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Physiological and proteomic responses to single and repeated hypoxia in juvenile Eurasian perch under domestication – Clues to physiological acclimation and humoral immune modulations

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ABSTRACT

We evaluated the physiological and humoral immune responses of Eurasian perch submitted to 4-h hypoxia in either single or repeated way. Two generations (F1 and F5) were tested to study the potential changes in these responses with domestication. In both generations, single and repeated hypoxia resulted in hyperglycemia and spleen somatic index reduction. Glucose elevation and lysozyme activity decreased following repeated hypoxia. Complement hemolytic activity was unchanged regardless of hypoxic stress or domestication level. A 2D-DIGE proteomic analysis showed that some C3 components were positively modulated by single hypoxia while C3 up- and down-regulations and over-expression of transferrin were observed following repeated hypoxia. Domestication was associated with a low divergence in stress and immune responses to hypoxia but was accompanied by various changes in the abundance of serum proteins related to innate/specific immunity and acute phase response. Thus, it appeared that the humoral immune system was modulated following single and repeated hypoxia (independently of generational level) or during domestication and that Eurasian perch may display physiological acclimation to frequent hypoxic disturbances.

1. Introduction

Most of the organisms need molecular oxygen to support major metabolic processes. Predominantly, oxygen serves as the final electron acceptor in oxidative phosphorylation resulting in ATP formation inside mitochondria. Ten to 15% of total intracellular oxygen is also consumed in numerous cellular reactions catalyzed by mono- and di-oxygenases, oxidases or peroxidases [1]. Therefore, exposure to hypoxic conditions may adversely affect a wide variety of biochemical and physiological processes. In comparison to terrestrial animals, oxygen availability is more problematic for aquatic organisms given that water oxygen capacitance and oxygen diffusion are only 1/30th and 1/10000th of those measured in air at the same partial pressure respectively [2]. Oxygen availability in water can change rapidly compared to the terrestrial environment. Fish are thus interesting model animals to study acclimation mechanisms to changes in environmental oxygen level.

Farmed fish regularly experience acute decreases in water oxygen concentration, especially during feeding and when reared at high density [3]. In many fish species, sub-lethal hypoxia causes a complex sequence of neural, behavioral and physiological modifications through the primary and secondary stress responses (*i.e.* allostasis) in an attempt to re-establish homeostasis [4]. Energetically, there are costs associated with allostatic mechanisms and particularly under conditions of repeated and chronic stress [5]. In the long-term, the reallocation of energy away from some energy-demanding functions may be needed to still ensure efficient stress-coping physiological responses [4,5]. Accordingly, stressful husbandry conditions may indirectly affect growth, reproduction and the immune system [6–16]. Innate and acquired immunity are of particular importance for the host resistance to infectious diseases. This is particularly true in breeding environments where spatial restriction limits the fish opportunities to select areas associated with lower infectious potential and where fish-to-fish

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proximity facilitates the spreading of pathogens between individuals [17,18]. Up to now, only one study reported immunosuppressive effects and increased susceptibility to high doses of *Edwardsiella ictaluri* in channel catfish *Ictalurus punctatus* subjected to short-term sub-lethal hypoxia [4].

Domestication could be defined as a micro-evolution process during which captive animals acclimate and adapt to the enclosed environment and human care [19,20]. Domestication thus implies gradual changes in behavior, morphology and/or physiology over generations so that captive populations progressively fit to the breeding environment [21]. In captivity, fish are usually confronted to repetitive and chronic stress situations (*e.g.* confinement, crowding, hypoxia or handling) from which they cannot escape, and have no other option than acclimation/adaptation to husbandry stressors. Consequently, modulation of stress sensitivity and/or stress-coping responses might be expected to be part of domestication. The Eurasian perch is a recently domesticated fish compared to some ancient farmed salmonid and cyprinid species. In perch, studies already reported that some traits related to growth, stress response and immune status may be modified after only four generations in captive conditions [22,23]. In other fish species, domestication also rapidly influenced stress physiology, growth, reproduction and behavior after less than 7 generations [24–28]. To date, no information is available regarding stress and immune responses to hypoxia in fish under domestication.

By providing information about gene expression at the protein level, proteomics appears as one of the strategies which can be used to get a better overall comprehension of underlying mechanisms and to complement the data obtained at higher levels of biological organization [29,30]. Up to now, proteomic studies have been performed in some fish species to evaluate the effects of variations in dissolved oxygen level (*e.g.* anoxia, hypoxia, hyperoxia) [31–33]. However, none of these studies examined changes in the serum proteome and whether they may be modulated along domestication.

This study aimed to investigate the physiological response to single or repeated hypoxia and subsequent effect on humoral immunity in the Eurasian perch (*Perca fluviatilis*), a percid species currently undergoing domestication. To evaluate whether domestication may imply changes in stress and humoral immune responses to hypoxia, captive fish from first and fifth generations were examined for usual stress markers (serum cortisol and glucose levels, spleen somatic index, muscle lactate concentration) and immune function (serum lysozyme activity and alternative complement pathway). Additionally, two-dimensional Differential In-Gel Electrophoresis (2D-DIGE) was used to resolve serum proteins. This work is the first to report changes in protein abundance in the serum of domesticated fish exposed to a hypoxic environment.

2. Materials and methods

The present protocol (FUN06/067) has been carried out in agreement with the local Ethics Committee for Animal Experiments in accordance with the European guidelines concerning animal welfare.

2.1. Fish generations and culture conditions

In the current experiment, we decided to compare only two filial generations (Filial 1:F1 *versus* Filial 5: F5) of one-year old juveniles. Firstly, we assume that comparison of a wild-close population *versus* a top-captive generation with a relatively long farming history can be representative of the changes occurring along domestication process. Besides, studies about the influence of domestication on fish biological functions and behavior often appeal to two contrasted domestication levels [24,25,34–36]. Secondly, covering all generational levels (*i.e.* F1, F2, F3, F4 and F5) was not an option because too many experimental conditions would have been included (limited facilities). Fish from the fifth generation (F5) originated from the reproduction of the fourth captive generation of breeders (F4) maintained under natural conditions in outdoor tanks. Those F4 breeders originated from successive reproductions of wild individuals (F0) from the Meuse River and their related captive offspring (F1, F2 and F3) in captivity. To obtain the first generation of captive fish (F1) used in the present study, another group of wild breeders (F0′) was captured from the Meuse River and allowed reproducing synchronously to the F4 breeders in the same photo-thermal conditions (Fig. 1). There are two reasons explaining the use of this new batch of wild breeders (F0′). Firstly, we wanted to compare fish which have been reared for several generations in captivity to fish obtained from wild individuals with a very short captive-life history (*i.e.* wild breeders captured at the onset of the reproductive period and spawning for the first time in captivity). In this context, the F0 fish were no longer suitable to produce the F1 progeny because they have been maintained for several years in the facility and were thus probably acclimated to captivity conditions. Secondly, a major part of the F0 breeders died after spawning in captivity so that we had to capture new fish from the wild at the time of producing the F1 juveniles. As a result, the F1 and F5 generations used in this study initially derived from two different groups of wild fish but which have been captured at an identical location (*i.e.* the "Tailfer" backwater) on the Meuse River in Belgium. According to Nesbo et al. (1999) [37], European populations of Eurasian perch showed very little genetic differentiation within drainages and regions and high levels of structuring only across drainages and regions. In view of the above, genetic background is expected to be similar between both generations at the start of the domestication process. From the fingerling stage, both generations were reared in duplicate under similar standardized conditions (4 m^2 tank, 15 kg/m^3, 23 °C, 16L:8O, $O_2 > 7$ppm) and fed once a day.

