AQUAEXCEL

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Glossary

AQUAEXCEL: Aquaculture Infrastructures for Excellence in European Fish Research

Acute stress: Brief or short term exposure to a stressor or set of stressors.

Allostasis: The process of achieving stability, homeostasis, through change. The process may include altered response of the HPI axis to stimuli.

Biomass per day = Biomass day x+1 (g) = Biomass day x (g) + (Feed intake day x (g) / FCR).

Chronic stress: Long term repeated or continuous exposure to one or several stressors often associated with chronic elevated levels of cortisol.

Condition factor (CF) = (100*body weight (g))/body length^3

Feed conversion rate (FCR) = Total feed intake (g) / Biomass increase (g); where the total feed intake and the biomass increase in a tank are calculated over the experimental period.

HPI axis: hypothalamus-pituitary-interrenal axis which is activated by stress.

Homeostasis: The control over internal processes following changes in, often, external stimuli.

Scan sampling: It is very similar to instantaneous sampling. In scan sampling the behaviour of all the individuals in a group of animals are recorded at predetermined time intervals.

Specific growth rate (**SGR**) (%/BW/day) = (ln(Wt) – ln(W_0)) * 100/T; where Wt is the average final body weight per tank (g), W_0 is the average initial body weight per tank (g) and T is the number of days of the growth period.

Stress: Events that activate catecholamine secretion and activation of the HPI axis for the synthesis of cortisol.

Stress response (%) (in behaviour studies) = Stress – Baseline; where Stress is the mean percentage of fish in a square after the onset of a stress moment and Baseline is the mean percentage of fish present in a square before the onset of a stress moment.

Oxygen consumption rate (VO_2) per minute/tank (mg O_2/kg fish/min);

$$VO_{2_t} = Vol \cdot Sol \cdot \frac{Sat_t - Sat_{t-1}}{\delta t \cdot 100} + Flow \cdot Sol \cdot \frac{100 - Sat_t}{100}$$

where Vol is the tank volume (500 litre), Sol is the solubility of oxygen at prevailing temperature and conductivity conditions (mg O_2/litre), Flow is the flow rate per tank (litre/min), Sat_t is the oxygen saturation at time t (mg O_2/litre) and since the oxygen saturation was monitored each minute δt = 1 min.

Summary

Objectives: to provide a set of methodologies for phenotyping the response of fish (gilthead sea bream, Atlantic salmon and rainbow trout) exposed to chronic environmental stressors.
Rationale: the effects of three different stressors applied during several weeks were studied with a multidisciplinary approach, integrating data on growth performance, feed intake, oxygen consumption, biochemical, haematological, feeding behaviour, gene expression and mucus proteomics.
Teams involved: CSIC, INRA, IMR, UoS
Tasks involved: Subtask 7.1.2
Geographical areas covered: all Europe

The aim of this deliverable was to phenotype the physiological and transcriptionally-mediated response of gilthead sea bream (GSB), Atlantic salmon (AS) and rainbow trout (RT) in front of chronic stress. Several environmental stressor models were established, some of them were shared by several species. GSB and AS were exposed to intermittent and repetitive environmental stressors for 21 days: (i) changes in water level and chasing (CHASE = C-ST) and (ii) multiple sensory perception stressors (AUTO = M-ST). GSB was also exposed to changes in water temperature (T-ST). RT was also exposed to repetitive multiple stressors for 10 days, but chronic stress was not induced. A model of chronic stress was established when RT was exposed to poor water quality (hypoxia) for 21 days. Skin mucus, blood, plasma and tissues samples were taken to analyse the invoked changes with biochemical, haematological, endocrinological and molecular tools (transcriptomics for the three species and also proteomics for GSB). Growth performance and feeding, stress and learning behaviours were analysed with different tests.

For GSB, gene expression profiling of liver was done using a quantitative PCR-array of 60 mitochondria-related genes, selected as markers of transcriptional regulation, oxidative metabolism, respiration uncoupling, antioxidant defence, protein import/folding/assembly, and mitochondrial dynamics and apoptosis. The mitochondrial phenotype mirrored changes in fish performance, haematology and lactate production. T-ST especially up-regulated transcriptional factors (PGC1a, NRF1, NRF2), rate limiting enzymes of fatty acid β-oxidation (CPT1A) and tricarboxylic acid cycle (CS), membrane translocases (Tim/TOM complex) and molecular chaperones (mtHsp10, mtHsp60, mtHsp70) to improve the oxidative capacity in a milieu of a reduced feed intake and impaired haematology. The lack of mitochondrial response, increased production of lactate and negligible effects on growth performance in C-ST fish were mostly considered as a switch from aerobic to anaerobic metabolism. A strong down-regulation of PGC1a, NRF1, NRF2, CPT1A, CS and markers of mitochondrial dynamics and apoptosis (BAX, BCLX, MFN2, MIRO2) occurred in M-ST fish in association with the highest circulating cortisol concentration and a reduced lactate production and feed efficiency, which represents a metabolic condition with the highest allostatic load score. These findings evidence a high mitochondrial plasticity against stress stimuli, providing new insights to define the threshold level of stress condition in fish. The proteomic study of the skin mucus of GSB allowed the mapping of more than 2,000 proteins and the identification of several candidate proteins which differed in M-ST fish and could be used as non-lethal indicators of chronic stress.

In AS, growth rate and feed intake was reduced in the CHASE model for 2 weeks, whereas full recovery occurred over a few days in the AUTO group. Appetite was recovered in the CHASE group by the end of trial. A stress-test at the end of trial revealed that neither treatment caused a down-regulation the HPI (hypothalamus, pituitary, interrenal) response (plasma cortisol). Consequently none of the groups were under chronic stress load at the

end of trial. Transcriptomic analysis of pituitary did not reveal major changes in gene expression following either stressor. However, minor changes were observed in oxidative phosphorylation, synaptic vesicle and phagosome regulation.

