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Cover or Velocity: What Triggers Iberian Barbel (*Luciobarbus Bocagei*) Refuge Selection under Experimental Hydropeaking Conditions?

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Abstract: The rapid river flow variations due to hydropower production during peak demand periods, known as hydropeaking, causes several ecological impacts. In this study, we assessed the potential of an overhead cover and velocity-refuge structure in an indoor flume as an indirect hydropeaking mitigation measure for the cyprinid species Iberian barbel (*Luciobarbus bocagei*). We designed a lab-scale LUNKERS-type structure using two different materials which were used separately: Wood (opaque) and acrylic (transparent), tested under hydropeaking and base-flow events. Physiological (glucose and lactate) and behavioral (structure use) responses were quantified. The structure use (inside and in the vicinity) and the individual and schooling behavior was assessed. Although there was no evidence that the hydropeaking event triggered a physiological response, the wood structure use was significantly higher than the acrylic one, where the metrics of use increased in the hydropeaking event. Differences between individual and group behavior were only higher for the entrances in the wood structure. The higher frequency of wood structure use under hydropeaking conditions suggests that the visual stimulus conferred by this shaded refuge enables fish to easily find it. The results suggest that the use of overhead and velocity-refuge structures may act as an effective hydropeaking flow-refuge mitigation measure.

Keywords: pulsed flows; fish behavior; flow-refuge; physiology; visual stimulus; cyprinids

1. Introduction

The operation of storage hydropower plants generally causes rapid and artificial flow fluctuations, due to turbines that are started up and shutdown according to the demand of the electricity market, often on daily or sub-daily time scales. This operation mode—called 'hydropeaking' [1], leads to rapid increases or decreases of river discharges, altering the velocity and water depth at the tailrace. The unpredictability and intensity of these discharge variations are rather permanent and frequent than those occurring due to natural flows, such as rapid snowmelt and precipitation [2]. In rivers affected by hydropeaking, hydromorphological characteristics may be significantly altered, including, e.g., substrate composition, banks wetted width and habitat suitability [3–5], with negative consequences for fish species, such as, e.g., stranding, dewatering of spawning grounds, and downstream displacement [6–9]. Those impacts are site-specific and, therefore, mitigation measures should be adapted considering the river morphology together with species–specific ecological requirements [6] such as life-stage [10]. Hydropeaking mitigation measures can be grouped into direct and indirect

measures [11]. Direct measures include specific operational schemes (e.g., decrease the flow ratio), as well as structural measures such as the construction of retention basins or hydropeaking diversion hydropower plants (e.g., [12,13]). Indirect measures address river morphological aspects, aiming to compensate specific negative impacts of hydropeaking by improving fish habitat, either by channel restructuring or velocity refuge implementation (e.g., [14,15]).

Experimental flume-based research has proposed indirect measures to mitigate hydropeaking consequences. For example, lateral refuges [14], deflectors [16], substrate heterogeneity [17], and other cover structures such as velocity refuge, visual isolation and overhead cover [15] have been studied as refuge alternatives for fish during hydropeaking events. Fish refuges have been used to mitigate the displacement and changes in movement patterns caused by hydropeaking on fish [18]. Korman and Campana [19] highlighted the importance of bank refuges to protect fish and other aquatic organisms from rapid flow variations to provide better growth conditions. Visual deterrent systems, such as strobe lights and bubble curtains, have also been studied to prevent fish entering hydraulic circuits of dams and in guiding them to fish passages to reduce fish mortality, but mainly on salmonid species [20]. Only recently, the behavioral sensitivity to light stimuli of cyprinid species has been studied [21]. Generally, fish rely on both visual and hydrodynamic cues to adapt their swimming movements to their surrounding environment [22], but the joining of both factors has not been reported in these studies.

Hydropeaking can be a threat to the fish homeostatic state as well. Changes in glucose and lactate levels have been widely used as secondary physiological indicators of stress to flow variability. Increases in the glucose levels are usually directly associated with primary responses to stress, and the lactate levels will likely increase when aerobic swimming is no longer sufficient to maintain sustained swimming, resulting in exhaustion [23]. Therefore, it is expected that physiological responses under hydropeaking conditions will be higher than those in base-flow conditions, though the direction and range of those responses is difficult to determine [24]. Recent studies demonstrated that hydropeaking triggers distinct physiological and behavioral responses, according to the combination of hydrodynamic conditions with the presence of lateral and instream structures (e.g., [16,25]). However, the potential of overhead cover and velocity-refuge structures to trigger physiological changes under similar conditions has not yet been tested.

