykiss* (Walbaum, 1792), *Micropterus salmoides* (Lacépède, 1802), *Sander lucioperca* (Linnaeus, 1758) and *Esox lucius* (Linnaeus, 1758), are present in the study area.

2.2. Environmental Variables

We described a total of ten environmental variables and gradient descriptors in the study area (Table 1). Water conductivity was measured in situ using a multi-parameter (340i WTW), and ecological status was assessed according to the EU Water Framework Directive, Fluvial Habitat Index (IHF) [55], Riparian Quality Index (RQI) [56] and altitude [57]. The Longitudinal Connectivity Index (ICL) [58] and free reach (length of reach available for free movement) were calculated using databases available online from the official monitoring service of the Segura Hydrographic Confederation (CHS) (https://www.chsegura.es/chs/cuenca/restauraciondерios/obstaculos/visorjs.html; accessed on 12 September 2018).

Water temperature and daily river discharge data were also obtained from the CHS online databases with data gathered by gauging stations distributed in the study area (https://www.chsegura.es/chs/cuenca/redesdecontrol/estadisticashidrologicas/; accessed on 12 September 2018). Mean monthly values of water temperature for 2009 and 2010 were calculated. Mean daily discharge (m^3 s^{-1}) over a 16-year period (1994–2010) was used to calculate the mean daily base flow (MDBF) as the total base flow component of the hydrograph divided by the number of recording days, and flow variability as the daily range between $Q_{10\%}$ and $Q_{90\%}$ discharge divided by the median value. These two flow metrics (MDBF and flow variability) were calculated using time series analysis (TSA) of the River Analysis Package (RAP version 3.0.7) [59].

Table 1. Environmental variables and gradient descriptors measured or calculated at sampling sites. Altitude (meters above sea level), ecological status (categorized as: 1 = high; 2 = good; 3 = moderate; 4 = poor), Fluvial Habitat Index (IHF) and Riparian Quality Index (RQI) (%), conductivity (µS cm^{-1}), free reach (km), Longitudinal Connectivity Index (ICL), water temperature (°C), mean daily base flow (MDBF) (m^3 s^{-1}) and flow variability (($Q_{10\%} - Q_{90\%}$)/median).

Sampling Site	Altitude	Ecological Status	IHF	RQI	Conductivity	Water Temperature	Free Reach	ICL	MDBF	Flow Variability
MU01	560	2	72	55	602	14.7	4.13	255	1.59	−2.44
MU02	540	2	81	85	619	14.7	2.43	255	1.59	−2.44
MU03	520	2	81	85	619	14.7	5.39	255	1.59	−2.44
MU04	480	3	69	43	639	14.7	3.25	205	8.58	−1.71
MU05	460	3	75	53	674	14.7	2.02	205	8.58	−1.71
MU06	430	3	70	64	688	14.7	3.67	205	8.58	−1.71
MU07	396	3	72	46	724	14.7	3.79	205	8.58	−1.71
MU08	354	3	70	34	1303	14.7	2.29	205	8.58	−1.71
MU09	350	4	67	39	1109	15.4	3.54	138	14.04	−1.62
SE01	860	3	78	94	364	14. 3	25.88	84	2.91	−3.14
SE02	685	1	61	98	387	14.3	32.33	84	2.91	−3.14
SE03	491	1	63	87	390	14.3	12.58	84	2.91	−3.14
SE04	470	1	67	78	340	13.8	30.02	0	4.14	−2.58
SE05	452	1	68	80	401	13.8	30.02	0	4.14	−2.58
SE06	432	1	78	98	401	13.8	30.02	0	4.14	−2.58
SE07	363	2	67	77	447	14.1	7.73	126	1.77	−13.10
SE08	325	2	73	66	492	14.1	5.96	126	1.77	−13.10
SE09	306	2	58	69	579	14.1	1.7	126	1.77	−13.10
SE10	290	2	62	32	756	15.9	7.18	126	14.04	−1.62
SE11	260	3	57	44	803	15.9	10.64	90	14.04	−1.62
SE12	200	2	66	70	861	16.4	4.98	106	14.22	−1.55
SE13	148	2	58	45	1139	16.4	6.88	365	15.14	−1.47
SE14	112	4	64	36	1157	16.8	4.36	365	2.46	−3.31
TAI	640	2	61	43	566	14.7	15.67	58	-	-
TUS	809	1	84	65	432	14.5	12.81	76	1.59	−2.44

2.3. Fish Sampling and Population Traits

Sites were sampled using electrofishing (working voltage between 200 and 350 V, 2–3 A), following the CEN standard protocol [60]. Each sampling site was considered an independent population, taking into account the distance between sampling sites, the presence of non-passable barriers (dams and weirs) and the biological characteristics of *L. sclateri*. Fish were collected in 100-m-long wadable sections blocked by nets that acted as barriers. Fish sampling sessions were carried out in 30–45 min. Sampling was conducted during October–November 2009 to prevent the capture of spawning fish [45] and to avoid variation in body condition due to gonad development [39,43,61]. Fish manipulation was carried out following the European Union Directive 2010/63/UE on the protection of animals used for scientific purposes and it was not necessary to obtain authorization from the research ethics commission. In concordance with administrative permits, a total of 1529 specimens of *L. sclateri* were caught, processed in the field and returned to their habitat. Fork length (FL ± 0.1 cm) and weight (TW ± 0.1 g) were measured. A subsample of scales (611 specimens) was taken and cleaned later in a laboratory to determine the age according to Herrera et al. [40] and Torralva et al. [45].

L. sclateri populations were evaluated at sampling sites to assess intra-specific variability. The fish population traits we studied were relative abundance, size-related parameters, age-related parameters, relative growth rates and somatic condition. Relative abundance was measured as the number of *L. sclateri* individuals caught per hour (catch per unit effort, CPUE) in a standardized sample area without significant differences in habitat complexity and, thus, assuming that catch efficiency remains constant. Size-related parameters included mean, maximum and range of fork length (FL), and a size diversity index calculated as a Shannon-Wiener modification using the number of size classes grouped in 2 cm length ranges. Age-related parameters included mean, maximum and range of age, determined for a subsample of *L. sclateri* scales. Back-calculated lengths were estimated by the Fraser-Lee equation following the methodology used by Torralva et al. [45] and Miñano et al. [62] based on the counting and measuring of scales' annuli, and checked according to Musk et al. [63]. Proportions (%) of back-calculated lengths for each age class were obtained from the Walford method [64] and used to calculate the mean individual growth index (GI). Relative growth rates at the site level were estimated from the mean individual growth index (GI). The methodology used to calculate the growth index (GI) is detailed in Masó et al. [65] and Amat-Trigo et al. [66], which followed the Hickley and Dexter procedure [67]. We used the mean values of GI at age 1 year, age 2 years and maturity (individuals older than 2 years) according to the age of maturity previously established for the species in the Segura River basin [43,45,61]. Somatic condition was expressed as predicted values of log-transformed weight (mean value at sampling site) obtained from the application of univariate analysis of covariance (ANCOVA) using total weight (WT) as the dependent variable and fork length (FL) as the covariate; differences in variation were tested by ANOVA and Tukey's HSD post hoc tests [68]. Individuals with an FL less than 75 mm were considered juveniles [43]. Bivariate relationships between population traits were analyzed using Spearman's rank correlations. Statistical analysis was performed with the SPSS software package v. 24.

2.4. Effect of Environmental Stress Factors on Population Traits

We conducted a model selection analysis based on the Akaike Information Criterion with a correction for small samples sizes (AICc) [69] to determine the stressors associated with the variability in *L. sclateri* population traits. To establish model ranking, the MuMIn (Multi-Model Inference) R Package was used [70]. Stress factors were obtained by the reduction in transformed environmental variables and gradient descriptors (log for numeric variables, arcsine square root for proportions) using principal component analysis (PCA) with varimax rotation [71] using R package *psych*. Spearman's rank correlation analysis was used to test the redundancy between the variables. Principal component axes scores were used as stressors. The ratio of the abundance of non-native fish species (abundance of non-native species/total abundance) was also considered a stressor. General linear model results (GLMs) for the best models (ΔAICc < 2.0) were used to describe the response of *L. sclateri* biological traits to the stress factors. These analyses were performed in the R statistical environment (Version 1.40.4).

3. Results

3.1. Relative Abundance and Population Traits

The abundance of *L. sclateri* showed high spatial variation, ranging from 13.33 to 150.00 catches per unit of effort (CPUE, Supplementary Table S1). The highest value was found in the Taibilla tributary (sampling site TAI) and the lowest was found at SE04, downstream of the Fuensanta reservoir (Figure 1).

The mean size fork length in the whole study area was 18.3 ± 6.55 cm FL, with the maximum value detected at MU03 (58.2 cm). The maximum validated age was 15+ years, with individuals this old detected at sampling sites SE04, SE07 and SE09; however, the mean age at the population level was 4.9 ± 1.43 years. The population size/structure differed among sampling sites, although a polymodal distribution pattern was evident in most sampling sites along the longitudinal gradient (Figure 3). Both size and age parameters displayed lower values in headwaters (Figure 3 and Table S1), with maximum fish sizes below 30 cm (FL), lower size ranges and lower size diversity index values (Figure 3a,b,c). The other sampling sites had specimens longer than 40 cm (FL), with higher values at sampling sites just downstream of non-passable obstacles (e.g., SE07, Figure 3) and upstream of large reservoirs (e.g., MU03, Figure 3).

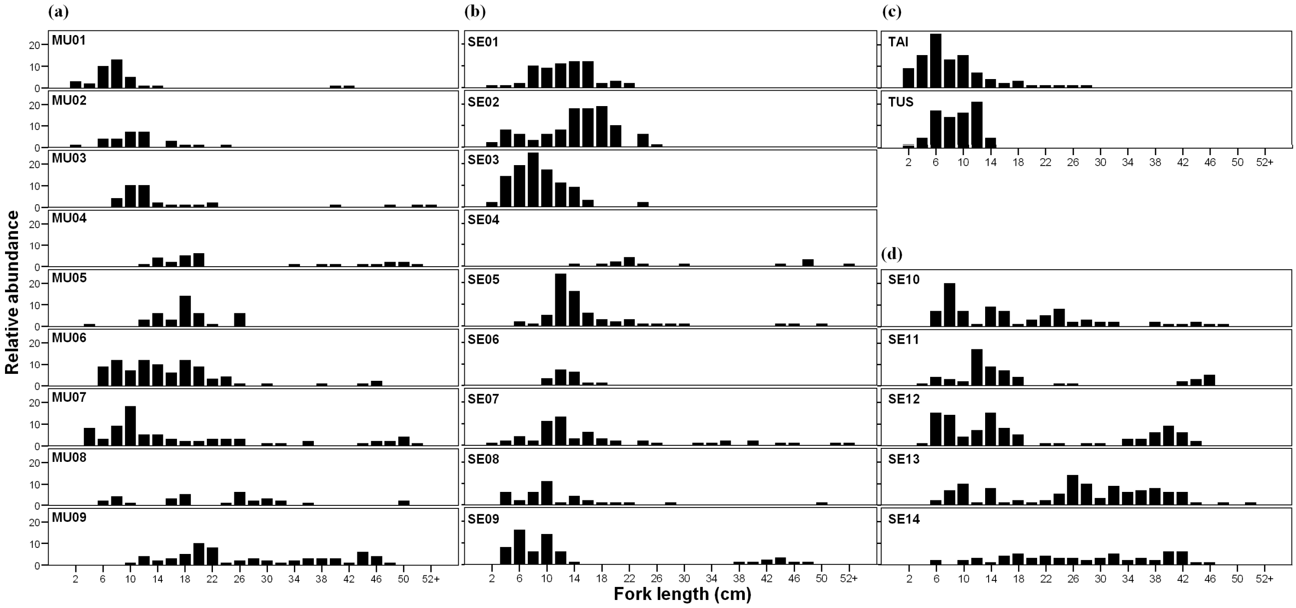

Figure 3. Size distribution (2 cm fork length (FL) groups) of *L. sclateri* at sampling site. Plot distribution: (**a**) Mundo River (MU) sampling sites, (**b**) sampling sites placed from Segura (SE) riverbed to Mundo River confluence, (**c**) sampling sites at tributaries Tus and Taibilla (TAI) Rivers and (**d**) sampling sites from Segura–Mundo confluence to the latest downstream sampling site (SE14).

The growth index (GI) displayed high variability for individuals of each age class between sampling sites. The mean GI value at age 1 year across the study area was 66.74 ± 1.28, with a maximum value of 82.47 detected at sampling site SE01, while the mean value of GI at age 2 years was 39.34 ± 1.13, with a maximum of 53.52 at sampling site MU09. For mature fish, the mean GI value in the study area was 17.69 ± 1.57, with a maximum of 32.53 detected at site MU05. The lowest GI values were detected in the TUS tributary (age 1 year = 45.72; age 2 years = 19.26; mature = 0.03).

Somatic condition was higher in mature individuals and also showed greater variability among sampling sites. The mean of predicted values of log-transformed weight for mature individuals was 1.87 ± 0.03, with a maximum value of 2.43 at sampling site MU04, whereas the mean value for immature individuals was 0.63 ± 0.03, with a maximum of 0.90 at sampling site SE10. Generally, lower values of somatic condition were detected in headwaters for both mature (TUS = 1.28) and immature (SE01 = 0.44) individuals. Population traits at the site level are shown in Table S1 (Supplementary Material). The relationships among population traits are presented in Table S2 (Supplementary Material).

3.2. Environmental Factors

The first three PC axes obtained from the dimension reduction of environmental variables and gradient descriptors explained 79.7% of the total variance (Table 2). PC1 was associated with habitat alteration and fragmentation, which were directly related to poor ecological status and low RQI, high values of conductivity and water temperature and low connectivity (high values for ICL and low values of free reach). PC2 was associated with the longitudinal gradient, which was directly related to high altitude, IHF and low water temperatures. PC3 was associated with flow regime alteration, which was directly related to flow variability and MDBF.

Table 2. Loadings and proportions of variance extracted by PCA of environmental variables with varimax rotation. Loadings > 0.50 are marked in bold.

Env. Variables	PC1	PC2	PC3
Ecological status	**0.817**	−0.150	0.202
Fluvial Habitat Index (IHF)	0.081	**0.882**	0.036
Riparian Quality Index (RQI)	**−0.648**	0.427	−0.286
Altitude	−0.339	**0.832**	−0.071
Free reach	**−0.909**	0.052	0.087
Longitudinal connectivity (ICL)	**0.869**	0.021	−0.068
Conductivity	**0.750**	−0.443	0.393
Water temperature	**0.520**	**−0.534**	0.466
Mean daily base flow (MDBF)	0.170	−0.473	**0.700**
Flow variability	−0.038	0.164	**0.950**
Proportion of variance	0.366	0.241	0.191

3.3. *Effects of Environmental Factors on Population Traits*

Table 3 displays the best models obtained from the model selection analysis based on AICc. The longitudinal gradient (PC2) was significantly linked with size-related parameters and somatic condition metrics. The gradient from the upper sampling sites to the downstream sites was associated with increment in population traits including the size range, mean and maximum size, size diversity index and somatic condition metric (longitudinal gradient axis, Figure 4). The longitudinal gradient interplayed with other stressors such as the non-native fish species, showing a significant effect on size range and maximum size (size-related parameters, Table 3). In addition, this environmental factor interplayed with the flow regime alteration (PC3), displaying a significant effect on mean size (size-related parameters, Table 3) and somatic condition for mature individuals (somatic condition, Table 3). Finally, the longitudinal gradient also interplayed with habitat alteration and fragmentation (PC1), displaying a significant effect on the size diversity index (size-related parameters, Table 3) and somatic condition in immature individuals (somatic condition, Table 3).

Table 3. General linear models (GLM) results for the best models obtained by model selection analysis based on the Akaike Information Criterion with a correction for small samples sizes (AICc). GLM analysis was conducted for each population trait: CPUE, size- and age-related parameters, growth and somatic condition. (● $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

SIZE-RELATED PARAMETERS					
Size range	**Longitudinal gradient + Non-native species**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	26.264	5.239	5.013	5.81×10^{-5}	***
Longitudinal gradient	−4.660	1.921	−2.426	0.024	*
Non-native species	18.278	8.792	2.079	0.050	●
Mean size	**Longitudinal gradient + Flow regime alteration**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	18.420	1.114	16.537	1.62×10^{-13}	***
Longitudinal gradient	−3.097	1.119	−2.768	0.0115	*
Flow regime alteration	2.578	1.117	2.307	0.0313	*

Table 3. *Cont.*

Maximum size	**Longitudinal gradient + Non-native species**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	29.456	5.913	4.981	6.27×10^{-5}	***
Longitudinal gradient	−4.812	2.168	−2.220	0.038	*
Non-native species	23.839	9.924	2.402	0.026	*
Size diversity index	**Longitudinal gradient + Habitat alteration and fragmentation**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	2.231	0.051	43.977	$<2 \times 10^{-16}$	***
Longitudinal gradient	−0.023	0.051	−4.434	0.0002	***
Habitat alteration and fragmentation	0.117	0.056	2.098	0.048	*
ABUNDANCE (CPUE)					
CPUE	**Null**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	61.525	7.786	7.902	5.3×10^{-8}	***
AGE-RELATED PARAMETERS					
Age range	**Flow regime alteration**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	8.765	0.519	16.900	4.36×10^{-14}	***
Flow regime alteration	−1.586	0.520	−3.048	0.006	**
Mean age	**Non-native species**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	3.237	0.749	4.324	2.73×10^{-4}	***
Non-native species	2.985	1.245	2.397	0.025	*
Maximum age	**Non-native species**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	7.206	1.658	4.345	2.59×10^{-4}	***
Non-native species	5.060	2.759	1.834	0.080	●
SOMATIC CONDITION					
Immature somatic condition	**Longitudinal gradient * Habitat alteration and fragmentation**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	0.642	0.023	28.162	9.97×10^{-14}	***
Longitudinal gradient	−0.119	0.028	−4.291	0.0007	***
Habitat alteration and fragmentation	−0.015	0.028	−0.552	0.589	
L. gradient: H. alter. and fragment.	0.110	0.043	2.578	0.022	*
Mature somatic condition	**Longitudinal gradient + Flow regime alteration**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	1.869	0.058	32.211	$<2 \times 10^{-16}$	***
Longitudinal gradient	−0.188	0.058	−3.228	0.004	**
Flow regime alteration	0.107	0.058	1.844	0.080	●

Table 3. *Cont.*

GROWTH					
Growth index (GI) at age 1	**Null**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	66.676	2.387	27.930	$<2 \times 10^{-16}$	***
Growth index (GI) at age 2	**Flow regime alteration**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	40.724	1.723	23.639	$<2 \times 10^{-16}$	***
Flow regime alteration	3.607	1.728	2.087	0.049	*
Growth index (GI) for mature	**Flow regime alteration**				
	Estimate	**Std. Error**	***t* value**	**Pr(>|t|)**	
(Intercept)	18.165	2.042	8.894	9.73×10^{-9}	***
Flow regime alteration	5.614	2.049	2.740	0.012	*

In addition to the interplay with the longitudinal gradient, the flow regime alteration (PC3) was linked to variability in the age range (age-related parameters, Table 3) and the growth index (GI) for mature fish and fish aged 2 years (growth, Table 3). An increase in the PC3 axis implied high flow variability and base flow (Impact 2, Figure 2c) and was associated with increased mean size and somatic condition of mature individuals, and GI at age 2 years and for mature individuals (flow regime axis, Figure 4). In contrast, a decrease in the PC3 axis implied low base flow and variability (Impact 1, Figure 2b) and was associated with a decreased age range (flow regime alteration axis, Figure 4).

In addition to the interplay with the longitudinal gradient, non-native fish species were linked to the variability in the mean and maximum age (age-related parameters, Table 3). An increased relative abundance of non-native fish species was associated with an increase in these two age-related parameters (non-native fish species axis; Figure 4).

Finally, habitat alteration and fragmentation (PC1) were linked to the size diversity index (size-related parameters, Table 3) and somatic condition of immature individuals (somatic condition, Table 3) and always exhibited an interplay with the longitudinal gradient. An increase in habitat alteration and fragmentation was associated with increased size diversity index (habitat alteration and fragmentation axis, Figure 4). Furthermore, the only interaction found in this study occurred between this stressor and the longitudinal gradient, which was associated with increased somatic condition for immature individuals (longitudinal gradient axis, Figure 4). Finally, the model selection analysis did not find any stress factors among those considered in this study to explain the variability in the growth index (GI) of fish at age 1 year or abundance (CPUE).

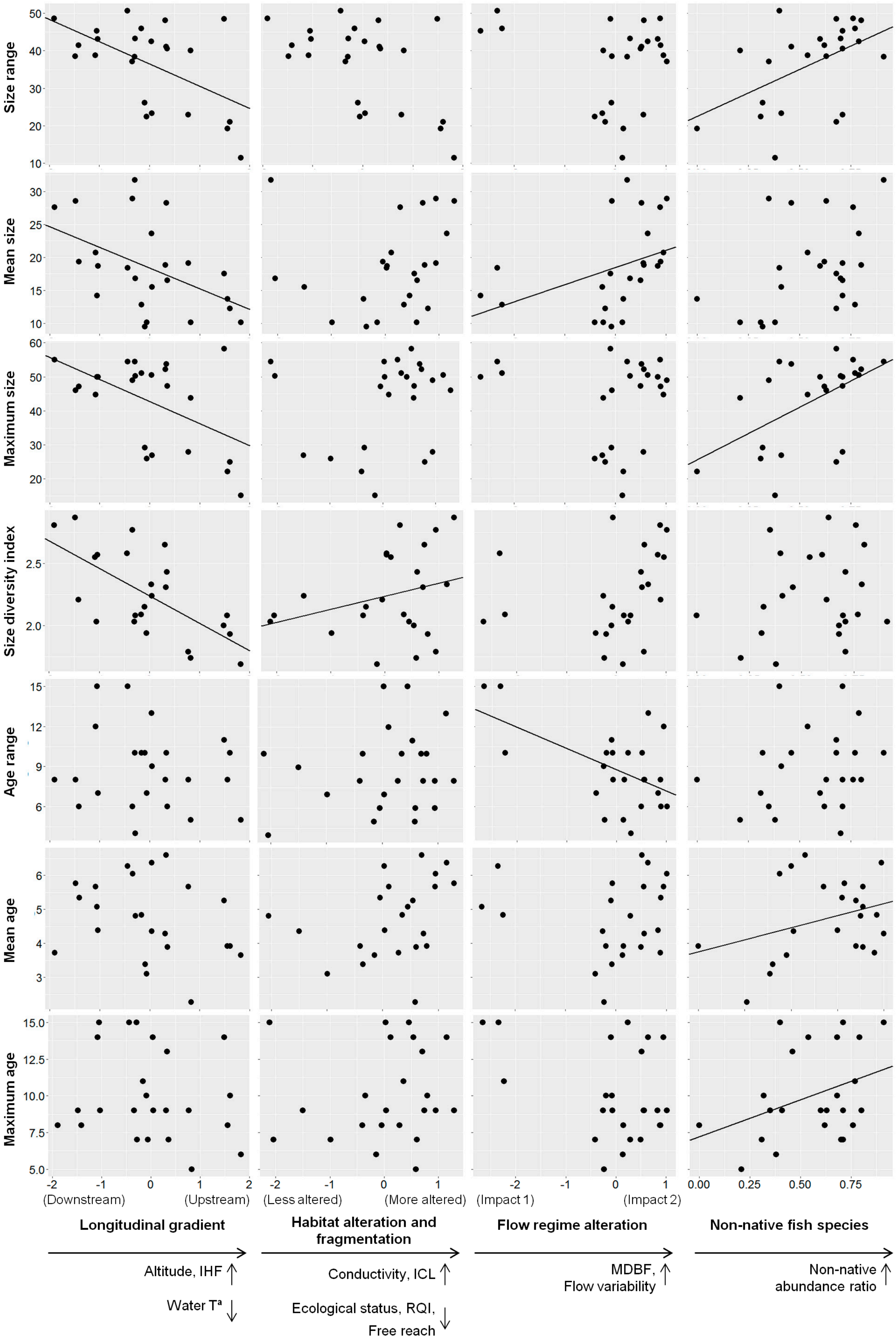

Figure 4. *Cont.*

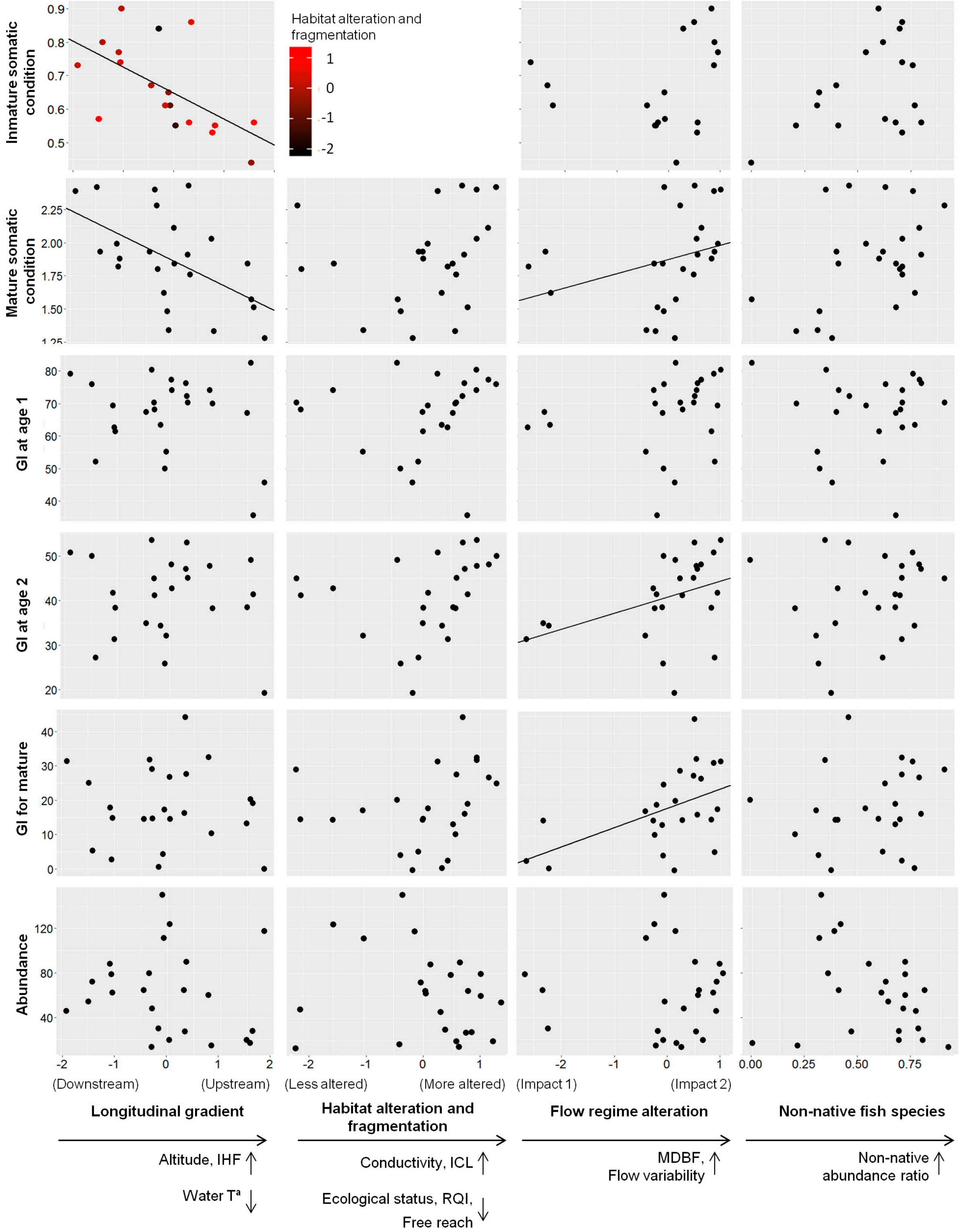

Figure 4. Relationship between environmental factors (longitudinal gradient, habitat alteration and fragmentation, flow regime alteration and non-native fish species) and *L. sclateri* population traits. Significant results (GLM p-value < 0.05) are represented with marked trend line on the plot. In x-axes, arrows pointing down indicate decrease in environmental variables (i.e., water temperature), and arrows pointing up indicate increase in environmental variables (i.e., altitude) and also increase in non-native abundance ratio.

4. Discussion

In this study, we analyzed the variation in population traits of *L. sclateri* in response to environmental factors in the fluvial reaches of the Segura River basin. Our results confirm significant variability in population traits along the longitudinal gradient due to the effect of human impacts, mostly those related to flow regulation such as fragmentation and habitat alteration, flow regime alteration and the relative abundance of non-native fish species. The use of biological traits related to size and age, relative abundance, growth and somatic condition allowed us to identify the population-level responses of this sentinel Iberian fish to different stressors and to verify the complex effect of multiple stressors in a heavily modified Mediterranean-type river.

The longitudinal gradient is a key factor in the structure and dynamic of freshwater ecosystems, so it is essential to consider its effects in the assessment of multiple stressors in river systems [72–75]. Environmental conditions along the longitudinal gradient determine the availability of resources such as food, refuges and breeding areas [34,76]. Consequently, a marked effect of the spatial variation on fish populations was expected, especially in Mediterranean-type rivers which exhibit a strong climatic and altitudinal gradient [10,77,78]. The increment of resources downstream along natural river systems promotes large sizes and a wider range of size classes in the fish fauna [73,76,79]. We found significant variability in the size parameters and somatic condition of *L. sclateri* populations associated with spatial variation, with an increase in these biological traits along the longitudinal gradient. The size population structure displayed a polymodal pattern, with high variability among sampling sites. The size distribution results highlighted a lack of some size groups, and this was more evident in sampling sites downstream of reservoirs. Small and medium-sized individuals were scarce downstream of operational dams (i.e., at sites MU09, SE04, SE09 and SE11). The GLM results (Table 3) showed that size and somatic condition parameters were significantly associated with the longitudinal gradient. In fact, habitat alteration and fragmentation, flow regime alteration and relative abundance of non-native fish species were all related to the variability of population parameters, reflecting the severe alterations that have affected the Segura River basin [34,37].

Human impacts increase along the longitudinal gradient as a consequence of the greater accessibility to water resources [77]. As a result, the interplay among spatial variation and human stress factors shaped the environmental conditions that act as a "filter" of biological traits [80]. The selected traits determine the biological responses of freshwater fauna to cope with altered conditions [18,75,81]. The Segura River basin shows a strong influence of human alterations mainly related to agricultural supply [33,37]. Agricultural practices involve strong hydraulic management that results in a loss of connectivity (habitat fragmentation), flow regime alteration and water quality and habitat degradation and encourages the establishment of non-native fish species [18,32,82,83]. Our results show the interplay between environmental stress factors, mostly those related to flow regulation, and the longitudinal gradient in some biological traits such as size and somatic condition. In addition, we found that age and growth variations were significantly associated with the isolated effect of the flow regime alteration and the relative abundance of non-native fish species. This finding suggests that the magnitude of human impacts in the study area could be masking some ecological responses to longitudinal gradients [84,85].

The flow regime is considered the main driver of freshwater ecosystems, defining the structure, function and dynamic of rivers, and affecting the individual fitness and growth rate of fish populations [80,86,87]. We found significant relationships between flow regime alteration and some population traits of *L. sclateri* including mean size, age range, somatic condition for mature individuals and growth variability. Our results show an increase in these population traits associated with fluvial sectors that had a high level of base flow throughout the year (reflected as Impact 2 in Figure 2) and a flow regime pattern that reduced the strong seasonal variability of Mediterranean-type rivers. Although these fluvial sectors display an inversion in flow seasonality related to agricultural water demands, they also provide an increased availability of water, refuge and food resources [25,76,88].

Furthermore, high flow levels promote changes in body shape and muscle development, which induce better swimming performance and increased somatic condition [89,90], a finding that was previously documented in the Segura River basin [43]. In contrast, we observed a decrease in population traits (mean size, age range, somatic condition for matures and growth variability) associated with strong inversion in flow seasonality and base flow reduction (Impact 1 in Figure 2). We found this flow pattern in sampling sites downstream of the Cenajo reservoir, a consequence of the operating characteristics of its dam [34,35,54]. The extreme hydrological conditions caused by this type of water regulation result in a poor-quality habitat, especially for adult barbels [91], and are considered a limiting factor for the growth of barbel species in the Iberian Peninsula [25,92]. These two contrasting results in the response of fish populations under different hydrological flow patterns have been described in other Iberian rivers as well [25,93].

Non-native fish species tend to be dominant in human-altered ecosystems, such as the Mediterranean rivers, where more stable environmental conditions that result from flow regulation measures encourage their establishment and spread [82,83,94]. Iberian fish communities have exhibited significant changes over recent decades as a consequence of the introduction of a wide range of non-native fish species [95–97], and these changes are especially evident in the Segura River basin [38]. The negative responses of native fish populations associated with the presence and abundance of non-native species in the fish assemblage are well documented [75,98,99]. Our results show an increase in the maximum size and size range, and the mean and maximum age of *L. Sclateri* populations associated with a higher ratio of non-native fish abundance. The proliferation of non-native species is generally a result of changes in the environmental conditions caused by flow regulation [4,8]. The lack of small size classes of fish at sampling sites placed downstream of reservoirs where the presence of non-natives is favored (i.e., SE04 in Figure 3) suggests an effect of the fish assemblage composition on the structure of *L. sclateri* populations. In general, predation by non-native fish could affect the population structures and dynamics of native fish species in the Iberian Peninsula [95]. Some studies from other Iberian rivers confirmed the inclusion of different barbel species in the diet of top predator fish such as *E. lucius* [100,101] and *S. Lucioperca* [102]. Predatory fish (*E. lucious, S. lucioperca* and *M. salmoides*) showed a higher occurrence in the lower reaches of the study area. They could be inducing higher predation pressure on certain size classes of *L. sclateri* and therefore affecting the population size structure as Bravo et al. [103] showed in the Palancar River, where *M. salmoides* predation was directly related to the lack of 0+ individuals of dominant species such as *L. sclateri*.

The results of this study highlight relevant associations between human impacts, most of which were related to flow regulation, and the population traits of *L. sclateri* along a longitudinal gradient, providing insights into the population-level responses of this sentinel Iberian fish to environmental conditions at the site level. The key role of the longitudinal gradient in driving the increase in human impacts as a result of greater accessibility related to the lower reaches of rivers is evident, since most of the stressors were related to the spatial variation, so this dependence makes it difficult to interpret the effect of isolated stressors. In addition, there is a wide variety of human impacts present in the study area that were not considered in this study. For example, pollution could be driving the response of *L. sclateri* to environmental conditions [32], or predatory mammals (*Lutra lutra*), whose predation on *L. sclateri* in the Segura River basin was recently confirmed [104].

The ability of this species to adapt to changes in local conditions has been shown by the variation in population size/structure along the longitudinal gradient. Although human impacts exerted significant effects on the biological traits we evaluated, our findings also suggest that the wide inter-population plasticity displayed by *L. sclateri* may be a mechanism for this species to successfully inhabit a highly modified Mediterranean-type river. Cyprinids in general show great adaptability to environmental alterations [12,25,92,105], and *L. sclateri* showed a tolerance to the effects of flow regulation previously studied in the same river basin [45,61].

In recent decades, declines in fish populations have been documented for several Iberian fish species and there have been drastic reductions in fish species that were previously widely distributed in the study area. Native fish species are vulnerable to the rapid increase in human pressure on the water resource; this is especially so in Mediterranean areas where an increase in the magnitude of extreme weather events is expected under climate change scenarios [106]. Therefore, the use of well-known and widely distributed sentinel species, such as *L. sclateri*, may prove a useful tool to increase the knowledge of the adaptability and population responses to gradients of single and multiple stressors, which is essential to establish and improve management actions to protect native fish species.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4441/13/6/747/s1, Table S1: Population traits of *L. sclateri* at sampling site. Table S2. Coefficients of Spearman rank correlation between population traits.

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Review

Scientific Methods to Understand Fish Population Dynamics and Support Sustainable Fisheries Management

Shewit Gebremedhin [1,2,*], Stijn Bruneel [1], Abebe Getahun [3], Wassie Anteneh [4] and Peter Goethals [1]

[1] Department of Animal Sciences and Aquatic Ecology, University of Ghent, 9000 Ghent, Belgium; stijn.bruneel@ugent.be (S.B.); peter.goethals@ugent.be (P.G.)
[2] Department of Fisheries, Wetlands and Wildlife Management, Bahir Dar University, P.O. Box 5501, 6000 Bahir Dar, Ethiopia
[3] Department of Zoological Sciences, Addis Ababa University, P.O. Box 1176, 1000 Addis Ababa, Ethiopia; abebe12002@yahoo.com
[4] Department of Biology, Bahir Dar University, P.O. Box 79, 6000 Bahir Dar, Ethiopia; wassie74@gmail.com
* Correspondence: shewitlove@gmail.com

Abstract: Fisheries play a significant role in the livelihoods of the world population, while the dependence on fisheries is acute in developing countries. Fisheries are consequently a critical element for meeting the sustainable development (SDG) and FAO goals to reduce poverty, hunger and improve health and well-being. However, 90% of global marine fish stocks are fully or over-exploited. The amount of biologically unsustainable stocks increased from 10% in 1975 to 33% in 2015. Freshwater ecosystems are the most endangered ecosystems and freshwater fish stocks are worldwide in a state of crisis. The continuous fish stock decline indicates that the world is still far from achieving SDG 14 (Life Below Water), FAO's Blue Growth Initiative goal and SDG 15 (Life on Land, including freshwater systems). Failure to effectively manage world fish stocks can have disastrous effects on biodiversity and the livelihoods and socio-economic conditions of millions of people. Therefore, management strategies that successfully conserve the stocks and provide optimal sustainable yields are urgently needed. However, successful management is only possible when the necessary data are obtained and decision-makers are well informed. The main problem for the management of fisheries, particularly in developing countries, is the lack of information on the past and current status of the fish stocks. Sound data collection and validation methods are, therefore, important. Stock assessment models, which support sustainable fisheries, require life history traits as input parameters. In order to provide accurate estimates of these life history traits, standardized methods for otolith preparation and validation of the rate of growth zone deposition are essential. This review aims to assist researchers and fisheries managers, working on marine and freshwater fish species, in understanding concepts and processes related to stock assessment and population dynamics. Although most examples and case studies originate from developing countries in the African continent, the review remains of great value to many other countries.

Keywords: life history traits; methods comparison; population imbalance; stock assessment

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1. Introduction

Human connections to fisheries have developed over thousands of years, underlining the notable contribution of fish and fisheries to human well-being. Globally, millions of people directly or indirectly depend on fisheries for their employment, income and food security [1–3]. This dependence is acute in developing countries, particularly for poor and marginalized people [4,5]. The opening up of global markets for fish and fisheries products have created multiple opportunities to increase employment and income from fisheries [6]. Total employment in fisheries grew increasingly from 28 million in 1995 to 39 million in 2010 [3] (Figure 1). In 2016, more than 40 million people were involved in fisheries, with 79% and 13% of these 40 million people living in Asia and Africa, respectively [3]. The involvement of people in fisheries in developing countries has been growing steadily, while it has been declining in most developed countries [7], indicating that fisheries play a significant role in developing countries. Whereas men are primarily involved in fishing, women are heavily involved in fisheries-related activities such as processing and trade [8]. In developing countries, particularly in Asia and Africa, millions of women are involved in fish processing, marketing, making and repairing nets, making baskets, pots, and baiting hooks [7].

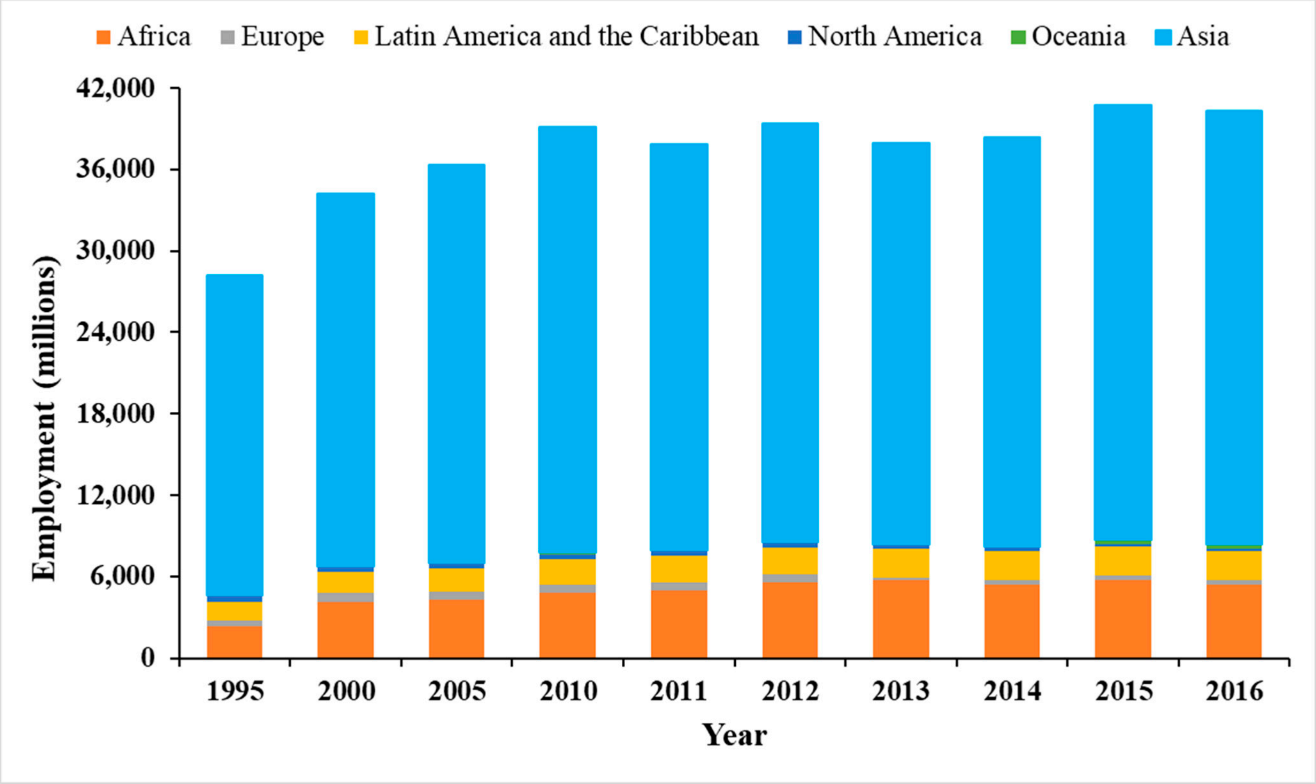


Figure 1. The global role of the fisheries sector for employment (Source: [3]).

Fisheries have an important economic contribution worldwide. Fisheries products are among the most traded food items and play a vital role in the global and local economy. For example, in 2016, approximately 60 million tonnes of fish and fish products (35% of global fish production) entered international trade in various forms [3]. This represents a total increase of 245% compared to 1976, but if we consider the trade in fish for human consumption alone the increase is more than 500% [3]. The value of global fish products also grew significantly from USD 8 billion in 1976 to USD 152 billion in 2017 [3]. This value surpasses the combined value of the net exports of rice, coffee, tea, tobacco and meat of that same year [3]. Fisheries play an important role in the national economies of many developing countries through the generation of foreign exchange derived from international trade. Fish production exports from developing countries account for approximately 60% of the total fish production being traded internationally [7]. Fish trade by developing countries increased from less than USD 4 billion in 1980 to USD 18 billion in 2001 [9]. Thus, fisheries are playing an increasingly important role in the national economy of many developing countries. Additionally, since the majority of the people involved in fisheries are from developing countries, fisheries are essential to keep households and communities out of poverty and improved fisheries management has the potential to further reduce poverty. The rural poor and marginalized people employed in fisheries could use the income earned from the sector to buy basic needs for living and to send their children to school. Fisheries have several valuable socio-cultural aspects. Understanding the socio-cultural values associated with fish and fisheries is, therefore, vital for effective management of the resources [10]. Ignoring these values could reduce the social acceptability of the management options [11].

Of the seven billion people in the world, one billion are officially designated as starving [12] and two billion people suffer from micronutrient deficiency [13]. Although the improvement of agriculture practices has been identified as essential to overcome the looming food security crisis, fisheries can also make a significant contribution [14]. Fish is an important and affordable source of protein, essential micronutrients, and fatty acids, especially for people in developing countries [15,16]. Fish consumption has been associated with various human benefits such as child mortality reduction, and maternal health improvement [7]. The annual global fish production for human consumption has increased from 67% of the total fish production in the 1960s to 88% in 2016 [3]. Fish accounted for 17% of animal protein consumed by the world population [3], the majority of which was consumed in low-income food-deficit countries [1,17,18]. Generally, fisheries are important to address hunger, micronutrient deficiencies and food insecurity [1], underlining its vital role in meeting the sustainable development goals (SDGs 1 = no poverty, 2 = zero hunger, 3 = good health and well-being) and FAO’s goal of a world without hunger and malnutrition.

Despite the significant contribution of fisheries to livelihoods, employment and income, many of the global fish stocks have been declining drastically. Successful management of the fish resources is therefore crucial. Failure to effectively manage world fish stocks can have disastrous effects on biodiversity and the livelihoods and socio-economic conditions of millions of people who are strongly dependent on these resources. Management strategies that successfully conserve the stocks and provide optimal sustainable yields are urgently needed. Successful management is possible when the necessary data such as age, growth, mortality and maximum yield are obtained, processed and interpreted and decision-makers are well informed. Fisheries managers, particularly from developing countries, are faced with many challenges due to lack of information on the past and current status of fisheries and the fish stocks. Although stock assessment modelling is necessary (i) to provide answers to questions about the current status of the stock, (ii) to predict the effect of current and future management measures and (iii) to support sustainable fisheries by providing fisheries managers with necessary advice to make informed decisions (Figure 2), such studies are limited in developing countries. To understand the factors affecting fish population imbalance, a good understanding of the wide range of age determination and validation techniques is required. They provide valuable input parameters for further stock assessment evaluation. However, the majority of young researchers in developing countries have limited skills and knowledge on how to select and prepare ageing hard structures and to validate measurements. Although there have been several well-documented methodological studies in developed countries that can be used as a reference, most of them are not open access. This hinders fish resources managers and young researchers in developing countries from accessing these documents. Therefore, the aim of this study is to provide an accessible review to fisheries managers and young researchers from developing countries. The review focuses on the factors that affect fish population imbalance, the different ageing hard structures, optimal otolith preparation and age validation techniques and their limitations and advantages. The review provides essential information to illustrate the need for reliable methods for life history trait estimation and evidence-based fisheries management. Therefore, this review aims to assist researchers and fisheries managers, working on marine and freshwater fish species, in understanding concepts and processes related to stock assessment and population dynamics. Although most examples and case studies originate from developing countries in the African continent, the review remains of great value to many other countries.

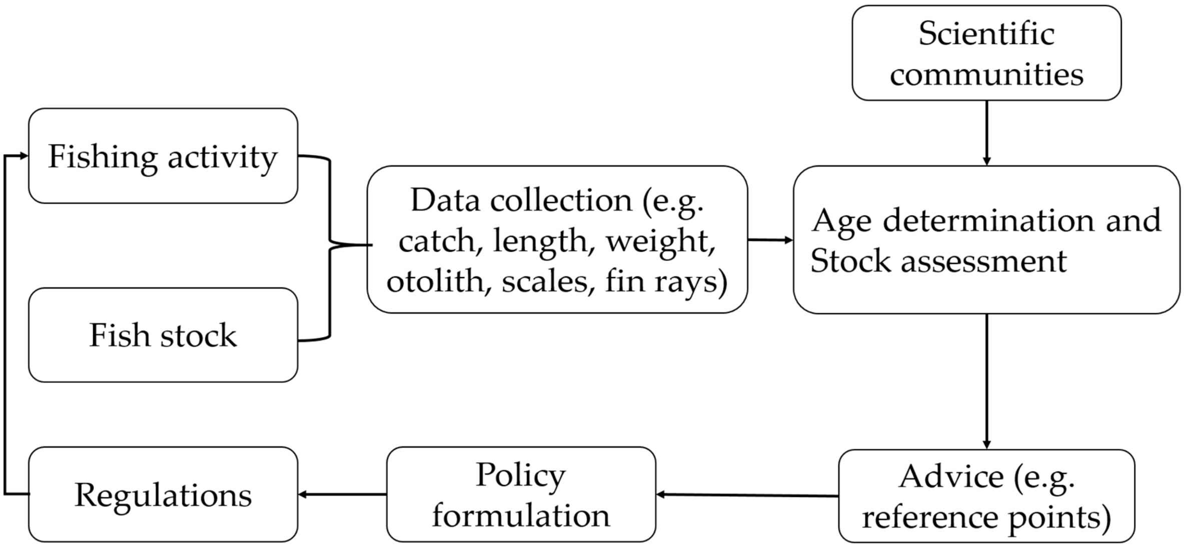
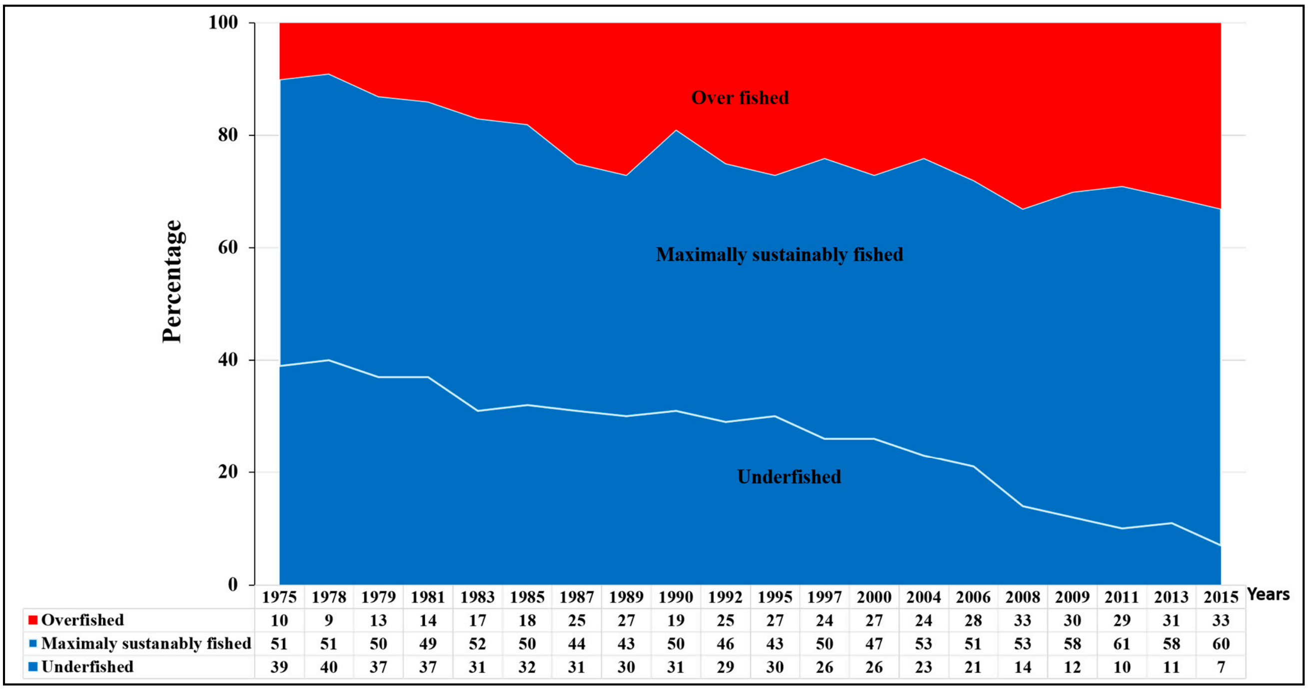


Figure 2. The process of fisheries data collection for age determination methods and stock assessment modelling to provide important advice to develop regulations and policies for sustainable fisheries management.

2. Global Fish Stock Status

Global fish stocks have been declining drastically. This decline has been attributed to several pressures, particularly the rapid increase of fishing efforts to feed the rapidly increasing human population [3]. Currently, 90% of global marine fish stocks are fully or over-exploited [3]. The size of biologically sustainable marine fish stocks (i.e., maximally sustainably fished and underfished stocks) decreased from 90% in 1975 to 67% in 2015, while the size of biologically unsustainable stocks (i.e., overfished stocks) increased from 10% in 1975 to 33% in 2015 [3] (Figure 3). Since there are no new fishing grounds to be exploited [3,19] and the current world human population is predicted to exceed nine billion by 2050, there will likely be more pressure on the stocks.



	1975	1978	1979	1981	1983	1985	1987	1989	1990	1992	1995	1997	2000	2004	2006	2008	2009	2011	2013	2015
🟥 Overfished	10	9	13	14	17	18	25	27	19	25	27	24	27	24	28	33	30	29	31	33
🟦 Maximaly sustanably fished	51	51	50	49	52	50	44	43	50	46	43	50	47	53	51	53	58	61	58	60
🟦 Underfished	39	40	37	37	31	32	31	30	31	29	30	26	26	23	21	14	12	10	11	7

Figure 3. The trend of the global marine fish stock (source: [3]).

Freshwater ecosystems, one of the most important life-support systems on Earth, are the most endangered ecosystems in the world [20]. Freshwater ecosystems are vulnerable to changes in the basin [21,22] originating from agriculture, mining, urbanization and dam and diversion weirs construction [23]. Anthropogenic pressures cause changes in the physical, chemical and/or biological components of the freshwater ecosystems when the carrying capacity of ecosystems decreases below the ability to absorb stress. Freshwater fish stocks are in a state of crisis worldwide [24]. The perceived increase is caused by changes in the monitoring and measuring rather than actual changes in fisheries production [3]. This is corroborated by the drastic decline in abundance and diversity of the different freshwater fish species due to the increased anthropogenic pressures [20,25–33]. Furthermore, ≥65% of the inland water habitats are moderately or highly threatened [27], suggesting that some of the fish species inhabiting these systems are at high risk of extinction. According to World Wide Fund for Nature (WWF [34]), the current rate of fish population decline in freshwater systems is two times the rate for marine systems.

There are many examples of excessive stressors with negative consequences. For example, the catches, diversity and composition of the fish communities, particularly the most commercially valuable species, in many African lakes such as Lake Malawi, Lake Tanganyika, Lake Victoria and Lake Tana have markedly declined due to overexploitation, illegal fishing, the introduction of exotic species and environmental degradation [31,33,35–38]. A recent assessment by the International Union for Conservation of Nature (IUCN) revealed that 9% of the 458 fish species inhabiting Lake Malawi are at high risk of extinction [39]. Studies indicated that in Lake Malawi, long-living, slow-growing and late-maturing species have been depleted [40–44] while the biomass of the endemic *Oreochromis* species (chambo), has been declining rapidly [45,46]. There are strong signs of overfishing for chambo, the most valuable component of the lake fishery [47]. The chambo fishery in Lake Malawi has declined from 5000 tonnes per year in 1992 to less than 2000 tonnes per year in 1999 [48]. Similarly, in response to intensified pressures, like eutrophication and overfishing, fish stocks in Lake Victoria have changed both in composition and abundance [49]. Currently, more than 76% of the fish species in Lake Victoria face extinction [50]. The abundance and diversity of fish species in Lake Turkana drastically declined due to degradation of the littoral habitats and flood pulse breeding areas caused by upstream development and climate change [51]. Fisheries in the major river basins such as the Zambezi River system have experienced drastic declines in catch rates, changes in fish communities and loss of valuable species [52]. Most of the fish communities in the inland waters of Ethiopia are showing signs of overfishing [25,53–55]. Anthropogenic pressures in and around the inland waters negatively affect the survival of fish species in Ethiopia. For example, the abundance and size of the fish populations in Lake Tana have been declining drastically due to overexploitation, agriculture and dam constructions [31–33]. As a result, the catch per unit effort (CPUE) of the endemic *Labeobarbus* in the lake decreased from 63 kg/trip in 1991–1993 [56] to 2 kg/trip in 2016–17 [55]. Currently, five *Labeobarbus* species are already reported as IUCN red-listed species [57]. This number will likely increase even more in the near future as the present status of many species have not been evaluated yet. Furthermore, the predominance of small-sized species with little economic importance over large-sized species with high economic importance has been reported [53]. This suggests overfishing of the system. Due to overfishing, the proportion of large and valuable species decreases in favour of small and less valuable species [58]. Therefore, the continuous fish stock decline, particularly in developing countries indicates that the world is far from achieving the SDGs. Especially, SDGs target 14.4 to end illegal, unreported and unregulated (IUU) fishing by 2020 will not be met at all. Additionally, the world human population is rapidly increasing, leading to a higher demand for fish, which poses a hurdle for the FAO's Blue Growth Initiative goal. This goal aims to maximize the goods and services provided by the different ecosystem types without compromising the social and economic benefits the systems offer [3].

3. Dynamics in Fish Population Size and Life History Traits

3.1. Factors Affecting Fish Population Imbalance

Fish population dynamics are primarily affected by three factors: (1) recruitment, (2) growth and (3) mortality rates [59]. The recruitment is defined as the number of individuals born within a given period. Growth is the increase in length and weight of the individuals of a population in a given period of time, and mortality is the number of individuals removed from the population within a given period of time (Figure 4). Recruitment and growth increase the fish population in number and biomass, while mortality, due to fishing and/or natural causes, decreases the population both in number and biomass. Illegal, unreported and unregulated (IUU) fishing increases fishing mortality and has adverse effects on the abundance and size composition of fish populations. Fishers often target the spawning biomass, causing high mortality rates which in turn leads to drastic reductions in the abundance of recruits and mega-spawners. Furthermore, small mesh sizes, typically used for illegal fishing, are known to have negative effects on the size distribution and maturation of fish [60,61]. For example, the majority of the specimens of *Labeobarbus* species in Lake Tana caught using $\leq$8 cm mesh size gillnets had fork length less than the size at first maturation ($FL_{50\%}$) [55]. The dominance of immature individuals ($<FL_{50\%}$) in the catch confirms the expected negative effect of small mesh size gillnets. Additionally, specimens of *Labeobarbus* with $\geq$40 cm fork length (FL) were more often recorded in the late 2000s [62,63] than in 2016/17 [55]. The absence of large-sized specimens (mega-spawners) is the result of destructive fishing activities targeting the spawning biomass and causing environmental degradation. Climate change also has the potential to affect freshwater fish resources, especially the mega spawners [64–67]. The reduction of mega-spawners in a stock is detrimental to the long-term survival of fish populations due to (i) their high fecundity, which creates a greater chance of survival to larvae [68,69], (ii) their ability to serve as reservoirs and distributors of desirable genes [70], and (iii) their ability to act as a natural safeguard against subsequent recruitment failure [71,72]. The presence of enough mega-spawners can be used as a simple estimator of the resilience of stocks against random disturbance events [70]. The presence of 30–40% of mega-spawners in stock indicates a healthy size and age structure of the population, while <20% could be a matter of concern [70]. In general, the fish population decreases if the addition to the population by recruitment and growth is smaller than the removal from the population due to mortality. Thus, the current drastic decrease in the global fish population suggests that mortality (fishing and/or natural) is exceeding recruitment and growth. Understanding the major factors that cause fish population imbalance is therefore vital for a sustainable exploitation of fisheries.

Size structure indices are useful to evaluate the status of the fish population and identify the pressures that affect the population dynamics [73–76]. For example, analysis of length-weight relationships for a species can provide fundamental insights into the ecology, population dynamics, and management of that species. Understanding how the weight of fish changes as a function of length is useful to predict weight from the length of the fish and determine the growth type and relative condition of the fish population. Use of the size structure indices as potential indicators of fish population imbalance has gained popularity because of their connections with recruitment, growth and mortality [73,75,77]. For example, the proportion of small size individuals in the population might be higher than the proportion of large size individuals due to high recruitment, slow growth and/or high mortality rates of large size classes and vice versa [73,74].

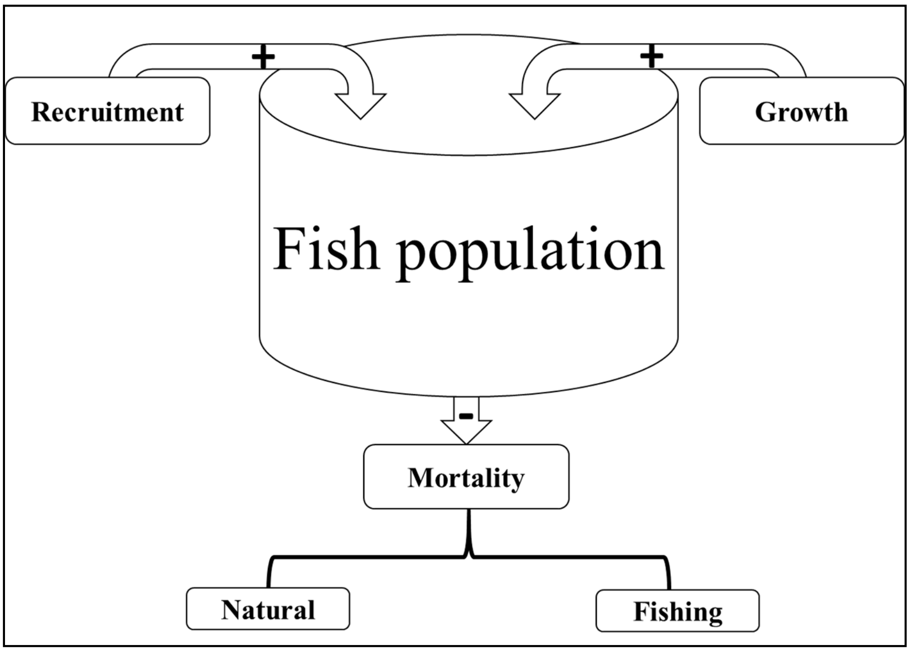


Figure 4. Illustration of the fish population dynamics. The "+" sign indicates an addition to the population, while the "–" sign indicates removal from the population.

3.2. Life History Traits as a Basis for Stock Assessment and Fisheries Management

The current world human population is rapidly increasing, although the world fish stocks have been declining drastically and there are no new fishing grounds to be exploited [3,19]. The rapid world human population increase will likely cause high fish consumption demand which in turn will increase the pressures on the stocks. Fisheries managers are facing many challenges as fish stocks continue to decline and IUU fishing undermines the sustainability of fisheries. The main problem of fisheries managers, particularly in developing countries, is the lack of appropriate fisheries data for accurate stock assessment modelling. Stock assessment models provide answers to questions about the current condition of the stock and allow for predictions about how the stock will respond to current and future management measures. Additionally, stock assessment supports sustainable fisheries by providing fisheries managers with vital advice to make informed decisions. However, stock assessment models require life history traits such as age, growth and mortality rates as input parameters [78–80]. In fisheries science, age is one of the most influential life history traits that is primarily used to estimate life history traits such as age at maturity, growth rate, mortality rate and population analysis [79]. The importance of life history traits for fisheries assessment and management is presented in Table 1 [59].

In fisheries science, the collection, preparation, and interpretation of different hard structures provide a means for age estimation [81]. Otoliths, scales and fin rays are the most commonly used hard structures for age estimation [79,82]. The choice of the most suitable hard structure for estimating age is guided by several factors: (1) the ease of obtaining the hard structure, (2) growth of the structure itself and the formation of growth zones on the structure, (3) difficulties in preparation of the hard structure and growth zones interpretation and (4) accuracy and precision of the age estimates derived from the hard structures [80]. Therefore, understanding the advantages and limitations of each structure (i.e., otoliths, scales and fin rays) is indispensable to make the correct choice. In the next sections these structures are discussed more in depth.

Table 1. The importance of life history traits for assessment and management use.

Life History Traits	Assessment and Management Use
Age data (i.e., individual age)	Used to determine the age at first maturity and to estimate growth parameters
Age and length at first maturity	Used to establish a minimum size limit to ensure that individual fish can reproduce at least once before being harvested
Maximum age (i.e., longevity)	Used to estimate the natural mortality rate and set the maximum age in the stock assessment models
Asymptotic length ($L\infty$)	Used to set size limits (i.e., restrict mesh sizes and prohibit fishing gears)
Growth rate (K)	Indicates how fast the fish will reach the fisher's minimum size limit
Mortality rates (i.e., natural and fishing) and spawning behaviour	Highlights the area and time of increased vulnerability to harvest and can be used for seasonal or area closures to protect the spawning biomass
Sex ratio	Used to understand the relationship between individuals, the reproductive potential of the species and the state of the population
Habitat preference	Helps to identify areas for protection

3.3. Advantages and Limitations of the Hard Structures Used for Age Estimation

3.3.1. Otoliths

Otoliths are calcium carbonate structures that aid in balance and hearing of fish [81]. Additionally, otoliths record a remarkable amount of information about the life history traits of the fish and the environments they are living in [83]. To better understand and manage the fish population, this information should be carefully assessed, interpreted and incorporated into fisheries management decision-making. Of the three existing types of otoliths, sagittal otoliths are used for the age estimation of most fish species [84–86], but asteriscus otoliths are the most suitable structure for the Cyprinidae family [87–89]. The calcium carbonate that is used to form the otoliths originates from the water and from the food of the fish. This process is influenced by fish metabolism. During seasons with adequate average temperatures and sufficient food availability, fish grow at a relatively fast rate causing formed rings to be widely spaced. However, during the colder months where there is limited food supply, particularly for fish species in temperate regions, growth is restricted leading to narrow ring formation. As a result, alternate opaque and translucent growth zones are formed, which are considered to have been formed in one year (i.e., annulus). The age estimates of the fish can be obtained by counting the number of annuli deposited on otoliths. Regardless of its effort and cost, ageing accuracy is much higher for analysis of growth zone deposition on otoliths compared to the length and otolith size-based methods [90].

