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How Does Season Affect Passage Performance and Fatigue of Potamodromous Cyprinids? An Experimental Approach in a Vertical Slot Fishway

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Abstract: Most fishway studies are conducted during the reproductive period, yet uncertainty remains on whether results may be biased if the same studies were performed outside of the migration season. The present study assessed fish passage performance of a potamodromous cyprinid, the Iberian barbel (*Luciobarbus bocagei*), in an experimental full-scale vertical slot fishway during spring (reproductive season) and early-autumn (non-reproductive season). Results revealed that no significant differences were detected on passage performance metrics, except for entry efficiency. However, differences between seasons were noted in the plasma lactate concentration (higher in early-autumn), used as a proxy for muscular fatigue after the fishway navigation. This suggests that, for potamodromous cyprinids, the evaluation of passage performance in fishways does not need to be restricted to the reproductive season and can be extended to early-autumn, when movements associated with shifts in home range may occur. The increased effort during the non-reproductive period suggests that adapting the operational regime of fishways, at biologically meaningful seasons in a year, should be assessed by considering the physiological state of the target species.

Keywords: vertical slot fishway; motivation; season; lactate; potamodromous; cyprinids

1. Introduction

In-stream physical structures, such as dams and weirs, cause the disruption of longitudinal river network connectivity [1,2]. Among the numerous impacts produced by these structures (e.g., water quality, sediment transport, biogeochemical cycles), they cause restrictions to fish movements by blocking the migratory pathways for fish populations [3,4]. Fishways are, generally, the main mitigation solution to reestablish longitudinal connectivity for fish, when obstacle removal is not feasible [5]. The vertical slot fishway (VSF) is considered a good option, particularly when several species are present, since different species can navigate through the slots at their preferred depths [6]. Additionally, the VSF remains operational across a wider range of upstream and downstream water levels, making this design a reliable option for managers [7].

The majority of fishway studies on potamodromous cyprinids are conducted during the reproductive migratory season [8,9], when fish are a priori physiologically motivated to swim upstream. Nevertheless, fish may also undergo considerable movements outside this period, in response to the flow volume and thermal cues or to search for food, evade predators and seek shelter [10–12].

Hence, it is imperative that connectivity is ensured throughout the year, to allow unrestricted fish movements [2].

Motivation, or the willingness to enter and navigate a fishway, is driven by internal and external factors. Internal factors include the physiological condition, such as fatigue level, migratory phase, age, and body size [13], as well as individual predisposition to move upstream. External factors include environmental cues which fish respond to, such as temperature, photoperiod, and flow velocity [14]. Motivation is, therefore, a key factor which drives attraction and passage performance and should be considered in fishway studies [15,16]. However, motivation cannot be measured directly since it depends on numerous factors [14,16]. It is not known how motivation affects attraction and passage efficiency [17], representing a major challenge to understanding fish movements. Further research is needed on the biological mechanisms that activate the migrations of fish to serve as a basis for fishway improvement [18].

The physiological state and the environmental conditions are key elements to evaluate fish passage performance [19]. Physiological methods, such as blood or muscle biochemistry, are valuable to measure fish fatigue [20]. The lactate concentration is indicative of anaerobic swimming and exhaustive exercise [21,22]. Hence, the collection of blood after exercise provides an approach to assess the intensity of passage efforts [23]. While there is extensive literature describing physiological processes occurring in salmonids during exercise, less information is available for potamodromous cyprinids [24,25]. It is known that salmon undergo physiological adjustments during the reproductive migration by increasing oxygen uptake and cardiac output to provide more oxygen to the locomotory muscles [26], while such research on cyprinids is lacking.

The present study focused on testing fish passage performance of the Iberian barbel, *Luciobarbus bocagei* (Steindachner, 1864), a potamodromous cyprinid fish, in a VSF during two distinct seasons: spring, the reproductive season for this species [27], and early-autumn, a non-reproductive season. For this, we hypothesized that passage performance metrics [28,29]—entrance time, entry efficiency, number of upstream movements and number of successes—will not vary in distinct seasons. We further hypothesized that post-exercise plasma lactate concentrations sampled in both seasons, used as a proxy to identify fish fatigue, will not vary between seasons and between the tested and the control fish.