LUNKERS (little underwater neighborhood keepers encompassing rheotactic salmonids) are wooden structures installed along the river bank to create overhead cover and velocity-refuge for fish, as an alternative for trout stream habitat improvement [26,27]. Inside the LUNKERS cavities, velocities are expected to be reduced up to around 50% [28]. LUNKERS require flow entering the upstream end of the structure and sweeping through them to maintain the void created by the spacer blocks of the structure, which should remain completely submerged as periodic wetting and drying will encourage premature decay and eventual failure. They are often used in a series along with other bank stabilization measures or riparian restoration projects, as they are beneficial for adding in-stream fish habitat in river reaches where it is challenging to add habitat by other means, also improving instream habitat conditions by providing a sanctuary for fish [27]. LUNKERS are also useful for habitat improving for nonsalmonid species, where cyprinid species, typically of lower swimming performance compared to salmonids [29], can find refuge under harsh flow conditions [30,31]. These structures have been also used for providing cover and shade along channel banks where riparian vegetation has been eliminated, which can also be referred to as *bankhides* [26].

To our knowledge, there are no studies addressing the potential of overhead cover and velocity-refuge structures for cyprinids under highly fluctuating flow environments. Furthermore, the interaction with additional stimuli sources (e.g., different materials) has been scarcely addressed. The study was carried out in an indoor flume, where we analyzed physiological and behavioural responses of the Iberian barbel species (*Luciobarbus bocagei*, Steindachner 1864). Young adults of *L. bocagei* were used for this study, as they are adapted to faster flow conditions, demonstrated to favor rheotactic behavior [25,32]. Thus, by subjecting fish to a hydropeaking event in the presence of structures that provide different hydraulic (base-flow vs. hydropeaking) and visual

(opaque vs. transparent) stimuli, our objective was to determine whether it was critical for *L. bocagei* to use the structure as a flow-refuging area. In this study, we addressed the following questions: (1) Are the blood lactate and glucose levels of *L. bocagei* altered under hydropeaking conditions in the presence of LUNKERS-type structures? (2) Does *L. bocagei* use the structures as areas of reduced flows under hydropeaking conditions? (3) Is the visual stimulus (opaque vs. transparent) critical to fish that use the structures as a refuge under rapid flow fluctuations?

2. Materials and Methods

2.1. Fish Sampling and Handling

The Iberian barbel is an endemic potamodromous cyprinid of the Iberian Peninsula, widely distributed in the river basins of northern and central Portugal [33]. The fingerlings, juveniles, and spawning adults are predominantly rheophilic, and outside the reproductive season the adults of *L. bocagei* tend to be limnophilic [34,35]. *L. bocagei* individuals were captured at the Sorraia River (39.011376° N, −8.357126° W), a left tributary of the Tagus River (central Portugal). The sampling site is not affected by hydropeaking which makes it a suitable source of fish that are unconditioned to artificial flow fluctuations. Fish were sampled once a week in four consecutive weeks between 9 and 13 November 2018 using a low-voltage (400 V) electrofishing gear (Hans Grassl IG-200, Schönau am Königsee, Germany), according to the norms defined by the European Committee for Standardization (CEN) [36] and national guidelines [37]. No more than 50 fish were captured per each sampling occasion, resulting in a total of 200 captured fish (mean total length ± SD; 13.9 ± 2.7 cm; mean total weight ± SD; 27.0 ± 14.8 g). After electrofishing, fish were transported to the laboratory in an aerated transport tank (Linn Thermoport 190 l, Lennestadt-Oedingen, Germany).

In the laboratory, *L. bocagei* were distributed between two 900 L holding tanks, with continuously aerated and biologically filtered water, and acclimated to ambient temperature and natural photoperiod for 72 h. Feeding took place only after the acclimation period. Water quality parameters (mean ± SD) were monitored in a daily basis using a multi parameter probe (YSI 556 MPS, Yellow Springs, OH 45387, USA) for temperature (18.36 ± 1.02 °C), pH (7.71 ± 0.13), dissolved oxygen (8.67 ± 0.54 mg/L), oxygen saturation (93.01% ± 6.06%) and conductivity (287.33 ± 66.1 μS/cm), and in a weekly basis using colorimetric methods for nitrites (0.02 ± 0.02 mg/L) and ammonia (0.001 ± 0.001 mg/L). Partial water changes (ca. 15%) were performed every other day. Each tank was covered with a sunshade mesh, and clay roof tiles and PVC pipes were placed in the bottom to provide refuge areas. The flume water temperature (20.21 ± 1.60 °C), pH (7.86 ± 0.12), dissolved oxygen (9.00 ± 0.49 mg/L), oxygen saturation (100.3 ± 8.03%) and conductivity (259.66 ± 9.79 μS/cm), as well as the air temperature (16.9 ± 1.36 °C) were monitored before and after each experiment. The water quality parameters were according to the national legislation for water quality standards to protect and improve the aquatic environment according to water use [38]. Fish were fed with a commercial diet for benthic species every night to avoid additional stress caused by food deprivation. During the experimental procedures no fish were sacrificed and all efforts were made to minimize stress during capture, transport, and handling. At the end of each week, fish were returned to their natural habitat.

