Article

Hematological and physiological responses under repeated acute hypoxia in rainbow trout (*Onchorynchus mykiss*)

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Simple Summary: The aim of this study is to determine the hematological and physiological response of rainbow trout under repeated hypoxic stress at different time points with the objective to observe if fish can habituate to these repeated shocks. Fish were subjected to three consecutive hypoxic shocks by subjecting the fish to 2mg/L oxygen in water for 1 hour, with 48h difference between shocks. Physiological and hematological changes were analyzed. Overall, our results indicate an ability of rainbow trout to cope with this type of hypoxic shocks.

Abstract: Oxygen is a limiting factor both in the environment and production systems, so reduction may become a stressor. Diel cyclic hypoxia occurs with varying frequency and duration in freshwater habitats. Under a stressful situation fish activate the hypothalamic-pituitary-interrenal axis (HPI) which triggers the release of cortisol that induces secondary and tertiary responses. The recovery of individuals depends on their ability to modulate physiological, and biochemical responses to maintain homeostasis. **The aim of this study is to determine the hematological and physiological responses of rainbow trout under repeated hypoxic stress in different time points**. The methodology of the experiment consisted of dividing the fish in 5 different treatment groups, 2 control groups and 3 hypoxia groups. Every exposure consisted in decrease the dissolved oxygen concentration from 8mg O_2/L to 2mg O_2/L for 1 hour. After the exposure the fish went to a recovery tank until the sampling procedure. Hematological and physiological results show a habituation of the fish to different parameters such as hematocrit, hemoglobin and mean corpuscular volume among others. Overall, our results indicate an ability of rainbow trout to resist this type of hypoxic exposures and a habituation of fish to repeated hypoxia as observed in the different measured parameters.

Keywords: repeated stress; hematology; fish; rainbow trout; hypoxia; dissolved oxygen concentration; RDW; cortisol

1. Introduction

The current context of climate change is expected to strongly affect fish because of its consequences on temperature and water conditions. The changes that will occur are related to abiotic factors (temperature, oxygen levels, salinity, and pH) and biological ones (primary production, pathogens, and food availability), affecting aquatic organisms in their distribution, growth, and size [1]. Wild populations may be exposed to many environmental stressors, such as pollution, temperature changes, hypoxia, among others, generating much uncertainty about the consequences of these stressors, and their potential synergy (e.g. hypoxia and temperature rise) [2]. As fish are aerobic organisms, the concentration of dissolved oxygen (DO) is a limiting factor both in the environment and in production

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systems, and their availability depends on their amount and solubility. Looking at the oxygen availability, there is a worldwide phenomenon which affects freshwater and coastal systems, namely aquatic hypoxia. In natural environments it can be a result of eutrophication and the increase of temperatures. In addition, this phenomenon is amplified by food production systems because of overstocking or overfeeding [3].

Hypoxia is defined as oxygen concentrations dropping low enough to cause negative physiological, immune, growth or behavioural effects on fish [4]. There are two types of hypoxias, acute and chronic which can occur due to changes in temperature, seasonality, water flow and/or composition [5]. In addition, as mentioned before the context of climate change increases the likelihood of these situations occurring more frequently and intensely [4]. Therefore, fish can be subjected to repeated episodes of hypoxia. Most species have a high ability to become habituate to fluctuating oxygen levels, but, if fluctuations are persistent, they can lead to increased mortality. Therefore, to maintain good fish performance, growth, and feeding, oxygen levels should be kept close to saturation, as the oxygen concentration of 5 mgO_2/L is the baseline for most species [4]. However, in some cases, culture conditions in aquaculture facilities are suboptimal, so that if fish are not able to adapt, they may develop a stress response [6].

The stress response is a very primitive and highly conserved response in extant species, since it preserves the organism's homeostasis [7,8]. activating primary, secondary, and tertiary responses [9]. In this sense, it has been shown that animals are able to adapt to different stressors, including hypoxia, that are repeated over time, reducing their physiological and behavioural responses [10]. However, the capacity for habituation depends on the species, as well as on the type and intensity of stress, as they can adapt to some stressors [11], but not to others [12].