In **RT**, at the behavioural level, focal observations indicated a dramatic decrease in group activity and the study of the learning capacity showed a significant reduction of the food anticipatory behaviour. An increase in the HPI axis responsiveness to acute stress was also observed. Transcriptomic analysis led us to suggest that such increase may be linked with regulation of cortisol by head-kidney as genes such as P450 or StAR were up-regulated. Surprisingly, no clear relationship with pituitary or brain functions was found. By contrast, a clear response was observed at gills. Despite absence of changes of plasma osmoregulatory parameters (ion levels), transcriptomic analysis of gill tissue reveal several hundreds of genes were significantly up- or down-regulated. These genes are involved in various biological functions such as defence against pathogen or oxidative stress, muscle contraction and cytoskeleton organization. The analysis of a candidate gene (Bcl2, anti-apoptotic) indicated a higher cell renewal. Overall, we have shown that various parameters related to behaviour, gill physiology and HPI axis are significantly modified after chronic stress exposure.

Therefore, we found clear species differences in terms of the response to environmental stress, the two salmonids species being more resistant than GSB. This comparison was possible because similar experimental set ups were used for the three species. The integration of data of growth performance, plasma biochemistry, haematology, behaviour and transcriptomics is necessary to get an accurate picture of chronic stress and allostatic load. Among the non-lethal methods, skin mucus proteomics in GSB and some behavioural tests in RBT and GSB are promising. By contrast, we have shown that cortisol responsiveness may not be always discriminant in some chronic stress situations and that it should be used associated with other biomarkers to assess chronic stress status. Concerning transcriptomic approaches, we have shown the usefulness of a panel of mitochondria-related genes for environmental stress in GSB, and highlighted the need to choose the correct target tissue for each type of stress. It appeared that pituitary did not give any relevant information in salmonids. However, the gills appeared to be the best tissue for analysing biological response in front of poor water quality.

1. Introduction and Rationale

The aim of this deliverable is to provide a better way to phenotype fish under the stressful environmental conditions in which fish are reared in aquaculture facilities, either at the industry or in research experimental procedures. The rationale behind this objective is that facilities and procedures have to adapt to fluctuating environmental situations, to reduce their impact on the environment, to select for those fish phenotypes better adapted to the changing environment, and to develop management procedures respectful with fish welfare.

Many studies have been done on the biological response in fish exposed to acute stress, whereas much less information is available on chronic exposure to stressors. It is well known that chronic stress in aquaculture can have negative effects on fish health and welfare, such as suppressed immune systems, decreased growth, swimming performance or reproduction (Portz *et al.* 2006). Chronic deterioration of water quality, by increasing density associated with ammonia increase, has been shown to affect food intake and growth in rainbow trout (; Person-Le Ruyet *et al.,* 2008) but also in turbot, sea bass or tilapia (Pichavant, 2001; Person-Le Ruyet et al., 2003; Lemarié et al., 2004 Santos *et al.,* 2010).

A multidisciplinary approach, from the whole organism to the molecular level has been used to find more accurate and relevant information from experimental fish integrating existing and new, non-invasive, non-lethal, cheaper, simpler and more focused methods available to the scientific community. The methodological panel includes analysis of behaviour, regulation of the HPI (hypothalamus, pituitary, interrenal) axis, physiological and transcriptomic profiles of the organs involved in the stress response (liver, pituitary, brain, headkidney, gills, etc.). Our working hypothesis is that such chronic stress status is a complex situation, cannot be assessed by only analysing cortisol, the most commonly measured stress hormone, which may not be elevated in chronically stressed fish due to a negative feedback mechanism of cortisol that causes a down regulation of the HP axis (Pickering and Stewart, 1984; Procarione et al., 1999). Such methodology will provide a refined way of phenotyping chronically stressed fish and will improve the quality of the conclusions drawn from experiments, when testing new husbandry technologies, systems or fish lines.

The first step for the integration of the obtained results was to harmonize the experimental procedures in the three species. For doing so, an internal debate was settled to define what chronic stress is and how to measure it without invoking further stress. One important and somewhat unresolved issue in the field of stress biology is whether every environmental change that causes a response in an organism represents a stressor or whether it is possible to clearly define a level of intensity and duration of exposure that results in an environmental factor being classified as either stressful or non-stressful (Schulte, 2014). Wild fish and fish in captivity will encounter many types of stressors throughout their life cycle. The stressors vary in form, severity and duration. While wild fish will normally move away from unfavourable conditions, fish in captivity have limited escape possibilities (Fernö *et al.,* 2007; Harper and Wolf, 2009; Bratland *et al.,* 2010; Folkedal, 2010). In aquaculture, many farm activities like people walking past tanks, noise, light changes and removal of dead fish result in sudden disturbances for the fish. All these activities give rise to a variety of stimuli. Most of the disturbances have a low predictability, therefore, habituation to most of these activities is often slow or absent (Bratland *et al.,* 2010; Nilsson *et al.,* 2012). Experimental approaches to study stress in fish have often made use of single acute stressors like chasing, lowering of water level etc. (Wendelaar Bonga, 1997). These approaches have significantly improved our understanding of acute stress responses. Until recently, chronic stress was not a major focus of research for fish biologists, and when explored, the common method was repeated exposure to a single stressor. This approach however, has several drawbacks. Most animals including fish have well developed high capacities to adapt to single stressors and down regulation of the responses may appear

within a week (Schreck, 2000; Folkedal, 2010). Rather, when exposed to several stressors over time in random fashion, the unpredictable stress load prevents adaptation and leads to chronic stress (Grissom and Bhatnagar, 2009). This approach has only recently been introduced in fish (Piato et al., 2011).

Establishing chronic stress models in fish is rather challenging. Normally, severe stressors in fish include some form of physical handling increasing the likelihood of skin, mucus or fin damages. Such damages could contaminate the chronic stress responses and lead to erroneous conclusions. Thus, developing automated systems minimizing the chances of damages will be important for future studies of chronic stress. Furthermore, many of the models include physical intervention by the researcher.