The use of otoliths for precise and accurate age estimation has several advantages: (1) otoliths grow continuously and form annuli even when the body growth slows down and the asymptotic length has been reached, (2) metabolically they are inert and not subject to resorption, (3) otolith growth varies between seasons leading to the formation of annual increments that can be used for age estimation, and (4) annuli reabsorption does not appear to occur during periods of food limitation or stress [79,88,91]. However, the use of otoliths also has limitations [92]. Age estimation using otolith is laborious, time-consuming, expensive and is dependent on the skills and experience of readers, which limits the sample size and prevents researchers with limited skills and experience to use otolith for age estimation [93,94]. Additionally, otoliths require sacrificing fish, which makes this approach difficult to be applied for threatened species or small populations [95].

3.3.2. Scales

The age of fish can also be determined by scales as the successive rings (circuli) are formed as the fish grow. The ctenoid and cycloid scales are most often used for estimating fish age [84]. Although scales from the whole part of the fish can be used, those that are found on the shoulder between the head and the dorsal fin are generally the best because of their relatively large size and low sensitivity to damage [80,84]. Traditionally, due to their non-lethal nature, scales have long been considered the most efficient and suitable structure for age determination, but more recent studies have revealed them to be inaccurate [96–99]. The limitations of scales to yield precise and accurate age estimates have been found most severe for slow-growing and older fish [79,98,99]. Scales have various inconsistencies, which make them difficult to read and interpret visually [100]. For example, well-defined marks on one scale might be absent on the neighbouring scales of the same fish [100]. Scales have several additional limitations: The first limitation is the dependency of scale growth and patterns of the circuli formation on fish growth. The variability in fish growth (i.e., between young and old fish) affects the scale growth and the appearance of the circuli. Scale growth is minimal or non-existent after the onset of maturity, particularly when fish growth is very low or ceases [84]. Thus, this causes underestimation of the actual fish age, particularly in older fish. In older fish, the circuli at the edge of the scale can be crowded making the circuli interpretation difficult. The second limitation is resorption causing some reworking or breaking of the circuli, leading to misinterpretation. The third limitation is transparency, which makes the circuli difficult to observe. The fourth limitation is that damaged or removed scales can be regenerated, resulting in growth patterns that do not accurately reflect the age of fish. The fifth limitation is that either some fish have no recognizable pattern on their scales or entirely lack scales. Therefore, when scales are used for age determination, either the age of all groups in the population should be validated, which is difficult if not impossible, or an alternative aging method should be used for older individuals in the population [80,84].

3.3.3. Fin Rays

Compared to otoliths and scales, fin rays are not frequently used for age estimation. However, the suitability of fin rays for some fish species is reported by some researchers [101–104]. The most commonly used fin rays are the dorsal, pectoral, and, pelvic fins [84]. Age estimates from fin rays have higher precision and accuracy compared to the estimates from scales [96,105,106]. Most importantly, using fin rays does not require the fish to be sacrificed [107,108] and the annuli remain representative for the age of older fish [109,110]. However, the low precision and accuracy of these structures have also been reported [111–113]. Fin rays provide inaccurate age estimates due to the following reasons: (1) difficult to read and interpret annular marks, (2) early marks are sometimes obscured by the vascular core of the fin rays, (3) irregular and unexpected spacing of annuli on the fin rays sections, which suggests resorption at different rates in different years, (4) difficult to distinguish between the true and false annulus and to correctly identify the first annulus, and (5) its preparation requires special technical skills [100,112,113].

4. Precision and Accuracy of Otolith Preparation Methods

Although there are several calcified structures available for age estimation [79,82], otoliths often provide the most precise and accurate age estimates [79,114]. However, age estimation based on the analysis of otolith growth zones involves judgment and subjective interpretation [79,81,115]. The subjective interpretation of otoliths contributes to two major sources of errors involving both processing and interpretation [79,116]. The first source of error relates directly to the nature of the otolith structure being interpreted. In this regard, otoliths must satisfy the criteria outlined in [117]: (1) otoliths must display growth increments that can be quantitatively resolved, (2) the formation of growth zones must conform to a regular and determinable time scale, and (3) otoliths must grow continuously throughout the lifespan of the fish. The second source of error can be traced back to the preparation of otoliths, interpretability of growth zones and reader experience [79,116,118,119]. The interpretation error can be minimized by describing a standardized, precise and accurate otolith preparation method and by training the age readers [79,116,118,119]. If the otolith images used for age estimation have the clearest view of the growth zones, core and edge, the bias between age readers should be minimal. It is, therefore, useful to describe the most adequate preparation method for each species. To this end, different otolith preparation methods such as transverse sections, staining, burn-and-breaking, polishing and whole otolith submerging in different substances including water and glycerol have been compared in attempt to describe the best method. Additionally, bias among hard structures such as scales, otoliths and fin rays has been compared to select the most appropriate structure. Such comparisons are especially important to approximate the accuracy of age estimates [84,120]. The precision and bias among different aging methods and age readers are usually done using statistical methods, graphical approaches, precision indices and qualitative expressions. Average percentage error (APE) [121] and coefficient of variation (CV) [122] are widely used and the most suitable and statistically sound measurements of precision [79]. The APE and CV are computed by the following formula:

$$\mathrm{APE} = \frac{100}{\mathrm{N}} \sum_{\mathrm{J}-1}^{\mathrm{N}} \left(\frac{1}{\mathrm{R}} \sum_{\mathrm{i}-1}^{\mathrm{R}} \frac{\left| \mathrm{X_{ij}} - \mathrm{X_j} \right|}{\mathrm{X_j}} \right) \tag{1}$$

$$\mathrm{CV} = \frac{100}{\mathrm{N}} \sum_{\mathrm{J}-1}^{\mathrm{N}} \left(\frac{\sqrt{\sum_{i=1}^{R} \frac{\left(\mathrm{X_{ij}} - \mathrm{X_j}\right)^2}{R-1}}}{\mathrm{X_j}} \right) \tag{2}$$

where N is the number of fish aged, R is the number of times fish are aged, $\mathrm{X_{ij}}$ is the ith age determination for the jth fish, and $\mathrm{X_j}$ is the average estimated age of the jth fish.

Although there is no rule of thumb, Campana [79] suggested CV $\leq$ 7.6% and APE $\leq$ 5.5% as reference values. The method with the smallest APE and CV values is, therefore, the most optimal method. The systematic bias between age readers, aging structures and aging time (i.e., if the reading is made two times by the same reader) can be described using a test of symmetry and it is best described through an examination of an age-agreement table [123]. The age estimates from the most experienced reader or the structure thought to be the most accurate should be used as the column variable in the age-agreement table. If the reading is made two times by the same reader, the first reading should appear as the column variable. Although several statistical methods were capable of detecting systematic aging differences, they were incapable of detecting both linear and non-linear biases in aging [115]. Some statistical methods, for example, were not sensitive enough to detect if the ages of younger fish were systematically over-aged or if the ages of older fish were systematically under-aged. To address this problem, Campana, et al. [115] introduced the age-bias plot to visually assess the differences in paired age estimates from two structures, two readers, or one reader at two times. Later, Ogle [124] modified the original age-bias plot in several ways. For the age-bias plot, one set of age estimates serve as reference age (x-axis). The age estimates that are thought to be most accurate are usually used as reference age. Thus, when the bias between age readers has comparable age estimates, the estimates of the most experienced reader should be used as reference age, whereas if the bias between two preparation methods or structures is compared, age estimates from the method or structure that is thought to be the most accurate should be used as reference age. However, the first reading should be used as reference age, if two readings from the same reader are made. Additionally, other factors such as qualitative expression may be involved as well. A quality control criteria (i.e., Q1 = readable otoliths with minimum bias, Q2 = readable otoliths with moderate bias and Q3 = unreadable otoliths) should be used to analyse readers' confidence. The method with the highest number of otoliths under Q1 has a higher readers' confidence than the other methods. The processing time and reading time should also be recorded. If there is no difference in precision and accuracy, the method that has the shortest processing time, the shortest reading time and the highest reader's confidence should be considered as the most optimal method.

The process of growth zone deposition on otoliths is affected by biological and environmental factors [125]. The rate of growth zone deposition on otoliths is either annual or biannual. Therefore, in age estimation studies, validation of the rate of growth zone deposition is essential. Although several methods to validate age or the rate of growth zone deposition are available, mainly marginal increment analysis and edge analysis are used [79]. For more detailed information, see literature elsewhere [79,81,84,115,124].

In developed countries, significant and extensive work has been done to standardize otolith preparation methods, validate age or the rate of growth zone deposition and estimate life history traits of fish [83,87,118,121,126–136]. Such studies are limited in developing countries such as African countries. Except for the limited efforts in South African and Ethiopian water bodies [85,86,89,137–146], many fish species in the African water bodies including the Great African Lakes remain poorly studied. Concerning the description of optimal otolith preparation methods, validation of the rate of growth zone deposition and estimation of life history traits. The present lack of information on life history traits of different fish species hinders scientists and fisheries managers from refining optimal strategies for their conservation. Thus, detailed information on the description of the optimal otolith preparation method and validation of the rate of growth zone deposition is crucial. The widely used methods to validate the rate of growth zone deposition are discussed in the next section.

5. Validation of the Rate of Growth Zone Deposition

Validation of the rate of growth zone deposition is indispensable for accurate age estimation. There are several validation methods including advanced methods such as radiochemical and bomb radiocarbon dating. However, since these advanced methods are very expensive and difficult to apply for short-living species, mark-recapture of chemically tagged fish, marginal increment analysis and edge analysis are often used to validate the rate of growth zone deposition [79]. In this section only these widely used methods are discussed, for information about the other validation methods see Campana [79], Green, et al. [81], Andrews, et al. [130] and Piddocke, et al. [147]. A summary of the different methods used to validate age or the rate of growth zone deposition is presented in Table 2.

Table 2. Advantages, limitations, precision, sample size and cost of the different methods used to validate age or the rate of growth zone deposition. Methods are listed regardless of any scientific value. (Source: [79,147]).

Methods	Advantages	Disadvantages	Precision	Sample Size	Cost
Mark-recapture	Validates the frequency of post-tagging growth increments.	Low or non-existent of recaptured fish at liberty (>1 year). Difficulty to identify a post-mark annulus	±1 year	>1	Minimal excluding cost of tagging cruise
Marginal increment analysis and Edge analysis	Validates frequency of growth increments formation.	Applicable to fast-growing or young fish. A year-round sample is required.	±1 year	>100	Minimal other than a fish collection
Radiometric	Validates absolute age. Applicable to any recently collected data. Suitable to long-lived fish	Can only distinguish between widely divergent age estimates	±25–50%	10–50	~$1000 per age category
Radiocarbon	Validate both absolute age and frequency of growth increments. Suitable for long lived fish. Does not require recently collected sample.	Some of the fish in the sample must be hatched before 1965.	±1–3 years	20–30	$700–$1000 per otolith

5.1. Mark-Recapture of Chemically Tagged Fish

At the moment this method is one of the best and most cost-effective methods available to validate the rate of growth zone formation [79]. It can be applied through various methods such as injection, immersion and feeding. Injection is the most common technique for tagging wild fish [148–150]. Fish species that are captured from the wild are injected with calcium-binding chemicals such as oxytetracycline (OTC), alizarine, calcein and strontium immediately at the time of tagging [125]. These chemicals are incorporated into otoliths shortly after injection. The permanent mark is visible under fluorescent light in the growth zone being formed at the time of tagging [79]. The rate of growth zone deposition can be determined based on the number of growth zones deposited distally to the mark in the recaptured fish and the time at liberty. If the difference in the time of injection and liberty is one year and one growth zone is deposited during this time, it means that the studied fish species deposited one growth zone per year. However, if two growth zones are deposited, it means the rate of growth zone deposition is biannual. This method has been applied to validate the periodicity of growth zone deposition in several fish species [141,143–145,151–154]. The growth zones being validated are formed while the fish is growing in the natural environment. This method is time-consuming, technically difficult to apply and the recovery rates of the tagged fish are usually low [155]. Additionally, since the numbers of growth zones formed after tagging are low, a wrong conclusion can be made on the rate of growth zone deposition, if one of the growth zones is misinterpreted.

5.2. Marginal Increment Analysis

Marginal increment analysis (MIA, linear-circular model) is the most widely used validation method due to its modest sampling requirements and low cost [79]. The MIA is based on the observed incremental patterns of growth zones throughout the year and assumes that the outermost increment displays a yearly sinusoidal cycle when plotted against months of capture [156,157]. It uses the ratio of the width of the last growing zone and the width of the last fully completed growth zone (MIR) as a dependent variable and months of capture as an independent variable [158–160]. The marginal increment ratio (MIR) is, therefore, computed as follows [161].

$$\mathrm{MIR} = \frac{\mathrm{R} - \mathrm{r_n}}{\mathrm{r_n} - \mathrm{r_{n-1}}} \tag{3}$$

where R is the distance from the core to the outermost of the edge, r_n is the distance from the core to the end of the growing zone and r_{n-1} is the distance from the core to the end of the last fully formed growth zone.

When the MIR value is equal to one, it indicates the completion of growth zone formation. Although the MIA is a useful method, especially when supported by other validation methods [159], it is also susceptible to bias and misinterpretation if not applied rigorously [79]. The approach has several limitations. The extended time of sample collection (monthly at least for one year), high possibility to collect small sample size per size classes within each month, difficulties to objectively classify the edge types and substantial inter-individual variation in marginal increment appearance [79,147]. These limitations are more pronounced in older fish where growth increments become very thin and packed together [118,162]. Therefore, when MIA is applied as age validation, the following protocols should be applied. (1) samples must be completely randomized when assigned to the examiner, (2) a minimum of two complete cycles need to be examined, in accordance with accepted methods for detecting cycles, and (3) the results must be interpreted objectively [79]. All the described protocols and encountered limitations for this technique here are also applicable for the edge analysis approach described below [79].

5.3. Edge Analysis

Similar to the MIA, edge analysis (EA, binary-circular model) is also based on examination of the marginal increments. Its dependent variable is binary, the otolith edge types either opaque or translucent, while the month of capture is the independent variable. Analysis of the EA can, therefore, verify the hypothesis that growth zone deposition is either annual or biannual. This approach assumes that the density of the outermost margin (i.e., proportion of the translucent zone) exhibits a sinusoidal cycle when plotted against the months of capture [79]. Several researchers found this approach useful for validating the periodicity of growth zone deposition [89,143,161,163]. For example, the Edge analysis revealed an annual growth zone deposition for *Labeobarbus platydorsus* in Lake Tana [140] (Figure 5). Although the EA approach is cheap and logically simple, it is susceptible to bias and misinterpretation if not applied rigorously [79].

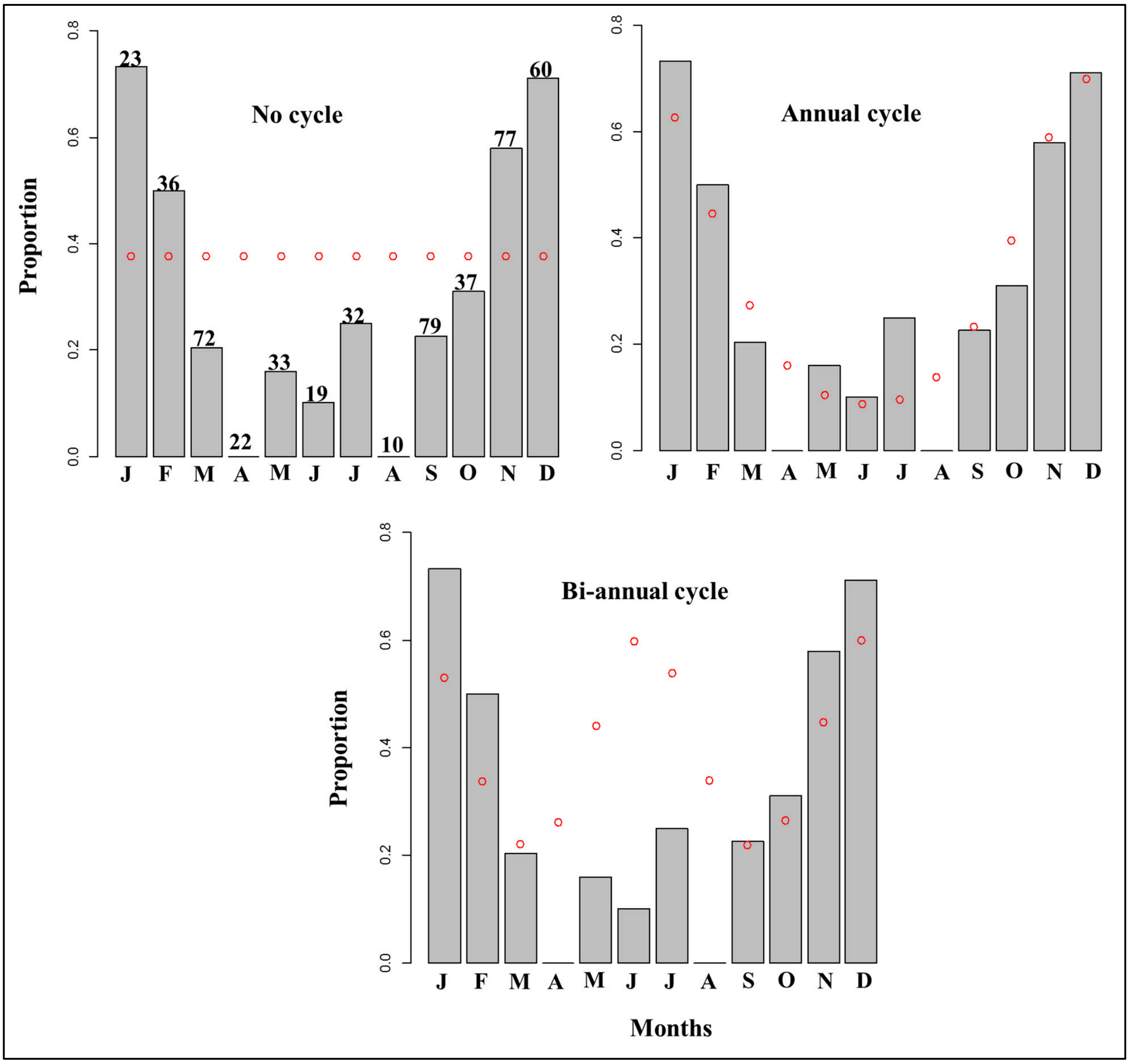


Figure 5. The proportion of asteriscus otoliths with a translucent growth zone on the edge for *Labeobarbus platydorsus* based on samples collected between May 2016 and April 2017 in Lake Tana, Ethiopia [140]. The bar graph denotes the proportion of the translucent growth zone and the open dot line represents the predicted model results. The numbers above the bars in the no cycle model are total sample size and the same sample size is used for the other models. The annual cycle mode best fit the data.

6. Conclusions

Fisheries management strategies must be developed to ensure that stocks are harvested at sustainable levels. Fisheries managers rely on age estimates to develop effective and sustainable management options. Accurate and precise age estimates can be obtained if and only if an appropriate otolith preparation method is described and the rate of growth zone deposition is properly validated. Age estimates combined with data such as fish length, weight and reproductive condition can be used to describe the structure and dynamics of the population considered to comprise the harvested stock. For example, longevity and growth rates are estimated using length and age data, while the combination of sex and reproductive condition with growth data are used to describe the age-fecundity relationship and sex-specific growth. Mortality rates are also computed by combining age estimates with counts of the number of fish per age class in a sample. These analyses provide researchers and fisheries managers with a range of information to derive sustainable harvest strategies through stock assessment evaluations. In order to avoid complete stock collapse, fisheries catch should not exceed the maximum sustainable yield of the stock (MSY). The MSY is an important tool to quantify the goal of a fishery and allows fisheries managers to evaluate the performance of the fishery. The comparison of the assessed state of the fish stock with the values of the fisheries reference points such as MSY supports the managers to make informed decisions. Thus, fisheries reference points should be calculated as correctly as possible. The most popular and widely used model to estimate the MSY is the yield-per-recruitment model, introduced by Beverton and Holt [164]. Understanding the population dynamics, age determination techniques, and the estimation of life history traits allow policymakers and fisheries managers to optimize future conservation strategies (Figure 6). Furthermore, monitoring and evaluating the effects of the major pressures such as pollution, habitat degradation and over utilization of aquatic resources is vital to provide insights into the changes of aquatic ecosystems and indicate their status (Figure 6).

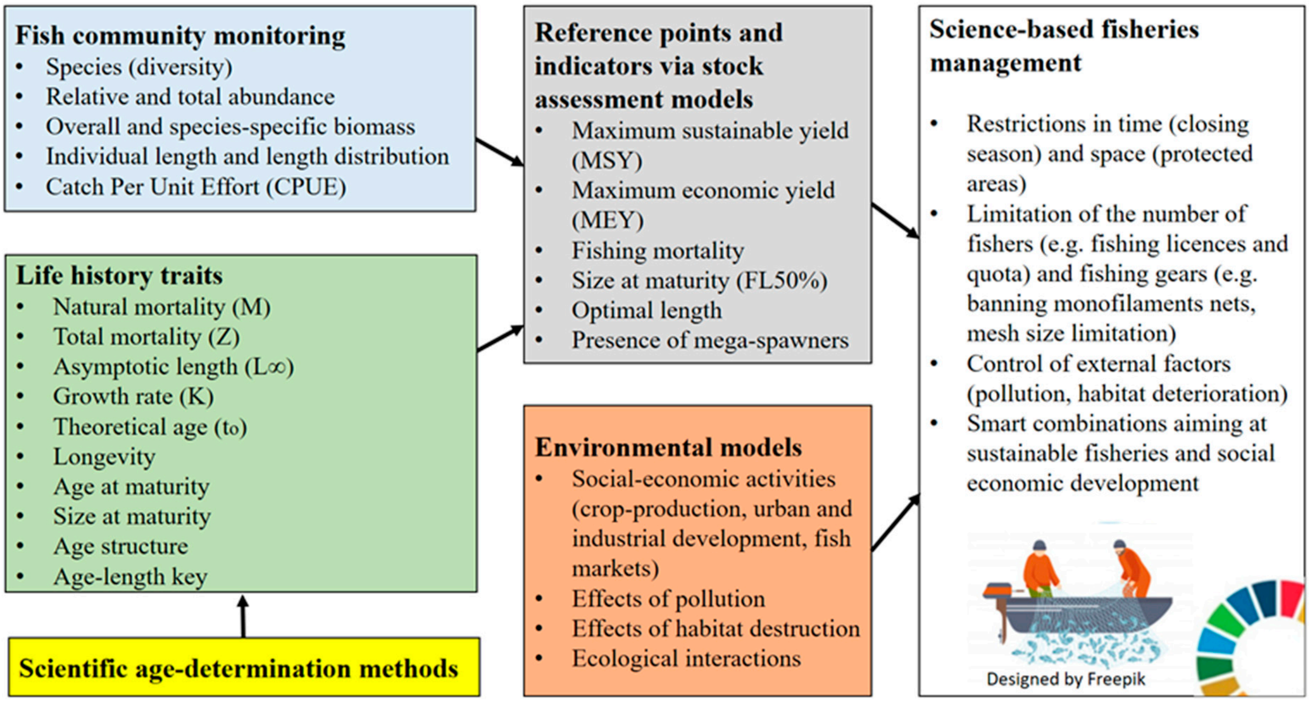


Figure 6. Schematic representation of fish community monitoring, fish stock assessment and environmental modelling to develop science-based fisheries management.

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Plasticity in Reproductive Traits, Condition and Energy Allocation of the Non-Native Pyrenean Gudgeon *Gobio lozanoi* in a Highly Regulated Mediterranean River Basin

Fátima Amat-Trigo [1], Mar Torralva [1], Daniel González-Silvera [2], Francisco Javier Martínez-López [2] and Francisco José Oliva-Paterna [1,*]

[1] Department of Zoology and Physical Anthropology, Faculty of Biology, University of Murcia, 30100 Murcia, Spain; fatima.amat@um.es (F.A.-T.); torralva@um.es (M.T.)
[2] Department of Physiology, Faculty of Biology, University of Murcia, 30100 Murcia, Spain; danielgs@um.es (D.G.-S.); javmaraq@um.es (F.J.M.-L.)
* Correspondence: fjoliva@um.es

Abstract: The invasion success of non-native fish, such as Pyrenean gudgeon *Gobio lozanoi* in several Iberian rivers, is often explained by the expression of its life history traits. This study provides the first insights into the reproductive traits, fish condition, and energy allocation (protein and lipid contents of tissues) of this species, along a longitudinal gradient in one of the most regulated river basins in the Iberian Peninsula, the Segura river. Larger sizes of first maturity, higher fecundity and larger oocytes were found in fluvial sectors with the most natural flow regimes, characterised by a low base flow with high flow peaks in spring and autumn. A delay in the reproductive period, lower fish condition and no differences in sex-ratio were observed in fluvial sectors with a high increase in base flow and notable inversion in the seasonal pattern of flow regime. Lipid contents in the liver and gonads were stable during the reproductive cycle and decreases in muscle were noted, whereas ovarian and liver proteins increased. In relation to energy allocation for *G. lozanoi*, an intermediate energy strategy was observed between income and capital breeding. Our results support the hypothesis that the high plasticity of *G. lozanoi* population traits plays a significant role in its success in a highly regulated Mediterranean river basin. Understanding the mechanisms by which flow regulation shapes fish populations in Mediterranean type-rivers could inform management actions.

Keywords: energy allocation; fecundity; flow regulation; Mediterranean-type river cyprinids; invasive fish

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1. Introduction

Flow regulation is one of the most widespread anthropogenic alterations in natural aquatic ecosystems and plays an important role in habitat development, food sources availability and the distribution of organisms [1,2]. There are many studies that confirm the impact of flow regulation (i.e., dams and weirs) on the structure and functioning of rivers, and in particular, how they affect populations of fish worldwide [3–5]. Stream flow is a factor which has been considered as an important force shaping fish population traits [6,7] and life-histories [8,9], and several flow alteration studies have already shown significant effects on population traits such as, for instance, growth and maturation [5], changes in the timing of spawning and spawning areas [10,11], recruitment failure [12,13] and even changes in reproductive traits [3,14].

In relation to reproductive strategies, nutrient acquisition and energy allocation to reproduction are essential for energy balance in order to meet survival, growth and reproduction demands and, consequently, to develop the most competitive strategy [7]. Thus, the management of energy reserves and allocation during the reproduction process determines the reproductive strategy [15]. Fish species that can use the energy previously stored in tissues for the development and maturation of reproductive features have been referred to as capital breeders [16,17], and this strategy is typical of total spawners or species with synchronous oocyte development [15]. Alternatively, income breeding strategists include species that are not able to store energy and where reproduction success is determined by the environmental resources at the time of reproduction [18]. This strategy is more common in many small, batch-spawning fishes with asynchronous oocyte development [15]. Between these two extreme strategies, some species show intermediate characteristics of both energy allocation strategies [19–21].

This study is focused on the reproductive strategy and energy allocation dynamics during the reproductive cycle of the Pyrenean gudgeon, *Gobio lozanoi* Doadrio and Madeira 2004 (Actinopterygii, Cyprinidae; Supplementary Material 1, Figure S1), which is an endemic species from the Iberian Peninsula and the south of France [22]. The species has been translocated into several Iberian catchments as live bait for angling and, nowadays, is widely distributed across the Iberian Peninsula, with established populations in many rivers [23,24]. Some authors consider this species as having a high capacity to spread and as being able to behave invasively, increasing its density rapidly and occupying new habitats [24]; a process which is probably favoured by river regulation and artificial impoundments [25]. It has already been suggested that this non-native species may have potential impacts on the environment and native species throughout several Iberian basins [23,25–27]. Some examples include interspecific competition for food resources [28,29] or disease transmission [30].

Freshwater biotas are especially vulnerable to new invasive fish, particularly in areas with high endemism, such as the Mediterranean basins [26,31,32]. The non-native populations of *G. lozanoi* have been previously classified as opportunistic strategists (sensu Winemiller and Rose [33]), but also as intermediate strategists because they use strategies ranging from periodic to opportunistic [34]. Thus, non-native populations of *G. lozanoi* are characterised by early maturity, low fecundity, multiple spawnings per year and have a long reproductive span [34]. However, there is a scarcity of studies that have dealt with the biology and reproductive traits of non-native populations of *G. lozanoi* [35–37]. Consequently, the negative effects of the species on native fish may not yet have been fully elucidated. In addition, no studies exist that have included a physiological approach to energy allocation dynamics in reproductive strategies.

A greater understanding of the phenotypic plasticity involved in the adaptation of non-native fishes to local conditions is an important tool for control programs [38]. According to Ribeiro and Leunda [39], there is a clear need for biological information about *G. lozanoi* population traits across the Iberian Peninsula and especially in its non-native river basins, which could be an important knowledge gap hampering effective control and management. Moreover, the life history variability of fish seems to play a key role in driving invasion success and significant intraspecific plasticity has often been observed in the process of acclimatization to new habitats [40,41]. However, nothing is known about the intraspecific variability of *G. lozanoi* along gradients in the same watershed or in terms of comparisons between populations located at different flow regimes. Taking into account that reproductive investment can be understood as the result of the energy balance between survival, growth and reproduction demands in order to achieve the most competitive strategy [7,15], the goal of this study was to analyse the reproductive traits and the energy balance of *G. lozanoi* in an invaded Mediterranean basin. The two main hypotheses proposed were: firstly, the reproduction strategy could show inter-population plasticity due to different flow scenarios and it is expected to be closer to an opportunistic strategy in fluvial sectors with the most unpredictable flow regimes. Secondly, energy allocation mechanisms should be closer to income breeding strategies according to its reproductive traits. For this purpose, the following specific objectives have been proposed: (a) to describe the reproductive and fish condition cycles of *G. lozanoi* in five fluvial sectors; (b) to describe the energy allocation (proteins and lipids contents) among tissues in this target fish; and (c) to analyse the relationships among reproductive traits, fish condition and patterns of energy allocation.

2. Materials and Methods

2.1. Ethical Information

The care and use of experimental animals complied with University of Murcia and Spanish Law 32/2007 and RD 53/2013 animal welfare laws, guidelines and policies, as approved by Ministry of the Presidency, Relations with the Courts and Democratic Memory. The specific permit AUF20150077 was approved by the Regional Ministry of Water, Agriculture and Environment of Murcia and Castilla-La Mancha and it allowed us to sacrifice the non-native species of the Segura River Basin.

2.2. Study Area

This study was conducted in the upper and middle parts of the Segura River Basin (drainage area of 18,870 km^2), a highly regulated river located in the southeast of the Iberian Peninsula (Figure 1). The Segura River Basin is characterised by a typical Mediterranean climate with a pronounced spatial and seasonal hydrological variability. Currently, this basin is highly regulated in terms of irrigation supply and human water demands, which have greatly modified the natural flow regime, resulting in changes in flow magnitude and a reverse seasonal flow pattern in some areas [42,43]. Supplementary Material 1 provides an accurate description about the flow regime characteristics of the sampled streams.

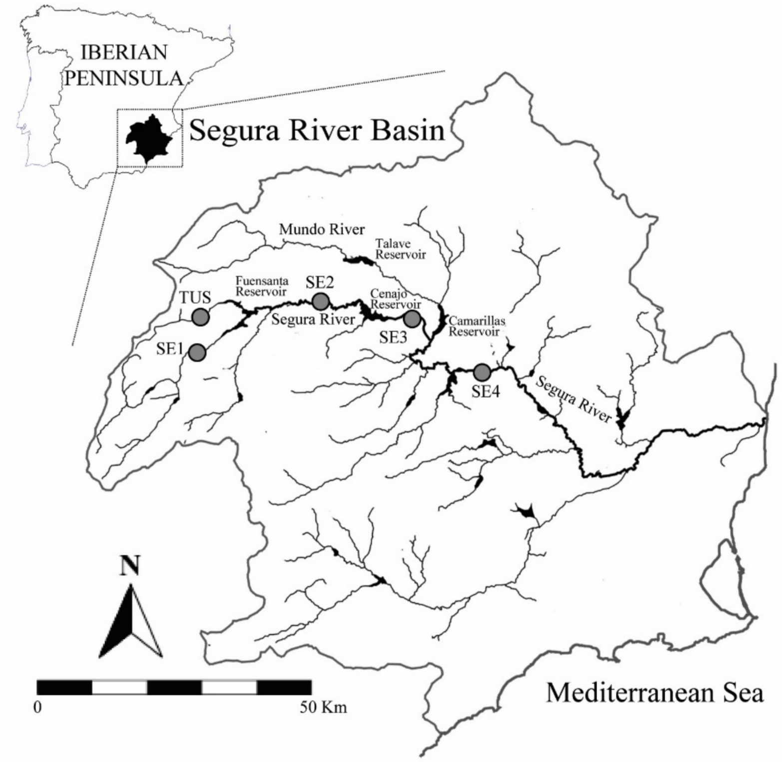

Figure 1. Sampling sites location for *Gobio lozanoi* in the Segura River basin at south-eastern Iberian Peninsula, Spain.

Sampling fluvial sites were selected following flow regime criteria. They were located along the longitudinal gradient of the basin in different hydrological sectors separated by large dams (Figure 1; Table 1). The flow characteristics ranged from natural (TUS) to reverse flow regimes (SE3 and SE4) (Supplementary Material 1, Figure S1 and Table S1). Each sampling site was characterised by the following six environmental variables (Table 1): altitude (Alt) (meters above sea level), ecological status sensu EU Water Framework Directive (Status) (with the following categories: 1 = high; 2 = good; 3 = moderate; 4 = poor), conductivity (μS cm^{-1}), Fluvial Habitat Index (FHI) [44], Riparian Quality Index (RQI) [45] and 2015 mean monthly temperature (°C). These six selected environmental variables are among the ecological drivers that play a significant role in the freshwater fish ecology, and also in biological invasion processes by fishes, of the Mediterranean-type rivers [39,40].

Table 1. Habitat variable values of each sampling site where *Gobio lozanoi* populations were assessed in the Segura River Basin.

Sampling Site	Code	Latitude	Longitude	Altitude (m.a.s.l.)	Status	Conductivity (μs cm^{-1})	IHF	RQI	Water Tempeture (°C, ± 95% Cl)
Tus	TUS	38°24′40.5″ N	2°19′01.3″ W	809	1	399.44 ± 9.89	84	65	15.63 ± 2.77
Camping	SE1	38°17′48.0″ N	2°24′42.3″ W	685	1	374.33 ± 8.06	61	98	14.71 ± 2.15
Letur	SE2	38°24′31.9″ N	2°06′33.5″ W	460	1	362.62 ± 4.21	68	80	13.04 ± 1.59
Bajo Cenajo	SE3	38°21′50.9″ N	1°46′17.2″ W	363	2	383.28 ± 3.73	67	77	12.49 ± 1.28
Hoya García	SE4	38°14′30.6″ N	1°32′35.7″ W	200	2	686.19 ± 55.20	66	70	16.25 ± 2.01

Altitude (meters above sea level), ecological status (1–4); water conductivity (±0.1); Fluvial Habitat Index (IHF); Riparian Quality Index (RQI), and water temperature (Mean and 95% Cl Confidence limits).

Since first recorded in the upper region in the 1980s, *G. lozanoi* has been registered in fluvial sectors and reservoirs along the Segura River Basin [23,27]. The ichthyofauna of this basin is characterised by low species richness and the fish assemblage composition is dominated by non-native species [27]. The studied species share resources and habitats with native cyprinids, such as the southern iberian barbel *Luciobarbus sclateri* (Günther, 1868) and the south iberian chub *Squalius pyrenaicus* (Günther, 1868), and with several non-native fish to the basin, such as the pumpkinseed *Lepomis gibbosus* (Linnaeus, 1758), bleak *Alburnus alburnus* (Linnaeus, 1758), common carp *Cyprinus carpio* (Linnaeus, 1758), iberian straight-mouth nase *Pseudochondrostoma polylepis* (Steindachner, 1864), northern pike *Esox lucius* (Linnaeus, 1758), pike-perch *Sander lucioperca* (Linnaeus, 1758) and largemouth black bass *Micropterus salmoides* (Lacepèd, 1802).

2.3. Field Sampling and Laboratory Procedures

Fish were collected by electrofishing (1800 W DC generator at 200–300 V, 2–3 A) during a one-year study period (January–December 2015). One fisherman with an electric dip-net, supported by two assistants each with a non-electric dip-net, removed fish following a zigzagging and upstream direction of each sampling stretch (100 m long), which was blocked off with barrier nets (samplings were carried out between 10 a.m. and 4 p.m.). Samples were taken once every two weeks in spring and summer, and monthly during the rest of the year (Supplementary Material 1, Table S2). A total of 2333 *G. lozanoi* were caught (TUS: 437; SE1: 478; SE2: 385; SE3: 485 and SE4: 548) and, in accordance with Spanish regulations, immediately sacrificed in a water tank with an overdose of anaesthetic solution (1:10 solution of clove oil dissolved in ethanol 70%), before being placed on ice and then they were stored at −20 °C in the laboratory.

Fork length ($L_F \pm 1$ mm), total and eviscerated masses (M_T and $M_E \pm 0.1$ g) and organ masses (hepatic and gonad, M_H and $M_G \pm 0.001$ g) of a subsample of 1982 fish were recorded (TUS: 382; SE1: 403; SE2: 365; SE3: 366 and SE4: 466). Gonads were visually inspected for sex determination (male, female or immature), and also to determinate the reproductive stage (i.e., quiescence, maturation, spawning and postspawning). A subsample of 133 mature specimens (110 females, TUS: 20; SE1: 25; SE2: 24; SE3: 17 and SE4: 24, and 23 males, TUS: 13 and SE4: 10), with fork lengths ranging from 7.2 to 11.2 cm was used to estimate fecundity, oocyte size and physiological macronutrients (protein and lipid content in tissues). Due to protein and lipid quantification methods, there were not enough testis masses to perform physiological analysis in every sampling site. To quantify protein and lipid content in the muscle, liver and gonad, samples were weighed and protein levels were determined using the method of Bradford (1976) [46] and expressed as percentages. Total lipids were extracted following the method of Folch et al. (1957) [47]. Samples were weighed and homogenized in 5 mL of chloroform/methanol (2:1 *v/v*) and washed with KCl (0.88% *w/v*). The weight of lipids was determined gravimetrically after evaporation of the solvent and expressed as percentages. Finally, these fish were aged, counting true annuli from scales taken between the lateral line and dorsal fin origin.

Ovarian development and fecundity were studied using the gravimetric method [48]. To make sure that the ovary was homogenous in structure (number and size of oocytes), small portions of anterior, middle and distal parts were compared, and no significant differences were found (ANOVA, $p > 0.05$). Therefore, all oocytes present in a subsample from the mid-region of the right ovarian lobe (5% of the total weight of the gonad) were placed in Gilson liquid, shaken periodically to soften gonadal tissue and to disperse oocytes, washed with distilled water and preserved in 70% ethanol for following analyses [37]. Image processing program ImageJ v1.80 (available at https://imagej.nih.gov/ij/) was used to count and measure oocytes. Fecundity was determined in 39 mature females caught from April to July. Fecundity was analysed at three levels: potential ($\mathrm{Fec}_{\mathrm{POT}}$), absolute ($\mathrm{Fec}_{\mathrm{ABS}}$) and batch fecundity ($\mathrm{Fec}_{\mathrm{BAT}}$). These levels were determined by counting the total number of opaque and vitellogenic oocytes, total number of vitellogenic oocytes and total number of vitellogenic oocytes of the last mode representing size before spawning, respectively [48,49]. Oocyte size at each level of fecundity ($Ø_{\mathrm{POT}}$, $Ø_{\mathrm{ABS}}$, $Ø_{\mathrm{BAT}}$) and maximum diameter ($Ø_{\mathrm{MAX}}$) were assessed.

2.4. Statistical Analyses

Sex-ratio was analysed for the whole population and in every sampling site. The degree of significance of the obtained results was established in χ^2 at a *p*-value of $p < 0.05$. Linear regressions of fecundity to fork length were fitted by least-squares method to log-transformed data.

Analyses of length-mass relationships were performed to study temporal variation in somatic condition (SC), hepatosomatic condition (HC) and gonadal activity (GSI) using the predicted values of M_{E}, M_{H} and M_{G} from analysis of covariance, respectively. The statistical approach included the application of a covariance analysis (ANCOVA) using M_{E}, M_{H} and M_{G} as dependent variables, L_{F} as the covariate (log-transformed data) and reproductive stage (quiescence, maturation, spawning and postspawning stage) as a factor. The analysis was developed by sampling site and sex separately. Differences between dependent–covariate relationships were tested to check that the covariate by-factor interaction (homogeneity of slopes) was significant ($p < 0.05$). If the covariate by-factor interaction was not significant, standard ANCOVA was applied to obtain predicted values (predicted M_{E}, M_{H} and M_{G} values). When differences were found, a post hoc Bonferroni test for multiple comparisons was performed. Student's *t*-test was used to evaluate differences in fish conditions (somatic and hepatosomatic condition), gonadal activity, and protein and lipid content between sexes.

Analyses of variance (ANOVA) were performed to determine differences in protein and lipid content among the different temporal phases and to evaluate differences among sampling sites at each reproductive stage in fish conditions, gonadal activity, fecundity, oocyte diameter and percentage of proteins and lipids in tissues, followed by the Tukey HSD (honestly significant difference) test post-hoc comparisons if significant differences among populations were found. When data did not show homogeneity of variances, Welch's analysis of variance (ANOVA) followed by T3 of Dunnett for pairwise multiple comparisons were used. The non parametric tests of the Kruskal–Wallis H-test and Mann–Whitney U-test were used when data did not fit normal distribution. Relations between fish condition, gonadal activity, fecundity, oocyte diameter and percentage of proteins and lipids by tissue were analysed using Spearman's correlation coefficients.

Size of first maturity was estimated after running binary logistic regressions (immature-mature individuals) for each sampling site by sex (Supplementary Material 2). Differences in first maturity among sampling sites were tested using generalised estimating equations (GEE), with binomial errors and the logit link function.

3. Results

3.1. Reproductive Cycle and Temporal Variation in Fish Condition

The results of the ANCOVA test to estimate the effects of the factor on the L_F-M_E, L_F–M_H and L_F–M_G relationships are shown in the Supplementary Material 3, Tables S3 and S4. In both sexes, significant differences were observed among reproductive stages in the five sampling sites for fish SC, HC and GSI (Figure 2; Supplementary Material 3, Tables S3 and S4).

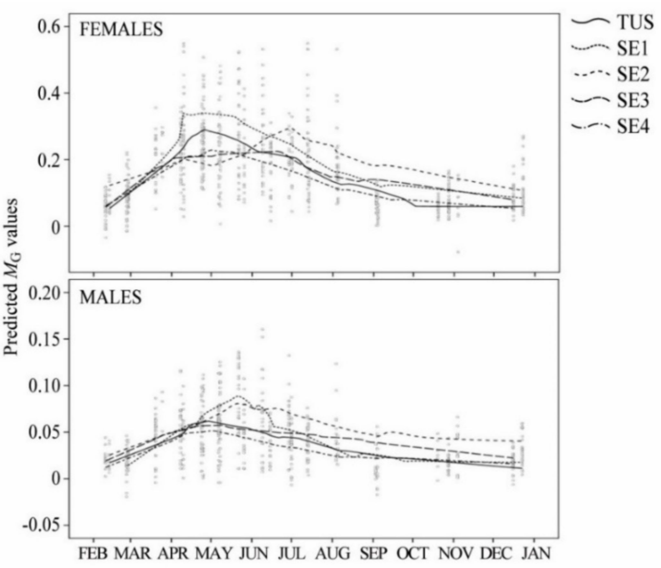

Figure 2. Temporal variation in gonad activity (predicted M_G values, M_G is gonad mass) along the study period for the five studied populations (TUS, SE1, SE2, SE3 and SE4) for both sexes of *Gobio lozanoi*. The lines represent the adjusted model *Loess* for each population.

The reproductive cycle was fitted by the ANCOVA predicted M_G values as a Gonadosomatic index (GSI) showing significant temporal differences in the gonadal activity (Figure 2). Both sexes showed a similar reproductive cycle in which four temporal stages were identified based on the GSI values (Figure 2): (1) the quiescence stage, with low values of GSI in winter; (2) the maturation stage, when GSI values rise up steeply—especially in March—and reach the maximum values at the beginning of May in females (except females from SE2) and also in males from TUS, SE3 and SE4, however, in males from SE1 and SE2, maturation was observed in late May; (3) the spawning stage, when GSI values are steady or decreasing moderately until late summer or early fall, and (4) the regression stage or postspawning, when GSI continues to decrease and reaches minimum values (Figure 2). The female gonadosomatic index was significantly higher than the male's in all reproductive stages (Student's *t*-test; quiescence stage: $t = -14.56$ $p < 0.001$; maturation stage: $t = -28.97$ $p < 0.001$; spawning stage: $t = -20.21$ $p < 0.001$; postspawning stage: $t = -11.54$ $p < 0.001$). Significant differences in the gonadal activity among reproductive stages were found in the total population (Figure 3) and when sampling sites were analysed individually for both sexes (Table 2). The GSI was significantly different among sampling sites for the total of fish (both males and females) (females Kruskal–Wallis, $\chi^2 = 46.17$, $p < 0.001$; males Kruskal–Wallis, $\chi^2 = 20.09$, $p < 0.001$), SE1 and SE2 populations showing higher GSI values and SE4 the lowest values in both sexes (Table 2).

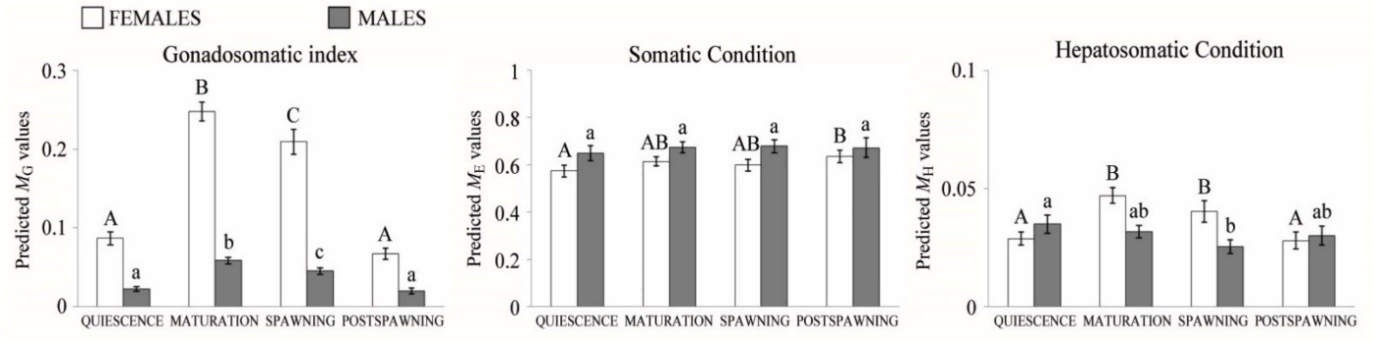

Figure 3. Mean predicted M_G, M_E and M_H values by ANCOVA (L_F as covariate) in each reproductive stage (quiescence, maturation, spawning and postspawning) for both sexes of *Gobio lozanoi*. Letters show significant differences (Welch's analysis of variance $p < 0.05$ and T3 of Dunnett post hoc tests) among reproductive stages in females (capital letters) and in males (lowercase letters).

Table 2. Mean predicted M_E, M_H and M_G values by ANCOVA (L_F as covariate) in each reproductive stage for both sexes of *Gobio lozanoi*. ANOVA results of comparison of somatic condition (SC), hepatosomatic condition (HC) and gonad activity (GSI) among reproductive stages in each sampling site are showed and significant *p*-values are included. Codes of sampling sites (TUS, SE1, SE2, SE3, and SE4) from the Segura River Basin were included.

	Sampling Site	Reproductive Stages					ANOVA		
		Total	Quiescence	Maturation	Spawning	Postspawning	df	F	*p*
FEMALES									
SC	TUS	0.651	0.569	0.679	0.683	0.629	3	4.851	0.004
	SE1	0.696	0.635	0.736	0.686	0.689	3	5.124	0.002
	SE2	0.634	0.664	0.625	0.605	0.665	3	0.908	0.443
	SE3	0.488	0.503	0.480	0.466	0.511	3	0.348	0.791
	SE4	0.583	0.543	0.598	0.567	0.638	3	4.656	0.004
HS	TUS	0.043	0.024	0.059	0.051	0.021	3	20.996	<0.001
	SE1	0.051	0.036	0.074	0.040	0.025	3	43.062	<0.001
	SE2	0.044	0.037	0.041	0.057	0.043	3	1.603	0.199
	SE3	0.025	0.020	0.027	0.029	0.023	3	1.888	0.135
	SE4	0.034	0.028	0.044	0.032	0.026	3	6.309	0.001
GSI	TUS	0.179	0.057	0.293	0.205	0.058	3	61.079 †	<0.001
	SE1	0.225	0.099	0.352	0.212	0.074	3	107.369 †	<0.001
	SE2	0.196	0.142	0.188	0.323	0.098	3	33.643	<0.001
	SE3	0.161	0.084	0.206	0.229	0.058	3	49.019	<0.001
	SE4	0.140	0.063	0.234	0.153	0.054	3	146.528 †	<0.001
MALES									
SC	TUS	0.697	0.641	0.747	0.725	0.586	3	3.409	0.022
	SE1	0.745	0.671	0.821	0.756	0.620	3	6.767	<0.001
	SE2	0.741	0.779	0.686	0.775	0.776	3	1.920	0.133
	SE3	0.610	0.578	0.621	0.585	0.690	3	1.407	0.249
	SE4	0.638	0.631	0.628	0.636	0.700	3	2.171	0.101
HS	TUS	0.031	0.034	0.036	0.030	0.021	3	1.561	0.206
	SE1	0.030	0.044	0.036	0.025	0.018	3	11.160	<0.001
	SE2	0.048	0.053	0.048	0.044	0.044	3	0.437	0.727
	SE3	0.026	0.023	0.028	0.021	0.037	3	4.472	0.007
	SE4	0.025	0.033	0.025	0.020	0.032	3	6.724	0.001
GSI	TUS	0.040	0.017	0.063	0.041	0.012	3	37.531	<0.001
	SE1	0.052	0.020	0.085	0.050	0.014	3	52.044	<0.001
	SE2	0.054	0.038	0.057	0.087	0.026	3	11.223	<0.001
	SE3	0.040	0.019	0.055	0.042	0.031	3	52.816	<0.001
	SE4	0.037	0.016	0.050	0.032	0.017	3	46.862 †	<0.001

† Means no normalised data and Kruskal–Wallis analysis of variance.

The somatic condition (SC) and hepatosomatic condition (HC) varied over the reproductive cycle with the exception of SC of males (Figure 3), and they showed significant differences among reproductive stages in both sexes in most sampling sites when they were analysed individually (Table 2). Male SC was significantly higher than for the females in most reproductive stages (Student's *t*-test; quiescence stage: t = 3.83, $p < 0.001$;

maturation stage: $t = 4.01$, $p < 0.001$; spawning stage: $t = 4.29$, $p < 0.001$), whereas female HC showed higher values than the males' during maturation and at spawning (Student's *t*-test; maturation stage: $t = -7.13$, $p < 0.001$; spawning stage: $t = -5.49$, $p < 0.001$). Fish conditions in all males showed significant differences among sampling sites (SC ANOVA, $F_{(4, 598)} = 15.56$, $p < 0.001$; HC Welch ANOVA, $F_{(4, 236.26)} = 15.24$, $p = 0.001$), SE3 and SE4 showed the lowest SC and HC values (Table 2). In all females, fish conditions also showed significant differences among sampling sites (SC Welch ANOVA, $F_{(4, 327.68)} = 39.99$, $p < 0.001$; HC Kruskal–Wallis, $\chi^2 = 73.99$, $p < 0.001$), SE1 showed the highest SC and HS values, while SE3 and SE4 showed the lowest in both conditions (Table 2).

3.2. Population Structure and Reproduction Traits

Gobio lozanoi fish ranged from 1.8 cm to a maximum L_F of 12.3 cm (a male caught in SE3). Total males (L_F 7.6 ± 1.6 cm) were significantly longer than females (L_F 7.1 ± 1.5 cm) (Student's *t*-test, t = 5.55, $p < 0.001$). Both sexes show significant differences among sampling sites in the total data (females ANOVA, $F_{(4, 922)} = 16.99$, $p < 0.001$; males Welch ANOVA, $F_{(4, 308.18)} = 2.57$, $p = 0.038$). Shorter females were found in TUS (6.5 ± 0.2) and SE3 (6.8 ± 0.2) and larger ones in SE1, SE2 and SE4 (7.5 ± 0.2, 7.4 ± 0.2 and 7.4 ± 0.2, respectively), while in males individuals in SE2 (7.9 ± 0.3) were larger than in TUS (7.2 ± 0.3).

The overall sex-ratio (696 males, 928 females) was significantly skewed towards females ($\chi^2 = 33.14$, $p < 0.001$) in the whole study period, with females being significantly more abundant in all sampling sites with the exception of SE3, which did not show differences between males and females ($\chi^2 = 1.43$, $p = 0.23$).

Length at first maturity in males ranged between 3.55 cm L_F in SE3 and 6.26 cm L_F in SE2, while female range was between 4.28 cm L_F in SE3 and 6.60 cm L_F in TUS. Above these lengths all individuals were considered mature (Figure 4). However, only significant differences in length at first maturity were found among sites in males (GEE: Wald-$\chi^2_{(4)} = 13.57$ $p = 0.009$), finding significantly larger fish at first maturity in TUS, SE1 and SE2 populations (Figure 4).

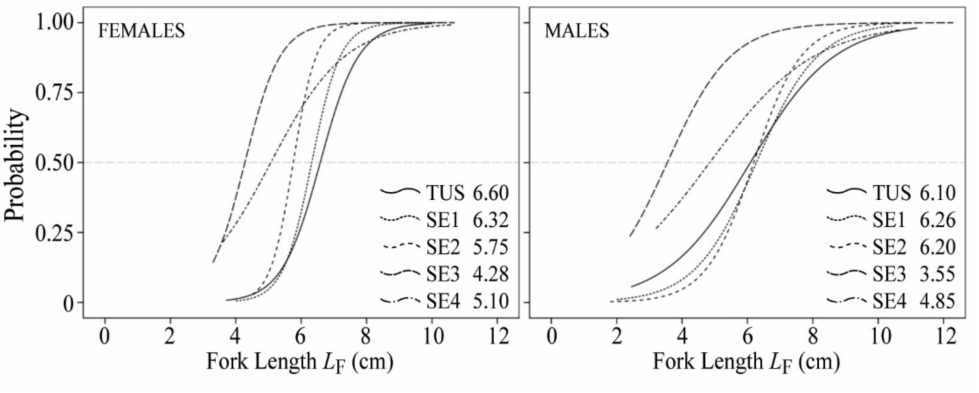

Figure 4. Predicted probability of maturity according to fork length for females and males for the five studied populations (TUS, SE1, SE2, SE3 and SE4) of *Gobio lozanoi*.

Oocytes larger than 0.25 mm in diameter were considered opaque and all oocytes above 0.55 mm of diameter were vitellogenic. Fecundity data from each sampling site are summarised in Table 3. No significant differences were found in fecundity and oocyte diameters by age (ANCOVA, $p > 0.05$) and fork length was not significant as a covariable when oocyte diameters were analysed, which indicates no effect of fish size on egg diameters in the studied fish. Significant differences were found in potential (ANCOVA $F_{(1, 4, 39)} = 3.27$, $p = 0.023$) and absolute fecundity (ANCOVA $F_{(1, 4, 39)} = 2.90$, $p = 0.037$) among sampling sites. SE1 showed the highest number of oocytes and SE3 showed the lowest ones at a given length (Bonferroni post hoc: $p = 0.023$ and $p = 0.037$, respectively). Only the diameter of batch fecundity showed significant differences among sampling sites (ANCOVA $F_{(1, 4, 37)} = 9.96$, $p < 0.001$). Batch oocyte diameters in TUS and SE1 populations were larger than in SE2, SE3 and SE4 (Bonferroni post hoc: $p < 0.001$) (Figure 5).

Table 3. Mean, minimum and maximum values of potential fecundity (Fec_{POT}), absolute fecundity (Fec_{ABS}) and batch fecundity (Fec_{BAT}) of *Gobio lozanoi*. Linear regression of fecundities and fork length (L_F) are shown (significant p-values are included). Codes of sampling sites (TUS, SE1, SE2, SE3, and SE4) from the Segura River Basin were included.

Site	Potential Fecundity			Absolute Fecundity			Batch Fecundity		
	Mean	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**
TUS	239.3	89	401	152.4	71	250	65.8	37	101
	$\text{LogFec}_{\text{POT}} = -3.64+ 6.30\ \text{Log}L_F$			$\text{LogFec}_{\text{VIT}} = -1.61 + 3.987\ \text{Log}L_F$			$\text{LogFec}_{\text{BAT}} = -1.942 + 3.988\ \text{Log}L_F$		
	$r^2 = 0.82$		$p = 0.001$	$r^2 = 0.54$		$p = 0.015$	$r^2 = 0.72$		$p = 0.004$
SE1	311.25	111	461	194.58	78	324	79	33	152
	$\text{LogFec}_{\text{POT}} = -4.337 + 7.045\ \text{Log}L_F$			$\text{LogFec}_{\text{VIT}} = -3.783 + 6.266\ \text{Log}L_F$			$\text{LogFec}_{\text{BAT}} = -4.511 + 6.628\ \text{Log}L_F$		
	$r^2 = 0.62$		$p = 0.007$	$r^2 = 0.56$		$p = 0.013$	$r^2 = 0.70$		$p = 0.003$
SE2	248.13	136	389	168.5	83	317	85	42	140
	$\text{LogFec}_{\text{POT}} = -2.023 + 4.634\ \text{Log}L_F$			$\text{LogFec}_{\text{VIT}} = -3.482 + 5.974\ \text{Log}L_F$			$\text{LogFec}_{\text{BAT}} = -3.444 + 5.575\ \text{Log}L_F$		
	$r^2 = 0.75$		$p = 0.012$	$r^2 = 0.77$		$p = 0.010$	$r^2 = 0.63$		$p = 0.018$
SE3	117.4	41	181	79	36	103	56	35	74
	$\text{LogFec}_{\text{POT}} = -3.085 + 5.509\ \text{Log}L_F$			$\text{LogFec}_{\text{VIT}} = -0.861 + 1.154\ \text{Log}L_F$			$\text{LogFec}_{\text{BAT}} = -4.143 + 6.165\ \text{Log}L_F$		
	$r^2 = 0.94$		$p = 0.033$	$r^2 = 0.34$		$p = 0.417$	$r^2 = 0.86$		$p = 0.073$
SE4	220.25	70	427	132.5	41	252	85.5	37	124
	$\text{LogFec}_{\text{POT}} = -3.671 + 6.115\ \text{Log}L_F$			$\text{LogFec}_{\text{VIT}} = -3.942 + 6.172\ \text{Log}L_F$			$\text{LogFec}_{\text{BAT}} = -2.105 + 4.119\ \text{Log}L_F$		
	$r^2 = 0.91$		$p = 0.044$	$r^2 = 0.95$		$p = 0.023$	$r^2 = 0.77$		$p = 0.121$

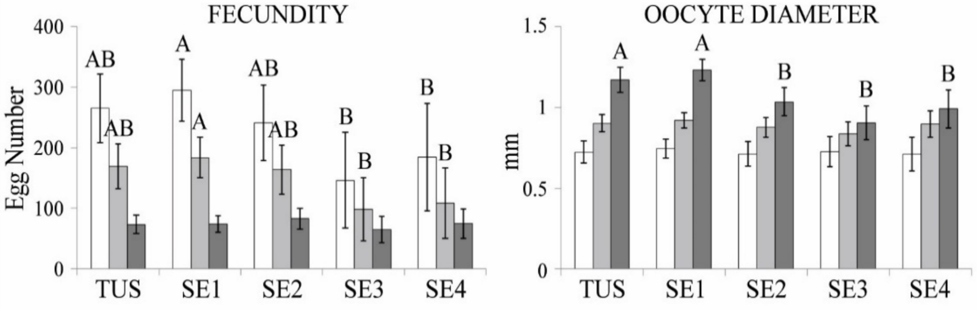

Figure 5. Estimated marginal means (by ANCOVA) ± IC 95% at 9.0 cm of fork length for oocyte number and diameter of opaque plus vitelogenic oocytes (potential fecundity; white bars), and of vitelogenic oocytes (absolute fecundity; grey bars) and oocytes of batch fecundity (dark grey bars). Letters show significant differences (ANCOVA, Bonferroni post hoc tests) among sampling sites.

3.3. Protein and Lipid Contents

Significant differences in the percentages of proteins and lipids were found in tissues during the whole period studied (Table 4). Females showed maximum protein values in the gonads and maximum lipid values in the liver, while males presented highest protein and lipid values in the liver (Table 4). Comparisons between sexes revealed that females showed significantly higher protein and lipid percentages than males in the muscles (Student's t-test, $t = -4.46$, $p < 0.001$; $t = -3.97$, $p < 0.001$, respectively), while the percentage of protein in the liver was higher in males (Student's t-test, $t = 4.51$, $p < 0.001$). In the gonads, the ovary protein content was higher (Student's t-test, $t = -9.75$, $p < 0.001$), but the testis showed higher values of lipid content (Student's t-test, $t = 3.14$, $p = 0.005$).

Percentages of protein and lipid content in tissues showed significant differences during the reproductive cycle and the lipid–protein dynamic was different between sexes (Figure 6). The lipid percentage in the muscle decreases from quiescence to spawning stages in both sexes (ANOVA: female $F_{(3, 106)} = 11.26$, $p < 0.001$; male $F_{(3, 19)} = 6.33$, $p = 0.008$)

(Figure 5). In the liver, protein percentages in females reached higher values at spawning (ANOVA $F_{(3, 104)} = 5.32$, $p = 0.002$), but no differences were found in the percentages of lipid contents in this tissue (Figure 6). No significant differences were found in the percentages of protein and lipid contents in the liver in males during the reproductive cycle. The protein percentage in the ovary increased until the spawning stage (ANOVA $F_{(3, 103)} = 8.73$, $p < 0.001$), but decreased in testis (ANOVA $F_{(2, 19)} = 9.670$, $p = 0.001$), whereas no significant differences were found in gonadal lipids during the reproductive cycle (Figure 6).

Table 4. Percentage of lipids and proteins in each tissue of *Gobio lozanoi*. All *p*-values in Kruskal–Wallis test are significant (<0.001).

			Muscle %			**Liver %**			**Gonad %**		**Kruskal–Wallis**
		n	**Range**	**Mean**	*n*	**Range**	**Mean**	*n*	**Range**	**Mean**	χ^2
Female	Protein	110	4.18–11.10	6.95	108	4.10–15.14	9.69	107	0.28–25.59	9.99	86.016
	Lipid	108	0.40–1.35	0.73	105	0.52–18.79	6.94	109	1.05–9.09	3.00	265.237
Male	Protein	23	3.49–8.18	5.67	23	9.26–18.13	12.51	23	0.16–8.99	2.68	54.916
	Lipid	23	0.39–0.97	0.58	23	2.45–22.42	7.18	21	1.84–8.73	4.65	46.484

n, Number of individuals, range of minimum and maximum values and Kruskal–Wallis test.

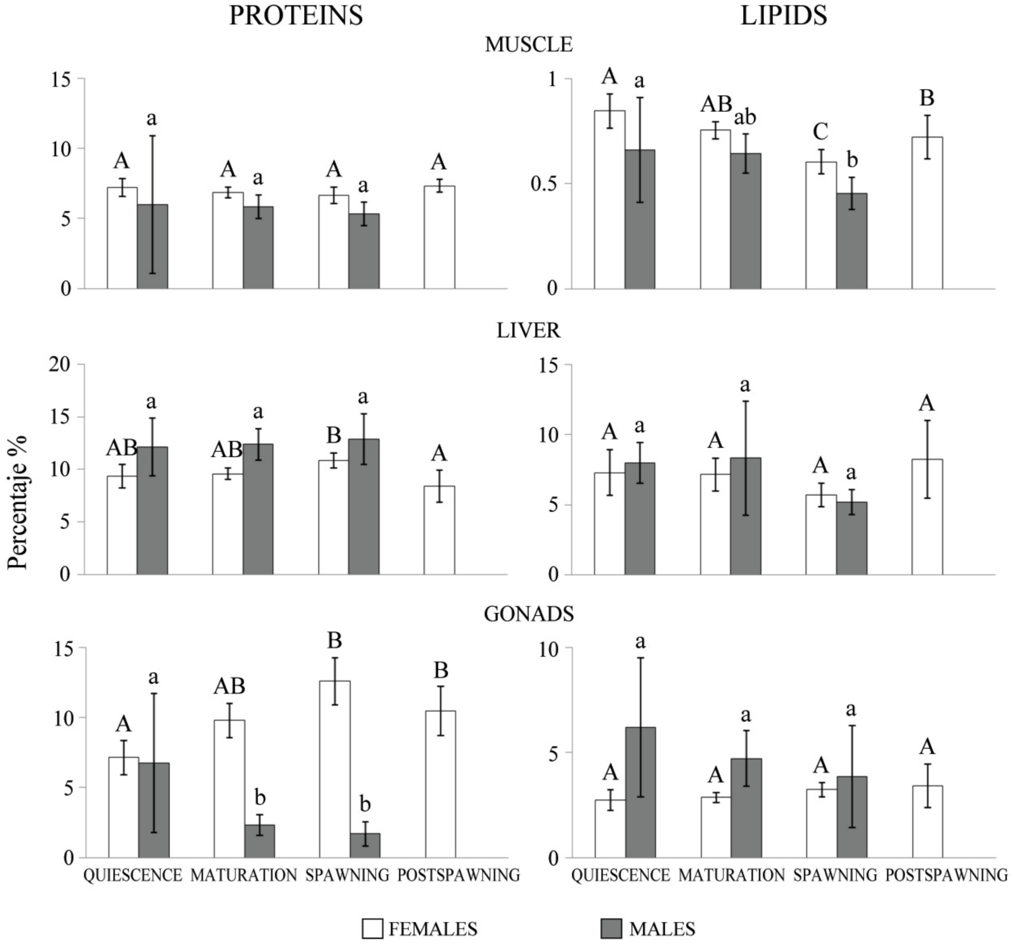

Figure 6. Mean and ± IC 95% percentages of proteins and lipids in muscle, liver and gonads by reproductive stages. White bars represent female values and grey bars represent the male ones. The letters show significant differences (ANOVA, $p < 0.05$) among reproductive stages by post hoc comparison Tukey test. Capital letters for female data and lower case letters for male data.