2.2. Experimental design of the single or repeated hypoxia

Whatever the type of hypoxia, four conditions were compared: F1 under normoxia, F1 submitted to hypoxia, F5 under normoxia, F5 submitted to hypoxia. For each of these experimental conditions, fish ($n = 35$ *per* tank; mean weight = 68 ± 28 g) were held in triplicate in 100 L tanks. Progressive hypoxia was induced by removal of the airstone and complete arrest of water inflow. As soon as the oxygen level reached the pre-established threshold (2 mg L^{-1}), hypoxia was maintained during 4 h via slight re-adjustments of water inflow. Dissolved oxygen concentration was continuously monitored and the current procedure allowed the stabilization of the hypoxic condition to 2.0 mg L^{-1} ± 0.5 mg L^{-1} throughout each hypoxia session. The normoxic condition was

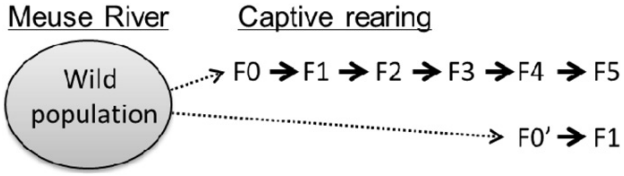

Fig. 1. This figure illustrates the way we obtained our captive F1 and F5 generations. Dotted arrows indicate capture of wild fish at the onset of the spawning period. Bold arrows indicate reproduction giving birth to the following progeny.

characterized by a stable dissolved oxygen level of 7.44 ± 0.37 mg L^{-1} throughout the experimental period.

2.2.1. Single hypoxia

Just after the first 4-h hypoxia period (Fig. 2), 5 fish *per* tank were harvested and anesthetized in an ethyl 3-aminobenzoate methane sulfonic acid salt 98% (MS-222, Sigma) solution (180 mg/L). Blood was rapidly collected from the caudal vein (within 5 min) to avoid the release of handling-induced cortisol. Blood was allowed to clot at 4 °C prior to centrifugation (7500 × g, 5 min) and aliquoted serum was then stored at −20 °C until measurements of cortisol, glucose, lysozyme and alternative complement pathway haemolytic activities and 2D-DIGE proteomic analysis. Spleen and a piece of dorsal white muscle were dissected. Fresh spleen was weighed and muscle tissue stored at −80 °C until lactate measurement was carried out.

2.2.2. Repeated hypoxia

Fish were submitted to a 4h-period of hypoxia twice a week during 46 days (*i.e.* 12 hypoxic stress sessions) (Fig. 2). On Day 46 and just after the last 4-h hypoxia was applied, 5 fish *per* tank were sampled in the same way as for the single hypoxia test to evaluate the response to repeated hypoxic stress using the same bio-indicators.

2.3. Cortisol and glucose assays

Serum cortisol was assayed in duplicate using a cortisol ELISA kit (DRG, EIA-1887) and following the manufacturer's instructions. The intra-assay coefficient of variation was 4.2%, the inter-assay coefficient of variation was 6.6% and the assay dynamic range was between 0 and 800 ng ml^{-1}. Serum glycemia was determined colorimetrically based on the glucose oxidase/peroxidase method described by Trinder (1969) [38].

2.4. Serum lysozyme activity

Lysozyme activity was evaluated in serum samples following the method of Siwicki and Studnicka (1987) [39] and Fatima et al. (2007) [40] with some modifications. Briefly, serum samples (7 μl) were mixed with 130 μl *Micrococcus luteus* (Sigma) solution (0.6 mg/ml 0.05M sodium phosphate buffer − pH 6.2). Lysozyme assay was performed in triplicate. Absorbance was measured at 450 nm every 5 min during 30 min at room temperature. Lysozyme activity (Units.ml^{-1}.min^{-1}) in serum was defined as the amount of enzyme causing a decrease in turbidity of 0.001.

2.5. Hemolytic activity of the alternative complement pathway (ACH50)

Following the method of Sunyer ant Tort (1995) [41] adapted by Milla et al. (2010) [42], ACH50 was evaluated using rabbit red blood cells (RRBC, Biomerieux, Craponne, France) as targets. Briefly, serial dilutions (from 1/3 to 1/300) of serum in veronal buffer (Biomerieux, Craponne, France) were performed and subsequently mixed with 10 μl of 3% RRBC suspension in veronal buffer (total volume *per* well = 70 μl). Plates were incubated for 100 min at 27 °C and centrifuged at 2000 g for 10 min at 4 °C. The spontaneous hemolysis was obtained by adding 60 μl of veronal buffer to 10 μl of RRBC and total lysis was obtained by replacing veronal buffer by distilled water. Absorbance was measured at 405 nm. Alternative complement activity was expressed as the 50% lysis dilution calculated by linear regression.

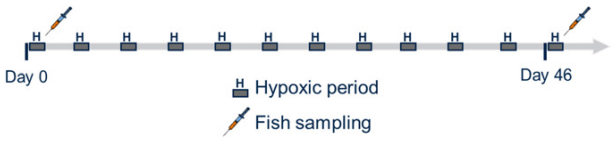

Fig. 2. Experimental protocol of single and repeated hypoxia. SINGLE hypoxia: the first hypoxia period was conducted on day 0 and fish ($n = 5$ *per* tank, 3 tanks *per* condition) were immediately sampled at the end of the 4-h period. REPEATED hypoxia: Fish were exposed to 4-h hypoxia, twice a week during 46 days. The last hypoxic stressor was conducted on day 46 and fish ($n = 5$ *per* tank, 3 tanks *per* condition) were immediately sampled at the end of the 4-h period.

2.6. Spleen-somatic index (SSI)

SSI was calculated according to the following formula: SSI (%) = fresh spleen weight (g)/total fish weight (g) × 100.

