Protein changes as robust signatures of fish chronic stress: a proteomics approach in fish welfare research

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Abstract

Background: In order to have a greater understanding of fish welfare, sensitive technological tools, such as proteomics, may assist the aquaculture industry as it allows an unbiased approach for the discovery of potential biomarkers for stress monitoring. Stress is characterized by a cascade of physiological responses that end-up inducing further changes at the whole-animal level that might either increase fitness or impair welfare. Monitorization of this dynamic process, up till now relies on indicators that are only a snapshot of the stress level experienced. Within this scope, using G ilthead seabream (Sparus aurata) as model, three chronic stress conditions, namely overcrowding, handling and hypoxia, were employed to evaluate the potential of the fish protein-based adaptations as reliable signatures of chronic stress, in contrast with the commonly used hormonal and metabolic indicators.

Results: A large spectrum of biological variation regarding cortisol and glucose levels was observed, which values rose higher in net handled fish. In this sense, a potential pattern of stressor-specificity was evidenced since the magnitude of response and tolerance varied markedly from a permanent (crowding) to a repetitive stressor (handling). Gel-based proteomics analysis of the plasma proteome also revealed that net handled fish had the highest number of differential proteins, compared to the other trials . M ass spectrometric analysis, followed by gene ontology enrichment and protein-protein interaction analyses, characterized those as humoral components of the innate immune system and key elements on the response to stimulus.

Conclusions: Overall, this study represents the first screening of more reliable signatures of physiological adaptation to chronic stress in fish, allowing the future development of novel biomarker models to monitor fish welfare.

Background

Managing welfare and preserving the well-being of fish in captivity is of increasing importance, whether by productivity concerns or sustainability issues (1). Welfare definition, apart from being complex and controversial, remains with no clear consent on how it should be defined or measured (2,3). Limitations like divergent coping mechanisms, the lack of complete knowledge regarding the nociceptive system of fish (e.g. emotional-like states; cognitive abilities, pain, suffering) (4–7) and the lack of reliable physiological indicators of fish welfare, make its investigation even more challenging (8).

The perception of threatening situations (stressors) by the fish is proceeded by a physiological cascade of events in the animal's biological system (9). This adaptive mechanism, known as stress response, enables the fish to cope with the stressor. However, when unpredictable and uncontrollable situations exceed the organism's natural regulatory capacity, consequent negative effects can impair animal's welfare (10,11). Such effects are commonly used as late indicators of the welfare condition of the individuals (12). The physiological stress response starts with the immediate activation of the sympathetic response, followed by a slightly delayed activation of the hypothalamo-pituitary-interrenal (HPI) axis. This result in the release of catecholamines and a corticosteroid hormone (cortisol in teleosts), respectively, into the bloodstream (13,14). These hormones lead to a series of downstream responses involving alterations in the energy metabolism and respiratory and immune functions (15). The rapid mobilization of energy substrates such as glucose (the fuel needed for the coping mechanisms) is caused by the activation of glycogenolysis in the liver or muscle, and hepatic gluconeogenesis, by the catecholamines and cortisol, respectively (16,17). Stressful stimuli can also lead to strenuous exercise fuelled by anaerobic glycolysis in the muscle, generating lactate which is released into plasma (18,19). In the case of prolonged exposure to the stressor, maladaptively alterations can be reflected in the whole-animal performance, like perturbations at the reproduction, immunological, growth and behaviour levels (14).

There are multiple stressors inherent to daily routines in an aquaculture rearing facility, ranging, in duration and severity (20). Recurrent acute or chronic stress can lead to physiological reactions distinct from those characterizing the response to a one-off stressful situation. The cortisol response, commonly along with glucose and lactate, are the most used physiological indicators of stress and welfare status in farmed fish (21). Nevertheless, several studies demonstrate already that these indicators can be unreliable in cases of chronic long-term stress, mostly due to: (i) high biological variability of responses; (ii) cortisol levels return to basal levels within minutes/hours after an acute stressor; (iii) in cases chronic stressors, fish can adapt, to certain extent, and the cortisol response is attenuated; and (iv) secretion might also be affected by intrinsic and extrinsic factors (e.g. age, sexual maturity, social status, level of domestication, prior experience, nutritional status) (22–27).

In this sense, behavioural observations and plasma physiological indicators need to be complemented to form a robust scientific welfare assessment, and more advanced and sensitive technologies are gaining popularity. Proteomics are promising alternatives for the discovery of candidate molecular markers that can indicate physiological alterations due to stress exposure (28). Despite the limitations

to the use of these technologies in the aquaculture field (29), several studies prove already the huge potential of proteomics for the identification of stress signatures (30–34).

Very little data is available concerning long-term coping with a chronic stressor and the consistency of the range of the universally used indicators in these cases. Considering this gap in research, we aim, in the present study, to assess comparatively the fish stress response at different levels (i.e., plasma stress markers, changes in plasma proteins' abundance and muscle biochemistry). Using Gilthead seabream (*Sparus aurata*) as model, three chronic stress conditions were employed, and proteomics was used to benchmark potential signatures of stress adaptation in the plasma proteome since several proteins resultant from physiological events are released into circulation. Gilthead seabream was the chosen species in this study since it is one of the most important species in European aquaculture with high commercial value. This work aims to pioneer a better understanding of the underlying molecular mechanisms behind the fish physiological adaptation to long-term stress. Additionally, it aims to bridge the gap between the scientific community and the industry by paving the way for the development of novel biomarkers to monitor fish welfare.

Results

Fish general condition

Fish were monitored every day during the trials reaching the end of the experimental period with a 100% survival rate. Fish overall condition and growth performance were also monitored (Additional file 1), and initial (IBW) and final body weights (FBW) were recorded for each experiment. Average body weights showed reductions at the end of the net handling (NET) and hypoxia (HYP) trials, in all groups, including the control. However, there were no significant differences in final body weights between the control and any of the stressed groups ($P > 0.05$), suggesting that weight reductions were unrelated to the stressor.

Plasma stress markers analysis

Circulating cortisol, glucose and lactate levels were measured either in Gilthead seabream submitted to different chronic stressors as in control fish (Fig.1). The overall levels of these metabolites presented high variability of biological responses for all trials, with several data points considered outliers (outside of the interquartile range). Cortisol levels presented the highest intervals of values. In the OC trial, only lactate plasma levels presented statistically significant differences, both between control and stressed groups ($Lactate_{CTRL}$ – 13.46 ± 4.07, $Lactate_{OC30}$ – 17.79 ± 5.29, $Lactate_{OC45}$ – 19.89 ± 6.19, $P = 2.89e^{-3}$). Curiously, in the case of cortisol, although not significant, stressed fish showed decreased levels compared to control ($Cortisol_{CTRL}$ – 28.03 ± 30.78, $Cortisol_{OC30}$ – 12.51 ± 13.13, $Cortisol_{OC45}$ – 8.54 ± 10.92, $P = 1.2e^{-1}$). In the NET trial, statistically significant differences were registered for the cortisol and glucose plasma levels, again between control and the stressed groups ($Cortisol_{CTRL}$ – 29.38 ± 38.06, $Cortisol_{NET4}$ – 55.69 ± 41.05, $Cortisol_{NET4}$ – 84.83 ± 50.77, $P = 5.15e^{-4}$; $Glucose_{CTRL}$ – 46.57 ± 6.58, $Glucose_{NET2}$ – 69.76 ± 12.90, $Glucose_{NET4}$ – 66.60 ± 13.74, $P = 2.06e^{-6}$). In the HYP trial, significant differences were observed exclusively for the glucose levels ($Glucose_{CTRL}$ – 55.85 ± 12.72, $Glucose_{HYP15}$ – 96.22 ± 45.53, $Glucose_{HYP15}$ – 79.30 ± 15.78, $P = 1.07e^{-4}$). Cortisol values are presented as mean ± standard deviation (S.D.) in ng/ml, and glucose and lactate values in mg/dl.

Post-mortem muscle biochemical changes

Muscle pH declined over 72 HAD, in Gilthead seabream stored in ice, ranging from an average of 7.4, 7.7 and 7.4 immediately after slaughtering, to 6.3, 6.5 and 6.4 at the last sampling time, in fish from OC, NET and HYP trials, respectively. Significant differences between conditions were found in NET and HYP trials at 4 ($P_{NET2\text{-}NET4} = 0.032$) and 72 HAD ($P_{CTRL\text{-}NET2} = 0.008$), and at 0 ($P_{CTRL\text{-}HYP15} = 0.021$), 8 ($P_{CTRL\text{-}HYP15} = 0.003$, also in HYP30-HYP15 with lower significance), 48 ($P_{CTRL\text{-}HYP15} = 0.006$) and 72 HAD ($P_{HYP30\text{-}HYP15} < 0.001$), respectively (Fig. 2).