2. Materials and Methods

Fishway navigation of Iberian barbel was assessed in a full-scale fishway facility (10 m long × 1.0 m wide × 1.2 m high) of a VSF, during spring and early-autumn. The fishway was built on a steel frame and had lateral glass walls, which allowed the observation of fish movements during the fish experiments (Figure 1), without interfering with their behavior [8,11,30,31]. The VSF had a total of six pools and the slope was set at 8.5%, which is common for this type of fishway [7]. The facility also included an upstream receiving chamber (1.8 m long × 1.0 m wide × 1.2 m high) and a downstream acclimation chamber (1.0 m long × 1.0 m wide × 1.2 m high). The VSF used in this study included a central and a lateral baffle, and was based on Design 1 proposed by Rajaratnam et al. [32]. The operating discharge was 0.11 $m^3 \cdot s^{-1}$ and the remaining parameters were: hm = 0.80 m (hm is the pool mean water depth); ΔH = 0.16 m (ΔH is the hydraulic head drop per pool); L = 1.88 m (L is the pool length); B = 1.0 m (B is the pool width), and b = 0.10 m (b is the slot width). The discharge was regulated by a pump speed controller and measured with a digital flow meter installed in the supply pipe. Maximum water velocity in the slots was 1.7 $m \cdot s^{-1}$, which roughly corresponds to the maximum theoretical velocity in the slot ($Vs = \sqrt{2g\Delta H}$) where $g = 9.8\ m \cdot s^{-2}$ is the gravitational acceleration [33,34]. At a water depth of 0.50 m, mean ± SE velocity magnitude in the pools was 0.51 ± 0.04 $m \cdot s^{-1}$ (Figure 2). For further details of the velocity measurements and turbulence conditions, see Romão et al. [28].

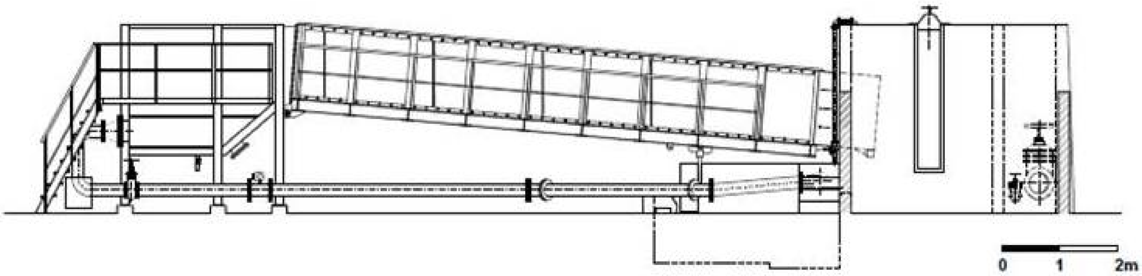

Figure 1. Vertical slot fishway (VSF) experimental flume, set on a slope of 8.5%. Flow runs from left to right.