2.7. Muscle lactate concentration

For muscle lactate extraction, 1 ml 0.9N perchloric acid was added to 200 mg of muscle tissue. Muscle was homogenized with Potter-Elvehjem homogenizer, pH was adjusted to 8–10 with potassium carbonate (1 M) and tissue was 20 times diluted in ice-cold phosphate buffer (100 mM; pH 7.4). Homogenates were centrifuged at 10 000 g for 5 min at 4 °C and supernatants stored at −20 °C until lactate assay was performed. Lactate concentration was measured using an L-lactic acid kit (Boehringer–Mannheim) following the manufacturer's instructions. Lactate concentration was expressed as mg g^{-1} muscle tissue.

2.8. Statistical analysis of physiological variables

Kolmogorov and Smirnov's test was used to assess the normality of data sets ($p < 0.05$) and Bartlett's test was conducted to evaluate variance homogeneity ($p < 0.05$). Results from single hypoxia and from repeated hypoxia were separately analyzed with two-way ANOVA ($p < 0.05$) taking dissolved oxygen (normoxia or hypoxia) and domestication level (F1 or F5) as two-modality factors. For both single and repeated hypoxia, the amplitude of glucose and SSI responses in F1 and F5 fish was further calculated as: Δ glucose = Glucose $level_{(hypoxia)}$ − glucose $level_{(control)}$ and ΔSSI = SSI $level_{(control)}$ − SSI $level_{(hypoxia)}$ Values were then analyzed using a two-way ANOVA ($p < 0.05$) with domestication level (F1 and F5) and stressor frequency (single or repeated) as two-modality factors. When significant, means were compared according to Tuckey's HSD post-hoc test ($p < 0.05$).

2.9. 2D-DIGE proteomic analysis of serum samples

2.9.1. Protein extraction and CyDye minimal labeling

For either single or repeated hypoxia experiment, serum samples from 4 fish *per* tank were uniformly pooled and proteomic analysis was conducted with triplicate gels (one gel *per* tank; 12 tanks). All serum pools ($n = 24$) were 6 times diluted in DLA buffer (7 M Urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, pH 8.5) and centrifuged for 15 min at 12 000 g. Protein concentration of each diluted serum pool was determined using a Bradford protein assay with BSA as a standard. Using appropriate amount of 50 mM NaOH, pH was adjusted to 8.5 and protein concentration was measured again with the Bradford protein assay. For CyDye labeling, 200 pmol of fluorescent CyDye (GE Healthcare) were added to 25 μg proteins. Cy3 and Cy5 were both used to label replicates of each experimental condition. A mixed sample composed of equal amounts of all pools ($n = 24$) was minimally labeled with Cy2 and used as an internal standard. Labeling was performed on ice in the dark and

quenched with 1 mM lysine for 10 min on ice. For each gel ($n = 12$), the three labeled mixtures were combined and total proteins (75 μg) were added to an equal volume of reduction buffer (7 M urea, 2 M thiourea, 2% CHAPS, 2% DTT, 2% $IPG_{4\text{-}7}$ buffer) for 15 min at room temperature.

2.9.2. Protein separation by 2D-DIGE

Prior to electrofocusing, IPG strips (pH 4–7; 24 cm; GE Healthcare) were rehydrated overnight into 450 μl of a standard rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 0.28% DTT, 0.5% IPG buffer pH 4–7 and a trace amount of bromophemol blue). The twelve sample sets containing the labeled mixtures were cup-loaded onto the strips and isoelectric focusing was run on an Ettan™ IGPphor II unit (GE Healthcare) with the following electrophoresis conditions: 20 °C, 300 V for 3 h, 1000 V for 6 h, 8000 V for 3 h, and finally 8000 V for 6 h for a total of 68 000 V-h. Afterwards, focused IPG strips were reduced (1% DDT) and alkalized (2.5% iodoacetamide) in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 0.002% bromophemol blue, 50 mM Tris pH 8.8) just before loading on 10% 24 cm, 1-mm-thick, SDS-PAGE gels. The strips were overlaid with 0.5% agarose in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) and run at a constant voltage (0.5 W/gel) during 17 h at 15 °C in an Ettan™ DALTsix electrophoresis unit (GE Healthcare) until the blue dye front reached the bottom of the gels.

2.9.3. Software image analysis and statistical tests

Labeled CyDye gels were scanned with a Typhoon 9400 scanner (GE Healthcare) at CyDye-specific wavelengths (488 nm, 532 nm and 633 nm for Cy2, Cy3 and Cy5 respectively). Resolution was 100 μm. Image analysis was carried out using the Decyder v5.0 software (GE Healthcare). At first, the DIA module detected and quantified the protein spots in each image using the internal standard sample as a reference to normalize the data. Second, the BVA module allowed multiple gel comparisons and ratio calculations by conducting a gel-to-gel matching of the internal standard spot map on each gel. The mean number of spots detected in the 12 gels was 1280 ± 94 and the mean number of spots matched across the gels was 939 ± 161. Representative gel for an overview of the 2D-DIGE is illustrated in Fig. 3. Results were analyzed by a two-way ANOVA ($p < 0.05$) and the multiple comparisons Tuckey Post-hoc test as previously described.

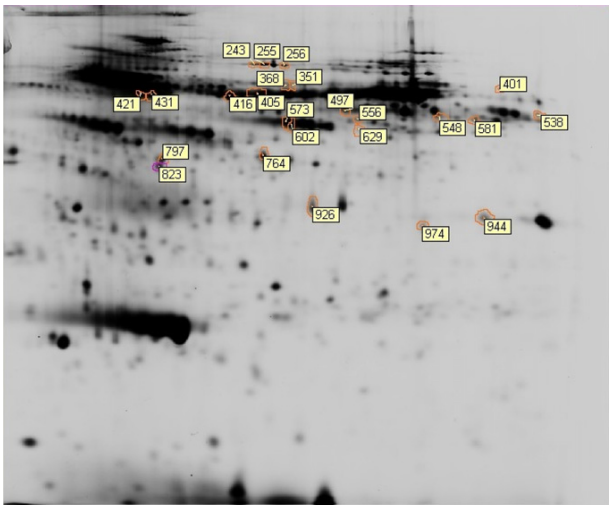

Fig. 3. Representative 2D-gel of serum proteome in Eurasian perch. Numbers assigned by Decyder software indicate identified protein spots with significant changes in intensity.

2.9.4. Mass spectrometry and protein identification

Preparative gels loaded with 250 μg of proteins of mixed samples were run following the protocol described above except they were post-stained with Krypton™ protein stain (N° 46630, Pierce) following the manufacturer's protocol. Preparative gels were scanned with the Typhoon 9400 scanner (GE Healthcare). Spots were excised from preparative gels using the Ettan™ Spot Picker (GE Healthcare), and proteins were digested with trypsin by in-gel digestion. The gel pieces were twice washed with distilled water and then shrunk with 100% acetonitrile. The proteolytic digestion was performed by the addition of 3 μl of modified trypsin (Promega) suspended in 50 mM NH_4HCO_3 cold buffer. Proteolysis was performed overnight at 37 °C. The supernatant was collected and the eluates were kept at −20 °C prior to analysis.