This deliverable shows the results obtained for three farmed species, rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) and gilthead sea bream (*Sparus aurata*). For each species several experimental trials were set out in which fish were exposed to different environmental stressors. An effort has been done on selecting similar experimental conditions, type of samples taken, sampling times and analyses. However, this has not been possible at all levels due to differences in domestication levels of the species, life cycles, or partner facilities/capabilities. In any case, as shown in the subsequent sections, integration of behavioural, physiological, endocrinological parameters have been obtained for most species.

2. Material and Methods

In the present project, we developed automated models for unpredictable stress mimicking daily handling activities in fish tanks to study the effect on fish metabolism, behaviour and stress responses. These protocols were then compared to classical stress models usually applied to fish, such as confinement. One additional temperature model was developed for gilthead sea bream. The gilthead sea bream and salmon trials were performed at the facilities of IMR in Matre (Norway), in an effort to standardize the procedures and to synergize the expertise of the different partners involved in this task. Rainbow trout trials were performed at the facilities of INRA in PEIMA (France).

Atlantic salmon and gilthead sea bream were exposed to CHASE and AUTO types of stressors. They were based on intermittent stressors similar to those resulting from husbandry processes, like cleaning of tanks, dead fish collection etc. To mimic these stressors fish were daily exposed to lowering of the water level in the tank in combination with chasing of the fish (CHASE). The AUTO stressor was designed to be operator independent, to cause minimum of harm to the fish, to replicate what would be recognized as normal handling routines in a fish farm, and to make use of the unpredictable chronic stress paradigm. An additional model was applied to gilthead sea bream, the thermal stress (TEMP), which was assumed to be similar to what fish encounter during winter and spring. Table 1A summarizes the experimental conditions for gilthead sea bream and salmon. In rainbow trout, a preliminary trial using repeated acute stressors were performed, but chronic stress was not achieved and therefore another experiment using low quality water was done. Table 1B summarizes the experimental set up and the procedures applied for each fish species, but the details on the particular materials and methods for each fish species are detailed in separate sections. As can be seen, several tissues were collected for the transcriptomic study, but not all of them were analysed due the analytical burden involved or to the fact that the RNA quality of some samples (AS) was degraded due to transport problems of the samples.

Table 1A. Summary of the experimental conditions of the stress trials run at IMR. For more detail, see sections 2.1.1 and 2.2.1.

Fish species	Data	CONTROL	CHASE	AUTO/ MULTIPLE	THERMAL
Sea bream	Fish/tank	27	27	27	27
	Replicas	3	3	3	3
	Mean initial weight (g)	274.1	268.5	272.1	265.7
	Stressors	None	Lowering water level (45 min)+ chasing (5 min)	Multiple automatic stressors (30 min)	Temperature changes (5 days cycle): 2 days at 12ºC 3 days at 21ºC
	Duration (days)	21	21	21	21
Salmon	Fish/tank	45	45	45	-
	Replicas	3	3	3	-
	Mean Initial weight (g)	559	562	561	-
	Stressors	None	Lowering water level (45 min)+ chasing (15 min)	Multiple automatic stressors (30 min)	-
	Duration (days)	21	21	21	-

Table 1B. Summary of the experimental conditions of the stress trials run at PEIMA (INRA) for rainbow trout. For more detail, see section 2.1.3. * Fish kept in a basket inside the tank.

Trial #	Data	CONTROL	CONFINEMENT	MULTIPLE	POOR WATER QUALITY
Trial 1	Fish/tank	80	80	80	-
	Replicas	3	3	3	-
	Mean initial weight (g)	100	100	100	-
	Stressors	None	200 kg/mm^{3*} (2 min)	Flash light/ hypoxia, intrusion object (2-3 times/day)	-
	Duration (days)	10	10	10	-
Trial 2	Fish/tank	84-89			84-89
	Replicas	3	-	-	3
	Mean initial weight (g)	150			150
	Stressors	3 renewals/day	-	-	0.8-1 renewals/day
	Duration (days)	21	-	-	21

Table 2. Summary of the experimental set up and the procedures applied for each fish species.

FISH SPECIES	TYPE OF STRESSOR	NON LETHAL SAMPLES				LETHAL SAMPLES
		Skin mucus	Blood	Plasma	Behaviour	Gene expression
Gilthead sea bream	Thermal	Proteomics*	Hb, Hc,	Hormones, metabolites	Feeding behaviour	Gills, **liver,** skin
	Chasing	Proteomics*	Hb, Hc	Hormones, metabolites	Feeding behaviour	Gills, **liver,** skin
	Multiple/Auto	Proteomics	Hb, Hc	Hormones, metabolites	Feeding behaviour	Gills, **liver,** skin
Salmon	Chasing	-	Hb, Hc	Hormones, metabolites, clinical chemistry	Feeding behaviour	Liver, head kidney, **pituitary**, muscle
	Multiple/Auto	-	Hb, Hc	Hormones, metabolites, clinical chemistry	Feeding behaviour	Liver, head kidney, **pituitary**, muscle
Trout	Confinement	-	-	Cortisol**	-	-
	Multiple	-	-	Cortisol**	-	-
	Poor water quality	-	-	Ions, osmolality, Cortisol***	Behaviour	**Gills, pituitary, brain,** liver

* Samples taken to map the mucus proteome, but not analysed for differences against control animals. ** After ACTH injection, *** After acute confinement stress. In bold, samples analysed in the lethal samples category.

Ethics statements

All procedures in gilthead sea bream and salmon experiments done at IMR were carried out according to the Norwegian National Ethics Board for experimentation with animals (ID No. 4007, 4763, respectively). Rainbow trout experiments were conducted at INRA facilities, with French authorization for animal experimentation (B29-777-02). All the experiments followed EU legislation on handling of experimental animals.