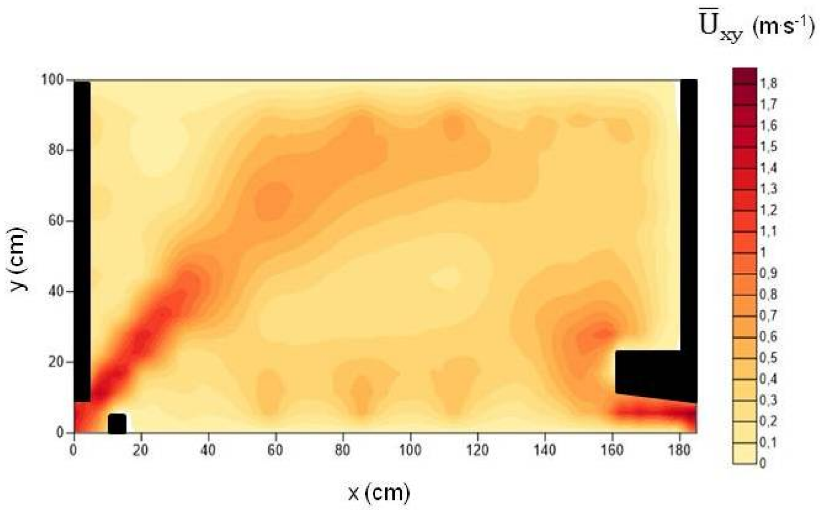

Figure 2. Horizontal mean water flow velocity $\overline{U}_{xy}$ (m·s^{-1}) contour lines at a water depth of 0.50 m. A ADV (Acoustic Doppler Velocimeter) was used to measure the instantaneous velocity components (x and y). Flow enters at the bottom left corner and exits at the bottom right corner.

During the fish experiments, the water quality in the fishway was measured daily using a multiparametric probe (HANNA, HI 9812-5). Water temperature in spring was 19.6 ± 1.2 °C (mean ± SD), pH 7.4 ± 0.2, and conductivity 135 ± 15.0 μS·cm^{-1}, while in early-autumn, the corresponding values were, 22.7 ± 1.1 °C, 8.3 ± 0.1, and 178 ± 4.2 μS·cm^{-1}.

Iberian barbel were captured at the Lizandro river, a coastal river located in Central Portugal. It is a medium-sized river (30 km long, 5–10 m wide) characterized by well-defined sequences of run and riffle sections, alternating with pool habitats. Fish capture was performed by using low-voltage backpack electrofishing (Hans Grassl IG-200) in a 150-m long segment during spring (17 May 2016), the reproductive migration period, and during early-autumn (25 September 2015) when movements, other than reproductive, associated with shifts in home range may occur [35]. Capture procedures followed CEN (2003) standards [36] to encompass multiple habitat types (runs, riffles, and pools). The fish selected for experimentation were chosen within the range of 15–30 cm TL (total length), which represents the typical size range of adult fish of this and other medium-sized benthic potamodromous cyprinids found in Iberian and European rivers (encompassing species from the genera *Barbus* and *Luciobarbus*, which share similar ecological guilds of physical habitat, reproduction, and migratory behavior) [37,38]. This range also ensured the fish were similar in size to avoid bias on swimming performance [39]. Sex was not determined, as previous studies on this species found no relation between swimming performance and sex [40].

In each season, thirty-five I. barbel individuals (mean ± SD: 18.5 ± 2.6 cm TL) were brought to the laboratory facilities in 190 L fish transport boxes (Hans Grassl), with aerated river water to minimize transportation stress. In the laboratory, fish were placed in 700 L holding tanks, with permanent filtration (turnover rate of 2300 L/h) under ambient temperature and natural photoperiod for a minimum of 48 h and a maximum of 72 h prior to the beginning of experiments [28,30]. Water quality in the holding tanks was monitored daily for temperature, pH, and conductivity. In spring, the temperature was 19.5 ± 0.3 °C (mean ± SD), pH 7.2 ± 0.3, and conductivity 158 ± 19.3 $\mu S \cdot cm^{-1}$, while in early-autumn, the corresponding values were 21.4 ± 0.4 °C, 8.0 ± 0.2, and 199 ± 15.2 $\mu S \cdot cm^{-1}$, respectively. During the experiments, the photoperiod (mean ± SD) in spring was 14:20:00 h ± 00:03:20 h, while in early-autumn it was 11:40:44 h ± 00:08:88 h [41].