Peptides were analyzed by using nano-LC-ESI-MS/MS maXis UHR-TOF coupled with a 2D-LC Dionex UltiMate 3000 (Bruker, Bremen, Germany). The digests were separated by reverse-phase liquid chromatography using a 75 μm × 150 mm reverse phase Dionex column (Acclaim PepMap 100 C18) in an Ultimate 3000 liquid chromatography system. Mobile phase A was 95% of 0.1% formic acid in water and 5% acetonitrile. Mobile phase B was 0.1% formic acid in acetonitrile. The digest (15 μl) was injected, and the organic content of the mobile phase was increased linearly from 5% B to 40% in 35 min and from 40% B to 100% B in 5 min. The column effluent was connected to an ESI nano Sprayer (Bruker). In survey scan, MS spectra were acquired for 0.5 s in the m/z range between 50 and 2200. The 3 most intense peptides ions 2^+ or 3^+ were sequenced. The collision-induced dissociation (CID) energy was automatically set according to the mass to charge (m/z) ratio and charge state of the precursor ion. MaXis and Dionex systems were piloted by Compass HyStar 3.2 (Bruker).

Peak lists were created using DataAnalysis 4.0 (Bruker) and saved as XML file for use with ProteinScape 2.0 (Bruker) with Mascot 2.2 as search engine (Matrix Science). Enzyme specificity was set to trypsin, and the maximum number of missed cleavages *per* peptide was set at one. Carbamidomethylation was allowed as fixed modification, oxidation of methionine and Gln → pyro-Glu were allowed as variable modification. Mass tolerance for monoisotopic peptide window was 7 ppm and MS/MS tolerance window was set to 0.05 Da. The peak lists were searched against the full NCBInr database (12852489 sequences downloaded on February 2011).

Scaffold (version Scaffold-2_06_01, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2) and X! Tandem (The GPM, thegpm.org; version 2007.01.01.). Peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm [43]. Protein identifications were accepted if they could be established at greater than 99% probability and based on preferentially more than one identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm [44]. When a protein spot could not be assigned to a single protein identification or if similar peptides were shared between several proposed proteins, all the protein hits were grouped to fit with the parsimony principle.

3. Results and discussion

3.1. Stress and immune responses to single hypoxia

In the present study, serum cortisol was used as a usual stress marker to evaluate whether Eurasian perch was sensitive to the

tested hypoxic conditions. Serum cortisol did not display significant differences with single hypoxic disturbance (Table 1). All treatments considered, cortisol concentration ranged from 10.28 to 21.54 ng ml^{-1} and was similar to levels observed for unstressed Eurasian perch in previous studies [22,23,42,45]. Similarly, Muusze et al. (1998) [46] investigated the effects of progressive and stepwise hypoxia in Amazon fish (*Astronotus ocellatus*) and detected no differences in cortisolemia while significant effects on other physiological parameters (lactate concentration and metabolic rate) were observed. Conversely, other studies on Siberian sturgeon (*Acipencer baeri*) and spotted wolffish (*Anarhichas minor*) reported rapid (after 30 min and 2 h 30 respectively) and significant cortisol elevation following a severe hypoxic disturbance [47,48]. In Eurasian perch, it has been demonstrated that cortisol peaked earlier than 1 h following an acute stressor and rapidly returned to pre-stress level within 1–6 h [11,42,45]. In the current study, we hypothesized that cortisol may not return to basal value throughout the hypoxic course since fish were continuously exposed to hypoxia during 4 h and immediately sampled after this period. However, we did not observe any prolonged cortisol increase in fish following a single 4-h hypoxia. In turbot, Pichavant et al. (2002) [49] did not observe any cortisol elevation during a 6-h period of slight or moderate hypoxia but an increase of cortisolemia was seen under severe hypoxia throughout the 6-h period and up to 6 h after recovery in normoxic water. Thus, the severity of hypoxic stress may be an important contributing factor of the cortisol release. Our results either suggest the absence of a cortisol response (which may be related to the intensity of hypoxic stress) or indicate that cortisol rise following hypoxia was of short duration (less than 4 h) and was not detected because it preceded blood sampling. We however detected elevated glycemia ($p < 0.01$) in the serum of single-stressed fish (Fig. 4A). As mentioned above, it is possible that we missed a rapid and transient cortisol rise which may be the trigger of such hyperglycemic response. For future research, it might be of interest to evaluate cortisol concentration as a function of time during hypoxia (*e.g.* 0.5, 1, 2, 3 and 4 h). An alternative explanation would be that hyperglycemia was induced by catecholamines. Several studies already demonstrated that situations like hypoxia requiring modulation of cardiocirculatory function and mobilization of energy reserves elicit catecholamine secretion [48–50]. Therefore, it might be interesting to measure the level of adrenalin/noradrenalin in Eurasian perch confronted to environmental hypoxia although this may be technically difficult. Indeed, use of cannulated fish would be required to perform proper sampling because of the very short time lag existing between stressor exposure and catecholamine release. More specific stress markers such as the hypoxia-inducible factor (HIF) 1α may also be assessed to reveal hypoxic stress in Eurasian perch [51]. When hypoxic conditions are severe, measures to maintain blood oxygen supply and to conserve energy (*e.g* reduced locomotion or feeding behavior) may not be sufficient. This can lead to the slowing-down of ATP production through aerobic respiration, forcing the animal to use anaerobic pathways to contribute to its energy requirements [49]. But anaerobic metabolism is not as efficient as aerobic respiration and a considerable increase in the glycolytic flux is needed to avoid a detrimental fall in cellular ATP [52,53]. As a result, blood glucose level increases for anaerobic fuel supply in many teleosts subjected to environmental and functional hypoxia and anaerobic metabolism further results in lactate accumulation in muscle tissue [49,53–55]. Interestingly in the present study, muscle lactate did not accumulate in muscle tissue (Table 1) while blood glucose concentration increased following exposure to the single hypoxic period. Perhaps hyperglycemia was not linked to the activation of anaerobic metabolism (see below: metabolism of erythrocytes) in the hypoxic conditions tested or lactate did not accumulate in muscle tissue. Literature supports the hypothesis that fish could be categorized as lactate "releasers" (*i.e.* lactate transfer from muscle tissue into the circulation) and "non releasers", depending on the species considered and the type/intensity of stressors [55,56]. It is not currently known if Eurasian perch belongs to the "releaser" category. To our opinion, it is essential to confirm any possible induction of anaerobic metabolism in Eurasian perch because this might bring important information on the tolerance level of this species to environmental hypoxia. Another possible cause for this hyperglycemic response would be linked to changes in the cellular compartment of the blood as suggested by SSI data. Indeed, SSI decreased ($p = 0.0002$) after a single hypoxia, indicating