The onset and resolution of *rigor mortis* (Fig.2) showed significant differences between treatments in the NET and HYP trials, specifically at 8 HAD ($P_{CTRL\text{-}NET4} < 0.001$), and at 8 ($P_{HYP30\text{-}HYP15} < 0.001$) and 24 HAD ($P_{HYP30\text{-}HYP15} = 0.020$), respectively. In the OC trial, all fish reached averaged maximum rigor strength at 24 HAD. In the NET trial, averaged maximum rigor strength was reached at 48 HAD in CTRL and NET2, and at 24 HAD in NET4 group. In the HYP trial, all groups reached averaged maximum rigor strength at 48 HAD.

Plasma proteomics analysis

A comparative proteomics analysis of the Gilthead seabream plasma between the control and the stress treatments detected, 681, 752 and 681 protein spots for the OC, NET and HYP trials, respectively, within the pH range of 4-7 and a molecular mass range of 11-114 kDa. After statistical analysis, 19, 360 and 34 protein spots within the OC, NET and HYP trials, respectively, were found to present significantly differential abundance (significance threshold at $P < 0.05$) between experimental conditions. From these, 7, 171 and 12 were manually excised from the 2D gels for MALDI-TOF/TOF MS analysis. No proteins were identified with significance for the OC trial. For the NET and HYP trials, 107 and 2 differential protein spots, respectively, were successfully identified by a combination of PMF and MS/MS search, with significant scores (protein score > 76, total ion score > 60, $P < 0.05$). Among the spots identified from the NET trial, 13 showed more than one significant protein identification (202, 326, 521, 559, 586, 604, 677, 877, 950, 959, 990, 996 and 1157), indicating that multiple proteins migrated to the same spots on the gel. The identified proteins are listed in an additional file (see additional file 2). A representative 2D-gel of the Gilthead seabream plasma proteome is shown in Fig.3.

Considering the number of identifications in each trial, only the 107 identified protein spots from the NET trial were considered for further statistical and bioinformatics analyses. At this step, a log-fold change cut-off of ± 1.0 ($P < 0.05$) was applied (Fig.4-A) and a total of 56 identified proteins spots (corresponding to 20 single entries) were considered significant. From these, 19 were up-regulated in stressed fish and 34 were down-regulated. Three spots (502, 990 and 1021) showed multi-expression patterns and could not be classified as up- or down-regulated. Seventeen protein spots (502, 715, 841, 905, 908, 919, 937, 939, 967, 997, 1004, 1016, 1021, 1151, 1221, 1238 and 1250) were identified as apolipoprotein A-I, whereas 13 were down-regulated in stressed fish. Four spots (864, 869, 990 and 996) were identified as apolipoprotein Eb and 2 were up-regulated. Complement factor B was identified in 4 spots (144, 146, 152 and 737) and complement component C3 in 5 spots (591, 593, 595, 1048 and 1083) from which 3 from each were up-regulated. Two protein spots (796 and 833) were identified as warm-temperature acclimation-related 65kDa, 1 down- and 1 up-regulated. Three spots (202, 206 and 209), identified as inter-alpha-trypsin inhibitor heavy chain H3, 2 spots (224 and 229) as alpha-2-macroglobulin and 5 (558, 751, 843, 904 and 1079) identified as transferrin were down-regulated. Two spots (663 and 710), identified as haptoglobin, were found to be up-regulated. Fibrinogen alpha-chain was identified in two spots (521 and 544) and were both up-regulated. Alpha-1-antitrypsin homolog, apolipoprotein B-100, beta-actin, calcium/calmodulin-dependent protein kinase type II, leucine-rich alpha-2-glycoprotein, fetuin-B-like, hemopexin-like, hyaluronic acid-binding protein 2 and pentraxin were identified in a single protein spot each.

Hierarchical clustering (HCA) and principal component (PCA) analyses were performed for the identified 107 proteins spots with differential relative abundance across NET groups to check how well the samples grouped based on the expression patterns of the protein spots. The PCA (Fig.4-B) showed two main clusters belonging to the control and NET4 samples, while 2 biological samples belonging to the NET2 group clustered together with the control samples and 1 with the NET4 samples. The 107 differential protein spots were centralized into two principal components (PC), PC1 and PC2, which represented the maximum variation (65.6%) and the next highest variation (5.5%), respectively. The HCA (Fig.4-C) revealed likewise two main groups regarding the biological replicates, as it is observed by the top dendrogram. The protein spots were also grouped in two main clusters, one displaying a pattern of higher and the other of lower relative abundance in stressed fish, when compared to control. As described above for the PCA, higher variability in NET2 was also shown in the HCA.

For the network and GO enrichment analyses the subset of 20 single protein identifications mentioned above was blasted against *Danio rerio* in the UniprotKB database. A PPI network (Fig.5-A) was generated on STRING web tool revealing 61 edges among 18 nodes/proteins (2 proteins had no interaction with the main network), with a clustering coefficient of 0.677 and a very significant enrichment value ($P < 1.0e^{-16}$). The analysis was performed on Cytoscape and specific topological parameters were selected to demonstrate the importance and distribution of the nodes in the network: a darker colour intensity of the nodes indicates higher degree, while the size was estimated using the variation in protein abundance (fold-change). For every single entry, one protein spot was chosen as the most representative of each protein (Table 1), based mainly on the protein score and experimental molecular weight and pI close to the theoretical ones. From these 18 spots, 11 were down- and 7 were up-regulated, however, these differences in abundance were mostly significant (log-fold change > 1.0 or < -1.0, *q-value* < 0.05) for the NET4 treatment (only 2 were exclusively significant for the NET2 treatment and 2 were significant for both treatments). Thus, the fold-change of these 18 spots between NET4 and CTRL groups was used to estimate the size of the nodes on the PPI network, which ranged from -4.04 to +2.78. SERPINC1 (antithrombin-III), TFA (transferrin) and FGA (fibrinogen alpha-chain) occupied the most central positions in the network having the highest number of interactions, while APOA1 (apolipoprotein A-I) showed the highest number of experimentally demonstrated interactions, mainly with

APOEB (apolipoprotein Eb), APOBB (apolipoprotein B-100) and FGA. GO Enrichment analysis (Fig.5-B) revealed 19 overrepresented (hypergeometric test, FDR < 0.05) GO Biological Process (BP) terms, mostly linked to the immune system and response to stimulus. No annotations were retrieved for alpha-1-antitrypsin, leucine-rich alpha-2-glycoprotein-like, apolipoprotein A-I, apolipoprotein B-100-like, haptoglobin, pentraxin and hyaluronic acid-binding protein 2. In the horizontal bar plot (Fig.5-B), only the 9 most significant terms are represented. GO Molecular function enrichment analysis accounted for 8 terms with 5 main proteins (alpha-1-antitrypsin, antithrombin-III, inter-alpha-trypsin-inhibitor, kininogen and alpha-2-macroglobulin) while GO Cellular component revealed 4 enriched terms with 2 main proteins (fibrinogen alpha-chain and alpha-2-macroglobulin). A complete list of all GO terms is described on the additional file 3.

Discussion

In this study the stress response of farmed Gilthead seabream adults to chronic stress conditions was primarily assessed through both changes in the concentration of routine plasma stress indicators, namely cortisol, glucose and lactate, and *post-mortem* biochemical parameters, explicitly pH and *Rigor mortis*. To evaluate the existence of unbiased and reliable markers of chronic stress, proteomics was used to verify the potential of fish protein-based adaptations in this endeavor.

Cortisol has been the most commonly used physiological indicator of the primary response to stress (21). However, there is a shred of evidence indicating that this corticosteroid is not a reliable biomarker of long-term stress exposure (27,35–37). In this study, Gilthead seabream exposed to high stocking densities during 54 days showed a possible reconfiguration of the cortisol response. This is supported by the observed downward trend of this metabolite, as compared to unstressed fish. Such consequence is suggestive of a non-activation or altered responsiveness of the HPI axis, which sometimes leads to the hyporeactivity of the corticosteroid response (38). The same outcome was observed in juvenile Gilthead seabream confined for 14 days at 26 kg/m^3 (39) and in meagre cultured at different stocking densities for 40 days (40). In NET trial, contrarily, and apart from the wide dispersion of observations, plasma cortisol levels were significantly higher in handled fish. This result suggests that the fish was not able to adapt to the handling stressor. The fact that it was not a constant stressor but instead repetitive, summed to its unpredictability and severity, could have prevented the possibility of habituation. Regarding the HYP trial, no systematic effect of the 48 h of hypoxia was observed in these fish. This suggests an acclimation to the low oxygen environment by a possible adjustment of the oxygen requirement (e.g. reduction of high energy behaviours).