Generally speaking, stress, age, sex, life stage, temperature, salinity, health and nutritional status, seasonality, species, or anaesthesia causes modulation of haematological parameters type and dose [13]. The number of red blood cells (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume of red blood cells (MCV), and mean haemoglobin concentration (MCH) are commonly used to evaluate the welfare and health status in fish [14]. In fact, under hypoxia, the hematological parameters are altered since they are indicators of oxygen uptake and distribution [4].

Also, the widening of the distribution of red blood cells (RDW), a parameter related to the axis of ionic erythropoiesis to measure the size and volume of the erythrocytes has been studied, as an additional indicator of health status as it has been shown in humans [15]. In addition, determination of the white blood cells (WBC) provides information on the animal's health related to immunity, as well as the percentage of heterophilic cells (neutrophils, basophils, and eosinophils) and mononuclear cells (lymphocytes and monocytes). Finally, the number of platelets (PLT) is also investigated [13].

Altogether, hematological parameters, as indicators of oxygen uptake and distribution [4] and their correlation with other physiological stress markers such as cortisol, glucose, and lactate in plasma [16] provide valuable insights on the welfare and health of an animal, if

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they are markers of a range of physiological variations and are becoming useful tools to detect and identify specific stressors.

In terms of the previous work done on hypoxia effects on fish, there are many studies that have addressed this subject in different species [5, 17] and particularly in rainbow trout *Oncorhynchus mykiss* [18-20], two aspects have been less taken into consideration, which are in the aims of this study. First, studies on hypoxia have dealt with fish that have been taken out of the water, so effectively subjected to anoxia, but, at the same time subjected to the consequent handling stress associated to catch and restriction of fish out of the water, and not always those two stressors have been experimentally separated. Second, studies on repeated hypoxia are less common, so the ability of fish to respond repeatedly to this stressor is less known.

Therefore, since hypoxic episodes may become more frequent, the aim of the present research was to know the effects of repeated hypoxia, as the fish were subjected to 1, 2 or 3 acute shocks, and to determine whether there is a cumulative effect, a certain adaptive capacity or, on the contrary, a constant response. In addition, the stress recovery phase, specifically at 1, 6 and 24 hours after the last shock was also analysed.

2. Materials and methods

2.1. Fish and rearing conditions

A total of 135 rainbow trout (Oncorhynchus mykiss) with a condition factor of 1.2 ± 0.1 were obtained from a local fish farm (Molinou, Rialb, Spain) and acclimated to a recirculation system (RAS) in the facilities of the Autonomous University of Barcelona (AQUAB) for two weeks. The RAS with water pump, recirculating chiller cooling system, sand filter, and biofilter had also an aeration system for the tanks that allowed to have a concentration between 7,20 - 8,10 mgO_2/L. The photoperiod was 12 L: 12 D and an average temperature of 14.6 ± 0.3 ^{o}C was maintained. All experimental procedures involving fish were submitted and authorized by the Ethics and Animal Care Committee of the "Universitat Autònoma de Barcelona" (permit number OH4218_4219 and DAMM 11251), that agrees with the international Guiding Principles for Biomedical Research Involving Animals (EU2010/63)

2.2 Experimental design

Five experimental groups were established, divided in two control groups and 3 groups that underwent a different number of hypoxic shocks. The first group was the absolute control (AC), which is the only group that was sampled only. The second group was the manipulated control (MC), since it is known that the manipulation of individuals, in this case the manipulation resulting from the administration of hypoxia, is an additional stressor.

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The remaining groups were the three hypoxic groups, all which were handled in the same manner as MC. The third group were individuals that were subjected to 1 hypoxia shock (H1). The fourth group suffered 2 hypoxia shocks (H2) and finally the fifth group suffered 3 hypoxia shocks (H3). Thus, H2 and H3 groups suffered repeated acute hypoxia stress, being the time between hypoxia shocks 48 h. In the groups where several hypoxia shocks were performed, the fish were returned to recovery tanks under normal oxygen and water physicochemical levels.