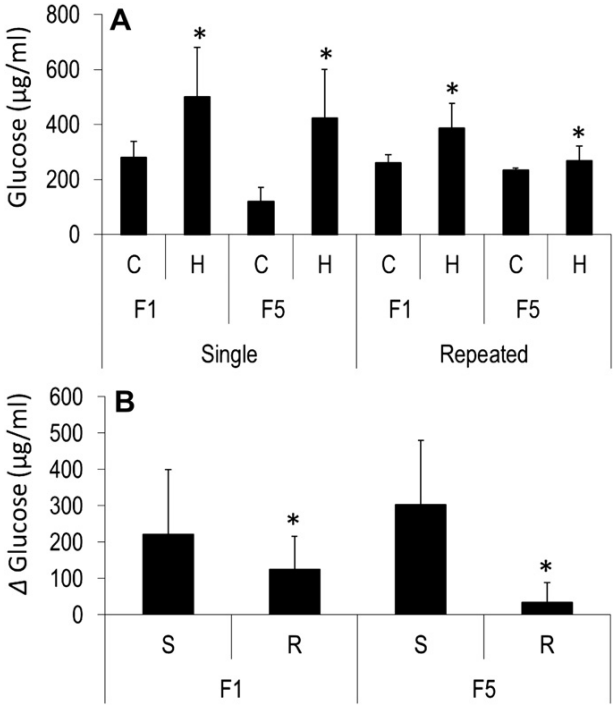

Fig. 4. Glucose response in the serum of Eurasian perch exposed to single or repeated hypoxia ($n = 3$). A) Total glucose level (µg/ml). C = control fish, H = hypoxic fish. Asterisk (*) indicates significant differences between C and H treatment. B) Δ glucose values (µg/ml). S = single hypoxia, R = repeated hypoxia. Asterisks (*) indicate differences between S and R treatment.

Table 1
Physiological stress indicators and immune parameters measured subsequently to single and repeated hypoxia in F1 and F5 generation.[a]

	Cortisol (ng/ml)	Lactate (mg/g)	Lysozyme (U/ml/min)	CH50
SINGLE hypoxia				
F1CTL	21.54 ± 8.68	2.78 ± 0.34	737 ± 72	42.59 ± 15.43
F1 HYP	12.95 ± 6.39	2.80 ± 1.01	639 ± 75	48.79 ± 11.90
F5 CTL	11.42 ± 2.79	2.20 ± 0.19	728 ± 146	31.66 ± 7.00
F5 HYP	10.28 ± 5.68	2.73 ± 0.40	593 ± 67	38.23 ± 14.01
REPEATED hypoxia				
F1 CTL	17.23 ± 11.09	2.00 ± 0.33	810 ± 111	90.67 ± 14.19
F1 HYP	15.79 ± 5.25	2.43 ± 0.79	693 ± 50[b]	93.41 ± 11.42
F5 CTL	11.11 ± 7.89	2.24 ± 0.46	745 ± 22	68.82 ± 22.36
F5 HYP	7.50 ± 0.99	2.20 ± 0.45	620 ± 36[b]	76.09 ± 14.25

[a] CTL, control treatment; HYP, hypoxia treatment ($n = 3$).
[b] Indicates significant differences between respective control and hypoxia treatments.

a significant spleen contraction in both generations (Fig. 5A). Likewise, other studies demonstrated that fish spleen contracted and released a considerable amount of erythrocytes into the circulating blood under both exercise and severe hypoxia [57,58]. Rapid increases in hematocrit and/or red blood cells (RBC) count were observed following hypoxia in Amazon fish, juvenile Tambaqui (*Colossoma macropomum*) and silver trevally (*Pseudocaranx dantex*) [46,55,59]. Although this should be verified in Eurasian perch, we speculate that spleen contraction following a single hypoxia resulted in the release of supplementary erythrocytes into the bloodstream to improve its oxygen carrying capacity. Interestingly, it appeared that red blood cells have high metabolic maintenance costs that are fuelled by both glucose and lactate [55]. Use of these molecules by metabolically active erythrocytes is expected to contribute to the maintenance of an efficient hemoglobin-oxygen transport system [55]. Thus, the elevated glycemia observed in the present study might also be explained by the release of spleen erythrocytes requiring glucose substrate for optimal oxygen transport activity. At the behavioral level, fish under hypoxia did not display any specific hyperactivity, trying to escape the hypoxic environment, and did not move upward to the water surface to resort to air breathing. Fish clearly adopted a resting behavior, remaining static at the bottom of the tank. Our observations are in accordance with data from the literature and suggest a shift towards energy saving strategies to face hypoxia [60,61]. We therefore assume that the above-described physiological responses were not induced by any potential hyperactivity of fish under hypoxia but by the hypoxic environment itself.

Pertaining to the effects of hypoxia on the immune system, serum lysozyme activity and ACH50 did not significantly differ following the single hypoxic period (Table 1). However, the proteomic analysis revealed that the single hypoxia challenge significantly influenced the abundance of some C3 complement proteins. Single hypoxia induced elevations of C3 complement proteins (spots n° 764 and 797) regardless of domestication level and up-regulated the abundance of distinct C3 complement proteins between both generations (spot n° 548 and 823 in the F1 and F5 generations, respectively). This suggests that domestication might be associated with few differences in the regulation of immune proteins following a single hypoxia (Table 2). Similarly to our previous study on chronic confinement [23], we found numerous protein spots identified as C3 complement. The presence of various C3 subunits is not surprising since multiple C3 isoforms have already been described for several fish species [62–64]. Moreover, it is well established that C3 components can be proteolyzed into several cleavage products, each with specific immune activities [62,63,65]. Another explanation could be the proteolytic degradation of serum samples. Although sample collection and storage were conducted to minimize ex-vivo changes in serum proteome profiles (*i.e.* clotting at 4 °C, rapidly followed by centrifugation and immediate freezing at −20 °C), we cannot exclude some protein degradation by endogenous proteolytic enzymes during the clotting process and following sample thawing since we did not add protease inhibitors to the serum. Following exposure to single hypoxia, up-regulation of these C3 proteins was however not associated with an increase in alternative complement hemolytic activity (ACH50). Perhaps, these fragments require equivalent modulation of other components to significantly influence the whole complement hemolytic activity. For example, C3b is an important fragment of the complement system, but other components (C5, C6, C7, C8 and C9) are essential to initiate the assembly of the membrane attack complex (MAC) responsible of cell lysis [62,63]. Second, studies on fish species reported the existence of several C3 isoforms differing in their binding and hemolytic efficiencies [62–64,66]. Although ACH50 was not affected in the present study, we cannot ascertain that the increased amounts in some of the C3 proteins have no effects on the overall immune response since C3 components are not only involved in hemolytic activity but also in the inflammation process, leucocyte chemotaxis, ROS production, B-lymphocyte activation, opsonisation of exogen particles and phagocytosis [62,63,66]. A better characterization of these C3 fragments is now needed to deepen the knowledge on how a single hypoxic stress influenced the abundance and activity of proteins from the complement system.