The experiments were performed between 20 and 27 May (spring) and between 29 September and 7 October (early-autumn). Each experiment was composed of 5 trials, and each trial monitored a school of 5 fish, yielding a total of 10 trials (5 trials × 2 seasons). The unit of analysis was a school of five adult I. barbel with similar size, because this species tends to move in schools, rather than individually, to increase hydrodynamic efficiency [42]; this behavior is advantageous for moving fish [43,44]. However, when working with fish schools, it is very difficult to analyze individuals, especially with similar body size, without increasing excessively the level of manipulation (tagging). Nonetheless, pooled movement data for the school has been used as a valid metric for fish preferences in previous studies [8,28,30,45]. Tested fish were used only once and were randomly selected from the tanks. Before each trial, fish were held 30 min in an acclimation area, which was created at the downstream end of the fishway by two mesh panels 1 m apart, to allow adaptation to the fishway flow conditions. After that period, the upstream mesh panel was removed and the fish were allowed to volitionally ascend the fishway for 90 min. Though motivation is difficult to measure [17,46], quantifying the number and rate of successes towards the fishway entrance and also the number of successful upstream movements to navigate the fishway can help to discern motivation to move upstream [47,48]. Therefore, the following variables were visually assessed during each trial: (i) the entrance time (i.e., the time taken by the first fish to enter the fishway once the trial started); (ii) the entry efficiency (i.e., number of successes in entering the fishway divided by the number of attempts to enter the fishway—when fish were in the direction of the jet passing through the slot while exhibiting their burst swimming mode); (iii) the number of upstream movements (i.e., upstream pool-to-pool displacements of a single individual); and (iv) the number of successes (i.e., number of times that any fish reached the top of the fishway). Video analysis was used to confirm the entry efficiency—the behavior of fish trying to enter and entering the first pool of the fishway.

Immediately after being tested in the VSF, a sample of blood was taken from every individual fish to measure the plasma lactate concentration ($mmol \cdot L^{-1}$)—an indication of muscular fatigue [21,49–51]—using a lactate reader (model Lactate Plus, Nova Biomedical, Walham, MA, USA). No anesthetics were used before the blood sampling, as recommended by some authors [21,52]. The use of lactate readers is a common practice, and it has been validated as a reliable tool for fish physiology [53–55]. For this procedure, fish were first restrained in a V-shaped padded bed where fresh water was continuously provided, and afterwards a syringe was used to remove a 0.1–0.5 mL blood sample from the caudal vasculature. Subsequently, the blood sample was inserted into the slot of the lactate reader. The procedure to collect the blood and insert it into the reader was conducted in less than 1 min [53]. Additionally, lactate concentration was measured in 10 control fish in each season. Control fish were not subjected to experimentation and were kept in the holding tanks (with the same water quality as the tested fish) throughout the experiments, to provide a baseline level (without fatigue) of lactate for each test season. Control individuals were captured from the same population, in the same river segment, and in the same fishing event. Fish were only fed (Tetra Pond sticks) after the end of each trial and blood sampling. After finishing the experimental trials, all fish were taken back and released in their natural habitat. All efforts were made to minimize stress on the tested fish. Table 1 presents: the fish total length, body mass, passage performance metrics (entrance time; entry efficiency; number of upstream movements; number of successes), and lactate concentrations in tested and control fish.

Table 1. Summary of the variables assessed in the Vertical Slot Fishway for the Iberian barbel, *Luciobarbus bocagei* (Steindachner, 1864), in both seasons (spring and early-autumn).

Season	Fish	Trials	Entrance Time (Minutes)	Entry Efficiency (%)	Number of Upstream Movements	Number of Successes	Lactate (mmol·L^{-1})	Total Length (cm)	Body Mass (g)
SPRING	*Tested*	A	14	9.1	35	13	2.5 ± 1.4	20.1 ± 1.8	84.4 ± 21.8
		B	4	8.6	37	4	2.4 ± 1.0	18.4 ± 2.8	70.2 ± 36.1
		C	32	5.8	15	1	3.8 ± 2.0	17.8 ± 1.8	57.0 ± 22.1
		D	7	14.8	36	5	3.6 ± 1.9	17.6 ± 1.5	52.6 ± 13.2
		E	34	8.5	24	4	2.9 ± 1.1	16.8 ± 1.2	45.3 ± 8.0
	Control	-	-	-	-	-	2.9 ± 0.8	19.8 ± 2.3	73.3 ± 30.0
EARLY-AUTUMN	*Tested*	A	24	19.3	20	3	8.0 ± 1.5	17.4 ± 1.7	45.0 ± 17.0
		B	4	10.2	26	7	5.0 ± 1.5	20.3 ± 2.7	71.9 ± 30.0
		C	11	21.7	25	5	7.2 ± 2.3	20.3 ± 1.2	69.4 ± 13.1
		D	11	20.0	23	4	8.3 ± 1.8	17.2 ± 1.5	43.4 ± 13.2
		E	23	16.1	18	5	5.6 ± 0.4	21.4 ± 2.2	83.6 ± 33.8
	Control	-	-	-	-	-	2.9 ± 1.2	17.5 ± 2.5	48.3 ± 21.8