2.2. Experimental Facilities

The experiments were conducted between 12 November and 6 December 2018, in an indoor flume located at the Laboratory of Hydraulics at the University of Lisbon, Portugal (Figure 1). The flume has a rectangular cross-section and is built on a steel frame with glass panels on both sides. An upstream reservoir controlled by a plane gate enabled rapid discharge variations, and a downstream flap gate controlled the water level. The usable flume length was limited to 6.5 m using two perforated metallic panels and the width was 0.7 m (Figure 1a,b).
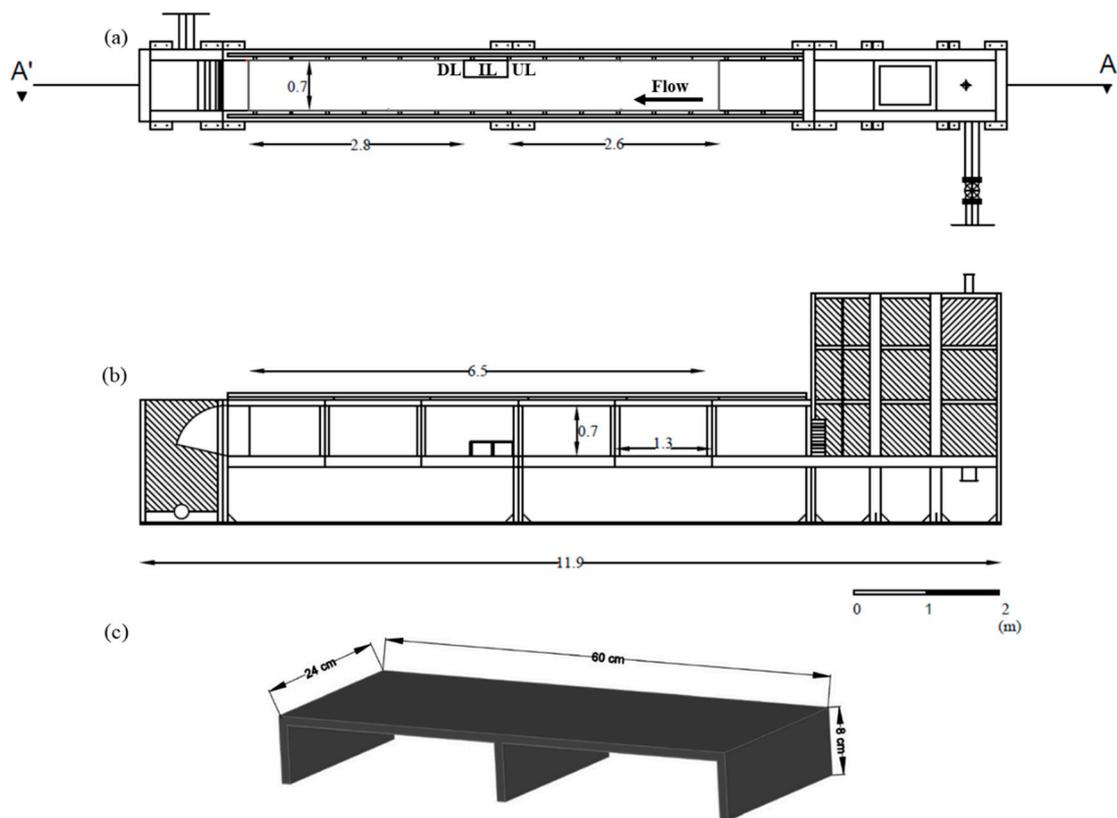
Figure 1. Top (**a**) and lateral (**b**) view of the indoor experimental flume with the dimensions (m), location of the L-structure and correspondent behavior observation areas (DL: Downstream L-structure; IL: Inside L-structure; UL: Upstream L-structure); (**c**) L-structure design and dimensions (cm).

To assess the potential of overhead cover and velocity-refuge to provide flow protection areas in hydropeaking conditions, we tested a LUNKERS-type structure, hereinafter L-structure. The study was divided into two experiments, according to the L-structure material: In the first, we tested a wood L-structure (LW) to create a shaded refuge; in the second we tested an acrylic L-structure (LA), creating a transparent refuge, this way testing fish visual attraction. Both wood and acrylic L-structures had the same dimensions with a 1 cm thickness (Figure 1c), were installed in the same flume area, and tested separately (Figure 1a,b).

2.3. Flow Events

Given the two discharges in the test, i.e., one peak discharge (60 L/s) and the base-flow discharge (7 L/s), the tested flow ratio, defined as the maximum flow divided by the minimum flow [39], was 1:8.6 (QBase:QPeak), which is considered high and above the national legal regulations from Switzerland (1:1.5), Austria, and Bolzano province, Italy (1:3) [6].