Overall, the aforementioned observations suggest that the cortisol response and the capacity of adaptation are modulated by the nature, duration and intensity of the stressor. However, other factors like species, age, sex and individual coping mechanisms seem to be ubiquitous and impact their adaptive processes (24,38,41). This process of stress habituation was already suggested and demonstrated in other studies (37,42), but this mechanism is not completely understood so far. High individual variability was also found in every trial, most likely due to individual differences in the stress response related with intrinsic factors to the animal (e.g. coping styles, cognitive perception) (39,43,44). Additionally, values registered for control fish, in every trial, are higher than the reference values reported in the literature for this species (45). These discrepancies can have several causes and that is the reason why cortisol should be used with caution when evaluating the magnitude of the stress response. Moreover, the difficulty of measuring the resting levels of this hormone is also acknowledged to be one of these causes. The lack of proper planning when sampling cortisol, or the manipulation needed to net and anaesthetize the fish, can result in high “control” cortisol levels that do not correspond to the “genuine” basal levels i.e., the non-manipulated fish levels. Also, it is well established that following the perception of an acute stressor, the levels of circulating stress markers increase within the first minutes or hours of stress response, returning to basal levels while time elapses, usually within 24 h (17,46,47).

Secondary physiological responses are characterized by an increase in glucose and lactate levels in blood plasma in order to satisfy the increased energy expenditure. Changes in glucose usually follow similar trends than cortisol after the stressor (14). This is corroborated in this study by the levels of plasma glucose registered in the fish from all trials (Fig.1). Glucose levels, besides following the same trend as cortisol levels, are, in general, below the basal values for this species (45). This could be related to the fish’s inability to maintain the same levels of glucose in the blood due to the high demand for glucose mobilization to other tissues. The decrease of plasma glucose levels in OC is consonant with the decrease in the cortisol levels, supporting the hypothesis of habituation or exhaustion of the endocrine system (27). The significant increases in the plasma glucose levels of stressed fish from NET and HYP trials are consistent with previous studies. These showed that glucose rises during air exposure or low oxygen levels, due to stimulation of muscle glycogenolysis and hepatic gluconeogenesis, where glucose is synthesized to maintain the energetic substrates’ demand (48). Similarly to cortisol, glucose and lactate circulating levels also return to basal levels within hours post-stressor, which also make of these metabolites weak reliable markers in case of prolonged stressors (49,50). Additionally, studies also demonstrate that glucose variations in the blood are not only

hormonal-induced due to stressful practices. Factors like variations in the water temperature and pH, anaesthesia, diet composition or fasting can also affect plasma glucose levels (51,52).

When insufficient oxygen is available to maintain the aerobic ATP production, fish resort to anaerobic metabolism to meet cellular requirements. This shift consequently leads to lactate accumulation in the muscle (19,53). In this study, changes in circulating lactate are not in agreement with cortisol and glucose variations. Statistically significant differences in the lactate levels were only observed in the OC trial. In this case, if the cortisol response is indeed lower due to HPI-axis acclimation, as suggested before, the lactate recycling rate in the hepatic glycogenolysis is reduced, explaining the significant plasma lactate increase in stressed fish. Additionally, previous studies show that during hypoxia or intense swimming activity, fish produce lactate in the muscle at a higher rate than it can be processed by other tissues (53).

Post-mortem muscle pH and *rigor mortis* have been used as tissue indicators of *ante-mortem* stress in numerous fish species (54–56). After the fish death, both blood circulation and oxygen supply cease. The major source of ATP to the muscle is thus lost, since glycogen can no longer be oxidized. However, for a limited time after death, ATP in the muscle is maintained at a definite level by creatine kinase. Consequently, the depletion of ATP reserves stimulates the breakdown of glycogen by anaerobic glycolysis in the muscle, in order to maintain the energy expenditure. This process results in the accumulation of lactic acid, generating H^+ ions and consequently lowering muscle pH (57). Glycolysis continues until all glycogen is consumed or the glycolytic enzymatic system is inactivated by the low pH. Hence, the magnitude and rate of this pH fall depend on the fish's energy reserves prior to death. These energy reserves can be influenced by the intensity and duration of the stress while fish is alive. To our knowledge, no studies were performed in this species regarding the effects of long-term chronic stressors on the evolution of *post-mortem* biochemical processes in the muscle. Results from this study (Fig.2) followed the same pH trends as previous studies on gilthead seabream (58,59), however, comparing with the existent studies on pre-slaughter stress (55,60,61), muscle pH values immediately after death are below the ones found in this study, suggesting that stress at slaughter was low in our fish. Poli et al 2005 state that in cases of exposure to a chronic stressor for a long time before death, the lactic acid produced can be gradually cleared from the muscle, but simultaneously the energy sources, like glycogen, will likewise become gradually exhausted. Hence, when the fish is killed, muscle pH do not suffer a dramatic fall due to an early end of *post-mortem* anaerobic glycolysis caused by energy source scarcity. This might explain the significant differences found in the HYP trial, where the highest pH values were observed in the highly stressed fish (HYP15), suggesting that these fish had lower energy reserves. Nevertheless, pH values registered after the 24 HAD, in every treatment, are in agreement with the reported by previous studies in this species at the same sampling times (58,62).

Rigor mortis is inextricably correlated with muscle ATP and the pH decline. The onset of *rigor mortis* occurs with ATP depletion. When ATP reaches low levels, actin and myosin in the muscle bind together forming the actomyosin complex and causing stiffness of the fish body (63). A strong relationship between low muscle pH immediately after death, and a rapid onset of the rigor state was demonstrated in a range of fish species (57,60). In this study, the evolution of *rigor mortis* (Fig.2) was similar between treatments and significant differences were only found in the NET and HYP trials at 8, and at 8 and 24 HAD, respectively. A delayed onset was observed, starting between 2 and 6 HAD in every trial and reaching the maximum rigor index between 24 and 48 HAD. This delay is consonant with the high muscle pH registered immediately after death, supporting the hypothesis of low energetic reserves in our fish at the time of death. Measuring glycogen and ATP content in the fish muscle and liver would be a complementary assessment to infer about the energetic reserves and corroborate our hypothesis.

Plasma proteins were evaluated in this study since blood plasma is a very informative biological fluid as it acts as a mirror of the physiological condition of the organism. Stress and stress-related hormones are recognized as modulators of the fish immune system (64), however, reactions depend on the intensity and duration of the stressor. The innate immune system is a fundamental defence mechanism in fish (65). The acute phase response is part of this system and it is mainly regulated by cytokines and glucocorticoids (66). This response is characterized by the release of acute-phase proteins (APP), by the hepatocytes, into circulation (67). APP can be classified as "positive" or "negative" depending on their plasma concentration increases or decreases during activation of this response (68). The response profile of our fish demonstrated the same tendency of protein changes.

In this study, protein changes observed in the plasma indicate that the fish's immune system was mainly affected by net handling and hypoxia stressors. Nevertheless, net handling was shown to be the most impacting. The levels of 20 different plasma proteins (distributed by 56 significantly differential spots), all related with immunological processes, were shown to be modulated by repetitive net handling, comparing to 2 proteins modulated by hypoxia. As mentioned, the same proteins were often detected from different spots on the 2D gels. Such a phenomenon can be due to existent isoforms or caused by adaptive changes of the proteome in an attempt to

maintain cellular homeostasis under stress. This adaptation, in addition to protein abundance, may involve changes at the protein degradation level, localization, function and activity – all of which can be modulated by post-translational modifications (PTMs) (69). PTMs can regulate fundamental biochemical processes and be more energetically efficient than altering protein abundance, constituting potential interesting signatures of stress. Studies on PTMs in fish are still scarce.

The protein changes detected (listed in additional file 2), along with the network and GO enrichment analyses (Fig.5) performed, confirmed the involvement of several components of the innate immune system in the physiological adaptation to these stressors. Proteins considered to be “positive” APP were likewise shown to be increased in abundance in the plasma of fish stressed by net handling (fibrinogen alpha-chain, complement component C3, haptoglobin, complement factor B, warm-temperature acclimation 65kDa protein, alpha-1-antitrypsin), while proteins considered as “negative” were decreased (transferrin, inter-alpha-trypsin inhibitor, apolipoprotein A-I) (70). A diverse number of proteins involved in the APR was also previously found to be modulated in chronically stressed gilthead seabream (71).