2.1 Gilthead sea bream (CSIC, IMR)

2.1.1 Fish and experimental set up

Juvenile gilthead sea bream (GSB) of Atlantic origin (Ferme Marine de Douhet, Ile d´Oléron, France) (5 g) were transported from the hatchery in France to the Institute of Aquaculture Torre de la Sal (CSIC, Spain). They were reared until they reached a weight of approximately 150 g and then in January 2012 were transported in truck, from Spain to Norway, to the Institute of Marine Research (IMR), Matre Research Station. Upon arrival, fish were acclimatized to these indoor experimental facilities for 2 months. Fish were then distributed into twelve 500 L tanks at a stocking density of 14–15 kg/m^3 (Table 1A). Each tank was closed with a lid fitted with two fluorescent light tubes (18W each) and one automatic feeder (ARVO-TEC T Drum 2000, Arvotec, Huutokoski, Finland). A 12D:12L photoperiod was maintained with lights on from 8:00 h to 20:00 h. All tanks were supplied with heated seawater (salinity 35‰) that was maintained at 20 ºC with a flow rate of 24–32 L/min. Fish were fed 4.5 mm dry pellets (EFICO YM 554, BioMar, Dueñas, Palencia, Spain) twice a day (11:00 h and 16:00 h) near to satiation 7 days/week. Feed intake was collectively and daily

monitored for each tank (experimental unit) through all the stress trial. Three weeks prior to the start of the stress trial, feed intake was also checked in order to ensure that there were no major tank effects in the trial. Each tank was equipped with a submerged camera positioned opposite the food entrance point adjusted so that the main feeding area was visible. All cameras were controlled via the DVR Server V6.67 Surveillance System (DVR Server, Norway, www.dvr.no). All tanks were also equipped with an oxygen probe (OxyGuard Standard Probe, OxyGuard International, Denmark, http://www.oxyguard.dk). The oxygen probe was positioned in the centre of each tank, just above the outflow. No substrate was present in the tanks.

Four groups, corresponding to control (CTRL) fish and three groups of stressed (ST) fish, were established in triplicate for an experimental period of 21 days. Fish assigned to the thermal stressed group (T-ST = TEMP) were under water temperature cycles of 2 days at 12 ºC to 3 days at 20 ºC. Regulation of water temperature was done manually in the morning (start time 9:00 h), lasting approximately 4 h. In the chasing stress group (C-ST = CHASE), water level in the tank was lowered twice a day (9:15 h and 14:15 h) to 10 cm and was kept at this level for 45 min. Thirty minutes after lowering water level, fish were intensively chased with a pole for 5 min. Fish assigned to the multiple sensory perception stressors group (M-ST = AUTO) were under a fast series of automated stressors for 30 min three times a day (9:30 h, 14:30 h and 18:30 h). During the stress time, fish were exposed to a short burst (10 s) of four different stressors in a random order: (i) a massage device shook the tanks and made a sound, (ii) a window wiper moved back and forth in the water, (iii) a pump reversed the water flow and (iv) a strobe light caused flashes of light.

At the end of the experiment, 6 fish per tank (18 fish in total per experimental condition) were randomly sampled and anaesthetized in a bucket containing 0.1 g/L of 3-aminobenzoic acid ethyl ester (MS-222; Sigma, Saint Louis, MO, USA). A skin mucus sample was collected for proteomics analyses. It was scrapped off the normal skin surface avoiding collection of blood along with mucus with sterile micro slides, transferred into eppendorf tubes and immediately frozen at -80 ºC until analyses. Blood was quickly drawn from caudal vessels. The total time including anaesthesia and blood withdrawal was 4 min for all sampled fish in a given tank. One aliquot of blood was used for haematocrit and haemoglobin measurements. Remaining blood was centrifuged at 3000g for 20 min at 4 ºC, and plasma samples were frozen and stored at 20 ºC until cortisol and metabolite analyses. Prior to tissue collection, fish were killed by cervical section. The liver was then rapidly harvested, frozen in liquid nitrogen and stored at -80 ºC until RNA isolation. All procedures were carried out according to the Norwegian National Ethics Board for experimentation with animals (ID No. 4007) and current EU legislation on handling of experimental animals.

2.1.2 Behaviour monitoring

To make individual behaviour measurements, all fish were marked three weeks before the onset of the current experiment. The marking consisted of coloured beads attached to the gill of each fish. However, the fish started to bite on each other's bead and after two days all fish had lost their marking. Therefore individual measurements on behaviour were not possible during the current experiment.

Stress behaviour: Video recording was made on day 0, 1, 2, 4, 8, 13, 16 and 20 of the experimental period. For the feed behaviour, all experimental tanks were recorded for 2 min before to 5 min after the onset of both feedings. For the stress behaviour, tanks of the AUTO treatment and tanks of the CHASE treatment were recorded for 2 min before to 5 min after the onset of the stress moment during both the morning (9:30h for AUTO and 9:15h for CHASE) and during the afternoon (14:30h for AUTO and 14:15h for CHASE). For the CHASE treatment lowering of the water level was chosen as the onset of the stress moment. Since there was no clear onset for the stress moment of the TEMP treatment and since no stressor was given to the CTRL treatment, for both treatments the same recording moments as for the AUTO treatment were chosen. All recordings were stored directly as AVI files for later analysis. Windows Media Player was used to analyse the videos.

Stress behaviour was monitored using submerged video cameras. As *S. aurata* immediately moves to the bottom of the tank during stress, a vertical distribution form was used to quantify the stress effect (Nilsson *et al*. 2008). Upon analysis the image was divided in four equal squares. The number of fish in each square was recorded on frozen video images at two sampling points; baseline and stress. Each sampling point consisted of three intra-trial periods:

- *Baseline*, 30 sec, 20 sec and 10 sec prior to the onset of the stress moment.
- *Stress*, 10 sec, 20 sec and 30 sec after the onset of the stress moment.