Before each event, fish acclimated in the flume for 120 min with a 7 L/s discharge (Table 1). During this period the flume upstream gate was open at a 10° angle. The downstream gate was fixed at a 76° angle during all experiments. The hydropeaking event consisted of a single upramping event where fish were subjected to the peak discharge for 40 min after the acclimation period. To simulate the hydropeaking event, the upstream gate was partially closed to fill the flume reservoir. The discharge was manually controlled until attaining 60 L/s. Afterwards, the upstream gate was opened to 10°, releasing the peak flow until reaching the permanent regime. The velocities in the flume [16] (i.e., 0.18 m/s for base-flow and 0.71 m/s for peak-flow) are in accordance with the suitability curves for juveniles and adults [34,40] and the sustained swimming ability [41] of *L. bocagei* (Table 1). As two

L-structures (LW and LA) and two flow events were tested (HP and BF), the following experiments were conducted: LWHP, LWBF, LAHP, and LABF.

Table 1. Flow events tested, base-flow (BF) and hydropeaking (HP), for each tested L-structure (wood and acrylic) with the respective discharge (L/s), time of exposure (min), water depth (cm), and maximum measured velocities in the flume (m/s).

Event	Number of Replicates	Discharge (L/s)	Time of Exposure [1] (min)	Water Depth (cm)	Maximum Registered Velocity [2] (m/s)
BF	9	7	40	8.5	0.18
HP	9	60	40	25	0.71

[1] After the 120 min acclimation period with a 7 L/s discharge; [2] From Costa et al. [16].

For each experiment, we tested a school of five *L. bocagei* individuals and replicated it nine times. Each school was tested only once. The selected number of replicates and group size is currently accepted for this type of research (e.g., [16,25,32,42–45]). Larger schools of *L. bocagei* likely occur in nature. However, this school size was selected to optimize the visual observations of structure use and to reproduce a representative group behavior [46].

2.4. Physiological Responses

For the present research we quantified *L. bocagei* physiological responses to find if there were differences between both L-structures, in hydropeaking and base-flow conditions. After each experiment, fish were dip-netted from the flume and transferred to individual recipients with continuously aerated water and placed in a v-shaped plastic trough in a supine position. Blood samples (0.1–0.5 mL) were collected via caudal puncture using 23 or 25 G preheparinized needles within 3 min of capture. It has been demonstrated that this timespan is not long enough to have a significant influence on primary stress responses (e.g., cortisol) [47]. The glucose and lactate levels were immediately measured using the portable meters Accu-check Aviva (Roche) and Lactate Plus (Nova Biomedical UK), respectively. These meters have been previously tested and validated for fish-stress research [48–50].

For each experiment, we conducted a Kruskal–Wallis analysis to verify whether the levels of blood glucose and lactate were different between replicates. Afterwards, when the assumptions for the application of parametric tests were verified, the one-way analysis of variance (ANOVA) was used to verify whether there were differences in the physiological responses between events for both L-structure [51]. When at least one of the parametric assumptions was not verified, the nonparametric Kruskal–Wallis test was used instead [51]. These statistical analyses were performed for $\alpha = 0.05$ using NCSS software version 11 [52].

2.5. Structure Use

To evaluate the potential of the L-structures to provide flow-refuging areas under hydropeaking conditions for *L. bocagei*, and the effect of the different materials, we quantified the frequency of the L-structure use and the time spent inside it. The frequency was defined as the number of occurrences, in absolute frequency, over the duration of the flow event (i.e., 40 min). We attributed each metric to a single fish (I) or to a group of two to five fishes (G). We considered a single structure use when one fish used the L-structure isolated from the group, whereas group behavior was considered when two to five fish used the structure as a school. We registered the frequency considering two areas of the L-structure: Outside and inside areas (Figure 1a). For the outside area two L-structure regions were considered: The upstream (UL) and downstream vicinities (DL). For the inside area (IL) we registered the frequency of entrances in the L-structure. The time spent inside the L-structure was quantified considering only when at least one individual spent five or more seconds inside it, otherwise the entrance was attributed to chance. The structure use was visually assessed by two observers (one for time and one for frequency), who registered the behavior occurrences on an ethogram sheet.

　　　We used a permutational multivariate analysis of variance (PerMANOVA) with the Euclidean distance considering two factors with two levels each: The L-structure material (LW and LA) and event (HP and BF). PerMANOVA was performed to test the null hypotheses that the number of approaches to the vicinities and the entrances to the L-structure were not significantly affected by those factors. PerMANOVA is a powerful nonparametric technique that relies on permutation to make significance tests of small-sized samples possible [53,54]. This approach offers the advantage that the null distribution of the statistical test is determined using permutations, thus not requiring the assumption of normally distributed data [55]. PerMANOVA tests were performed for $\alpha = 0.05$ with the package "PerMANOVA" for PRIMER v 7.0 [56]. The t-test pairwise analyses were computed in the same software following the same assumptions.