Entrance time (i.e., time taken by the first fish to pass the first slot); entry efficiency (i.e., number of successes in entering the fishway divided by the number of attempts); number of upstream movements (i.e., upstream pool-to-pool displacements of a single individual); number of successes (i.e., number of times any fish reached the top of the fishway); mean [lactate], total length and body mass are presented as mean ± standard deviation (SD).

A Permutational Multivariate Analysis of Variance (PerMANOVA) using the Euclidean distance was performed to search for significant differences, between distinct seasons, in the entrance time, the entry efficiency, the number of upstream fish movements, and the number of successes. Differences in lactate levels between different seasons and between tested and control fish were also explored using PerMANOVA. This approach has the advantage over traditional parametric methods (e.g., ANOVA), as the null distribution of the test statistic is determined using permutations, hence not requiring the often ecologically unrealistic assumption of normally distributed data [56]. It is also a powerful test, which enables significance tests, even for small sample sizes [30,57]. This statistical analysis was used to test the null hypotheses that: (i) season had no effect on the entrance time and entry efficiency; (ii) season had no effect on the number of upstream movements of the I. barbel; and (iii) season had no effect on the number of successes. Additionally, the following null hypotheses were tested: (iv) season had no influence on the measured levels of plasma lactate concentration and (v) plasma lactate concentration was similar between control and tested fish. PerMANOVA tests were performed with the package PerMANOVA for PRIMER +v6.0 [57].

3. Results

The analysis of the passage performance metrics, showed that no significant differences were found in the entrance time (Figure 3a) between seasons ($F = 0.24$, $df = 9$, $p = 0.634$). Therefore, in both seasons, fish displayed similar times to enter the facility, suggesting that season add no effect on this metric. However, significant differences were found in the entry efficiency ($F = 10.42$, $df = 9$, $p = 0.017$), which was higher in early-autumn than in spring (Figure 3b). Concerning the number of upstream movements (Figure 3c), no significant differences were detected neither between spring and early-autumn ($F = 2.36$, $df = 9$, $p = 0.172$), nor in the number of successes ($F = 0.08$, $df = 9$, $p = 0.785$) (Figure 3d). Fish tested in spring and in early-autumn presented similar TL ($F = 3.22$; $df = 49$; $p = 0.084$) and body mass ($F = 0.01$; $df = 49$; $p = 0.915$), suggesting that passage performance was not biased by the size effect, since fish presented similar total length and body mass.