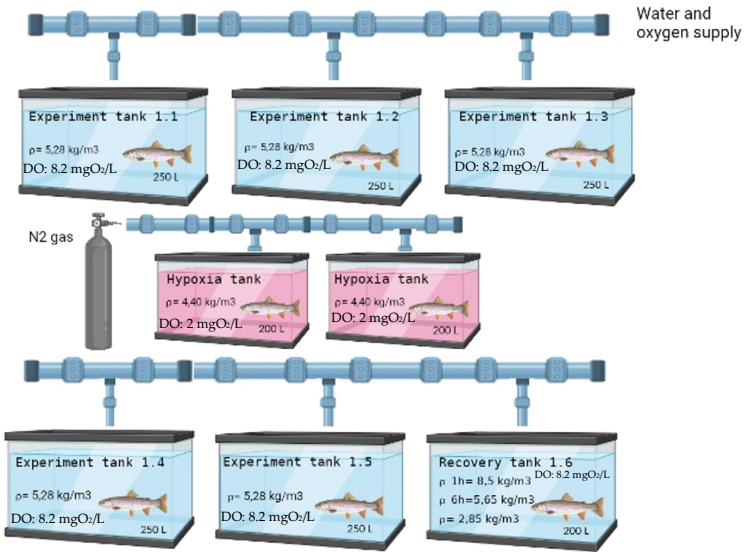

Figure 1. Graphical abstract of the design of the experiments

2.3 Hypoxic shock and sampling procedure

To perform the hypoxia shock, 2 tanks were used to replicate the treatment procedure. After the procedure all fish were placed in the same recovery tank to avoid the tank effect. The shock consisted of catching the fish from the experimental tanks to put them in the hypoxia tanks where the DO concentration was decreased from 7.5 ± 0.5 to 2.2 ± 0.5 mgO_2/L by adding nitrogen gas (N_2). The fish were introduced once the oxygen level had been decreased and remained there for 1 hour, the duration of the shock. It should be added that during the hypoxia shock. dissolved oxygen was continuously monitored to check that the levels were within the study range, and to avoid a decrease below 1,5 mgO_2 /L.

Following the hypoxia shock, fish were transferred to a recovery tank with 7.2 – 8.1 mg O_2/L which was isolated from the system to avoid any possible circulating cortisol levels affecting the other fish, as there is a possible chemical detection by peripheral cortisol receptors that may affect fish response [21] Sampling took place once the fish were in the recovery tank. By the time the sampling of the experimental group was finished, oxygen and water turnover recovered the optimal levels.

Nine fish per time (1, 6 and 24 h) post hypoxia and treatment were anaesthetised with sublethal doses of MS-222 buffered with sodium bicarbonate. Rainbow trout were weighed and measured, and blood samples were taken.

2.4 Hematological analysis

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Blood collection was performed with a heparinized syringe through caudal puncture. 500 µL of blood were added to Eppendorf tube with heparin (1:40) for hematological parameters determination. The second aliquot was centrifuged at 1500 g for 10 minutes and immediately after plasma was separated and frozen at - 20 ºC until the physiological analysis performance.

Hematological analysis were performed within 2-12 hours after blood sampling using the automated flow cytometer blood cell analyser Sysmex XN-1000V for veterinary use (Sysmex corporation, Kobe, Japan). Internal quality control (QC) was performed daily using three levels of commercially available QC material (Sysmex XN Check Level 1 or low range, Level 2 or normal range, and Level 3 or abnormal high range (Sysmex corporation, kobe, Japan).

2.6 Physiological analysis

For cortisol analysis, the plasma was first diluted in the analysis buffer and the aliquots were frozen at - 20 ºC for at least 24 h until analysis with an ELISA test using a commercial EIA kit (Cortisol ELISA KIT; Neogen® Corporation, Ayr, UK) following the manufacturer's instructions. This kit was already validated and used in previous experiments [22].

Glucose and lactate analyses were performed using a colorimetric test with kits (LO-POD glucose and LO-POD lactate, Spinreact, Spain) following the manufacturer's instructions.