3.4. Fish Metrics Relationships

Fish conditions (SC and HC) and GSI were positively correlated with fecundity and the percentage of ovary proteins. SC had a positive relationship with batch oocyte diameter only, whereas HC and GSI were positively correlated with potential, absolute and maximum oocyte diameters (Table 5). Fish conditions and GSI were positively correlated with the percentage of ovary protein, whereas SC and HC had a negative relationship with the percentage of muscle lipids. Moreover, the percentages of ovary proteins and lipids were positively correlated with absolute fecundity and oocyte diameters (Ø$_{\mathrm{POT}}$, Ø$_{\mathrm{ABS}}$, Ø$_{\mathrm{BAT}}$, Ø$_{\mathrm{MAX}}$). Absolute oocyte diameter (Ø$_{\mathrm{ABS}}$) was positively related to the percentage of proteins in the liver and negatively related to the percentage of muscle lipids (Table 5).

Table 5. Correlation matrix of fish somatic condition (SC), hepatosomatic condition (HC), gonad activity (GSI), fecundity, oocyte size and proteins and lipids percentages by tissues in females of *Gobio lozanoi*. Spearman's coefficient above the diagonal and *p*-values below the diagonal.

	SC	**HC**	**IGS**	**Fec$_{\mathrm{POT}}$**	**Fec$_{\mathrm{ABS}}$**	**Fec$_{\mathrm{BAT}}$**	**Ø$_{\mathrm{POT}}$**	**Ø$_{\mathrm{ABS}}$**	**Ø$_{\mathrm{BAT}}$**	**Ø$_{\mathrm{MAX}}$**	**% P$_{\mathrm{MUS}}$**	**% P$_{\mathrm{LIV}}$**	**% P$_{\mathrm{OVA}}$**	**% L$_{\mathrm{MUS}}$**	**% L$_{\mathrm{LIV}}$**	**% L$_{\mathrm{OVA}}$**
SC	-	0.713 **	0.426 **	0.491 **	0.386 **	0.749 **	0.032	0.155	0.347 *	0.142	−0.054	0.014	0.234 *	−0.267 **	−0.146	−0.037
HC	<0.001	-	0.816 **	0.415 **	0.635 **	0.755 **	0.465 **	0.472 **	0.186	0.556 **	0.016	0.038	0.435 **	−0.215 *	−0.031	−0.019
IGS	<0.001	<0.001	-	0.267**	0.611 **	0.748 **	0.570 **	0.470 **	0.103	0.627 **	−0.017	0.041	0.282 **	−0.130	−0.101	−0.060
Fec$_{\mathrm{POT}}$	<0.001	<0.001	0.005	-	0.695 **	0.744 **	0.079	0.136	0.401 **	0.228 *	0.087	−0.259 **	0.173	0.041	0.161	0.073
Fec$_{\mathrm{ABS}}$	<0.001	<0.001	<0.001	<0.001	-	0.768 **	0.732 **	0.680 **	0.466 **	0.780 **	0.088	−0.126	0.467 **	−0.092	0.167	0.233 *
Fec$_{\mathrm{BAT}}$	<0.001	<0.001	<0.001	<0.001	<0.001	-	0.015	0.058	0.173	0.212	0.116	−0.308 *	−0.054	−0.030	−0.018	−0.038
Ø$_{\mathrm{POT}}$	0.744	<0.001	<0.001	0.413	<0.001	0.924	-	0.925 **	0.383 *	0.874 **	−0.029	0.071	0.587 **	−0.111	0.055	0.565 **
Ø$_{\mathrm{ABS}}$	0.138	<0.001	<0.001	0.192	<0.001	0.720	<0.001	-	0.570 **	0.922 **	−0.005	0.216 *	0.682 **	−0.326 **	0.093	0.661 **
Ø$_{\mathrm{BAT}}$	0.023	0.246	0.520	0.009	0.002	0.279	0.013	<0.001	-	0.896 **	−0.190	0.107	0.384 **	−0.234	−0.030	0.284
Ø$_{\mathrm{MAX}}$	0.141	<0.001	<0.001	0.016	<0.001	0.184	<0.001	<0.001	<0.001	-	−0.034	0.054	0.605 **	−0.174	0.132	0.583 **
% P$_{\mathrm{MUS}}$	0.581	0.870	0.860	0.368	0.387	0.470	0.760	0.959	0.234	0.724	-	−0.070	−0.054	0.096	0.175	0.134
% P$_{\mathrm{LIV}}$	0.888	0.695	0.675	0.006	0.216	0.050	0.460	0.036	0.507	0.574	0.466	-	0.119	−0.255 **	−0.279 **	0.030
% P$_{\mathrm{OVA}}$	0.015	<0.001	0.003	0.072	<0.001	0.739	<0.001	<0.001	0.013	<0.001	0.574	0.217	-	−0.213 *	0.143	0.368 **
% L$_{\mathrm{MUS}}$	0.005	0.025	0.178	0.669	0.365	0.852	0.250	0.001	0.141	0.060	0.317	0.007	0.026	-	0.240*	−0.083
% L$_{\mathrm{LIV}}$	0.139	0.754	0.303	0.099	0.104	−0.910	0.576	0.380	0.855	0.179	0.073	0.004	0.146	0.013	-	0.141
% L$_{\mathrm{OVA}}$	0.707	0.847	0.533	0.450	0.020	0.813	0.003	<0.001	0.025	0.001	0.162	0.753	<0.001	0.389	0.148	-

Fec$_{\mathrm{POT}}$, potential fecundity; Fec$_{\mathrm{ABS}}$, absolute fecundity; Fec$_{\mathrm{BAT}}$, batch fecundity; Ø$_{\mathrm{POT,}}$ oocyte size at potential fecundity; Ø$_{\mathrm{ABS}}$, oocyte size at absolute fecundity; Ø$_{\mathrm{BAT}}$, oocyte size at batch fecundity; Ø$_{\mathrm{MAX,}}$ maximum oocyte size; % P$_{\mathrm{LIV}}$, percentage of proteins in liver; % P$_{\mathrm{MUS}}$, percentage of proteins in muscle; % P$_{\mathrm{GON,}}$ percentage of proteins in gonads; % L$_{\mathrm{LIV}}$, percentage of lipids in liver; % L$_{\mathrm{MUS}}$, percentage of lipids in muscle; % L$_{\mathrm{GON,}}$ percentage of lipids in gonads; ** significance level of $p < 0.01$; * significance level of $p < 0.05$.

4. Discussion

Reproductive cycles of freshwater fish depend on a set of environmental factors and rheophilic fish, such as the target species, usually need flow requirements to activate migration processes, gonadal maturation and spawning success [50]. Reproduction is related to stream flow, photoperiod and temperature cues [8,51] and there must be optimal conditions for all these variables to coincide in time for gonadal activation to begin. Similar temporal dynamics of the gonadosomatic index were found in another studied population of *G. lozanoi* in an upper fluvial sector of the Segura River Basin [37]. However, in other non-native populations of *G. lozanoi* located more to the north of the Iberian Peninsula, where environmental factors are different, shorter maturation and spawning periods were observed [35,36].

During this study, several intraspecific differences in reproductive traits among populations inhabiting different hydrological sectors have been observed. Indeed, temporal dynamics of the gonadosomatic index showed two different patterns: one of them increased steeply, reaching a peak GSI value in April and May, just in the most upper sites, and the other one showed a slight GSI increase until June and July. The maturation delay, found mainly in SE2 and SE3 fluvial sectors, could be related to the lack of flow cues, such as spring peak flows present in TUS and SE1. Hydrological sector SE2 did not show any high flow peak during the year and SE3 is located right below the Cenajo dam which starts to release water in March, while other hydrological sectors showed high flows in early February (Supplementary Material 1; Figure S1). Thus, the increase in flow stability or reduction in natural flow disturbances, together with an imbalance between temperatures and flow peaks may be affecting the onset of the gonadal activation [8,52]. Furthermore, the spawning delay observed below the Cenajo dam (SE3) and the disruption of temperature increase (due to hypolimnetic cold water selective releases from the reservoir) may cause gonadal regression or failed oocyte development [9], which could explain the lower GSI values observed in fish inhabiting this fluvial sector.

Fish conditions (somatic and hepatosomatic) showed different patterns between sexes, suggesting that condition investment was not the same for both sexes during the reproductive cycle. The SC patterns of both sexes were not observed in other non-native populations of *G. lozanoi* in the Iberian Peninsula, which showed two peak values of somatic condition at the beginning and at the end of spawning, with minimum values in October [35]. Temporal dynamics of the HC of females were similar to the GSI pattern, increasing at the beginning of the activation stage and decreasing at the end of spawning. In indeterminate batch spawners such as *G. lozanoi* [35,36], oocyte recruitment is continuous during the spawning season and high liver mass (high HC) could confirm an intense liver activity for vitellogenesis, while in determinate batch spawners or total spawners the hepatosomatic activity decreases during the reproductive cycle due to the completion of vitellogenesis prior to spawning [53,54]. Moreover, fish conditions showed differences among different flow regimes during the reproductive cycle. In fact, only in the hydrological sector where flow was constant all year round (SE2) was no significant variability observed for these parameters. No drastic flow events and homogenization of flow conditions may favour the stability of fish investment because fish inhabiting unstable environments with seasonal flash floods may require high levels of energy reserves, such as high somatic and hepatosomatic conditions; a high investment to increase reproductive success [8,55,56]. On the other hand, the unnatural concurrence of very high flows during the reproductive event (maturation and spawning) implies energy redistribution in fish between survivals in suboptimal environments and reproductive investment [7], which could explain the lower values of fish conditions and GSI in these hydrological sectors (SE3 and SE4).

Previous studies of *G. lozanoi* in the Iberian peninsula observed maximum size ranges between 10.1 to 14.0 [23], whereas the population studied here showed a maximum length (FL) of 12.3 cm. The smallest individuals were observed in sampling sites located in the most natural flow conditions (TUS), and the largest ones were observed in SE4 and SE2. Larger maximum sizes of fish populations usually correlate with more stable environments, where abiotic fluctuations, such as flow peaks, are less significant and food sources are more readily available, so lower mortality rates can be observed [57]. Furthermore, low-flow periods, typical of dry summers in the Mediterranean basins, reduce food and habitat availability and may be affecting growth rates as has been observed in other Mediterranean cyprinids [8].

Hydrologic conditions are, for sure, one of the main drivers responsible for shaping the reproductive success and reproductive strategy used [6]. In this study, the analysed reproductive traits showed significant intraspecific variability which could be related to flow conditions at the basin scale. There were a higher number of females in all populations, apart from directly below the Cenajo dam (SE3). Thus, very high flows could be increasing female mortality rates after reproductive investment, which was higher than male investment; this has been recorded in other non-native populations of *G. lozanoi* in the Iberian Peninsula [36]. In addition, variability in sexual maturity is a compensatory population response to different environmental factors [58,59]. Many studies relate high mortality in populations to early maturation in order to compensate the decrease observed in the number of adults and maximize the egg production capacity [59,60]. In general, sexual maturity of non-native populations of *G. lozanoi* in the Iberian Peninsula was reached at a young age and most of the individuals aged 2+ or greater than 7.0 cm in fork length were mature [35–37]. In this study, the shortest mature males were 3.55 cm (L_F) and females were 4.28 cm (L_F), representing the smallest sizes found in the whole Peninsula. Highly disturbed areas or extreme environmental conditions and unregulated flow conditions (natural hydrological sectors in this study) are expected to be associated with early maturation, which is typical of opportunistic strategies [3,33]. However, in this study, a shorter length of first maturation was observed in flow regulated sectors, where natural disturbances are buffered, although some other environmental perturbations may be acting as well.

The studied population showed a lower absolute fecundity compared to other populations of *G. lozanoi* in the Iberian Peninsula [35,36]. In spite of this, oocyte diameters (between 0.84 and 0.92 mm) were larger than in other Iberian populations, which scattered oocyte diameters of 0.76, 0.76 and 0.73 mm in the Matarraña, Moros and Ucero rivers, respectively [35,36]. These results could indicate that a trade-off between egg size and fecundity is established [58]. Furthermore, higher fecundity and larger oocytes were observed in more natural flow areas, while populations inhabiting more altered flows and with reverse regimes showed a lower number and size of oocytes. Production of larger oocytes could be a compensatory strategy to produce larger larvae which will be more resistant to low flow stress factors in dry summers, while the production of a high number of eggs can ensure the survival of the species against the high mortality rates of eggs and larvae in areas with very variable and unpredictable flows [58,59]. On the one hand, the first hypothesis of this study suggested an opportunistic life-history strategy (small size, short longevity, early maturity, low fecundity, multiple spawnings per year and long reproductive periods) (*sensu* Winemiller and Rose, 1992 [33]) in more natural flow conditions, because this strategy is more associated with Iberian native and non-native species inhabiting unregulated Mediterranean rivers with strong seasonal flow patterns [61]. On the other hand, characteristics closer to a periodic life-history strategy (large body size, late maturation, high fecundity and a reduced spawning period) would be expected in more predictable and hydrologically stable environments. However, later maturity and a higher number and size of oocytes were observed in more natural flow sectors; later maturity, higher fecundity and small oocytes were found in the most stable flow sector, and populations with earlier maturity and lower number and size of oocytes were found in hydrological sectors with reverse flow regimes. We hypothesised that our studied populations would show intermediate characteristics between opportunistic and periodic life-history strategies, as previously described [34]. The results were not conclusive enough to establish strong correlations between the life strategy of *G. lozanoi* and flow conditions. Although reproductive strategies and reproductive success are directly related to hydrologic conditions [6], the flow effect is probably not strong enough to drastically change the reproductive strategy of populations of *G. lozanoi* that live in the same basin and may share the same genetic pool. The intraspecific variability observed in some reproductive traits suggests a certain degree of effect caused by flow regimes, however, its plasticity allows the species to survive flow regulation events at several scales, as well as to resist the long-term environmental stress typical of Mediterranean-type rivers [62].

Percentages of lipid contents in liver and gonads were stable during the reproductive cycle in both sexes. The increase in protein contents in females' livers at the beginning of reproduction could suggest synthesis activity in the liver of the yolk and eggshell proteins which are transported to the ovarian tissues for oocyte vitellogenesis and maturation [58,63]. Ovarian protein content also increased during the reproductive cycle in response to the oocyte development and maturation [64,65]. In females, during gonadal maturation ovarian and liver lipid contents did not vary and protein content increased in both tissues. This could indicate that enough energy was available during the spawning to develop gonads and store energy in the liver. Additionally, in the absence of other energy sources, feeding during the reproductive season might provide energy for vitellogenesis [66–68]. The reproduction investment of studied populations strongly depends on food availability and provides rapid transport from ecosystem productivity to reproduction, which may allow continuous adjustments of the reproductive effort to food intake [15,69]. These findings provide evidence of the energy intake of *G. lozanoi* during the reproductive season which is typical of income breeders. However, the decrease in lipid percentage content in muscles during the reproductive cycle in females could be suggesting energy allocation to reproduction from muscle. The use of muscle tissue as an energy source has been documented for several fish, such as salmonids [70]. This pattern could suggest that *G. lozanoi* exhibits an intermediate strategy (income–capital breeding strategy) in which stored energy in muscles is also required for reproduction [71,72].

Higher fecundity and oocyte diameter were correlated with better somatic and hepatosomatic condition, as well as gonadosomatic index, suggesting fish condition plays an important role during recruitment and development of oocytes [73]. Moreover, proteins and lipids of ovary tissue also showed a positive correlation with absolute fecundity and oocyte sizes supporting the fact that a greater mobilization of macronutrients to gonads favours recruitment and oocyte quality. Thus, food availability has an important influence on reserves of protein and lipid in the tissues and there is a food-dependent variation in stored lipid energy which affects the reproductive potential of individual fish [16,74].

5. Conclusions

In summary, flow conditions have an important effect on some reproductive traits of *G. lozanoi*, reflected as intraspecific variations in the most of studied parameters. However, this intraspecific variability was not conclusive enough to classify populations either as opportunistic strategists in unpredictable flow sectors, or as periodic strategists in areas that show stable flow regimes. Moreover, protein and lipid contents in tissues during the reproductive cycle provide some insight into the energy allocation during the reproduction of this species, which suggests that current food intake is the main—but not only—energy source for this species to reproduce, since they also use part of the energy supplement they store. Hence, *G. lozanoi* can be classified as an income–capital breeder. Comparative studies of reproductive traits and energy balance are a powerful approach to understanding life-history trade-offs of species, and they may serve as excellent models for studies of plasticity and adaptation of breeding strategies to new environmental conditions in exotic species. Further studies are needed to increase the knowledge about phenotypic plasticity in species that may be potential invaders since the lack of information could be hiding negative effects on other species, as well as on the environment.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4441/13/3/387/s1. Supplementary Material 1: Flow Characterisation of Sampling Sites. Supplementary Material 2: Immature-Mature Determination. Supplementary Material 3: ANCOVA Results. Supplementary Material 4: Picture of the target species.

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Fish Rescue during Streamflow Intermittency May Not Be Effective for Conservation of Rio Grande Silvery Minnow

Thomas P. Archdeacon [1,*], **Tracy A. Diver** [1] **and Justin K. Reale** [2]

[1] U.S. Fish & Wildlife Service, New Mexico Fish & Wildlife Conservation Office, Albuquerque, NM 87131, USA; tracy_diver@fws.gov

[2] U.S. Army Corps of Engineers, Albuquerque District, Albuquerque, NM 87131, USA; justin.k.reale@usace.army.mil

* Correspondence: thomas_archdeacon@fws.gov

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Abstract: Streamflow intermittency can reshape fish assemblages and present challenges to recovery of imperiled species. During streamflow intermittency, fish can be subjected to a variety of stressors, including exposure to crowding, high water temperatures, and low dissolved oxygen, resulting in sublethal effects or mortality. Rescue of fishes is often used as a conservation tool to mitigate the negative impacts of streamflow intermittency. The effectiveness of such actions is rarely evaluated. Here, we use multi-year water quality data collected from isolated pools during rescue of Rio Grande silvery minnow *Hybognathus amarus*, an endangered minnow. We examined seasonal and diel water quality patterns to determine if fishes are exposed to sublethal and critical water temperatures or dissolved oxygen concentrations during streamflow intermittency. Further, we determined survival of rescued Rio Grande silvery minnow for 3–5 weeks post-rescue. We found that isolated pool temperatures were much warmer (>40 °C in some pools) compared to upstream perennial flows, and had larger diel fluctuations, >10 °C compared to ~5 °C, and many pools had critically low dissolved oxygen concentrations. Survival of fish rescued from isolated pools during warmer months was <10%. Reactive conservation actions such as fish rescue are often costly, and in the case of Rio Grande silvery minnow, likely ineffective. Effective conservation of fishes threatened by streamflow intermittency should focus on restoring natural flow regimes that restore the natural processes under which fishes evolved.

Keywords: thermal stress; salvage; climate change; drought; hypoxia

1. Introduction

Intermittency is a common and natural condition for many of the world's streams [1]. However, many regions of the world are predicted to experience decreased precipitation, altering historical streamflow regimes [2]. Currently, climate change coupled with human-mediated water abstraction has already increased the frequency of streamflow intermittency in some areas [3,4], threatening endemic fishes [5]. These species now face increasing water temperatures and risk of stranding in isolated pools [6–8]. Some stream temperatures are projected to exceed the thermal critical maxima for many fishes [9], which may lead to temperature-dependent mortality, population declines, or other sublethal effects [10–12]. Exposure to intermittent habitats and elevated water temperatures can reshape fish assemblages [13,14], which may result in shifts towards more homogenous assemblages dominated by extremophile species [15]. Understanding the long-term consequences of drought on fish communities, including the effects of water temperature and streamflow intermittency, will likely be an ever-increasing challenge for native fish conservation [16,17].

Streamflow intermittency can directly impact fish communities when historically perennial systems become disconnected, forcing individuals to seek refuge in more contracted and often less hospitable habitats [14,15,18]. The Middle Rio Grande (MRG) covers ~330 km through central New Mexico, USA (MRG; Figure 1). This reach is affected by seasonal and supra-seasonal drought that often results in streamflow intermittency. Historically, the MRG was a large, wide, and shallow river dominated by sandy substrates with considerable intra-annual variation in flows with peak runoff driven by spring snowmelt and low summer flows [19]. Historical accounts of streamflow intermittency prior to the 1890s occurred, but intermittency was not observed annually [19]. Low-flows and intermittency became more common in the MRG in the 1900s as more water management infrastructure was constructed in New Mexico and Colorado [19]. Indeed, the MRG fish assemblage formerly included species like shovelnose sturgeon *Scaphirynchus platorhynchus* and American eel *Anguilla rostrata* that would be intolerant of frequent drying [20]. More recently, frequent supra-seasonal drought [21], declines in snowpack [22,23], and human-mediated water abstraction of up to 95% [24,25] in the MRG Basin have resulted in long periods of summer streamflow intermittency, e.g., >100 days and >80 km in extreme years, averaging around 38 days and 35 km annually [18].

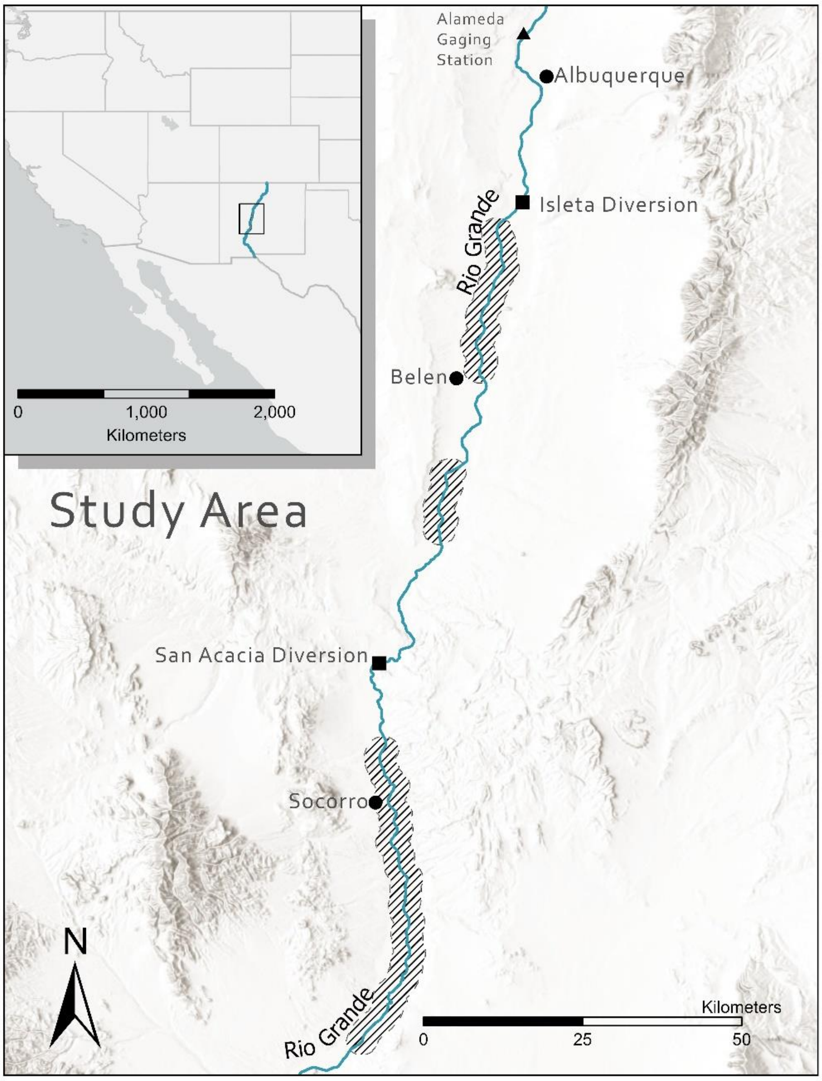

Figure 1. Areas of streamflow intermittency (crosshatched areas) in the Middle Rio Grande Basin of New Mexico, where temperatures of isolated pools were collected from 2011 to 2016. Circles represent metropolitan areas, squares are surface flow diversion structures, and the triangle is a streamflow and water quality gauging station.

The MRG is designated as a critical habitat for the only remaining wild population of the endangered Rio Grande silvery minnow *Hybognathus amarus*, a small-bodied member of the family Cyprinidae [26,27]. Rio Grande silvery minnow were once widespread throughout the mainstem Rio Grande and Pecos Rivers, from north central New Mexico to the Texas coast, but now occurs only in the MRG [28,29]. Rio Grande silvery minnow exhibits an opportunistic life-history strategy [30]. Fishes with this life-history strategy are typically small, short-lived, have high reproductive output, and maintain high demographic resilience [31]. Rio Grande silvery minnow have a relatively short lifespan, with few living > 2 years [32]; they reach maturity in the first year of life [33] and have relatively high fecundity for their body size [34]. Rio Grande silvery minnow spawn non-adhesive, neutrally buoyant ova directly into the water column [35], a mode of reproduction found in several other cyprinid species in the Great Plains of the western United States [36]. For Rio Grande silvery minnow, annual reproductive success is highly variable and is tied to high flows in spring [18,33,37], likely through creation of nursery habitats for larvae and juveniles [38,39]. Rio Grande silvery minnow were listed as endangered in 1994. Prior to Rio Grande silvery minnow being listed as endangered, four other species of minnow with similar reproductive biology were extirpated from the MRG during the 20th century [28].

Rio Grande silvery minnow are directly impacted by intermittent streamflow. During periods of river drying, Rio Grande silvery minnow are forced into isolated pools [37]. Compared to other intermittent streams in the American Great Plains region [15,40] and around the world [41], isolated pools in the MRG are shallow (<0.6 m) and short-lived, with only a small percentage persisting > 4 days [37]. Globally, isolated pools can last for weeks to years and allow the persistence of fishes and other organisms [42–44]. Isolated pools that support fish can be important for fishes that inhabit seasonally intermittent streams by providing habitat patches that reduce mortality compared to areas with no surface water [45]. However, in parts of the MRG, the river channel is perched above irrigation canals built for downstream water deliveries [46], which has resulted in rapid onset of intermittent conditions and evaporation of isolated pools due to a lack of connection to groundwater. In the absence of fish rescue, stranding in isolated pools in the MRG would result in almost certain mortality of fishes during summer months [37]. Thus, immediate rescue of Rio Grande silvery minnow, i.e., collection from isolated pools and translocation to areas with perennial surface flow, is performed before complete desiccation of isolated pools occurs as a conservation action to mitigate for the negative effects of streamflow intermittency.

Fish rescue and translocation are commonly used for mitigating the negative effects of stranding and streamflow intermittency [8,47,48]. However, rescue efforts are costly and rarely evaluated for effectiveness [8]. Exposure to stressors prior to translocation, as well as capture and transport stress, may limit survival after rescue. Evaluation of rescue efforts has focused on salmonids and the economic cost–benefit of translocating stranded fish [49,50]. Benefits to the population, simulated or realized, require that rescued fish survive to reproduce [50]; however, this assumption is likely both species and season dependent. Within the MRG, rescue and translocation of Rio Grande silvery minnow stranded during streamflow intermittency has been used as a management action for conservation since the early 2000s [18]. Fish are rescued and transported to areas with perennial flow each day after intermittency begins. However, the short-term survival of these fish after rescue and, ultimately, the effectiveness of rescue for conservation of the species is unknown. Many factors may decrease the effectiveness of fish rescue in the MRG. Pools may experience extreme temperature or dissolved oxygen fluctuations during streamflow intermittency in summer months [51]. Even though fish are typically rescued and translocated within 24 h of being stranded, they are likely exposed to high, and possibly lethal, temperatures, hypoxic conditions, and other stressors, such as crowding and predation prior to rescue. All of these factors may reduce the short-term survival of Rio Grande silvery minnow rescued from isolated pools.

Acute temperature and dissolved oxygen tolerances for larval, juvenile, and adult Rio Grande silvery minnow have been quantified in a laboratory setting [52]. Temperature tolerance was determined through the incipient lethal temperature technique [52]. This method requires an abrupt transfer of fish to temperatures above or below the acclimation temperature, and lethal thresholds are determined when 50% mortality occurs within a set timeframe [53–55]. Acute dissolved oxygen tolerance was determined similarly [52]. For adult Rio Grande silvery minnow, the 50% lethal temperature threshold was 32.8 °C within a 24-h period; however, mortality began to occur at temperatures > 30 °C. Juveniles had higher 50% lethal temperature thresholds of 36.7 °C, with mortality beginning at about 34.5 °C within a 24-h period. For dissolved oxygen, juveniles experienced 50% mortality at 0.7 mg/L with no mortality at concentrations > 1.9 mg/L. For adults, these limits were 0.8 mg/L for 50% mortality and 1.1 mg/L for no acute mortality. While these types of tests can be critical for defining critical limits, the effects of chronic exposure to sublethal or fluctuating water temperatures or hypoxic conditions remains largely unknown. Chronic exposure to high, sublethal temperatures can affect metabolic rates [56], slow growth, and reproduction, and can alter behavior and increase stress and susceptibility to disease in other fishes [12]. Chronic hypoxia can also cause stress, affect swimming performance and metabolic functions [57], and result in transgenerational reproductive impairment [58]. Thus, understanding both the risk and effects of exposure to poor water quality may help managers determine the value of fish rescue and improve our understanding of the effects of rising water temperatures on fish assemblages [16].

Rescue efforts remain a major management strategy for Rio Grande silvery minnow during periods of streamflow intermittency and have been employed every year since 2009 [37]. In order to evaluate the severity of thermal conditions fish are exposed to prior to rescue, we used multi-year data collected during fish rescue to estimate hourly and seasonal changes in pool temperatures during streamflow intermittency. We compared this to the thermal regime of an upstream perennial reach. We also examined the number of Rio Grande silvery minnow exposed to elevated water temperatures or hypoxic conditions prior to rescue to assess the extent to which individuals in this endangered population are subjected to damaging water quality conditions. Finally, we estimated post-rescue survival of Rio Grande silvery minnow collected from isolated pools formed during streamflow intermittency as a preliminary evaluation of the effectiveness of fish rescue. Our results will help provide realistic estimates of the water quality conditions fishes are exposed to during streamflow intermittency, which will inform future studies examining lethal and sublethal effects of water temperature and hypoxia. Further, our results will inform conservation actions aimed at mitigating the effects of streamflow intermittency and stranding.

2. Materials and Methods

During irrigation season, March through November, surface water is diverted at multiple diversion dams within the MRG. During warm summer months, surface flow diversions can result in streamflow intermittency in areas below the Isleta and San Acacia Diversion dams (Figure 1). Typically, flows are decreased over several days and constant bypass is held at a diversion dam. Intermittent sections expand over these days, then remain relatively constant until further streamflow diversion is needed, reducing flows, or precipitation increases flows. Intermittency occurs in a mid-reach pattern, with perennial areas upstream and downstream of drying [59]. The pattern of drying is due to upstream diversions, irrigation return flows, channel perching, and groundwater influences. Multiple drying and wetting cycles can occur annually due to monsoon rain events or irrigation demands [18]. Observations of the channel conditions are made daily during this period of time; crews are dispatched to rescue fish when new areas of isolated pools form. The amount of newly dewatered channel is variable, ranging from 0.1 km up to 8 km [37], resulting in up to 130 new isolated pools per day. Fish rescue occurred within one day of streamflow intermittency, usually the morning through afternoon after isolated pools formed. During our study period from 2011 to 2016, we visited all isolated pools that occurred due to streamflow diversions. We recorded counts of Rio Grande silvery minnow collected in each pool, dissolved oxygen, temperature, maximum depth (0.1 m), date, and time of day. Fishes were sampled using 3.0 m by 1.0 m seines (3.2 mm mesh size). All Rio Grande silvery minnow collected were transported in ~130-L fiberglass tanks mounted on all-terrain vehicles. We supplied pure oxygen to transport tanks to maintain ~100% saturation. Fish were released the same day in nearby areas with surface flows that were not expected to become intermittent. Depending on the number of pools that formed, fish spent 1 to 6 h in transport tanks before being released.

We measured water temperature (±0.01 °C) and dissolved oxygen (±0.01 mg L^{-1}) of all pools with a multi-parameter probe (YSI 556 MPS; Yellow Springs Institute) where maximum depth was recorded at the time of fish rescue. We were not able to collect temperature or dissolved oxygen at every pool due to equipment malfunction or data recording errors. We assumed isolated pools were thermally unstratified because most were <0.60 m in depth [37]. Previous studies support this assumption: in 2007, continuous (e.g., 15-min intervals) water quality monitoring conducted in five isolated pools over several days showed less than a 1 °C difference between surface temperature and temperature above the substrate [60].

We set a range of water temperature criteria based on preliminary laboratory data for Rio Grande silvery minnow [52] and similar species found in North America [55,61]. Based on these studies, we classified temperatures into four broad categories: <30 °C—no adverse effects, 30–33 °C—minimal adverse effects, including loss of equilibrium and muscle spasms, >33–36 °C—lethal for adults and sublethal for juveniles, and >36 °C—lethal for all age classes. These broad categories represent a continuum of possible adverse effects, but chronic, sublethal effects of elevated water temperature on Rio Grande silvery minnow are unknown. In order to place these effect thresholds into context with water temperatures experienced during flowing conditions, we compiled a 15-min interval temperature record over the same time period on the MRG at Alameda Gauging Station (Figure 1) from two locations c. 400 m from one another. Specifically, records collected by the University of New Mexico [62] and the U.S. Geological Survey (Gage No. 08329918) were combined to minimize temporal gaps. This location is within the occupied Rio Grande silvery minnow critical habitat that also maintains perennial surface flow. Similarly, we defined broad categories for dissolved oxygen: >2.0 mg L^{-1}—no adverse effects, 1.0–1.9 mg L^{-1}—minimal mortality for adults but potentially lethal for juveniles, and <1.0 mg L^{-1}—potentially lethal for both adults and juveniles.

We estimated average pool temperatures or stream temperatures by date and time of day from 2011–2016. We used a linear mixed-effects model with a sinusoidal response. We used both first and second harmonic sine and cosine terms for time of day (period = 24) and ordinal day of year (period = 365) to model the cyclical annual and diel temperature fluctuations. We treated these as continuous fixed effects and year as a random effect to predict the mean temperature of isolated pools over time. We used the same model on each water temperature recorded at the Alameda Gauging Station and independently of isolated pools. Statistical models were run in the program R version 4.0.2 using the package "lme4" [63,64].

We also estimated post-rescue survival of Rio Grande silvery minnow. Rather than immediately returning fish to perennial areas, we retained smaller samples of Rio Grande silvery minnow from isolated pools and held them in sterile laboratory conditions for 5 to 7 weeks. Additional fish were collected from areas with surface flow to serve as control groups (3 replicates, 311 total fish) and compared to those collected in isolated pools (9 replicates, 2289 total fish) from March through August in 2018 and June through August in 2020 (Table 1). Fish spent approximately one hour longer in transport tanks—compared to those released directly in areas with surface flows—while being transported back to laboratory aquaria. Fish were acclimated to water in aquaria by slowly replacing tank water with aquarium water over 15–45 min, which was similar to fish released in areas with surface flow. Fish were held indoors in a 5100-L indoor recirculating system consisting of seven individual fiberglass tanks, one of which functioned as a sump and contained filtration and aeration equipment to maintain oxygen levels at 100% saturation. Each tank measured 180 × 75 × 60 cm. The system was filled with municipal water passed through a reverse-osmosis filter. Water temperature in tanks was not continuously monitored and fluctuated with ambient indoor air temperatures. However, discrete temperature measurements in holding tanks were between 19 and 23 °C. Water drained through a standpipe to a sand filter and ultraviolet sterilizing filter. Flow to each tank was approximately 1000 L/h. This system has held a variety of fish species with minimal mortality of control groups [65,66]. We fed fish flake food specifically designed for Rio Grande silvery minnow [67] twice daily to satiation, and excess food and waste were siphoned from tanks twice per week for the duration of the experiment. We recorded mortality daily.

Table 1. Dates, stream conditions, number, and survival of Rio Grande silvery minnow rescued in the Middle Rio Grande, New Mexico.

Date	Conditions	Number	Survival
26 March 2018	Continuous	102	76.4
24 April 2018	Continuous	67	100
9 May 2018	Continuous	142	73.9
3 April 2018	Intermittent	250	24.4
10 April 2018	Intermittent	300	43.7
8 July 2018	Intermittent	226	2.6
4 August 2018	Intermittent	267	6.6
11 June 2020	Intermittent	45	8.9
12 June 2020	Intermittent	290	1.4
14 June 2020	Intermittent	101	8.9
14 July 2020	Intermittent	389	5.4
7 August 2020	Intermittent	421	5.0

3. Results

We sampled 7597 pools during the six-year study period. We measured temperature in 6780 pools and dissolved oxygen in 5555 pools. Pool temperatures at the time of rescue ranged from 6.4 to 41.0 °C. Dissolved oxygen ranged from 0.01 to 21.35 mg L^{-1}. Across all days, pools were hottest each day from ~13:00 to 18:00 (Figure 2). Isolated pools rarely exceeded effect levels of >30 °C prior to 1000 h. The model results (Table A1) show that the estimated mean water temperatures began exceeding 30 °C in mid-June and persisted through August (Figure 3), with the largest daily duration at or above this temperature occurring in August. The highest mean pool temperatures occurred in August between ~14:00 and 16:00 (Figure 3). Isolated pools had diel water temperature fluctuations of >10°C, with these pools cooling at a faster rate in evenings compared to rates of warming in the mornings (Figure 3). In the perennial flow reach, maximum estimated mean water temperatures were <28 °C and exhibited diel fluctuations of ~5 °C (Figure 3).

Dissolved oxygen was also variable among pools. Generally, dissolved oxygen in the pools decreased as temperature increased. However, values above and below the temperature-dissolved oxygen solubility curve [68] (Figure 2), suggesting other physical (e.g., groundwater contributions) and biological controls (i.e., gross primary productivity and ecosystem respiration), are influencing the dissolved oxygen among pools.

From 2011 to 2016, we rescued 32,951 Rio Grande silvery minnow from pools where water temperature was measured. The majority of fish were juveniles (63.9%). A similar number of both juveniles (N = 10,041) and adults (N = 10,251) were collected in pools that were <30 °C (5688 isolated pools), which represented the majority (61.6%) of rescued fish. We found a substantial portion (10,895 juveniles, 1179 adults) of Rio Grande silvery minnow in pools above the 'no adverse effects' threshold of >30 °C (643 isolated pools). Of those, 351 isolated pools had water temperatures > 33 °C and contained 85 juveniles and 311 adults. Ninety-nine isolated pools had lethal water temperatures ≥ 36 °C and contained four juveniles and 74 adults.

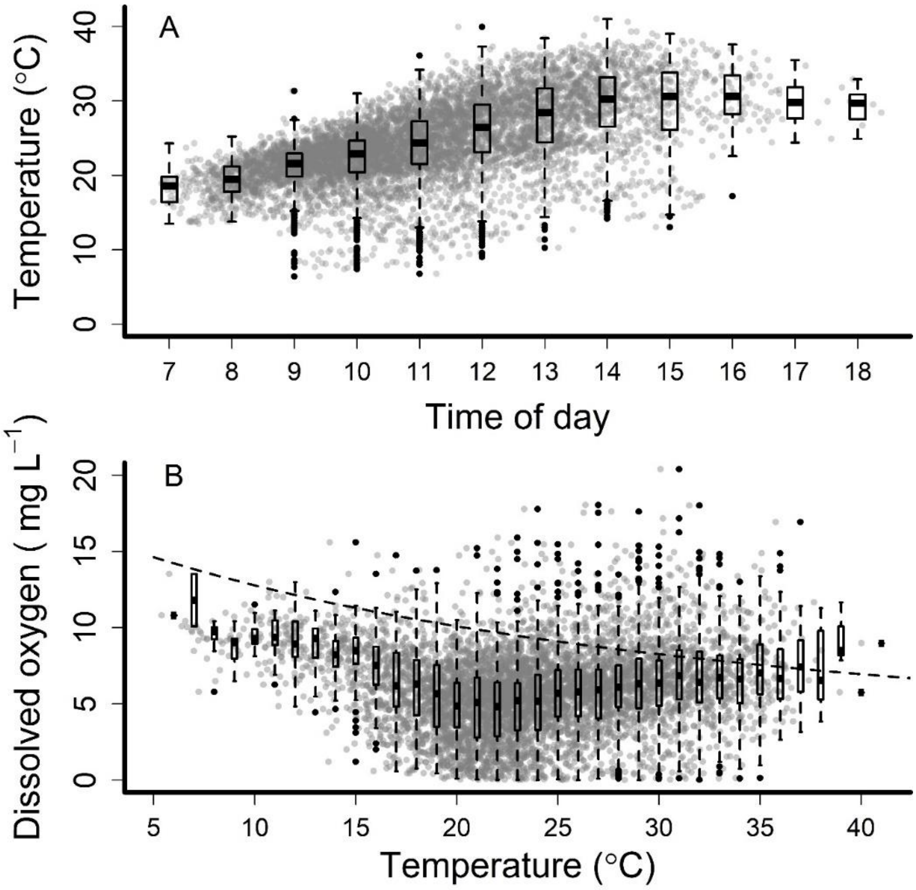

Figure 2. Water temperature of isolated pools by time of day (**A**) and dissolved oxygen by water temperature (**B**) that formed during streamflow intermittency in the Middle Rio Grande, New Mexico, June through October from 2011 to 2016. Individual pools are represented by gray dots and box-and-whisker plots depict the median (bar), interquartile range (box), points within 1.5 times the interquartile range (whiskers), and outliers (black dots). The dashed trendline represents the effect of temperature on solubility of oxygen in freshwater (chlorine and salinity = 0 ppm) at 760 mm Hg [68].

The majority of isolated pools (N = 5059) had dissolved oxygen concentrations ≥ 2.0 mg L^{-1} and contained the majority of Rio Grande silvery minnow: 9960 juveniles and 3383 adults. Fewer isolated pools had dissolved oxygen concentrations between 1.0 and 2.0 mg L^{-1} (N = 272) and had 364 juveniles and 1051 adults. Finally, we found 224 isolated pools with <1.0 mg L^{-1} of dissolved oxygen and containing three juveniles and 323 adults.

Survival of Rio Grande silvery minnow rescued from isolated pools was generally poor. Fish rescued during surface flow conditions early in the year had higher survival than those rescued during streamflow intermittency, ≥74% compared to <50% (Figure 4). Further, among groups rescued from isolated pools, those collected earlier in the year had 3 to 20 times higher survival compared to those rescued in June and later (Table 1).

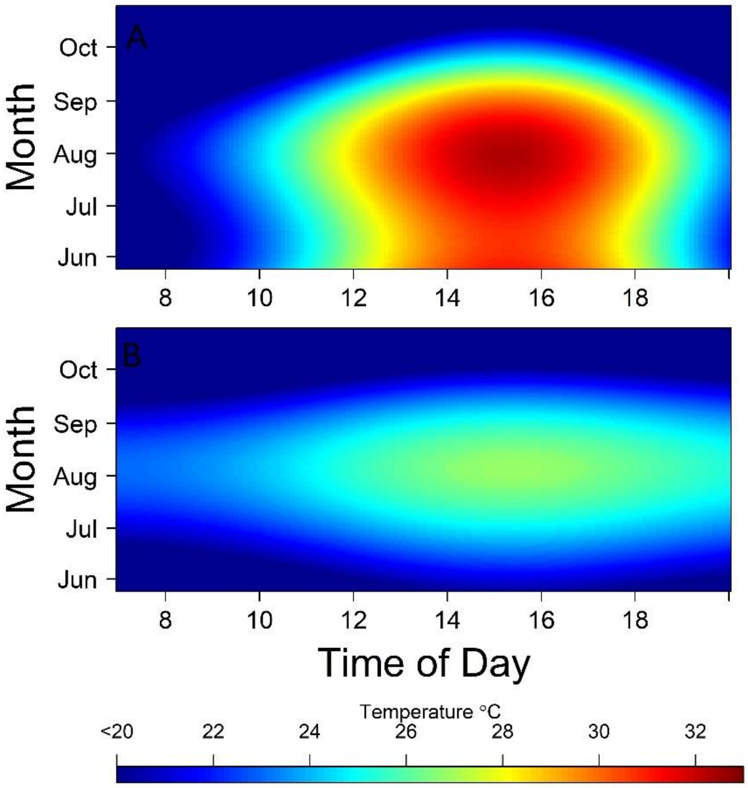

Figure 3. Heat map showing estimated mean temperature of isolated pools (**A**) or areas with perennial flow (**B**) from June through October, 2011 to 2016 in the Middle Rio Grande, New Mexico, by time of day and date, with cooler temperatures shown in blue.

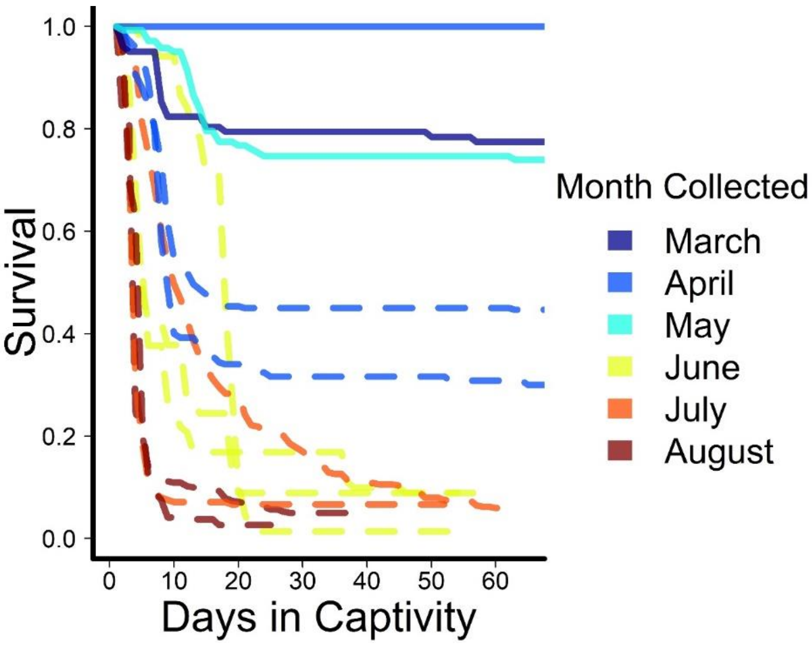

Figure 4. Daily cumulative survival of Rio Grande silvery minnow captured during surface flows (solid lines) or during streamflow intermittency (dashed lines) and held in captivity in 2018 and 2020.

4. Discussion

As expected, we found a substantial effect of time of day and ordinal day on pool temperature, with the hottest recorded water temperatures occurring in the late afternoon of June through August. Pool temperatures regularly exceeded levels found in an upstream section of the MRG that maintains continuous streamflow, suggesting that Rio Grande silvery minnow are exposed to unusually high temperatures during streamflow intermittency. Indeed, the maximum water temperature we recorded from isolated pools was >10 °C higher than the maximum temperature recorded in the connected upstream reach. Although these water temperature comparisons are somewhat confounded due to their spatial separation, it is likely that Rio Grande silvery minnow would experience lower and more stable water temperatures if continuous streamflow was maintained downstream. However, during the summer of 2018, diel fluctuations of >10 °C and values that regularly exceeded 30 °C were observed during low-flow but continuous conditions (~0.03 $m^3 s^{-1}$) on the MRG between 3 and 10 km downstream of San Acacia Diversion Dam [51]. Although continuous flows alone likely do not provide thermal refuge, they can allow individuals the opportunity to seek refuge in upstream areas below diversion dams or near irrigation return outfalls, which may be cooler and have dissolved oxygen concentrations > 2.0 mg L^{-1} [51], whereas the opportunity to do so during intermittency is eliminated. Although they are difficult to implement in a water-scarce basin, Rio Grande silvery minnow and other fishes in the MRG would benefit from development of water-use strategies that enhance connectivity among habitats during all seasons, allowing fish greater access to refuges during warm periods.

Relatively few Rio Grande silvery minnow were exposed to lethal water temperatures at the time of collection. Exposure to lethal water temperatures within isolated pools was likely limited because the majority of rescue efforts took place rapidly (within 24 h of drying) and during the early hours of the day before pool temperatures increased above critical thresholds. A slower response to new areas of drying or increased rates of channel drying would lead to more individuals being exposed to lethal temperatures. While the acute effects of water temperature on mortality are relatively well understood, the effects of chronic exposure to sublethal temperatures and rapid fluctuations in temperature on fishes is largely unknown [12,55]. We found that a large number of rescued fish experience both sublethal temperatures and relatively large temperature fluctuations. The effects of sublethal temperature exposure on Rio Grande silvery minnow have not been evaluated, but laboratory studies conducted on the regionally similar endangered loach minnow (*Tiaroga cobitis* Family: Cyprinidae) showed that sublethal static temperatures of 28 and 30 °C reduced growth rates compared to fish held at 25 °C [69]. These reduced growth rates suggest that fish experienced chronic stress at these thermal thresholds. Although lethal temperature tolerances have been evaluated for Rio Grande silvery minnow at varying life stages, it is unknown how chronic temperature stress affects this species.

The ability of an organism to respond to thermal change likely depends on the magnitude of the temperature shift, the frequency of thermal change, and the ability of individuals to acclimate to constant or fluctuating diurnal cycles [70]. While it is common for streams to experience daily fluctuations of ~4°C [12,71,72], our model results show that, on average, isolated pool temperatures fluctuated by >10 °C within a relatively short 8 to 12 h period. Our estimated diel fluxes are consistent with previous studies where temperature loggers were deployed in isolated pools within the MRG [51,60]. Growth rates in salmonids are reduced when subjected to sublethal, dynamic water temperature regimes [72,73], though these have focused on much lower temperature changes than what Rio Grande silvery minnow experience in isolated pools.

The observed dissolved oxygen values within the pools can largely be attributed to temperature controls of the solubility of oxygen in the water [68]. An inverse relationship between temperature and dissolved oxygen has been observed during short-term (3 days) deployments of high-frequency sensors within isolated pools in the MRG [51]. The reduction of biological controls on dissolved oxygen, via a reduction in rates of gross primary productivity and ecosystem respiration, has been observed in other rivers following pool isolation [74]. However, we observed that dissolved oxygen values elevated above the temperature–dissolved oxygen solubility curve indicate that inputs from primary production [75] could be contributing, as considerable periphyton biomass was observed in a small number of pools during rescue and during summer low-flow conditions in the MRG [76]. The dissolved oxygen concentrations observed below the temperature–dissolved oxygen solubility curve may be a result of deep groundwater inputs within the MRG [77–79], upwelling of hyporheic waters [80], or high rates of heterotrophic metabolism within the hyporheic zone [81].

Regardless of the mechanism controlling diel dissolved oxygen dynamics, we observed that 496 pools that contained 367 juvenile and 1387 adult Rio Grande silvery minnow were <2.0 mg/L, whereas locations below diversion dams and irrigation return drains during low-flow conditions provided more suitable dissolved oxygen conditions for Rio Grande silvery minnow [51]. Although periods of acute hypoxia have also been observed within the MRG during periods of connectivity [51,82,83], periods of acute hypoxia in isolated pools have been observed in groundwater-dominated headwater streams [15,84]. However, native cyprinid species with low tolerance to hypoxia in the laboratory have been found to persist in isolated pools [15]. This variation in laboratory and field observations suggests that hypoxic conditions may not result in mortality of native species within isolated pools, but may favor non-native extremophile fishes [15]. Non-lethal effects of short-term hypoxia include transgenerational reproductive impairments [58], swimming performance, and behavior [85,86]. As a result, a higher threshold for sub-lethal effects of hypoxia on fish is recommended [87], and we propose that it should be further evaluated and implemented for Rio Grande silvery minnow.

Despite relatively few Rio Grande silvery minnow being exposed to elevated water temperatures, survival of rescued fish was context dependent. Survival was low in the weeks following capture and transport to a laboratory setting for fishes rescued in June through August. The markedly higher overall survival of Rio Grande silvery minnow collected under lotic conditions compared to isolated pools and lower survival of fishes collected in summer months demonstrates the cumulative stressors of confinement, temperature, and otherwise declining water quality conditions through the year during streamflow intermittency [51]. In both 2018 and 2020, almost no young-of-year fish were collected due to low spring runoff, resulting in failed recruitment [33]. Body condition, age, and season can all affect thermal tolerance in fish [88]. Possibly, adult fish are in poor condition after spawning and may have died regardless of exposure to high water temperatures. Future research should focus on both improving survival after rescue and examining differential effects on survival of adults and young-of-year. We also stress that these are likely conservative estimates of post-rescue survival: Fish were held in sterile, predator-free conditions, with optimal water quality, and were provided access to food resources.

As river drying occurs, it is likely that increased temperatures and reduced flows limit the ability of individuals to seek thermal refuge. Thus, it is likely that Rio Grande silvery minnow are exposed to lethal and sublethal temperatures for several days prior to stream intermittency and could be physiologically compromised before being rescued [51]. Handling and transport alone are significant stressors on healthy Rio Grande silvery minnow [89]. During rescue, mitigating stress to increase overall survival would prove difficult to implement. Fish transported in live wells mounted on the back of all-terrain vehicles cannot reasonably be acclimated to the broad daily temperature range from which Rio Grande silvery minnow are collected. The inability to properly acclimate fish during rescue along with additional handling stress may further limit the ability of individuals to survive once translocated. Water in holding tanks is generally around 23 °C early in the day and increases to about 27 °C at the time of release. Given the large number of pools that can be encountered daily (>100) and the long distances covered (>10 km), acclimating fish from individual pools would not be feasible. Rescuing a smaller number of Rio Grande silvery minnow per day with the intention of increasing post-rescue survival may be possible. However, this may be ineffective, as pool size does not necessarily predict numbers of stranded Rio Grande silvery minnow [37]. Rescuing fish prior to drying, while increasing survival, is also more difficult, as sampling efficiency is much lower compared to collecting from isolated pools. Further, this period of time overlaps with Rio Grande silvery minnow spawning [33]. Handling of fish during that time could not only affect their ability to spawn but would not likely prevent their offspring from being stranded in isolated pools, as they produce neutrally-buoyant, non-adhesive eggs that passively disperse downstream [35,90].

Given that Rio Grande silvery minnow are a relatively short-lived species that rarely live longer than two years in the wild, the effects of streamflow intermittency on survival and growth warrant concern. Immediate effects of river drying include direct or indirect mortality, which could reduce population size. Further, little is known about the effects of chronic stress, including sublethal stress and its effects on reproduction. Increased mortality of young life stages or reproductive impairment could reduce demographic resiliency and eliminate reproductive contributions in subsequent years. Coupled with a 90% reduction in geographic range and fragmented habitats in the remaining population [26,90], streamflow intermittency further hinders recovery of Rio Grande silvery minnow, an outcome likely to be shared by many similar species with opportunistic life histories unless effective conservation actions are implemented [91,92].

Recovery of Rio Grande silvery minnow and other imperiled freshwater fishes will depend on proactive actions as opposed to reactive actions. Our results show that only a small portion of fishes rescued from isolated pools survive in the short term and that rescue is likely ineffective at mitigating the negative effects of streamflow intermittency on the population over the long term. For Rio Grande silvery minnow, rescue and translocation represent a conservation trap, in which concerted conservation efforts to offset species' declines result in actions that are perpetuated and unsustainable in the long term [93]. While fish rescue and translocation may be useful in dire conditions, their use as a regular conservation action to offset widespread, frequent streamflow intermittency has hindered recovery of Rio Grande silvery minnow. Rather than reactively rescuing stranded fish during intermittency, conservation actions need to examine ways to limit the ultimate causes of streamflow intermittency in order to achieve recovery goals. Such proactive efforts face multiple challenges in the MRG, chiefly declining precipitation [23,94] and surface flows [25,95] coupled with over-appropriation of water [96]. However, proactive actions are likely more effective than reactive actions. Proactive conservation actions may be less expensive over the long term and may prevent "conservation-reliant species" [93,97,98].

Climate change and increased human demands for freshwater will likely increase the frequency, duration, and spatial extent of streamflow intermittency, resulting in more fish stranding, greater fragmentation of habitats, and higher water temperatures. It is possible that similar rescue efforts will become more common in other systems. Thus, the effectiveness of rescue efforts must be evaluated. Population simulations may prove useful for modeling the overall population-level impact of fish rescue; however, the effect is likely minimal unless post-rescue survival can be increased. Arguably, the most prudent management action should be to maintain a known level of base flow that maintains suitable water quality, which, in turn, supports the survival of fishes and does not induce unnecessary stress. Recovery of threatened fishes will be difficult in the absence of proactive conservation efforts designed to promote self-sustaining populations.

Appendix A

Table A1. Model parameters relating temperature of isolated pools or stream temperatures in a perennial section of the Rio Grande, New Mexico, to time of day and day of year in a linearized sinusoidal mixed-effects model.

Model	Parameter	Estimate	SE	*t*-Value
	Isolate Pools			
	Intercept	16.9809	1.2183	13.938
	sine time	−5.1871	0.3277	−15.83
	cosine time	−8.495	0.8906	−9.539
	sine time (2nd harmonic)	1.2147	0.2123	5.722
	cosine time (2nd harmonic)	−1.537	0.3304	−4.652
	sine day	6.1214	1.0853	5.64
	cosine day	−5.3918	1.1174	−4.825
	sine day (2nd harmonic)	4.4314	0.5364	8.262
	cosine day (2nd harmonic)	−1.926	0.1974	−9.758
	Perennial Flow			
	Intercept	14.17188	0.103947	136.34
	sine time	−1.55107	0.00554	−279.97
	cosine time	−0.55226	0.005598	−98.65
	sine time (2nd harmonic)	0.479119	0.005553	86.29
	cosine time (2nd harmonic)	0.169554	0.005589	30.34
	sine day	−3.68166	0.005618	−655.37
	cosine day	−9.53207	0.005798	−1643.96
	sine day (2nd harmonic)	1.024666	0.005507	186.05
	cosine day (2nd harmonic)	−0.83944	0.005737	−146.32

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Factors Influencing Abundances and Population Size Structure of the Threatened and Endemic Cyprinodont *Aphanius iberus* in Mediterranean Brackish Ponds

Serena Sgarzi [1,*], Sandra Brucet [1,2,*], Mireia Bartrons [1], Ignasi Arranz [1,3], Lluís Benejam [1] and Anna Badosa [1]

[1] Aquatic Ecology Group, University of Vic—Central University of Catalonia, 08500 Barcelona, Spain; mireia.bartrons@uvic.cat (M.B.); ignasi.arranz-urgell@univ-tlse3.fr (I.A.); lluis.benejam@uvic.cat (L.B.); anna.badosa@uvic.cat (A.B.)

[2] Catalan Institution for Research and Advanced Studies (ICREA), 08010 Barcelona, Spain

[3] Laboratoire Evolution et Diversité Biologique (EDB UMR 5174), Université de Tolouse, CNRS, IRD, UPS, 118 Route de Narbonne, F-31062 Tolouse, France

* Correspondence: serena.sgarzi@uvic.cat (S.S.); sandra.brucet@uvic.cat (S.B.); Tel.: +34-93-881-5519 (S.S. & S.B.)

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Abstract: *Aphanius iberus* is an endemic cyprinodontoid fish species of Mediterranean ponds in danger of extinction. In this study, we studied some abiotic and biotic factors that can influence *A. iberus*'s size structure and density in Mediterranean brackish ponds. We sampled fish using fyke nets in 10 ponds of Empordà (Spain) during the spring season. Our results showed that a better ecological status (according to the Water Quality of Lentic and Shallow Ecosystems (QAELS) index), pond's depth and pond's isolation (reflected by an increase in total nitrogen) were related to larger individual sizes and more size-diverse populations. Increasing the salinity is known to help the euryhaline *A. iberus* acting as a refuge from competitors. Nevertheless, our results showed that higher conductivities had a negative effect on *A. iberus*'s size structure, leading to a decrease in the mean and maximum size of the fish. Fish abundance (expressed as captures per unit of effort (CPUE)) seemed to increase with increasing the pond's depth and total nitrogen (the latter reflecting pond isolation). In conclusion, our results suggest that achieving a better pond ecological status may be important for the conservation of endangered *A. iberus*, because better size-structured populations (i.e., larger mean and average lengths) were found at higher water quality conditions.

Keywords: Mediterranean ponds; fish; *Aphanius iberus*; size structure; ecological status

1. Introduction

Aquatic ecosystems of the Iberian Peninsula are a hotspot for endemic freshwater fish fauna; still, most of the fish species are critically threatened by habitat destruction, intensive agricultural activities or the introduction of exotic species [1,2]. The Spanish toothcarp (*Aphanius iberus*, Valenciennes, 1846) is a small cyprinodont (up to 6 cm in length) endemic from the Eastern Mediterranean lowland waters of the Iberian Peninsula [3–5] and in danger of extinction [6,7]. As other cyprinodonts, it is characterized by fast growth, early maturity, high reproductive effort and multiple spawnings [8,9], which implies a short longevity (age up to two+). *A. iberus* is an eurytherm and euryhaline species, well-adapted to changes in environmental conditions [10] such as sudden alterations in temperature and salinity due to marine intrusions or freshwater floodings [11]. This cyprinodont originally inhabited a wide range of lowland waterbodies, but now, its geographical distribution is limited to brackish and hypersaline coastal waterbodies [1,5,8,12] due to habitat degradation (e.g., intensive agriculture, water pollution and wetland desiccation) and the introduction of invasive species, which usually act in an additive manner, since habitat degradation facilitates biological invasions [13,14]. The high degree of isolation among the remaining populations also poses a threat to their conservation, as they show higher rates of extinction than populations in well-connected locations [5].

Some studies have shown that the abundance and size structure of *A. iberus* depends on the ecological status of the ponds, with larger individuals and higher densities found in ponds with a higher water quality [15]. Indeed, in Italy, another species of the *Aphanius* genus (*Aphanius fasciatus*) has been proposed as an indicator of the ecological status of salt marshes [16], suggesting that those fish are sensitive to changes in the ecological status of their environments. More confined and less accessible ponds also seem to host populations of *A. iberus* more abundant and stable over time [17]. Another variable that may influence the density and size structure of *A. iberus* is the pond morphometry (area and depth), because it has a strong impact on the structural complexity and niche availability, as has been found for other fish species and communities [18–20]. However, to our knowledge, there have been not studies in this respect.

The Eastern mosquitofish (*Gambusia holbrooki*) [21,22] is an invasive species that very often interacts with *A. iberus*, because both share similar habitats [23] and compete for the same resources [24,25]. Both fish species are zooplanktivorous, but *G. holbrooki* consumes mainly cladocerans, ostracods and copepods [26,27], and *A. iberus* prefers harpacticoid copepods, copepod nauplii and detritus [24]. Sometimes, *G. holbrooki* can act in an aggressive way against *A. iberus,* and this behavior seems to be inversely proportional to the salinity, as well as its ability to capture prey [28]. Young individuals of *A. iberus* have been found to capture less prey in the presence of conspecific adults and *G. holbrooki*, suggesting both strong intraspecific and interspecific competition [12,29]. Currently, *A. iberus* has disappeared from fresh and oligohaline waters, and its habitat is restricted to salt marshes, coastal lagoons and river mouths [24,30], where the invasion success of the mosquitofish is limited due to the high salinity fluctuations [24,31,32]. Nevertheless, although *A. iberus* tolerates high salinity conditions, its metabolism may be affected when the salinity levels in the location are high [33–35]. Physiological functions such as oxygen consumption, critical swimming speed and routine activity level show a general decrease at the extreme salinity in *Aphanius dispar* [36], although the spawning efficiency seems not to be significantly affected by the changes in salinity [37].

Identifying the key factors that influence the population structure of *A. iberus* is relevant to develop efficient conservation and management plans for this endangered species. The body size of *A. iberus* has been used to assess growth-related parameters, such as age [8], fecundity and sexual maturity [38], as well as ammonia excretion rates [39]. Although, in Mediterranean brackish ponds, trophic interactions are very often body size-dependent [40–42], studies about the size structure of *A. iberus* and the factors that determine it are scarce.

The present study aims to identify the factors influencing the abundances and population size structure of A. *iberus* in the north-east of the Iberian Peninsula in late-spring (i.e., when this species finishes the first period of annual reproduction). Specifically, we assessed whether abiotic factors (i.e., conductivity, nutrient concentrations and pond morphology); the ponds' ecological status; food resource availability (zooplankton biomass) and the presence of the main competitor, *Gambusia holbrooki*, are correlated with the size structure and abundance of this endangered species in 10 coastal brackish and hypersaline ponds. We assessed the size structure using the size diversity index [43], in addition to several size metrics, such as the maximum size, mean size and size range.