Apolipoprotein A-I (Apo-AI) was only modulated by net handling stress and 17 proteoforms were identified in the plasma proteome map, being mostly decreased in abundance. Apo-AI is the main protein constituent of the high-density lipoprotein (HDL), playing a role in lipid metabolism and participating in the reverse transport of cholesterol from tissues to the liver (72,73). Apo-AI was also found to be decreased in abundance in crowded Atlantic salmon (74). In cod (*Gadus morhua*) it acted as a negative regulator of the complement system (75). Other two apolipoproteins were also found to be down-regulated in the plasma of fish from NET2 and NET4 groups (Apolipoprotein Eb and apolipoprotein B-100).

The complement system is an essential part of the innate immune system which can be activated through three pathways: the classical, alternative and lectin pathways (76). Fish display a plethora of complement components, mainly complement component C3 (C3), which may present around five proteoforms in a single species (77). C3 is one of the most abundant proteins in the plasma and plays a central role in the innate immune system, supporting the activation of all three pathways (76). In this study, C3, identified in 5 proteoforms, and complement factor B (Bf), identified in 4, were found to be increased in abundance by net handling. Contrarily, C3 was down-regulated in fish exposed to low oxygen levels. Bf also plays a role in complement activation by acting as the catalytic subunit of C3 convertase, an enzyme responsible for the proteolytic cleavage of C3, in the classical and alternative pathways (76).

Several metal-binding proteins, existent in the plasma of vertebrates, can chelate iron, zinc and copper, which are essential elements for the virulence of bacteria (78). Alpha-2-macroglobulin (A2M) is a multifunctional protein (79) found to be down-regulated in the plasma of fish submitted to handling stress. It is mostly known to act as a broad range serine proteinase inhibitor and to bind metal ions (78). Contrarily, haptoglobin, which is also responsible for the sequestration of iron by binding to hemoglobin, was found to be increased in the plasma of handled fish. Similarly, warm-temperature acclimation-related 65 kDa protein (Wap65), which is involved in the scavenging of free heme (80), was increased in abundance by net handling and hypoxia stressors. Wap65 in fish is the homologue of mammalian hemopexin (81) and in most teleosts presents two proteoforms (82). In this study, two spots were also matched to this protein suggesting the presence of these two proteoforms. Transferrin (Tf) decreased in abundance in the plasma of fish stressed by net handling. Tf is a plasma protein also capable of binding iron and an important constituent of the iron homeostasis (33).

In fish, antiproteases are important participants of the non-specific humoral immune defence mechanism (70). A2M is an important factor in this mechanism. Alpha-1-antitrypsin is a serine protease inhibitor, up-regulated in net-handled fish, which is responsible to negatively regulate blood clotting molecules to prevent thrombosis (83). Inter-alpha-trypsin inhibitor H3 is also a serine protease inhibitor, which was found to be down-regulated in the plasma of fish from NET groups. The same pattern of protein changes was verified for fetuin-B, a cysteine proteinase inhibitor recently described in teleosts (84). Finally, fibrinogen alpha-chain, a beta-globulin involved in blood clotting, an integral part of innate immunity (83), was found to be up-regulated in the plasma of fish belonging to NET groups.

Conclusions

In summary, the results suggest that physiological changes were higher in fish exposed to repeated handling, while mild and permanent stressors may allow the fish to refine their physiological processes and adapt to certain challenges. The mechanism of habituation, along with the return to basal levels after a few hours and all the other factors affecting the release of cortisol, glucose and lactate, demonstrate that these indicators may not be the most robust in case of chronic stress monitoring. On the other hand, plasma proteomics allowed to detect, in stressed fish, a cohesive network of protein changes associated with essential immunological pathways. These proteins will be useful in understanding the biological processes behind protein-based stress adaptation in fish and may, therefore, represent the first screening for potential biomarker candidates of chronic stress in gilthead seabream. However, this work

is the first step of a major core, since a reliable assessment of fish welfare requires a multidisciplinary approach, and the study of the stress response from the molecular to the behavioural level might just be the holistic approach needed to achieve such goal.

Methods

Animals

Gilthead seabream (*Sparus aurata*) were obtained from a commercial fish farm (Maresa, Mariscos de Estero S.A., Huelva, Spain) and kept under quarantine conditions for a 2-week period at the Ramalhete Research Station (CCMAR, University of Algarve, Faro, Portugal). The fish were then individually weighed and distributed among conical fiberglass tanks (500 L), according to the density requirements of each trial. The tanks were supplied with natural flow-through seawater from Ria Formosa, and kept under natural temperature (13.4 ± 2.2 °C) and photoperiod, salinity at 34.7 ± 0.8 ‰, and artificial aeration (dissolved oxygen above 5 mg.L -1). Fish were fed by hand once a day, with a diet manufactured by AquaSoja Portugal, following the species' nutritional requirements.

Experimental design

The study was performed in three separate trials: (1) Overcrowding (OC), (2) Net Handling (NET) and (3) Hypoxia (HYP), due to logistic issues. Each trial followed a 2-week acclimation period and the initial rearing density was established at 10 kg/m^3 (except in the experimental groups of high stocking densities). In the OC trial, during the 54 days of experiment, fish (initial body weight (IBW) = 372.33 ± 6.55 g) were stressed using different high stocking densities, by increasing the number of fish in the tanks. Three different experimental groups were tested in triplicate: Control – 10 kg/m^3 (OC_{CTRL}), medium density – 30 kg/m^3 (OC_{30}), high density – 45 kg/m^3 (OC_{45}). The NET trial lasted for 45 days and the fish (IBW = 375.69 ± 11.88 g) were stressed by 1-min air exposure, using nets designed to fit inside the tanks and to be lifted to perform the stressful event. The experimental groups were established, in triplicate, as follows: Control – undisturbed fish (the net was also placed in the tanks but not lifted) – (NET_{CTRL}), fish air-exposed twice a week (NET_{2x}) and fish air-exposed four-times a week (NET_{4x}). In the HYP trial, fish (IBW = 397.99 ± 16.56 g) were subjected to low levels of saturated oxygen, by injection of nitrogen in the water, for 48 h, according to the following experimental groups (in triplicate): Control – 100% saturated oxygen – (HYP_{CTRL}), 30% saturated oxygen (HYP_{30}) and 15% saturated oxygen (HYP_{15}). Different trial times are due to differences in the nature and severity of the stressor, to which rearing protocols had to be adjusted accordingly.

Sampling procedure

Prior to the sampling day, fish were starved for 48 h to clean the digestive tract. Nine random fish per tank were lethally anaesthetized with tricaine methanesulfonate (MS-222; Sigma Aldrich, St. Louis, Missouri, USA) for the following sampling procedures: 3 fish for *rigor mortis* index assessment, 3 fish for muscle pH measurement and 6 fish for blood collection. Blood samples of approximately 2 ml were collected from the caudal vein with a heparinized syringe and immediately centrifuged at 2,000 g for 20 min. Plasma samples were immediately frozen at -80°C until posterior analyses. Fish for the measurement of *post-mortem* biochemical changes (pH and *rigor mortis*) were stored in polystyrene boxes with ice during the sampling period (72 h). All fish were weighed and measured.

Plasma stress indicators' measurement

Plasma cortisol levels were quantified using a commercial Cortisol ELISA kit RE52061 (IBL International, Hamburg, Germany), following the manufacturer's instructions. Measurements were registered at 450 and 620 nm along with a prepared standard curve on a microplate reader Biotek Synergy 4 Hybrid Technology™ (Biotek Instruments Inc, Winooski, USA). Plasma glucose and lactate levels were assessed through commercial colorimetric kits (Spinreact, Girona, Spain), following the manufacturer's instructions.

Biochemical and quality characterization of fish muscle

Muscle pH measurements were performed (n = 3 per tank), using a waterproof pH spear for food testing (Oakton® Instruments, Nijkerk, Netherlands), in the dorsal muscle, at 0, 1, 2, 4, 6, 8, 24, 48 and 72 hours after death (HAD), approximately 1-2 cm apart. At the same *post-mortem* periods, *rigor mortis* was assessed (n = 3 per tank) by the rigor index (RI), as previously described (85), using the formula:

$RI\ (\%) = [(L_0-L_t)/L_0] \times 100$

L_0 (cm) refers to the vertical distance between the base of the caudal fin and the table surface (where the anterior half of the fish is placed), measured immediately after death, whereas L_t (cm) corresponds to the same distance, however at selected time intervals. Fish were carefully handled during the measurements to avoid any interference with the *rigor* onset.