2.7 Statistical analysis

The statistical test used in this study was a generalized linear model (GzLM) using the software RStudio Version (R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). The factors analysed were hypoxia treatments, time and the interaction between them. The significance level used was $p \leq 0,05$. Once this statistical test had been carried out, pairwise comparisons by Tukey correction were applied.

3. Results

Along this section results are presented according to significant effect of each factor and the significant interaction between them. Therefore, treatment is first presented, followed by time, and finally the interaction between the two factors is shown.

3.1. Treatment effects

The number of red blood cells (RBC), the amount of hemoglobin (HGB), platelets (PLT) and the mean concentration of hemoglobin (MCH) were affected by treatment (Table 1). An increase in RBC and HGB was observed by handling in MC compared to AC group, whereas hypoxia tended to decrease these values, although it is observed that from H2 onwards the most marked effect was handling, showing H3 intermediate values between absolute control and manipulated control.

Instead, for platelets (PLT), there is no significant effect of treatment nor time, but there was a trend to a decrease ($p<0.07$), as more hypoxic shocks were experienced (Table 1).

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Finally regarding MCH it can be observed that the mean concentration of hemoglobin increased in H1, but recovered the control level in H2 group, whereas in H3 there were lower values in comparison with absolute control (Table 1).

Group	RBC (10^3/µL)	HGB (g/dL)	PLT (10^3/µL)	MCH (pg)
AC	0.961 ± 0.0224 ^{b}	5.57 ± 0.108 ^{ab}	2.34 ± 0.140	50.8 ± 0.770 ^{ab}
MC	1.058 ± 0.0229 ^{a}	5.78 ± 0.110 ^{a}	2.34 ± 0.162	48.3 ± 0.770 ^{bc}
H1	0.939 ± 0.0224 ^{b}	5.61 ± 0.108^{ab}	2.38 ± 0.149	52.8 ± 0.770 ^{a}
H2	1.031 ± 0.0229 ^{a}	5.77 ± 0.123 ^{ab}	2.28 ± 0.154	49.5 ± 0.846 ^{bc}
H3	0.975 ± 0.0224 ^{ab}	5.35 ± 0.108 ^{b}	2.61 ± 0.154	47.6 ± 0.770 ^{c}

Table 1. Red Blood Cells Number (RBC), Haemoglobin (HGB), Platelet number (PLT) and mean hemoglobin concentration (MHC) in rainbow trout. Data are represented as mean ± SEM (n=9 per sampling time-). Significant differences between treatments are marked with different letter (p-valor <0.05). Abbreviations: absolute control (AC); manipulated control (MC); 1 hypoxic shock (H1), 2 hypoxic shocks (H2): 3 hypoxic shocks (H3).

Regarding the percentage of heterophilic and mononuclear cells, there is an effect of hypoxia treatment and time, but there is not an interaction between the two factors. It should be noted that H2 is the treatment that shows the highest heterophile levels compared to the control group at 6 hours after exposure (Figure 2). Moreover, a clear effect of time was found at 1h, showing significantly higher mononuclear cells and, therefore, a lower population of heterophiles in blood circulation.

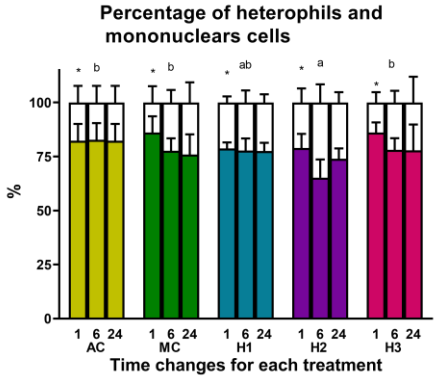

Figure 2. Percentage of mononuclear cells (color bars) and heterophils (white bars) in rainbow trout blood 1, 6, and 24h after treatment. Data are represented as mean ± SEM (n=9 per sampling time-point). Significant differences between treatments are shown with different letters (p< 0.05) and the significant differences in sampling time are indicated by an asterisk (p< 0.05).