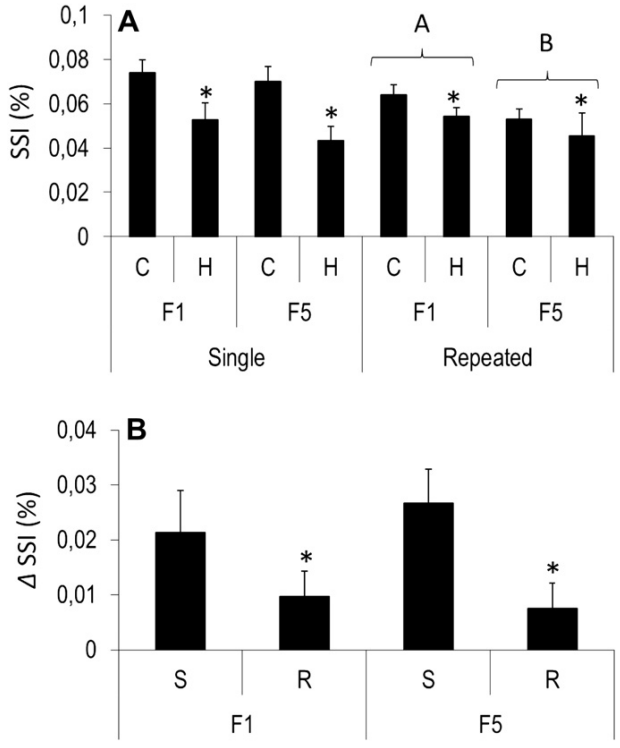

Fig. 5. SSI response in the serum of Eurasian perch exposed to single or repeated hypoxia ($n = 3$). A) SSI (%). C = control fish, H = hypoxic fish. Asterisk (*) indicates significant differences between C and H treatment. B) Δ SSI (%). S = single hypoxia, R = repeated hypoxia. Asterisks (*) indicates differences between S and R treatment. Capital letters indicate differences between F1 and F5 generations.

3.2. Repeated hypoxic stress: clues to acclimation mechanisms and humoral immune modulations

Similarly to what happened after the single hypoxia experiment, fish exposed to repeated hypoxic disturbances did not display significant differences in the level of serum cortisol in comparison to unstressed fish, for both generations (Table 1). Once more, we detected elevated glycemia in the serum of repeatedly-stressed fish (Fig. 4A). However, the magnitude of hyperglycemia lowered ($p = 0.048$) in all generations in comparison to fish stressed only once (Fig. 4B). Such reduction in hyperglycemic response may either indicate the development of acclimation mechanisms (at the physiological level and/or behavioral level, e.g. energy-saving behavior of more quiet fish) or exhaustion of the energetic metabolism. Indeed, decrease in stress response to long-term disturbance is usually associated with fish habituation [67,68]. But response attenuation may also be attributed to the exhaustion/breakdown of the physiological system, when the animal is too fatigued to maintain an efficient stress response to the repeated stimuli [68]. This alternative explanation is rarely considered in

Table 2
List of identified proteins differentially expressed in serum of F1 and F5 *P. fluviatilis* following exposure to single hypoxia.

Spot no.	Accession no.[a]	Protein name	Species	Matching Peptides[b]	Theoretical pI/Mw (kDa)	Fold change [c] between treatments[d]
764	gi|226731843	Complement component C3	*Perca flavescens*	2	5.7/65	1.30[e] in HYP *vs* CTL
974	gi|146447341	Fibrinogen beta chain precursor	*Paralichthys olivaceus*	3	6.5/56	1.75[e] in F5HYP *vs* F1HYP
548	gi|12957116	Complement component C3	*Anarhichas minor*	1	6.3/186	1.25[e] in F1HYP *vs* F1CTL 1.37[e] in F5CTL *vs* F1CTL
823	gi|58373439	Complement component C3	*Hippoglossus hippoglossus*	2	5.7/59	1.47[e] in F5HYP *vs* F5CTL 1.47[e] in F5HYP *vs* F1HYP
797	gi|6682835	Complement component C3	*Paralichthys olivaceus*	2	6.1/184	1.33[e] in HYP *vs* CTL
	gi|58373439	Complement component C3	*Hippoglossus hippoglossus*	2	5.7/59	1.57[g] in F5 *vs* F1
	gi|226731843	Complement component C3	*Perca flavescens*	2	5.7/65	
401	gi|225056702	Transferrin	*Dicentrarchus labrax*	2	5.9/74	1.42[e] in F5 *vs* F1
556	gi|225056702	Transferrin	*Dicentrarchus labrax*	3	5.9/74	−1.29[e] in F5 *vs* F1
405	gi|33340571	Immunoglobulin heavy chain	*Siniperca chuatsi*	2	5.4/63	1.45[e] in F5 *vs* F1
416	gi|33340571	Immunoglobulin heavy chain	*Siniperca chuatsi*	2	5.4/63	−1.58[e] in F5 *vs* F1
255	gi|6682835	Complement component C3	*Paralichthys olivaceus*	4	6.1/185	1.96[f] in F5 *vs* F1
	gi|226731843	Complement component C3	*Perca flavescens*	2	5.7/65	
497	gi|12957116	Complement component C3	*Anarhichas minor*	1	6.3/186	1.50[f] in F5 *vs* F1
368	gi|6682835	Complement component C3	*Paralichthys olivaceus*	1	6.1/184	−1.19[e] in F5 *vs* F1
243	gi|6682835	Complement component C3	*Paralichthys olivaceus*	4	6.1/184	2.13[g] in F5 *vs* F1
	gi|226731843	Complement component C3	*Perca flavescens*	3	5.7/65	
581	gi|6682835	Complement component C3	*Paralichthys olivaceus*	6	6.1/184	−1.26[e] in F5 *vs* F1

[a] NCBI database accession number.
[b] Refers to the number of sequenced peptides exclusively assigned to the protein.
[c] Positive and negative values indicates up- and down-regulation respectively.
[d] CTL, control treatment; HYP, hypoxia treatment.
[e] $p < 0.05$.
[f] $p < 0.01$.
[g] $p < 0.001$.