Plasma proteomics analysis

Protein labelling

Plasma samples were diluted 80x in DIGE buffer (7M urea, 2M thiourea, 4% CHAPS, 30 mM Tris pH 8.5) and the protein content measured with Bradford assay using the BioRad Quick Start Bradford Dye Reagent 1X (Bio-Rad Laboratories, Hercules, California, USA) and bovine serum albumin (BSA) as standard, BioRad Bovine Serum Albumin Standard Set (Bio-Rad Laboratories, Hercules, California, USA). Samples' pH was checked with a pH-indicator paper, Sigma-P4536 (Sigma Aldrich, St. Louis, Missouri, USA) and adjusted to 8.5 using 0.1 M NaOH. DIGE minimal labelling of 50 µg of protein was carried out using the CyDye™ DIGE fluor minimal labelling kit 5 nmol (GE Healthcare, Little Chalfont, UK), with 400 pmol fluorescent amine reactive cyanine dyes freshly dissolved in anhydrous dimethylformamide (DMF), following the manufacturer's instructions. Labelling was achieved on ice for 30 min, in the dark, and the reaction quenched with 1 mM of lysine for 10 min. For each trial, six samples per experimental condition were labelled with Cy3 and six with Cy5 to reduce the impact of label difference, while an internal standard consisting of a pool of all samples, with equal amounts, was labelled with Cy2. Samples were randomly sorted to avoid labelling bias.

Protein separation by 2DE

For each strip, 150 µg of protein (50 µg from each dye) were loaded along with rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.001% bromophenol blue, 0.5% Bio-lyte 3/10 ampholyte (Bio-Rad Laboratories, Hercules, California, USA) to complete 450 µl. Passive rehydration was conducted for 15 h on 24 cm Immobiline™ Drystrips (GE Healthcare, Little Chalfont, UK) with linear pH 4-7, on an IPG Box (GE Healthcare, Little Chalfont, UK). Following, isoelectric focusing (IEF) was performed in 5 steps: 500 V gradient 1 h, 500 V step-n-hold 1 h, 1000 V gradient 1 h, 8000 V gradient 3 h and 8000 V step-n-hold 5h40 for a total of 60.000 Vhr using Ettan IPGphor at 20°C (GE Healthcare, Little Chalfont, UK). Focused strips were reduced and alkylated with 6 ml of equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol and 2% SDS) with 1% (w/v) dithiothreitol (DTT) or 2.5% (w/v) iodoacetamide (IAA) respectively for 15 min each, in constant agitation. Strips were then loaded onto 12.5% Tris-HCl SDS-PAGE gels and ran in an Ettan DALT*six* Large Vertical System (GE Healthcare, Little Chalfont, UK) at 10 mA/gel for 1 h followed by 60 mA/gel until the bromophenol blue line reaches the end of the gel, using a standard Tris-Glycine-SDS running buffer.

Image acquisition and analysis

CyDye-labeled gels were scanned on a Typhoon™ laser scanner 9400 (GE Healthcare, Little Chalfont, UK) at 100 µm resolution, with the appropriate laser filters for the excitation and emission wavelengths of each dye (i.e., Cy2-488/520 nm; Cy3-532/580 nm; and Cy5-633/670 nm), according to the manufacturer's recommendations. The voltages of the Photo Multiplier Tube (PMT) were adjusted to obtain a maximum image quality with minimal signal saturation and clipping. Gel images were checked for saturation during the acquisition process using the ImageQuant TL software (GE Healthcare, Little Chalfont, UK). The final images were analysed with SameSpots software (Totallab, Newcastle, UK), including background subtraction (average normalized volume ≤ 100,000 and a spot area ≤ 500), filtering, spot detection, spot matching, normalization and statistical analysis. Spot volume ratios that showed a statistically significant difference (abundance variation of at least 1.0-fold, $P < 0.05$ - one-way ANOVA on log2-transformed normalized

spot volumes) were processed for further analysis. Protein spots with statistically different intensities were manually excised from preparative gels and identified by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS).

Protein identification by MALDI-TOF/TOF MS

Spots from SYPRO® Ruby-stained (InvitrogenTM, Carlsbad, CA, USA) gilthead seabream plasma 2D gels were picked and subjected to in-gel tryptic digestion, similar as reported before (86). In this study, gel plugs were washed twice with 50 mM ammonium bicarbonate solution in 50 % v/v methanol (MeOH) for 20 min and dehydrated twice for 20 min in 75 % acetonitrile (ACN). Proteins were then digested with 8 µL of a solution containing 5 ng/µL trypsin (trypsin Gold, Promega) in 20 mM ammonium bicarbonate for 6 h at 37°C. A 0.1% trifluoroacetic acid (TFA) solution in 50% ACN and a solution of 7 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN/0.1% TFA were used for peptide extraction and spotting respectively. MALDI TOF/TOF analysis was performed with a TOF/TOF™ 5800 (AB SCIEX, Redwood City, CA, USA) mass spectrometer in MS and MS/MS mode. For each spot, the 10 most intense peaks of the MS spectrum were selected for MS/MS acquisition. Database interrogation was carried out over with ProteinPilot v4.5 (AB Sciex) on an in-house Mascot server version 2.6.1 (Matrix Science Ltd., London, UK).

Mass lists were searched against NCBInr database restricted to the taxonomy “other Actinopterygii” (tax ID 7898 excluding 31033 and 7955) with the following parameters: maximum 2 missed cleavages by trypsin, peptide mass tolerance ± 100 ppm, fragment mass tolerance set to 0.5 Da, carbamidomethylation of cysteine selected as fixed modification and tryptophan dioxidation, histidine, tryptophan and methionine oxidation, and tryptophan to kynurenine as variable modifications. Protein hits not satisfying a significance threshold ($P < 0.05$ and a total ion score > 60) were further searched against vertebrate EST (expressed sequence tags) database also restricted to the taxonomy “other Actinopterygii”.

Protein-protein interaction (PPI) network and gene ontology (GO) enrichment analyses

The theoretical molecular masses and isoelectric points (pI) of the MS identified proteins were calculated using the amino-acid sequences (in one-letter code) on the ProtParam Tool (http://us.expasy.org/tools/protparam.html). A significance cutoff was applied for the identified proteins at log-fold change ± 1.0. Following, the identified proteins were blasted against *Danio rerio,* on the UniprotKB database, using the FASTA protein sequences as queries. The orthologues were mapped using STRING web tool v11.0 (https://string-db.org/) to screen for protein-protein interactions (PPI). Gene ontology (GO) enrichment analysis and network visualization and analysis were performed on Cytoscape v3.7.1 (http://www.cytoscape.org/) with the BiNGO plug-in. Important hub proteins were screened by counting the degree of connectivity of each node in the network. Over-represented GO terms were identified, using *B. rerio* as reference, by selecting the hypergeometric test with a significance threshold of 0.05 after Benjamini & Hochberg FDR correction.

Statistical analyses

All univariate and multivariate statistical analyses were performed using R v3.5.3 for MacOSX (https://www.r-project.org). Statistical analyses of the plasma parameters and the *post-mortem* muscle biochemical changes were performed using plasma cortisol, glucose and lactate levels, muscle pH and *rigor* index as dependent variables, and the stress treatment as factor. Statistical differences between treatments were analysed independently for each trial (OC, NET and HYP). For *rigor* index and muscle pH, data were processed separately for each sampling time. Differences in plasma and muscle parameters between treatments were assessed by a one-way analysis of variance (one-way ANOVA) on log10-transformed data, except for *rigor mortis* data, which was transformed by arcsine square root. Multiple comparisons were carried out by the post-hoc Tukey HSD test. When transformed data failed the Shapiro-Wilk normality test, the non-parametric Kruskal-Wallis on ranks was used, followed by Dunn’s test. When transformed data did not verified homoscedasticity assumption by Levene’s test, statistical significance was analysed by Welch’s ANOVA, followed by Games-Howell. A significance level of $\alpha = 0.05$ was used in all tests performed. Experimental data is expressed as mean ± standard deviation (SD). Principal component analysis (PCA) and hierarchical clustering analysis of the identified proteins were performed on the log2-transformed normalized spot volumes obtained from SameSpots software, with autoscaling. Heatmap was generated by comparing Z-

scores of normalized spot volumes and hierarchical clustering of samples and protein spots was performed using the Euclidean distance and the maximum cluster agglomeration method as distance metrics.