3.2. Time effects

A time effect was detected in HCT. Interestingly, an increase was observed at 1 hour (mean 1h = 37.9 ± 0.871; mean 6h = 34.9 ± 0.847; mean 24h = 35.6 ± 0.867).

3.3. Interaction effects

A significant interaction between hypoxia stress and time was found in MCV, RDW-SD, WBC, glucose, lactate, and cortisol (Figure 3).

In H1 and MC groups, a gradual reduction of the MCV can be observed over time. In addition, H1 also presented higher MCV levels at 1 and 6 hours than those found in both AC and MC and the other groups that underwent hypoxic shock (H2 and H3) (Figure 3A).

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RDW showed no difference when comparing AC group and the rest of the experimental groups. What can be observed is that H1 had the highest values at each time point, and the MC had the lowest, except at 6 h, and the rest of the groups showed intermediate values (Figure 3B). The most relevant difference regarding time occurs in MC and H1 groups, showing lower values 1 and 24h after treatment than at 6h. This trend was also found when comparing all experimental groups versus AC, being higher in H1 and therefore, suggesting a greater anisocytosis 6h after treatment.

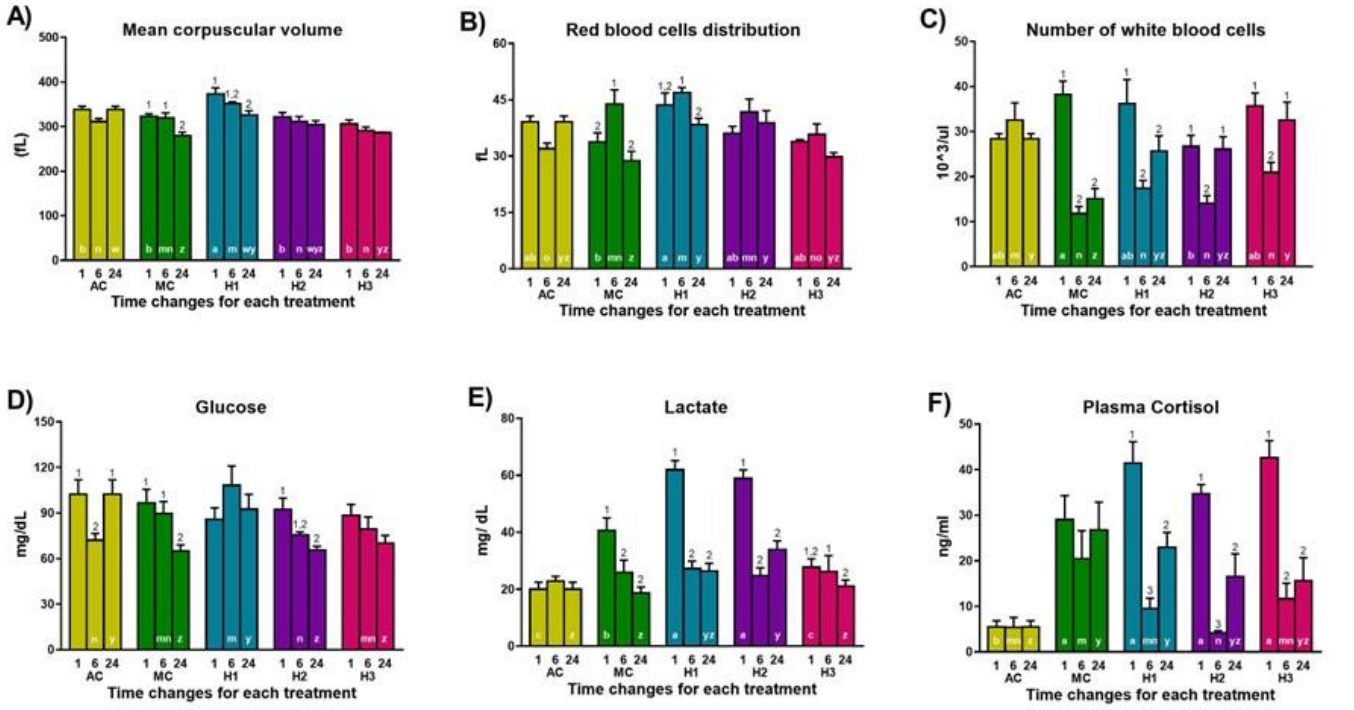

Figure 3. A) Mean Corpuscular volume, B) Erythrocyte distribution width (RDW-SD), C) Number of white blood cells, D) Plasma glucose, E) Plasma lactate ad F) Plasma cortisol in rainbow trout blood 1, 6, and 24h after treatment. Data are represented as mean ± SEM (n=9 per sampling time). Significant differences ($p< 0.05$) between treatments at the same sampling time are shown with different letters (a/b at 1h, m/n at 6h and y/z at 24h after treatment). Significant differences in sampling time among the same treatment are shown by numbers ($p< 0.05$).

Regarding immune cells, the lowest WBC levels occur 6 hours after manipulation or hypoxic exposure when compared with AC (Figure 3C). In MC and H1 these differences are maintained even after 24 hours; however, this is not observed in the groups subjected to repeated hypoxia.

In terms of physiological response, plasma glucose levels tended to increase 6h after treatment in H1, being significantly higher. On the contrary, glucose values were reduced at 24h, except for AC, (Figure 3D). Lactate levels clearly increased 1 hour after exposure and then decreased until recovering basal levels, except for the H2 group, that presented higher levels at 24h than AC (Figure 3E). It should be highlighted that lactate did not peak at 1h in H3 group comparing to H1 and H2.