　　　After the PerMANOVA analysis, Kruskal–Wallis multiple comparison tests were computed for each of the six response metrics for all experimental sets. The pairwise comparisons analysis for each metric was performed with a Dunn's post-hoc test.

　　　Finally, to assess the statistical differences between group and individual responses, a Wilcoxon test for two-sample analysis was used [51]. These statistical analyses were performed for $\alpha = 0.05$ using the NCSS software version 11 [52].

3. Results

3.1. Physiological Responses

　　　After the Kruskal–Wallis analysis to verify whether the levels of blood glucose and lactate were different between replicates, there was not statistical evidence supporting this hypothesis, thus the physiological responses of each *L. bocagei* were considered as true replicates.

　　　Considering the wood L-structure (LW), the mean (± SD) levels of blood lactate in *L. bocagei* were 3.0 ± 1.6 and 3.1 ± 1.6 mM for the base-flow (LWBF) and hydropeaking (LWHP) experiments, respectively. In the acrylic L-structure experiments (LA), the mean (± SD) lactate levels were 3.3 ± 1.4 and 3.4 ± 2.0 mM for the base-flow (LABF) and peak-flow (LAHP) experiments, respectively (Figure 2a).
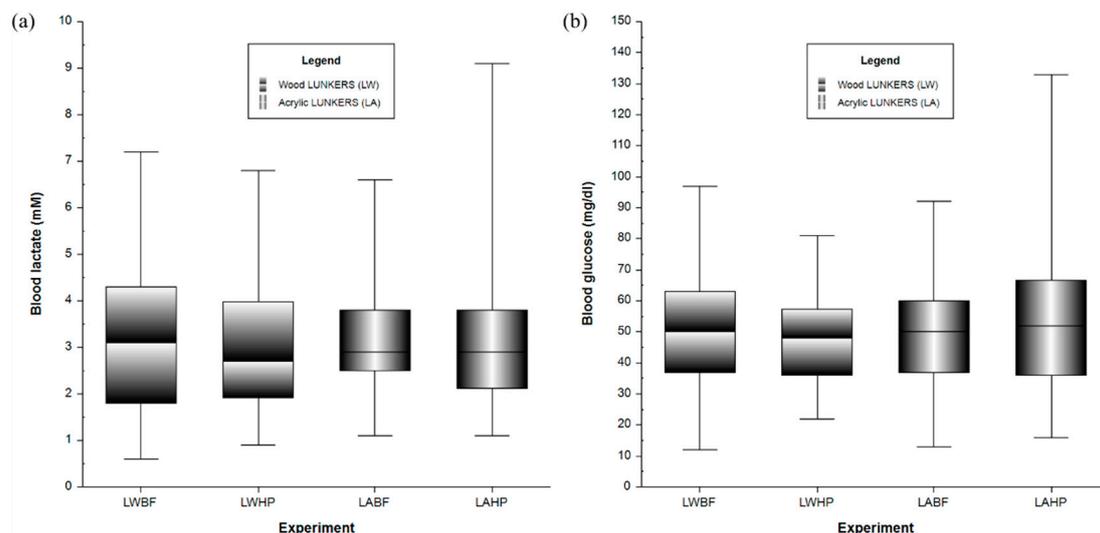

Figure 2. Boxplots of the variation of (**a**) blood lactate (mM) and (**b**) blood glucose (mg/dL) levels for *L. bocagei* (n = 45) for both base-flow (BF) and hydropeaking (HP) events, and for both L-structure experiments (LW: Wood and LA: Acrylic).

　　　The mean (± SD) levels of blood glucose in *L. bocagei* individuals were 51.7 ± 21.3 and 47.8 ± 15.1 mg/dL for LWBF and LWHP, respectively. The mean (± SD) glucose levels in *L. bocagei* were 48.3 ± 17.4 and 54.4 ± 24.1 mg/dL for LABF and LAHP, respectively (Figure 2b).

When comparing the results from both L-structure experiments, the lactate levels were not significantly different ($\chi^2 = 0.465$, $p = 0.926$), neither the glucose levels (F = 1.003; $p = 0.393$).