List Of Abbreviations

ANOVA: Analysis of variances

DIGE: Differential Gel Electrophoresis

FC: Fold-change

FDR: False discovery rate

GO: Gene ontology

HAD: Hours after death

HCA: Hierarchical clustering analysis

HYP: Hypoxia

IEF: Isoelectric focusing

MALDI-TOF/TOF: Matrix-assisted-laser-desorption-ionization time-of-flight/time-of-flight

MS: Mass spectrometry

NET: Net handling

OC: Overcrowding

PCA: Principal Component Analysis

pI: Isoelectric point

PPI: Protein-protein interaction

RI: Rigor index

SD: Standard deviation

Declarations

Ethics approval and consent to participate

This study was approved by the ORBEA Animal Welfare Committee of CCMAR and the Portuguese National Authority for the Animal Health (DGAV) on August 26th 2019. The experiment described was conducted in accordance with the European guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU) and the Portuguese legislation for the use of laboratory animals, under a “Group-1” license (permit number 0420/000/000-n.99-09/11/2009) from the Veterinary Medicine Directorate, the competent Portuguese authority for the protection of animals, Ministry of Agriculture, Rural Development and Fisheries, Portugal and following category C FELASA recommendations.

Consent for publication

Not applicable.

Availability of data and materials

The authors declare that all relevant data supporting the findings of this study are available within the article (and its additional files).

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

CRM carried out the animal experiments, the biochemical and the proteomics analyses, performed the statistical analyses and wrote the manuscript. DS assisted with the animal experiments and the proteomics analyses. APF advised and assisted on the data bioinformatic analyses. DR, AK and SP performed the mass spectrometry analyses and participated in the interpretation. PMR designed the experiments and was a major contribution to the paper’s writing. MC designed and assisted with the experiments and was a major contribution to the paper’s writing. All authors provided a critical review and approved the final manuscript.

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Table

Spot[a]	Accession no.[b]	Protein ID[c]	FC[d]		Danio rerio homolog (UniprotKB identifier)	String annotation
			NET2	NET4		
152	XP_008277007.1	PREDICTED: complement factor B-like [Stegastes partitus]	1.72	2.54	F1QFT0	zgc:158446
202	XP_010753395.2	PREDICTED: antithrombin-III [Larimichthys crocea]	-0.36	-1.61	Q8AYE3	serpinc1
209	XP_019111370.1	PREDICTED: inter-alpha-trypsin inhibitor heavy chain H3-like [Larimichthys crocea]	-0.49	-1.76	F1QTF9	zgc:110377
224	XP_017260893.1	alpha-2-macroglobulin, partial [Kryptolebias marmoratus]	-0.62	-1.74	A0A0R4IDD1	a2ml
316	AWP20152.1	putative apolipoprotein B-100-like isoform 2 [Scophthalmus maximus]	-0.88	-1.46	Q5TZ29	apobb
367	XP_023285742.1	alpha-1-antitrypsin homolog [Seriola lalandi dorsalis]	1.17	2.78	Q6P5I9	serpina1
544	KKF22678.1	Fibrinogen alpha chain [Larimichthys crocea]	1.33	2.26	B8A5L6	fga
556	XP_018550494.1	PREDICTED: leucine-rich alpha-2-glycoprotein-like [Lates calcarifer]	0.51	1.20	Q5RHE5	LRG1
558	AEA41139.1	transferrin [Sparus aurata]	-0.46	-2.18	A0A2R8RRA6	tfa
595	ADM13620.1	complement component c3 [Sparus aurata]	1.08	2.06	Q3MU74	c3b
710	ARI46218.1	haptoglobin [Sparus aurata]	1.39	1.53	F8W5P2	ENSDARG00000051890

[a] Spot no. – number of the spot in the 2D gel (Fig. 3), attributed by the SameSpots software

[b] Accession number – NCBI accession number

[c] Protein ID – protein identification by MALDI-TOF/TOF MS

[d] FC - Log2(fold-change) - significant changes in protein abundance (treated/control). Bold lettering indicates significant fold-changes (> 1.0 and < -1.0).

Spot[a]	Accession no.[b]	Protein ID[c]	FC[d]		Danio rerio homolog (UniprotKB identifier)	String annotation
			NET2	NET4		
736	AJW65884.1	Hyaluronic acid binding protein 2 [Sparus aurata]	-0.33	-1.53	Q1JQ29	habp2
796	ACN54269.1	warm temperature acclimation-related 65 kDa protein [Sparus aurata]	0.85	1.13	Q6PHG2	hpx
877	BAM36361.1	pentraxin [Oplegnathus fasciatus]	-1.24	-0.73	Q7SZ53	crp2
	XP_022604055.1	kininogen-1-like [Seriola dumerili]	-1.24	-0.73	Q5XJ76	kng1
996	APO15792.1	apolipoprotein Eb [Sparus aurata]	-0.10	-3.04	O42364	apoeb
997 1072	XP_010742296.3	apolipoprotein A-I [Larimichthys crocea]	-0.92	-4.04	O42363	apoa1a
	XP_020489366.1	fetuin-B-like [Labrus bergylta]	-0.47	-1.07	E7FE90	fetub

[a] Spot no. – number of the spot in the 2D gel (Fig. 3), attributed by the SameSpots software

[b] Accession number – NCBI accession number

[c] Protein ID – protein identification by MALDI-TOF/TOF MS

[d] FC - Log2(fold-change) - significant changes in protein abundance (treated/control). Bold lettering indicates significant fold-changes (> 1.0 and < -1.0).

Table 1
String annotations and fold-changes of the proteins in the PPI network. Bold lettering in the “FC” column indicates significant fold-changes (> 1.0 and < -1.0). List is given in ascending order of spot number.

Figures

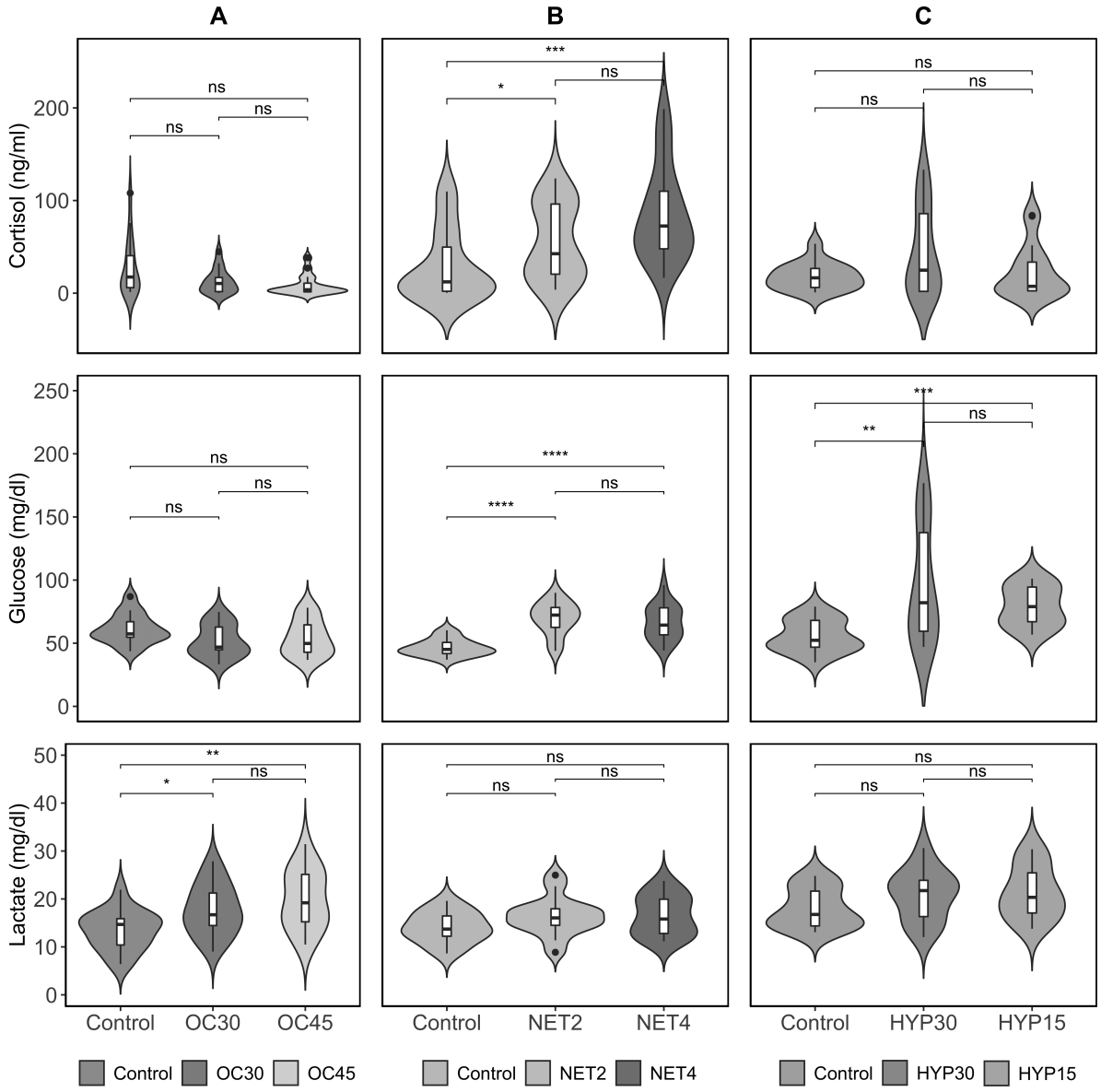

Figure 1

Violin plots showing the distributions of plasma cortisol (ng/ml), glucose (mg/dl) and lactate (mg/dl) levels of gilthead seabream (Sparus aurata) submitted to different chronic stressors (A – overcrowding, B – net handling, C – hypoxia), in two intensities, and unstressed fish (control) (n = 18). The box-plot inside includes observations from the 25th to the 75th percentiles as determined by R software; the horizontal line indicates the median value. Whiskers extend 1.5 times the interquartile range. Single data points are outlying data. *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.0001. NS (not significant) indicates a P-value greater than 0.05.