Finally, a significant rise in plasma cortisol levels at 1h post exposure was observed in rainbow trout that underwent hypoxic shock (H1, H2 and H3) and in MC group versus AC, then dropping at 6h, and interestingly rising again at 24h (Figure 3F). When looking at 6- and 24-hours differences between treatments, they were maintained in MC versus AC, but not in H1, H2 and H3, which showed intermediate values.

4. Discussion

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The overall results of our experiment on induced hypoxia suggest that trout subjected to 1 hour repeated acute hypoxia could cope with oxygen levels down to 2mg/L in water up to 24 hours after the stressor, and that subjecting the fish again to the same stressor, trout shows a trend for habituation.
Thus, among the hematological variables red blood cells (RBC) and hemoglobin (HGB) showed an increase in the MC group, presumably due to handling of the animals as observed in [23]. On the contrary, as observed in H1, hypoxia produces a decrease in RBCs, probably because the oxygen delivery system is excessively altered and then the overall metabolism and activity decreases [24]. In the case of H2, RBC levels increase, which would help to improve the transport and oxygen content of the blood [25] being the start of the habituation of the animals. The hypothesis of habituation would be also reinforced by the results observed in H3 that showed intermediate values.

The Increase of hematocrit (HCT) after 1 hour could be associated to balancing out the extra need for oxygen which involve the increase of the number of red blood cells, therefore improving the oxygen delivery capacity to the tissues under hypoxia [26]. Although the difference is not significant, the later tendency towards hematocrit decrease was observed as exposures increased, which suggests an adaptation resulting in a lower need for oxygen in habituated fish [27,28].

Regarding the mean corpuscular volume (MCV), there are quite a few differences between groups and time. The highest MCV is observed in the group that has undergone 1 hypoxia shock, which indicates that the erythrocyte volume increases. This fact is probably due to two possible mechanisms that have been previously demonstrated. First, the osmoregulatory changes that occur over time to increase the efficiency of oxygen transfer through the cell membrane [25]. Second, the increase of HCT and MCV values, without much change in RBC may suggest the swelling of the erythrocytes [25]. The swelling of the erythrocytes has been observed as a result of catecholamines release after stress, trying to compensate the efficiency of cell oxygen uptake due to the increased demand of oxygen, in this case caused by the hypoxic stress [29].