We hypothesized that a good ecological status, together with a larger pond dimension (depth and area), would increase the possibilities to find well size-structured populations with a higher size diversity, as well as higher densities of fish, as these two factors are supposed to set good conditions for the fish growth. Concerning conductivity, we expected a decrease in the size-related variables of *A. iberus*, because the high conductivity negatively affects its metabolism. In contrast, locations at higher conductivity levels could host higher *A. iberus* densities, because high conductivity may prevent the colonization of invasive species, such as *G. holbrooki*. We also hypothesized that the presence of *G. holbrooki* (main competitor of *A. iberus*) would lead to lower densities of *A. iberus*, as *G. holbrooki* have been observed to outcompete *A. iberus* [30]. Finally, we would expect that the abundance of *A. iberus* would be negatively correlated with the zooplankton biomass due to fish predation on zooplankton.

2. Materials and Methods

2.1. Study Area

The studied ponds are located in two protected areas of the Empordà coastal wetlands (Figure 1), between 42°01′42″ N–3°11′18″ E and 42°15′58″ N–3°08′17″ E of the Ter River Basin (NE Iberian Peninsula). Eight of the ten ponds were located in the "Aiguamolls de l'Empordà" Natural Park, and two were located south in the "El Montgrí, Illes Medes i el Baix Ter" Natural Park (some examples of individual ponds are shown in Figure S1. The climate is Mediterranean, with hot, dry summers and mild, wet winters. The hydrology of these Mediterranean coastal wetlands is characterized by a prolonged confinement period during warm seasons without water inputs, followed by irregular flooding events (i.e., rainfall or marine intrusions during sea storms, the latter no more often than twice a year) [44]. Hydrological connections among ponds and/or to rivers and the sea take place only during such irregular flooding events [45]. The studied ponds are characterized by their shallowness and cover a range of morphometry (e.g., pond area and depth) and conductivity [46]. Concentrations of inorganic nutrients (nitrates and phosphates) in late-spring are low due to the scarceness of water inputs, but concentrations of total nutrients, especially total nitrogen, are high due to a concentration effect produced by a high evaporation rate [44,45,47]. In these confined coastal environments, nitrogen, rather than phosphorous, usually limits the primary production [41,48,49]. For more details about the environmental characteristics and planktonic composition of the studied ponds, see [46].

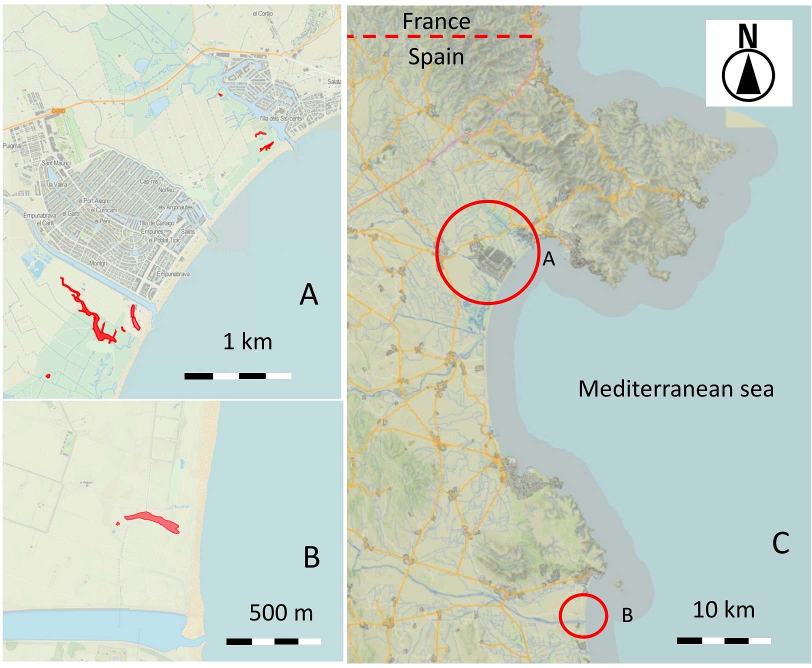

Figure 1. Location of the study region: Empordà coastal wetlands (Ter River Basin in the NE of the Iberian Peninsula) (**C**), with the detailed geographical position on the studied ponds in red (**A**,**B**). Eight of the studied ponds were located in the "Aiguamolls de l'Empordà" Natural Park (**A**), and two were located south in the "El Montgrí, Illes Medes i el Baix Ter" Natural Park (**B**). This map was produced with the online software ArcGIS (version 10.5.1, 2017, ESRI Environmental System Research Institute, Redlands, CA, USA) (https://www.arcgis.com).

Fish community in the studied ponds is mainly composed by *Aphanius iberus* and the invasive fish species *Gambusia holbrooki* [46]. Whereas the former is, overall, more abundant in ponds of higher salinity, the latter is more abundant in oligohaline ponds [46]. The rest of the community is composed by the *Atherina boyeri* (Risso, 1810), *Pomatoschistus* sp. (Gill, 1863), *Mugil cephalus* (Linnaeus, 1758), the marine fish *Solea solea* (Queusel, 1806), *Anguila anguila* (Linnaeus, 1758) and the invasive *Lepomis gibbosus* (Linnaeus, 1758), although the last three species are very scarce (see [46] for more details on the relative abundance of the fish species).

2.2. Field Sampling and Analysis

The 10 ponds studied were sampled once during the end of the spring season (from May to early June 2016). Conductivity ($mS \cdot cm^{-1}$) was measured using a multiparameter probe (Hanna Instruments, Woonsocket, RI, USA). Total area (m^2) of each pond was estimated by using the "Google Maps Area Calculator Tool" [50] (reference 58, old paper), while the mean water column depth (cm) was calculated from in situ repeated measures obtained with a two-meter rule. Total nitrogen ($mg \cdot L^{-1}$) was measured according to Koroleff, 1973 [51], adapted by Seal Analytical to an integrated system of a CFA (Continuous Flow Analysis) digester.

Two ecological indices related to the pond's ecological status were used in each pond: (1) the ECELS (Conservation Status of Lentic and Shallow Ecosystems) estimates the conservation status of lentic ecosystems based on morphological aspects, type of aquatic vegetation and human impacts [52]. The ECELS categories range from bad (0–30 out of 100), deficient (30–50 out of 100), mediocre (50–70 out of 100), good (70–90 out of 100) and very good (90–100 out of 100), and (2) the QAELS (Water Quality of Lentic and Shallow Ecosystems) index evaluates the water quality based on the composition of microcrustacean assemblages and taxonomic richness of aquatic insects and crustaceans in the Mediterranean wetlands [53]. The QAELS categories range from bad (<0.46), deficient (0.46–0.55), mediocre (0.55–0.62), good (0.62–0.72) and very good (≥0.72). The QAELS index was calculated after the observation of macroinvertebrate samples under optic microscope and a stereoscope. Samples were obtained through a dip net (mesh size 250 μm) following standard protocols [53].

Zooplankton samples were taken from each pond by mixing subsamples from five different sites in order to overcome the expected patchy distribution of plankton. Five liters of mixed water samples were filtered through a 50-μm mesh size net and preserved in 4% Lugol's acid solution. Zooplankton individuals collected (including rotifers, copepods and cladocerans) were counted, identified and measured using a stereoscope and an inverted microscope (Utermöhl method), as was described in [46]. To estimate the zooplankton biomass, the total length (μm) of the first 100 individuals (when possible) was measured assuming that all individuals were equally distributed in the observed sample. Individual biomasses were then calculated using approximation to shape formulas.

Fish were caught by fyke nets set for 24 h, a common and widely used method in coastal lagoons [17,54,55]. Fyke nets consisted of a semicircular entrance ring followed by three smaller circular rings surrounded by a net (3.5-mm mesh) with two consecutive funnels (120 mm of funnel diameter, 1050 cm^2 of interception area, 98 cm of length, 30 cm of height and 95 cm of wing length). The total number of fyke nets set in each pond varied according to its area and depth (Table S1). A total of 49 fyke nets were set in all the ponds. All captured fish were sexed (except juveniles < 13 mm), measured for total length (mm) and released. We measured all the individuals of *Aphanius iberus* in each sample, in order to minimize the error estimation.

2.3. Aphanius iberus Abundance and Size Structure

In each pond, the *Aphanius iberus* abundance was calculated by dividing the total captures by the number of fyke nets set in each pond (captures per unit of effort; CPUE). The size structure of *A. iberus* in each fyke net was assessed using four size-based metrics: (1) the maximum size, (2) mean size (computed as the geometric mean), (3) size range (the difference between the maximum and the minimum size) and (4) size diversity index (μ). For each fyke net, the size diversity was calculated using individual size (i.e., length) measurements, as proposed by [43]. Size diversity is based on the Shannon–Wiener diversity index [56] adapted for a continuous variable, such as body size. This index is the continuous analog of the taxonomic Shannon diversity index, and it produces values in a similar range to those of the Shannon index. In our case, it integrates the amplitude of the length range and relative abundance of the different lengths. Thus, the high values of the size diversity would indicate a high diversity of sizes with an equitable numerical frequency of sizes along the distributions [57,58]. In contrast, the low values of size diversity (rarely taking negative values) would indicate a low diversity of fish sizes with an inequitable numerical frequency of sizes along the distribution [43].

2.4. Data Analysis

We used mixed linear models (MLMs) to test the effects of abiotic and biotic factors on the abundance and size metrics of *A. iberus*. We considered captures of each fyke net as an observation unit (N = 49), and "pond" was introduced as a random effect to deal with pseudoreplication. As predictor variables, we considered conductivity, pond area and mean water depth, total nitrogen, ECELS and QAELS indexes (as estimates of ecological status) and zooplankton biomass (as food resource availability). Pearson's r index revealed correlations among some of those variables. We applied Bonferroni correction to counteract the multiple comparisons issue and, finally, removed the variables that were highly correlated (>0.6). As *G. holbrooki* was not present in all the ponds (it was absent in 7 ponds out of 10), we could not include its abundance in our MLMs. Instead, we performed an ANOVA, comparing the situations "with" and "without" the presence of *G. holbrooki* for the abundance and for each of the size-related variables of *A. iberus* considered in this study.

We looked for the most parsimonious model from the full models by using a stepwise (backward) selection. The most parsimonious model was chosen using the Akaike information criteria (lowest AIC), which represents the best at explaining the data with the lowest combination of variables. We also calculated the standardized (beta) coefficients for the significant predictors included in the best models by using the R package "QuantPsyc" version 1.5 [59]. Predictors were previously checked for normality and homogeneity of variance, and, if variables did not meet the assumptions, base 10 logarithmic transformations were applied. Additionally, a visual inspection of the residual plots was done to detect any violation of the regression assumptions. In order to improve homoscedasticity, we used the function "varPower" of the package "nlme" [60]. For the creation of the boxplots, we used the "ggplot2" package [61]. All analyses were done with the software R version 3.4.2 (R core Team, 2017, Boston, MA, USA).

3. Results

3.1. Description of the Local Characteristics in the Mediterranean Ponds

There were wide ranges of environmental and ecological conditions across the study area (Table 1). Abiotic and biotic factors measured, as well as ECELS and QAELS indexes of ecological status, are shown in Table 1. The 10 studied ponds during spring showed conductivity values ranging from 10.7 $mS\cdot cm^{-1}$ to 69.10 $mS\cdot cm^{-1}$ and mean water column depths ranging from 16 cm up to 150 cm. The ponds differed quite a lot in their areas, with values ranging from 147.90 m^2 to 68,150 m^2. Total nitrogen, which includes organic and inorganic nitrogen compounds, showed the lowest value at 55.58 $\mu mol\cdot L^{-1}$ and the highest value at 234.40 $\mu mol\cdot L^{-1}$. The zooplankton biomass ranged from 1.13 $\mu g\cdot L^{-1}$ up to 4840.48 $\mu g\cdot L^{-1}$. Regarding the ponds' ecological status, ECELS index values ranged from 43 to 98, indicating a "bad" and "mediocre" status, respectively. Whereas the QAELS index ranged from 0.25 to 0.56, also indicating a "bad" and a "mediocre" status, respectively (Table S1). As an average, ponds showed a "deficient" status according to the mean index value.

Table 1. Mean, standard deviation (SD), minimum and maximum values of the abiotic and biotic factors and ecological status indexes measured in the study ponds (N = 10). ECELS: Conservation Status of Lentic and Shallow Ecosystems and QAELS: Water Quality of Lentic and Shallow Ecosystems.

	Mean	**SD**	**Minimum**	**Maximum**
Mean water column depth (cm)	59.30	41.93	16.00	150.00
Pond area (m^2)	10,873.60	20,463.50	147.90	68,150.00
Conductivity (mS·cm^{-1})	46.14	17.63	10.07	69.10
Total nitrogen (µmol·L^{-1})	92.01	51.63	58.55	234.40
Zooplakton biomass (µg·L^{-1})	498.17	1313.92	1.13	4840.48
ECELS index	77.00	18.70	43.00	98.00
QAELS^e 2010 index	0.47	0.12	0.25	0.56

3.2. Variation of the Population Structure of A. iberus across Mediterranean Ponds

The mean, standard deviation and minimum and maximum values calculated for the different size metrics of *A. iberus*, as well as the abundance (expressed as CPUE), are shown in Table 2. Maximum length values ranged from 19 mm to 54 mm, while the mean length had a minimum of 16 mm and a maximum of 41 mm. This last value coincided with the maximum value of the length range, whereas the minimum length range value was 6 mm. Size diversity showed a wide range of values, from 0.27 to 2.34. *A. iberus* abundance (in CPUE) also varied largely among fyke nets, from two individuals to 525 individuals.

Table 2. *A. iberus* size metrics and abundance (CPUE) obtained/computed per each sample (N = 49). The descriptive statistics are the mean, standard deviation (SD), minimum and maximum.

	Mean	**SD**	**Minimum**	**Maximum**
Aphanius iberus maximum length (mm)	41.08	8.06	19.00	54.00
Aphanius iberus mean length (mm)	29.21	5.36	16.00	41.00
Aphanius iberus length range (mm)	21.67	7.38	6.00	41.00
Aphanius iberus size diversity	1.15	0.36	0.27	2.34
Aphanius iberus capture per effort unit (CPUE)	59.49	91.68	2.00	525.00

3.3. Main Drivers Affecting the Fish Population Size Structure and Density

The MLMs identified the most important drivers influencing the fish population size structure and density across all ponds. The most parsimonious significant models for each fish metric mentioned above (Table 2) as dependent variables are shown in Table 3. The results showed that the maximum length was negatively related to the conductivity and zooplankton biomass but positively related to the total nitrogen and QAELS index. The zooplankton biomass was the predictor with the strongest effect on the maximum length of *A. iberus* (Table 3).

Concerning the mean length of the fish, the MLM model showed similar results as when considering the maximum length of *A. iberus* as a response variable. The mean length of *A. iberus* significantly decreased with the increasing conductivity and zooplankton biomass but showed a positive relation with the ecological quality index QAELS.

Table 3. Results of the linear mixed models (N = 49 fyke nets) showing the predictor variables that significantly relate with *Aphanius iberus* size metrics and abundance (expressed as CPUE). Only the most parsimonious significant models were shown for each response variable. For each model, the intercept (estimated and standard error, S.E.), beta coefficients (standardized), *t*-value, significance (*p*-value) and degrees of freedom (df) are also reported.

Response Variable	Predictor	Estimate	S.E.	Beta Coefficients	*t*-Value	*p*-Value	df
Aphanius iberus MAXIMUM length	Conductivity	−19.49	4.69	−0.47	−4.15	<0.01	5
	Log Total Nitrogen	2325.09	564.20	0.45	4.12	<0.01	5
	QAELS index	3894.26	690.27	0.59	5.64	<0.01	5
	Zooplankton biomass	−0.22	0.05	−0.69	−4.51	<0.01	5
Aphanius iberus MEAN length	Conductivity	−0.15	0.05	−0.42	−3.35	0.02	6
	QAELS index	31.94	6.76	0.55	4.73	<0.01	6
	Zooplankton biomass	−0.01	−0.01	−0.59	−4.01	0.01	6
Aphanius iberus length RANGE	Log Pond Mean Depth	14.45	5.36	0.42	2.70	0.03	7
	Log Total Nitrogen	36.88	8.93	0.48	4.13	<0.01	7
Aphanius iberus SIZE DIVERSITY	Conductivity	<0.01	<0.01	0.29	2.21	0.06	7
	Log Total Nitrogen	0.93	0.37	0.30	2.51	0.04	7
Aphanius iberus capture per effort unit (CPUE)	Log Pond Mean Depth	1.46	0.57	0.55	2.56	0.04	7
	Log Total Nitrogen	2.15	0.97	0.36	2.23	0.06	7

The length range of *A. iberus* was found to be positively related to the pond mean depth and total nitrogen. Beta coefficients of this model (0.42 and 0.48) showed similar effects of both predictor variables on the length range. The same results were found for fish density, with the CPUE positively related to the mean depth and total nitrogen (Table 3), suggesting that a higher number of individuals inhabited more nutrient-rich and larger ponds. However, the pond mean depth exhibited a stronger correlation on the fish density than the total nitrogen (beta coefficients of 0.55 and 0.36, respectively; Table 3).

Concerning the size diversity, it was positively related to the total nitrogen and only slightly related with the conductivity (Table 3). In this case, the beta coefficients for the two main drivers were similar (0.29 for conductivity and 0.30 for total nitrogen; Table 3). Finally, the ECELS index and pond area were the only variables not retained in any of the models selected (see the full models in Supplementary Table S2).

3.4. Influence of the Presence of *G. holbrooki* on the Size Structure and Density of *A. iberus*

Boxplots showed the CPUE and size metrics of *A. iberus* in the presence and absence of *G. holbrooki* in the pond (Figure 2). Overall, the presence of *G. holbrooki* in the pond did not significantly modify the size structure and density of *A. iberus* (*p*-values > 0.39; Figure 2). However, the mean size of *A. iberus* was significantly higher when *G. holbrooki* was present in the pond (Figure 2), indicating an unexpected increase of body size with the presence of the main competitor.

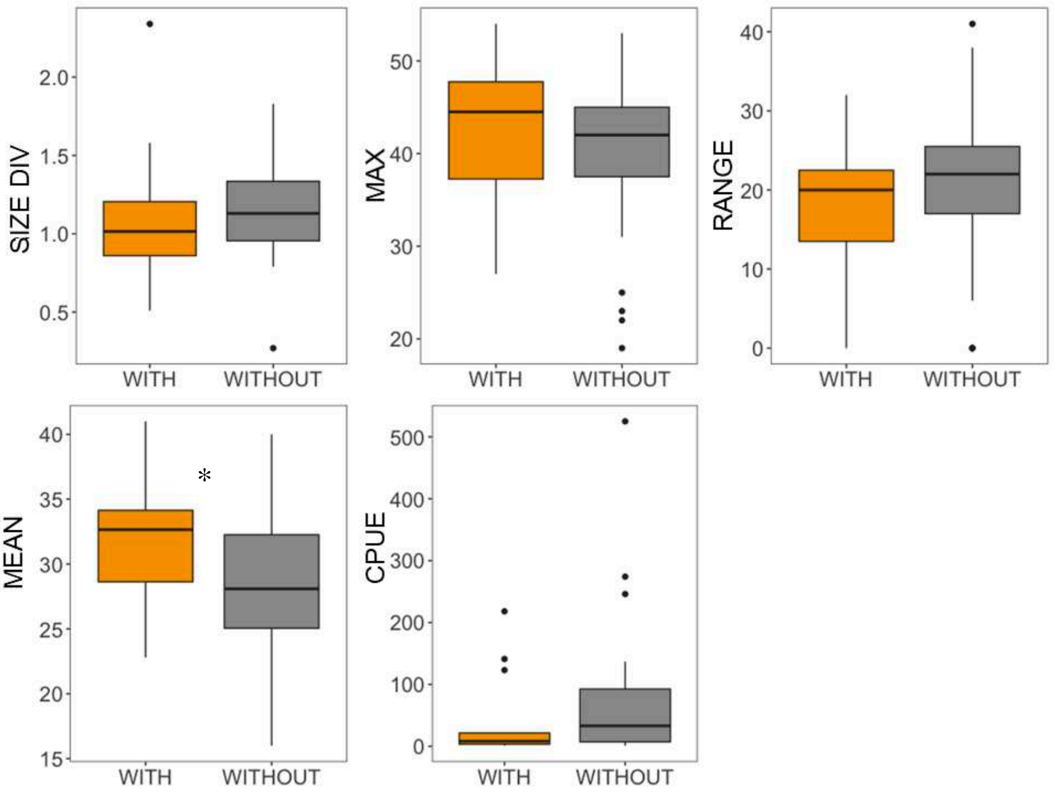

Figure 2. Boxplots showing the distribution of *Aphanius iberus* capture per unit of effort (CPUE) and size-related variables according to the presence ("with Gambusia") or absence ("without Gambusia") of *Gambusia holbrooki*. Significant differences are marked with the asterisk symbol (*).

4. Discussion

Our results suggested that both the maximum and mean sizes of *A. iberus* increased with the increasing pond's water quality (QAELS index) and decreased with the increasing conductivity and zooplankton biomass. The size range, maximum size, size diversity and CPUE of *A. iberus* were positively related to the nutrient concentration (i.e., total nitrogen), while the size range and CPUE were also larger in deeper ponds. In contrast to our hypothesis, the presence of *G. holbrooki* seemed not to affect negatively the population structure of *A. iberus*.

We found larger maximum and mean lengths of *A. iberus* in locations with better water quality (i.e., the QAELS index). These results support a previous study on multiple water bodies in the southernmost distribution area of *A. iberus*, showing its preference for ponds of better ecological status [15]. Although the results showed that the average of the studied locations had a deficient ecological status, higher values of the QAELS index are usually associated with the predominance of large zooplankton (such as big copepods) over small rotifers (that are more linked to eutrophic and hypoxic conditions; [53,54,62]. Adults of *A. iberus* (with larger body lengths) are usually associated with glasswort habitats (highly productive and occasionally inundated environments) where big zooplankton is more abundant, and this may support the positive relationship between *A. iberus* size and the ecological status of the ponds found in our study. In contrast, younger and smaller individuals positively select more eutrophic algal mats, associated with a bad ecological status, where small rotifers dominate [24].

The total nitrogen was found to be related with the CPUE, as well as with all size metrics, expect the mean length. In Mediterranean salt marshes, the concentration of total nitrogen in the water is an indicator of a pond's confinement or isolation [44], and during the late-spring and summer season, the total nitrogen is more concentrated because of evaporation processes [47]. Our results are in accordance with previous studies that showed that, in more confined and less accessible ponds, the populations of *A. iberus* are more abundant and stable over time, probably due to a lack of external perturbations, such as isolation from invasive species [17] or fewer entries of freshwater inputs. Since nitrogen is typically the limiting nutrient in these ponds, a higher total nitrogen concentration could also be associated with higher production rates of *A. iberus*, which, in turn, may favor larger populations.

Our results also showed, as expected, a negative relationship among conductivity and the maximum and mean sizes of *A. iberus.* In high saline habitats, conductivity can act as a "refuge" for the *A. iberus* to avoid the colonization of less salt-tolerant fish species, such as *G. holbrooki* [63]. However, high salinity levels may also have negative effects on the metabolism of cyprinodontoids [5,36], because the energy used for osmoregulation is not available for their growth performance and survival [33–35]. This could explain the presence of smaller fish in ponds at higher levels of conductivity.

In our study, the *A. iberus* CPUE, along with the *A. iberus* size range, were positively related to the pond depth. Similarly, studies from European wetlands and lakes found that wider and deeper waterbodies hosted greater biomass and sizes of fish [17,64], with the consequent higher probability to find a wider range of fish sizes [20]. Additionally, individuals of *A. iberus* trapped in brackish ponds due to competition exclusion and habitat degradation can, in some cases, reach unnaturally high densities [17].

Our results also showed that the zooplankton biomass is negatively correlated to the *A. iberus* maximum and mean sizes. Other studies on *A. iberus* observed that both juveniles and adults of this species have similar food preferences, as they mainly feed on harpacticoids, copepods and nauplii, detritus and diptera larvae [24]. Still, smaller individuals prefer feeding on small-sized prey, while larger fish show a greater preference for large-sized prey [24]. Larger individuals have higher feeding rates [34,65]. Thus, the presence of larger fish (expressed by higher mean and maximum sizes) may imply a lower zooplankton biomass, as it increases the consumption rates with the fish body sizes. In addition, previous studies observed that, when the potential resource availability is low, the fish size distribution tends to be more diverse, suggesting that competitive interactions for resources promote diversification by size [19,66,67].

As for the influence of *G. holbrooki* on *A. iberus* abundance and size structure, the results suggested that both the CPUE and size metrics of *A. iberus* were not affected by the presence of this allochthone fish. Only the mean size seemed to be slightly positively affected by the presence of the competitor. This result differed from our expectations, in which *A. iberus* would be smaller and less abundant in the presence of *G. holbrooki*. This apparent inconsistency could be explained by the fact that *G. holbrooki* was found just in few of the studied ponds, the ones with lower conductivity, and what we observed could be an indirect effect of environmental conditions that favor *A. iberus* development more than the effect of direct competition. Thus, the low number of ponds with *G. holbrooki* in our study did not unable us to derive strong conclusions about the influence of the *G. holbrooki* presence on *A. iberus* abundance and size structure.

In conclusion, our results suggest that the ponds' ecological status (as shown by the QAELS index), depth, conductivity and nutrient concentrations are key variables that determine the variations of the size structure and abundance of *A. iberus* in Mediterranean brackish ponds. Achieving a better pond ecological status seems to be important for the conservation of endangered *A. iberus,* because better size-structured populations (i.e., larger mean and average lengths) are found at higher water quality conditions. In addition, a pond's isolation may also be an advantage to preserve *A. iberus* populations.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/11/3264/s1: Figure S1: title, Table S1: Main geographic and morphometric characteristics of the studied ponds, along with the number of traps used and the ecological status values (QAELS and ECELS) for each pond, Table S2: Results of the MLMs (N = 49) showing the predictor variables that affect size-related variables and abundance (CPUE) of *Aphanius iberus*. Both Full models and Best models are presented. For each one, intercept (estimate and standard error, S.E.), Beta coefficients (standardized), *t*-value, significance (*p*-value), and df are shown.

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Fish Biodiversity Conservation and Restoration, Yangtze River Basin, China, Urgently Needs 'Scientific' and 'Ecological' Action

Jianhua Li [1], Xiaochen Chen [2,*], Xinliang Zhang [3], Zhenjia Huang [2], Liang Xiao [2], Liangliang Huang [4], Yuichi Kano [5], Tatsuro Sato [5], Yukihiro Shimatani [5] and Chunpeng Zhang [6]

[1] Key Laboratory of Yangtze Water Environment, Ministry of Education, Tongji University, Shanghai 200092, China; leejianhua@tongji.edu.cn
[2] Fujian Provincial Engineering Research Center of Rural Waste Recycling Technology, College of Environment and Resources, Fuzhou University, Fuzhou 350108, China; zhuang@fzu.edu.cn (Z.H.); n190627056@fzu.edu.cn (L.X.)
[3] Crawford School of Public Policy, Australian National University, Canberra, ACT 2601, Australia; adam.zhang@alumni.anu.edu.au
[4] College of Environmental Science and Engineering, Guilin University of Technology, Guilin 541004, China; llhuang@glut.edu.cn
[5] Department of Urban and Environmental Engineering, Graduate School of Engineering, Kyushu University, Fukuoka 819-0395, Japan; kano@species.jp (Y.K.); sato@civil.kyushu-u.ac.jp (T.S.); shimatani@civil.kyushu-u.ac.jp (Y.S.)
[6] Key Laboratory of Groundwater Resources and Environment (Ministry of Education), Jilin University, Changchun 130021, China; zhang_cp@jlu.edu.cn
* Correspondence: chenxiaochen@fzu.edu.cn

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Abstract: Although many significant policies and legislation have been put forth by the Chinese government aiming toward "ecological civilization" for the aquatic environment, in practice, the situation is still undesirable. A pioneering multi-year study has since been conducted on the East Tiaoxi River (a major tributary of the renowned Yangtze River), focusing on fish distribution, influencing factors and habitat requirements, and river health as well. This revealed certain key species, hotspot protection areas, negative impacts of local anthropogenic activities, and inappropriate perceptions and practices of conservation and restoration. We found that not all fish species were equally conserved and that the supposedly ecological engineering measures were initiated without regard for ecological integrity. Our exemplary study appreciates scientific basis and truly ecological notion, and urgently advocates comprehensive and continuous basin-scale scientific investigation of fish biodiversity, and "ecological river"-oriented conservation and restoration action for the Yangtze River Basin and broader areas of China.

Keywords: conservation; ecological integrity; fish biodiversity; restoration; river basin management; Yangtze River

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1. Introduction

The number and biodiversity of fish are the most significant indicators of the health of a waterbody. In China, there are more than 1100 species of freshwater fish, 70% of which inhabit the rivers [1,2]. Although the total number is notable among worldwide statistics, the biodiversity has been continuously declining with increasing anthropogenic and economic development. In fact, as early as 1998, 92 species of the freshwater fish were listed in the China Red Data Book of Endangered Animals: Pisces [3], which shows that four species unfortunately have gone extinct and 28 species have become endangered. The loss of biodiversity greatly exceeds the world average [4]. It is the adverse anthropogenic impacts on the freshwater ecosystem that must first be to blame. Both notorious industrialisation and urbanisation are major hindrances to sustainable development in China. Against the background of abrupt population expansion, many irrational activities associated with urbanisation have led to deterioration of the aquatic environment, such as unsustainable exploitation of water resources, improper river modification, pollutant discharge and exotic species invasion.

In 2018, China's 13th National People's Congress inserted the term "ecological civilization" into the country's constitution for the very first time, indicating that the notion has now been raised up to the national strategy level as a main focus paralleling economic and social development [5]. In the field of aquatic environment, Chinese government has established a series of policies aimed toward balanced water resources exploitation and aquatic ecosystem protection and restoration. In April 2015, the State Council of China promoted the "Action Plan for Prevention and Control of Water Pollution," a milestone also known as the "Ten-point Water Plan," the primary objective of which was to substantially improve the country's water quality and aquatic ecological situation by the year 2020 [6]. More recently, legislation also has been enhanced. In 2018, the revised Water Pollution Prevention and Control Law came into force, aiming at boosting aquatic environmental protection and restoration from a legal standpoint [7]. The effects of water quality on aquatic biodiversity have been comprehensively reviewed, and accordingly a series of protection areas have been established throughout the country. All these actions demonstrate that the priority of public policy has shifted from mainly social and economic progress to also include environmental conservation for healthy aquatic ecosystems and sustainable hydrologic cycles.

However, the practical situation suggests an aquatic ecological deficit would remain. What is worse, knowledge of the ecosystemic constitution of freshwater fish (including migratory fish) of river basins over much broader areas of China, as well as specific factors influencing the biodiversity, is far from sufficient. The reported monitoring data over the past few decades were either not comprehensive or lacked continuity. As a result, the conservation and restoration of aquatic ecosystems have been performed nationwide without a reliable scientific basis and have even tended to be too formalistic and/or disorganised. To make contributions for sound river basin management in China, a pioneering study on the ecological situation (especially the fish biodiversity) was initiated in the Yangtze River Basin. The necessity and significance of the scientific basis and the truly ecological notion would be emphasized. Besides, the comprehensive and continuous basin-scale scientific investigation, as well as the "ecological river" concept-oriented conservation and restoration action, would be timely advocated.

2. Materials and Methods

As shown in Figure 1, the Yangtze River originates from glaciers on the Qinghai–Tibet Plateau at its westernmost point, heads toward the East China Sea and eventually enters the Pacific Ocean. Along the way, it flows across the southwestern and central parts of China before reaching its outlet in Shanghai (the economic centre of the country). With a length of 6300 km and a catchment area of 1.8 million km^2, the Yangtze River is the third largest river in the world. Having the biggest discharge volume, it also supports the world's largest hydroelectric power station, i.e., the Three Gorges Dam. Designated as part of the latest national development strategies, the Yangtze River Economic Belt includes about 40% of the country's population and aggregate economy and is on the way of high-speed development. In its development planning outline issued in 2016, "ecology first" and "green development" form basic principles. Unfortunately, the Yangtze River Basin had long been sustaining extremely severe destruction of aquatic habitats [8]. In a multi-year study undertaken by the China Europe Water Platform (CEWP) project, a China–Austria cooperating project, etc., representative tributaries of the lower basin of the Yangtze River were thoroughly and continuously investigated. The main study area was the East Tiaoxi River Basin, one of the best epitomes of the environmental issues of the Yangtze River Basin (Figure 1). With a mainstream length of 152 km and a catchment area of 2267 km^2, the East Tiaoxi River is the largest river flowing into Lake Taihu, the third largest freshwater lake in the Yangtze River Basin. This lake with scenic beauty has been drawing focuses of worldwide researchers, ironically due to its notorious chronic water pollution problems such as eutrophication [9].

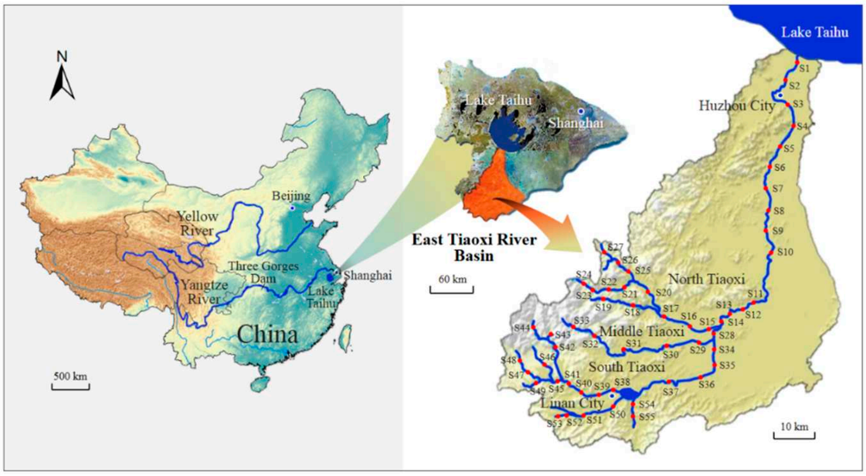

Figure 1. Study area and sampling sites.

Regarding fish species and communities, the joint research focused primarily on the following: (1) spatiotemporal distribution, (2) natural and anthropogenic influences, (3) habitat requirements of certain key species and (4) river health assessment based on fish-based index of biological integrity (FIBI). Regarding FIBI system development, 24 metrics were selected as candidate metrics, which covered five attributes of fish assemblage, i.e., species richness and composition, trophic composition, tolerance/intolerance, reproductive guilds, and fish abundance and health condition. Two FIBI systems were then established for the upper reaches (eco-zone of evergreen broad-leaf forest) and middle-lower reaches (eco-zone of urban and suburban agricultural areas), respectively. The detailed system composition, as well as the criteria of metric scoring and assessment, was described by Huang et al. [10] Geographic Information System (GIS) was one of the major tools used to facilitate the entire research. Detailed field investigation was carried out at 55 sampling sites of the East Tiaoxi River Basin (Figure 1), by wading in the upper reaches (reservoirs excluded) and by boat in the middle-lower reaches, respectively. In the shallow reaches fish were sampled using specific backpack electrofisher at the main habitat units such as riffle, glide and pool, while in the middle-lower reaches local boat electrofisher was used. The samples were identified to species level, followed by data processing using methods such as sample-based rarefaction curve and cluster analysis to reveal fish distribution patterns [11–13]. The spatiotemporal variation of fish communities and the associations of fish density and richness with environmental variables (influencing factors) were also investigated using methods such as non-metric multidimensional scaling and canonical correspondence analysis based on fish abundance data [11,13–15]. Note that the major natural factors focused on included distance to source, altitude, stream width, stream velocity, stream depth and pH, and the major anthropogenic factors covered residential area, revetment, farmland and factory.

3. Results

In this study, we recorded 84 species of fish, 35 of which were endemic. The main results are presented in Table S1, Figures S1 and S2. Inspiringly, certain endangered species in the neighbouring country Japan were found to be locally flourishing (Figure 2). Owing to the devastation of habitats as well as the blockage of breeding migration routes, the once common *Leptobotia tchangi* Fang, 1936 has almost disappeared and hence was called a "natural monument" by the Japanese Ministry of the Environment.

For similar anthropogenic reasons, *Vanmanenia stenosoma* Boulenger, 1901 and *Acheilognathus chankaensis* Dybowski, 1872 are both at risk of extinction in Japan. The detailed investigation of their habitat requirements (stream depth, stream velocity, pebble size, etc.) provided valuable information for further protection and restoration initiatives in both China and Japan. Taking *Leptobotia tchangi* for instance, it was found that its density had positive correlation with stream velocity, while its body size positively correlated with pebble size (see Figures S3 and S4). On the other hand, the once-famous *Coilia ectenes* Jordan & Seale, 1905 of high culinary and economic value now can rarely be observed, as the deterioration of water quality, devastation of habitats and overharvesting in the Yangtze River led to the drastic decline of this diadromous fish in the past few decades [16]. Meanwhile, another variety featuring freshwater migration—*Coilia nasus taihuensis* Yuan, Lin, Liu & Qin, 1977—has emerged in Lake Taihu and tributaries of the East Tiaoxi (Figure 2), reminding people of the dramatic ecological change induced by human activities. Furthermore, according to the results of river health assessment (Tables S2 and S3), 24 out of 45 monitoring sites were determined to be "healthy" or "fair," while the rest were either "poor," "very poor" or "null." Based on the strategy of identifying conservation priority areas first, 21 locations with high fish biodiversity were determined to be hotspot protection areas, including the habitats of all the above-mentioned key species (see Figure 2). GIS-based tools significantly increased the accuracy of protection activities.

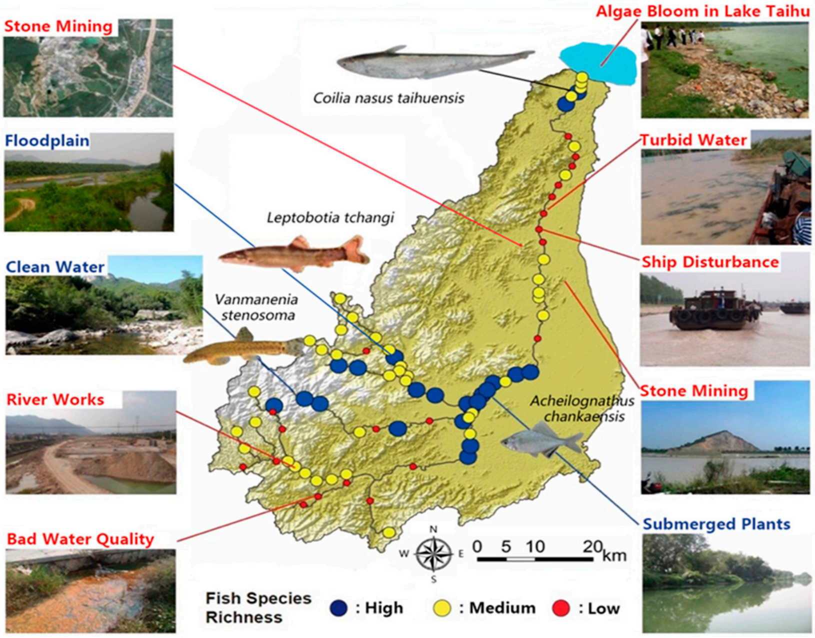

Figure 2. Map of fish biodiversity hotspots and influencing factors for East Tiaoxi River.

By comprehensively researching the influences on the richness of fish species, natural factors were identified, including distance to source, altitude, stream width, stream velocity, stream depth and pH (Table S4, Figure S5). More importantly, the study also revealed the impacts of anthropogenic activities (Figure S6, Table S5), especially the negative influences (Figure 2). In addition to common adverse effects such as from water pollution and ship disturbance, we found the impact of local privatised stone mining to be non-negligible. With no regard for the aquatic environment, the stone powder and other residuals from mining and transportation freely entered the adjacent waterbodies, which seriously suffocated the fish by getting stuck in their gills. Besides, other ubiquitous practices such as river training works and water pollution control projects have been continuously converting river environments from their original states to artificial states, thereby causing serious loss of the basic ecological functions of the rivers. The entire study area has locally been subjected to various negative impacts by human activities, such as the expansion of floodways, constrictions caused by bridge crossings, river realignments due to cutoffs and restoration activities, changes in resistance of dikes, weirs and riprap, river diversion for water intakes, dams and grade control structures, sediment supply from deforestation, increased runoff due to impervious cover, increased nutrient loads and aggravated bank erosion (see Figure 3 for examples). From one October to the next, engineering construction had degraded a hotspot with 15 fish species and 216 individuals to a silent-spot with only 6 species and 20 individuals in just a year. Only where the aquatic habitats were maintained in a good state and rich with hydrophytes was satisfactory fish biodiversity observed (Figure 2). Ironically, the "submerged plants" area would have been reclaimed without this study at river basin scale. The flora presents relevant ecological significance, including the ability to mitigate negative impacts resulting from stone mining. However, it was completely neglected in previous conservation and restoration schemes, as regular surveys on water quality and fish biodiversity were conducted randomly and only at the local level, thus failing to draw the whole picture.

Figure 3. Threat to riverine fish biodiversity of Yangtze River posed by engineering construction.

We have also noticed inappropriate perceptions and practices of protection and restoration, which require urgent correction. For example, only fish regarded as flagship species and/or included in the national protection list draw broad attention, such as *Acipenser sinensis* Gray, 1835 and *Neophocaena* Palmer, 1899. Normal fish species, including ones presented in Figure 2, are usually ignored. However, based on the theory of a healthy ecosystem as well as the experience and lessons of many developed countries, all the species within a specific ecosystem must be equally conserved, as they all interrelate and interdepend. Swayed by the impractical expectation of instant effects as well as influenced by the urbanisation tendency in river basin planning, various kinds of supposedly ecological engineering measures are prevailing in riparian areas. Energy-consuming modes of wastewater treatment plants and facilities have been transplanted to the riparian areas. Since they fulfil the dream of ameliorating water pollution overnight, such palliatives make it seem to be quite desirable for local government to handle the inspection and assessment based on directives from higher authorities. Also, popular artificial wetlands have been constructed here and there, with the main focus being on water purification and aesthetics rather than biodiversity conservation. Even worse, a large amount of money has been invested to the artificial landscape projects with no true ecological idea involved, such as a hydrophilic platform (Figure 4). These supposedly ecological measures swiftly satisfy human desires but go against the laws of nature. With dramatically changed land cover and land use in the riparian areas, the vulnerable habitats for fish and other living creatures are apt to be even further degraded. According to the theory of ecological integrity, it is a natural and undisturbed state with physical, chemical and biological integrity that should be the right goal of ecological restoration [17].

Figure 4. Hydrophilic platform, an example of supposedly ecological measures.

4. Discussion

The master plan for the development of the Yangtze River Economic Belt has become one of China's principal national strategies. Because the primary goal of water-related environmental policies in China has fundamentally changed as conservation and restoration gain top priority, some measures have been taken as essential, such as a 10-year ban on fishing in the Yangtze River starting in 2020. However, this case study revealed that current practices apparently fail to keep pace with the central government's directives. The present situation in the Yangtze River Basin is still sounding the alarm and causing critical concern among the public. As the biodiversity of freshwater fish keeps on decreasing mainly due to anthropogenic impacts, it is apparent that there has been a serious lack of scientific basis and truly ecological action for sound river basin management.

Comprehensive and continuous scientific investigation at river basin scale is proposed to track riverine fish biodiversity and to further understand the influencing factors. We believe that accurate identification of ecological protection red lines and relevant hotspot protection areas, more targeted action plans and technical guidelines for normalising conservation and restoration activities would be achieved only on the basis of data from scientific study in accordance with the theory of ecological integrity. The results of this exemplary study reveal significant insight into the highly anticipated nationwide practices. To support the initiative, we recommend that the relevant government bodies—such as the Ministry of Science and Technology and the affiliated National Natural Science Foundation of China—set up special funds for long-term fundamental research on the ecological environment and biological resources of the Yangtze River Basin. All involved local authorities should also take responsibility for routine environmental monitoring and assessment. Further, we advise that the supposedly ecological engineering measures for conservation and restoration, together with the destructive pollution control and treatment practices, be strictly prohibited by policy and institutional means [18], which recently have received positive reply from the prime minister. Meanwhile, scientific data and the know-how for effective environmental conservation and restoration must be delivered to and shared among all the stakeholders, especially the government decision-makers in this Chinese case. From the viewpoint of ecological integrity, the real concept of "ecological river" needs to be advocated and put into practice, which aims at recovering the original structure and ecological functions of a river and hence make it regain the resilience and stability in order to maintain acceptable quality under long-term or sudden disturbance [13]. Last but not least, to ensure the success of the whole cause, more stringent legislation and regulations must be enacted and enforced.

Water resources management is the epitome of China's environmental issues, and significant aquatic biodiversity loss in the major river basin shows the severity of the challenges. Heading toward sustainable development, China must make its great power accountable and lead the way by taking "scientific" and "ecological" action.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/11/3043/s1, Figure S1: Fish species composition in the East Tiaoxi River [13], Figure S2: Fish species richness in each stream order of the East Tiaoxi River [13], Figure S3: The relation between *Leptobotia tchangi*'s density and stream velocity at different sampling sites [11], Figure S4: The relation between *Leptobotia tchangi*'s body size and pebble size at different sampling sites [11], Figure S5: Canonical Correspondence Analysis (CCA) of environmental factors and fish assemblage [11], Figure S6: Impacts of anthropogenic activities to non-parametric multidimensional scaling (NMDS) ordination of fish assemblages [11], Table S1: The list of fish species and their distribution in the East Tiaoxi River [13], Table S2: Criteria for river health assessment based on ecological integrity of fish in the East Tiaoxi River [11], Table S3: River health condition based on Fish Integrated Biotic Index [11], Table S4: Matrix of Spearman r_s correlation coefficients of environmental variables [11], Table S5: One-way ANOSIM for analysing the impacts of human activities on fish communities [11].

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Article

First Captive Breeding Program for the Endangered Pyrenean Sculpin (*Cottus hispaniolensis* Bacescu-Master, 1964)

Joan Manubens [1,*], Oriol Comas [1], Núria Valls [1] and Lluís Benejam [2]

[1] Associació de Defensa i Estudi de la Fauna i Flora Autòctona (ADEFFA), Camadoca, Santa Maria de Merlès, 08517 Catalonia, Spain; a.adeffa@gmail.com (O.C.); hola@adeffa.cat (N.V.)
[2] Aquatic Ecology Group, University of Vic—Central University of Catalonia, Vic, 08500 Catalonia, Spain; lluis.benejam@uvic.cat
* Correspondence: joanmanubensgil@gmail.com

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Abstract: The strong decline of freshwater fish species in Europe implies that further ex-situ conservation plans should be implemented in the near future. The present study reflects our experience with the Pyrenean sculpin (*Cottus hispaniolensis* Bacescu-Mester, 1964)—a small cottid endemic to the Hispano-French Garona River basin. In recent years, the Spanish Pyrenean sculpin population has reached a limit situation. Because of that, the non-profit association ADEFFA—with support from the public administration—started the first captive breeding program for this species in 2006. Fourteen years later, this study presents the results and evaluates the different steps of the program, with the aim of discussing and improving the ex-situ conservation plans for this and other cold freshwater species. There is a description and a comparison between six consecutive phases during the captive breeding process: nesting behaviour, courtship, egg fixation, parental care (incubation), hatching and survival during juvenile development. The purposes of this project are to: (1) identify the most determining phases for a successful captive breeding; (2) identify the factors that had a major influence to the success of the critical phases; and (3) increase the number of the offspring. This study is based on thirty-three wild individuals collected from Garona River (Val d'Aran, Spanish Pyrenees). During the program, twelve couples spawned in captive conditions, with around 2300 eggs laid. Eight couples bred successfully, with 751 hatched individuals and 608 juveniles reared. The analysis of each step of the captive breeding does not reveal significant differences between phases, so it can be concluded that they are all critical at the same level. In the literature, similar study-cases of captive breeding programs identify incubation and survival phases as the most critical. Consequently, the management made for this project has probably allowed to overcome in part the main impediments described in other similar programs.

Keywords: threatened species; conservation planning; climate change; captive breeding; *Cottus hispaniolensis*

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1. Introduction

Freshwater ecosystems are some of the most threatened ones in the world. Over 37% of European freshwater fishes are threatened—which is one of the highest threat levels in any major taxonomic group [1]. Especially the Iberian Peninsula is experiencing a critical conservation status of its ichthyofauna, with 52% of species now catalogued as critically endangered, endangered or vulnerable, according to the IUCN criteria. This condition is related to a high degree of endemism (among native species, 65% are endemic to the Iberian Peninsula) and a long history of human impact in these areas [2,3].

In Europe freshwater species of the genus *Cottus* (family Cottidae, order Scorpaeniformes) are found in a variety of cold-water habitats such as well-oxygenated headwaters, lakes and channels. They are generally nocturnal and feed on wide variety of benthic invertebrates. The egg-laying period occurs between March and April (when the water temperature rises above 12 °C). The eggs are adhesive, laid in a compact clutch on the ceiling of small cavities, so they need gravel or rocky river beds. Females spawn once a year and most individuals spawn for several years. Males guard the eggs until hatching, while they do not feed and may lose 20% of their body weight [4–6].

Recently, a total of 16 *Cottus* species have been described in Europe [4,5,7]. Several of these species are endemic of certain river basins and isolated in their particular habitats, as ecological barriers do not allow them to migrate across main rivers [4,5]. These speciation events occurred due to glacial periods [8,9]. These range-restricted species have been reported as endangered during the past few years, mostly due to habitat fragmentation [10–13]. The habitat fragmentation is aggravated by the intense human pressure, with additional threats such as habitat reduction caused by urban and agricultural pollution; water extraction for agriculture and damming; or unnatural flow regimes resulting from flow regulations which meet electrical power demands—often resulting in low water levels, followed by instantaneous high flow regimes [6,10,11,13]. This impact has reduced the distribution areas into smaller and isolated populations, which face serious risks such as environmental changes, demographic stochasticity, natural catastrophes and reduced genetic variability. Therefore, the combination of these threats leads to population decline and, eventually, to extinction [14–16].

This study focuses on the Pyrenean sculpin (*Cottus hispaniolensis* Bacescu-Mester, 1964), a small cottid (up to 100–150 mm, total length) endemic to the Hispano-French Garona River basin and restricted to the southern part of the drainage—in the central part of the Pyrenean mountain range [4,5]. This species shows a discontinuous presence along its distribution range [17], with an apparent (but unknown) degree of isolation.

Freyhof & Kottelat [18] catalogued the Pyrenean sculpin as of *Least Concern* according to the IUCN guidelines. Afterwards, Doadrio et al. [19] reported an accentuated population decline and proposed to catalogue it as *Critically Endangered* (B1bc+2bc), which was supported again by Sousa-Santos et al. [13]. The Pyrenean sculpin is included in the Annex II of the European Habitats Directive 92/43/EEC [20] and in the Annex I (for endangered species) of the Spanish National Catalogue of threatened species [21]. In the French Red List, it is included as Data Deficient [6].

During the summer of 2013, there was a devastating flood in the Garona River—caused by heavy rainfall and a big fast snow melt—with an estimated return period (RP) of 30–50 years [22–24]. Changes in the river course and the subsequent arrangement by the machinery led the Pyrenean sculpin populations to a limit situation: only 16 individuals were captured during several samplings along most of the Spanish distribution area in 2014 [17].

The delicate situation of the Pyrenean sculpin has been known for a long time. The Conselh Generau d'Aran (local administration) and the LIFE LimnoPirineus project (NAT/ES/001210) have been working with the species. They have been sampling the population of the Pyrenean sculpin since 2001, in different points all along the Spanish part of the Garona and its tributaries, in order to determine its size, density and trends. Further actions need to be implemented—such as genetic studies [17]. Knaepkens et al. [25] have suggested that in-situ actions are required to increase the population size and range of endangered populations. It is therefore necessary to restore the longitudinal functionality of rivers and to guarantee its dilution capacity, by: improving water management policies—in order to mimic the natural hydrologic regime; improving the connectivity with well-designed fish ladders; and reducing pollution—in order to improve water and ecosystem quality [2,13].

When endangered species are incapable of surviving in their natural habitat despite all the in-situ efforts, and effective alternatives are unavailable in the short term, ex-situ conservation can play a crucial role [26–28]. The strong decline of freshwater fish species implies that further ex-situ conservation plans should be implemented in the near future [2]. However, it is important to underline that captive breeding is a last resort and it should not be a long-term conservation strategy which excludes other in-situ actions [26,28–30], because it is typically associated with many limitations such as high economic costs, diseases, adaptation to captivity, relaxation of adaptation, inbreeding and loss of genetic diversity—with consequent inbreeding depression and fitness reduction [26–29,31–33].

In 2006, the non-profit association ADEFFA, with the support from the public administration, started the first captive breeding program for this species in Camadoca wildlife centre (Santa Maria de Merlès, Barcelona). The main purposes of the breeding program were to complete the ex-situ breeding cycle, to maintain a genetic stock, and to study the biology, reproduction and behaviour of the Pyrenean sculpin. Other actions such as habitat restoration, population studies in-situ, environmental education and divulgation were also implemented to complement the breeding program [34].

This study describes the results and the evaluation of fourteen years of captive breeding of the Pyrenean sculpin, as an ex-situ conservation program. It describes and compares six consecutive breeding phases during the breeding period: nesting behaviour, courtship, egg fixation, parental care (incubation), hatching and survival during juvenile development, in order to: (1) detect which one of these phases of the reproductive cycle is most determining with respect to the success of the captive breeding; (2) observe the variation into this critical phase/s and the factors influencing its success; (3) determine key factors that influence the success of the captive breeding and the number of offspring produced; and, finally, (4) develop possible improvements for the critical steps of the ex-situ program. The results of other captive breeding programs with cold freshwater species have shown that the incubation and the juveniles' survival are the most critical phases [35–37]. Therefore, the hypothesis is that these two phases could be the most determining ones to the success of the captive breeding program for the Pyrenean sculpin.

2. Materials and Methods

2.1. Brood-Stock Transportation and Maintenence

Thirty-three wild samples of Pyrenean sculpin (18 males and 15 females) were collected along the Spanish part of the Garona River (Figure 1) by electrofishing during spring in 2006, 2009, 2014, 2017 and 2018, with the collaboration of the Conselh Generau d'Aran forestry guards. Breeders collected in 2014 were maintained in captivity until 2015 breeding season. Fish total length ranged from 70 mm to 120 mm and weight ranged from 9 g to 26 g at the time of collection. They were carried in 25 L plastic tanks with oxygenation, inside isolation boxes, to the fauna conservation centre Camadoca (Santa Maria de Merlès, Barcelona). The care and use of all specimens had complied with local animal welfare laws, with the corresponding administration permissions. The facility counts with two 450 L rectangular glass tanks for the breeders without direct natural illumination and another 250 L rectangular glass tank for the juveniles and hatched larvae (rearing tank). Since 2015, there is a new and more efficient facility to improve the maintenance, with four more 250 L rectangular glass tanks for the breeders and direct natural illumination (from a window). In 2018, the frontal part of these tanks was covered partially in order to reduce the direct illumination from the window. The bottom of all the tanks (except the rearing tank) is covered with a gravel layer. Each tank is provided with stones or curved clay tiles as a substratum on which the fish can hide or make nest to spawn. Before every stocking event, water is changed, all the material is renewed and tanks are cleaned and disinfected.

Each tank counts with mechanic filter system (50 L/h), water cooling system equipped with thermostat (except in 2006) and continuous aeration. The rearing tank counts with an external UV-irradiation lamp (without direct contact with the tank). A single 40 W fluorescent suspended at 20 cm above the water surface of each tank provides a natural photoperiod (13L:11D). Except in 2006 (when there wasn't cooling system), water temperature is maintained between 8 °C and 10 °C for 20–40 days after the stocking. At this time, it is gradually increased to 11 °C. Adults are fed every evening with alive *Artemia sp.* nauplii, chironomids, tubifex and other freshwater macroinvertebrates *ad libitum*. Juveniles are first fed with decapsulated *Artemia sp.* during two weeks approx.

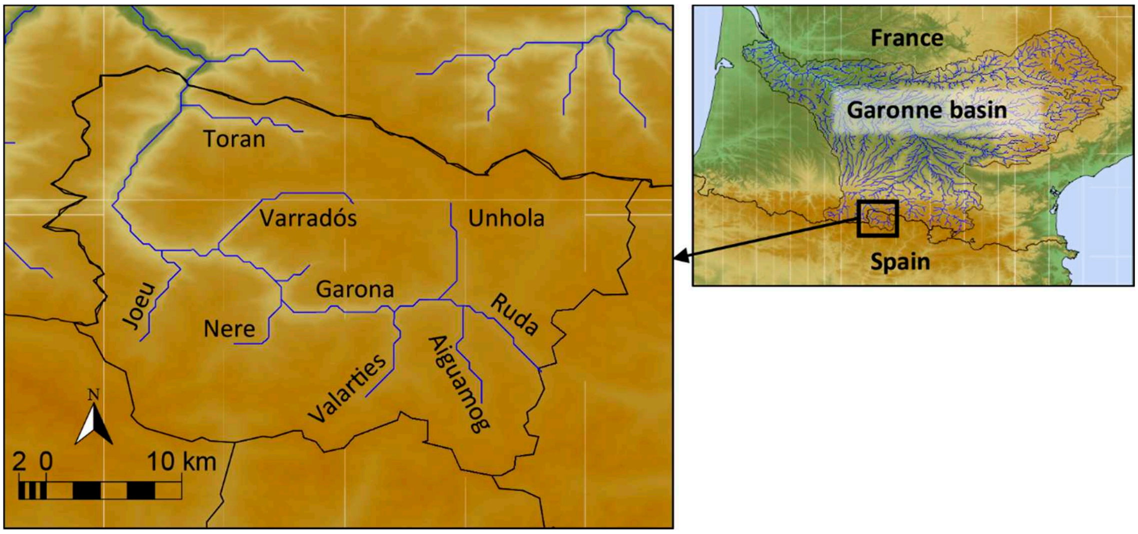

Figure 1. Map of the Garona drainage in Val d'Aran (Catalonia, Spain) and its main tributaries (left) and map of the Garona River basin (right).

2.2. Reproductive Cycle and Data Analysis

In order to evaluate which phases of the captive breeding are the most critical (with the objective of improving the breeding techniques effectiveness), the process is divided in six phases (1 = "nesting"; 2 = "courtship"; 3 = "fixation"; 4 = "incubation"; 5 = "hatching"; 6 = "survival"). The description of each phase and the success criteria used are attached in Table 1, in concordance with [38]. Each phase is evaluated with success (yes = 1) or failure (no = 0), depending on whether the couple finishes the phase successfully. It cannot be either success or failure if the previous phase is failure. Then, the number of couples that succeed in a phase is divided by the initial number of couples (percentage of success from initial couples) and by the number of couples that succeeded in the previous phase (percentage of success from the previous phase). These percentages of the phases considered to be most critical will be analysed.

Table 1. Description of each phase and the success criteria used. The male naturally guards the eggs until the hatching (ca. 30 days), but most times it happens that the clutch is removed from the breeding tank to be put it in the rearing one.

Phase Name	Description	Success Criteria
Nesting behaviour	In males: when the yellow margin of the first dorsal fin contrasts against the black overall coloration. In females: when ovulation process occurs.	If male constructs and guards the gravel cavity (the cave) under the stones or tiles. If female is gravid (full of completed eggs).
Courtship (sexual behaviour)	When the male attracts the female to the cave and 'hugs' her during hours until laying finishes.	If sexual behaviour occurs (external and internal courtship) and the eggs are fertilized.
Fixation	While female is laying, eggs are fixed by gelatinous matrix in an oval compact clutch.	If the clutch is fixed on the ceiling of the cave (fixation substrate is indicated to evaluate this phase).
Parental care (incubation)	The male ventilates (fanning activity) and guards the eggs from predators and infections.	If the male takes care of the eggs at least some days (number of days are indicated to assess this success).
Hatching	When larvae go out of the egg (it takes hours). They are little mobile and feed on the vitelline sack few days until they become juveniles.	If at least one individual hatches. It is assessed with the hatching percentage. $(N_{laid\ eggs}/N_{live\ hatched\ alevins})$
Survival	Survival during the juvenile development, 3 months after the hatching.	If at least one juvenile survives. It is assessed with the survival rate.

Each couple is also categorized by two additional variables, in order to control its effects upon success in the subsequent analysis and distinguish them from the phases effects upon success: couple type (1 = "first year in captivity"; 2 = "one year in captivity at least"; 3 = "born in captivity"; 4 = "combination of 2 and 3") and breeding year (1 = "2006"; 2 = "2009"; 3 = "2014"; 4 = "2015"; 5 = "2017"; 6 = "2018"). Then, a logistic regression is performed to test the relation between the binary outcome (“Success”) and the categorical variables (“Phase”, “Type” and “Year”) with a Generalized Lineal Model (GLM, family = binomial, link = logit) in R i383. Four different GLM models are performed to evaluate whether success is related to the different fixed factors (phase, type or year). The first one, considers the interaction between all three factors; the second and the third one, considers the interaction between two of them (the phase with the other two factors); and the last one, considers only the phase. Finally, the most parsimonious model is chosen, by comparing the Akaike Information Criteria (AIC). Significance level for this study is set to 5%.

During the six breeding seasons performed since 2006, twenty-two couples are formed including five individuals (one male and four females) born in captive conditions (Table 2). There are three males present in two couples (14–15, 16–17 and 19–20). Breeders of 2014 are the same individuals in 2015, but any combination of individuals in the couples is the same.

Table 2. Final results summary per year with total number of breeders present in the season and numerical code of each formed couple. Couples 14–15, 16–17 and 19–20 are formed with the same three males and different females. Where F = female, M = male. Apart from the success (Yes or No) it is indicated in parentheses the fixation substrate, the number of days during which the male takes care of the eggs, the hatching rate and the survival rate. † Individuals maintained at least one year in captivity. ‡ Individuals born in captivity. § Only female nesting behaviour is considered as success.

Year	No. Breeders	Code Couple	Reproductive Phases						No. Fitness
			Nesting	Courtship	Fixation	Incubation	Hatching	Survival	
2006	4	1	Yes	Yes	Yes (stone)	Yes (21 days)	Yes (49%)	Yes (14%)	14
		2	No	-	-	-	-	-	-
2009	8	3	Yes	Yes	Yes (stone)	Yes (4 days)	No (0%)	-	-
		4	Yes	Yes	Yes (stone)	Yes (3 days)	No (0%)	-	-
		5	No	-	-	-	-	-	-
		6 (F ‡, M ‡)	Yes §	Yes	Yes (glass)	No	-	-	-
2014	7	7	Yes	Yes	Yes (tile)	Yes (12 days)	Yes (73%)	Yes (94%)	170
		8	No	-	-	-	-	-	-
		9	No	-	-	-	-	-	-
2015	8	10 (F †, M †)	Yes	Yes	Yes (stone)	Yes (7 days)	Yes (7%)	Yes (100%)	4
		11 (F †, M †)	No	-	-	-	-	-	-
		12 (F †, M †)	Yes	Yes	No	-	-	-	-
		13 (F ‡, M †)	Yes §	Yes	No	-	-	-	-
2017	9	14	Yes	Yes	Yes (tile)	Yes (11 days)	Yes (36%)	No (0%)	-
		15 (F ‡)	Yes	Yes	Yes (tile)	Yes (11 days)	Yes (29%)	Yes (100%)	16
		16	Yes	No	-	-	-	-	-
		17	Yes	Yes	Yes (tile)	Yes (5 days)	Yes (41%)	Yes (93%)	75
		18	No	-	-	-	-	-	-
2018	9	19 (F ‡)	Yes	Yes	Yes (stone)	Yes (5 days)	No (0%)	-	-
		20	Yes	Yes	Yes (stone)	Yes (9 days)	Yes (9%)	Yes (14%)	2
		21	Yes	Yes	Yes (tile)	Yes (22 days)	Yes (56%)	Yes (95%)	212
		22	Yes	Yes	Yes (tile)	Yes (13 days)	Yes (60%)	Yes (96%)	115
% initial success			73%	68%	59%	55%	41%	36%	
% phase success			73%	94%	87%	92%	75%	89%	

3. Results

The results of GLM analysis show that proportion of success and failure is not significantly different among phases of the reproductive cycle (Table 2, Figure 2). Any of the four GLM analysis performed does not show a statistically significant relationship. The best performed model is the one that considers the binary outcome (success) and the effect of different phases (*p*-value = 0.595; d.f. = 5; AIC = 72.91). As it is shown in Table 3, models including the effect of the couple type or the year have a higher AIC value, so it can be considered that the best model has to exclude these effects. Anyway, as no model has a significance level low enough, the success is not significantly different between phases.

Considering that all phases are critical at the same level (no differences among phases), it is analysed the success percent and the variation observed into all of them. The lowest percent observed is in nesting behaviour (73% of the couples), including two couples (6 and 13) in which males did not present nesting behaviour (these couples continued to the next phase and completed it successfully). So, nesting behaviour is observed only in 27 breeders from the 45 present in the program (60%). On the other hand, 94% of the couples with success in the first step go on courtship and spawn—the higher percent observed. Only couple 16 failed in this phase (the female laid the clutch without interaction with the male). During 2017 and 2018, there were three males that spawned with two females (Table 2).