field studies. As a result, it is often falsely concluded that fish with a lower stress response habituate to the long-term stressor while, in fact, they display a reduced capacity of response and remain repeatedly/chronically stressed [68]. Similarly to the single hypoxia experiment, we did not detect any significant lactate elevation in muscle tissue in relation to repeated hypoxia (Table 1). For future studies, evaluation of muscle and blood lactate concentration might be very informative with respect to the possible induction of anaerobic metabolism and "lactate releasing" activity in our studied species. In both generations, SSI decreased after repeated ($p = 0.046$) hypoxic disturbances, indicating a significant spleen contraction (Fig. 5A). The amplitude of SSI decrease clearly attenuated ($p = 0.0019$) at the end of the repeated hypoxia experiment (Fig. 5B). But, conversely to hyperglycemia, this attenuation is likely to be due to SSI reduction in control fish rather than to a reduced contraction in repeatedly hypoxic fish. The 2D-DIGE proteomic analysis indicated that transferrin (spot n°351) was significantly up-regulated in both generations following repeated hypoxic periods (Table 2). Similar findings were already reported in human cells, rat and mice exposed to hypoxic environment [69]. During hypoxia, it is conceivable that an elevation in plasma iron transport is required to ensure sufficient oxygen supply and illustrated by an increase in transferrin abundance [69–71]. Transferrin is also an acute phase protein that rapidly reduces iron availability to bacteria following injury, trauma or infection [71,72]. Together with this increased transferrin level, the reduced hyperglycemia would finally suggest the development of acclimation mechanisms to repeated hypoxic disturbances rather than fish exhaustion. For future research, it might be interesting to evaluate indicators such as haematocrit, haemoglobin and erythropoietin levels, ventilatory frequency and swimming behavior to confirm this hypothesis.

Serum lysozyme activity significantly decreased ($p = 0.012$) in repeatedly-stressed fish from both generations (Table 1). The proteomic analysis also revealed that several C3 proteins were modulated following repeated hypoxic stress. Spot n° 764 was the only one to be up-regulated following both single and repeated hypoxia (Table 2). After repeated hypoxic disturbances, down-regulation was reported for several C3 complement proteins (spot n° 823 in both generations and spot n° 926 in F5 fish only) (Table 2). Once more, modulations of C3 complement protein amounts did not induce changes in ACH50. Immuno-suppressive effects of stressors have already been reported in many studies *e.g.* [9,10,12,42,73,74]. Reduction in lysozyme activity and C3 component abundance in repeatedly-stressed fish may result from the energetic demand associated with long-term stress responses. Indeed, if a portion of the fish's energy budget is required to cope with stressors, then less energy will be available for other biological functions, including immunity [5]. It now remains to be determined whether these immune changes would have deleterious effects on the fish overall resistance to disease through achievement of bacterial challenge tests. Moreover, since the effects of stressors might be tissue-specific [42], not only serum immune actors but defense mechanisms in internal organs (*e.g.* head kidney, spleen, thymus) and on the primary barrier (*i.e.* gut, gills, mucus and skin) should also be studied to go through the immunological consequences of hypoxia. Even if deleterious immune changes occur at the circulatory level, this may not necessarily result in an increased susceptibility to disease depending on the integrity and immune capabilities in these above-mentioned tissues.

3.3. Domestication and its influence on stress and immune responses to hypoxia

It seems that domestication was of limited influence on the stress and immune responses of fish to both single and repeated hypoxia (*i.e.* only significant divergence in the abundance of three C3 proteins: spots n° 548, 823 and 926). Our previous studies on chronic confinement suggested that domestication could be associated with a clearer divergence in stress responsiveness after only four generations reared in captivity [22,23]. To elucidate why

domestication resulted in a better tolerance to chronic confinement and not to hypoxia, we propose that this may depend on the stressor capacity to severely threaten internal homeostasis. Indeed, stress response is first an adaptive mechanism to promote the best chances of survival towards hostile situations. In this context, low-level stress responses may not constitute a systematic advantage and the preservation of optimal stress responses may be necessary for immediate survival under severe conditions. This might be particularly true during exposure to physico-chemical, mechanical or thermal stressors which constitute real threats to homeostasis. We assume that severe environmental hypoxia belongs to this stressor category. On the other hand, certain stressors represent a perceived danger rather than a real hazard to life and body integrity (*e.g.* chronic confinement). Under such cognitive and/or mild stressing situations, reduction in stress sensitivity may not be deleterious since individuals are not really in danger. On the contrary, reduction in stress responsiveness may be profitable since energy expenditure associated with allostatic load would be avoided. The influence of domestication on physiological processes has not been widely studied so far, particularly concerning stress responses to diverse sub-optimal farming conditions. We are convinced that the biological consequences of such adaptive process to captive life can be significant and that this area of research would deserve further consideration.

Independently of any stressor exposure, domestication significantly influenced the size of the spleen as well as the abundance of several serum proteins with different roles in the acute phase response and immune defense. Firstly, a lower SSI ($p = 0.030$) was observed in the F5 generation in comparison to the F1 counterparts at the end of the repeated hypoxia period (Fig. 5A). After the single hypoxia experiment, a lower SSI (although not significant) was also observed in the F5 generation. Our previous study already reported significant SSI reduction with domestication level in Eurasian perch [23] but the causes for such spleen size reduction are not currently known. The 2D-DIGE approach indicated that anserinase (spot n°538) was under-expressed in the F5 generation at the end of the experimental period (Table 3). Anserinase is a metallodipeptidase hydrolyzing anserine to histidine and which is believed to be universally distributed in poikilothermic animals [75]. To our knowledge, there is currently no information on the biological roles of anserinase in poikilotherms. Studies mainly focus on the corresponding carnosine/carnosinase pathway in higher vertebrates and humans and contradictory results have been reported so far [75,76]. Domestication influenced the expression of warm temperature acclimation proteins (Wap65) present in spots n°573 and n°602 since a lower abundance was observed in the F5 fish group on day 46 (Table 3). Wap65 are glycoproteins present in plasma and liver of teleost fish and two different isoforms (Wap65-1 and Wap65-2) have been isolated from several fish species [77,78]. Due to sequence homology and highly conserved domains, it is suggested that teleost Wap65 proteins are homologous of mammalian hemopexin (Hpx), a plasma glycoprotein functioning as a high-affinity scavenger of free heme released from damaged/senescent erythrocytes [78–80]. In mammals, Hpx is also an acute phase protein induced during inflammation to sequester heme-associated iron away from bacteria [80]. It has also been proposed that Wap65 proteins might be involved in the immune response [77,78,81]. We therefore suggest that the observed down-regulation of Wap65 level in serum is not positive in the context of domestication since it can result in oxidative stress, higher iron availability for invading

Table 3
List of identified proteins differentially expressed in serum of F1 and F5 *P. fluviatilis* following exposure to repeated hypoxia.