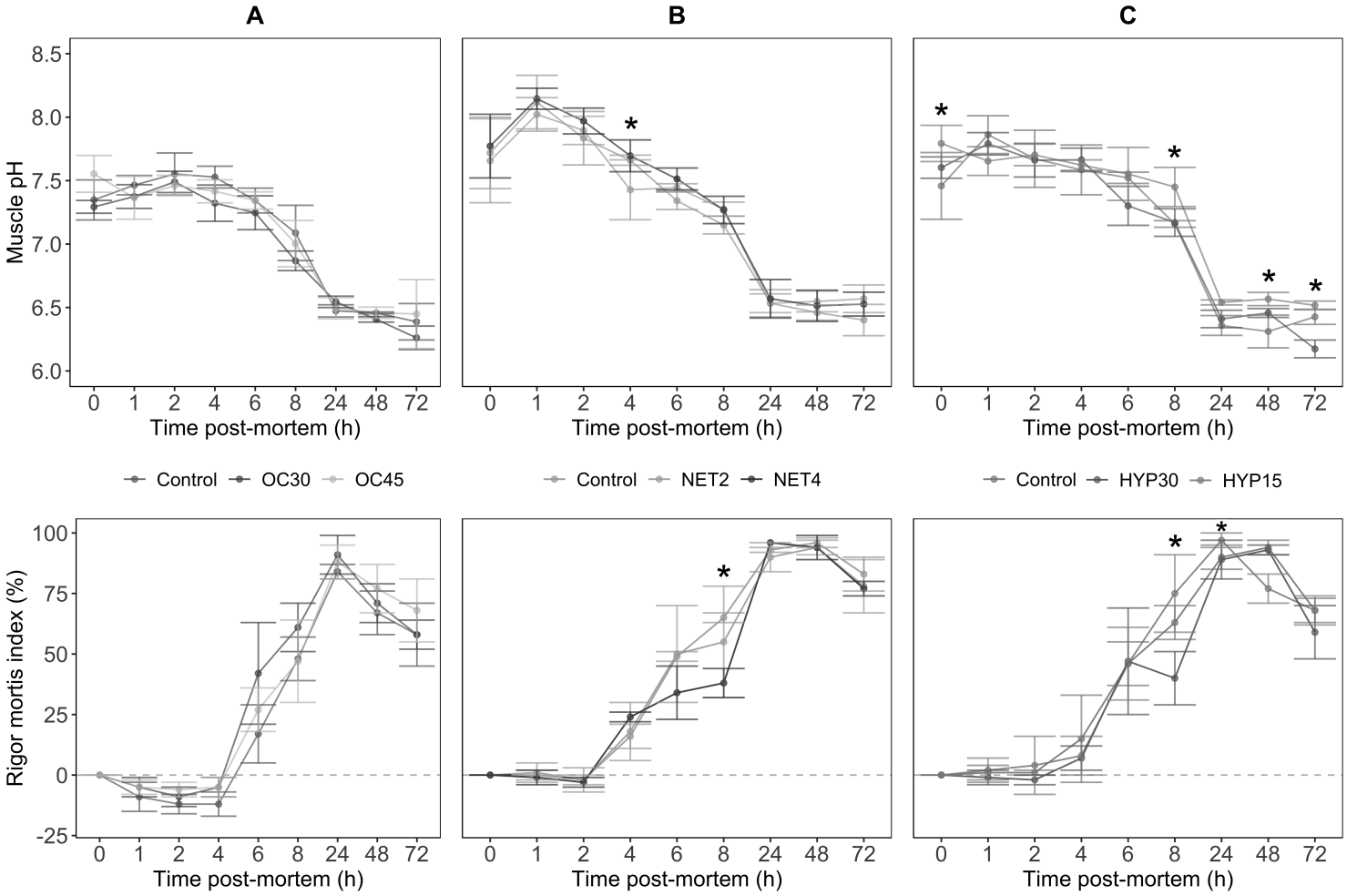

Figure 2

Post-mortem changes in muscle pH and rigor mortis of gilthead seabream (Sparus aurata) submitted to different chronic stressors (A – overcrowding, B – net handling, C – hypoxia), in two intensities, and unstressed fish (control), stored in ice for 72 h. Data points are the mean ± S.D. of n = 9 for each sampling time. Means labelled * are different at $P < 0.05$.

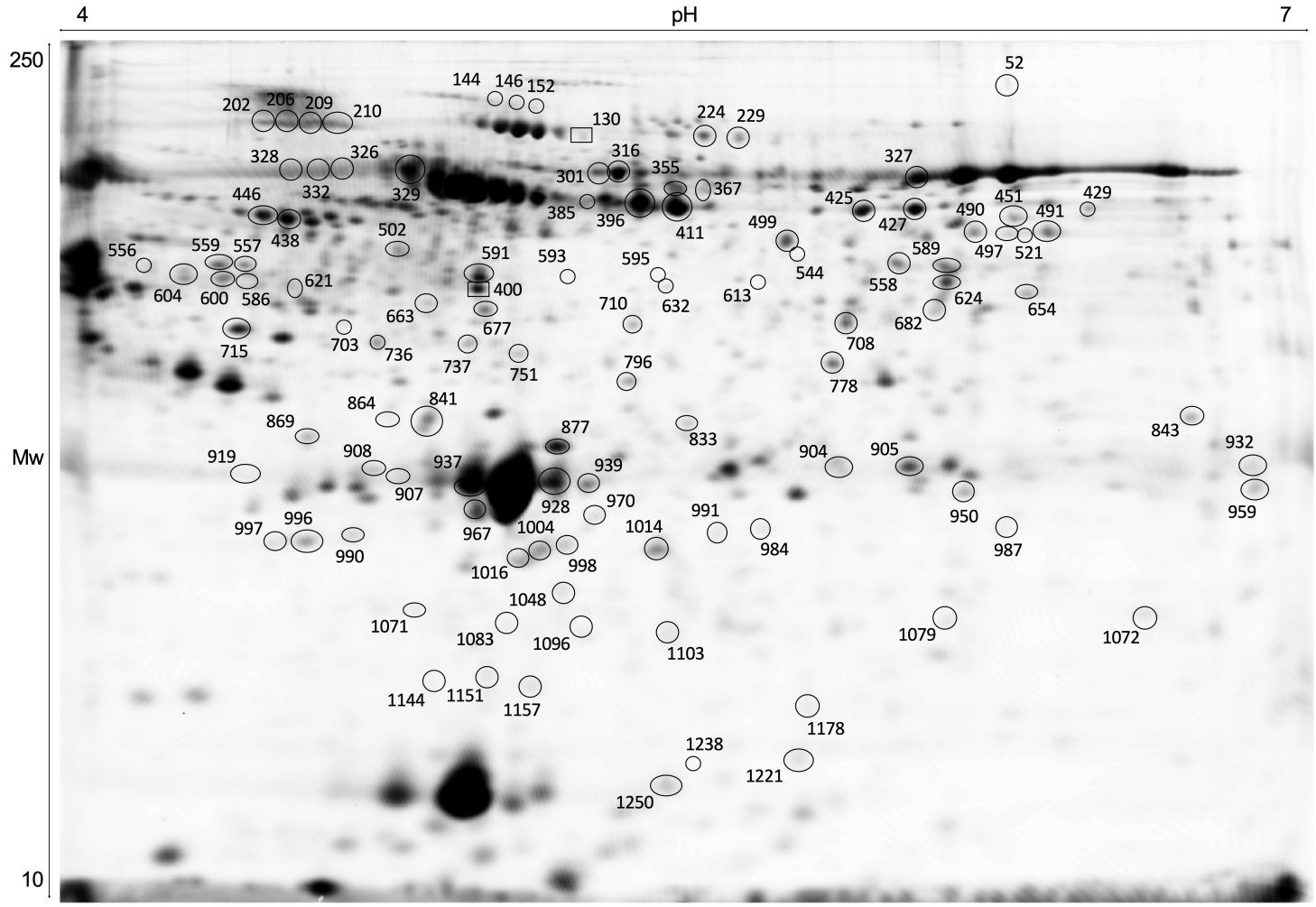

Figure 3

Representative pattern of gilthead seabream (Sparus aurata) blood plasma on a 12.5% polyacrylamide 2D gel. Black circles represent the 107 proteins identified by MALDI-TOF/TOF MS with significant differences in abundance in NET groups and black squares the 2 proteins with significant differences in abundance in HYP groups ($P < 0.05$).