3.2. Structure Use

The mean frequency (± SEM) of the number of occurrences of structure use for the nine replicates at all four experiments is presented in Figure 3. All measured metrics mean frequencies were higher in the hydropeaking (HP) when comparing with the base-flow (BF) experiments for both tested structures. In the LA structure the mean frequency values were always lower than in the LW, except for upstream approaches in group (UL_G) results (Figure 3).
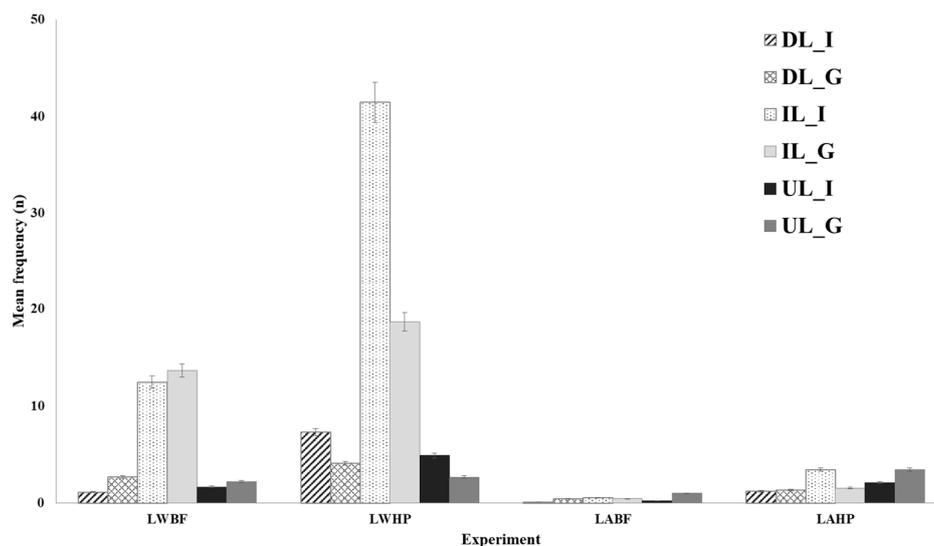
Figure 3. Mean frequency (± SEM) for the wood (LW) and acrylic (LA) L-structures use by *L. bocagei* (n = 45) for base-flow (BF) and hydropeaking (HP) events at the three structures areas: Downstream (DL_I and DL_G) and upstream (UL_I and UL_G) approaches and inside the L-structure (IL_I and IL_G) (I: Individual; G: Group).

The PerMANOVA analysis showed a significant effect of the structure factor (F = 4.68; $p = 0.002$). Although individual (I) and group (G) results were higher in the peak event (Figure 3), there was no effect of the event factor (F = 2.30; $p = 0.087$). The interaction of both factors also showed no effect (F = 1.48; $p = 0.251$) on the number of approaches to the outside areas (UL and DL) and in the entrances to the L-structures (IL). Pairwise comparisons performed for the structure factor showed that for both events (BF and HP) the number of approaches and entrances differed significantly (Table 2).

Table 2. Pairwise comparisons after the main test (PerMANOVA) on the frequency of the number of approaches to the outside areas (UL and DL) and entrances (IL) in the L-structures by *L. bocagei* (n = 45) regarding the structure factor.

Factor	Pairwise Comparison	Results	
		t	p
Structure	LWHP vs. LAHP	1.70	0.007
	LWBF vs. LABF	2.00	0.003

Regarding each structure use metric, the results from the Kruskal–Wallis analysis showed that, for downstream approaches (DL) and the structure entrances (IL), there were significant differences ($p < 0.05$) among events for both individual (I) and group (G) behavior (Table 3). For the upstream approaches, only the group results (UL_G) did not present significant differences ($\chi^2 = 4.41$, $p = 0.22$). In Table 3 the

significant different pairs, resulting from the Dunn's post-hoc test for pairwise comparisons (z-value > 1.96), are also presented. When assessing the pairwise results between events for the same L-structure, there were only significant differences for DL_I in the LW structure (LWBF vs. LWHP). For the other pairwise comparisons, significant differences were obtained only between the two L-structures (Table 3).

Table 3. Kruskal–Wallis results for the behavioral responses from *L. bocagei* (n = 45) in each L-structure (LW and LA) and event (BF and HP) and the correspondent pairwise significant differences (Dunn's z-value > 1.96).

Tested Metrics	Kruskal–Wallis Multiple Comparison Tests		Pairwise Significant Differences
	χ^2	p	
DL_I	19.145	0.0002	LWBF vs. LWHP LWHP vs. LABF LWHP vs. LAHP
DL_G	9.901	0.01	LWHP vs. LABF LWBF vs. LABF
IL_I	21.217	0.00009	LWBF vs. LABF LWHP vs. LABF LWHP vs. LAHP
IL_G	23.114	0.00004	LWBF vs. LABF LWBF vs. LAHP LWHP vs. LABF LWHP vs. LAHP
UL_I	11.891	0.007	LWHP vs. LABF
UL_G	4.41	0.22	-

After the Wilcoxon test for two-sample analysis, only in LWHP the individual entrances (IL_I) were significantly higher in comparison with group entrances (IL_G) (Z = 2.428; p = 0.015), and there were no significant differences between group and individual entrances (IL) and approaches (DL and UL) for the other events and structures.