Spot no.	Accession no.[a]	Protein name	Species	Matching Peptides[b]	Theoretical pI/Mw (kDa)	Fold change[c] between treatments[d]
351	gi|225056702	Transferrin	*Dicentrarchus labrax*	2	5.9/74	1.22[f] in HYP *vs* CTL
764	gi|226731843	Complement component C3	*Perca flavescens*	2	5.7/65	1.30[e] in HYP *vs* CTL
823	gi|58373439	Complement component C3	*Hippoglossus hippoglossus*	2	5.7/59	−1.37[e] in HYP *vs* CTL
629	gi|150036374	Transferrin	*Chaenocephalus aceratus*	1	6.4/74	1.37[e] in F5CTL *vs* F1CTL
926	gi|6682835	Complement component C3	*Paralichthys olivaceus*	2	6.1/184	1.98[e] in F5CTL *vs* F1 CTL
	gi|58373439	Complement component C3	*Hippoglossus hippoglossus*	2		−1.36[e] in F5HYP *vs* F5CTL 1.53[g] in F5 *vs* F1
556	gi|225056702	Transferrin	*Dicentrarchus labrax*	3	5.9/74	−1.25[e] in F5 *vs* F1
538	gi|76362267	Anserinase	*Oreochromis niloticus*	3	5.4/55	−1.53[f] in F5 *vs* F1
573	gi|66267674	Warm temperature acclimation related 65	*Oryzias latipes*	3	5.5/48	−1.44[g] in F5 *vs* F1
602	gi|66267674	Warm temperature acclimation related 65	*Oryzias latipes*	2	5.5/48	−1.24[e] in F5 *vs* F1
421	gi|283362240	L-amino acid oxidase	*Platichtys stellatus*	1	5.8/58	1.13[e] in F5 *vs* F1
431	gi|283362240	L-amino acid oxidase	*Platichtys stellatus*	1	5.8/58	1.32[g] in F5 *vs* F1
944	gi|146447341	Fibrinogen beta chain precursor	*Paralichthys olivaceus*	7	6.5/56	1.22[e] in F5 *vs* F1
	gi|218665023	Fibrinogen beta chain precursor	*Larimichthys crocea*	4	5.9/56	
974	gi|146447341	Fibrinogen beta chain precursor	*Paralichthys olivaceus*	3	6.5/56	1.40[e] in F5 *vs* F1
243	gi|6682835	Complement component C3	*Paralichthys olivaceus*	4	6.1/184	1.90[f] in F5 *vs* F1
	gi|226731843	Complement component C3	*Perca flavescens*	3	5.7/65	
255	gi|6682835	Complement component C3	*Paralichthys olivaceus*	4	6.1/184	2.07[f] in F5 *vs* F1
	gi|226731843	Complement component C3	*Perca flavescens*	2	5.7/65	
797	gi|6682835	Complement component C3	*Paralichthys olivaceus*	2	6.1/184	1.70[f] in F5 *vs* F1
	gi|58373439	Complement component C3	*Hippoglossus hippoglossus*	2	5.7/59	
	gi|226731843	Complement component C3	*Perca flavescens*	2	5.7/65	
548	gi|12957116	Complement component C3	*Anarhichas minor*	1	6.3/186	1.26[e] in F5 *vs* F1
256	gi|6682835	Complement component C3	*Paralichthys olivaceus*	6	6.1/184	−2.53[g] in F5 *vs* F1
581	gi|6682835	Complement component C3	*Paralichthys olivaceus*	6	6.1/184	−1.24[e] in F5 *vs* F1

[a] NCBI database accession number.
[b] Refers to the number of sequenced peptides exclusively assigned to the protein.
[c] Positive and negative values indicates up- and down-regulation respectively.
[d] CTL, control treatment; HYP, hypoxia treatment.
[e] $p < 0.05$.
[f] $p < 0.01$.
[g] $p < 0.001$.

pathogens and/or alteration of immune responses to infection. Further investigations are now needed to assess the physiological effects of Wap65 down-regulation in domesticated fish. Domestication was also associated with increases in L-amino acid oxidase (LAAO) abundance (spots n° 421 and 431) in the F5 individuals at the end of the experimental period (Table 3). LAAO has been reported in several fish tissues (viscera, skin mucus and gills) as well as in the serum where four isoforms have already been identified in the rockfish *Sebastes schegeli* [82,83]. It has been reported that LAAO has antiviral, antibacterial and anti-protozoal activities in a variety of animal fluids [83,84]. In serum, it seems that LAAO exhibits a broad-spectrum antibacterial activity against both gram-positive and negative bacteria, most potently against *Aeromonas* spp which are common pathogen species in aquaculture facilities [82]. Consequently, we propose that up-regulation of two serum LAAO isoforms in F5 individuals might be advantageous given the efficient role of this enzyme in bacteria killing. Changes in the abundance of fibrinogen molecules (spots n° 944 and 974) were observed as well with respect to domestication level. Serum fibrinogen abundance was higher in F5 individuals at the end of the experimental period (Table 3). Fibrinogen is a key molecule in the blood clotting process and an acute phase protein whose plasma concentration increases in any condition that causes inflammation or tissue damage [85–87]. It has also been suggested that fibrinogen participates to immune function in both invertebrate and vertebrate animals [87,88]. Further investigations are needed to evaluate whether elevated fibrinogen levels positively contribute to the blood clotting process and immune defence mechanisms in domesticated fish. In the current study, domestication was associated to conflicting changes in the abundance of several transferrin molecules (up-regulation of spot n° 401 and down-regulation of spot n° 556), immunoglobulin heavy chains (up-regulation of spot n°405 and down-regulation of spot n° 416) and C3 complement components (up-regulation of spots n° 243, 255, 497, 548, 797, 926 and down-regulation of spots n° 256, 368, 581) (Tables 2 and 3). For now, it is not possible to determine the physiological causes and consequences of up- and down-regulations of anserinase, Wap65 proteins, LAAO, fibrinogen, transferrin isoforms, Ig heavy chains and C3 complement components observed between F1 and F5 generations and it is indispensable to deepen our knowledge on the regulation and activity of these proteins differentially expressed in the serum of each generation.

4. Conclusions

This study demonstrated that exposure of Eurasian perch to both single and repeated environmental hypoxia induced energetic readjustments and spleen contraction (probably coming from a rapid release of spleen erythrocytes into the bloodstream) as well as immune changes in serum proteome (*i.e.* C3 components) following either single or repeated hypoxia. Under repeated hypoxia, data suggested the development of acclimation mechanisms and showed immunosuppressive effects on lysozyme activity and abundance of C3 components in serum. Domestication however did not induce marked divergence in physiological and immune responses to both single and repeated hypoxia. Independently of stressor exposure, numerous conflicting changes in the abundance of proteins related to acute phase response and innate/specific immunity were observed according to the generational level of fish but causes and consequences are currently unknown. Further investigations are now required to support these findings and to get a more comprehensive view of how hypoxia and/or domestication affect the fish physiology and immune system. With respect to the latter, it might be interesting to test several successive offsprings (*e.g.* F1, F2, F3, F4 and F5) to assess the progression of physiological and immune changes from one generation to the next.

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