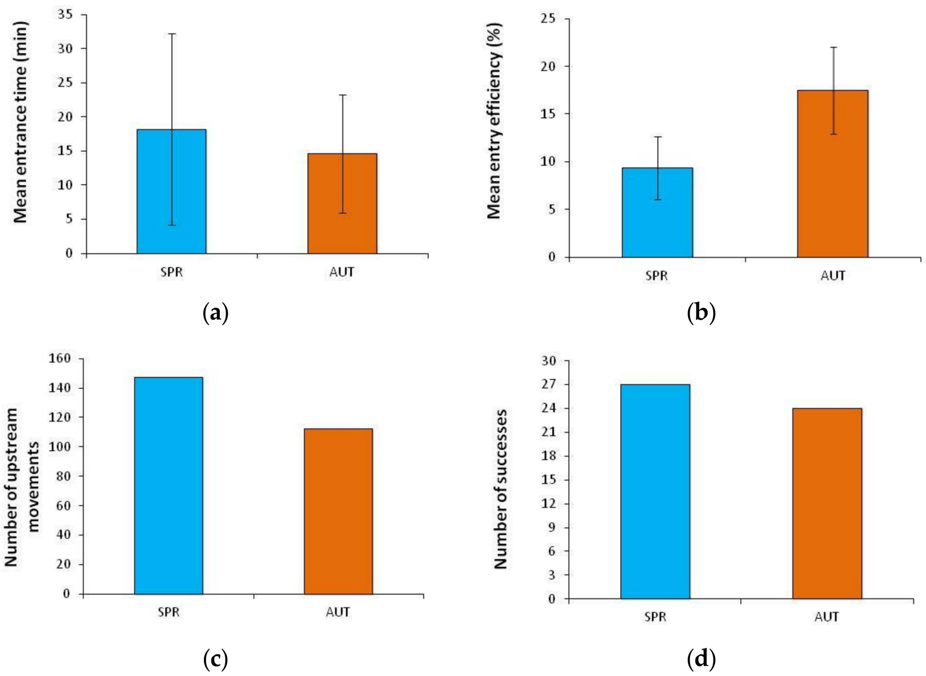

Figure 3. (**a**) Mean entrance time ± SD; (**b**) mean entry efficiency ± SD; (**c**) number of upstream movements; (**d**) number of successes performed by the I. barbel in both seasons (spring-SPR and early-autumn-AUT). Significant differences were found between seasons in the metric, "mean entry efficiency" ($p < 0.05$).

Considering the results from the lactate concentrations, significant differences were detected between seasons (F = 49.95, df = 49, p = 0.001) in the lactate concentrations measured (mmol·L^{-1}). In early-autumn, the lactate concentrations were higher than in spring, however, no differences were found when control fish were compared between seasons (F = 0.62, df = 19, p = 0.429) (Figure 4). When tested fish were compared with control fish, significant differences were found in early-autumn (F = 36.69, df = 34, p = 0.001), although in spring, no significant differences were found (F = 0.26, df = 34, p = 0.603) in the lactate concentrations. Control fish in spring and in early-autumn presented similar TL (F = 3.79; df = 19; p = 0.069) and body mass (F = 3.86; df = 19; p = 0.071), indicating that the differences detected were not due to the size or body mass effect.

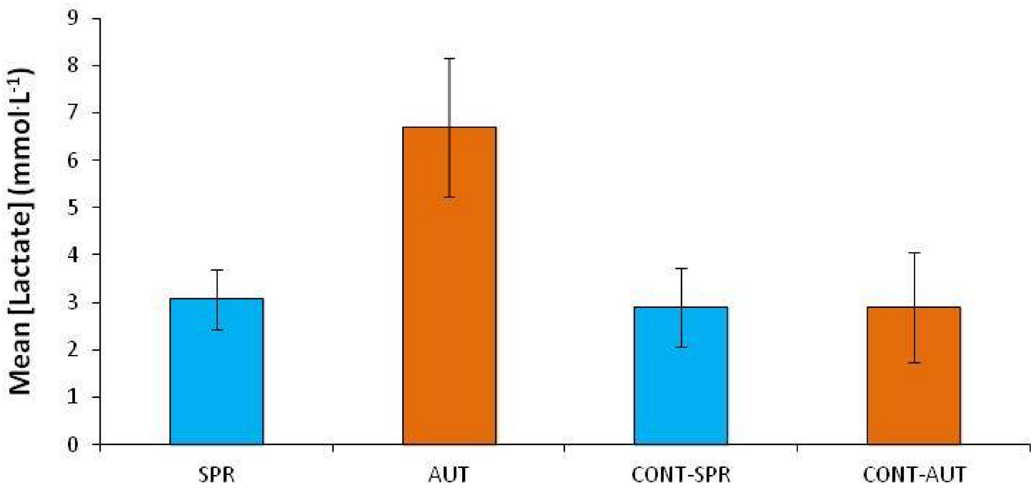

Figure 4. Mean lactate concentration ± SD (mmol·L^{-1}) measured in the I. barbel in both seasons (spring-SPR and autumn-AUT) and in control fish, spring (CONT-SPR) and autumn (CONT-AUT). Significant differences were found between lactate concentrations of tested fish in autumn (AUT) and control fish in autumn (CONT-AUT) (p < 0.05).