When a fish was half in one square and half in the other square, this was counted as a half fish for each of the two squares. Otherwise one fish was assumed to be in the square holding more than 50% of the fish, and 0 in the other. Video analyses were made on day 0, 2, 13 and 20 for both the stressors in the morning and the afternoon.

After counting the number of fish in each square, for both the Baseline and the Stress sampling point an average of the three intra-trial periods was calculated per day, per square. To correct for the number of fish, this average was converted to a percentage of the total number of fish in the tank. For each tank, per stress moment of the analysed days the stress response for the separate squares was measured as the mean difference between the percentages of fish present before the onset of the stress moment (*Baseline*) and the percentage of fish present after the onset of the stress moment (*Stress*).

Feeding behaviour: The determination of the position of the fish in the tank occurred in the same way as for the stress behaviour. Again the tank image was divided in four equal squares and the number of fish in each square was recorded on frozen video images. The food entrance point was above square 2 and before the experimental period started it was observed that the fish crowded into this square directly after the onset of feeding. There were also two sampling points for the feed behaviour; Baseline and Feed. Every feed moment had a duration of one hour. During this hour, the automatic feeders delivered the food in small portions with some minutes interval in between the given food portions. The response of the fish to a food portion was very quick and therefore it was chosen to have only one intra-trial period for the Feed sampling point. However, the Baseline sampling point still consisted of three intra-trial periods: Baseline, 50 sec, 40 sec and 30 sec prior to the onset of feeding; Feed, 1 sec after the onset of feeding. When a fish was half in one square and half in the other square, this was counted as a half fish for each of the two squares. However, when a fish was unequal divided over two squares this fish was counted as being part of the square which contained the largest part of the fish.

Videos were analysed on day 0, 1 and 20. Furthermore, for all experimental tanks, video recordings of the morning feed moments, were also made four days and five days before the experimental period of the current experiment started (day -5 and day -4). These videos were also analysed, to study the feed behaviour when they were not exposed to the stress treatments.

After the number of fish in each square was counted, for the Baseline sampling point an average of the three intra-trial periods was calculated per day, per square. To correct for the number of fish, this average was converted to a percentage of the total number of fish in the tank. Also for the Feed sampling point, the number of fish in each square was converted to a percentage of the total number of fish in the tank. For each tank, the feed response per square was measured for the different days, as the mean difference between the percentage of fish present before the onset of feeding (*Baseline*) and the percentage of fish present after the onset of feeding (*Feed*).

2.1.3 Blood haematology and biochemistry

Plasma glucose and lactate were analysed using a Maxmat PL II autoanalyzer (ERBA Diagnostics, Montpellier, France). Plasma cortisol levels were analyzed using an EIA kit (Kit RE52061, IBL, International GmbH, Germany). The limit of detection of the

assay was 2.46 ng/mL with intra- and inter-assay coefficients of variation lower than 3% and 5%, respectively. Haematocrit was measured using heparinized capillary tubes centrifuged in a Compur M1100 Microspin centrifuge (Bayer, Germany). Haemoglobin was assessed using a colorimetric kit (No. 700540, Cayman Chemical Company, MI, USA).

2.1.4 Gene expression analysis

The gene expression analysis was focused on mitochondria, as this organelle resulted to be the best candidate after the meta-analysis performed with the "Fish and Chips" tool developed also within WP7 and which is part of D.7.1. RNA from liver was extracted using a MagMAX™-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, USA). RNA yield was 50–100 µg with 260 and 280 nm UV absorbance ratios (A260/280) of 1.9–2.1 and RIN (RNA integrity number) values of 8–10 as measured on an Agilent 2100 Bioanalyzer, which is indicative of clean and intact RNA. Reverse transcription (RT) of 500 ng total RNA was performed with random decamers using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Negative control reactions were run without reverse transcriptase and real-time quantitative PCR was carried out on a CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using a 96-well PCR array layout designed for simultaneously profiling a panel of 60 mitochondria related genes under uniform cycling conditions (Supplementary Table 1, Annex 1). Among the 60 genes, 40 genes were novel for GSB and their sequences were uploaded to GenBank (JX975224–JX975265) (Supplementary Table 2, Annex 1). These genes were selected as markers of nuclear transcriptional regulation (5 genes), oxidative metabolism/respiration uncoupling (13 genes), antioxidant defence (7 genes), protein import/folding/assembly (23 genes), and mitochondrial dynamics and apoptosis (12 genes). These markers were selected on the basis of the transcriptionally-mediated responses of GSB to crowding stress (Bermejo-Nogales *et al.,* 2008; Saera-Vila *et al.,* 2009; Calduch-Giner *et al.,* 2010), and literature references in other animal models, including rodents and humans (Manoli *et al.,* 2007; Liesa *et al.,* 2009; Ljubicic *et al.,* 2010; Wenz, 2013).

Four housekeeping genes and controls of general PCR performance were included on each array, all the pipetting operations being performed by means of the EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany). Briefly, RT reactions were diluted to convenient concentrations and the equivalent of 660 pg of total input RNA was used in a 25 µl volume for each PCR reaction. PCR-wells contained a 2X SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 µM were used to obtain amplicons of 50–150 bp in length. The program used for PCR amplification included an initial denaturation step at 95 ºC for 3 min, followed by 40 cycles of denaturation for 15 s at 95 ºC and annealing/extension for 60 s at 60 ºC (Supplementary Table 3, Annex 1). The efficiency of PCR reactions was always higher than 90%, and negative controls without sample templates were routinely performed for each primer set. The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5 ºC/10 s over a temperature range of 55–95 ºC), linearity of serial dilutions of RT reactions, and electrophoresis and sequencing of PCR amplified products. Fluorescence data acquired during the PCR extension phase were normalized using the delta–delta Ct method (Livak and Schmittgen, 2001). β-Actin, elongation factor 1, α-tubulin and 18S rRNA were tested for gene expression stability using GeNorm software, but the most stable gene was β-actin (M score = 0.21) and, thereby, it was used as housekeeping gene in the normalization procedure. Fold-change calculations were done in reference to the expression ratio between ST and CTRL fish (values >1 indicate stress up-regulated genes; values <1 indicate stress down-regulated genes). For comparing the mRNA gene expression level of a panel of genes in a given stress condition, all data values were in reference to the expression level of proliferator-activated receptor gamma coactivator 1 β (PGC1β) in CTRL fish, which was arbitrarily assigned a value of 1.