Related to HCT and MCV we found a relationship with the average concentration of cellular hemoglobin (MCH). In this case the groups with higher levels of HCT and MCV present higher MCH. This could be explained because during the first hour after the hypoxic shock cellular hemolysis may occur as it has been previously shown [26, 29]. Furthermore, this effect is reduced over time suggesting recovery. This idea of hemolysis was initially proposed by [30] as HCT and MCV values increase because of cell swelling and further hemolysis. Afterwards, differences were also observed between treatments and, as in the rest of the erythrocyte parameters, a certain habituation of the hypoxia groups can be observed, So, the more hypoxia shocks, the lower response, as it has seen during a chronic exposure [29].

Concerning the distribution by size and volume of the cell, i.e., Red Cell Width distribution (RDW), it has been shown that other stressors, such as after infection by trematode metacercaria, entailed a decrease in this parameter [31]. In nutritional studies, an inverse relationship between this hematological parameter with others, such as MCV or HGB, was observed [32]. In the present study after hypoxia shocks, the RDW was higher at 6 hours suggesting greater heterogeneity, therefore greater anisocytosis, since some cells are recovering while others are not, thus increasing the heterogeneity levels at this time point. After 24 hours, homogeneity is restored, but since the cells are smaller because no swelling is produced, the values at 24 hours are lower than those at 1 hour. It should be added that this parameter has been little studied in the case of fish, but in the case of humans a decrease in RDW levels is related to anemia, so that erythrocytes do not have the capacity to transport enough oxygen [33]. As has been seen with the rest of the parameters, in the case of H3, RDW recovers the control levels, at 1 and 24 hours, suggesting again habituation.

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For white blood cells, all treatments showed a decrease in white blood cells number at 6 hours respect to the control group, especially fish subjected to 2 hypoxic shocks that presented a significant decrease also at 1 hour after treatment. These results agree with those found by [34] in the face of repeated stress. Moreover, H3 rainbow trout showed an ability to recover WBC levels suggesting habituation. The explanation for this decrease maybe related to cell damage detection, one of the functions of WBC [34, 35].

Under the name of white blood cells two different cell types can be distinguished depending on their nuclear morphology: heterophils and mononuclear cells. Among heterophils neutrophils, eosinophils, and basophils are found. Most of them are neutrophils, which do not show relevant changes when there is circulating cortisol, as it has been known from early studies [36]. On the other hand, in the face of a stressful situation, eosinophils have been shown to decrease, whereas no changes have been observed in basophils population. In this sense, the depletion of eosinophils would match with the peak of plasma cortisol [37]. So, as for the mononuclear cells, monocytes and lymphocytes, the same authors showed no changes in monocytes in front of a stressful situation, but lymphocytes do significantly decrease their abundance. However, since the effect on the eosinophils is greater due to the respective proportions, this decrease in monocytes could be masked [37]. Further studies are needed to ascertain the less known consequences of hypoxia on white blood cells.
The main function of platelets (PLT) is related to clotting, so no differences should be expected for such thrombocyte cells, since no lesions were observed at any point of the experiment. In addition, it has been seen in other studies that cortisol levels did neither affected thrombocyte levels [37]. Although a trend is suggested, differences were not significant.

4.3. Physiological response

The variation observed in cortisol levels between treated groups and control ones is commonly found in stress works, particularly associated to handling procedures [38]. This may be the case also in our results under hypoxia exposure since, as observed in [39], neither acute nor chronic exposure to hypoxia caused significant differences with respect to the control groups, whereas handling is a stressor to which the fish shows an increased cortisol response although they can later become habituated [40]. In addition, looking also at the dynamics of the number of white blood cells, it is observed that the WBC levels seem to mimic the rise of the cortisol levels and therefore, the activated response of the hypothalamic-pituitary – interrenal axis [41], as thus, cortisol and WBC show a high correlation (figures 3C and 3F). Similar results have been observed in other studies such as in [22], where the WBC were higher in a polluted environment under sub-optimal conditions involving neutrophilia and/or lymphopenia in fish. In addition, the increase of WBC is related to the migration of these cells from the spleen to the blood [42].