4. Discussion

Fish motivation, in combination with environmental and hydraulic conditions, is a key element for an effective passage performance evaluation in fishway studies [20,58]. Ineffective fishways may relate to lack of understanding on how motivation or biological stimuli influence fish passage performance [14,16,58]. In the present study, the fish passage performance of a potamodromous cyprinid was evaluated in two distinct seasons and related to the performance of fish entering and navigating a VSF.

Considering the metrics used to quantify motivation in both seasons, no significant differences were observed on the number of upstream movements and of successes in ascending the fishway, suggesting that motivation to navigate the fishway, and thus passage performance, was similar between early-autumn and spring, the usual period of reproductive migration [27,38,59]. Indeed, in the spring tests, fish exhibited nuptial tubercles, a signal that they were mature and motivated to move upstream as they were within the peak of their spawning season. Results from entry efficiency confirmed that I. barbel were also motivated to pass the VSF outside of their reproductive period. In fact, during the non-reproductive period, I. barbel showed a higher entry efficiency, indicating that motivation to enter the VSF extended beyond the reproductive migration period. According to Roscoe and Hinch [18], the number and rate of attempts to enter a fishway, especially under difficult hydraulic conditions in the field, can help determine motivation to pass the fishway. However, differences in water temperature may bias these results, since temperature influences fish swimming performance [39,60–62]. Even so, Kieffer [63] found that the anaerobic capacity of rainbow trout (*Oncorhynchus mykiss*) was not distinct according to varying water temperatures. So, movements may occur beyond the spawning season, e.g., during early-autumn, even if the photoperiod is shorter and water temperatures higher than those observed in the spring; this is concurrent with their apparent propensity to move upstream outside the spawning period, and it may possibly be related to an active search for refuge, feeding, and exploratory behaviors [35]. Similar results were found by other authors [64] for the European barbel *Barbus barbus* (Linnaeus, 1758), outlining the period between early-summer and early-autumn as a secondary upstream migration period of the adults. When considering the passage performance and motivation of semelparous anadromous fish, mainly salmonids, the passage assessment is much more unambiguous, since fish are highly motivated to pass; however, for potamodromous cyprinids, such as the I. barbel, the circumstances are not so obvious [18]. Furthermore, fishway uses can vary significantly for potamodromous fish among seasonal reproductive migration and other life-cycle activities throughout the year [35]. Fish migrations involve spatial and temporal movements, between spawning, feeding, and searching for refuge habitats [65,66]. Hence, it is necessary to better understand the role of motivation, because some fish may simply lack the motivation to migrate, and bias efficiency estimates in fishway studies [16].

Physiological tools can support the identification of behavioral cues and have the potential to explain passage performance behaviors [20]. Measuring the lactate concentration can be useful to identify the extent to which fish may use anaerobiosis during the fishway ascent, given that, during anaerobic swimming, fish produce metabolic wastes that can be measured in blood samples [20,22–24,60]. Results from plasma lactate measurements in tested and control (untested) I. barbel suggest that physiological adjustments may occur in potamodromous cyprinids in distinct seasons. Significant differences were observed between seasons and between tested and control fish, with the exception of the spring experiments. In this particular case, fish lactate levels were similar to control levels. The I. barbel exhibited a lower concentration of plasma lactate in spring, suggesting that fish could have a higher aerobic swimming capacity during the reproductive migration or a physiological mechanism that inhibits the production or enhances the disposal of metabolic wastes. According to Cooke et al. [26], during migration, the wild adult Pacific salmon (genus *Oncorhynchus*) increases the oxygen uptake and cardiac output to provide oxygen to locomotory muscles. On the other hand, rainbow trout (*Oncorhynchus mykiss*) respond to intense swimming by strongly stimulating lactate disposal. Both strategies seem to carry an equally important role in reducing the lactate produced by fish glycolytic white muscles (produced at higher rates than it can be processed by aerobic tissues such as red muscle and heart) from accumulating in circulation [22]. Additionally, the stage of maturation and the season seem to have affected the swimming performance of Pink salmon (*Oncorhynchus gorbuscha*), where gravid fish were stronger swimmers, and in sockeye salmon (*Oncorhynchus nerka*), where fish tested in summer displayed a higher swimming capacity than that of fish tested in winter [60]. Furthermore, during riverine migration, American shad (*Alosa sapidissima*) also increased the red muscle capacity, as a result of the aerobic needs during this part of their life-cycle [67]. Though it seems evident that physiological adjustments occur in anadromous fish, such information is rarer for potamodromous fish, which might experience similar processes while migrating, as the results from this study suggest.