2.1.5 Proteomics

Gel electrophoresis: The protein composition of mucus was first analysed by one-

dimensional gel electrophoresis. A pool from all sampled animals from CTRL and the three stressed groups was made, and triplicate samples (54-56 µg) were separated by polyacrylamide gel electrophoresis (SDS-PAGE) using an Any KD precast gel (Bio-Rad) at 200V for 25 min, and stained overnight with colloidal Coomassie (Bio-Rad). The gel lane was then divided into ten slices that were analysed independently. Proteins in gel were digested with protein grade trypsin (Promega) as described elsewhere (Shevchenko *et al.,* 1996) and concentrated by speed vacuum at a final volume of 12 µl for mass spectrometry.

For identification of differentially abundant proteins due to multiple sensorial stress, individual samples of CTRL and M-ST (n=8 for each group) were precipitated by means of the 2-D Clean-Up kit (GE Healthcare), and then solubilized in labelling buffer (7 M urea, 2 M thiourea, 4 % w/v CHAPS, 20 mM Tris). The N-hydroxysuccinimide ester dyes Cy2/3/5 were used for minimal labelling using the mixed internal standard methodology of Alban *et al*. (2003) following manufacturers' protocol (GE Healthcare). Fifty µg of each of the 16 experimental samples were individually labelled with 400 pmol of either Cy3 or Cy5 such that half of the members of each group were labelled with Cy3 and the other half were labelled with Cy5 to compensate for any dye-specific labelling artefacts. In parallel, a mixed internal standard was generated by combination of equal amounts of each experimental sample and labelled with 400 pmol of Cy2. Labelling was performed for 60 min on ice in the dark after which the reaction was quenched by the addition of 10 nM lysine for 10 min.

For each gel, a total protein amount of 150 µg was run (50 µg of a Cy3 labelled sample, 50 µg of a Cy5 labelled sample and 50 µg of the Cy2-labelled internal standard). Immobiline DryStrips (pH 3-11 NL, 24 cm) were rehydrated overnight in 8 M urea, 4 % (w/v) CHAPS, 12 µl/ml DeStreak reagent, 1 % ampholytes (pH 3-11 NL). Samples were applied near the basic end of the strips by cup-loading after being incubated in 65 mM DTT, 1 % ampholytes. After focusing at 32 kvh at 20 ºC, strips were equilibrated prior to SDS-PAGE, first for 15 min in reducing solution (6 M urea, 50 mM Tris-HCl, 30% v/v glycerol, 2% w/v SDS, 2 % w/v DTT) and then in alkylating solution (6 M urea, 50 mM Tris-HCl, 30 % v/v glycerol, 2 % w/v SDS, 2.5 % w/v iodoacetamide) for 15 min. Second-dimension SDS-PAGE gels were run at 20ºC at a constant power of 2 W per gel for 60 min followed by 15 W per gel until the bromophenol blue tracking front had run off the end of the gel (6 hours).

Data acquisition and mass spectrometry: Fluorescence images were obtained on a Typhoon 9400 scanner (GE Healthcare). Cy2, Cy3 and Cy5 images were scanned at excitation/emission wavelengths of 488/520 nm, 532/580 nm and 633/670 nm, respectively, at a resolution of 100 µm. Images were cropped to remove areas extraneous to the gel image using ImageQuant Tools (Amersham Biosciences) prior to analysis. Both image analysis and statistical quantification of relative protein levels were performed using DeCyder v. 6.5 software (GE Healthcare). Proteins of interest were manually excised from analytical gels, digested with sequencing grade trypsin, and subject to LC-MS/MS analysis.

For each sample, 5 µl were loaded onto a trap column (NanoLC Column, 3µ C18-CL, 350 µm x 0.5 mm, Nikkyo) and desalted with 0.1 % TFA at 3 µl/min during 10 min. Peptides were then loaded onto an analytical column (LC Column, 3 µ C18-CL, 75 µm x 12cm, Nikkyo) equilibrated in 5 % acetonitrile, 0.1 % formic acid. Separation was carried out with a linear gradient of 5-40 % acetonitrile gradient with 0.1 % formic acid at a flow rate of 300 nl/min. Peptides were analysed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX). The tripleTOF was operated in information-dependent acquisition mode, in which a 0.25-s TOF MS scan from 350–1250 m/z, was performed, followed by 0.05-s product ion scans from 100– 1500 m/z on the 50 most intense 2-5 charged ions.

Protein identification: Protein identity was determined using ProteinPilot v4.5 (ABSciex) that incorporated the Mascot search algorithm (v2.2, Matrix Science). ProteinPilot default parameters were used to generate peak list directly from 5600 TripleTof wiff files. Mascot was used to search into Expasy protein database or the GSB nucleotide database (www.nutrigroup-iats.org/seabreamdb; Calduch-Giner *et al.,* 2013) with the following parameters: trypsin specificity, carbamidomethyl C to fix modification, deamidated (NQ), Gln->pyro-Glu (N-term Q), Glu->pyro-Glu (N-term E), oxidation (M) to variable modification,

75 ppm as peptide mass tolerance and 0.6 Da as fragment mass tolerance. Proteins showing score higher than homology or significance threshold were identified with confidence ≥ 95%.

For mapping the reference proteome of skin mucus, identified proteins against the GSB nucleotide database were considered only for functional analysis when they showed a protein score higher than 30. Pathway analysis with this set of proteins was performed by means of the IPA software (www.ingenuity.com). For each protein in the analysis, the Uniprot accession equivalent for one of the three higher vertebrates model species in IPA (human, rat or mouse) was searched as previously done for the functional analysis of GSB transcriptome (Calduch-Giner *et al.,* 2013).