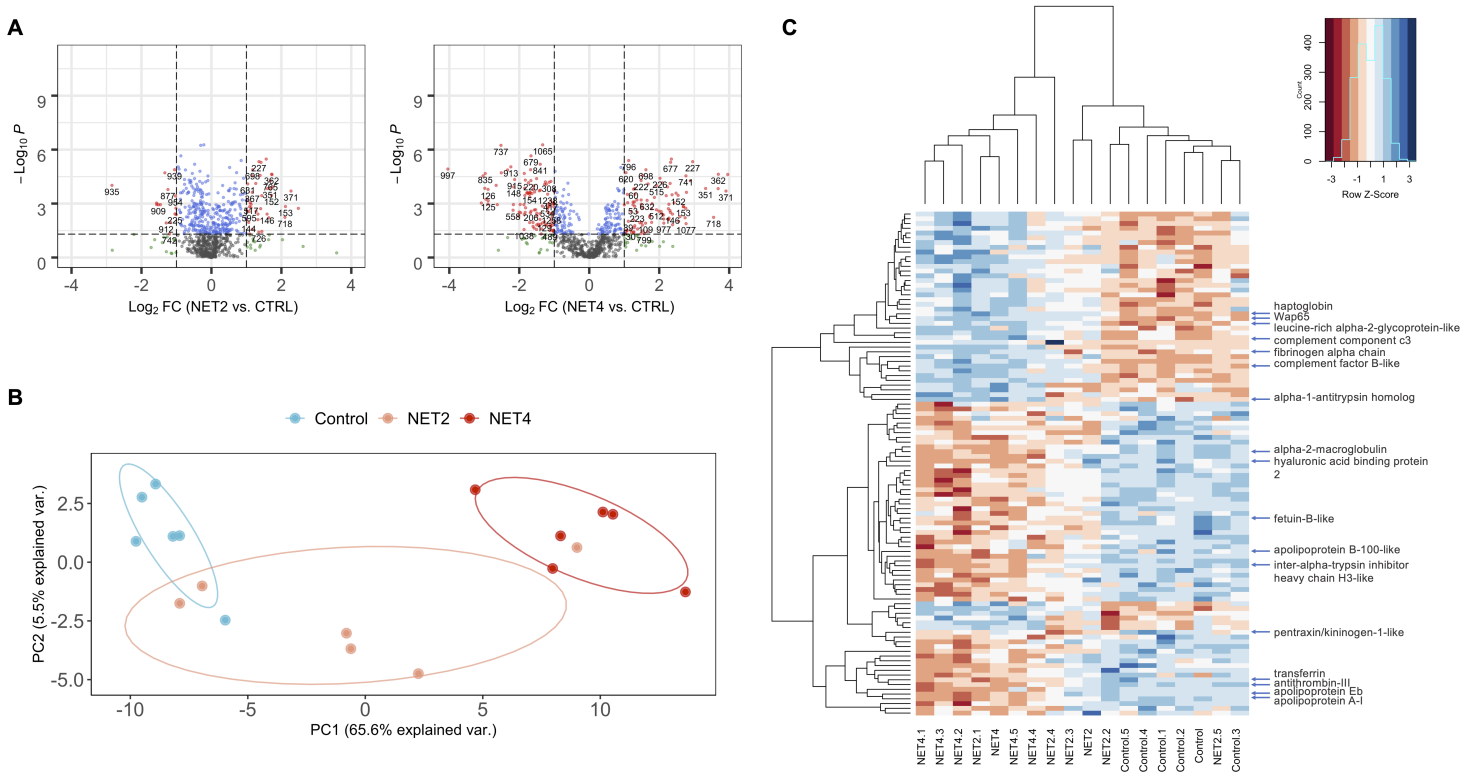

Figure 4

A – Volcano plots of the entire set of plasma proteins detected by DIGE analysis on the NET trial samples. Each point represents the difference in abundance (fold-change) between stressed fish (NET2 on the left; NET4 on the right) and control fish plotted against the level of statistical significance. Dotted vertical lines represent a 2-fold variation in abundance, while dotted horizontal line represent the significance level of $P < 0.05$. Red dots represent proteins significantly up- and down-regulated. B – Principal component analysis performed with the normalized spot volumes of the 107 identified proteins in the plasma samples of gilthead seabream from the NET trial (n = 6). Blue, orange and red dots represent CTRL, NET2 and NET4 groups, respectively. C – Hierarchical clustering of 107 significantly differential proteins identified in the plasma samples of gilthead seabream from net handling (NET) trial. Rows represent expression patterns of individual proteins, while each column corresponds to a biological replicate (fish). Cell colour indicates the normalized Z-scores of the spot volumes.

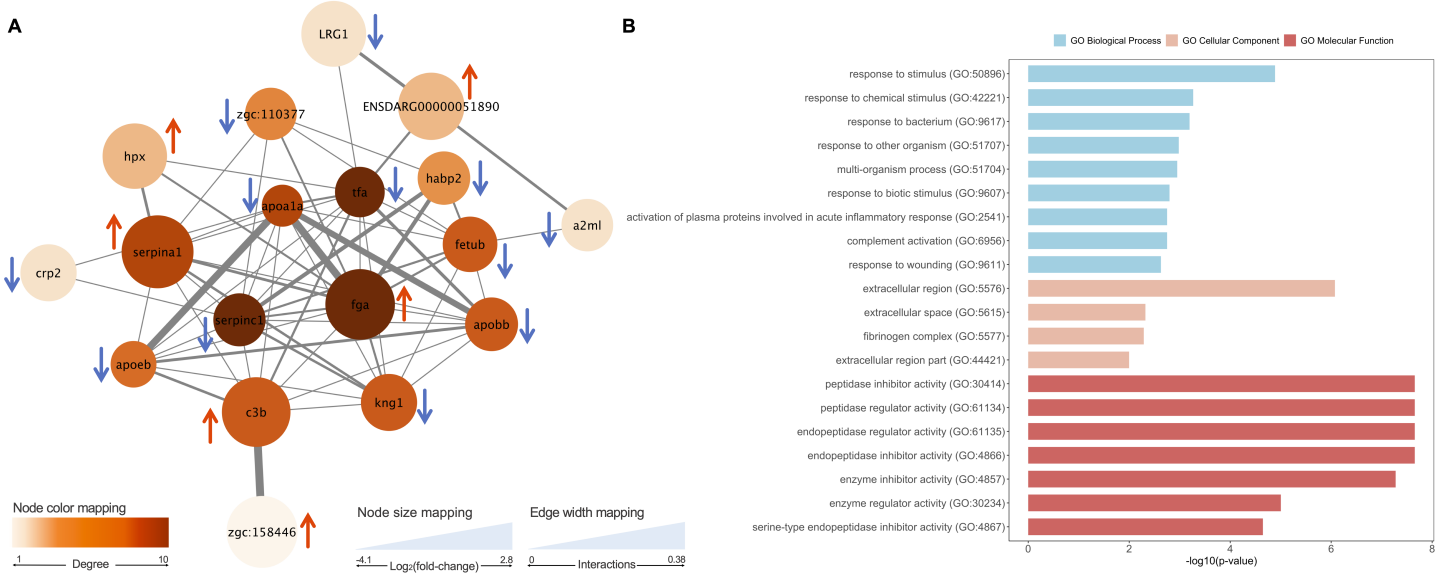

Figure 5

A – Protein-protein interaction network generated with 18 differential proteins identified in the plasma of fish from NET trial. Nodes represent proteins and edges the functional associations between them. STRING annotations are described in Table 1. Red arrows represent up-regulated proteins in both treatments; blue arrows represent down-regulated proteins in both treatments. D – GO Enrichment analysis of the 18 proteins showing significantly differential abundance between control and NET treatments (hypergeometric test, FDR < 0.05).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile3.pdf
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