Water temperature should also be considered when analyzing fish lactate levels in response to stress (i.e., exhaustive exercise; [24]). According to Pottinger [68], when exploring the role of confinement stress response in cyprinids, a clear lactate response was experienced in carp (*Cyprinus carpio*) when the water temperature was between 4 and 8 °C. Nevertheless, when water temperature was 15 °C, few changes in lactate were evident, suggesting a higher capacity to recover from metabolic acidosis or a faster disposal of metabolic waste. Conversely, in this study, the higher lactate levels were exhibited by I. barbel in autumn, when water temperature was higher. However, the mean lactate levels in control fish were similar between both seasons, suggesting that the water temperature had no effect on the measured lactate levels. Consequently, it is worth considering that some species may respond differently to the same stressor (i.e., exhaustive exercise; [69]). According to Kieffer [24,63], the temperature could have a more pronounced effect on the recovery process from a post-exercise response; for instance, the rate of disposal of lactate from the blood, in rainbow trout, was considerably affected by acclimation temperature.

5. Conclusions

Differences were detected between seasons through a physiological component, since I. barbel exhibited a higher concentration of post-exercise lactate levels in early-autumn. Interestingly, the metrics used to characterize motivation to enter and navigate the VSF did not exhibit significant differences between the two seasons, except for the entry efficiency, which was higher in early-autumn. For passage performance studies, several factors, such as behavior, endocrinology, physiology, hydraulics, and spawning habitat distribution, could affect the results, and a better understanding of these factors is required to recognize the mechanisms driving passage success or failure in these type of studies [14,16,20,58,70]. Considering the findings from the present study, fishway studies on potamodromous cyprinids may be extended from the typical reproductive period (spring-early summer) to the early-autumn, when motivation to enter and navigate fishways is still evident, though fish may exert a higher effort during the latter. In addition, fishway monitoring concerning these species should account for motivation beyond the migratory season. This may lead to adapting the operating regime of fishways to be compliant with the behavior and physiological requirements of the target species. Future studies, in the laboratory and in the field, should focus on how the physiological parameters of potamodromous cyprinids, such as the I. barbel, affect the passage performance throughout the year, because this information is still scarce compared to that available for salmonid species.

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Author Contributions: All authors listed have contributed substantially to the manuscript to be included as authors. António N. Pinheiro and Christos Katopodis had a substantial involvement in the conception, guidance, and revising of the manuscript. Filipe Romão, José Maria Santos, and Paulo Branco were involved in the design and data acquisition, both in the laboratory and field work. The data analysis and interpretation was done in collaboration with all authors, as was the final approval of the version to be published. Filipe Romão, Paulo Branco, and José M. Santos wrote the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Ethical Statement: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Animal trials and sampling were conducted in agreement with national and international guidelines to maintain the welfare of the tested fish. Fish samplings were obtained from the Institute for Nature Conservation and Forests (ICNF). Fish experiments were carried out with strict adherence with the recommendations of the "protection of animal use for experimental and scientific work" of the Department for Health and Animal Protection (Direcção de Serviços de Saúde e Protecção Animal) that authorizes animal experiments to be conducted in the experimental facility, and fish to be maintained in the laboratory. All efforts were made to minimize stress, and no fish were sacrificed during the experiments.

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