2.1.6 Statistical analysis

Data on fish performance, biochemistry and gene expression were analysed using one-way analysis of variance (ANOVA-I), followed by a Student–Newman–Keuls post hoc test. When the test of normality or equal variance failed, a Mann–Whitney Rank Sum test or a Kruskal–Wallis ANOVA on ranks followed by Dunn's method was applied instead, respectively. The significance level was set at $P < 0.05$. All analyses were conducted using SPSS package version 19.0 (SPSS Inc., Chicago, IL, USA).

Other statistical analyses were performed using Microsoft Excel 2010 and IBM SPSS Statistics 19. Tanks were considered as the experimental units for the current research. Differences in growth performance, total feed intake and oxygen consumption rates between the treatments were tested with a non-parametric Kruskal-Wallis test. When a significant treatment effect or a trend was found, Mann-Whitney U tests were applied *post-hoc*. Statistical significance was taken at $P < 0.05$, except when a Bonferroni correction was applied ($P < 0.0167$). Data shown in tables are reported as mean ± SEM, while data shown in figures are reported as mean ± SD.

2.2 Atlantic Salmon (IMR, UoS)

2.2.1 Fish and experimental setup

Atlantic salmon (LA strain- Norwegian breeding programme) were hatched and kept in seawater at 10 ± 1.5 °C under a continuous simulated daylight regime at Matre Aquaculture Research Station, Norway. At 450 g, 50 fish were transferred into each of nine 1.5 x 1.5 x 0.6 m standard fiberglass tanks supplied with 800 litres of aerated seawater (9± 1.0 °C 20L/min) and continuous light (24:0 L:D) supplied by fluorescent light tubes (18 Watt each) (24:0 L:D). Each tank was supplied with an automatic feeder (ARVO-TEC T Drum 2000, Arvotec, Huutokoski, Finland, www.arvotec.fi) and a feed collector. All tanks were also equipped with an oxygen probe (OxyGuard Standard Probe, OxyGuard International, Denmark, http://www.oxyguard.dk) positioned in the centre of each tank, just above the outflow. To enable individual measurements on behaviour, all fish were marked three weeks before the onset of the current experiment. This marking consisted of a coloured bead, which was attached to the pectoral fin of each fish. However, the fish started to bite on each other's bead and after one week all fish had lost their marking. Therefore individual measurements on behaviour were not possible during the current experiment. The fish remained in the tanks for 4 weeks until an average weight of 561 g was achieved. Two days before the start of the experiment the fish were anaesthetized in 0.4% benzocaine and weight and length measured. Of the 9 tanks, 3 were control treatments given as little handling as possible, the remaining 6 tanks were divided into a water level and chasing group (CHASE) and an automatic stressor group (AUTO). An overview of the tank setup is given in Table 1 and the CHASE and AUTO stressors are equivalent to those applied to GSB (see section 2.1.1).

The diets were commercial pellets for the size of fish (EWOS Innovation). The fish were overfed by 20 % and surplus feed collected in automated feed collectors to enable

accurate estimation of feed conversion and appetite. Throughout the experimental period video recordings were made of times before feeding, during feeding, before stress and during stress. Video recordings of these moments were only made on days 0, 1, 2, 4, 8, 13, 16 and 20 of the experimental period. Oxygen was recorded continuously throughout. At the end of the experiment 5 fish were anaesthetized in MS-222 and sampled for biochemical and molecular measurement, and remaining fish subjected to confinement stress (100 L) tank with flow through water for 1h and then resampled.

2.2.2 Blood analysis

At T = 0 and T = 1h, blood was withdrawn from the dorsal aorta using heparinized needles. Hematocrit (Hc) was obtained using heparinized microcapillary tubes and a Compur M1100 haematocrit centrifuge. Plasma was prepared immediately by centrifugation at 11,000 rpm for 1 min and stored at –80 ºC prior to analysis. Plasma samples were analysed for lactate and glucose using MaxMat PL (MaxMat S.A., Montpellier, France). Plasma cortisol was analysed using a Cortisol ELISA kit (RE52061) (IBL International GmbH, Hamburg, Germany). Osmolality was measured in 20 µl plasma using a Fiske-210 Micro-Sample Osmometer (Advanced Instruments, Inc., Norwood, MA, USA) and ions were analysed with Cobas c 111 System (Roche Diagnostics Ltd., Rotkreuz, Switzerland).

2.2.3 Gene expression analysis

Immediately after blood sampling, pituitaries were removed and stored in RNAlater for transcriptomics analysis and stored at -80 ºC until analysis. It was conducted using a custom-made 4 x 44K Atlantic salmon oligo microarray (Agilent Technologies, Wokingham, UK) described in detail previously (Taggart *et al.,* 2008). The salmon custom array and laboratory procedures utilized in this study have been widely used and validated in many previous studies (Morais *et al.,* 2011; Martínez-Rubio *et al.,* 2012; Tacchi *et al.,* 2012). Briefly, total RNA was extracted from individual samples using TRI Reagent essentially according to manufacturer instructions (Sigma-Aldrich, Dorset, UK), and including a high salt precipitation as recommended for polysaccharide-rich tissues such as liver (Chomczynski and Mackey, 1995). RNA quantity, integrity and purity were assessed by agarose gel electrophoresis and spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, USA). Pools of RNA samples from two individuals were amplified using a Target-Amp 1 round aRNA amplification kit (Cambio Ltd, Cambridge, UK) following recommended procedures and purified with RNeasy Mini Kit (Qiagen, Manchester, UK) and analysed as a single biological replicate. Aminoallyl-amplified RNA (aRNA) samples were labelled with Cy3 dye (GE HealthCare Life Sciences, Buckinghamshire, UK) while a pool of equal amount of all aRNA samples was labelled with Cy5 dye (GE HealthCare Life Sciences) and used as a common reference. Unincorporated dye was removed by purifying the aRNA samples with Illustra AutoSeq G-50 dye terminator columns (GE HealthCare Life Sciences). Dye incorporation rate was calculated using NanoDrop ND-1000 and sample integrity assessed by agarose gel electrophoresis.