Egg fixation was completed in 87% of the couples in the last step: six fixed the clutch on the stone, six on a tile and one on the glass (couple 6). Couple 13 did not fix it. Female of couple 12 died in this phase without laying. The death cause determined in the necropsy was an ovarian obstruction. 92% of the fixed clutches were incubated at least few days for the male (parental care). Only two clutches (couple 1 and 21) were incubated until the hatching and the other ten were situated in controlled conditions (in rearing tanks) at 5–13 days after fixation. The second lowest percent is observed in hatching, with 75% of success of the incubated clutches. Moreover, the hatching rate is very low many times (with a reduced number of hatched larvae). Survival during juvenile development is a success 89% of the times, with only one failure.

From the eight couples that had brood with success, only four (7, 17, 21 and 22) had reared a considerable number of juveniles. In 2006 (couple 1), there was high mortality due to an infection. The diagnostic analysis—both of breeders and eggs—carried out in 2006 by doctor Francesc Padrós (Servei de Diagnòstic Patològic de Peixos, UAB) shows that infections during parental care were caused by fungus of the *Saprolegnia* genus. During the rearing in 2006, an *Ichthyophthirius multifiliis* infection (white spot disease) was also detected. Since 2015, it is observed that couples situated in the new facilities did not succeeded (11, 12, 13, 18 and 19) or had the lowest fitness number (10 and 20).

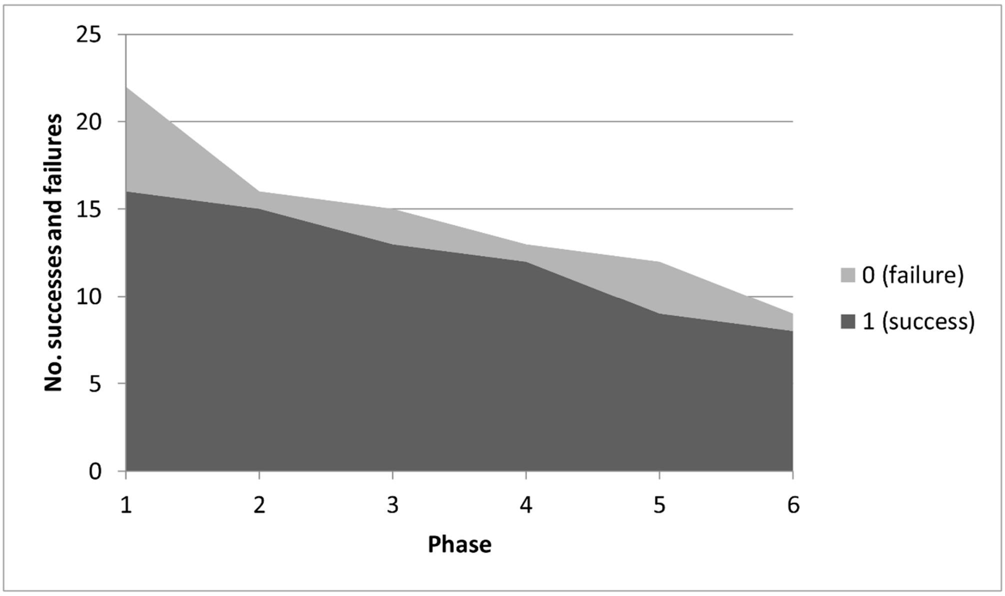

Figure 2. Number of successes (1) and failures (0) in each phase (1 = "nesting"; 2 = "courtship"; 3 = "fixation"; 4 = "incubation"; 5 = "hatching"; 6 = "survival").

Table 3. Results of the performed GLM analysis, with the factors considered for each model, its *p*-value (from a Chi square test), its freedom degrees and its AIC value.

Model	*p*-Value	d.f.	AIC
Exit ~ Phase + Type + Year	0.442	12	82.232
Exit ~ Phase + Type	0.817	8	77.976
Exit ~ Phase + Year	0.769	9	79.094
Exit ~ Phase	0.595	5	72.913

As general results of the breeding program, twelve couples spawned in ten nests with around 2300 eggs laid (mean = 212; SD = 118), 751 individuals hatched and 608 juveniles reared. Individuals (juveniles and breeders) of the captive breeding seasons of 2014, 2015 and 2017 were released in different points along the Garona River and its tributaries in collaboration with the LIFE LimnoPirineus project (NAT/ES/001210), a Fecsa ENDESA project and the Conselh Generau d'Aran, in order to support declining populations. There was no post-releasing monitoring program.

4. Discussion

As significant differences on the success among phases cannot be determined, the conclusion is that there is no phase more related to success than others. However, in most of the captive breeding programs with cold freshwater species, it is widely known that incubation and juveniles' survival are the most critical phases on its management. This is the case of the well-known salmonids hatcheries [35,39–41]. In the same way, it is also observed in little literature on the ex-situ breeding of the genus *Cottus* [36,37]. A comparison between the bibliography study-cases and results obtained during this project, leads us to discuss possible success/failure causes, factors that influence success and possible improvements for each phase. The aim is to improve program's management efficacy and finally to collaborate with the species conservation.

4.1. Analysis of Variation into Phases

Referring to nesting behaviour, factors that cause differences between couples are unknown. They may be intrinsic factors to the funding individuals (age, size, sexual maturity, life history, etc.) or they may also be factors related to the species' reproductive behaviour (social behaviour, sex ratio, relative size, etc.)—all of them related to mating success in behaviour studies [42–46], so specific behaviour studies (ex-situ and in-situ) should be developed in order to improve captive breeding effectiveness [47]. However, it is observed that there is a limiting factor in this step. The number of gravid females marks the number of couples that can breed, as far as one male can breed with more than one female. For that reason, it would be recommendable to increase the ratio of females per male in the event that there are more females than males in the brood-stock or if males do not present nesting behaviour. The female of the only couple that failed during courtship (couple 16) was bigger than the male, so relative size could be taken into account in future behaviour studies (also suggested by Brown [48] and Bisazza and Marconato [42]). Fixation is probably related to the type of breeders and the time during which they are maintained in captivity, as far as the female of couple 12 did not spawn the previous year (probably due to the ovarian obstruction). Fixation success is also probably related to behaviour, because when males failed in the first step, this phase was never completed correctly (if the clutch was fixed, it was fixed on the glass). Until further behavioural studies still lack, these types of breeders in the program should be avoided. However, the rate of couples that finally fixed the egg-laying (50%) is considerably higher than the 20% described in Piccinini et al. [37].

When fixation is completed properly, parental care also succeeds (except couple 6, commented above). Even though, success and quality of successive phases indicate that major problems occur during parental care, due to stress and infections. For its importance, those key factors will be discussed latter in detail. This tendency during parental care—as said before—coincides with problems described in the two study-cases of *Cottus* species breeding. However, they describe this phase as critical, together with survival—a phase described as successful in the present study. There is only one case in which this phase failed (couple 14), due to a human error in the maintaining. White spot disease problem was practically solved during the following breeding seasons—as the results show—working on the same key factors affecting incubation discussed below. In fact, after 2006, survival is considerably higher comparing with Vught et al. [36]. They describe problems in intensively larvae rearing with tanks.

4.2. Key Factors Affecting Success

There are some principal factors considered to be important for the success of the captive program that have influenced its development. These factors are stress, infections, type of breeders and time during which they are maintained in captivity.

It is well-known that stress negatively affects captive breeding [49]. During the first season of the program, remarkable territorial behaviours with aggressivity between males were observed. In order to avoid these stress inputs, since 2006, males are situated in different tanks during breeding period. It is also important to minimize noise in facilities and handling during all breeding phases. During 2009, some stress factors that could be related to the breeding failure were observed (illumination and noise due to an extensive monitoring with research and education purpose). Moreover, there are many factors such as illumination conditions that have to be considered as stress factors affecting reproduction, as indicate Raghavan et al. [50]. In that way, some of the differences between facilities (such as background coloration, illumination type or intensity) could affect reproduction success, as observed with couples situated in the new facilities. In fact, with a little modification done during 2018 by reducing the impact of overall illumination inside tanks, an improvement in the couple's behaviour situated there (19 and 20) was noted. Although it has to be studied in detail, any relation with smaller tank sizes in the new facilities could be discarded, because other breeding programs such as Vught et al. [36] describe a much larger brood fish density per tank.

Saprolegnia sp. infection is the main cause of problems in the incubation of several captive breeding programs and hatcheries, and it has a direct effect in their success [35,37,39–41]. The male Pyrenean sculpin naturally takes care of the clutch in order to protect it from fungal infections, but their appearance is still inevitable. With the management developed, there are three key factors used to control its expansion. The first one is temperature. In the first year, when there were not coolers, the water temperature was higher during summer, rising at 16 °C—when average temperature does not reach 11 °C during June in Garona River. Coldwater fish are more resistant to infection at lower temperatures [40]. Conversely, higher temperatures increase *Saprolegnia*'s growth rate [39,51] and favour rapid appearance of fungus while incubating. This can be prevented by maintaining temperature at a relatively low level (considering 11 °C as optimum for that species). The second key factor is feeding. High level of organic particles and nitrogenous waste in the water may predispose the fish to saprolegniasis [40] and reduce efficiency of disease treatments [52]. Although it is important to feed with organisms similar to the ones that we find in the species natural habitat [29], feeding with organisms from totally different environments may be safer to prevent infections, especially during incubation. It has been shown that high salinity prevents saprolegniasis [40,53] and withe spot disease [54], which could imply that the *Artemia sp.* is a less probable vector. For that reason, since 2009, during critical periods, we feed exclusively with *Artemia sp.* Finally, the third key factor: the number of days and the kind of treatment during incubation in controlled conditions (artificial incubation) are probably highly related to the clutch degree of affectation. It has been observed in some occasions (e.g., couple 14 and 15) that if the male incubates during a longer time, the number of viable eggs can be considerably reduced due to a longer exposition to infection. This situation may be also related to filial cannibalism [55]. Otherwise, when the infection is detected in its start (e.g., couple 17) it can be successfully stopped with antifungal treatments in controlled conditions [40,52]. Although the risk of manipulation, infections have been controlled in a more efficient way by removing the clutch from the breeding tank and putting it in the rearing tank in controlled conditions (with also different antifungal treatments, not tested in this study).

The last factor to discuss is the captive breeding method employed. During the captive program, it was observed that captive born females could breed successfully, and wild breeders could be maintained (with breed success as well) more than a year in captivity. However, in order to avoid problems related to captivity, it is important to choose a qualitatively and quantitatively appropriate number of breeders. Thus, the genetic diversity of the captive stock can be maximized [56,57]. It is also important to consider population genetics structure (variability within and between populations), with the aim of maintaining offspring genetic variability [2,29]. Supportive breeding method is based on the introduction of new genotypes that increase variability and fitness [33,58]. This method raises the demographic size in the post-releasing censuses, but it may result in genetic risks which would also imply problems in terms of fitness reduction if we do not work with some important parameters [10]. In addition, captive populations are frequently established when wild populations of the species are at risk or have already suffered significant reductions in population size, which limits the number of individuals that can be collected, with a small fraction of the genetic diversity [59]. In fact, in some cases, it is not possible to achieve a level of genetic diversity high enough to obtain a self-sufficient captive population without compromising wild populations [60]. In such a case, it is fundamental to choose the most appropriate method of intervention [33].

With the aim to avoid the accumulation of all these problems related to the conditions in captivity over time and to reduce the cost of this complex maintenance, as well as to reduce the risk of accidents (like the one of the death female in 2015), it is recommendable to opt for a system that reduces the duration of the breeding period—as pointed out by Philippart [29]—with wild adults that are sexually mature and that have been collected just before or during the reproduction period. Besides, considering the limited number of individuals of the species of study (due to threat level and the current situation of the population), it could be positive to return both adults and offspring to wild population after the breeding (as has been done in 2017 and 2018). Thus, the total number of breeders of the breeding program will progressively increase over the necessary seasons and it will not have too much impact on the current population.

Precisely, due to this limitation, it has been impossible to carry out replies or different treatments of the factors previously discussed. It has been done this way to avoid risks for breeders and offspring. When the species status improves, it will be possible to develop experimental designs that better explain the influence of the different factors, and it would underline possible differences between phases (that are not significant in the current model).

5. Conclusions

It has not been possible to: (1) determine the critical phases of the reproductive cycle under captive conditions—as the expected differences between phases are not significant. Despite that fact, (2) observing variation into phases, many factors that might be related to its success are detected—such as the ratio of females per male, relative size between individuals of the couple and proper courtship. Avoiding stress factors (by reducing illumination and noise); controlling infections by keeping temperature at 11 °C with coolers, by providing accurate feeding (e.g., *Artemia* sp.) during critical periods, and by incubating artificially as soon as diseases appear; and reducing as much as possible the period in captivity of the brood-stock and offspring are (3) described as key factors determining breeding success and number of offspring produced. Working upon the factors as soon as they have been detected (improving progressively the program) has probably (4) allowed to overcome main impediments of the most critical steps of the reproductive cycle described in other similar programs. It is true that it has been impossible to carry out replies or different treatments of the factors previously discussed. When the species status improves, it will be possible to develop experimental designs that better explain the influence of the different factors, and it would underline the possible differences between phases (that are not significant in the current model). So, as a general conclusion, the initial aim of the captive breeding program (i.e., to complete the ex-situ breeding cycle) is accomplished with encouraging results in fishes' maintenance, that may be a little step forward for the captive breeding of all endangered *Cottus* species and its long-term conservation.

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Interpopulation Variability in Dietary Traits of Invasive Bleak *Alburnus alburnus* (Actinopterygii, Cyprinidae) Across the Iberian Peninsula

Dani Latorre [1], Guillem Masó [1,2], Arlo Hinckley [3,4], David Verdiell-Cubedo [1], Gema Castillo-García [5], Anni G. González-Rojas [5], Erin N. Black-Barbour [5], Anna Vila-Gispert [1], Emili García-Berthou [1], Rafael Miranda [6], Francisco J. Oliva-Paterna [7], Ana Ruiz-Navarro [7], Eduardo da Silva [8], Carlos Fernández-Delgado [9], Julien Cucherousset [10], José M. Serrano [3] and David Almeida [1,5,*]

[1] GRECO, Institute of Aquatic Ecology, University of Girona, 17003 Girona, Spain; danilatorre1@gmail.com (D.L.); guillem.maso@gmail.com (G.M.); verdiell@um.es (D.V.-C.); anna.vila@udg.edu (A.V.-G.); emili.garcia@udg.edu (E.G.-B.)
[2] Department of Biodiversity and Ecological Restoration, Instituto Pirenaico de Ecología (IPE-CSIC), 22700 Jaca, Spain
[3] Department of Biodiversity, Ecology and Evolution, Complutense University of Madrid, 28040 Madrid, Spain; arlo@ebd.csic.es (A.H.); jomserra@ucm.es (J.M.S.)
[4] Conservation and Evolutionary Genetics Group, Estación Biológica de Doñana-CSIC, 41092 Sevilla, Spain
[5] Department of Basic Medical Sciences, USP-CEU University, 28925 Alcorcón, Madrid, Spain; g.castillo6@usp.ceu.es (G.C.-G.); ag.gonzalezrojas@usp.ceu.es (A.G.G.-R.); e.black@usp.ceu.es (E.N.B.-B.)
[6] Department of Environmental Biology, University of Navarra, 31080 Pamplona, Spain; rmiranda@unav.es
[7] Department of Zoology and Physical Anthropology, University of Murcia, 30100 Murcia, Spain; fjoliva@um.es (F.J.O.-P.); anaruiz@um.es (A.R.-N.)
[8] Department of Anatomy, Cell Biology & Zoology, University of Extremadura, 06006 Badajoz, Spain; edasilva@unex.es
[9] Department of Zoology, University of Córdoba, 14071 Córdoba, Spain; ba1fedec@uco.es
[10] CNRS, Université Toulouse III Paul Sabatier, ENFA, UMR 5174 EDB (Laboratoire Évolution & Diversité Biologique), 31062 Toulouse, France; julien.cucherousset@univ-tlse3.fr
* Correspondence: david.almeidareal@ceu.es

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Abstract: The bleak *Alburnus alburnus* is native to most of Europe. This cyprinid fish is a successful invader in the Iberian Peninsula. No studies exist on its foraging strategies on a large scale for this ecoregion. The aim of the present study was to compare dietary traits of invasive bleak among the main Iberian rivers and a 'reference' native bleak population from France. Bleak were sampled during May–June 2019 from the Iberian Rivers Ebro, Tagus, Guadiana, Segura and Guadalquivir and the River Saône (France). Diptera larvae and zooplankton were common food categories in the River Saône. Insect nymphs were more important in the River Ebro. The intake of plant material was higher in the River Tagus. Flying insects were more consumed in the River Guadiana. Nektonic insects were important in the River Guadalquivir. Detritus was a frequent food category for all populations, in terms of occurrence and mass. Dietary parameters followed a unimodal response in relation to the latitudinal gradient, with the maximum values for the Tagus and Guadiana populations. Overall, results suggest that this wide interpopulation variability will contribute to the species' successful establishment throughout Mediterranean Europe, which poses a serious risk to its highly valuable native fish fauna.

Keywords: gut content analysis; Mediterranean rivers; prey richness; trophic niche breadth; Spain

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1. Introduction

The disruptive effect of fish invasions is of particular conservation concern in the Iberian Peninsula, which is rich in endemisms. Indeed, >50% of native fish species are unique to this region [1,2], but the proportion of non-native fishes (>30%) continues to increase [2,3]. The bleak *Alburnus alburnus* (L., 1758) is a cyprinid species native to most of Europe, from the northern Pyrenees to the Urals. In its native area, bleak inhabit lakes or still-waters in medium/large rivers and feed chiefly on zooplankton [4–6]. In the Iberian Peninsula, this species was mainly introduced in reservoirs during the 1990s as a 'forage fish' for non-native piscivorous fishes, such as northern pike *Esox lucius* L., 1758, largemouth bass *Micropterus salmoides* (Lacepède, 1802) or pikeperch *Sander lucioperca* (L., 1758) [7]. Since its introduction, the bleak has displayed a strong invasive character throughout Spain and Portugal, taking advantage of anthropogenic disturbances on Mediterranean freshwater ecosystems [8–10]. Moreover, this species threatens several Iberian fishes, mainly through hybridization, trophic competition and behavioral interference (i.e., aggression) [3]. This is because Iberian fish communities have low diversity and are poorly adapted to a such invasion level of non-native fishes [1], which usually displace native species from available resources. As an example, direct observations by snorkeling have clearly shown that foraging behavior of endemic Ebro nase *Parachondrostoma miegii* (Steindachner, 1866) is disturbed by bleak [11]. All this information on bleak traits (e.g., spread capacity, environmental impacts) allows to consider this species as a clear invasive fish of high ecological risk in Iberia [2,12]. However, the available information on bleak dietary traits in the Iberian Peninsula corresponds to a few particular sites only [13,14], with no study comparing data at the scale of this entire ecoregion. This would be of particular interest to reveal macroecological patterns (e.g., latitudinal gradients). Indeed, dietary traits of non-native fish can show wide variability under contrasting ecological conditions [14–16] and this has implications from a conservation perspective. Thus, a better understanding of dietary data can be used by policy-makers and environmental managers to assist monitoring programs in identifying which other areas are likely to be colonized by this invasive species [2]. Therefore, the information in the present paper is highly relevant to understand invasion features of this fish species throughout Iberian freshwaters.

Consequently, the aim of the present study was to assess the interpopulation variability in dietary traits of invasive bleak across the Iberian Peninsula. For this purpose, bleak populations were compared among the main rivers within this region, along with a native bleak population from France. Specifically, diet composition was analyzed by means of two overall indices: percentages of occurrence and ingested mass; and four dietary parameters were examined: ingested mass, prey richness, trophic diversity and trophic niche breadth.

2. Materials and Methods

2.1. Study Areas

Five main Iberian rivers were sampled for bleak along a latitudinal gradient (from North to South): Ebro (41°47′30″ N–1°05′24″ W and 41°26′39″ N–0°28′26″ W), Tagus (39°49′07″ N–4°20′25″ W and 39°58′12″ N–4°42′17″ W), Guadiana (38°59′58″ N–5°51′52″ W and 38°50′18″ N–6°13′53″ W), Segura (38°06′05″ N–1°17′50″ W and 38°04′54″ N–0°53′37″ W) and Guadalquivir (37°37′01″ N–5°35′45″ W and 37°30′46″ N–5°56′38″ W). These geographic coordinates correspond to two sampling sites per river (first coordinates are for sites located upstream). For comparative purposes, we surveyed a main river within the bleak's native range close to the Pyrenees, i.e., historically a potential 'donor region' for non-native fish introductions to the Iberian Peninsula along the so-called 'Perpignan–Barcelona corridor' (see Clavero and García-Berthou [17] for details on invasion routes). Thus, the River Saône (Rhône drainage, eastern France) was selected as a 'reference' population, although only one site (47°02′13″ N–5°06′52″ E) was finally surveyed. In any case, a comparable number of bleak specimens (200 individuals, see below) were collected from the River Saône and data from the two sampling sites in Iberian rivers were pooled for statistical analyses, as no 'site effect' was found (see Data Analyses below). All the study rivers were selected because they represent large water courses (i.e., >500 km river length), where bleak are widely distributed and reach relatively high abundances within the local fish assemblages. A final key point for selection of these particular Iberian rivers was that bleak were sequentially introduced into these different catchments and thus, their populations will potentially reflect specific 'invasion stages' on the examined dietary traits (e.g., Závorka et al. [18]). Specifically, bleak were introduced into the study rivers in the following years (first mention) [7]: Ebro in 1992, Guadiana in 1999, Segura in 2004, Tagus in 2005 and Guadalquivir in 2006. The climate in the study area of the River Saône is Temperate Oceanic (800–1000 mm of mean annual rainfall, 10–13 °C mean annual temperature) [19], the most common climatic conditions for the bleak's native range in Europe [6]. The climate regime for the study Iberian rivers is typical Mediterranean, with rainfall concentrated in autumn–winter (≈500 mm) and intense summer drought (<100 mm). The mean annual temperature ranges between 15–18 °C. The lowest temperatures occur in winter (down to −5 °C) and the highest in summer (>35 °C) [20]. The bed geomorphology was similar between rivers, mainly consisting of pebbles and gravel, although the fraction of silt was slightly higher in the River Saône.

2.2. Field and Laboratory Work

Fish were collected from May to June 2019, just before the spawning period of bleak for each study area (authors, pers. obs.), thereby avoiding any effect of the 'reproductive status' on foraging habits. Moreover, the year 2019 is considered to have been hydrologically 'average' in the study areas [19,20]. As a result, the effects of particular dry or wet years on dietary traits are avoided within our study. Sampling sites (n = 11, two per Iberian river plus one in the River Saône) were selected to encompass similar environmental conditions. Specifically, sites were located in well-regulated middle reaches of the main channel, where water level fluctuations are controlled throughout the year, avoiding the effects of strong increases/decreases in river discharge. Water velocity was registered in each site to check the values were similar among populations for a better comparability. These habitat conditions are representative of large rivers in both the native and Iberian ranges. Also, sampling sites were located in the vicinity of similar surroundings (e.g., land use for agricultural exploitation) and far from the influences of main tributaries and towns. Sites were separated >50 km within each Iberian river to ensure the data were more representative from the study areas and also to minimize data dependence among sampling sites per river. Given that water temperature has a strong influence on fish metabolism and food digestion, this parameter was measured per site to check that variability was in accordance with the study latitudinal gradient (i.e., colder waters to the North and warmer waters to the South). A variety of consistent sampling protocols was followed by wading and from boats according to the European legislation (CEN/ISO Standards, EC Directive 2014/101/UE [21]). This allowed obtaining a representative sample of bleak across the broadest possible body size range from each river. Catch methods consisted of following a zigzagging and upstream direction in both banks at each site (100-m river length) by electrofishing (2000 W pulse DC generator at 200–250 V, 2–3 A, 30 min per bank), dip nets (1.5-m-long pole, 30-cm-diameter net, 10-mm mesh size), seine nets (20 × 2 m, 10-mm mesh size) and gill nets (20 × 1 m, 10-mm mesh size, 50% hanging ratio, 1.5-m deep). All surveys followed the same sampling protocols (e.g., proportional effort in terms of people and time) to ensure comparability among the study rivers. Additionally, a professional fisherman sampled for bleak in the River Saône. To encompass the existing environmental variability, fish were collected from all meso-habitats present in the study rivers (e.g., runs, pools, shallows), as bleak can be found under contrasting river conditions in the Iberian Peninsula [10,14].

After each survey was concluded, bleak were immediately euthanized by immersion in an overdose solution of anesthetic (MS-222) for 15 min, followed by severance of the spinal cord. Fish were stored on ice during transport to the laboratory (<2 h within the same sampling date). Individuals of the remaining fish species were identified and counted. Native species were kept in a tank with supplied oxygen (two battery-operated aerators with portable pumps) until fully recovered before being released. Other non-native species were euthanized (see details above). All field procedures complied with animal use and care regulations of Europe (specific licenses were granted for scientific field research). Fishes were collected by trained personnel and thus, no adverse effects were caused on the wildlife/habitat in the sampling sites, with all native fish being fully recovered.

On arrival at the laboratory, bleak individuals (n = 1200, 100 ind. × 2 sites × 5 Iberian rivers + 200 ind. × 1 site from the River Saône) were measured for standard length (SL, ±1 mm). This particular fish length was selected because it avoids 'noise' given by variation of caudal fin length not related to body size (e.g., wounds and cuts in the fish skin and rays). Specifically, bleak size ranged between 40–195 mm SL. Fish were dissected to examine the sex and collect the digestive system. Only the anterior one-third of the intestinal tract was preserved in 4% formalin for subsequent examination to avoid food remains severely digested (see a similar procedure in Latorre et al. [13]). Food items were identified to the lowest possible taxonomic level (e.g., Tachet et al. [22]) using a dissecting microscope (40×) and weighed using an electronic balance (±0.1 mg).

2.3. Data Analyses

Preliminary analyses did not find any difference between the sexes for the examined dietary variables (all p-values > 0.05). Consequently, this categorical factor was not included in subsequent data analyses to simplify the analytical models and thus, increase the statistical power of the remaining sources of variation, which would otherwise be seriously compromised (see a similar procedure in Alcaraz and García-Berthou [23]). Given that percentages of empty stomachs were similar across populations (25–35%), this parameter was not statistically analyzed. In addition, data were pooled per Iberian river because the effect of 'sampling site' within each water course was not significant for any dietary variable (all p-values > 0.05), after performing Generalized Linear Mixed Models (GLMMs) with 'site' as the random factor (see a comprehensive review of this statistical technique in Johnson et al. [24]). This analytical approach (i.e., pooled data) considerably improved the clarity of results and also facilitated biologic interpretation.

Two overall dietary indices were calculated (omitting empty guts) and expressed as a percentage for each food category (e.g., Latorre et al. [13]): 'occurrence' (frequency of fish guts in which a particular food category occurred relative to the total number of fish individuals) and 'ingested mass' (frequency of the mass of a particular food category relative to the total ingested mass in all the examined intestinal tracts). Both of these percentages indicate whether a given food item is commonly eaten within the population and whether this food category is energetically important to the population. Furthermore, four dietary parameters were also calculated for each fish (e.g., see Almeida et al. [16] for details on the particular formulae): ingested mass (mg), prey richness (S), trophic diversity (Shannon index, H') and trophic niche breadth (standardized Levin's index, B). These parameters were selected as different measures of trophic plasticity to avoid potential bias of using only one parameter. Consequently, any macroecological pattern (e.g., latitudinal gradient) in dietary traits will be much clearer revealed if profiles among rivers are similar for several parameters.

Given that previous studies revealed significant relationships between dietary parameters and bleak size (an approach of age for fish) in Iberian waters (e.g., Almeida et al. [14]), linear regressions were used, which showed similar slopes among the populations (all p-values > 0.05), with low and positive values. Consequently, analysis of covariance (ANCOVA) was used to reveal significant differences between populations (i.e., rivers) for the four dietary parameters. The effect of body size was controlled by using SL as the covariate. ANCOVAs were followed by a post hoc Tukey–Kramer honestly significant difference (HSD) test. Data were transformed by using ln (x + 1). Assumptions of normality of residuals and homogeneity of variances were verified through residual plots. Statistical analyses were performed with R version 3.1.3 [25]. The significance level was set at $\alpha = 0.05$. Sequential Bonferroni corrections were performed for every set of multiple tests.

3. Results

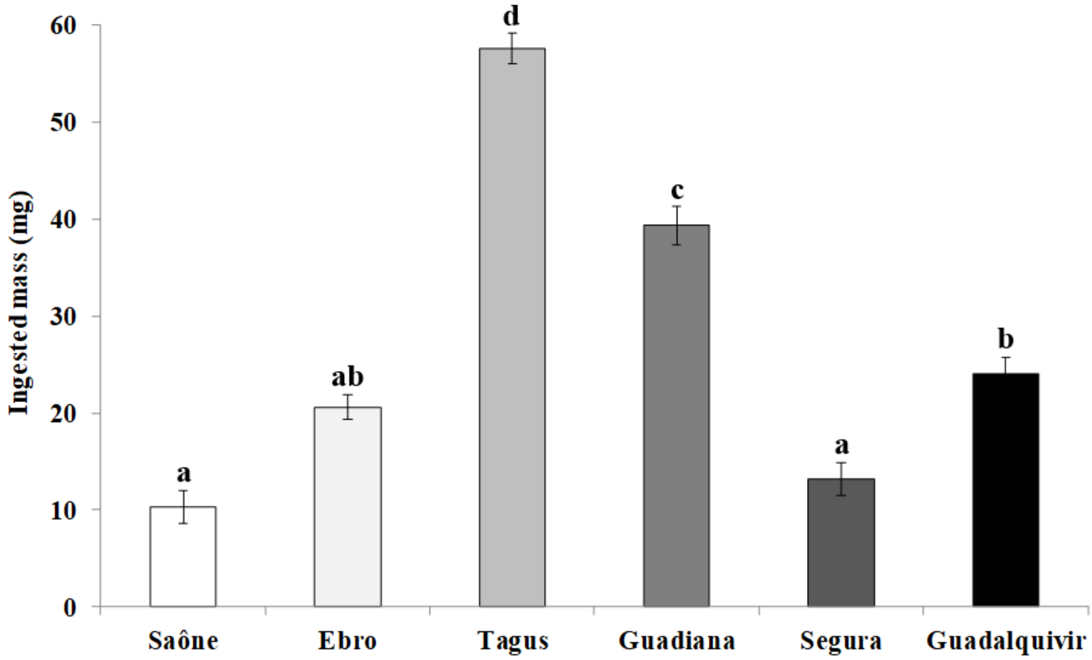
Diptera larvae (e.g., Chironomidae and Simuliidae), zooplankton (e.g., Cladocera water fleas) and detritus were the most frequent food categories for the reference native population (i.e., the River Saône), both in terms of occurrence and ingested mass (Table 1). The Ebro population showed a similar diet composition as per native bleak, although Ephemeroptera and Plecoptera nymphs were also important prey items. For the rest of study rivers, percentages of food categories were highly variable (Table 1). More in detail to be highlighted per river: vegetation and other benthic invertebrates (e.g., freshwater snails) were frequent in the River Tagus; flying insects (e.g., wasps, mosquitoes or butterflies) and Diptera larvae were important in the Guadiana and Segura populations, respectively; benthic invertebrates (e.g., Diptera larvae, insect nymphs, Mollusca) and nektonic insects (e.g., water boatmen or predatory beetles) were very important in terms of ingested mass (>40% and ≈20%, respectively) for the River Guadalquivir. Detritus was an important food category for all bleak populations: 21–42% in occurrence and 13–27% as ingested mass (Table 1).

Table 1. Diet composition of bleak *Alburnus alburnus* in the study rivers. Percentages of occurrences (Oc., %) and ingested masses (Mass, %) are presented.

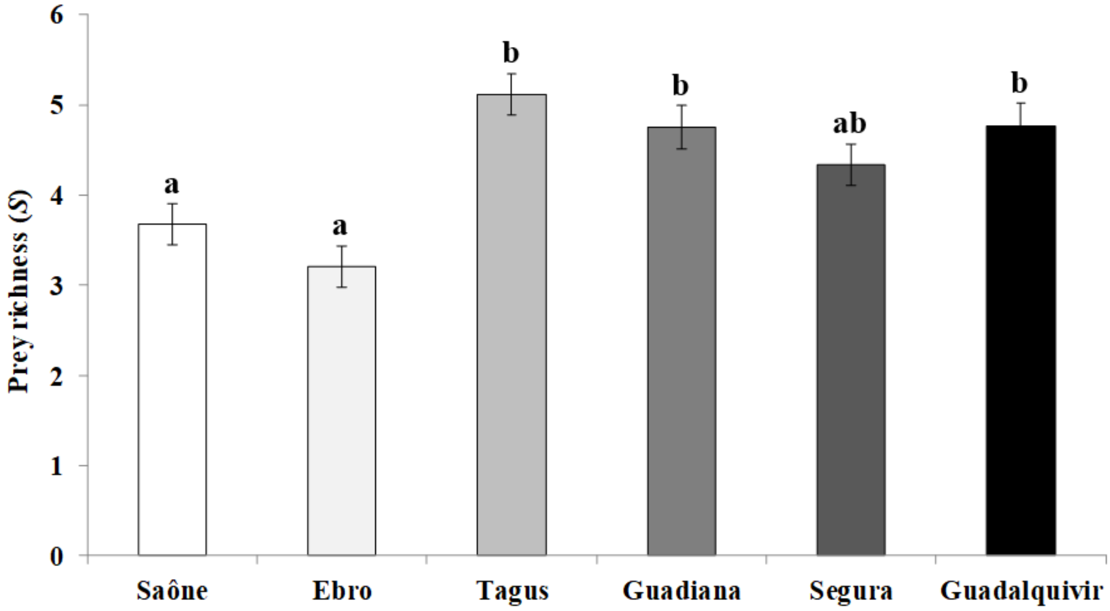
River:	Saône		Ebro		Tagus		Guadiana		Segura		Guadalquivir	
Food Category	**Oc.**	**Mass**	**Oc.**	**Mass**	**Oc.**	**Mass**	**Oc.**	**Mass**	**Oc.**	**Mass**	**Oc.**	**Mass**
Algae and plant debris	3	<1	2	5	29	17	5	3	3	<1	8	9
Zooplankton [a]	50	17	40	15	11	8	1	<1	5	3	10	6
Ephemeroptera and Plecoptera nymphs	5	9	33	21	5	10	20	23	7	21	5	12
Odonata nymphs	1	2	11	6	1	5	5	9	1	<1	1	3
Diptera larvae	69	39	27	15	3	15	3	1	59	31	3	16
Trichoptera larvae	6	1	5	<1	1	<1	15	13	6	11	1	5
Other benthic invertebrates [b]	12	4	3	3	53	13	1	<1	12	1	71	14
Nektonic and neustonic insects [c]	1	<1	1	<1	1	<1	1	<1	1	<1	20	19
Flying insects [d]	3	<1	5	8	35	7	61	23	3	19	–	–
Terrestrial arthropods [e]	1	<1	1	<1	–	–	1	9	1	<1	7	2
Detritus	31	27	25	26	42	24	24	18	31	13	21	14

[a] Acari, Cladocera and Copepoda; [b] Mollusca, Oligocheta and Coleoptera larvae; [c] Corixidae, Gerridae and Dytiscidae adults; [d] Hymenoptera, Diptera and Lepidotera; [e] Araneae and Formicidae.

Regarding dietary parameters and after accounting for fish length, significant differences were found between bleak populations for ingested mass ($F_{5,812} = 32.94$, $p < 0.001$). Four distinct groups were found (from low to high adjusted mean): Saône/Segura (≈12 mg), Guadalquivir (≈25 mg), Guadiana (≈40 mg) and Tagus (almost 60 mg) (Figure 1a). Differences were also found for prey richness ($F_{5,812} = 18.68$, $p < 0.001$). Two river groups were found: Ebro/Saône ($S \approx$ 3–4 prey items) and Guadiana/Guadalquivir/Tagus ($S \approx 5$ prey) (Figure 1b). Significant differences were found between populations for trophic diversity ($F_{5,812} = 19.76$, $p < 0.001$). Two river groups were found: Saône/Ebro/Guadalquivir ($H' \approx 1$) and Guadiana/Tagus ($H' > 1.5$) (Figure 1c). Significant differences were also observed between rivers for trophic niche breadth ($F_{5,812} = 20.49$, $p < 0.001$). Two distinct groups were found: Saône ($B \approx 1.4$) and Tagus/Guadiana ($B > 2$); while the remaining three rivers showed intermediate B values (1.6–1.7) (Figure 1d).

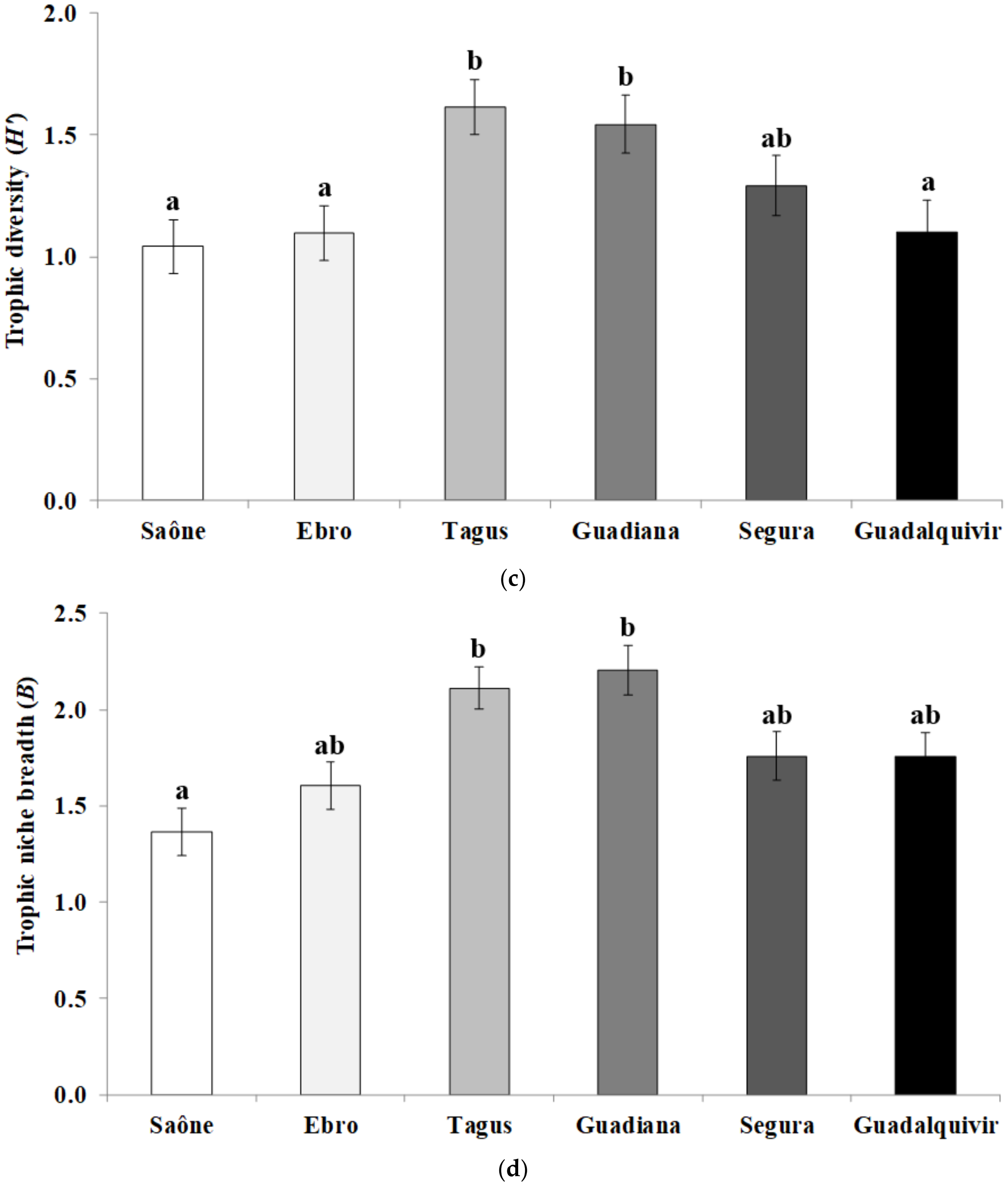


(**a**)



(**b**)

Figure 1. *Cont.*



(**c**)

(**d**)

Figure 1. Comparison of dietary parameters for bleak *Alburnus alburnus* among the study rivers. Results are adjusted means ± SE (ANCOVA, covariate: SL). Letters above bars indicate significant differences between bleak populations (Tukey's HSD tests, $p <$ critical p-value from Bonferroni correction). (**a**): ingested mass. (**b**): prey richness. (**c**): trophic diversity. (**d**): trophic niche breadth.

4. Discussions

Given that the bleak mainly inhabits stillwaters in its native area, this species is well-adapted to play an ecological role as an openwater feeder, with diet being chiefly based on zooplankton [4,5]. Similarly, planktonic Crustacea were very important for native bleak in the River Saône. However, the main food categories were Diptera larvae and detritus (i.e., benthic food items) for this reference population. This discrepancy between scientific literature and the present results for the native area was probably because most studies on bleak have been performed in lakes and reservoirs (e.g., Vinni et al. [4]; Vašek and Kubečka [5]), whereas bleak diet has received less attention in flowing waters.

However, even in lentic environments, bleak can modulate this zooplanktivorous strategy at each particular habitat patch to increase resource partitioning and consequently, reduce competition with coexisting cyprinid fishes, such as roach *Rutilus rutilus* (L., 1758) or freshwater bream *Abramis brama* (L., 1758) [4,5]. Dietary indices were more similar between the River Saône and the River Ebro, the (geographically) closest study area to the reference population. This was also observed by Latorre et al. [26]) for other biologic traits (i.e., growth and reproduction), which was explained in terms of climatic and hydrological 'proximity'. However, the intake of Diptera larvae and insect nymphs was different between the Saône and Ebro populations. In the River Saône, particle size was finer (see Methods section), with these bed conditions being very suitable for Diptera larvae (J. Cucherousset, pers. obs.), whereas substrate was coarser in the River Ebro (see Methods section), providing a high availability of nymphs (R. Miranda, pers. obs.). Other food items were prominent among the study populations, including a wide variety of trophic resources in terms of contrasting ecological features: vegetation, neuston, nekton and benthos. Thus, consumption of algae and plant material was higher in the River Tagus. In this respect, Vinni et al. [4] demonstrated that intra- and mainly inter-specific competition were key causes to understand this result (i.e., high use of vegetation) in the native area. In relation to this finding, Tagus River showed the highest value for ingested mass, resulting in only one 'statistical group'. This may compensate a higher proportion of vegetation in the diet (see Results above). As a support for this interpretation, Latorre et al. [26] did not find any effect of this 'low nutritious' feeding type (vegetation) on the growth rate in the River Tagus, with this population showing the highest values. Particularly for detritus, bleak showed a high consumption in the study Iberian rivers, which was also found elsewhere within this ecoregion, from streams to reservoirs [13,14]. As a potential explanation, bleak may accidentally ingest plant material and detritus while feeding on sheltered invertebrates (D. Verdiell-Cubedo, pers. obs.). In the Guadiana population, bleak took advantage of prey from the water surface, such as fallen flying arthropods (D. Almeida, pers. obs.), which results in a low effort in terms of energy investment. Bleak caught prey from the water column in the River Guadalquivir, with this population displaying a 'costlier' foraging alternative, as nektonic insects (e.g., adult Dytiscidae beetles) require higher pursuit and capture efforts. However, more important, bleak fed on a great variety of benthic prey, from insect nymphs and larvae to mollusks and annelids, in all river populations. This finding indicates an elevated capacity of this fish to use food resources apparently less suitable to its morphologic adaptations (e.g., conspicuous superior mouth). Overall, these results suggest that bleak could deeply change the foraging strategy, from a 'pelagic' to a 'benthic' feeder, even within the same population (see Results for benthos and nekton in the River Guadalquivir). This capacity may aid bleak to better thrive in contrasting Iberian rivers, where zooplankton availability may be a more limited trophic resource [14]. Such a significant dietary shift has been also observed in the Iberian Peninsula for other invasive fishes adapted to a more stable hydrological regime in their native ranges, although they are phylogenetically and geographically very 'distant' to the bleak (e.g., North American centrarchids, see Almeida et al. [16]).

Regarding dietary parameters, variation ranges for the four examined predictors were similar as per other study areas in the Iberian Peninsula (Latorre et al. [13] in the NE; Almeida et al. [14] in the SW). The present data were obtained from large rivers and the just mentioned studies included streams and reservoirs. Thus, these overall results indicate that Iberian bleak populations possess a high capacity of adapting to the particular habitat conditions where this invasive fish inhabits. No clear pattern was observed for any dietary parameter in relation to the year of introduction, which was in accordance with Latorre et al. [26] for growth and reproduction traits. This lack of relationship is probably because the establishment stage was reached in a few years after bleak introductions (e.g., Bøhn et al. [27]). As an example, a rapid and wide establishment of this species has been recently demonstrated in the River Segura [10]. Nevertheless, it must be clearly stated that data were limited (n = five Iberian rivers). However, unlike the year of introduction, the foraging strategy appeared to show a slight relationship with the latitudinal gradient, specifically a unimodal response [28], with Guadiana and mainly Tagus reaching the maximum values for all dietary parameters. In addition, the Tagus population showed the highest backcalculated lengths at all ages and growth index among the same study rivers (see Latorre et al. [26]). A potential explanation is that bleak may be more favored by a moderate Mediterranean climate in central Iberia during the prespawning period (warmer) than temperate conditions in the native area (colder). Yet, bleak are affected by slightly more 'severe' ecological conditions in southern Iberia, e.g., oxygen level was lower because of higher temperature (physicochemical data not shown, but in accordance with the expected latitudinal gradient) [29]. More in detail, these 'benign' conditions in central Iberia may improve the physiological status (e.g., metabolic rate) of bleak, which facilitates this fish species to use alternative prey and widen its diet, with the corresponding increase in the trophic variability. In contrast to this 'generalist' strategy, more northerly and southerly populations (i.e., distant from the optimum in terms of habitat quality, see theoretical aspects in Oksanen and Minchin [28]) displayed a more 'specialist' strategy, reducing the complexity of diet composition. Nevertheless, these ecological interpretations must be understood with caution, as similarly to the previous statement, data were limited to a few locations (n = five Iberian rivers) and moreover, the assessed latitudinal amplitude was relatively narrow (only 10° of Latitude, 37–47° N).

Ecological responses such as dietary traits usually show wide variability in non-native species when invading new habitats, with this being particularly clear in freshwater fishes [13,15,16]. In the Iberian Peninsula, invasive fishes usually display wide trophic plasticity under contrasting environmental conditions, which contributes to improve their invasion process [3,13,14]. Accordingly, dietary traits were clearly variable among the study rivers, indicating a wide 'flexibility' in bleak foraging strategies dependent on particular river conditions (i.e., habitat heterogeneity, food supply). Studies on bleak diet exist across Iberia in which resource availability were not assessed [13,14]). However, other studies on feeding habits of invasive fishes truly estimated food supply as a measure of trophic resources in this ecoregion (e.g., Almeida et al. [16,30]), comparing this availability to the use (i.e., bleak diet), and specifically calculating electivity indices (i.e., selection). The present study did not assess resource availability because of technical restrictions (in terms of materials and personnel) to properly quantify the biomass of plant, benthos and plankton in large rivers, which poses a strong study limitation. Thus, abundance of contrasting food items is a key factor to be analyzed for future research on bleak invasion. Similarly—as per other biologic attributes (e.g., growth and reproduction)—the high interpopulation variability is considered a mechanism for bleak to successfully invade novel Mediterranean freshwater ecosystems, from streams to large rivers [8,10,26]. Indeed, Iberian endemic species are specialized to narrow trophic niches [1] or alternatively, these fishes may display high plasticity to the natural variability in Mediterranean rivers. Irrespective of this specific mechanism, the low level of coevolutionary competition is a potential cause why Iberian fish fauna is not adapted to strong invasive competitors, such as bleak. This is one of the main reasons to consider this fish species as a bioinvader of high risk in the Iberian Peninsula (see Table 1 in Almeida et al. [2]). Consequently, environmental managers should apply urgent conservation measures to control bleak populations across Iberian freshwaters. First, these measures should focus on the target species (i.e., fish culling). Second—and according to other studies on bleak in Iberian waters [9]—actions on aquatic habitats should be implemented to restore the 'Mediterranean conditions' of natural flow regimes that benefit native fish communities and hamper invasive species.

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water

Article

Assessing the Fish Stock Status in Lake Trichonis: A Hydroacoustic Approach

Triantafyllia-Maria Perivolioti [1,*], Jaroslava Frouzova [2,3], Michal Tušer [2] and Dimitra Bobori [1]

[1] Department of Zoology, School of Biology, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; bobori@bio.auth.gr
[2] Institute of Hydrobiology, Biology Centre of the Czech Academy of Sciences, 370 05 České Budějovice, Czech Republic; jaroslava.frouzova@hbu.cas.cz (J.F.); michal.tuser@hbu.cas.cz (M.T.)
[3] Institute for Environmental Studies, Faculty of Science, Charles University, Benátská 2, 128 43 Prague, Czech Republic
* Correspondence: triaperi@bio.auth.gr

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Abstract: Fish stock monitoring is an important element for the sustainable management of inland water resources. A scarcity of data and the lack of systematic monitoring for Lake Trichonis precludes an up-to-date assessment. To assess the current status of pelagic fish stock, a hydroacousting survey was conducted for the first time in Lake Trichonis, Greece. In October 2019, the lake was acoustically surveyed with two, horizontally and vertically mounted, 120 kHz transducers during day and night. A decreasing gradient in pelagic fish density from the western to the eastern shores of the lake was observed. Fish density was significantly higher in the intermediate layers of the water column, in the eastern region, compared to the western region. The lake appears to host primarily communities of small-sized fish (TL: 0–5 cm), whereas larger fish (TL: 5–50 cm) are a small minority of the total fish stock. The overall average estimated fish length was approximately 2.4 cm. The adoption of routine inland fish stock monitoring through hydroacoustic methods could be a promising step in the effort to improve the understanding of unique inland water ecosystems with minimum impact on endemic species, as well as to mitigate human impact and achieve long-term sustainable management.

Keywords: acoustics; fish density; fish distribution; Mediterranean lake

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1. Introduction

Within Europe, in order to eliminate or mitigate freshwater degradation, the Water Framework Directive (WFD) calls for all the natural aquatic ecosystems to achieve a "good" ecological status. To date, inland fisheries management is focused on improving the aquatic environment for biodiversity, allowing the sustainable exploitation of the resources and verifying conservation and protection of fish and fisheries [1]. However, the methodologies adopted by researchers for the monitoring of freshwater fish stocks, such as gillnetting, are often unable to support an ecosystem–based management approach [2], as they fail to comprehensively address the spatiotemporal variability of fish stocks [3], require high effort [4] and often result in a reduction of fish biomass [5].

In this context and considering the proposed WFD goals, there is a strong demand for standardized, reliable, reproducible and non-invasive methods for routine monitoring programs. Satellite-based Earth Observation data provide safe and cost-effective approaches for the systematic monitoring of water bodies [6] and fish environmental preferences but fail when it comes to the direct detection of fish [7]. On the other hand, hydroacoustic methods have been applied in all kinds of aquatic ecosystems in order to acquire detailed information about aquatic fauna, and especially about fish [8].

In freshwater ecosystems, hydroacoustic methods are used for assessing fish biomass, fish behaviour, stock abundance and fish size distribution [8]. The most widely used scientific echosounders in freshwaters operate at single frequencies of 38, 70, 120 and 200 kHz [9]. The main advantage of hydroacoustics is the capability to remotely sample large volumes of water in a relatively short amount of time, providing high spatial resolution in both the horizontal and the vertical domain. In addition, acoustic measurements are non-invasive and non-extractive [8,10]. Moreover, the technological improvements in this field and their increased precision have contributed to their extensive use in both marine and freshwater ecosystems [11–13]. Although there are several limitations of current hydroacoustic methods, e.g., lack of species identification, difficulties in shallow waters, and limitations in noisy environments, and despite the considerable need for further research, the application of hydroacoustics is based on a well-established, standardized methodology for inland fishery production assessment and management [14].

Geographically, hydroacoustics research in freshwater ecosystems has so far been concentrated in North America and Northern and Central Europe (83% of the studies reviewed in [15]). Although the Mediterranean Basin is found to be globally important for its freshwater biodiversity [16], few studies have been published with respect to the application of hydroacoustic methods (e.g., [17]). In Greece, hydroacoustics research has so far been applied in marine systems, mostly focusing on the application of acoustic techniques to monitor marine fisheries resources (e.g., [18,19]). Nevertheless, hydroacoustic research on Greek inland waters has not been conducted before.

Inland fishery production in Greece is impacted by overfishing and habitat alteration, as well as pollution [20]. Professional fisheries are operated primarily in large lakes. Lake Trichonis, the largest natural lake in Greece, bears very high economic, cultural and ecological significance. The lake is important for its fish fauna since 20 species have been recorded in its waters, 11 of which are endemic (see detailed list in [21,22]), among which there are unique species such as *Economidichthys trichonis*. The species present in the lake are classified into 8 different systematic families, which, ecologically, span all available ecological niches (herbivores, benthivores, planktivores and carnivores). Among them, there are carnivorous species such as *Anguilla anguilla* and *Silurus aristotelis* that feed at night, predominantly on fish [23,24]. The majority of fish species inhabiting Lake Trichonis belong to Cyprinidae and spawn during spring to early summer [25–28]. Moreover, small gobies are reproducing in the same period [25]. Additionally, the lake belongs to one of the most important fishing attractions of the country. In fact, a land-locked, not overexploited population [29] of *Atherina boyeri* is also present in the lake, which offers, with the exploitation of other species such as *Rutilus panosi* and *Scardinius acarnanicus,* economical support for professional fishers. According to the local Fisheries Department, the primary catch composition for the period of 1989–1998 is made up of small species. In particular, in October 1989, *A. boyeri* (L_{max} = 13.5 cm [30]; L_m = 5.8 cm [31]) comprised approximately 64%, while *Tropidophoxinellus hellenicus* (L_{max}= 9.3 cm [31]) and *Scardinius acarnanicus* (L_{max} = 35.4 cm [32]; L_m = 14–18 cm [31]) contributed each 17% of fisheries production (calculated as the percentage, per weight, of all catches). In 1990, 47% of the production consisted of *T. hellenicus* (L_{max} = 9.3 cm [31]), while 22% of production was *R. panosi* (SL = 12.0–14.0 cm [31]). In 1998, 56% of the annual production of the lake consisted of *Atherina boyeri* while 20% of the production was *Scardinius acarnanicus* (L_{max} = 35.4 cm [32]; L_m = 14–18 cm [31]). Other species (*Silurus aristotelis, Luciobarbus albanicus, Carassius gibelio, Tropidophoxinellus hellenicus*) comprised approximately 3% of the total production. A similar species composition was found to occur during the contemporary fishing catches (local professional fishermen, pers. comm).

Despite its significance, because of limited staff, management authorities are having a hard time monitoring fish harvests of individuals for the prevention of illegal fishing in Lake Trichonis. Furthermore, the lack of comprehensive long-term studies has resulted in the limited availability of information about fish stock status in the lake. Although historical data exist as a result of research projects carried out by universities, institutes and organizations, it is scarce and, in practice, the status of inland fisheries is largely unknown. Taking all the aforementioned into account, it is important to adopt a standardized, effective and non-invasive method for the assessment and monitoring of inland fish stocks.

Hydroacoustic techniques could serve as a non-destructive tool for inland fish monitoring. The purpose of this study is to use a hydroacoustic method for obtaining a detailed overview of the freshwater fish stock status in Lake Trichonis and highlight the hindrances and improvements that need to be considered, in order to adopt this methodology as a common practice in Greek lakes. In particular, we aimed to (i) quantify freshwater fish biomass and fish density, (ii) assess fish size distributions, and (iii) study the variation of fish vertical distribution in relation to water abiotic parameters. Therefore, despite ongoing progress in hydroacoustics, the present work comprises a first-step towards the attempts that should be made for fish stock assessment in larger biogeographical scales [15], and especially in the Mediterranean countries, where high levels of endemicity are observed.

2. Study Area

Lake Trichonis, located in the central-western part of Greece, has a surface area of 96.9 km^2, a maximum length of 19 km, and is the deepest (maximum depth of 58 m, average depth of 30 m) natural lake in Greece (Figure 1). The trophic status of the lake is characterized as oligotrophic [33]. Lake Trichonis belongs to the Natura 2000 network established under the Habitats Directive 92/43/EEC and is characterized as an area with high potential research value [34]. Responsible for the protection, management and monitoring of the lake is the Management Authority of Messolonghi Lagoon.

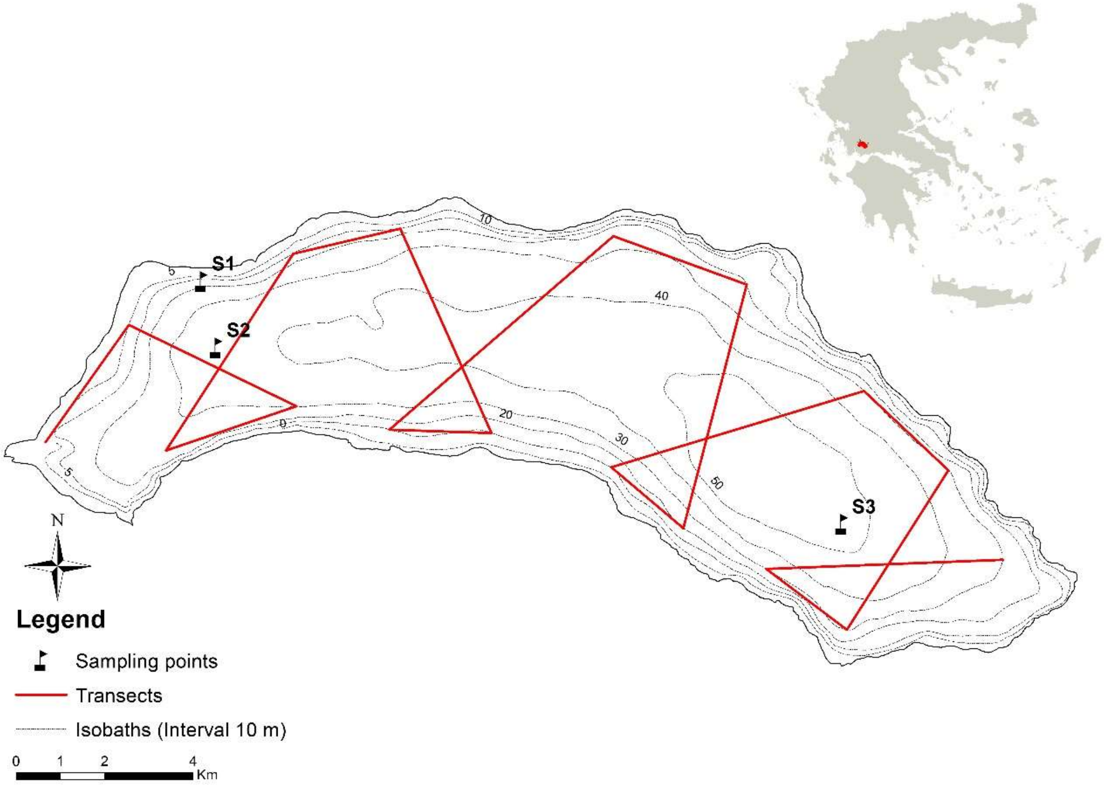

Figure 1. Depiction of the study area and lake bathymetry (derived from the digitization of 1:50,000 scale topographic maps, which constitute an appropriate reference source for such geomorphometric information [35,36]) with the hydroacoustic survey and the sampling points overlaid.

3. Materials and Methods

3.1. Hydroacoustic Survey Design

The hydroacoustic survey was carried out in Lake Trichonis during the period of 3–10 October 2019. The pattern of the survey transects was designed in such a way, as to include fish communities along areas of identical or similar depths (i.e., along depth contours) (Figure 1). Horizontal and vertical recordings were carried out simultaneously using a suitable arrangement.

In order to assess the variation in diurnal estimates of fish biomass and distribution, the survey was conducted during both day and night [37]. The day survey commenced one hour after local sunrise, whereas the night survey commenced one hour after local sunset. Considering the high sensitivity of horizontal recording to the noise introduction into the hydroacoustic records, the survey was carried out on windless days. In total, two hydroacoustic surveys were needed to cover the whole lake; one during daytime and one during nighttime. The total length of each survey was ~40 km, resulting in a coverage coefficient of 4 per survey [38,39]. Surveys were grouped per time of day, into daytime and nighttime surveys.

A Simrad EK60 echosounder with a frequency of 120 kHz, equipped with two simultaneously operating transducers (SIMRAD ES120-7C), was used to collect the acoustic data. The transducers were mounted at a depth of about 1 m, at the front of the boat. The first transducer was vertically oriented and the second one horizontally oriented, enabling fish detection in the surface zone. The horizontal transducer was oriented perpendicularly with respect to the vertically oriented transducer and was facing towards the side of the vessel (perpendicular to the sailing direction). The echosounder was driven by the Simrad ER software. The ping interval was set to 0.2 s. Each transducer transmitted 2–3 pings per second, alternating between transducers (known as multiplexing), for a total ping rate of 5–6 pings per second. During surveying, the boat maintained a constant speed of 6 km/h and a volume of 3.7×10^6 m^3 of water was explored. For the full calibration of the system, a standard copper sphere of 23 mm in diameter was used and gains were calculated according to Demer et al., 2015 [40]. The sphere was placed at approximately 9.4 m of depth from the transducer, which was aimed vertically towards the sphere. The calibration was performed before starting with the first day survey. The effect of background (passive) noise was removed from all files by subtracting passive listening data collected before the survey. The background noise was checked by stopping pulse transmission and putting the equipment into a passive listening mode. Passive listening was performed on a windless day before the acoustic survey.

Survey positioning was assisted by a Garmin GPSMAP 60CSx GPS receiver (Geolocation/horizontal accuracy of 3–5m—95%).

3.2. Hydroacoustic Data Processing

Acoustic raw data were converted to compatible formats and processed using the Sonar5 Pro software (CageEye A/S, Oslo, Norway). Fish density values (number of individuals per hectare) were calculated using the S_V/TS scaling method [8], which uses volume back-scattering strength, S_V, and the mean target strength (TS) to calculate fish density.

Acoustic biomass was expressed as the volume backscattering strength (S_V), which is the equivalent logarithmic measure of the volume backscattering coefficient (s_V) obtained through echo-integration [8]. S_V has been found to be a good expression of biomass distributions, especially for small targets [8].

Mean target strength (TS) was estimated based on single echo detections (SED) [41]. In order to express TS into total length measurements (cm) rather than dB, empirical TS-length relationships were used. Equations for a 120 kHz echosounder given by [42] in the case of vertical and [43] in the case of horizontal recordings were applied. Fish Total Length distribution was categorized into 50 classes by 1 cm step, starting from zero.

During the data post-processing, the threshold for target strength (TS) was set to −65 dB and for S_V to −71 dB, in order to cover the entire possible size spectrum of fish targets [44]. The collected data were visually examined to remove subsets with noise and non-fish targets, in order to improve the signal-to-noise ratio of the dataset. A layer of 0.5 m above the detected bottom layer was excluded in order to eliminate the effects of bottom back-scattering. A surface layer of 4 m was applied in horizontal and vertical recordings in order to exclude the near field. Each transect in both the horizontal and the vertical survey was divided into 200-m segments. In order to assess the vertical distribution of fish, the water column recorded in the vertical survey was divided into 1-m thick depth layers. The vertical data were processed up to a depth limit equal to the maximum depth up to the bottom, depending on the position of the segment within the transect. The horizontal data were processed up to a range equal to the maximum depth of the lake. The surface layer ranging from 0–4 m was covered by the horizontally-aimed transducer and compensates for the lack of data in the near field of the vertical transducer. Data recorded horizontally were processed using deconvolution procedure. Deconvolution is a procedure employed in the processing of horizontally recorded data and is especially used for the determination of target size. It is based on stochastic assumptions of random aspect orientation [45].

The data were taken along the transect, which was divided in segments. In charts of vertical distribution, the horizontal layer was taken to describe the entire surface layer and is represented by a single depth range. The rest of the vertical layers have been accordingly matched and aligned, below the horizontal layers. The overall arrangement represents a division of the total insonified lake volume in cells, each of which spans 200 m × 1 m (along-transectx vertically), with a variable athwart dimension for each depth, as a result of the conically-shaped beam. Exceptionally, the cells of the surface layer were sized 200 m × 4 m (along-transect x vertically). The data were either summed or averaged vertically (for vertical analyses) and averaged or summed horizontally (i.e., along the transect) for horizontal or lengthwise analyses.

Due to the potential effects of multiple scattering and acoustic shadowing caused by very dense aggregations of fish on TS and the consequent density estimates, additional measures were checked and examined. These include the share of single echoes (the ratio between volume backscattering strength resolved as single echoes and the total volume backscattered strength) and the Sawada index N_V (the number of fish per acoustic sampling volume where TS is estimated) [46] (see Supplementary Materials).

3.3. Environmental Parameters

In situ measurements of physico-chemical parameters were conducted during the day survey in Lake Trichonis at three sampling stations (Table 1 and Figure 1). In order to estimate the temperature (°C) and dissolved oxygen (DO) (mg/L) vertical profiles, the measurements were carried out at 1-m depth intervals using a YSI multiparameter probe (YSI Incorporated, Ohio, USA).

Table 1. Locations of the 3 sampling stations in Lake Trichonis.