Figure 4 presents the measured time from all nine replicates for both BF and HP events, for each L-structure. Although the BF event in the wood structure presented a lower number of entrances (IL) in both individual (I) and group (G) results (Figure 3), the average time (mean ± SD) fish spent inside it was higher (12.52 min ± 14.32) than in the HP events (9.58 min ± 11.06) (Figure 4). Fish were never inside the acrylic L-structure for more than five consecutive seconds.
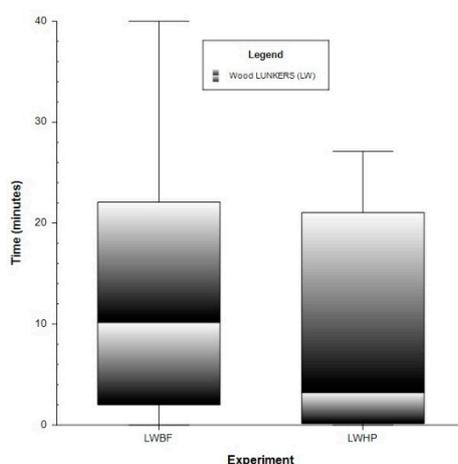

Figure 4. Boxplots of the time (min) that *L. bocagei* individuals have continuously spent inside the wood L-structure.

4. Discussion

In this study we assessed the potential of wood and acrylic LUNKERS-type structures as an indirect hydropeaking mitigation measure for the *L. bocagei* in an indoor flume. We used a multidisciplinary approach that combined physiological and behavioral responses. With the two L-structures materials, it was possible to additionally test the effect of cover type in the velocity-refuge.

There was no statistical evidence that, in hydropeaking conditions, the different visual stimulus conferred by opaque (LW-wood) or transparent (LA-acrylic) structures triggered physiological changes. The results suggest that the created hydraulic conditions were not severe enough to trigger physiological adjustments. Similar results were obtained by Costa et al. [32] and Flodmark et al. [57] where no statistical differences in the blood lactate or glucose responses were found. The results for blood glucose and lactate were similar between both events with the wood L-structure (LWBF and LWHP), suggesting that the opacity of this structure may prevent physiological adjustments. The direction and range to establish a causal relation between flow variability and potential stress responses is difficult to determine. Reported explanations for this difficulty include the time from stimulus perception to sample collection, the effects of other environmental and biological factors and the experimental conditions (laboratory vs. field experiments) [24]. Thus, performing indoor experiments that minimize the confounding effects of external factors, and combine them with adequate physiological indicators, has been encouraged [9,57] and further research may reveal the thresholds which trigger physiological responses on fish species subject to hydropeaking conditions.

The structure use results indicate that *L. bocagei* responses are more related to the combination of the flow increase and the tested L-structure material, rather than solely to the hydropeaking event itself. Although *L. bocagei* used more the assessed structure areas (outside and inside), during the hydropeaking event in comparison with the base-flow event in both L-structures, this distinction was clearly evident in the LW results (Figure 3). This suggests that the fish used the low velocity areas to recover from the harsh hydropeaking conditions. Alexandre et al. [58] studied *L. bocagei* movement patterns, home range size and habitat selection in regulated and nonregulated Portuguese rivers, and found out that in the regulated river these fish seemed to occupy habitats with coarser substrate, often located near the margin, contrarily to the individuals inhabiting the nonregulated river. Authors assumed it was a behavior to avoid displacement during artificial high flow events, where fish could use solid substratum and marginal cover as velocity-refuge areas, which is in line with our results. Increases in behavioral metrics associated with peak discharges have also been reported in indoor and outdoor experimental flumes (e.g., [14,16,25,32,59]), as well as in rivers affected by hydropeaking (e.g., [60–63]).

In the acrylic L-structure (LA), the approaches for both DL and UL vicinities were similar to the IL results (Figure 3). However, the frequency differences between IL and both DL and UL approaches in the wood LW structure during HP, evidences *L. bocagei* being triggered by the visual stimulus (i.e., shaded cover), in addition to the velocity-refuge conditions provided by the L-structure. As well, the LA structure use presented lower values in all metrics when compared to the LW results except for the UL_G (Figure 3), with several pairwise significant differences between structures (Table 3). This was particularly clear in both group and individual entrances (IL_I and IL_G). The PerMANOVA statistical analysis also supports the clear evidence that fish were triggered by the cover protection from the opaque wood refuge. This statistical result showed that the structure factor produced a significant effect on fish approaches and entrances between the two L-structures. Although the structure use frequencies were higher in the HP results for all metrics when compared to BF values, particularly for the LW (Figure 3), PerMANOVA showed no significant effect between the two events. In nature, the adult *L. bocagei* individuals, outside of the reproduction season, are known to be a limnophilic benthic species that uses cobbles and vegetation as shelter [34,35], being in line with our results regarding fish being attracted by the shaded refuge. These results are also in accordance with Dijkgraaf [64], where the author concluded that the visual system appears to be the most likely sensory channel to provide cyprinid fish with a reference point as an indicator of body displacement, since with given

visual cues, the presence or absence of a functional lateral line made no difference on triggering a rheotactic response. Nevertheless, the use of cover is dependent on the season and ontogeny [15,40,65]. Santos et al. [40] found *L. bocagei* to use more covered areas in autumn than in warmer seasons and microhabitat use was affected by *L. bocagei* differently depending on the size class. In our study, which reports the fragment associated with young adults of *L. bocagei* in the autumn season, the same pattern was observed.