At this point, it is interesting to emphasize that in the present work hypoxia was achieved by reducing the oxygen concentration in water, which is rather different than the hypoxia caused by air exposure. The latter, involves a concurrent stressor such as a significative manipulation plus the maintenance of fish out of its water environment. As observed in the case of [43], which performed an aerial exposure experiment on trout, cortisol levels are much higher in percentage than in the present case, in which hypoxia was achieved by reducing the levels of oxygen from the water by displacement with nitrogen. It should be noted that the physiological stress response is effectively and quickly detected as plasma cortisol increase already after 3 minutes, although the maximum peak is detected after approximately 1 h post stress [44, 45].
Fish that have suffered some type of stress (manipulation or hypoxia shocks) show a progressive decrease in glucose levels since it is used as a substrate for glycolytic activity

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leading to the release of cell energy [46]. In the present work, this is not observed in the case of the control group so that, as time progresses these reserves are used. Furthermore, in most cases an acute stress induces an increase in plasma glucose, in part because of the energetic needs derived from the stress situation and the mediating effects of cortisol and catecholamines [4]. In addition, the absence of significant differences between the different groups at 1 hour indicates that the animals have the capacity to recover their reserves between the 48h recovery from the different shocks.
Moreover, the product generated due to the glycolytic activity is lactate [46], and facing a situation of hypoxia, lactate levels increase to maintain cellular energy balance [46, 47], as in the present study. This happens because oxygen-independent energetic mechanisms are needed since oxygen-dependent mechanisms are 15 times lower under hypoxic conditions [47]. In this sense, the group subjected to one hypoxia is the one with the highest values, so that it would be the group that activated anaerobic metabolism the most. What is important to highlight is the fact that the group subjected to 3 exposures did not increase lactate levels, which again suggests habituation by the fish in the same way that occurs with the hematological parameters. This habituation may occur since the stress to which they are subjected is not really that severe and the stressor was applied for short periods of time [48].

5. Conclusions

The overall results show a tolerance and a capacity for a certain habituation of an oxygen sensitive species, such as rainbow trout, to this type of repeated hypoxia. Respect to haematological parameters, we can observe initial differences compared to the control group, but after 3 shocks these differences are reduced and therefore a habituation of the fish can be seen, both in red blood cells functions related to oxygen supply and in white blood cells related to immune and cell repair functions. With respect to cortisol, although the values of hypoxic groups are not recovered compared to the control group, cortisol levels decrease as the number of shocks increases, It is possible that in the long term fish can recover the basal levels provided that the stressor maintains the same characteristics. Regarding glucose and lactate, after an initial alteration associated to changes in aerobic/anaerobic metabolism, with a peak of lactate at 1 hour after hypoxic shock, this effect disappears in fish subjected to repeated hypoxia, which again indicates that a certain habituation is taking place.

As a general conclusion, we could say that rainbow trout are capable of habituating to these temporary hypoxia episodes without having negative consequences at the functional level. However, to affirm this, it would be necessary to analyze how gene and protein expression is modulated, since it may help to understand basic molecular and cellular changes associated to the tolerance of hypoxia.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, Nuria Ruiz, Lluís Tort and Irene García-Meilán.; methodology, Nuria Ruiz, Ali Rheza Khansari, Josep Pastor, Lluís Tort, Mariana Teles and Irene García-Meilán .; formal analysis, Nuria Ruiz and Irene García-Meilán, Josep Pastor; investigation, Lluís Tort; resources, Lluís Tort; data curation, Irene García-Meilán.; writing—original draft preparation, Nuria Ruiz; writing—review and editing, Mariana Teles, Ali Rheza Khansari, Josep Pastor, Lluís Tort and Irene García-Meilán; visualization, Irene García-Melilán; supervision, Lluís Tort.; project administration, Lluís Tort.; funding acquisition, Lluís Tort. All authors have read and agreed to the published version of the manuscript."

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Informed Consent Statement: "Not applicable."

Data Availability Statement: We encourage all authors of articles published in MDPI journals to share their research data. In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Where no new data were created, or where data is unavailable due to privacy or ethical restrictions, a statement is still required. Suggested Data Availability Statements are available in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics.

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Conflicts of Interest: Declare conflicts of interest or state "The authors declare no conflict of interest."

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