Lake Location	Latitude (deg)	Longitude (deg)	Sampled Depth Range (m)
Sampling point S1	38.58625	21.48000	22
Sampling point S2	38.57250	21.48301	34
Sampling point S3	38.53671	21.61033	50

3.4. Statistical Analysis

The calculated parameters were mapped along the transect, as well as depth-wise, and were explored for differences between day and night. Mean S_V (dB), share of single echoes (%) and fish density (inds./ha) were averaged per layer, mapped by depth and compared for differences between day and night. Cross-correlation analysis was performed on the average fish density depth-wise distributions using depth-lag to determine the mean depth difference between day and night fish density distributions. Fish density was explored for spatial distribution differences, while it was also color-mapped both along transect and depth-wise, in the West-East direction, in order to visualize the variation in both dimensions at the same time, where transect mileages were matched to lake regions based on the transect path. Finally, fish density was also summed depth-wise at each segment and interpolated spatially to create total-water-column fish density maps for day and night.

To study fish density in combination with size composition, the targets were separated in two classes, ≤5 cm (small fish) and >5 cm (large fish) and density was re-calculated for small and large fish, respectively, using the corresponding target count ratios. The target count proportions for small and large fish were also mapped independently as stacked absolute values vs. depth (excluding layers with a total of <100 targets), separately for day and night, to study variations due to time of day, depth and size class simultaneously. Targets that were classified in 1 cm sized classes were separated by orientation (surface layer vs. vertical data layers) and time of day (daytime vs. nighttime) and were weighed by average class size, to calculate weighted average fish sizes. Specifically, the middle of each class was multiplied by the total target count of the class; the products were summed and divided by the total targets from all classes.

The segments of the transect closest to each sampling point were determined, and fish density was determined by depth for each of those segments (summing fish density values for all layers). Thereafter, the average fish density based on these segments was determined. A circle of specific radius was used to locate the closest segments, and segments were included wherever they intersected this circle. For two of the points, a radius of 1 km was used, whereas a radius of 2.5 km was used for the third point, because no segment was close enough to intersect a circle of 1 km radius around that point.

Data parsing and post-processing was carried out using custom scripts in "R" (R Development Core Team, 2020), as well as LibreOffice Calc and Microsoft Excel. Thematic maps were extracted using ARCGIS and QGIS. Interpolation for the creation of Raster Grids from the scattered processed hydroacoustic data was carried out using Multilevel B-splines [47]. Comparisons between series were made using pairwise t-tests (or 1-sigma confidence intervals where noted), and distributions were compared using Kolmogorov–Smirnov tests. For all tests, a significance level a = 0.05 was used. In some results, marginal hypothesized differences were also explored as the mean difference leading to a *p*-value approximately equal to a.

4. Results

4.1. Acoustic Biomass and Fish Density

Acoustic biomass detected per sampling unit (Figure 2) did not exhibit diurnal heterogeneities. During the day, the average S_V per depth layer was −65.9 ± 9.1 dB, ranging from −87.6 dB to −49.4 dB, while during the night, the average S_V per depth layer was −66.1 ± 10.1 dB, ranging from −83.8 dB to −49.3 dB. No significant difference was found ($p = 0.92$). Concerning the vertical distribution, during the day and night survey, the highest biomass was observed in the layer 16–30 m, (average value −55.8 dB in day survey and −55.6 dB in night survey). The coefficient of variation (CV) revealed greater heterogeneity in the vertical distribution during night rather than during the day survey (CV = 15% against 13%).

The share of single echoes did not exhibit large fluctuations comparing between acquisition times (Figure 3). Specifically, the day average was 17 % ± 20.2 %, ranging from 0 % to 91.9 %, while the night average was 15 % ± 11.3 %, ranging from 0 % to 44.8 %. Day-to-night difference was not statistically significant ($p = 0.39$). Most of the values were relatively low, typically less than 40% in most layers, during both day and night. The layers close to the surface (<8 m depth) were exceptions to this observation, with the night measurements exhibiting a share of single echoes up to approximately 45% at night versus approximately 90% during the day for those layers.

Fish density layer average was approximately 1226 ± 1608 inds./ha, ranging from 8 to 7984 inds./ha during the day. During the night, the fish density layer average was 3473 ± 3994 inds./ha, ranging from 20 to 16,384 inds./ha. The vertical distribution of fish density was significantly different between day and night ($p < 0.001$). An assumed mean difference of 1250 inds./ha less during the day produces a *p*-value of 0.05 for the t-test, meaning that this is a marginal difference that could be statistically accepted. Moreover, average fish density is more than double at night than during the day in the surface layer (0–4 m) and 5 times higher at 15 m (Figure 4). Application of a cross-correlation model between day and night vertical distributions and using depth as the lag parameter, reveals an average depth-wise offset of ~9 m between the two distributions, with a maximum correlation coefficient of 0.724 at dh = 9 m (Figure 5). This means that the distributions are very similar but translated with respect to each other by approximately 9 m of depth.

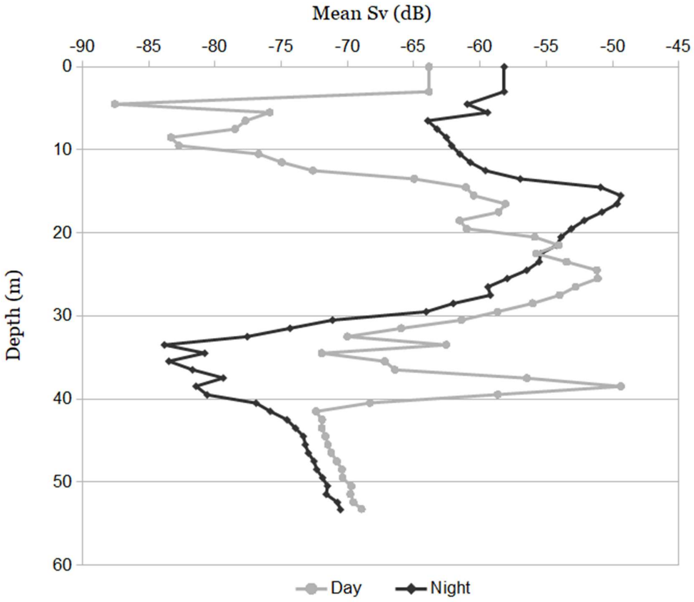

Figure 2. S_V (dB) vertical distribution during the day and night survey in Lake Trichonis.

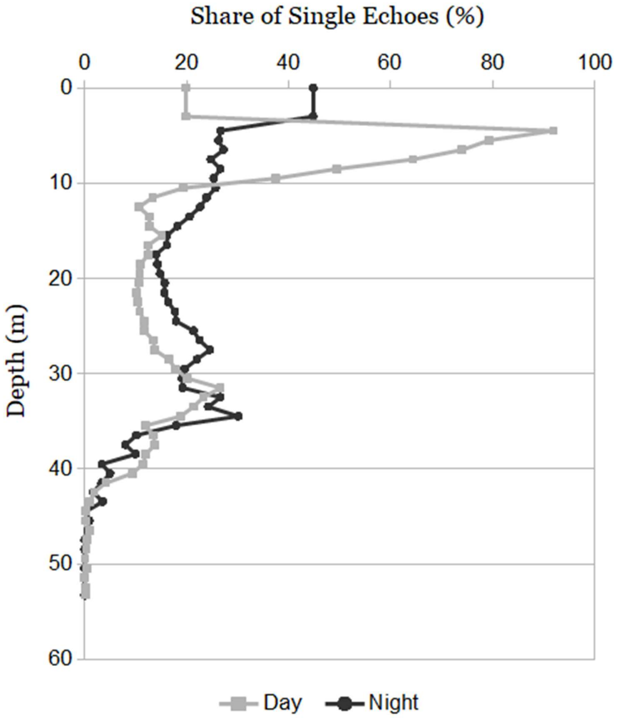

Figure 3. Differences in share of single echoes (%) between different layers as well as measurement acquisition time (daytime, nighttime).

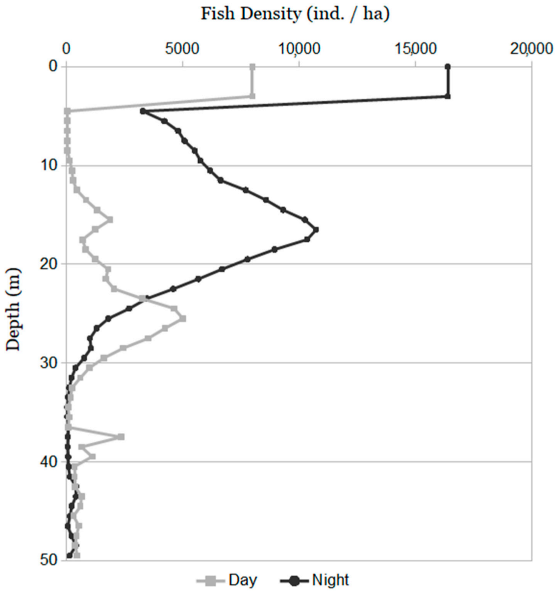

Figure 4. Vertical distribution of average density (inds./ha) for each water column layer during day and night surveys.

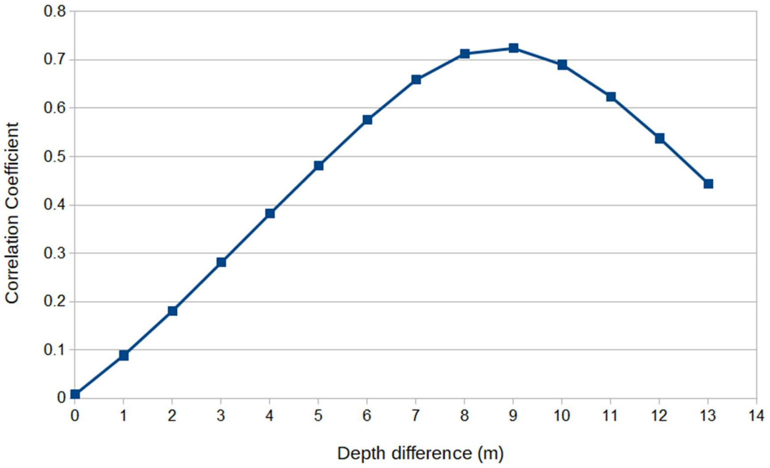

Figure 5. Cross-correlation between day vs. night, using depth (h) as the lag parameter. A depth difference of dh = ~9 m produces the maximum correlation between day and night fish density distributions.

Total water column fish density exhibited significant differences between the shallow and the deep areas of the lake, separating at a longitude decimal degree of 21.55 ($p < 0.0001$, Figure 6). Average fish density during the day was 41,925 ± 18,735 inds./ha in the shallow areas (western side of the lake) and 32,424 ± 13,548 inds./ha in the deep areas of the lake (eastern area) (total water column sums). During the night, fish density was 174,986 ± 69,521 inds./ha in the western region and 127,930 ± 79,934 inds./ha in the eastern region (total water column sums). An assumed mean day fish density difference of 8880 inds./ha between regions produces a marginal value of $p = 0.05$, while for the night, a marginal $p = 0.05$ value is produced by an assumed mean night fish density difference of ~45,750 inds./ha. The fish density depth-wise and spatial distribution along the transect, as well as the differences between day and night are shown in detail in Figures 7 and 8. A horizontally interpolated spatial distribution of fish density is shown in Figures 9 and 10.

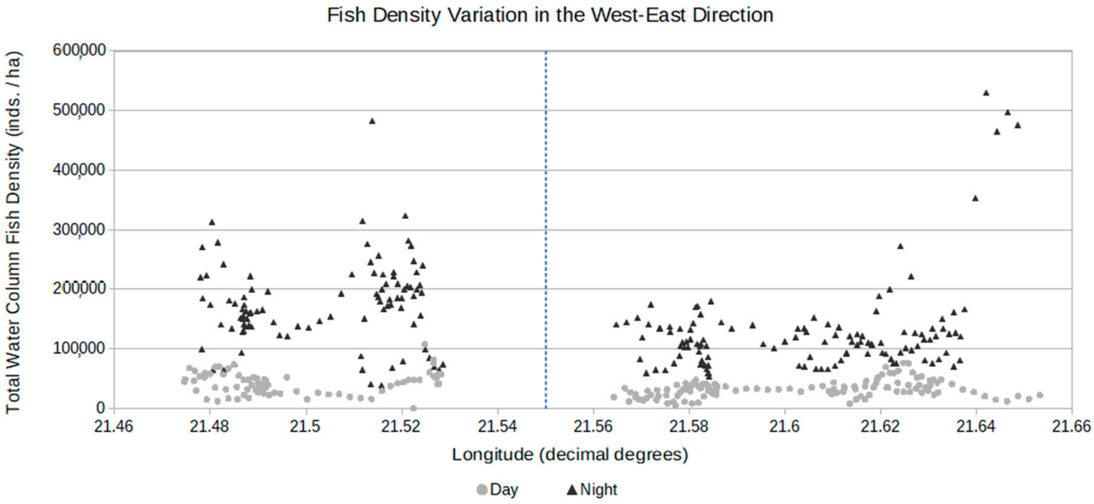

Figure 6. Total water column fish density vs. longitude of averaged-cell midpoint. Significantly different fish density clusters are visible between the regions for both day and night.

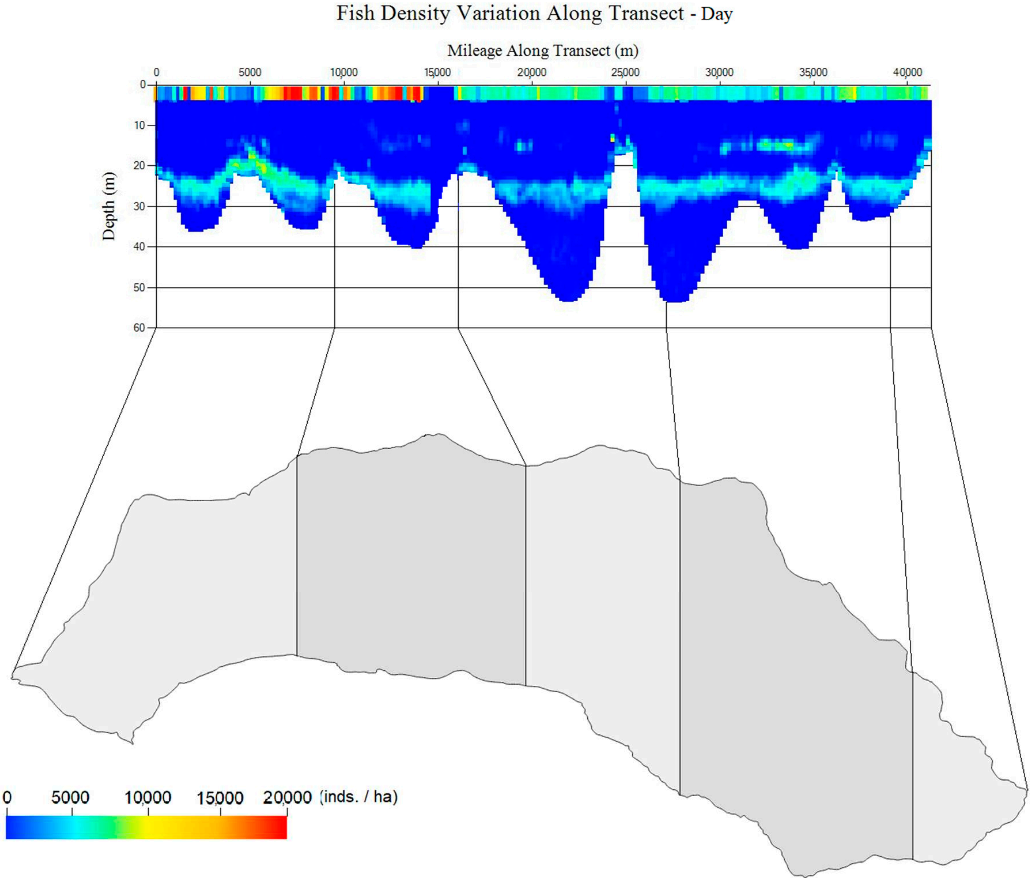

Figure 7. Color map of daytime fish density depth-wise and along transect variations. Figure is spatially referenced with respect to the lake.

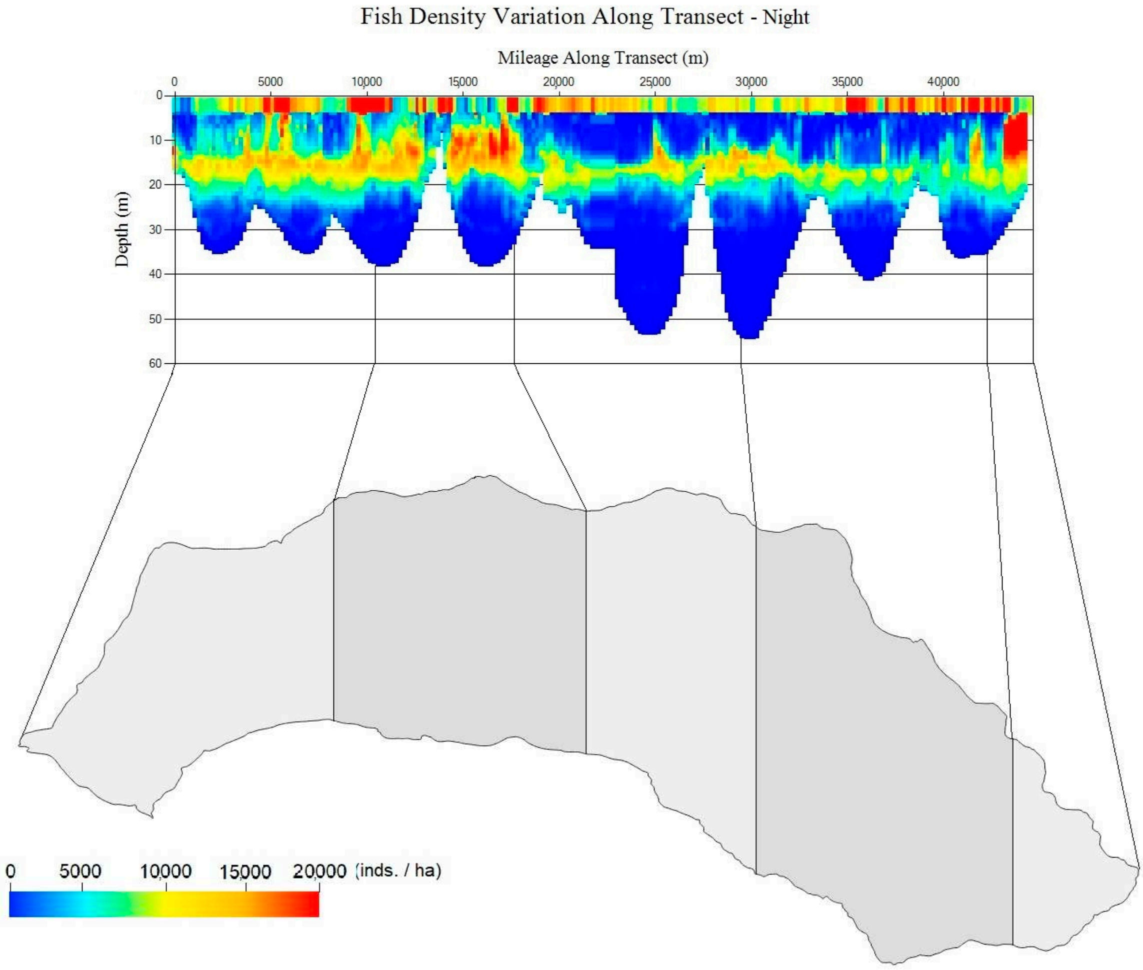

Figure 8. Color map of nighttime fish density depth-wise and along transect variations. Figure is spatially referenced with respect to the lake.

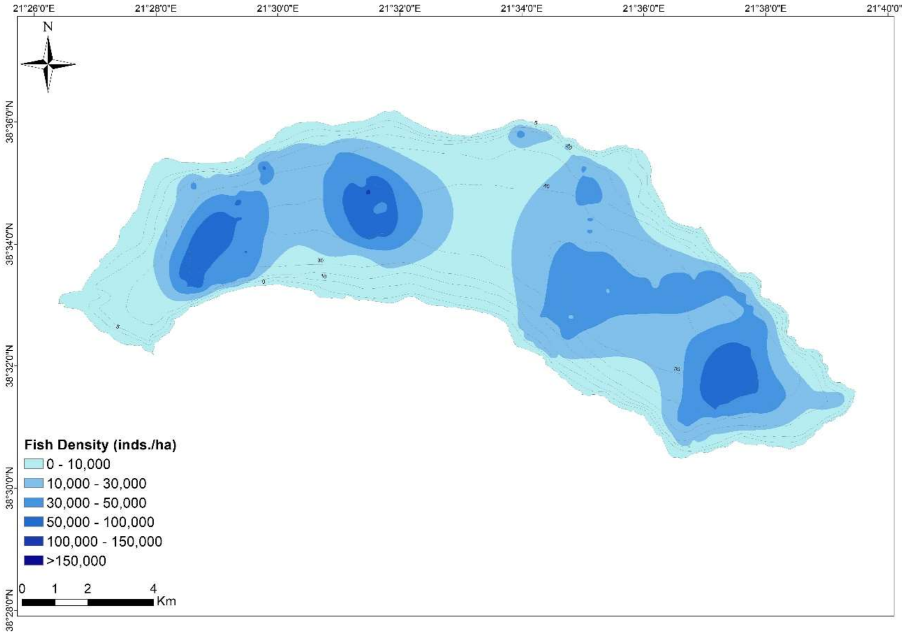

Figure 9. Fish density (inds./ha) distribution during the day survey in lake Trichonis.

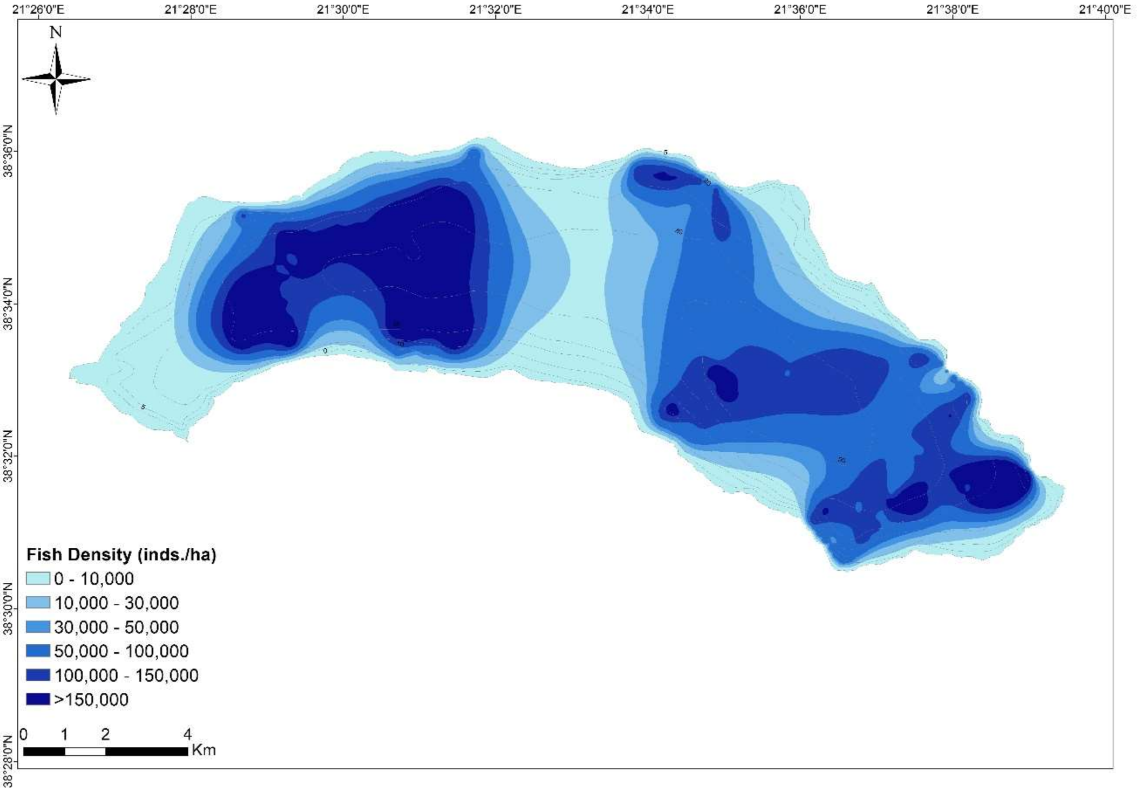

Figure 10. Fish density (inds./ha) distribution during the night survey in lake Trichonis.

4.2. Fish Size Composition and Distribution

Individual fish lengths, converted from TS data, ranged up to 50 cm. The length of most of the detected fish is not larger than 5 cm (Figures 11 and 12). Smaller fish, with lengths less than 5 cm dominate the total counts and observed fish density diminishes as target size increases. After separating by orientation (surface layer vs. vertical data layers) and time of day and weighing for class size, weighted mean fish size for the surface layer was ~1.6 cm for the day survey and ~2 cm for the night survey, while for the layers from 4 m to the bottom, mean fish size was ~4.5 cm for the day survey and 2.9 cm for the night survey.

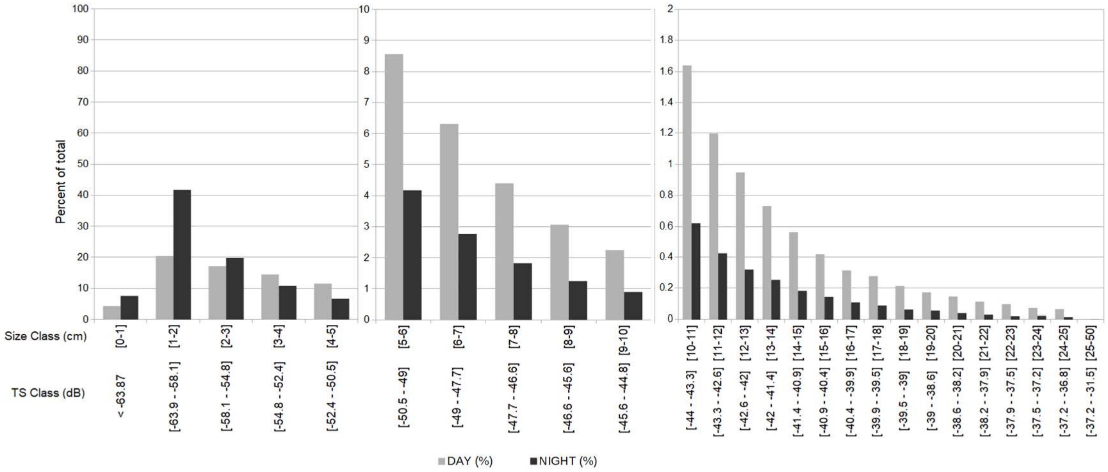

Figure 11. The size (total length, cm) and Target Strength (dB) distribution of pelagic fish recorded during night and day vertical surveys in Lake Trichonis.

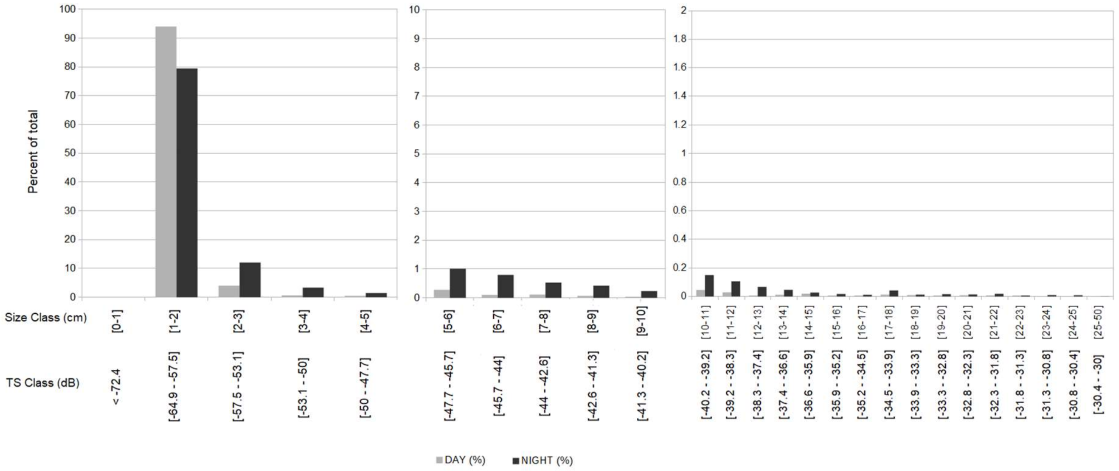

Figure 12. The size (total length, cm) and Target Strength (dB) distribution of pelagic fish recorded during night and day horizontal surveys in Lake Trichonis.

Small fish density during the day was 922 ± 1324 inds./ha, while during the night, it was 3068 ± 3496 inds./ha. Correspondingly, large fish density during the day was 304 ± 463 inds./ha, whereas during the night, large fish density was 405 ± 808 inds./ha. Small fish density was significantly larger during the night ($p < 0.001$), with a marginal $p = 0.05$ being produced by an assumed mean difference equal to 1220 inds./ha (of size < 5 cm). Large-fish density distribution by depth was not significantly different between day and night (Figure 13). Horizontal layer fish density exhibits fish density values more than an order of magnitude higher than the vertical layers, for both day and night (Figure 14).

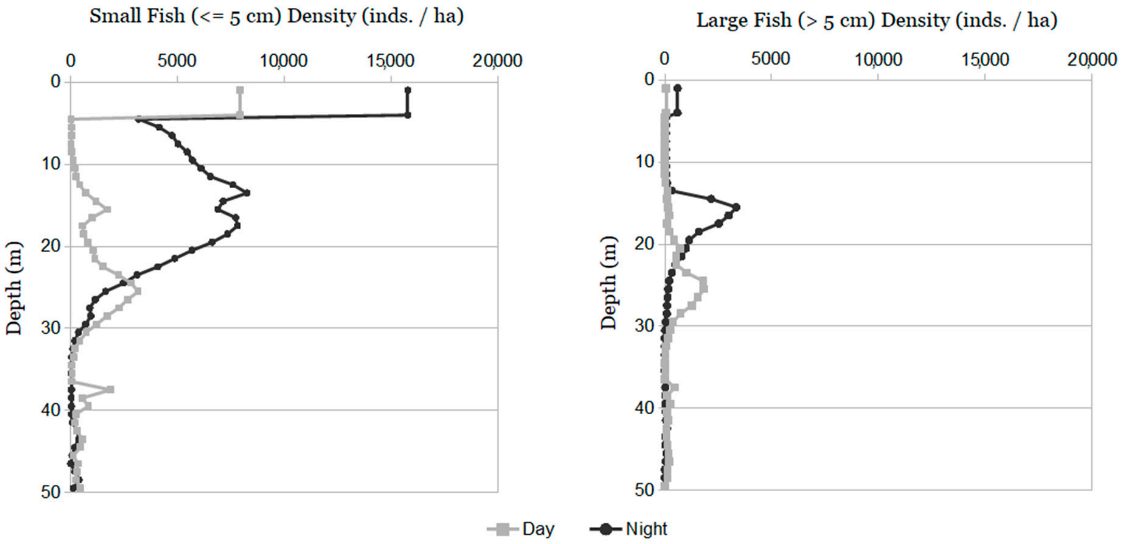

Figure 13. Vertical distribution of density (inds./ha) of the detected target size classes (small: <5 cm, large: 5–50 cm) during the day and night surveys in Lake Trichonis.

4.3. Temperature and Oxygen Stratification

The water temperature in the surface layer varied between 23.5 and 24.7 °C in the three sampling points. In the eastern region (deepest point), a thermocline at around 18–28 m was recorded, while temperature was almost constant in the hypolimnion (11.6–11.9 °C). During the survey, differences in temperature and oxygen profiles were observed between the western and eastern region (Figure 15). In the deep point of the western region, the thermocline was recorded at around 15–31 m, while the temperature in the hypolimnion was 12.7 °C. In the shallow point of the western region, temperature was almost constant up to a depth of 14 m, after which it exhibited a noticeable gradual drop, reaching its lowest value (14.3 °C) at 22 m.

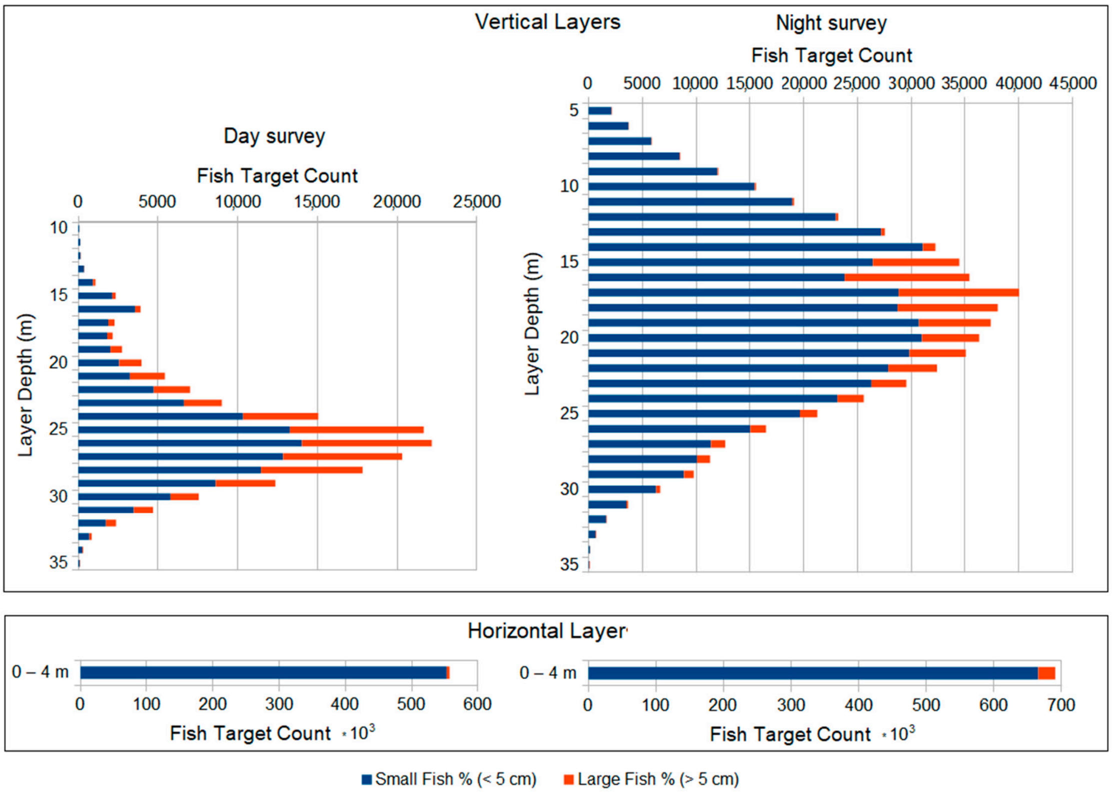

Figure 14. Proportional fish distribution by size, depth and time of day. Layers with <100 detected individuals excluded for clarity.

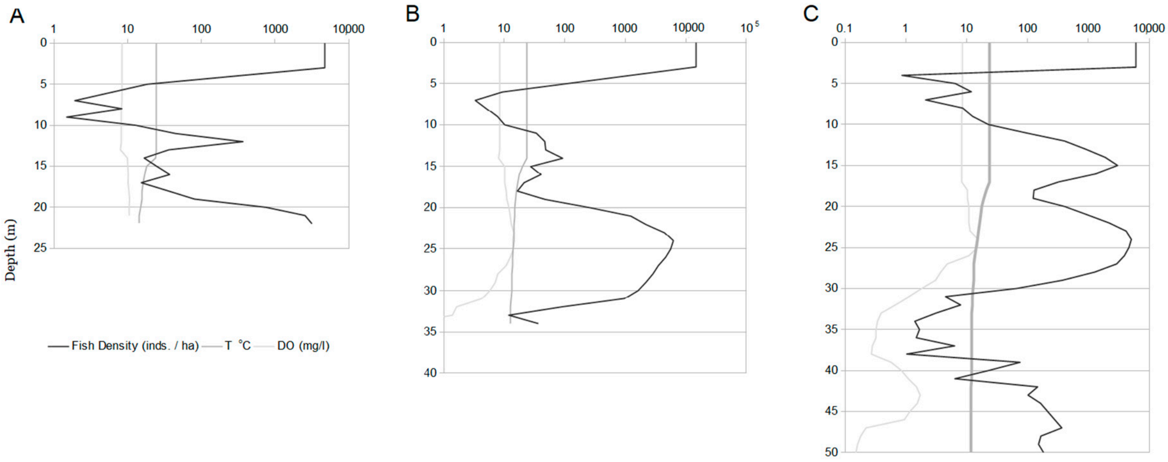

Figure 15. Variations of density (inds./ha), dissolved oxygen (mg/L) and water temperature (°C) with depth (m) at the three different sampling locations of Lake Trichonis ((**A**). Sampling point S1 (**B**). Sampling point S2 (**C**). Sampling point S3).

The horizontal variations of DO concentrations in the epilimnion from the eastern to the western region of the lake were weak. In the eastern region (deepest point) the DO concentration remained at approximately 8.3 mg/L in the upper 17 m mixing layer and significantly increased to 10–11 mg/L at a depth of 18–26 m. An anoxic layer was recorded after a depth of 30 m, as DO concentration fell below 2 mg/L [48]. Namely, the recorded DO values ranged from 1.82 to 0.15 mg/L. In the shallow point of the western region, the DO concentration was higher (approximately 10.5 mg/L) at around 16–22 m of depth than in the surface layer (approximately 8.3 mg/L). In the deep point of the western region, the highest concentration of DO (approximately 11.9 mg/L) was recorded at a depth around 15–27 m. An anoxic layer was also recorded after the depth of 32 m.

The vertical distribution of fish during the day survey varied according to thermal stratification and seemed to roughly follow the vertical variations of DO concentrations (Table 2). Based on the results of correlations between fish density and dissolved oxygen, it appears that as the depth of the sampled water column increases, a strong correlation manifests as the relationship between these two parameters. An apparent rise in correlation coefficient absolute values was observed between fish density and dissolved oxygen, especially around the area of the deepest point of the lake, whereas anoxic conditions occurred in the bottom layers.

Table 2. Correlation coefficients between fish density (inds./ha) and DO (mg/L)/Temperature (°C) for the three sampling points in Lake Trichonis.

	Sampling Point S1		**Sampling Point S2**		**Sampling Point S3**	
	Temperature	**Dissolved Oxygen**	**Temperature**	**Dissolved Oxygen**	**Temperature**	**Dissolved Oxygen**
R	−0.56	0.54	−0.52	0.53	0.018	0.61
R^2	0.31	0.29	0.27	0.28	0.00032	0.37
***p*-value**	0.013	0.018	0.0026	0.0023	0.91	5.9×10^{-6}
DF	17	17	29	29	45	45

5. Discussion

Volume backscattering strength (S_V), was used in this study as a proxy variable for fish biomass [8]. The calculation of biomass based on acoustic recordings requires knowledge of an overall fish SL–W (Standard Length to Weight) relationship, particularly for a mixed-species community, as well as TS estimates for fish fauna [49]. Given that this was the first hydroacoustic survey in Lake Trichonis, it was not feasible to obtain the aforementioned values. S_V values were compared among the different layers of the water column [50]. The average S_V values in Lake Trichonis −66.1 dB in the night and −65.9 dB in the day were slightly lower than in other 18 European lakes (on average −62.8 dB) [51]. The recorded range of S_V values in the different layers was also comparable with values of monomictic alpine lakes with similar morphology (ranges from −62.3 to −67.4 dB in different layers and lakes) [9] and with the results of Cech et al. [52], who studied distributions of juvenile fish (range of S_V was measured from −71.3 to −45.7 dB). Similarly to this last study, in Lake Trichonis, fish formed more or less dense layers; the type of aggregations is probably dependent on the kind of species.

Fish density was found to be lower in the deep areas (eastern area) than the shallow areas (western area) of the lake. Additionally, fish density was higher in intermediate depth locations, indicating that fish targets are generally distributed in the water column at larger depths. An offset between day and night was observed in the vertical distribution of fish, estimated at approximately 9m (fish were located deeper during the day). Fish were more closely aggregated during the day and more dispersed at night. Vertical migration behavior with diel periodicity is well described [53] and is linked to predation avoidance and reduction of competition [54].

A statistically significant difference was observed in vertical distribution between day and night in the pelagic zone. The overall recordings revealed a relatively high degree of fish aggregation (low share of single echoes), especially during daytime, which, in turn, makes it harder to discern and count single targets and decreases the reliability of density estimations. According to Appenzeller and Leggett [55], the estimation of the number of targets can be biased towards smaller values when fish form dense aggregations, due to acoustic shadowing. Precise determination of fish target counts should coincide with the time of their maximal dispersion [37]. The share of single echoes did not present significant changes in depth distribution between day and night. Nevertheless, during the night, significantly larger fish densities were observed at smaller depths, thereby observing a wider size spectrum of fish living in the lake. In this respect, it may suggest that night hydroacoustic surveys in Lake Trichonis could be more suitable. Nonetheless, we recommend that diel differences be further investigated by carrying out additional day-night comparisons, also using fish catches. The use of hydroacoustics in a future setting over the same study area would be useful to investigate the possible bias and aggregation-related behavior and simultaneously also the true size composition by the use of appropriate fishing gear.

In relation to higher fish densities, values of the Sawada index were also taken into account to consider reliability of the density estimates. In most of the analyzed cells, values of N_V were below the commonly accepted limit of 0.1 by Parker-Stetter et al. [56], only exceeding 0.1 in the layers with the densest aggregations, with a few or no single echo detections. Nevertheless, TS distribution was examined. There was no statistically significant difference in the two distributions (TS for all N_V and TS only for $N_V < 0.1$) in the lake, neither for the day (K-S D-value = 0.2143, $p = 0.9205$), nor for the night (K-S D-value = 0.167, $p = 0.9985$) (for details, see Supplementary Materials).

The majority of all detected targets have a small size, while larger targets only comprise a minimal fraction. The presence of small-sized fish [21] is also a reason for the threshold used in this study, which may seem to be lower than other hydroacoustic studies using a frequency of 120 kHz. The lake hosts 20 fish species [21], most of which are of small size, such as the big-scale sand smelt *Atherina boyeri*, which occupies the pelagic regions of the lake and its population is extremely abundant, supporting a valuable commercial purse-seines fishery [29]. It is important to note that the targets observed could not be other than fish, as no big zooplankton, which could overlap with small fish, was observed in the lake [22], nor invertebrates [57] could be expected in this autumn time. Nevertheless, we cannot exclude the possibility of juvenile fish being mixed into the composition of the recorded populations. However, due to the lack of catch data in the present study, as well as due to the lack of corresponding historical data, it is not possible to identify the smaller fish target species, thus precluding a conclusive interpretation of the results. Consequently, provided that the detected fish target species composition, biology and behavior are not known, further investigation into the variations in acoustic parameters across an annual cycle is warranted [58]. It is also necessary to use control catches to enable proper interpretation of acoustic targets.

A general estimation of the fish length, even roughly, is crucial to the understanding of an aquatic ecosystem [9]. Fish length from TS data can be calculated based on an equation that expresses this relationship [8]. The most important factor affecting acoustic estimates appears to be the appropriate relationship between the fish length and TS for a given population. Equations of Love [42] and Frouzova et al. [43] were derived from different fish species and size ranges, and therefore, the estimation of fish length based on those equations ought to be further assessed with data from catches regarding their use for the endemic species of the lake.

The vertical distribution of the fish in the lake appears to have been primarily driven by water temperature and oxygen stratification. In the present study, the surface layer density does not seem to be correlated to dissolved oxygen and water temperature, but other factors, such as predation risk, may play an important role. Similarly to Doulka and Kechayias [59], Lake Trichonis showed a minimum saturation value of dissolved oxygen in the lower hypolimnion in autumn. However, in contrast to the study of Doulka and Kechayias [59], in the present study anoxic conditions were recorded in the lake's hypolimnion, rendering the DO concentration a limiting factor for the vertical dispersion of fish. As a result, in the vertical domain, fish distribution was highly correlated with oxygen stratification, i.e., it decreased as the dissolved oxygen also decreased. This correlation between density and dissolved oxygen is well established, while the relation can be explained, in part, by the fact that there is a natural threshold of oxygen, beyond which survival very quickly becomes non-viable [60]. It is also important to note that the presence of larger abundances of large fish in intermediate layers is in agreement with the results of Breitburg et al. [61], according to which the response of predators and prey to oxygen distribution variations strongly influenced the spatial focus of trophic interactions.

Consequently, the metalimnion in Lake Trichonis is the most productive depth stratum, due to the intense variation of abiotic and biotic variables [59].

The methodology presented and the results obtained through this research were the first step to present and analyze the challenges for optimizing a hydroacoustic approach for monitoring freshwater fish stock in Greek lakes. This methodology could be incorporated in the decision-making process towards the improvement of the implementation of the EU Water Framework Directive 2000/60. The use of hydroacoustic techniques can eliminate some distortions traditionally contained in censuses and studies of fish fauna in lakes. Lake Trichonis is traditionally exploited by commercial fishermen, most of which use purse-seine fishing. The knowledge of feeding and nursery grounds where fish are gathered during their vertical and horizontal movements through the day will help and support local authorities and contribute to decision making in applying restrictions for a more sustainable fisheries management. The potential adjunct usage of common fishing gear might not be able to adequately reveal the potentially essential role of small fish in the open waters of Lake Trichonis. As a result, the proposed methodology can provide new insights for enhancing fisheries monitoring.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/6/1823/s1. Figure S1: Empirical distribution functions for TS, categorized in 2-dB bins separately for day and night, as well as for the sets of all sampled cells, and of only cells with $N_V < 0.1$. Figure S2: Sawada N_V index vertical distribution with 1-sigma confidence intervals (left) and day-night difference, i.e., day values minus night values (right).

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water

Article

Changes in the Fish Community of a Western Caribbean Estuary after the Expansion of an Artificial Channel to the Sea

Juan J. Schmitter-Soto * and Roberto L. Herrera-Pavón

El Colegio de la Frontera Sur, Av. Centenario km 5.5, Chetumal 77014, Quintana Roo, Mexico; rherrera@ecosur.mx
* Correspondence: jschmitt@ecosur.mx; Tel.: +52-983-835-0440 (ext. 4302)

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Abstract: Increased connectivity between coastal lagoons and the sea is expected to entail a greater proportion of marine species in the former. Chetumal Bay, estuary of the Hondo river into the Caribbean, had a limited access to the sea until the opening of the Zaragoza Canal. We sought changes in the fish community from 1999–2001 (just after an expansion of the canal) to 2015–2018. The same fishing gear was used, in the same localities, during all seasons. Total fish abundance and mean local richness decreased, although total abundance increased in the polyhaline zone. Diversity was greater in the oligohaline zone in 1999–2001, and in the mesohaline zone in 2015–2018. Three guilds were absent in 2015–2018: Medium-sized herbivores, large piscivores, and medium-sized planktivores. Abundance of small benthivores decreased by decade; medium-sized piscivores and small planktivores became more abundant in 2015–2018 in the polyhaline zone. These changes may be due to the opening of the channel, but illegal fishing outside the bay may explain the decrease in juveniles of large piscivores, and erosion in the innermost part may be destroying important habitats. Our findings can be a reference for similar situations, as coastal development and climate change interact and affect tropical estuaries.

Keywords: ichthyofauna; trophic guilds; salinity-tolerance; connectivity; Chetumal Bay; Mexico

1. Introduction

Chetumal Bay (known as Corozal Bay in Belize) is the estuary of the Hondo River and minor affluents, connecting them to the Caribbean Sea, at the border between Mexico and Belize. This large water body (about 3500 km^2 in area) is protected by both countries [1]. For thousands of years, the narrow and winding natural passage of Bacalar Chico was the connection of the Mexican part of the bay to the Caribbean Sea; a much wider opening exists near the Belize–Guatemala border, about 300 km south. However, between 1999 and 2004 an artificial channel was dredged and expanded for navigation, the Zaragoza Canal, opened initially in 1901, but never made deep enough for larger vessels. This direct communication started having a strong influence on the abiotic and biotic conditions of the system, including the intrusion of corals and other reef organisms to formerly brackish areas of the bay [2].

Many of the fishes found in this system are important resources. Most relevant for recreational fisheries in Belize and Mexico are bonefish (*Albula vulpes*), but also permit (*Trachinotus falcatus*), snook (*Centropomus undecimalis*), and tarpon (*Megalops atlanticus*) [3]. Other species are fished mostly for local markets: Snappers (*Lutjanus* spp., especially *L. griseus* and *L. apodus*), mojarras (*Gerres cinereus*, *Eugerres plumieri*), mackerels (*Scomberomorus maculatus* and other species), and barracuda (*Sphyraena barracuda*) [4]. Other mojarras (*Eucinostomus* spp.) are also abundant, as are needlefishes (*Strongylura* spp.), pupfishes (Cyprinodontidae), flatfishes (several families), sea catfishes (Ariidae), gobies (Gobiidae), puffer (*Sphoeroides testudineus*), and, under the influence of freshwater, also cichlids, poeciliids, and tetra (*Astyanax bacalarensis*), among others [5]. A recent addition to this ichthyofauna is an exotic invader, lionfish (*Pterois volitans*) (unpublished observation).

Qualitative and quantitative data on the ichthyofauna of Chetumal Bay are available from the time just before the expansion in depth and width of the Zaragoza Canal [5,6]. Our aim in this paper is to evaluate ichthyological changes almost two decades later, in terms of composition, diversity (including richness, evenness, beta-diversity), abundance of species, trophic guilds, and salinity-tolerance categories, maximum length, and distribution through the salinity gradient of the bay, from the river mouth to the Zaragoza Canal. We discuss possible processes to account for the observed patterns.

2. Materials and Methods

2.1. Study Area

The region has a warm humid climate, with summer rains and an average temperature greater than 26.5 °C, with an annual precipitation of 1000–1500 mm; the bay is shallow, its maximum depth 5 m (mean 3.2 m), with scattered, much deeper, sinkholes [7]. Most of the bay is mesohaline (13 to 22 psu), with freshwater conditions occurring near the mouth of the Hondo River, and marine salinity close to the Zaragoza Canal [8]. Salinity varies seasonally, increasing in the dry season (February to April) and decreasing during the rains (May to October) and during the colder "north winds" season (November to January). However, three areas can be recognized year-round, and we term them here Oligohaline, Mesohaline, and Polyhaline (Figure 1). Except in winter, southeasterly trade winds predominate, with a mean wind speed of 3.1 $m \cdot s^{-1}$ [7].

Tides are semidiurnal, with excursions ≥ 0.5 m but, nevertheless, inducing inward or outward currents at the Zaragoza Canal and minor channels. Water temperature fluctuates between 22 °C at the peak of the north-winds season and 31 °C in August [7]. Bottoms are mostly sandy or sandy-muddy, except near rocky points, usually without vegetation but often with seagrass and algae. Mangrove rims the coast, with *Avicennia germinans* reaching heights of 10 m [9], and occasional patches of exotic *Casuarina equisetifolia* trees.

There are no human settlements in the northern half of the bay. Chetumal City lies at the mouth of the Hondo river (Figure 1), and just to the south is the Belizean town of Sarteneja. Pesticides from agricultural fields along both banks of the river and organic matter from Chetumal represent environmental stressors [10], although largely confined to the river mouth by winds and currents.

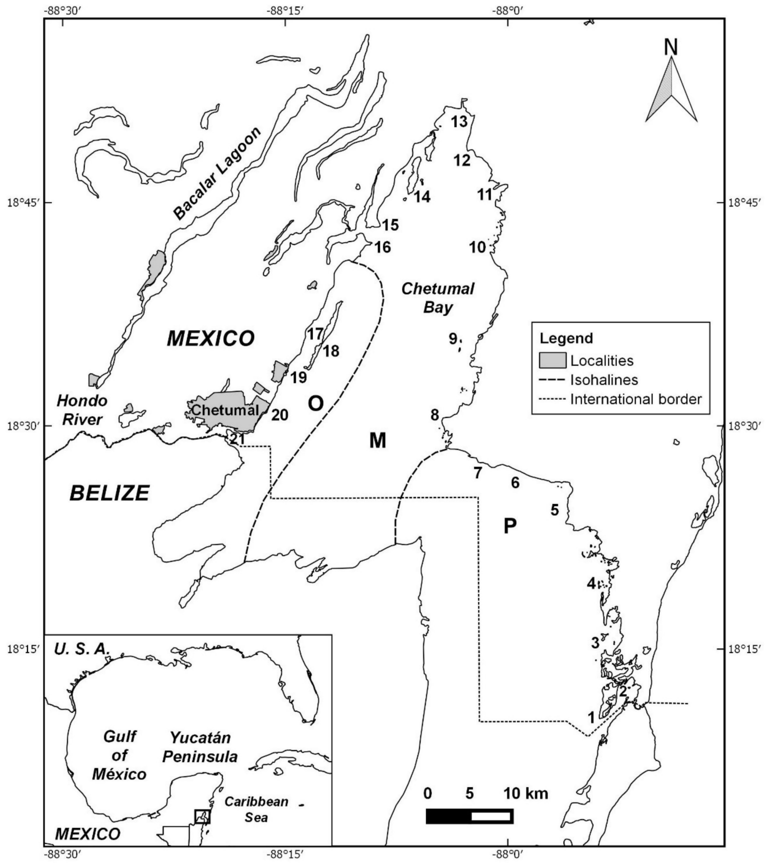


Figure 1. Study area, the northern (Mexican) part of the Bay of Chetumal (known as Bay of Corozal in Belize). Localities: 1 Cayo Chelem; 2 Zaragoza Canal; 3 Dos de Abril; 4 Mala Noche; 5 Punta Jas; 6 Mainada; 7 Punta Calentura; 8 Punta Flor; 9 Dos Hermanos; 10 Mogote Barlovento; 11 Siete Esteros; 12 Tollocan; 13 Río Krik; 14 Cayo Venado; 15 Punta Pol Box; 16 Punta Lagarto; 17 Isla Tamalcab (leeward); 18 Isla Tamalcab (windward); 19 Punta Catalán; 20 UQROO; 21 ECOSUR. Abbreviations for salinity zones: O—oligohaline; M—mesohaline; P—polyhaline (see text). Map by Janneth Padilla.

2.2. Field and Laboratory Work

We sampled the bay for fishes at the same 21 localities explored by us about 15 years before [6] (Figure 1); the same procedure described below was applied. Expeditions took place in April 13–15 (dry season) and July 6–8, 2016 (wet season), and in April 23–24, 2018 (same as in 1999–2001), and more than 13,000 specimens were collected from 162 samplings, using the same fishing gear and effort (a beach seine, 20 m long, 1.2 m tall, 1 cm diagonal mesh, 2–5 seinings per site, until no new species were found, each seining lasting ca. 3 min). The contagious distribution of some species could be a source of heteroscedasticity even within one same site, but usually abundant species continued to be present in a similar proportion in all subsequent seinings.

Fish were identified and counted in situ and then freed; voucher specimens were kept only for those species which could not be determined in the field, such as specimens of *Eucinostomus*, which were deposited in the fish collection of ECOSUR (acronym ECO-CH). Abundance was controlled by the number of seinings per site/date, as catch per unit effort (CPUE). Collected specimens were measured (standard length) to the nearest millimeter with a vernier caliper or an ichthyometer.

Composition (i.e., the species list) included data from the literature [11,12], revision of collection specimens, and observations or captures outside seinings.

2.3. Data Analysis

We compared the following variables between decades (1999–2001 vs. 2015–2018) and by salinity areas (Oligohaline, Mesohaline, and Polyhaline—hereafter zones O, M, P): (a) composition (i.e., presence/absence of species); (b) richness; (c) diversity, both alpha and beta; (d) abundance (i.e., CPUE) of dominant species; (e) abundance by guild (see below); (f) abundance by salt-tolerance category [13]: marine stenohaline, marine euryhaline, estuarine resident, freshwater primary or secondary; (g) maximum observed length of selected species; (h) frequency (i.e., proportion of localities where present); (i) environmental abiotic variables, mainly temperature, salinity, and wind at the time of capture. We did not compare biotic data by month because an earlier analysis found no seasonal difference in 1999–2001 [6]. Differences in salinity (refractometer, to 1 psu), temperature (digital thermometer, to 1 °C) and wind force (Beaufort scale) were examined controlling by season (see Study Area) and time of day (morning 07–10:59, noon 11–14:59, afternoon 15–19 h: time when sampling started). Fourteen guilds were defined as combinations of feeding habits and three body-size categories [14–16].

In addition to analyzing pooled data, the variables listed above were examined separately by salt-tolerance category of the fishes. Abundance was log-transformed to achieve homoscedasticity and normality of the data. Only species that made up 75% of total abundance were analyzed separately. The test used was a two-way ANOVA (by decade and by salinity area), with an interaction term; post-hoc comparisons were performed using Tukey Honest Distance. Frequencies were compared by a Kolmogorov–Smirnov test (KS). Diversity (H'n, Shannon index, in nats) and its components (richness, as number of species; J'n, Pielou's equity) were calculated by locality and controlled by rarefaction. Beta-diversity as turnover was explored via the Sørensen index and plotted in the triangular graph suggested by Koleff et al. [17]. The software used to analyze data and prepare graphs was R [17], with $p < 0.05$ (instances where $p > 0.05$ but $p < 0.10$ are also mentioned and discussed); the package "vegan" was used for diversity analyses [18].

We sampled under Permit PPF/DGOPA-053/15 from the Comisión Nacional de Pesca (the Mexican Commission for Fisheries), with further authorization by the protected area "Bahía de Chetumal Santuario del Manatí". Accession to our database will be provided during review.

3. Results

Sampling effort, with emphasis on the mesohaline zone (samplings: 140 O, 261 M, 215 P) due to the greater number of localities (5 O, 8 M, 7 P), did not vary between decades (KS, D = 0.67). Every locality was sampled on average 29 times between both decades. The mean number of fish by species per sampling was 9; the maximum, 294.

Salinity varied from 1 to 36 psu (mean 10.9 psu); water temperature, 25–34 °C (mean 29 °C). Wind force in the Beaufort scale was 1 to 4 (mode 2). Controlling by season, salinity varied significantly between decades (F = 10.9), changing from a mean of 10.4 psu in 1999–2001 to 11.8 psu in 2015–2018. Controlling by season and by time of day, water temperature was also significantly different between decades (F = 6.9), from 29.2 °C to 28.7 °C, as well as wind force (F = 21.6), from 1.9 to 2.4 in the Beaufort scale.

General abundance of fish decreased from 1999–2001 to 2015–2018, except in zone P (F = 5.82 by decade, F = 8.05 for the interaction decade:zone; Figure 2, Appendix A). By zone, an "estuary effect" is apparent, with greater abundance in zone P than in O, but greater in O than in M (F = 5.97).

There were few changes in composition (Appendix B). The most conspicuous novelty is the lionfish *Pterois volitans*, absent in 1999–2001, now confirmed in the zone P, closest to the Zaragoza Canal. Among the species that were captured in 1999–2001 but not in 2015–2018 are *A. vulpes*, *Ariopsis assimilis*, *Centropomus undecimalis*, *Jenkinsia lamprotaenia*, *Opsanus beta*, and *Opisthonema oglinum*; however, all of them are still present in the area, as observed outside our seinings. On the contrary, species that were not seined in 1999–2001 but that were collected in 2015–2018 include *A. bacalarensis*, *Corvula sanctaeluciae*, *Hypanus* spp., *Diapterus auratus*, and *Lutjanus* spp. Juveniles of *Trachinotus falcatus*, not found in the quantitative samplings in 1999–2001, were captured in all three salinity zones in 2015–2018. Three species of *Strongylura* were seined in 1999–2001, but in 2015–2018 only *S. notata*. Also exclusive to the older decade were usually reef-dwelling species, as *Holocentrus rufus* and *Sparisoma rubripinne*, freshwater secondary species, as *Poecilia kykesis*, and others (*Syngnathus* spp., *Selene vomer*, *Haemulon* spp., *Chaetodipterus faber*, *Chilomycterus schoepfi*, *Bothus ocellatus*, and *Acanthurus* spp.). One case of concern was *Gobiosoma yucatanum*, the only species endemic to Chetumal Bay [19,20], which we found in 1999–2001, but not in the most recent expeditions.

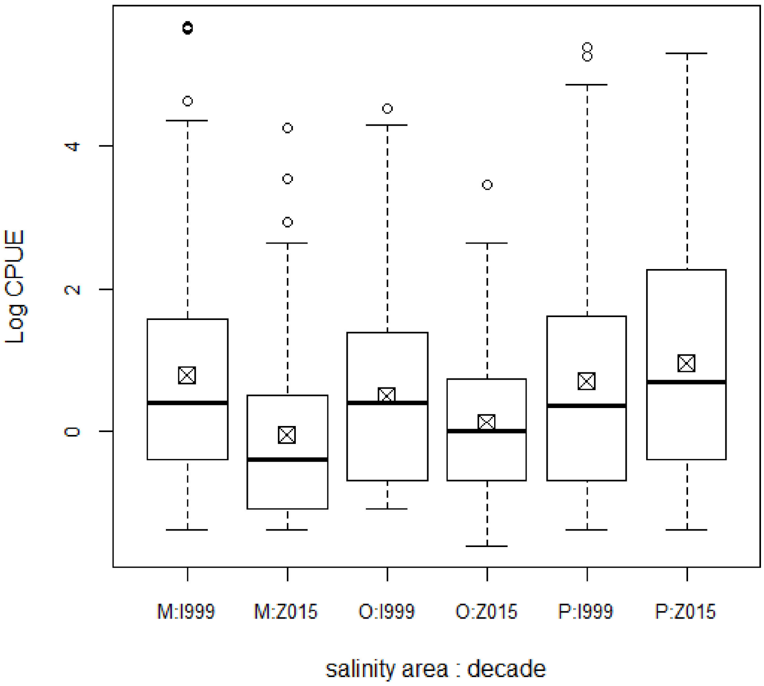


Figure 2. General abundance of fishes by salinity area and decade in Chetumal Bay. Abundance (CPUE) log-transformed. Posthoc significant differences between M:1999 and M:2015, P:1999 and M:2015, M:2015 and P:2015, and O:2015 and P:2015. Abbreviations for salinity zones: O—oligohaline; M—mesohaline; P—polyhaline; "1999" is 1999–2001 and "2015" is 2015–2018.

Changes in dominance were clearer than changes in composition. *Atherinomorus stipes* and *Harengula jaguana* were always abundant and frequent, the former especially in the windward (sandy) side of mangrove islands, whereas *Floridichthys polyommus*, in both decades, predominated in the leeward (silty) side. *Bairdiella ronchus* and *Cyprinodon artifrons* decreased (in frequency, 3 to 1); in 1999–2001, *C. artifrons* was more abundant than the ecologically similar *Jordanella pulchra*, which increased in frequency from 1 to 3. Concerning another ecologically similar pair, *Eugerres plumieri* was more abundant than *Gerres cinereus* in 1999–2001 (especially in zone O), but not in 2015–2018 (*G. cinereus* increased in frequency from 2 to 3). The five species of *Eucinostomus* kept their relative abundances similar, except that *E. melanopterus* was not seined in 1999–2001.

Few species displayed significant changes by decade or salinity zone (Table 1). Most increases occurred in zone P, most decreases in zone M. The flatfish *Achirus lineatus* decreased in abundance in all three salinity zones; among zones, it preferred O to P, in spite of being a marine species. The silverside *Atherinomorus stipes* was almost absent from zone O back in 1999–2001, and in 2015–2018 it preferred P to M. As stated above, *E. plumieri* decreased, especially in zone O, which it used to prefer in 1999–2001. The needlefish *Strongylura notata* became more abundant in zone P in 2015–2018.

Table 1. Fish species whose abundance changed significantly by decade (1999–2001 vs. 2015–2018) and/or between salinity zones (Oligohaline, Mesohaline, Polyhaline) in Chetumal Bay.

Species	F	Difference
Achirus lineatus	12.70	Decreased by decade at all zones
Achirus lineatus	3.41	Greater abundance in O than in P
Atherinomorus stipes	4.97	Decreased by decade in M
Eugerres plumieri	6.11	Decreased by decade in O and M, not in P
Gerres cinereus	3.44	Decreased by decade in M
Harengula jaguana	7.97	Greater abundance in P in 2015–2018
Strongylura notata	2.47	Greater abundance in P in 2015–2018

Diversity was not uniform by locality. In 1999–2001 most of the sites with greater diversity belonged in zone O, such as Punta Catalán (H'n = 2.47 nats) and UQROO (2.14 nats), same as equity (J'n = 0.84, 0.97, respectively) and richness (up to 19 spp.), whereas in 2015–2018 most diverse localities were in zone M, e.g., Siete Esteros (2.32 nats) and Punta Flor (2.19 nats), same as equity (0.90, 0.88, respectively) and richness (up to 14 spp., although Chelem, in zone P, reached 15 spp.).

Total diversity or equity did not differ between decades (t = 0.36 and t = 1.10, respectively), but richness yes (t = 2.30), decreasing from a mean of 11.8 spp. in 1999–2001 to 9.3 spp. in 2015–2018. Beta-diversity (turnover) did not differ between decades, although the graph shows a somewhat greater dispersion in 2015–2018 than in 1999–2001 (Figure 3). Turnover varied slightly between decades, from 0.78 to 0.79, and nestedness from 0.06 to 0.08.