The time *L. bocagei* individuals have spent inside the structure also indicates a clear distinction between L-structures material selection, and less pronounced between the studied events. In the LW, the higher activity induced by the hydropeaking events decreased the continuous time that fish spent inside the overhead cover and velocity-refuge structure. In the BF experiments, fish spent the total time of the experiment inside the structure in at least one of the replicates, which did not happen in any hydropeaking event where fish were more active, spending less time inside it (Figure 4). On the opposite, in the LA structure we did not register a continuous stay of any individual for more than five consecutive seconds.

The differences observed in both individual and group behavior between HP and BF events showed that the flow conditions were not always favorable to maintain schooling (Figure 3). Group disruption was particularly marked in the entrances frequency (IL) in the LW hydropeaking experiments (LWHP), where the statistical analysis resulted in significant differences between IL_G and IL_I. Similar results were obtained by Costa et al. [16,25,32], where authors observed an increase of schooling disruption with the peak discharge. The advantages of schooling behavior have been demonstrated to reduce the total swimming costs [22,66], including during reproductive migrations [67,68]. The schooling disruption may also impose an additional stress for fish to cope with hydropeaking conditions [32].

The ecological impacts of hydropeaking can result in the reduction of fish suitable habitat and favorable areas for refuge [5,6,69]. Under those conditions, fish may benefit from the presence of instream structures for flow-refuging [11,15,25]. Thus, when designing morphological mitigation measures, it is strongly recommended to create habitats that remain stable during rapid flow variation, while assuring flow refuge areas during high velocities and water connectivity with the main channel [14]. The L-structure proposed in our study seemed to provide the suitable velocity-refuge for fish regarding the frequency of use during the hydropeaking event. Thus, the studied L-structure, acting as an overhead and velocity-refuge instream structure, may be considered as a potential indirect hydropeaking mitigation measure. The introduction of such structures in nature should therefore take into account the interaction between the river stretch hydromorphology and the hydropeaking-induced water level variation, since this structure should be always submerged to assure its durability and effectiveness [27]. Iberian rivers are influenced by the Mediterranean climate, corresponding to low water availability in summer compared with winter conditions, where environmental flows cannot exceed those of the natural flow regime [70]. During summer periods the flow ratio can be up to eight-fold, the daily peak frequency two-fold, and peak duration lasts from 1 to 2 h [65], which should also be considered for the implementation of such mitigation structures. Although we have studied the potential of the structure's upstream and downstream vicinities for flow-refuging as well, the known case studies where the LUNKERS are implemented did not consider these areas in its uses. Nevertheless, we have found that it may increase the LUNKERS-type structures potential for hydropeaking mitigation purposes.

5. Conclusions

Our study provided a novel insight for alternative fish refuge solutions, based on an overhead and velocity-refuge LUNKERS-type structure, to mitigate hydropeaking impacts for an endemic cyprinid species. With our experimental results it was clear that, under the simulated conditions, there were no advantages on using only a hydraulic mitigation structure for *L. bocagei*, without creating an opaque refuge acting as a shaded shelter. Likewise, the wood L-structure proved to trigger flow-refuging behavior on *L. bocagei* individuals during the hydropeaking events, promoting, as well, sheltering during

the low-flow periods. As well, the versatility of LUNKERS-type overhead and velocity-refuges should enhance river habitat heterogeneity and quality, since it may provide additionally to velocity-refuge an overhead cover sheltering and sanctuary for fish decreasing the predation risk, at the same time supporting bank stabilization in river restoration projects [27]. Further research may reveal to which extent this overhead cover and velocity-refuge can act as, for instance, an efficient multi-species flow-refuging structure, which life-stages it may benefit more, or the hydraulic thresholds concerning hydropeaking mitigation purposes. Although such laboratory studies cannot fully replicate natural conditions, they do provide the opportunity to minimize biased results concerning the multiple variables under investigation, encouraging the development of new approaches and solutions for the impacts under concern.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or 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. Fish trials and sampling were supervised in agreement with national and international guidelines to maintain the welfare of the tested animals. Fish experiments were carried out with strict agreement with the guidelines of the "protection of animal use for experimental and scientific work" of the Department for Natural Resources and Nature Conservation (Departamento de Recursos Naturais e Conservação da Natureza) from the Portuguese Institute for Nature Conservation and Forests (ICNF), that authorized fish sampling and experiments to be completed in the experimental facility, and the studied individuals to be held in the laboratory. All efforts were made to minimize stress and no fish were killed during the experiments.

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