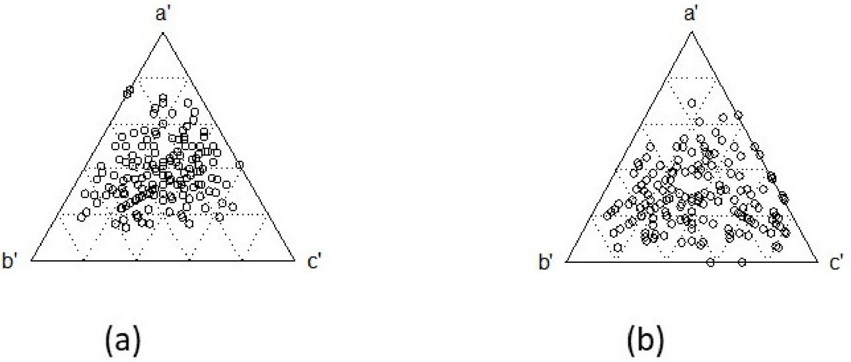


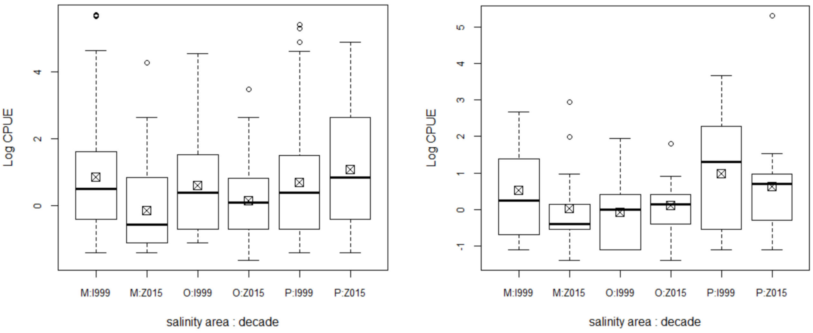
Figure 3. Beta-diversity of fishes in Chetumal Bay by decade: (**a**) 1999–2001; (**b**) 2015–2018. Circles are pairwise comparisons of species turnover derived from the Sørensen formula, where a′ are shared species, b′ are species exclusive to the focal locality and c′ are species exclusive to the compared locality.

The most frequent guilds were benthivores, small and medium-sized, followed by small planktivores and medium-sized piscivores. No large benthivores occurred in our samplings in 1999–2001 and three guilds were absent in 2015–2018: Medium-sized herbivores, large piscivores, and medium-sized planktivores. Although medium-sized and small benthivores always predominated, the proportions for the 14 guilds changed significantly between decades (χ^2 = 106.75; Table 2). However, significant changes in abundance by guild between decades and/or zones occurred only for small benthivores (F = 5.27: decreased by decade), medium-sized piscivores (F = 4.03: greater in zone P than zone M in 2015–2018), and small planktivores (F = 8.00: greater in zone P than in zones M and O in 2015–2018, mostly due to juveniles of *H. jaguana*).

Table 2. Fish guild occurrences in Chetumal Bay in 1999–2001 and 2015–2018. Figures are number of samplings.

Guild	1999–2001	2015–2018
Large benthivores	0	4
Middle-sized benthivores	63	46
Small benthivores	157	97
Medium-sized detritivores	8	1
Small detritivores	8	6
Medium-sized herbivores	1	0
Small herbivores	9	3
Medium-sized omnivores	3	2
Small omnivores	11	12
Large piscivores	1	0
Medium-sized piscivores	45	42
Small piscivores	1	6
Medium-sized planktivores	1	0
Small planktivores	63	26

By salinity-tolerance, the most frequent species were marine euryhaline, followed by resident estuarine, freshwater, and very few marine stenohaline, the latter only in the most saline localities of zone P. Freshwater fishes appeared not only in zone O, but also in stream mouths within zone M (not only secondary freshwater species, but also primary, i.e., *A. bacalarensis*), especially during the rainy season. The proportions did not change between decades (KS, D = 0.25). Marine euryhaline species decreased between decades in zones O and M, but increased in zone P (F = 9.54; Figure 4a). Estuarine species decreased in zones M and P, and increased in zone O, but, in spite of that trend, they always tended to prefer areas of greater salinity (F = 2.85, p = 0.06; Figure 4b).



(**a**) (**b**)

Figure 4. Abundances of salinity-tolerance groups of fishes by decade and salinity area in Chetumal Bay. Abundance (catch per unit effort (CPUE)) log-transformed; (**a**) marine euryhaline fishes; (**b**) estuarine resident fishes. Posthoc significant differences for (**a**): M:2015 against M, O, and P:1999 and P:2015; P:2015 against O:2015; (**b**) P vs. O and M. Abbreviations for salinity zones: O—oligohaline; M—mesohaline; P—polyhaline; "1999" is 1999–2001 and "2015" means 2015–2018.

Only two species exhibited significant differences in maximum length observed in seinings. *Strongylura notata* decreased in size between decades from 268.9 to 181.7 mm (F = 6.15). *Eucinostomus gula* increased (F = 8.49), but only in zone O, from a mean maximum length of 56.4 mm in 1999–2001 to 87.3 mm in 2015–2018; by zones, only in 2015–2018, it was larger in zone O than in zone P (50.8 mm), whereas in 1999–2001 there was no spatial difference in size. Another mojarra, *E. argenteus*, also seemed to increase marginally in size in all zones, from 42.4 mm in 1999–2001 to 50.0 mm in 2015–2018 (F = 3.6, *p* = 0.07).

4. Discussion

Given that our study features no parallel "control" for the "impacted" area, ours is not a true B.A.C.I. (Before/After-Control/Impact) design [21]. Moreover, the "before" data belong in fact to a period *after* the initial enlargement of the canal. Nevertheless, we expected the expansion of the Zaragoza Canal after 1999–2001 to induce changes in composition and diversity of fishes in Chetumal Bay, some of them perhaps positive for the environmental integrity (greater access of marine fishes, at least to zone P, and better development of sea grasses due to increased salinity [22]), others probably negative, not just because of the entrance of lionfish and sargasso (unpubl.), but also due to the methods required for the expansion. The bottom of the canal is mostly bedrock, so explosives are needed to deepen it, resulting in sediment suspension likely affecting habitats in the bay and adjacent reef; moreover, widening the canal implies destroying mangrove, an important habitat for fishes within and outside the bay [23].

The increase in abundance between decades specific to the polyhaline zone, as well as the increase of the guilds of medium-sized piscivores and small planktivores, and the group of marine euryhaline fishes, were in line with our expectations, given the expansion of Zaragoza Canal and its interaction with other factors (discussed below). When an artificial channel was opened in the El Carmen-Machona coastal lagoon system in the southern Gulf of Mexico, the composition of the estuary included more marine and fewer estuarine species over the course of one decade [24].

Large schools of the sardine *Harengula jaguana*, a planktivore that we classified as "small" because we captured mostly juveniles, occurred in zone P in 2015–2018 apparently as often as they do in the adjacent coast, usually over seagrass meadows (pers. obs.), whereas in former decades they were more abundant in zones O and M (Table 1, Appendix A). In addition, the oligohaline zone was the most diverse area in 1999–2001, but the mesohaline zone was most diverse in 2015–2018, also shifting dominance towards the seaward opening of the system. Notwithstanding, no general change in composition or diversity was apparent, and several trends that may cause concern were detected, e.g., decreases in total abundance, mean local richness, and number of guilds.

Excessive illegal fishing outside the bay may be one factor that helps explain the decrease in juveniles of large piscivores. The negative trend for this guild exists as well in the reefs adjacent to Chetumal Bay [21]. The probable shift in dominance from *E. plumieri* to *G. cinereus* may also reflect the fact that the former is preferred as a fishery resource in the bay [4].

The increased size of *E. gula*, and perhaps also *E. argenteus*, especially in zone O, could be a by-product of the decline in abundance of other benthivores in that area, possibly because of less competition. In contrast, *S. notata* became smaller in length in zone P, where diversity increased. Body size strongly influences trophic level and habitat use of fishes [25].

The changes in zone P may reflect the salinity increase in Chetumal Bay during the last decade, a process that had been noted already by other researchers [26]. However, the reason for this salinization is not only the expansion of Zaragoza Canal: There is a general trend in the region for the dry season to be longer, thus increasing evaporation and decrease freshwater input from rains (Carrillo, pers. comm.). As for the lower local water temperature in the face of increasing regional heat [27], the increased wind force may offer a straightforward explanation, although an interacting factor could be the canal expansion itself, i.e., greater tidal movement of water, fewer shallow sites with slow-moving water susceptible to heating.

The differences in freshwater and estuarine species are more difficult to explain, except for the catfish *A. assimilis*, which in 1996 suffered a massive mortality that was attributed to pollution and specific pathogens [28]; the species seemingly has not recovered ever since. The interdecadal decrease for the guild of small benthivores, many of them estuarine, especially strong in zone M, is not explainable by predation, because medium-sized and large piscivores also declined. We speculate that this decline could be accounted for, at least in zone O, by the loss of habitat due to the continued erosion of the western coast of the bay. This process that has been ongoing for centuries, as attested by

Classic Mayan fishery structures that should have been in contact to the shore, but now are several meters away from the coast [29].

The man-made opening of seaward channels strongly alters benthic communities of plants and invertebrates in lagoons [30] by promoting the entrance of marine organisms, including stony corals [2], into Chetumal Bay. This obviously affects herbivore and benthivore species, also accounting for the spread of the invasive lionfish [31]. The zooplankton can be affected as well, especially if the salinity and the trophic state of the estuary change [32]. However, as stated above, we are cautious to ascribe this change solely to the expansion of the canal, given the mentioned regional trend towards less precipitation in the region, related to global climate change.

On the other hand, and although we did not detect any interdecadal difference in beta-diversity, the increased abundance in zone M of freshwater species, even primary, and of marine species in zone O, may signal a faunistic homogenization of the bay. In fact, part of our original rationale for defining these three areas was to follow approximately the isolines of 9 psu, upper salinity tolerance of the primary freshwater characid *A. bacalarensis* and lower limit for juveniles of the marine euryhaline snapper *L. apodus*, and 19 psu, upper bound for the secondary freshwater molly *P. mexicana* [33], and yet, in 2015–2018, many of these species were recorded outside their preferred salinity area.

As observed by Rahel [34], "[h]abitat and flow homogenization are major drivers of biotic homogenization." At a planetary level, biotic homogenization implies invasion by nonnative species [35], a process enhanced by global warming [36]. Locally, however, impact factors, such as hurricanes, can also make the fauna more homogeneous [37]. In any case, it is clear that the canal expansion is not an isolated factor for this process.

The expansion of the Zaragoza Canal may be ethically ambiguous. On the one hand, it favors the entry of invaders and could induce faunal homogenization, as well as the probable loss of such important guilds as large piscivores. On the other hand, making the opening wider and deeper might play a positive role for the seasonal migratory movements of bonefish [12], barracuda [23], and other species between the Caribbean sea and the bay. However, if habitat destruction is not controlled, especially the flats and mangrove cays that are favorite feeding grounds for bonefish and permit, the damage would be both ecological and socioeconomical, by impinging detrimentally on such species that support an important fishery in Belize and Mexico [3].

In spite of the methodological limitations of this study, and perhaps the idiosyncratic nature of Chetumal Bay and its artificial canal, our findings can be a useful reference for similar sites elsewhere. For example, coastal works in Spain's Mar Menor [38] have brought positive changes (increased benthic biodiversity due to greater availability of hard bottoms), but also negative ones (altered sediment quality and vegetation cover), and canals are globally a major pathway for biological invasions [39]. Examining a variety of outcomes for comparable situations should be necessary for environmental managers and ecologists alike.

Author Contributions: Conceptualization, J.J.S.-S.; methodology, J.J.S.-S. and R.L.H.-P.; software, J.J.S.-S.; validation, J.J.S.-S. and R.L.H.-P.; formal analysis, J.J.S.-S.; investigation, J.J.S.-S. and R.L.H.-P.; resources, J.J.S.-S. and R.L.H.-P.; data curation, J.J.S.-S.; writing—original draft preparation, J.J.S.-S.; writing—review and editing, J.J.S.-S. and R.L.H.-P.; visualization, J.J.S.-S.; supervision, J.J.S.-S.; project administration, J.J.S.-S.; funding acquisition, J.J.S.-S.

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Appendix A

Quantitative sampling: Fish species of Chetumal Bay by salinity zone (Oligohaline, Mesohaline, Polyhaline) and decade. Figures are mean abundances (CPUE).

Species Zone	1999–2001	2015–2018
Anchoa cayorum	0.5	0.5
O	0	0.5
P	0.5	0
Achirus lineatus	68.3	13.4
M	45.4	2.0
O	19.0	9.7
P	3.8	1.7
Albula vulpes	3.5	0
P	3.5	0
Anchoa colonensis	0.3	0
M	0.3	0
Anchoa cubana	19.2	10.0
M	3.0	0
O	11.7	0
P	4.5	10.0
Ariopsis assimilis	2.3	0
M	1.0	0
O	1.3	0
Astyanax bacalarensis	0	45.2
M	0	38.0
O	0	1.2
P	0	6.0
Atherinomorus stipes	2306.3	590.7
M	1542.2	90.8
O	167.7	0.3
P	596.5	499.5
Bairdiella ronchus	43.3	2.0
M	21.5	0
O	21.3	0
P	0.5	2
Bathygobius curacao	0	1.2
M	0	1.2
Bothus ocellatus	0.3	0
P	0.3	0
Canthigaster rostrata	0	0.3
P	0	0.3
Centropomus undecimalis	1.0	0
M	1.0	0
Chriodorus atherinoides	9.8	2.5
M	8.2	0
P	1.7	2.5
Corvula sanctaeluciae	0	0.7
P	0	0.7
Cyprinodon artifrons	45.2	6.0
M	1.5	0.7
O	0	1.5
P	43.7	3.8

Species Zone	1999–2001	2015–2018
Diapterus auratus	0	23.3
P	0	23.3
Eucinostomus argenteus	34.2	63.6
M	9.5	3.3
O	4.5	0.5
P	20.2	59.8
Eucinostomus gula	269.8	53.8
M	38.8	12.3
O	9.5	5.0
P	221.5	36.5
Eucinostomus harengulus	15.7	30.7
M	3.3	11.2
O	0.3	1.0
P	12	18.5
Eucinostomus jonesii	257.5	72.8
M	155.3	13.8
O	45.3	34.1
P	56.8	24.8
Eucinostomus melanopterus	0	1.7
M	0	0.3
P	0	1.3
Eugerres plumieri	78.4	7.8
M	23.6	1.0
O	52.3	2.9
P	2.5	3.8
Floridichthys polyommus	133.4	266.7
M	78.3	34.4
O	12.5	5.5
P	42.7	226.8
Gerres cinereus	1.8	31.2
M	1	4.3
O	0	20.3
P	0.8	6.5
Gobiosoma yucatanum	1.5	0
O	1.5	0
Gambusia sexradiata	0.5	0
O	0.5	0
Gambusia yucatana	9.8	1.5
M	8.5	1.0
P	1.3	0.5
Harengula clupeola	9	30
P	9	30
Harengula humeralis	6	0.3
P	6	0.3
Harengula jaguana	174.7	149.0
M	37.3	1.0
O	74.3	0
P	63.0	148.0
Hippocampus erectus	0.3	0
P	0.3	0
Hypanus americanus	0	0.3
M	0	0.3

Species Zone	1999–2001	2015–2018
Hyporhamphus roberti	1.5	0.25
M	0	0.25
O	1.5	0
Jenkinsia lamprotaenia	2.5	0
P	2.5	0
Jordanella pulchra	8.0	8.9
M	8.0	2.0
O	0	6.25
P	0	0.7
Lophogobius cyprinoides	13.8	2.8
M	7.3	0
O	6.5	2.8
Lutjanus griseus	0	13.3
P	0	13.3
Mayaheros urophthalmus	4.2	1.5
M	4.2	0
O	0	1.5
Monacanthus tuckeri	0	0.3
M	0	0.3
Oligoplites saurus	1.2	0.3
M	0.7	0
P	0.5	0.3
Oostethus lineatus	0	0.3
M	0	0.3
Opisthonema oglinum	9.8	0
O	5	0
P	4.8	0
Opsanus beta	1.7	0
O	1.7	0
Paraclinus fasciatus	0.5	0
O	0.5	0
Poecilia mexicana	20.2	0.4
M	19.2	0
O	2.0	0.4
Sphoeroides testudineus	36.0	47.8
M	6.0	27.4
O	25.2	11.7
P	4.8	8.7
Sphyraena barracuda	24.8	12.0
M	8.8	4.6
O	5.2	3.5
P	10.8	4.0
Strongylura marina	0.5	0
P	0.5	0
Strongylura notata	51.9	150.5
M	25.3	27.2
O	5.8	1.5
P	20.8	121.8
Strongylura timucu	1.5	0
M	1.5	0
Syngnathus floridae	0.25	0
M	0.25	0

Species Zone	1999–2001	2015–2018
Syngnathus scovelli	0.5	0
O	0.5	0
Trachinotus falcatus	0	1.2
M	0	0.3
O	0	0.5
P	0	0.3
Trichromis salvini	0	3.5
O	0	3.5
Vieja melanurus	2.0	0
O	2.0	0

Appendix B

Qualitative records: Fish species of Chetumal Bay by salinity zone (Oligohaline, Mesohaline, Polyhaline) and decade (before 2005 vs. 2006 up to now). Voucher numbers (at ECO-CH, except one from the Colección Nacional de Peces, acronym CNPE) or literature sources (in *italics*) given, when the species was not recorded in this study. Records not assignable with precision to one of the three salinity zones, or known only from ichthyoplankton, not considered.

Species	O	Before 2005 M	P	O	2006 up to Now M	P
Acanthurus bahianus			4222			
Achirus lineatus	X	X	X	X	X	X
Aetobatus narinari			pers. obs.			
Albula vulpes	4221		X		[12]	[12]
Anchoa cayorum			X	X		
A. colonensis		X				
A. cubana	X	X	X			X
Archosargus probatocephalus	4219					
A. rhomboidalis	4217					
Ariopsis assimilis	X	X	4261			
Astyanax bacalarensis	7815	1301		X	X	X
Atherinomorus stipes	X	X	X	X	X	X
Bagre marinus		3143				
Bairdiella ronchus	X	X	X			X
Bathygobius curacao					X	
Bothus ocellatus			X			
Canthigaster rostrata						X
Caranx latus	4214				X	
Centropomus undecimalis	4216	X				
Chaetodipterus faber		4213				
Chilomycterus schopfi		4047				
Chriodorus atherinoides		X	X	8020		X
Conodon nobilis			CNPE 3221			
Corvula sanctaeluciae		1703				X
Cribroheros robertsoni	X					
Cyprinodon artifrons	X		X	X	X	X
D. guttata		4194				

Species	O	Before 2005 M	P	O	2006 up to Now M	P
Diapterus auratus						X
Echeneis neucratoides		2785	X			
Elops saurus			[38]			
Epinephelus itajara		pers. obs.	pers. obs.			
Eucinostomus argenteus	X	X	X	X	X	X
E. gula	X	X	X	X	X	X
E. harengulus	X	X	X	X	X	X
E. jonesii	X	X	X	X	X	X
E. melanopterus					X	X
Eugerres plumieri	X	X	X	X	X	X
Floridichthys polyommus	X	X	X	X	X	X
Gambusia sexradiata	X					
G. yucatana		X	X		X	X
Gerres cinereus		X	X	X	X	X
Gobiosoma yucatanum	X					
Gymnothorax funebris						pers. obs.
Haemulon sciurus			pers. obs.			
Harengula clupeola		2061	X			X
H. humeralis			X	7806		X
H. jaguana	X	X	X		X	X
Hippocampus erectus			X			
Holocentrus rufus		4224				
Hypanus americanus					X	
Hyporhamphus roberti	X				X	
Jenkinsia lamprotaenia			X			
Jordanella pulchra		X		X	X	X
Kyphosus incisor						pers. obs.
Lachnolaimus maximus						pers. obs.
Lobotes surinamensis	4218					
Lophogobius cyprinoides	X	X		X		
Lupinoblennius vinctus	4134					
Lutjanus analis			[38]			
L. apodus			4236			
L. cyanopterus			pers. obs.			
L. griseus			4384			X
L. jocu						[39]
L. mahogoni			pers. obs.			
L. synagris			X			
Mayaheros urophthalmus	X	X		X		
Megalops atlanticus		3139				4381
Monacanthus tuckeri					7838	
Mugil cephalus		pers. obs.				
Narcine brasiliensis						pers. obs.
Ocyurus chrysurus						[39]
Oligoplites saurus		X	X			X
Oostethus lineatus					X	
Opisthonema oglinum	X		X			
Opsanus beta	X					
Paraclinus fasciatus	X	3169				
Poecilia kykesis		1246				
P. mexicana	X	X		X		
Pristis sp.	pers. obs.		pers. obs.			

Species	O	Before 2005 M	P	O	2006 up to Now M	P
Pterois volitans						pers. obs.
Scomberomorus maculatus		4768			pers. obs.	
Selene vomer		4215				
Sparisoma viride		pers. obs.				
Sphoeroides testudineus	X	X	X	X	X	X
Sphyraena barracuda	X	X	X	X	X	X
Strongylura marina			X			
S. notata	X	X	X	X	X	X
S. timucu	5063	X		8025		
Styracura schmardae			3799			
Symphurus diomedeanus	4137					
Syngnathus floridae		X				
S. scovelli	X					
Trachinotus falcatus				X	X	X
Trichromis salvini				X		
Vieja melanurus	X	4302				

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Article

Hepatic Steatosis in a Bullhead (*Cottus gobio*) Population from a High-Mountain Lake (Carnic Alps): Adaptation to an Extreme Ecosystem?

Paolo Pastorino [1,2,*], Marino Prearo [2], Elisabetta Pizzul [1], Marco Bertoli [1], Danila Raffaella Francese [2], Vasco Menconi [2], Davide Mugetti [2], Elena Bozzetta [2] and Katia Varello [2]

[1] Department of Life Sciences, University of Trieste, via Giorgieri 10, 34127 Trieste, Italy; pizzul@units.it (E.P.); marco.ber3@gmail.com (M.B.)
[2] Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Via Bologna 148, 10154 Torino, Italy; marino.prearo@izsto.it (M.P.); danilaraffaella.francese@izsto.it (D.R.F.); vasco.menconi@izsto.it (V.M.); davide.mugetti@izsto.it (D.M.); elena.bozzetta@izsto.it (E.B.); katia.varello@izsto.it (K.V.)
* Correspondence: paolo.pastorino@izsto.it; Tel.: +39-011-268-6295

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Abstract: The aim of this study was to describe liver alterations observed in a bullhead (*Cottus gobio*) population from a high-mountain lake (Dimon Lake; 1857 m a.s.l.) located in Carnic Alps (Northeast Italy). Two fish sampling campaigns (summer and autumn) were performed in 2017 to explore the possible causes of this phenomenon. In addition, to investigate the typical liver features of *C. gobio*, control specimens were captured from Degano Creek in the same seasons. Total length, weight, and liver weight were recorded in fish from both sampling sites. In addition, Fulton's condition factor (K) and hepatosomatic index (HSI) were calculated. Stomach contents were also analyzed. Liver from each specimen was sampled, and histological examination was carried out. Liver steatosis (L) (nuclear displacement and cytoplasm vacuolization) were histologically evaluated by assigning a semiquantitative severity score. A significant difference in L was found between Dimon Lake and Degano Creek in both summer (Mann–Whitney test; $p = 0.0001$) and autumn (Mann–Whitney test; $p < 0.0001$). Regarding HSI, a significant difference was also recorded between Dimon Lake and Degano Creek in summer (Mann–Whitney test; $p < 0.0001$) and also in autumn (Mann–Whitney test; $p < 0.0001$), but no seasonal change in K values was recorded between the two sites in both summer (Mann–Whitney test; $p = 0.8589$) and autumn (Mann–Whitney test; $p = 0.6415$). A significant positive correlation between HSI and L was found (ρS 0.573). The causes of this abnormality might be related to adaptation by the fish to the high-altitude environment, accumulating lipids in the liver to tackle the adverse season, which lasts for 6 months of the year. Nonetheless, our study does not preclude steatosis induced by a nearly exclusive chironomids diet in bullhead from Dimon Lake.

Keywords: adaptation; alpine lakes; *Cottus gobio*; hepatic steatosis

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1. Introduction

Alpine lakes are extreme ecosystems with harsh climatic conditions. Though located in remote areas, they may be affected by local anthropogenic impacts, such as fish introduction. Most alpine lakes were, in fact, originally fishless, isolated ecosystems. Owing to the relatively small number of native species, the lakes have low resilience to disturbances and can be especially sensitive to the introduction of alien fish for recreational fishing. During the greater part of the year, snow and ice cover prevents sunlight from penetrating the lake water column [1]. Without the penetration of light, photosynthesis is not possible, and the lake waters remain in a long period of darkness [2], becoming a heterotrophic system isolated from the surrounding area. In early summer, when the snow melts, the lakes quickly shift from extremely low to extremely high solar irradiance correlated with altitude. Low oxygen availability and low temperatures make life at such altitudes challenging, though many species have adapted successfully via physiological changes. Unlike short-term acclimatization (immediate physiological response to a changing environment), high-altitude adaptation means irreversible, evolved physiological responses to high-altitude environments, associated with behavioral and genetic changes.

Published data on fish adaptation to high-altitude environments are scarce. For example, some studies have focused on metabolic rate, as reported in highland westslope cutthroat trout (*Oncorhynchus clarkii*) as compared with introduced lowland rainbow trout (*Oncorhynchus mykiss*) in the Oldman River basin (Canada): Fish at high altitudes have a lower metabolic rate [3]. Kang and co-workers [4] investigated the genetic mechanism of high-altitude adaptation in *Creteuchiloglanis macropterus* (Siluriformes) and found that many genes were involved in energy metabolism and hypoxia, which might be an important resource for studying adaptations to a high-altitude environment. Li and co-workers [5] characterized the mitochondrial genomes of two species ("morphologically primitive" clade and "morphologically specialized" clade) of schizothoracine fishes. They reported evidence for positive selection acting on the protein-encoding genes in the mitochondrial genomes of the "morphologically specialized" clade, implying a possible genetic basis for high-altitude adaptation in this derived lineage of cypriniform fish.

Other studies on macrobenthic invertebrates in streams have showed a decrease in species richness with increasing altitude due to lower oxygen saturation rather than low temperature [6]. These factors may reduce productivity in high-altitude ecosystems, meaning there will be less energy available for consumption, growth, and activity, which provides a survival advantage to fish with lower metabolic demands. During 2017, two fish sampling campaigns were conducted in a high-mountain lake (Dimon Lake) located in the Carnic Alps (northeast Italy). Liver examination revealed alterations in specimens of the bullhead (*Cottus gobio*). The aim of this study was to describe the liver alterations observed in *C. gobio* and discuss the possible causes of this abnormality.

2. Materials and Methods

2.1. Study Sites

The main study site was Dimon Lake (46°34′05.4″ N; 13°03′45.8″ E), a high-mountain lake located in the Carnic Alps (municipality of Ligosullo, Udine Province, Friuli Venezia-Giulia Region, northeast Italy) at 1857 m a.s.l. The Carnic Alps are among the most remote areas in Italy. Anthropic impacts are very limited, except for pasturing activity. Dimon Lake is a typical glacial-origin lake, classified as a Site of Community Interest and Special Areas of Conservation (SCI/SAC-IT3320002 "Monti Dimon e Paularo"). Originally a fishless lake, fish were introduced for recreational fishing in the past [7]. The lake lies on sandstone and volcanic rocks; it measures 376 m in perimeter, 0.6 ha in surface area, and has a maximum depth of 4.27 m. For the purposes of comparison in this study, *C. gobio* individuals were captured in Degano Creek (46°26′21.9″ N; 12°52′43.6″ E), a sub-alpine watercourse near the municipality of Raveo (520 m a.s.l.), about 40 km from Dimon Lake, with scarce human impacts (Figure 1). The comparison of lake vs. stream populations was dictated by the absence of *C. gobio* in other high-altitude lakes in northeast Italy.

Figure 1. Location of fish sampling sites in Friuli Venezia-Giulia, Italy. Degano Creek (pictured on the left) and Dimon Lake (pictured on the right).

2.2. *Fish Sampling*

In 2017, two fish sampling campaigns (July and October) were performed. These two months were chosen because of accessibility, which is usually limited by snowfall from early November to June. Fish were sampled using an electrofishing boat in both the littoral and the deep zones (maximum depth 4.27 m). The fish were identified and measured for length (TL; cm) and weight (W; g). Fulton's condition factor (K) was also calculated for each specimen. The K factor was derived from the following formula [8]:

$$K = (W/TL^3) \times 100,$$

where W is the weight (g) and TL is the total length of the fish (cm). A total of 35 fish (15 in the summer and 20 in the autumn campaign) were sampled, euthanized with an overdose of tricaine methanesulfonate MS-222 (50 mg kg^{-1}), and then necropsied. The liver from each specimen was weighted to calculate the hepatosomatic index (HSI) using the formula [9]:

$$HSI\ (\%) = (LW/FW) \times 100,$$

where LW is the liver weight (g) and FW is the whole weight of the fish (g). Liver was sampled for histological evaluation. Only larger fish were selected in order to have enough material for all histological analyses (see Section 2.4). Moreover, as control fish, we also performed two sampling campaigns in the same periods (July and October) using electrofishing in Degano Creek. Only 10 individuals both for summer and autumn were captured to avoid putting pressure on the wild native population. TL, W, and LW were determined and HSI and K were calculated. Fish sampling campaigns were performed by permission of Ente Tutela Patrimonio Ittico del Friuli Venezia-Giulia (authorization n. 11/DIR/17/01/2017).

2.3. *Analysis of Stomach Contents*

In both sampling campaigns the stomach contents of the fish from Dimon Lake were analyzed to obtain more detailed information about their diet. The stomachs were preserved in alcohol 70% and the content was identified by optical microscope (Olympus BX40, Olympus, Tokyo, Japan) and stereomicroscope (Zeiss Stemis V8, Zeiss, Shangkeheng, Germany). An attempt was made to identify the ingested prey to the lowest recognizable taxa, but accurate identification was often not possible due to digestion of the prey. Stomach content analysis was also performed on the fish from Degano Creek. In order to describe the diet, the prey frequency of occurrence (Fi) [10] was calculated for both seasons.

2.4. Histological Evaluation

A total of 35 liver samples were partly fixed in 10% neutral buffered formalin and partly frozen. The fixed tissues were dehydrated in a gradual series of alcohols and embedded in paraffin. The paraffin blocks were cut into 4 ± 2 mm sections and stained with hematoxylin and eosin (HE) for microscopic evaluation. Histological changes (nuclear displacement and cytoplasm vacuolization) to define liver steatosis were evaluated and a semiquantitative severity score was assigned: 0—not observed; 1—mild; 2—moderate; 3—severe [11]. Histochemical stains, Periodic-acid Schiff (PAS) to detect mucopolysaccharides and Sudan III to detect lipids, were also performed [12]. Slides were evaluated microscopically at increasing magnification (10×, 20×, 40×) on a Zeiss Axio Scope.A1 microscope. Samples were considered positive for the presence of red-purple glycogen or orange lipidic deposits in hepatocyte cytoplasm for PAS and Sudan III stain, respectively.

2.5. Statistical Analysis

The Wilcoxon test was performed to compare (a) the liver steatosis score (L), (b) the hepatosomatic index (HSI), and (c) the K values between seasons in Dimon Lake and in Degano Creek. Mann–Whitney U tests were used for pairwise comparison of L, HSI, and K values of *C. gobio* in Dimon Lake vs. Degano Creek in both summer and autumn. Spearman's rank correlation coefficient (ρS) was used to test correlations between L, W, HIS, and K. Differences were considered significant with a *p* value <0.05. All statistical analyses were performed using GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Fish Sampling and Anatomopathological Features

Values of TL, W, K, LW, and HIS for *C. gobio* captured in Dimon Lake and Degano Creek are reported in Tables 1 and 2, respectively.

Regarding Dimon Lake, the average TL for *C. gobio* captured during the summer campaign was 13.88 ± 1.12 cm, with an average W of 31.20 ± 7.54 g. The average TL of individuals captured during autumn was 13.25 ± 1.37 with an average W of 26.70 ± 6.89 g. K values ranged between 1.15 ± 0.12 in summer and 1.14 ± 0.15 in autumn. LW ranged between 0.85 ± 0.14 in summer and 0.82 ± 0.24 in autumn. HSI ranged between 2.80 ± 0.38 in summer and 3.08 ± 0.39 in autumn (Table 3). At necropsy, the livers appeared variably pale to yellowish in color and friable in consistency; no visible lesions in the other visceral organs were observed (Figure 2).

In Degano Creek, the average TL of individuals captured during summer was 14.24 ± 0.86 cm with an average W of 33.60 ± 6.15 g. Instead, during autumn, individuals showed a TL of 12.36 ± 1.76 cm with a W of 23.10 ± 9.04 g. K values ranged between 1.16 ± 0.14 in summer and 1.17 ± 0.14 in autumn. LW ranged between 0.54 ± 0.12 in summer and 0.36 ± 0.14 in autumn. HIS ranged between 1.60 ± 0.15 in summer and 1.60 ± 0.10 in autumn (Table 3). No lesions in organs were observed, liver included (Figure 3).

Statistical analysis showed no seasonal differences in the liver steatosis score in Dimon Lake (Wilcoxon test; $p = 0.6719$), but a significant difference was found between Dimon Lake and Degano Creek in both summer (Mann–Whitney test; $p = 0.0001$) and autumn (Mann–Whitney test; $p < 0.0001$) (Table 3). Regarding HSI, no seasonal differences were observed in Dimon Lake (Wilcoxon test; $p = 0.1173$), but a significant difference were recorded between Dimon Lake and Degano Creek in summer (Mann–Whitney test; $p < 0.0001$) and also in autumn (Mann–Whitney test; $p < 0.0001$) (Table 3). No seasonal change in K values was recorded in Dimon Lake (Wilcoxon test; $p = 0.5696$), not even between Dimon Lake and Degano Creek in both summer (Mann–Whitney test; $p = 0.8589$) and autumn (Mann–Whitney test; $p = 0.6415$). A significant positive correlation between HIS and L was found (ρS 0.573).

Table 1. Total length (TL), weight (W), liver weight (LW), hepatosomatic index (HSI), Fulton's condition factor (K), and liver steatosis score (L) of *Cottus gobio* (N) captured during summer and autumn in Dimon Lake.

Season	N	TL (cm)	W (g)	LW (g)	HSI	K	L
Summer (July)	1	14.0	29	0.803	2.77	1.06	2
	2	15.3	38	0.927	2.44	1.06	1
	3	15.3	37	0.925	2.50	1.03	2
	4	14.9	29	0.913	3.15	0.88	2
	5	13.1	27	0.867	3.21	1.20	3
	6	15.4	46	1.090	2.37	1.26	0
	7	14.5	41	0.914	2.23	1.34	2
	8	13.5	32	0.877	2.74	1.30	3
	9	13.2	26	0.874	3.36	1.13	3
	10	15.0	39	0.905	2.32	1.16	2
	11	13.4	31	0.964	3.11	1.29	3
	12	13.5	27	0.877	3.25	1.10	2
	13	12.2	20	0.594	2.97	1.10	3
	14	12.5	25	0.767	3.07	1.28	3
	15	12.4	21	0.540	2.57	1.10	1
Autumn (October)	16	14.3	37	1.424	3.85	1.27	3
	17	12.3	19	0.507	2.67	1.02	0
	18	14.5	37	0.884	2.39	1.21	3
	19	12.4	26	0.946	3.64	1.36	2
	20	12.0	17	0.530	3.12	0.98	3
	21	14.5	31	0.973	3.14	1.02	3
	22	15.5	33	0.861	2.61	0.89	2
	23	15.0	32	0.896	2.80	0.95	1
	24	15.5	39	1.197	3.07	1.05	3
	25	13.5	23	0.655	2.85	0.93	3
	26	14.5	34	1.231	3.62	1.12	3
	27	13.0	26	0.738	2.84	1.18	2
	28	13.5	28	0.717	2.56	1.14	2
	29	11.0	20	0.578	2.89	1.50	2
	30	12.5	24	0.838	3.49	1.23	3
	31	13.5	27	0.896	3.32	1.10	3
	32	11.8	19	0.606	3.19	1.16	3
	33	12.0	20	0.674	3.37	1.16	3
	34	11.7	19	0.589	3.10	1.19	3
	35	12.0	23	0.718	3.12	1.33	3

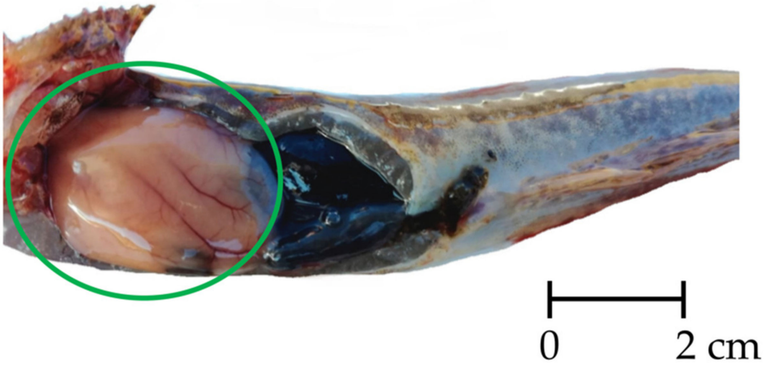

Figure 2. Macroscopical aspect of liver from *Cottus gobio* captured in Dimon Lake during autumn 2017. It appears pale to yellowish in color and friable in consistence (green circle).

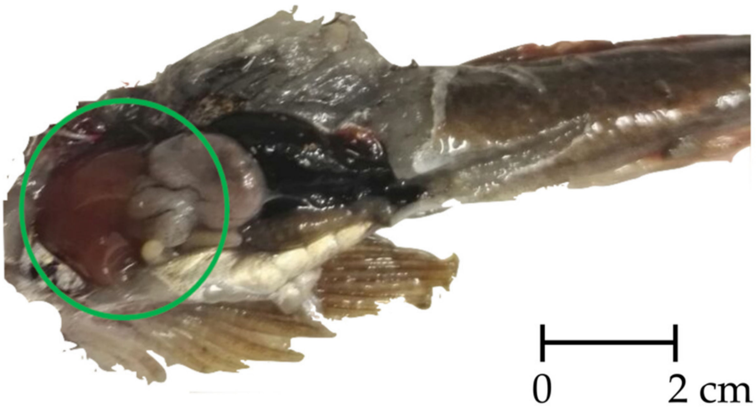

Figure 3. Macroscopical aspect of normal liver (green circle) of *Cottus gobio* captured in Degano Creek during autumn 2017.

Table 2. Total length (TL), weight (W), liver weight (LW), hepatosomatic index (HSI), Fulton's condition factor (K), and liver steatosis score (L) in individuals of *Cottus gobio* (N) captured during summer and autumn in Degano Creek.

Season	N	TL (cm)	W (g)	LW (g)	HSI	K	L
Summer (July)	1	13.5	27	0.407	1.509	1.10	0
	2	14.2	40	0.768	1.920	1.40	1
	3	15.4	38	0.566	1.490	1.04	0
	4	14.9	31	0.478	1.543	0.94	0
	5	15.1	37	0.661	1.789	1.07	1
	6	13.1	25	0.390	1.560	1.11	1
	7	14.5	41	0.598	1.458	1.34	0
	8	13.5	32	0.530	1.657	1.30	0
	9	13.2	26	0.429	1.652	1.13	1
	10	15.0	39	0.575	1.475	1.16	0
Autumn (October)	11	11.2	16	0.282	1.766	1.14	0
	12	13.2	26	0.402	1.547	1.13	0
	13	10.7	15	0.244	1.631	1.18	0
	14	14.1	38	0.622	1.639	1.36	1
	15	13.0	24	0.416	1.735	1.09	0
	16	10.6	14	0.216	1.542	1.14	0
	17	10.7	16	0.253	1.584	1.29	1
	18	14.4	28	0.462	1.651	0.93	0
	19	10.7	17	0.251	1.479	1.39	1
	20	15.0	37	0.532	1.437	1.10	0

Table 3. Mean and standard deviation of total length (TL), weight (W), liver weight (LW), hepatosomatic index (HSI), Fulton's condition factor (K) and median values of liver steatosis score (L) in individuals of *Cottus gobio* (N) captured during summer and autumn in Dimon Lake and Degano Creek. Lowercase letters indicate statistically significant differences between sites in summer (a) and autumn (b) ($p < 0.05$).

Site	Season	N	TL (cm)	W (g)	K	LW (g)	HSI	L
Dimon	Summer	15	13.88 ± 1.12	31.20 ± 7.54	1.15 ± 0.12	0.85 ± 0.14	2.80 ± 0.38 ^{a}	2 ^{a}
	Autumn	20	13.25 ± 1.37	26.70 ± 6.89	1.14 ± 0.15	0.82 ± 0.24	3.08 ± 0.39 ^{b}	3 ^{b}
Degano	Summer	10	14.24 ± 0.86	33.6 ± 6.15	1.16 ± 0.14	0.54 ± 0.12	1.60 ± 0.15 ^{a}	0 ^{a}
	Autumn	10	12.36 ± 1.76	23.1 ± 9.04	1.17 ± 0.14	0.36 ± 0.14	1.60 ± 0.10 ^{b}	0 ^{b}

3.2. Stomach Contents Analysis

The analysis of the stomach contents showed that *C. gobio* from Dimon Lake fed chiefly on Chironomidae larvae in both summer (96.18%) and autumn (98.30%). Other taxa were present in extremely low proportions (Chironomidae pupae: 3.18% in summer and 0.79% in autumn; Tricoptera: 0.64% in summer and 0.39% in autumn; Tricoptera Limnephilidae: 0% in summer and 0.52% in autumn). Stomach content analysis of the Degano Creek fish revealed Tricoptera (42.63% in summer; 34.03% in autumn), Ephemeroptera (7.5% in summer; 5.8% in autumn), and Plecoptera (4.1% in summer and 2.5% in autumn).

3.3. Histological Analysis

Histology of the liver samples of *C. gobio* from Dimon Lake showed a range of cytoplasm vacuolization of hepatocytes from mild (score 1) to severe (score 3) in 33 specimens, with multifocal to diffuse localization (Figure 4). Only two specimens showed no hepatic lesions (score 0). Nuclear displacement and cytoplasm vacuolization are depicted in Figure 5. Table 1 presents the liver steatosis score (L) for each specimen examined. Table 3 presents median values of L for both seasons.

Sudan III was positive in all the samples with a high vacuolization score (2–3) (Figure 6a). PAS stain positivity was inversely proportional to the histological score (Figure 6b). Fish with grade 3 showed only rare residual glycogen deposits in hepatocytes.

Finally, individuals used as the control and sampled from Degano Creek showed no hepatocyte vacuolization (score 0; Figure 4a) in both seasons, except a few cases with a score of 1. Median values of L are also reported in Table 3.

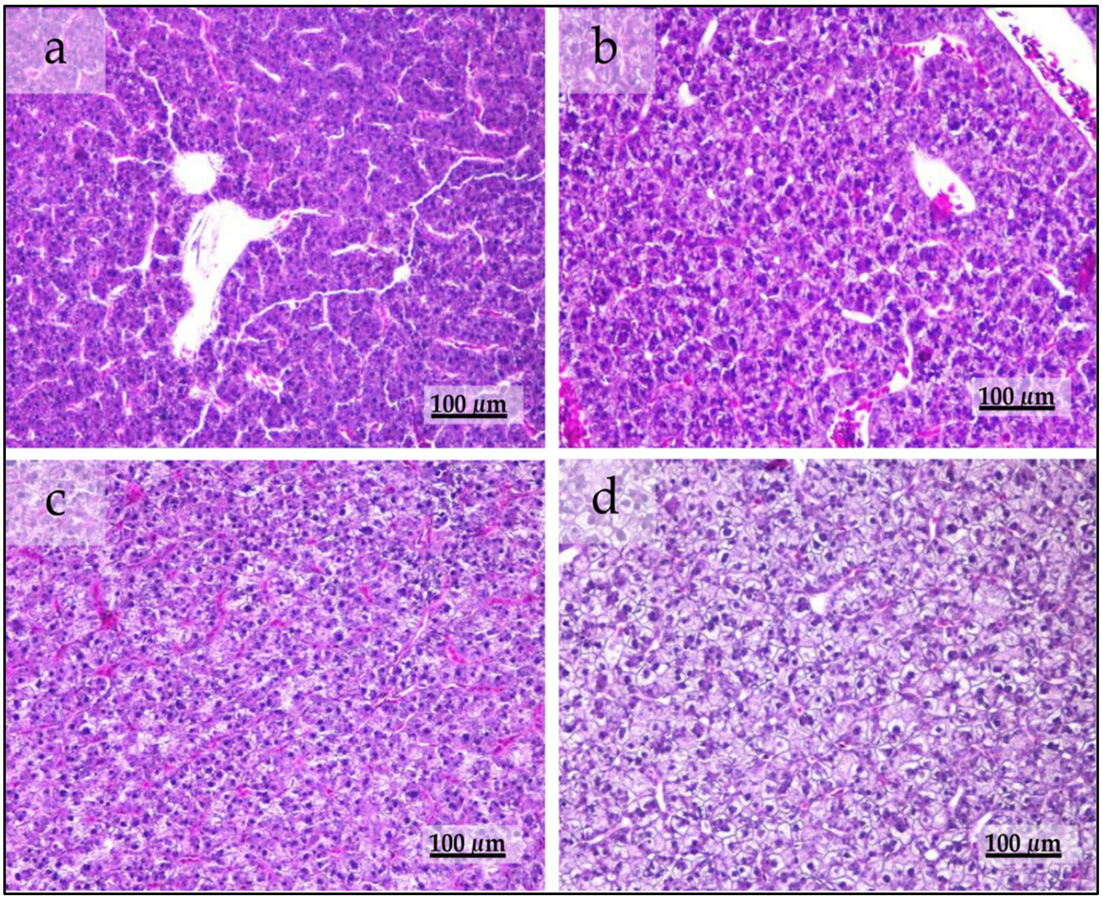

Figure 4. Liver sections of *Cottus gobio* (HE): (**a**) no vacuolization observed in hepatocytes (control); (**b**) mild vacuolization; (**c**) moderate vacuolization; (**d**) severe, diffuse vacuolization.

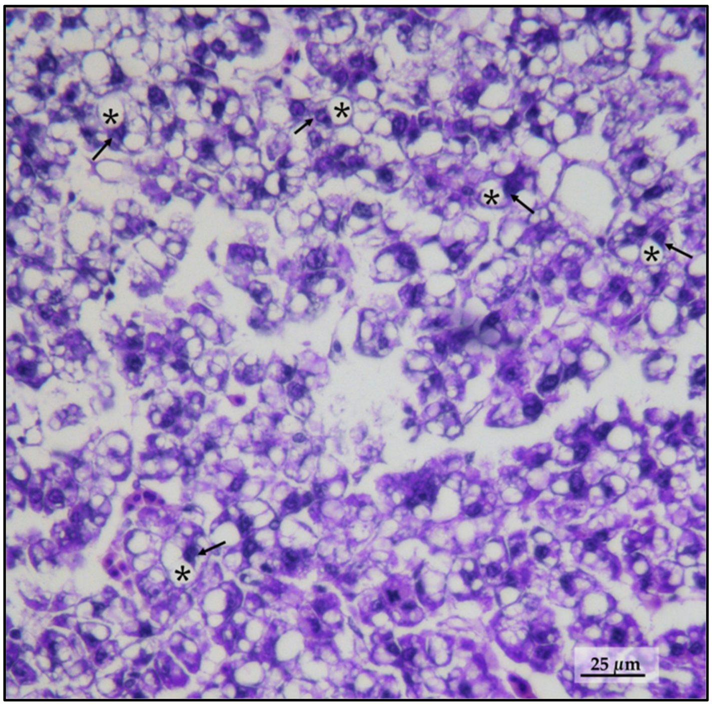

Figure 5. Liver section of *Cottus gobio* (HE) from Dimon Lake: detail of Figure 4d with nuclear displacement (black arrow) and cytoplasm vacuolization (asterisk).

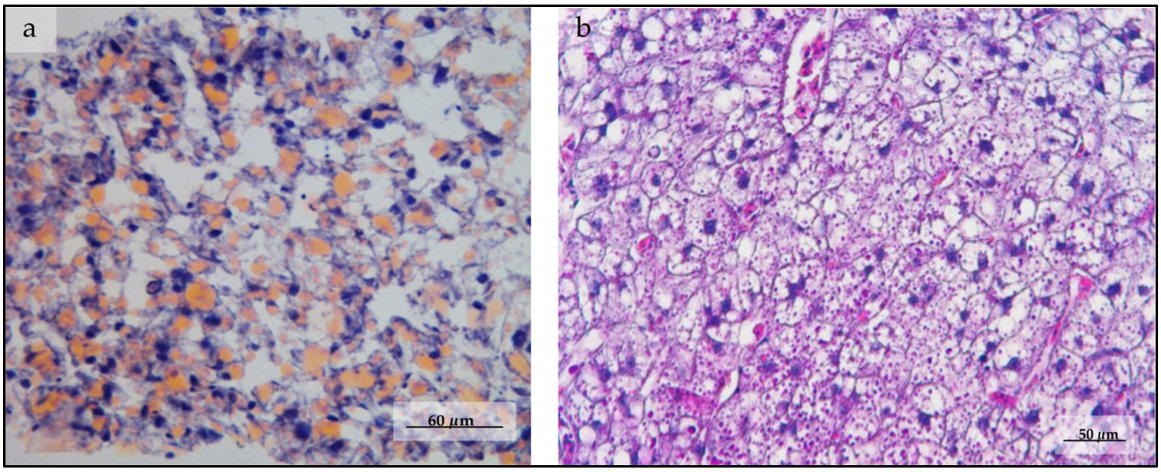

Figure 6. Liver sections of *Cottus gobio* from Dimon Lake. (**a**) Sudan III stain; orange lipidic deposits in hepatocyte cytoplasm of a subject with a high vacuolization score (3); (**b**) Periodic-acid Schiff (PAS) stain: red-purple residual glycogen deposits in hepatocyte cytoplasm of bullhead classified as grade 3.

4. Discussion

High-altitude ecosystems are rare and important habitats that host endemic and endangered species characterized by specific adaptations to extreme conditions. These ecosystems are often subject to direct anthropic pressure, such as the introduction of alien species. For example, fish species introduced for recreational fishing in originally fishless alpine lakes in Italy [13] have become a serious threat to aquatic biodiversity, especially for amphibian communities. Species (predators, such as salmonids, but also forage fish) reared in aquaculture facilities were transported by fishermen or fishing associations, placed in these environments, and forced to live in oligotrophic waters with scarce prey [14]. Bullhead (*Cottus gobio*) naturally inhabits stony-bottom, oxygen-rich, cool-water streams and rivers. The altitude ranges from high-altitude streams to lowland rivers. *Cottus gobio* is a retiring fish [15]. This relates to its high vulnerability to predation; for this reason, cover and shade are extremely important habitat features for bullhead. Generally, the bullhead diet is based on benthic invertebrates and its feeding activity is concentrated around dawn and dusk because it is susceptible to predators during the day. While autochthonous to the Padano-Veneto district (Friuli-Venezia Giulia included) [16], the species was introduced accidentally with brook trout (*Salvelinus fontinalis*) to Dimon Lake in the 1980s [17]. *S. fontinalis* is presumably no longer present in the lake since we caught none.

Our study started with the aim of exploring the fish community of the lake. After discovering bullhead specimens with pronounced steatosis, we decided to investigate this abnormal condition and pose a hypothesis for its cause. Stomach contents analysis was performed to understand nutritional requirements, trophic and energy dynamics, food webs, food chains, material, and energy transfers between and within ecosystems [18]. This is an important part of studying feeding habits and, in general terms, a necessary step in research into the more complex questions of freshwater fish ecology. We analyzed the stomach contents of *C. gobio* sampled during two seasons both in Dimon Lake and Degano Creek to determine whether there were differences in diet across the two seasons [18]. Stomach content analysis showed that *C. gobio* from Dimon Lake feed mostly on Chironomidae larvae. This could be justified by the relative, great abundance, and almost exclusive presence of Chironomidae larvae in the lake [19], and it could also be explained by the feed ecology of this species, since this taxon is most easy to prey than other aquatic macroinvertebrates. In fact, *C. gobio* is a stationary benthic feeder, which shows a well-developed homing instinct [20]. Indeed, Hirudinea and Oligochaeta taxa are also present in this lake [19], but more endobenthic than Chironomidae, thus more difficult to hunt, justifying the bullhead preference for Chironomidae. Generally, *C. gobio* prey on a wide range of insect larvae as Plecoptera, Trichoptera, Ephemeroptera, and Diptera [21–24], as recorded in Degano Creek. Nevertheless, bullhead was found to be predominantly food generalists, with an ability to shift their feeding strategy towards specialization when exposed to environmental disturbances [25]. Comparison of the histological evaluations on fish from the two different sites (lake vs. stream) showed that only *C. gobio* from Dimon Lake was affected by medium-severe (score 2–3) liver steatosis. Lipid accumulation in the hepatocytes in this species or in fish living in other alpine lakes has never been reported in the literature to date. Our study is the first report of this phenomenon. Two hypotheses for the causes of these histological alterations can be offered. The first posits for adaptation of the fish to the winter season, which in this type of environment lasts for 6 months of the year. Our results clearly reveal that HSI values were significantly lower in Degano Creek compared to Dimon Lake in both seasons. Furthermore, a slight increase in the hepatosomatic index was recorded in autumn compared to summer in Dimon Lake. In this situation, it can be assumed that the bullhead from Dimon Lake accumulates lipids in the liver and then use them as fuel for energy in the adverse season (winter).

Adaptive processes are often based on biochemical and physiological adaptations. Lipids play a key role in biochemical adaptation in animals, especially in aquatic organisms, and they are of pivotal importance for long-term energy storage. Adaptations provide an effective response to stressors (e.g., changes in water temperature or oxygen). In some cases, fish may be able to adapt by changing their behavior, but more often it is through physiological adaptations, especially at the cell membrane level. Temperature, for instance, can be considered a stressor that acts to establish a new balance between the environment and the biochemical properties of cell membranes. Fish respond to environmental temperatures by activating a suite of compensatory mechanisms operating mainly at the cellular and subcellular levels of organization [26]. For example, carps progressively cooled from 30 °C to 23, 17, and 10 °C showed substantial changes in the fatty acid composition of the endoplasmic reticulum phospholipids, with increasing proportions of unsaturated fatty acids and changes in the activity of other biosynthetic enzymes [27,28].

Animals develop a complicated metabolic system to adapt to changes in nutritional states. In nature, when food is plentiful, animals eat and store the excess energy as lipids. This is a survival strategy for animals when food is not readily available [29]. Lipids are the most important biochemical compounds of fish [30]. Fish store lipids in muscles and liver [31] in contrast to mammals, which store lipids in adipose tissue. Most of these lipids are transferred to different parts of the body for various physiological actions [32]. In some fish species, extensive lipid infiltration into the liver is not considered pathological, since the liver works as a major lipid storage organ, especially in autumn [33,34]. However, in our study, we found no significant statistical difference in liver alterations between summer and autumn seasons. Kandemir and Polat [31] investigated seasonal and monthly variations in the amount of total lipid and fatty acids in the muscle and liver of reared rainbow trout (*Oncorhynchus mykiss*). Lipid levels were higher in summer, autumn, and winter than in spring. Furthermore, the amount of total lipid and fatty acid was higher in the liver than in muscle tissue. Active fish store their lipids in muscle tissues, but fish living at the bottom store their lipids in the liver [35], as seen in the *Cottus gobio* in our study. These considerations seem to corroborate our hypothesis. However, values of K did not show significant difference between fish from Dimon Lake and Degano Creek, indicating a robustness and good state of nutrition of both populations [36]. In this case, the use of Fulton's condition factor did not allow us to predict the lipid accumulation, as also previously suggested [37].

The second hypothesis rests on the diet of bullhead, and consequently the lipid content of chironomids, which are the only trophic resource available for this species. Bogut and co-workers [38] showed how *Chironomus plumosus* larvae represent a potential suitable natural component of farm fish, with a crude fat content of 1.3% and 9.7% in fresh larvae and dry matter, respectively. Timon-David [39] reported a similar percentage of lipids (8.3%) for adults of *Chironomus* spp. and larva. Lipids are high-energy nutrients and typically make up about 7% to 15% of a fish diet [40]. Nevertheless, an adult diet of Chironomid larvae may induce liver steatosis in reared male *Nothobranchius furzeri* [41]. For this reason, we cannot exclude that the presence of only chironomids as feed could be the cause of the steatosis observed in the bullhead samples.

Steatosis can be also induced by environmental contaminants, such as cadmium [42], but we excluded this possibility since Cd concentrations in bullhead from Dimon Lake were under the limit of quantification (0.02 mg kg^{-1}) as reported by Polazzo [19].

Morphological modifications, such as hepatocyte vacuolization, may also be interpreted as a response to a pathological process caused by infections or parasites [43,44], but we did not find bacteria or parasites in the fish during a survey performed in the same animals as reported by Pastorino and co-workers [7].

Further investigations are needed to explore this phenomenon, for example, by analyzing the composition of the total body fat in chironomids present in the lake or by determining the lipid profile in the livers of bullhead specimens.

All organisms need to adapt to their habitat if they are to survive. Our results suggest that fish may also implement strategies, such as physiological changes (lipid accumulation) or changes in feed preferences, to survive in adverse conditions.

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water

Article

Comparison of Otolith Readability and Reproducibility of Counts of Translucent Zones Using Different Otolith Preparation Methods for Four Endemic *Labeobarbus* Species in Lake Tana, Ethiopia

Shewit Gebremedhin [1,2,*], Karen Bekaert [3], Abebe Getahun [4], Stijn Bruneel [1], Wassie Anteneh [5], Peter Goethals [1] and Els Torreele [3]

[1] Department of Animal Science and Aquatic Ecology, University of Ghent, 9000 Ghent, Belgium
[2] Department of Fisheries, Wetlands and Wildlife Management, Bahir Dar University, Bahir Dar 6000, Ethiopia
[3] Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), 8400 Ostend, Belgium
[4] Department of Zoological Sciences, Addis Ababa University, Addis Ababa 1000, Ethiopia
[5] Department of Biology, Bahir Dar University, Bahir Dar 6000, Ethiopia
* Correspondence: shewitgebremedhin.kidane@ugent.be; Tel.: +25-192-051-8412

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Abstract: The analysis of fish age data is vital for the successful conservation of fish. Attempts to develop optimal management strategies for effective conservation of the endemic *Labeobarbus* species are strongly affected by the lack of accurate age estimates. Although methodological studies are key to acquiring a good insight into the age of fishes, up to now, there have not been any studies comparing different methods for these species. Thus, this study aimed at determining the best method for the endemic *Labeobarbus* species. Samples were collected from May 2016 to April 2017. Asteriscus otoliths from 150 specimens each of *L. intermedius*, *L. tsanensis*, *L. platydorsus*, and *L. megastoma* were examined. Six methods were evaluated; however, only three methods resulted in readable images. The procedure in which whole otoliths were first submerged in water, and subsequently placed in glycerol to take the image (MO1), was generally best. Except for *L. megastoma*, this method produced the clearest image as both the coefficient of variation and average percentage error between readers were lowest. Furthermore, except for *L. megastoma*, MO1 had high otolith readability and no systematic bias. Therefore, we suggest that MO1 should be used as the standard otolith preparation technique for the first three species, while for *L. megastoma*, other preparation techniques should be evaluated. This study provides a reference for researchers from Africa, particularly Ethiopia, to develop a suitable otolith preparation method for the different tropical fish species.

Keywords: count bias; growth zone counts; precision; otolith preparation methods

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1. Introduction

The endemic *Labeobarbus* species in Lake Tana are the only remaining known intact species flock of large cyprinids in the world [1]. *Labeobarbus* species are the most economically and ecologically important fishes in Lake Tana. However, because of their migratory and spawning aggregation behaviours [2] and specialized endemic characteristics [3], *Labeobarbus* populations are highly susceptible to illegal fishing and environmental degradation. As a result, their abundance has markedly declined [4–7] and six of the seventeen species have already been listed in the IUCN (International Union for Conservation of Nature) Red List as threatened species [8,9]. Therefore, the conservation of these species is urgently needed.

In this regard, the local fisheries authorities have taken the responsibility to conserve these species. However, their attempts are hampered by a lack of information. Although there have been reports on some biological aspects and anthropogenic pressures [2,3,10–17], little is known about the life-history traits of the endemic *Labeobarbus* species in Lake Tana. Information about age, age at maturity, growth, mortality, and longevity of *Labeobarbus* spp., which are important indicators of the health of fish stock [18], is virtually non-existent.

To understand the current stock status of the *Labeobarbus* species, investigation of their life-history traits is crucial. In studying the life history traits of the *Labeobarbus* species, knowledge of the age structure of fish populations, which is the basis of quantitative stock assessments, is vital [19]. Age structures allow the determination of the age at maturity, growth and mortality rates, which are essential input parameters for stock assessment models [19]. Inaccurate age estimations result in biased stock assessment outputs, which in turn lead to incorrect implementation of fisheries management strategies [20,21]. Several hard structures such as otoliths, scales, vertebrae, and fin rays can be used for fish age determination, although otoliths and scales are most frequently used. Because of their non-lethal nature, scales have long been considered the most efficient and practical structures for age determination, but later, several studies have revealed them to be inaccurate [22–25]. The limitations of scales to reveal all growth zones, particularly for slow-growing and older fish, have been proven by various studies [19,24,25]. Even though they require sacrificing the fish, otoliths generally provide precise and accurate counts of growth zones [26].

As it is well known that the most adequate otolith preparation methods differ from one species to another, a description of the most suitable method is indispensable for precise and accurate age estimation. In developed countries, validated age determination methods have been established for many fish species. However, this has not been the case in developing countries like Ethiopia. This study is part of a large study about the stock assessment of the endemic *Labeobarbus* species in Lake Tana. The aim of the study is to compare different otolith preparation methods in order to select the method with the best contrast between translucent and opaque growth zones. Therefore, this study is a prerequisite to undertake more detailed studies on age validation, growth, and mortality. The findings of this study are the first attempts to describe the most suitable method for the four *Labeobarbus* species, and these will be adopted as a standard protocol in further age validation studies.

2. Materials and Methods

2.1. Study Area

Lake Tana is situated on the basaltic plateau of the north-western Ethiopian highlands at 12° N, 37°15′ E and at about 1800 m above sea level (Figure 1). Lake Tana, covering an area of approximately 3050 km^2, is Ethiopia’s largest lake and contains half of the country’s surface freshwater. The climate is typical for a semi-arid region close to the equator. Lake Tana, as a tropical lake, has a relatively low temperature ranging from 20 to 27 °C [27].

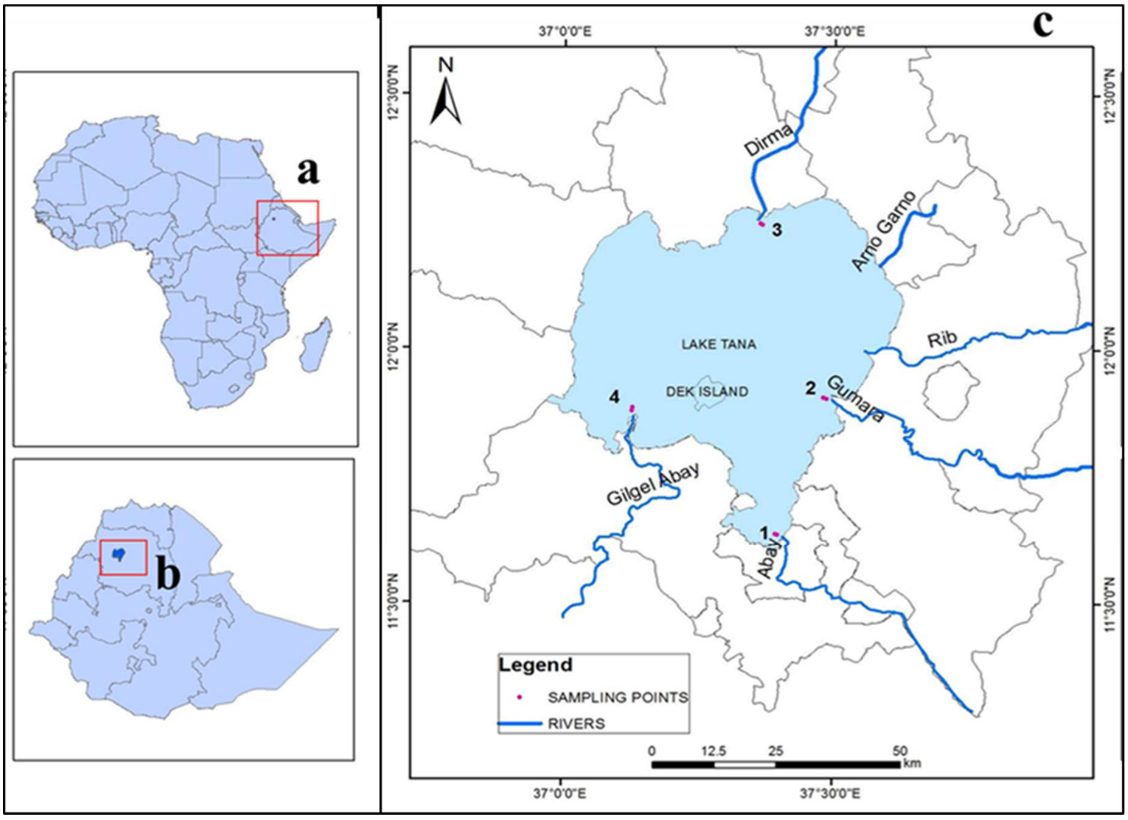

Figure 1. (**a**) The location of Ethiopia and Lake Tana in Africa, (**b**) the location of Lake Tana in Ethiopia, and (**c**) a map of the Lake Tana watershed showing the four study sites.

2.2. Sampling Design

Four sampling sites were systematically distributed over the lake and together represent the open-water zone of the whole lake. For site selection, we considered the cardinal directions north, south, east, and west of the lake; presence of the major tributaries, which are an ideal breeding grounds of the migratory *Labeobarbus* species in the lake; and the major fishing grounds. Sampling was conducted each month from May 2016 to April 2017 at all four sites. We used 10 multi-mesh gill nets consisting of 6, 8, 10, 12, and 14 stretched mesh sizes and monofilament gill nets of 4 and 6 cm stretched mesh size. Multifilament gill nets were set for 16 h, around 16:00 to 08:00, and were lowered to the bottom using anchors. The monofilaments were set during the daytime from 08:00 to 10:00. The identification of *Labeobarbus* spp. to species level using keys took place immediately after capture [11]. The majority of the *Labeobarbus* spp. caught by gill net died, but those that were caught without major injury were quickly identified and returned to the lake. Fork length of each specimen (to the nearest 0.1 cm) and total weight (to the nearest 0.1 g) were measured in the field using a measuring board and a precision balance. After dissection, the gonad maturity of each specimen was determined using a seven-point maturity scale [28] and each fish was sexed. For growth zone counts, four species—*L. intermedius*, *L. tsanensis*, *L. platydorsus*, and *L. megastoma*—were selected. Species selection was based on their prevalence in the commercial catch. All specimens selected for growth zone counts were beheaded and samples were transported using an ice-box to the fisheries laboratory in Bahir Dar University for otolith extraction.

2.3. Otolith Preparation Methods

Of the three otolith pairs within the Cyprinidae family, asteriscus otoliths are the largest and have the most suitable hard structure for aging [29–31]. A total of 150 asteriscus otolith pairs were collected from each of the four *Labeobarbus* species. Otoliths were cleaned by removing the attached tissues and were preserved in 45% glycerol using labelled micro-centrifuge tubes. In order to ascertain the best method to obtain a clear view of the core, edge, and growth increments, we tried out a number of different otolith preparation methods. These preparation methods included (1) whole otoliths submerged in glycerol and water and (2) otoliths that were sectioned and stained. A detailed description of the different otolith preparation methods is given in the following sections. The left otolith was generally used, but if it was broken or lost, the right otolith was used instead.

2.3.1. Whole Otolith Submerged in Glycerol and Water

The effect of glycerol and water to enhance the visibility of the growth zones was evaluated. Whole otoliths were first immersed in glycerol at different time intervals. For example, the whole otoliths were immersed in glycerol for 2, 4, 6, 8, 12, 14, and 16 hours and the visibility of the growth zones at each time interval was examined. Similarly, the same otoliths were submerged in water following the same procedures used for glycerol. In order to remove the effect of glycerol, otoliths were rinsed using acetone, cleaned, and dried prior to submergence in water. The combined effect of glycerol and water was also evaluated. The whole otoliths were first submerged in glycerol before transferring them to a water medium to take the image for growth zone count, or vice versa. Whole otoliths were placed in a black bowl filled with glycerol or water using forceps and an image was taken using an AxioCam MRc camera attached to a stereo-microscope using reflected light at 10X magnification.

2.3.2. Sectioned and Stained Otoliths

Prior to sectioning, whole otoliths were cleaned using water, dried, and embedded in black resin. A mould with six rows was used to embed the otoliths. The black resin was first poured on each row and then, depending on the size of the otolith, 9–10 whole otoliths were placed on each row using forceps. The embedded otoliths were allowed to dry for 12 h. After fully drying, a top layer was added and allowed to harden for an extra 12 h before sectioning. Finally, an approximately 0.45–0.50 mm thick transverse section was cut through the nucleus using a high-speed saw rotating at 3000 rpm (ATM BRILLANT250).

After sectioning, otoliths were stained using 100% of a neutral red solution and the enhancement of growth zone visibility was checked at 1-minute intervals for 30 min. As this trial did not give a good result, we also tried to stain the otoliths using 50% of the solution. Moreover, as an alternative to the neutral red solution, alizarin solution was used. Using this solution, otoliths were stained for 30 min and growth zone enhancement was checked at 1-minute intervals. Additionally, otoliths were also stained for 1 h without interruption. Besides the sectioned otoliths, we also stained the whole otoliths using both solutions. Both reflected and transmitted light was used to take images of the sectioned and stained otoliths at 16X magnification.

2.3.3. Preliminary Evaluation of Otolith Preparation Techniques

A preliminary evaluation was made based on the clarity and contrast of the images (Figure 2). Of all the examined otolith preparation methods, three methods did not produce distinguishable growth zones. More specifically, (1) the method using sectioned otoliths, (2) the method using stained otoliths, and (3) the method using otoliths submerged in water and remained in the water to take the image. Thus, these methods were omitted at this stage and otolith preparation was continued using only the three methods that produced distinguishable growth zones, core, and edge. These methods are the following: (1) method 1: whole otoliths were first submerged in water for 16 h and the image was taken while the otolith was placed in glycerol (MO1), (2) method 2: whole otoliths were first submerged in glycerol for 14 h and the image was taken while the otolith was placed in water (MO2), and (3) method 3: whole otoliths submerged in glycerol and remained in glycerol to take image (MO3). For MO1, whole otoliths submerged in water for about 16 h resulted in clear images, whereas for MO2 and MO3, whole otoliths immersed in glycerol for about 14 h produced relatively good images. The data from these three methods were retained for data analysis

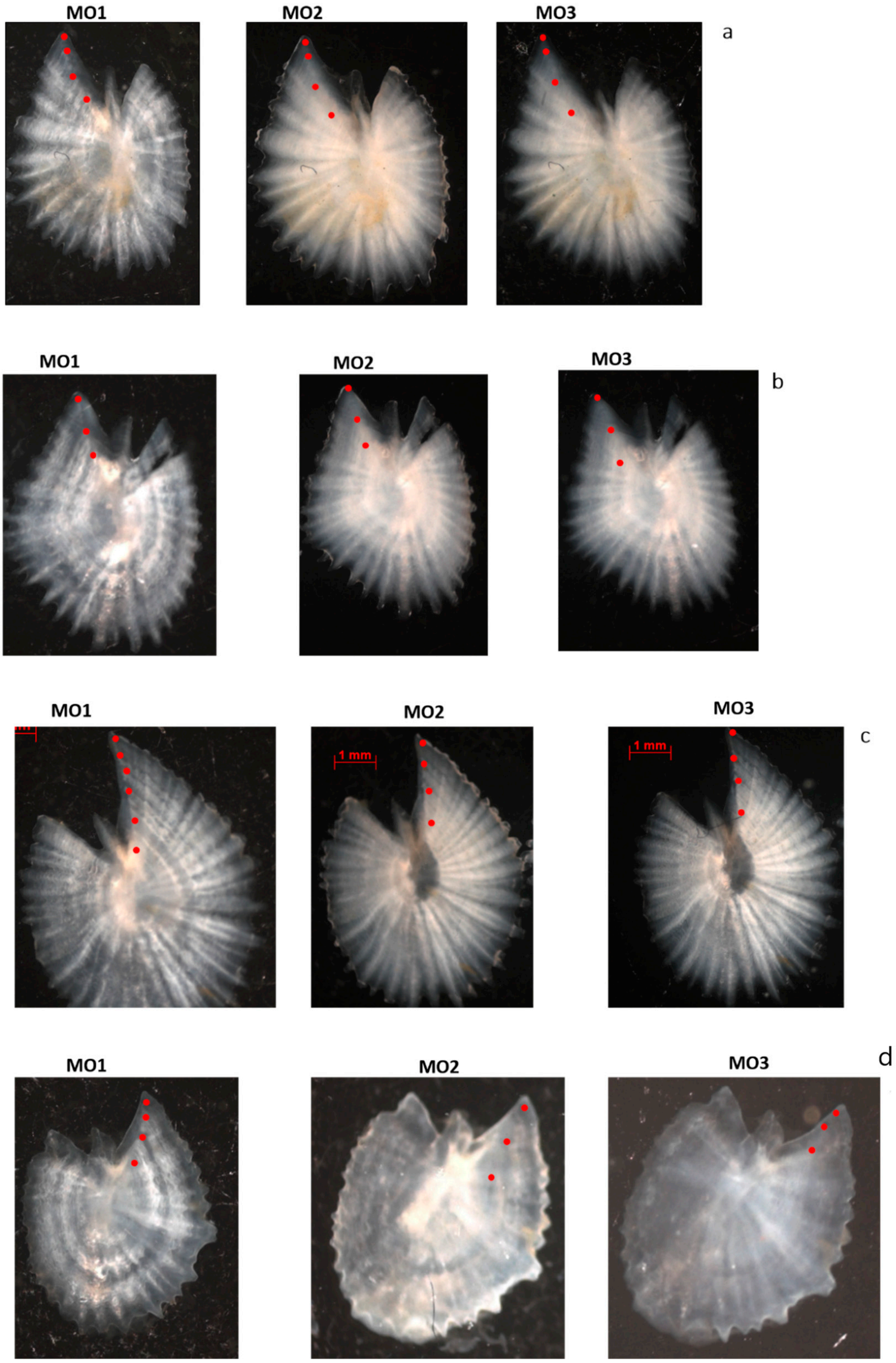

Figure 2. Otoliths images prepared by the three different aging methods for *L. intermedius* (**a**), *L. tsanensis* (**b**), *L. platydorsus* (**c**), and *L. megastoma* (**d**). MO1, MO2, and MO3 represent aging method one, two, and three, respectively. Red dots are a number of growth zones.

Otolith preparation and examination was undertaken in the Flanders Research Institute for Agriculture, Fisheries, and Food (ILVO) in Belgium. Growth zones were annotated on the digital otolith image using SmartDots software (www.smartdots.ices.dk). Growth zones were visible as translucent (dark) and opaque (light) zones when examined under reflected light. Counts of translucent zones were made along an anterio-dorsal transect situated on the otolith rostrum. All otoliths were examined by two experienced readers unfamiliar with these species and working independently. Readers had no previous knowledge on the date of capture, size, and sex of the fish. Each reader read each otolith once and the reader's confidence to otolith readability was assigned as follows: easily readable (Q1), moderately readable (Q2), or unreadable (Q3).

3. Statistical Analysis

Translucent Zone Count Precision and Bias

Both statistical and graphical methods were used to compare the bias and precision between readers and methods. The precision between readers and methods was measured using the average percentage error (APE) [32], coefficient of variation (CV) [33], and percentage of agreement (PA). The APE and CV are the most suitable and statistically sound measurements of precision [19]. Although PA can be used as an index of precision, it is often not considered as a suitable measure as it widely varies among species as well as among ages within a species [32]. The PA is the ratio of the number of agreements between the paired readings to the total number of readings made by the two independent readers. The APE and CV were computed by the following formula:

$$\mathbf{APE} = \frac{\mathbf{100}}{\mathbf{N}} \sum_{\mathbf{J-1}}^{\mathbf{N}} \left(\frac{\mathbf{1}}{\mathbf{R}} \sum_{\mathbf{i-1}}^{\mathbf{R}} \frac{\left| \mathbf{X_{ij}} - \mathbf{X_{j}} \right|}{\mathbf{X_{j}}} \right), \quad (1)$$

$$\mathbf{CV} = \frac{\mathbf{100}}{\mathbf{N}} \sum_{\mathbf{J-1}}^{\mathbf{N}} \left(\frac{\sqrt{\sum_{i=1}^{R} \frac{(\mathbf{X_{ij}} - \mathbf{X_{j}})^2}{R-1}}}{\mathbf{X_{j}}} \right), \quad (2)$$

where N is the number of fish whose otoliths were read, R is the number of times otoliths were read, X_{ij} is the ith read for the jth fish, and X_j is the average estimated growth zone count of the jth fish.

Count-bias plots were produced to visualize the deviation of readings between the methods and readers from the 1:1 equivalence line [34]. Although none of the three methods was validated, otolith images from MO1 were clearer and sharper, which would likely lead to fewer errors in growth zone count. Additionally, the precision of MO1 was better than the other methods for all four species. On this basis, we assumed that growth zone counts from MO1 are reliable and, therefore, we compared growth zone counts from MO2 and MO3 with those from MO1, which is considered as a reference count. Similarly, growth zone counts from MO2 were compared with those from MO3, because the precision for MO3 was better than for MO2. The null hypothesis that the mean of the none-reference counts did not significantly differ from the reference counts for each reference count was tested using a paired t-test. The mean difference of the growth zone counts assigned by the two readers for each method was determined using an independent t-test. One-way analysis of variance (ANOVA) was used to determine if there were significant differences among mean growth zone counts derived from the three methods. The null hypothesis that there is no symmetric difference in growth zone counts between readers and methods was tested using a Bowker's chi-square symmetry test [35].

In order to reveal if the difference between the growth zone counts of the two readers and among methods was consistent, a linear regression was computed. The slope and intercept of the regression line were tested for significant difference from 1 and 0, respectively. For the regression analyses between readers, growth zone counts from reader 1 (R1) and reader 2 (R2) were used as an independent and a dependent variable, respectively. As both readers had relatively the same otolith reading experience and both of them were unfamiliar with these species, we randomly chose growth zone counts from R1 as the independent variable. The mean of the paired reads was used for the regression analyses between methods. For the regression analyses between MO1 and the other methods, growth zone counts from MO1 were used as an independent variable, while growth zone counts from MO3 were used as an independent variable for the regression analysis between MO2 and MO3.

The null hypothesis that the mean difference between paired growth zone counts is zero was tested using a paired t-test. Otolith readability based on the confidence rankings assigned by the two readers was tested using a Mann–Whitney U test. A Kruskal–Wallis test was used to determine otolith readability among methods. Confidence rankings from R1 were used for this analysis. The pairwise comparison of the otolith readability between methods was made using Dunn's test with *p*-values adjusted with the Benjamini–Hochberg method. When a reader's confidence to otolith readability was assigned as Q3 (an unreadable), it was discarded from the analysis to ensure equal sample sizes for the three methods. The periodicity of growth zone deposition was validated and the growth zone deposition rate in asteriscus otolith of the studied species was proven to be annual [36]. Data analysis was undertaken using R software (version 3.5.0, R Developer Core Team, R Foundation for Statistical Computing, Vienna, Austria).

4. Results

4.1. Length Frequency

Fish size ranged from 10–65 cm fork length (FL) for *L. intermedius*, 12–45 cm FL for *L. tsanensis*, 12–65 cm for *L. platydorsus*, and 12–57 cm for *L. megastoma*. The majority of the specimens of *L. intermedius*, *L. tsanensis,* and *L. platydorsus* were within the length groups of 15–20 cm (18 cm mid-length) and 20–25 cm (23 cm mid-length), while specimens with a fork length of more than 33 cm of were rarely caught (Figure 3). For *L. megastoma*, a high length frequency was recorded for specimens that were included within the length groups of 25–30 cm (28 cm mid-length) and 30–35 cm (38 cm mid-length), whereas specimens larger than 38 cm were rare (Figure 3).

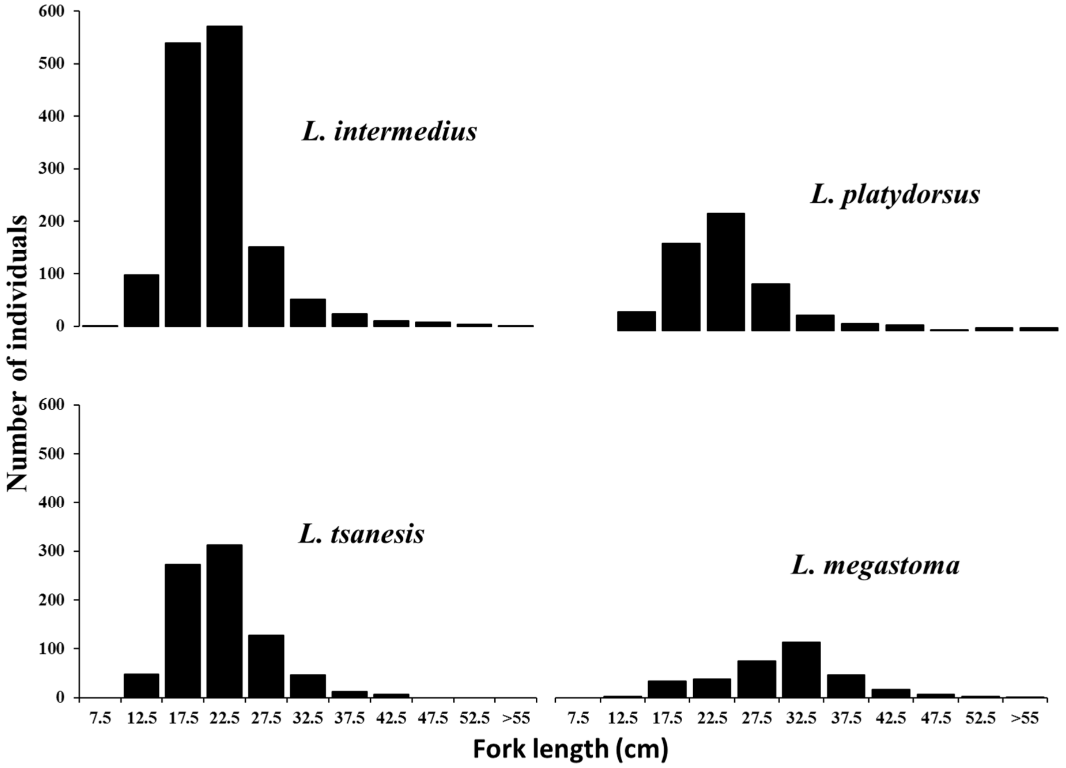

Figure 3. Length frequency distribution of the four dominant *Labeobarbus* spp. in Lake Tana.

4.2. Otolith Readability

For both readers, otoliths prepared using MO1 resulted in higher readability than the other methods for the four species (Figure 4). Readability of the growth zones of otoliths prepared using MO2 and MO3 was inconsistent. It was often difficult to delineate the growth zones around most of the sections, which likely led to misinterpretations. Regarding the otoliths prepared using MO1, both readers were able to read the growth zones (Q1 and Q2) in 100% of the samples for *L. intermedius* and *L. tsanensis* and in 99% of the samples for *L. platydorsus* and *L. megastoma*, while there was a considerable number of unreadable (Q3) otoliths prepared by MO2 and MO3 (Figure 4). A Mann–Whitney U test indicated that the confidence rankings between readers for the three methods were not significantly different for *L. intermedius* and *L. tsanensis* (Figure 4). However, for *L. platydorsus* and *L. megastoma*, confidence rankings between readers for MO1 were significantly different. Confidence rankings among methods showed a significant difference (Table 1). For all four species, confidence rankings for MO1 were significantly different from the other methods, while there was no significant difference between MO2 and MO3 (Table 1). Therefore, the high level of readers' confidence for MO1 likely led to a reliable count of growth zones with lower error levels.

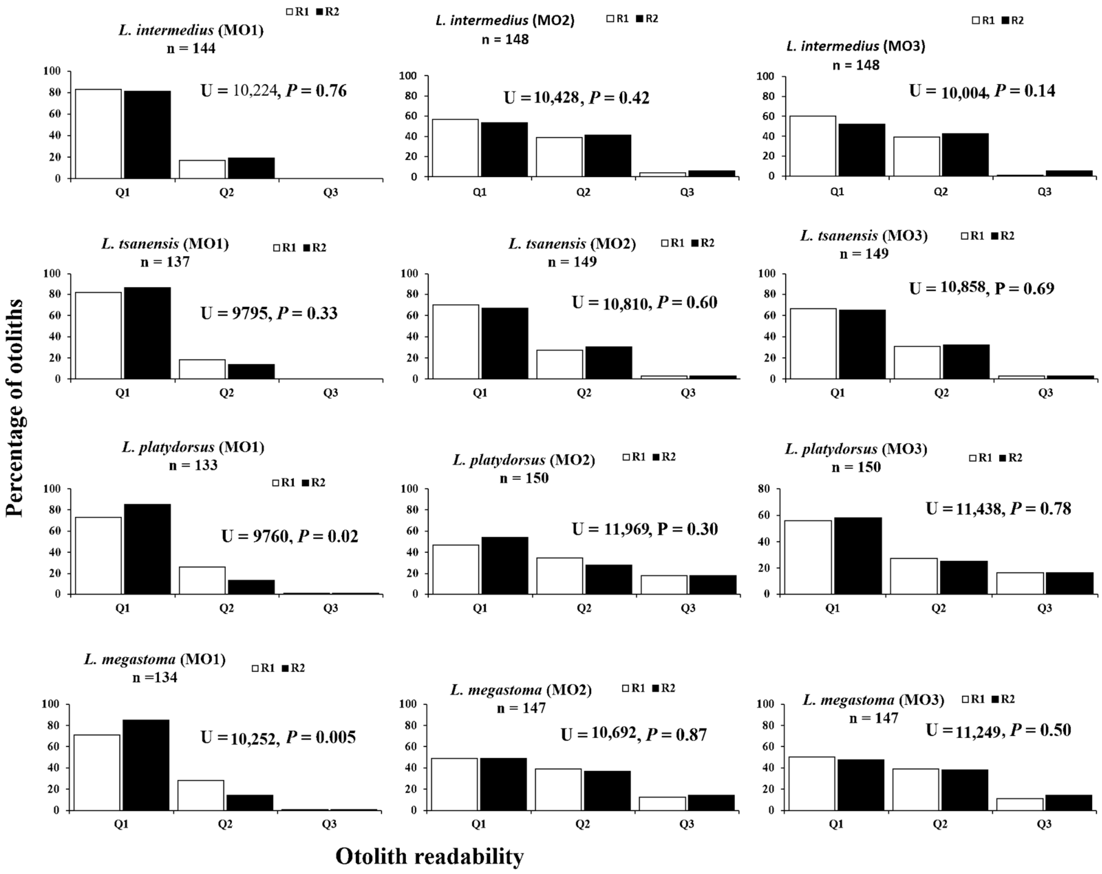

Figure 4. The bar graphs indicate the percentage of otoliths at each level of otolith readability (Q1 = readable, Q2 = moderately readable, and Q3 = unreadable). R1 refers to reader 1 and R2 refers to reader 2. The Mann–Whitney U test, which compares the confidence rankings between readers for each method, is also included.

Table 1. Comparison of reader confidence among the three methods for the four endemic *Labeobarbus* species in Lake Tana. Q1 = readable, Q2 = moderately readable, and Q3 = unreadable. Numbers refer to the number of otolith under each readability level. Different superscript letters for the methods for each species indicate a significant difference. Confidence rankings from reader 1 (R1) were used for this analysis.

Aging Methods	**Readers' Confidence**			**Kruskal–Wallis Rank Sum Test**		
	Q1	**Q2**	**Q3**	**Chi-Square**	**df**	***p*-Value**
			L. intermedius			
MO1^a	119	25	0	26.25	2	0.000
MO2^b	85	58	5			
MO3^b	88	58	2			
			L. tsanensis			
MO1^a	112	25	0	9.69	2	0.008
MO2^b	104	41	4			
MO3^b	99	46	4			
			L. platydorsus			
MO1^a	97	35	1	27.10	2	0.000
MO2^b	70	52	28			
MO3^b	84	41	25			
			L. megastoma			
MO1^a	95	38	1	23.97	2	0.000
MO2^b	72	57	18			
MO3^b	74	57	16			

4.3. Precision and Bias between Readers

Age composition of the studied fish species based on the three methods did not exhibit large variation (Figure 5). Although growth zone counts distribution derived from MO3 was only slightly wider for *L. intermedius*, *L. tsanensis*, and *L. platydorsus*, while MO1 and MO2 resulted in the same growth zone counts distribution. For *L. megastoma*, growth zone counts distribution derived from MO1 was slightly wider than the other methods. Growth zone counts of 4 and 5 were dominant for *L. tsanensis* and *L. platydorsus*, while growth zone counts of 5 and 6 were dominant for *L. intermedius*, and 3–5 for *L. megastoma*. The relationship between length and growth zone counts for the four studied species is depicted in Figure 6.

The reproducibility and uncertainty of the growth zones counts from all three methods were evaluated by means of CV, APE, and PA with respect to comparisons between readers. The CV and APE values between readers were generally low for all methods for *L. intermedius*, *L. tsanensis*, and *L. platydorsus*, while these were relatively high for *L. megastoma* (Table 2). MO1 resulted in more precise age readings for *L. intermedius*, *L. tsanensis*, and *L. platydorsus* than did MO2 and MO3. This is indicated by the low CV and APE values (Table 2). For *L. megastoma*, MO1 and MO3 exhibited equal precision, while MO2 yielded relatively low precision (Table 2). In all three methods, the CV and APE values were the lowest for *L. intermedius* and generally the highest for *L. megastoma*. Growth zone count assignments by the two readers had a relatively poor percentage of agreement for all three methods and four species (Table 2). Compared with the PA of the other methods (Appendices Figures A1 and A2), the PA between readers for MO1 was relatively high for *L. intermedius* (69%), *L. tsanensis* (60%), and *L. platydorsus* (68%). Additionally, the PA for MO1 at age 1 and ≥2 absolute differences, respectively, was low for *L. intermedius* (25%, 6%), *L. tsanensis* (34%, 5%), and *L. platydorsus* (29%, 3%) (Figure 7a,c, Figure 8a, histograms in the y-axis), while these values were relatively high for MO2 and MO3 (Appendices Figures A1 and A2, histograms in the y-axis). However, for *L. megastoma*, the PA at age zero and 1 absolute differences was relatively the same for all three methods (Figure 8c and

Appendices Figure A2, histograms in the y-axis). The PA for this species was low compared with the other species.

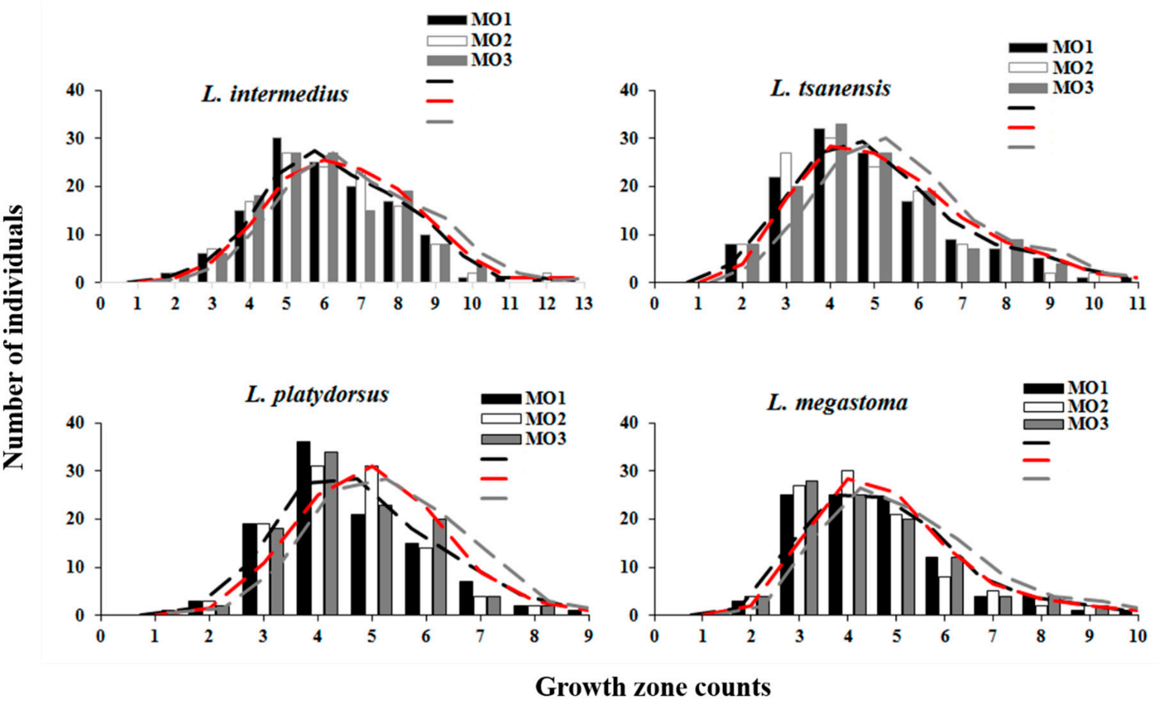

Figure 5. A number of individuals with the same number of growth zones in their otoliths examined using the three methods: MO1, MO2, and MO3 for the four dominant *Labeobarbus* species in Lake Tana. Mean of the paired reads from the two independent readers was used. The dotted black, red, and grey lines indicate the trend in the distribution of translucent growth zones counts for MO1, MO2, and MO3, respectively.

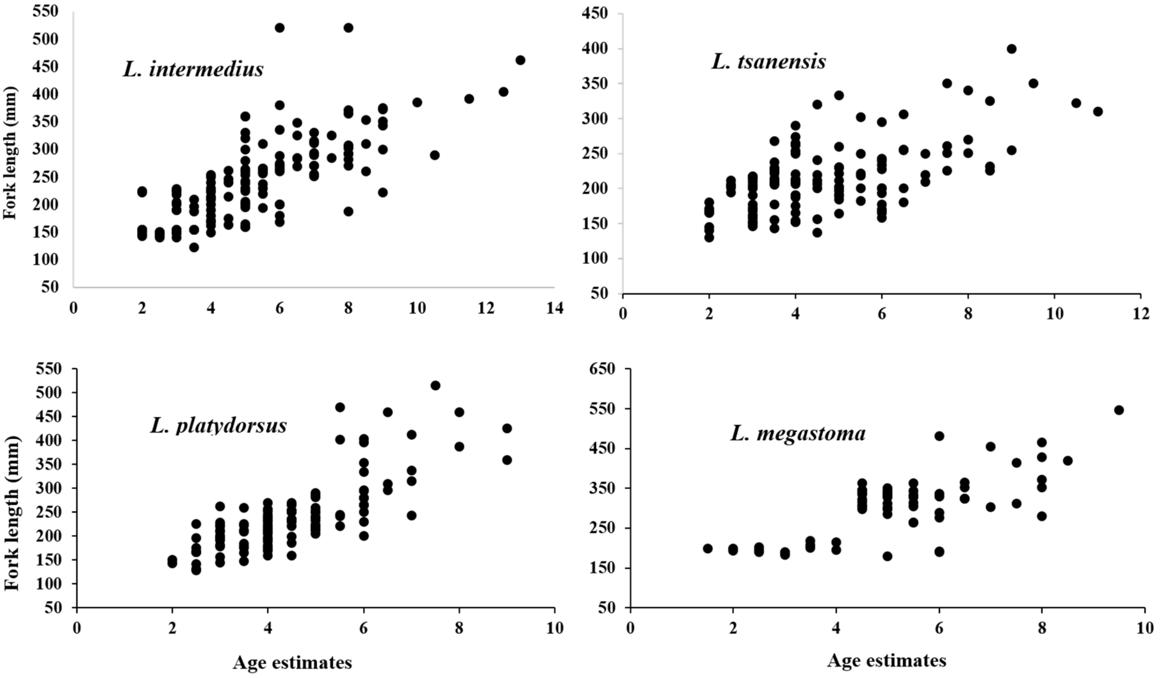

Figure 6. Relationship between fork length and the count of the number of translucent zones in the otoliths for the four studied *Labeobarbus* species in Lake Tana.

Table 2. Average percentage error (APE, %), coefficient of variation (CV, %), and percentage of agreement (PA, %) to determine the precision of age readings. n = sample size and R = number of readings.

	Between Readers														
Species	**MO1**					**MO2**					**MO3**				
	n	**R**	**CV**	**APE**	**PA**	**n**	**R**	**CV**	**APE**	**PA**	**n**	**R**	**CV**	**APE**	**PA**
L. intermedius	144	2	4	3	69	139	2	7	5	57	144	2	6	4	64
L. tsanensis	137	2	7	5	60	143	2	9	6	56	143	2	9	6	56
L. platydorsus	133	2	7	5	68	119	2	9	6	55	123	2	9	6	62
L. megastoma	133	2	11	8	45	125	2	13	9	42	123	2	11	8	47
	Between Methods														
	MO1 vs MO2					**MO1 vs MO3**					**MO2 vs MO3**				
L. intermedius	128	2	3	2	77	128	2	4	3	67	128	2	3	2	77
L. tsanensis	129	2	4	3	73	129	2	5	4	71	129	2	4	3	78
L. platydorsus	104	2	5	4	71	104	2	5	4	75	104	2	3	2	81
L. megastoma	99	2	4	3	71	99	2	4	3	77	99	2	4	3	79

Overall, the mean non-reference growth counts did not significantly differ from each corresponding reference counts for all four species (paired t-test, $p > 0.05$). However, the mean difference between non-reference and reference growth zone counts (y-axis) plotted against the reference counts (x-axis) exhibited a minimal relative bias from the line of the agreement for each method and species (Figure 7a,c and Figure 8a,c and Appendices Figures A1 and A2). For MO1, the mean non-reference growth zone counts for the young specimens of *L. intermedius*, *L. tsanensis*, and *L. platydorsus* were only slightly higher than the reference counts, but the reverse was true for the old specimens (Figure 7a,c, Figure 8c). However, for *L. megastoma*, mean non-reference growth zone counts for both the young and old specimens were higher than the reference counts (Figure 8c). In general, for *L. intermedius*, *L. tsanensis*, and *L. platydorsus*, differences from the line of the agreement were relatively small for MO1 (Figure 7a,c, Figure 8c) compared with the other methods (Appendices Figures A1 and A2).

4.4. Precision and Bias among Aging Methods

The precision of age readings derived from the three methods for the four *Labeobarbus* species was generally good (Table 2). The lowest CV and APE values were computed from MO1 versus MO2 and MO2 versus MO3 for *L. intermedius*, *L. tsanensis*, and *L. megastoma*, while these values were low only between MO2 and MO3 for *L. platydorsus* (Table 2). The PA values between MO2 and MO3 were slightly higher than the other methods (Table 2). For all comparisons between methods, the discrepancy at a zero difference was high for all four species (Figure 7b,d and Figure 8b,d, and Appendices Figure A1 & Table A1, right, histograms in the y-axis), although the discrepancy at age 1 absolute difference was also high in some cases.

Even though there was no significant difference between the mean non-reference growth zone counts and each corresponding reference count (paired t-test, $p > 0.05$), the age bias plots for comparison of the three methods revealed a discrepancy from the line of agreement (Figures 7 and 8 and Appendices Figures A1 and A2, right). For most species, MO2 and MO3 underestimated the growth zone counts of old individuals relative to MO1. These trends were also apparent in plots of MO2 and MO3 counts of growth zones. Differences between the mean non-reference growth zone counts and the reference count increased with the number of growth counts for all three methods and four species. The discrepancy began at count 4 for *L. megastoma*, at count 6 for *L. platydorsus*, and at count 8 for *L. intermedius* and *L. tsanensis* (Figures 7 and 8 and Appendices Figures A1 and A2, right). This suggests that the level of count precision among methods could be lower when counting growth zone from old fish.

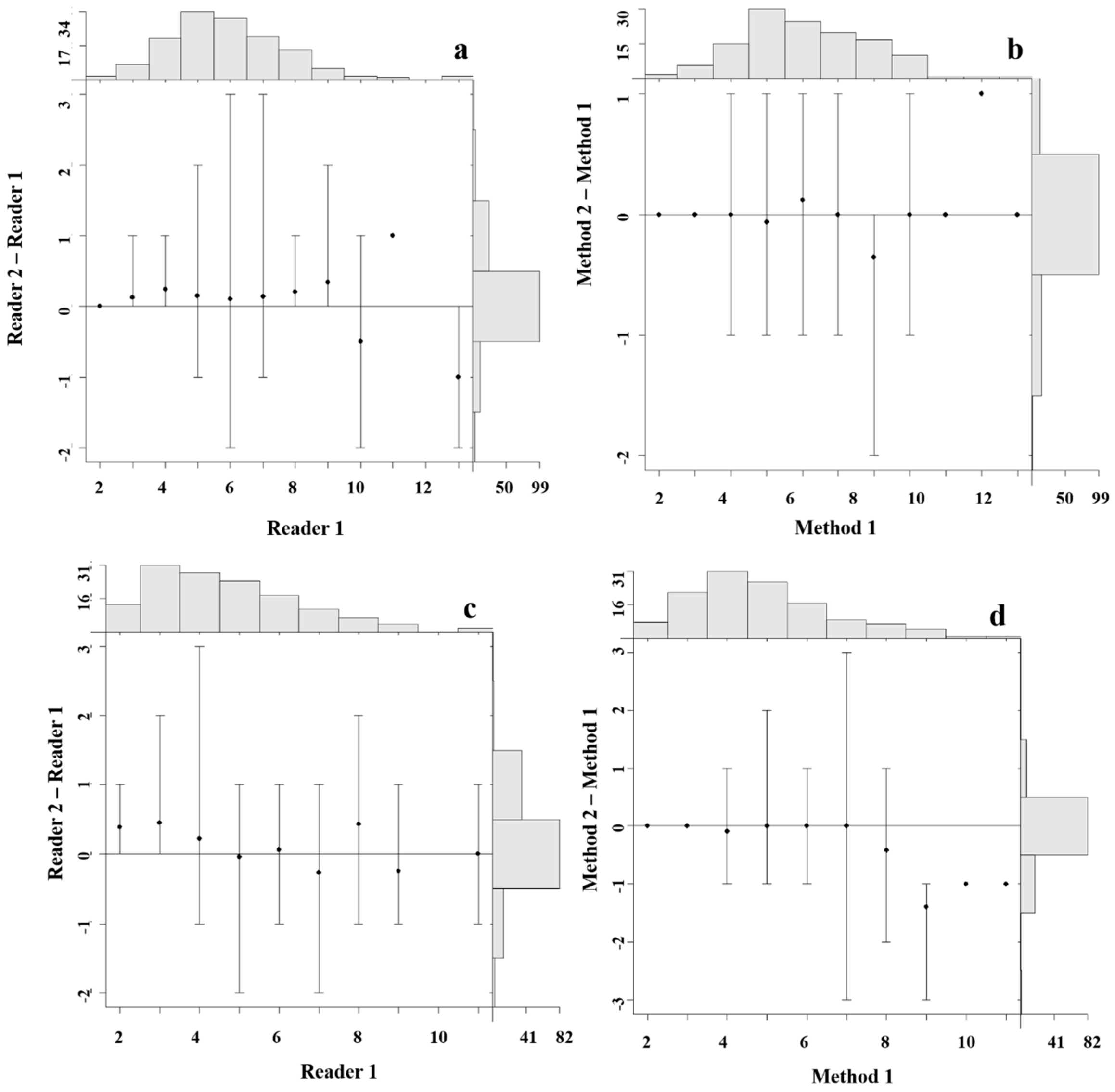

Figure 7. Mean (dots) and 95% confidence intervals of differences in counts of growth zones derived from the best method (MO1) between two independent readers at the readings for the first reader and difference between methods (MO1 and MO2) at the readings for MO1 for *L. intermedius* (**a** & **b**) and *L.tsanensis* (**c** & **d**) in Lake Tana. The horizontal solid line is the 1:1 equivalence line and marginal histograms are for counts of the first reader (top) and differences in counts between readers and methods.

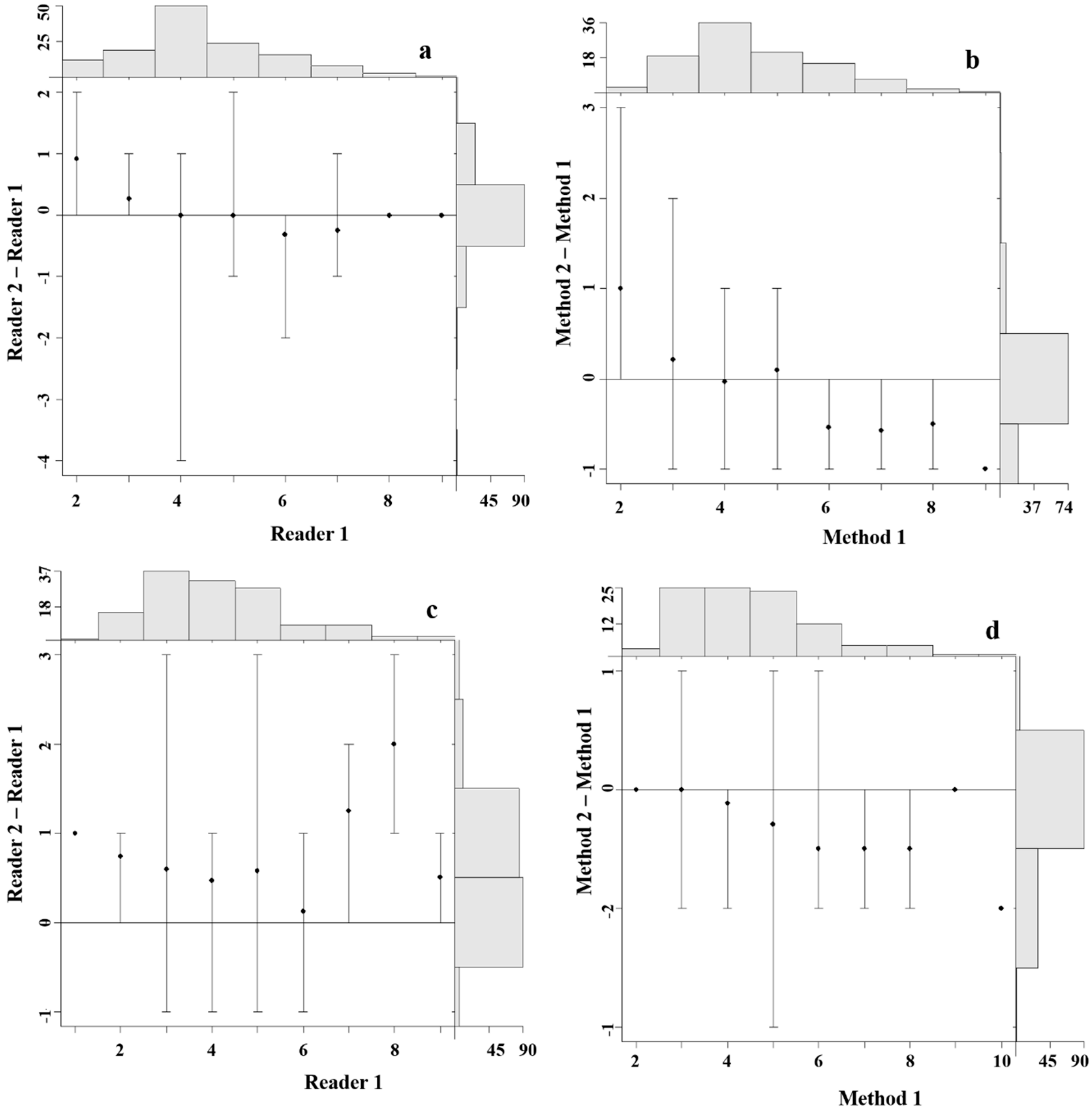

Figure 8. Mean (dots) and 95% confidence intervals of differences in counts of growth zones derived from the best method (MO1) between two independent readers at the readings for the first reader and difference between methods (MO1 and MO2) at the readings for MO1 for *L. platydorsus* (**a** & **b**) and *L. megastoma* (**c** & **d**) in Lake Tana. The horizontal solid line is the 1:1 equivalence line and marginal histograms are for counts of the first reader (top) and differences in counts between readers and methods.

4.5. Statistical Comparisons of Growth Zones Counts between Readers and Methods

A one-way ANOVA test revealed no significant difference among mean growth zone counts derived from the three methods for all four species (*L. intermedius*, *F*(2, 381) = 0.04, *p* = 0.97; *L. tsanensis*, *F*(2, 384) = 0.23, *p* = 0.80, *L. platydorsus*, *F*(2, 309) = 0.05, *p* = 0.95; and *L. megastoma*, *F*(2, 294) = 0.48, *p* = 0.62). Except for *L. megastoma*, growth zone counts between readers for the three methods and three species were not significantly different (Table 3). This suggests that there was no significant count bias between readers, except for *L. megastoma*. Moreover, for *L. intermedius, L. tsanensis*, and *L. platydorsus*, there was no evidence of systematic disagreement for growth zone counts assigned between R1 and R2 using MO1 and MO3, while this was significantly different for *L. megastoma* (Table 4). However, the growth zone counts assigned between R1 and R2 using MO2 revealed symmetric bias for all four species (Table 4). No systematic bias in growth zone counts of the different methods was found, except those between MO1 and MO2 for *L. megastoma* (Table 4).

Table 3. The mean number of growth zones counted by reader 1 (R1) and reader 2 (R2) for each of the three methods of four *Labeobarbus* species in Lake Tana.

Methods	**Reader**		**t-Value**	**df**	***p*-Value**	**95% Confidence Intervals**
	R1(Mean)	**R2 (Mean)**				
L. intermedius						
MO1	5.9	6.1	−0.61	285	0.54	−0.59–0.31
MO2	5.8	6.1	−1.45	276	0.15	−0.81–0.12
MO3	5.8	6.1	−1.22	277	0.22	−0.75–0.17
L. tsanensis						
MO1	4.7	4.9	−0.75	272	0.46	−0.64–0.29
MO2	4.5	4.8	−1.06	284	0.29	−0.66–0.20
MO3	4.8	4.8	−0.28	284	0.78	−0.50–0.38
L. platydorsus						
MO1	4.4	4.5	−0.39	263	0.70	−0.41–0.27
MO2	4.1	4.3	−1.45	236	0.15	−0.56–0.08
MO3	4.2	4.3	−0.94	244	0.35	−0.50–0.18
L. megastoma						
MO1	4.1	4.7	−2.97	259	0.003	−1.01–(−0.20)
MO2	3.8	4.6	−3.42	229	0.000	−1.20–(−0.32)
MO3	4.0	4.6	−3.03	233	0.003	−1.09–(−0.23)

Table 4. Analysis of Bowker's chi-square symmetry test, comparing pairs of growth zone counts between readers and methods applied to the four *Labeobarbus* species in Lake Tana. "ns" refers to no significant difference. "*" and "**" refers to significant difference at $p = 0.01$ and 0.001, respectively.

Between Readers									
Species	**MO1**			**MO2**			**MO3**		
	df	**Chi-sq**	***p***	**df**	**Chi-sq**	***p***	**df**	**Chi-sq**	***p***
L. intermedius	17	19	ns	19	28	**	15	24	ns
L. tsanensis	14	22	ns	15	30	*	15	21	ns
L. platydorsus	10	19	ns	10	20	*	10	15	ns
L. megastoma	15	58	**	17	59	**	13	58	**
Between Methods									
	MO1 vs. MO2			**MO1 vs. MO3**			**MO2 vs. MO3**		
L. intermedius	9	7	ns	10	8	ns	10	9	ns
L. tsanensis	13	14	ns	16	12	ns	13	15	ns
L. platydorsus	9	15	ns	11	10	ns	4	6	ns
L. megastoma	8	16	*	7	11	ns	7	9	ns

The regression and paired t-test analyses between readers and methods showed significant differences in some cases, generally indicating minimal bias between readers and methods (Appendix C). For MO2 and MO3, the slope and intercept of the linear regression between readers were significantly different from 1 and 0, respectively, for all four species, with the exception of the intercept for *L. megastoma*. Similarly, the mean difference of the paired readings for MO2 and MO3 showed a significant difference from 0 for the four species. However, for MO1, the slope and intercept of the linear regression between readers did not significantly differ from 1 and 0, respectively, for *L. intermedius* and the slope was not significantly different from 1 for *L. megastoma*. Additionally, the mean difference of the paired readings obtained from MO1 was not significantly different from 0 for *L. platydorsus*.

Between readers, the mean paired difference for the four species was generally small for MO1 (<0.18), compared with MO2 and MO3.

With respect to the methods, the slope and intercept of the linear regression between MO1 and MO2 showed a significant difference from 1 and 0, respectively, for *L. tsanensis*, *L. platydorsus*, and *L. megastoma*. Similar results were observed from the regression analyses between MO1 and MO3 for *L. tsanensis* and *L. platydorsus* and between MO2 and MO3 for all four species. The comparisons between methods did not reveal significant mean paired differences from 0 for most of the cases (Appendix C).

5. Discussion and Conclusions

Age estimation based on the interpretation of growth zones usually involves judgment and subjective interpretation [19]. Errors in age determination are mainly related to the inconsistent periodicity of growth zones and systematic errors due to the validity of the otolith preparation method, interpretation problems of the aging structure, or experience level of age readers [21,37,38]. The latter could be minimized by describing a precise and accurate otolith preparation method [19]. The precision of otolith preparation methods can be computed using graphical and statistical methods, while a validated method for all age groups in the population is required to estimate accuracy [39]. Because validating all the proposed otolith preparation methods for all different age groups in the population is logistically challenging, it may be more efficient to select the most accurate technique first. The best method should, therefore, be validated for the rate of growth zone formation.

The process of growth zone formation is affected by a combination of factors including physical, chemical, environmental, and physiological aspects. Depending on these factors, different otolith preparation techniques may be considered as optimal [40]. Consequently, optimal otolith preparation techniques differ markedly among species. In order to assist the fisheries biologists and managers in selecting the best technique, we compared different otolith preparation techniques for the *Labeobarbus* species in Lake Tana. Selection of the appropriate otolith preparation method is dependent on the balance between readability (ease of annulus interpretation) and consistency (repeatability of growth zone counts) of the aging structures [41]. In this study, selection of the most suitable otolith preparation methods, out of the six evaluated methods, was undertaken based on clarity and contrast of the images. These methods that produced distinguishable growth zones, core, and edge were selected and compared to find the optimal method. Three methods including (1) the method using sectioned otoliths, (2) the method using stained otoliths, and (3) the methods using whole otoliths submerged in water and remained in the water to take the image did not produce readable structures and were omitted from the comparison, while the other three methods (MO1, MO2, and MO3) produced distinguishable growth zones, core, and edge. Similar to the findings of this study, problems with sectioned asteriscus otoliths to produce clear and readable growth zones were reported in previous studies [31,42–46]. Higher readability and consistent age estimates were produced from whole asteriscus otoliths immersed in methyl salicylate than sectioned otoliths [43,45]. Furthermore, both whole and sectioned asteriscus otoliths from *Cyprinus carpio* resulted in similar interpretation [47], but the authors noted that sectioned asteriscus otoliths obscured the visibility of opaque zones close to the margin.

A comparison of growth zone counts from the three suitable otolith preparation methods within this study revealed that MO1 produced readable and consistent results for *L. intermedius*, *L. tsanensis*, and *L. platydorsus* in Lake Tana. Concerning the reproducibility of growth zone counts derived from MO1 by the two readers, the CV and APE values for *L. intermedius*, *L. tsanensis*, and *L. platydorsus* were lower than the average values in the literature (CV 7.6%, APE 5.5%) [19], while these values calculated from MO2 and MO3 were slightly higher for most species. For *L. megastoma*, the CV and APE values between readers for the three methods were relatively high compared with average values in the literature. With respect to the methods, the CV and APE estimates were lower than the average values in the literature for the four species. The precision of the comparison between methods was generally higher than the precision of the readers. Additionally, except for *L. megastoma*, the count bias plots between readers for MO1 revealed less bias relative to MO2 and MO3. Furthermore, MO2 and MO3 underestimated actual growth zone counts, particularly for old individuals, relative to MO1. This is most likely because of the poor clarity of the images, suggesting that using these methods for precise and accurate growth zone counts of the *Labeobarbus* species is difficult. In contrast, the precision estimates and count bias plots indicated that MO1 provides consistent counts of growth zones for *L. intermedius*, *L. tsanensis*, and *L. platydorsus*. These results sustain the choice of this method for age determination of these species.

The suitability of MO1 for growth zone counts of the three *Labeobarbus* species was further supported by the statistical comparisons and by the assigned scores for readability, which were best for MO1. Except for *L. megastoma*, there was no evidence of a symmetric difference between R1 and R2 for MO1 and MO3, while this was significantly different for MO2 for all species. Although the regression and paired t-test analyses revealed significant difference for some of the cases, the slope and intercept of the linear regression for MO1 were much closer to 1 and 0, respectively, compared with the other methods. Moreover, the mean difference was smaller than 0.18 for MO1, while this difference was generally higher for the remaining methods. Otoliths prepared using MO1 were perceived to have good readability, as indicated by the quality scores given by readers, showing that growth zone interpretation was relatively easy for this method. Both readers felt growth zones on otoliths prepared by MO1 were better defined and relatively easy to interpret and had higher confidence than in growth zone counts they made from MO2 and MO3. More than 99% of the otolith samples prepared using MO1 were readable at AQ1 and AQ2 confidence rankings.

The underestimation of the growth zone counts, particularly for the older fish using MO2 and MO3 relative to MO1, occurs because of the presence of false rings, difficulty in following the growth zones around the otolith, and invisibility of growth zones at the edge of the otolith leading to misinterpretation of the growth zones. Imprecise and inaccurate interpretation of growth zones leads to incorrect estimation of growth and mortality parameters, which in turn may have drastic implications for fish stock assessment and fisheries management. The information generated will be useful to fisheries managers and researchers to select the most appropriate otolith preparation method for age determination of the selected fish species. Therefore, as MO1 resulted in less error, we conclude that this method should be accepted as standard protocol for preparation of the otolith of *L. intermedius*, *L. tsanensis*, and *L. platydorsus* for reading. Concerning *L. megastoma*, more research is needed before a conclusion can be drawn regarding the most suitable method. Description of the best otolith reparation method is the first step in the extraction of useful information about a fish. Although there have been efforts to compare different otolith preparation methods for some of the South African fish species [43], a lot still needs to be done to ascertain optimal method development and method selection for the tropical freshwater fish species. The aim of this study is to fill this gap by describing the best method for the *Labeobarbus* species in Lake Tana and providing a reference for researchers from Africa, particularly Ethiopia, to develop or select suitable otolith preparation methods for the different tropical fish species.

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Appendix A

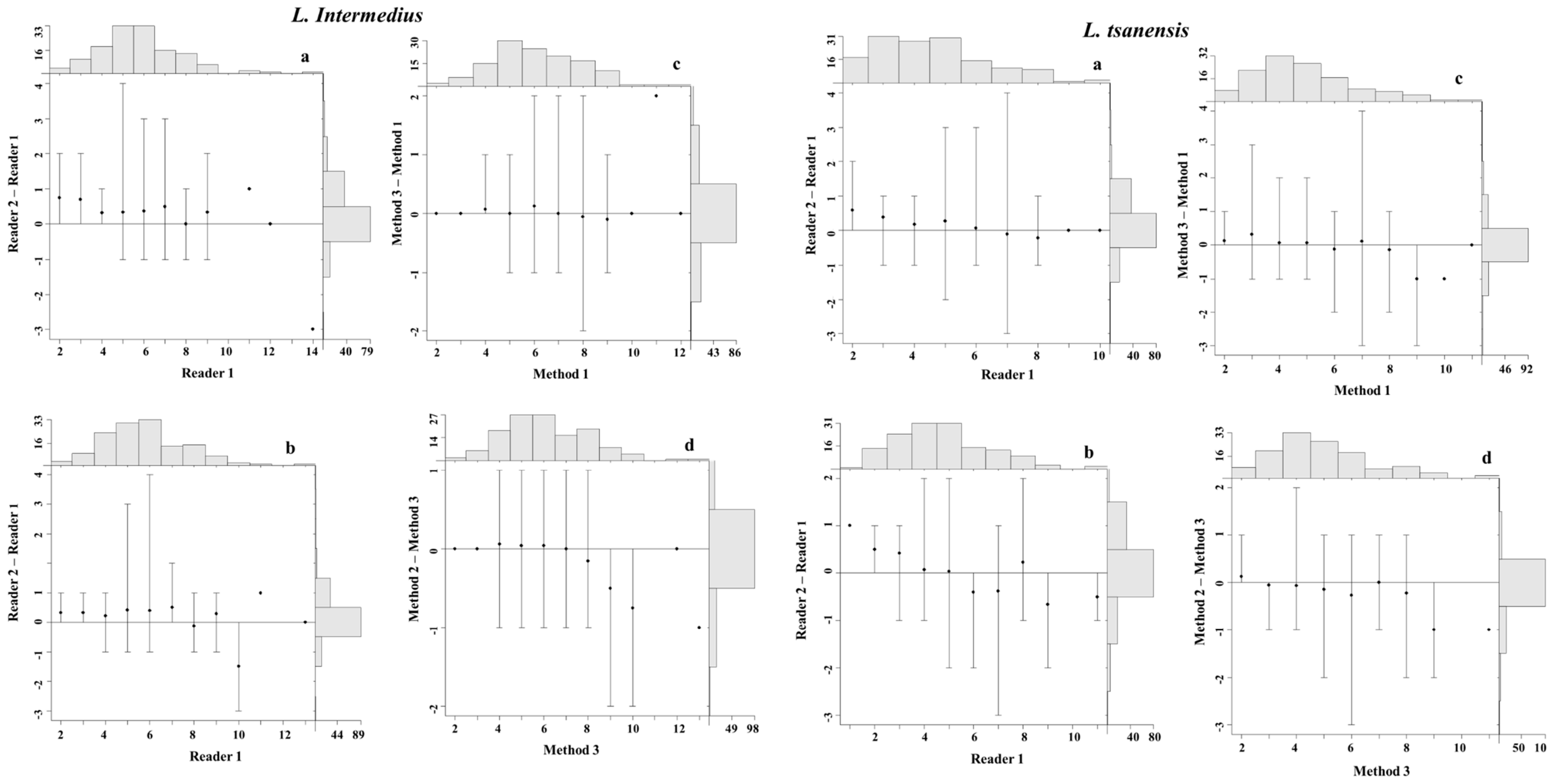

Figure A1. Mean (points) and range (95% confidence interval) of differences in growth zone counts derived from MO2 (**a**) and MO3 (**b**) between two independent readers at the counts for the first reader (**left**) and difference between methods at the counts for MO1 (**c**) and at the counts for MO3 (**d**) for *L. intermedius* and *L. tsanensis* in Lake Tana (**right**). The horizontal solid line is the 1:1 equivalence line and marginal histograms are for growth zone counts of the first reader (**top**) and differences in growth zone counts between readers and methods.

Appendix B

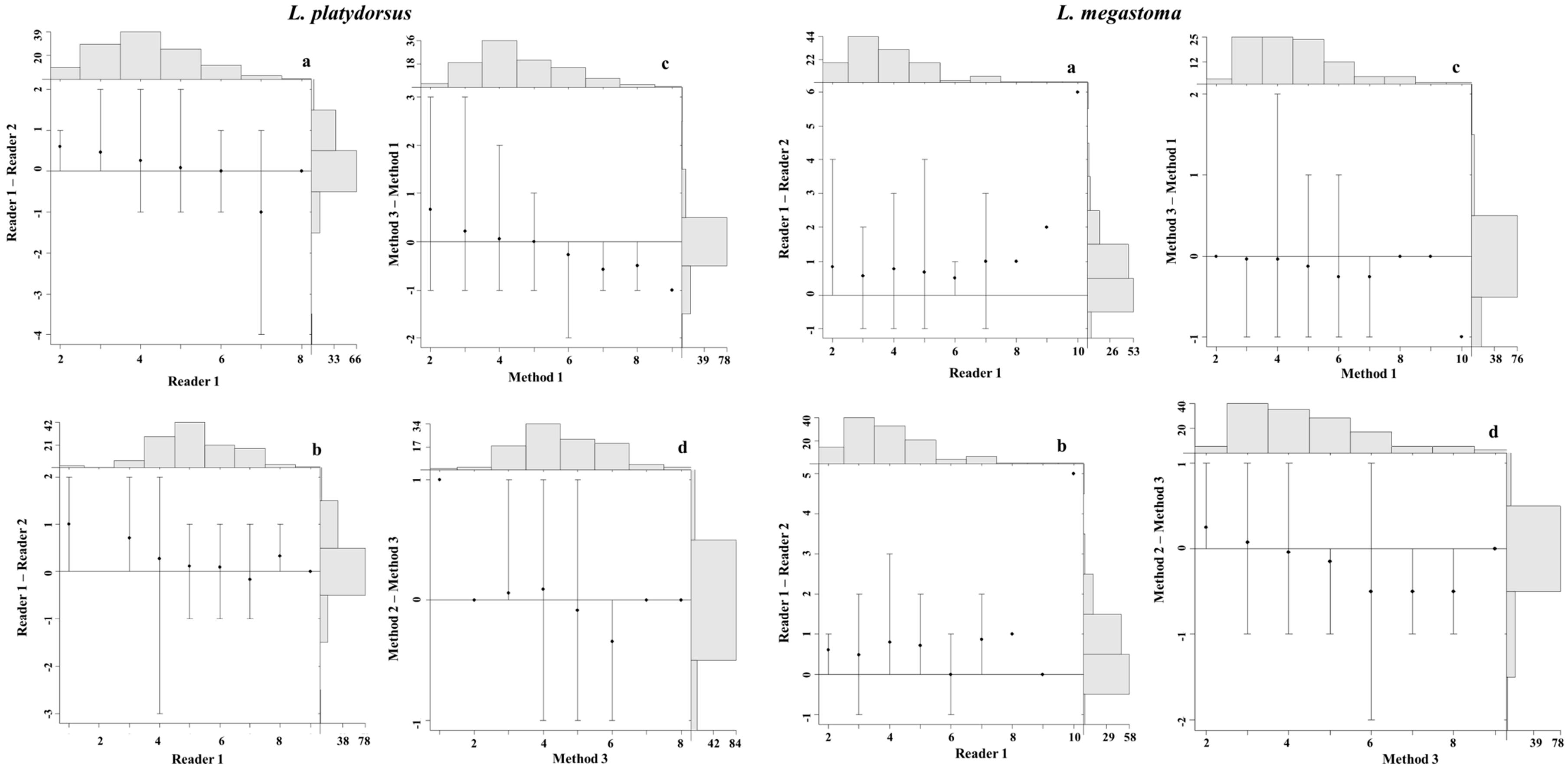

Figure A2. Mean (points) and range (95% confidence interval) of differences in growth zone counts derived from MO2 (**a**) and MO3 (**b**) between two independent readers at the counts for the first reader (**left**) and difference between methods at the counts for MO1 (**c**) and at the counts for MO3 (**d**) for *L. platydorsus* and *L. megastoma* in Lake Tana (**right**). The horizontal solid line is the 1:1 equivalence line and marginal histograms are for growth zone counts of the first reader (**top**) and differences in growth zone counts between readers and methods.

Appendix C

Table A1. Statistical tests (regression and paired t-test) for the detection of bias of growth zone counts of the endemic *Labeobarbus* species between readers and methods.

Statistics	**Readers and Methods Pairs**					
	Reader 1 vs. Reader 2 (MO1)	**Reader1 vs. Reader 2 (MO2)**	**Reader 1 vs. Reader 2 (MO3)**	**MO1 vs. MO2**	**MO1 vs. MO3**	**MO2 vs. MO3**
			L. intermedius			
			Regression			
Slope	0.97 ± 0.03	0.92 ± 0.04	0.95 ± 0.04	0.99 ± 0.02	1.00 ± 0.03	0.92 ± 0.02
p	0.392	0.033	0.248	0.660	0.856	0.000
Intercept	0.31 ± 0.20	0.84 ± 0.23	0.57 ± 0.26	0.03 ± 0.15	−0.01 ± 0.21	0.42 ± 0.15
p	0.137	0.000	0.029	0.826	0.979	0.005
			Paired t-test			
Mean difference	−0.139	−0.345	−0.286	0.031	−0.031	−0.063
p	0.027	0.000	0.000	0.482	0.608	0.184
			L. tsanensis			
			Regression			
Slope	0.92 ± 0.03	0.89 ± 0.04	0.87 ± 0.04	0.90 ± 0.03	0.90 ± 0.04	0.91 ± 0.03
p	0.012	0.007	0.000	0.002	0.009	0.002
Intercept	0.56 ± 0.16	0.72 ± 0.19	0.68 ± 0.18	0.39 ± 0.17	0.54 ± 0.21	0.29 ± 0.15
p	0.000	0.000	0.000	0.021	0.010	0.060
			Paired t-test			
Mean difference	−0.175	−0.231	−0.063	−0.116	0.031	0.147
p	0.006	0.002	0.372	0.063	0.682	0.007
			L. platydorsus			
			Regression			
Slope	0.83 ± 0.04	0.81 ± 0.56	0.87 ± 0.05	0.79 ± 0.04	0.82 ± 0.05	0.90 ± 0.03
p	0.000	0.000	0.004	0.000	0.000	0.002
Intercept	0.09 ± 0.17	1.02 ± 0.24	0.73 ± 0.20	0.91 ± 0.20	0.81 ± 0.22	0.43 ± 0.15
p	0.000	0.000	0.000	0.000	0.000	0.006
			Paired t-test			
Mean difference	−0.068	−0.235	−0.163	0.058	0.019	−0.038
p	0.273	0.001	0.012	0.368	0.774	0.374
			L. megastoma			
			Regression			
Slope	1.04 ± 0.04	1.17 ± 0.06	1.12 ± 0.05	0.89 ± 0.03	0.95 ± 0.03	0.88 ± 0.03
p	0.378	0.006	0.018	0.000	0.145	0.000
Intercept	0.45 ± 0.19	0.12 ± 0.25	0.18 ± 0.21	0.30 ± 0.15	0.12 ± 0.16	0.41 ± 0.15
p	0.020	0.618	0.391	0.048	0.460	0.007
			Paired t-test			
Mean difference	−0.609	−0.760	−0.658	0.212	0.101	−0.111
p	0.000	0.000	0.000	0.000	0.049	0.033

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