Labelled aRNA samples were hybridized to the custom-made array. A dual-label common reference design was adopted, where equal amounts of each individual aRNA sample and the common reference pool were competitively hybridized to one array. Samples were processed with the Gene Expression Hybridization Kit (Agilent Technologies), applied to the arrays and immediately incubated using SureHyb hybridization chambers in a DNA Microarray Hybridization Oven (Agilent Technologies) for 17h. Throughout the experiment samples were always randomized, avoiding samples from the same treatment being overrepresented in a particular batch in order to avoid unintentional biases. Scanning was performed using a GenePix 4200 AL Scanner (Molecular Devices (UK) Lid., Wokingham, UK) and the resulting images analysed with Agilent Feature Extraction Software v.9.5 (Agilent Technologies) to extract the intensity values and identify the features. The foreground intensity was computed as the mean value of pixels, considered a better estimator as being less susceptible to distortion from outlier values (Russell *et al.,* 2009),

while background intensities were computed as the median value of pixels.

Differential expression analysis: Transcriptomic data analyses were performed using R v.3.0.1 and Bioconductor v.2.12 as described in De Santis *et al.* (2015). Features of the array were annotated using BLAST 2.2.29+ (blastx) against the entire non-redundant protein database as well as using the KEGG Automatic Annotation Server to obtain functional annotations. Differentially expressed features between treatments were extracted using the package limma (Smyth, 2004) as indicated in De Santis *et al.* (2015).

2.2.4 Data analysis

Growth and blood data were expressed as group means and standard errors of means (SEM). Data were subjected to Cochran's test for equal variances and in cases of data sets showing heteroscedasticity a log-transformation was performed resulting in equal variances. Between group differences in responses (fed versus fasted fish) were analysed by means of ANOVA. Null hypotheses were rejected at a probability level of $P < 0.05$.

Specific Growth Rate (SGR) was calculated for the period in between the two weight measurements which include 10 days before the onset of the stress period and the stress period itself. Because the fish lost their individual tags, all growth values were calculated at tank level. During the experimental period some fish died and so weight data of these dead fish were not used. Feed Conversion Ratio (FCR) and biomass per day were calculated.

Oxygen consumption rate (VO2) was measured at tank level with each tank being used as an open respirometer, by measuring the difference in oxygen saturation between the influent and effluent water. The oxygen saturation of the influent water was assumed to be 100 % during the whole experimental period. Measurements in the inflow of each experimental tank also showed that on average the oxygen saturation in these inflows was 100 %. The probes monitored the oxygen saturation for each minute, starting from the day before the onset of the current experiment (day -1).

2.3 Rainbow trout (INRA)

2.3.1 Fish and experimental setup

Rainbow trout stress trials were performed at the facilities of PEIMA (INRA fish farming in Brittany, France), using fish hatched and reared in an open flow system. Two experiments were performed:

Preliminary trial 1: to validate a chronic stress protocol. It consisted of a combination of acute stressors applied daily during 10 days. Fish (~70g, autumnal spawning line) were exposed to i) acute confinement stress (2 min at 200kg/m^3); ii) daily repetitions of several acute stressors (flash light, hypoxia, intrusion of a new object in the tank, 2-3 times per day) and iii) control group, undisturbed. At the end of the experiment, response of the HPI axis was evaluated in control and chronically stressed fish. Ten fish from each group were caught and injected intracelomically with saline (NaCl 0.9%) or with ACTH (Porcine $ACTH_{1-39}$ Sigma Aldrich) at a concentration of 5 IU/Kg fish. Response of the HPI axis was evaluated before and after each acute stressor. At 1 h and 3 h after this injection, fishes were euthanized with a lethal dose of phenoxy ethanol and blood was taken to analyse plasma cortisol.

Trial 2: the stressing factor was poor water quality (mainly hypoxia). Fish (~ 150g) were distributed into 18 tanks and acclimatized to a 50 kg/cm^3 density (84-89 fishes/tank) for 2 weeks, with 3 water renewals/h. Then, water renewal was reduced to 0.8-1/h in 9 tanks for 3 weeks (Stress group = S), whereas it was kept as initially in the remaining 9 tanks (CONTROL group = CTRL). Fish
were fed 3 times/day *ad libitum*. Oxygen and temperature were continuously measured in 4 tanks (2 CTRL and 2 S) (Orion equipment). Oxygen was check several times per day in the other tanks. N-NH4 was measured every week in at least 4 tanks (Ammonium kit, Hach-Lange, France). Twelve tanks (6 CTRL + 6 S) were dedicated for behaviour observations

and six other tanks (3 CTRL + 3 S) were used to measure endocrinological and physiological parameters. After 2, 10 and 21 days in these conditions, 9 fish of NS and S groups were euthanized with a lethal dose of phenoxy-ethanol and weighed. Blood, gill, brain section containing (hypothalamus, mid brain, telencephalon, preoptic area), pituitary, head kidney (which includes interrenal cells) and liver tissue were sampled for the molecular analysis of the HPI axis and gill functions. Plasma and tissues were stored at -20°C and at -80°C, respectively until used. Moreover, on day 21, after sampling tissues and blood, 20 fish from each group were exposed to a standardized acute confinement stress (2 minutes) and plasma cortisol levels were measured 1h and 3h following acute stress.

2.3.2 Behavioural studies

Focal observations: After the 2 wk-acclimation period, behavioural parameters were video-recorded for 10 min within the CTRL and S groups at Day-1 (the day before the beginning of the chronic stress), D1, D4, D8, D11, D16 and D18, 3 times/day (9.30 before the first meal, 11.00 during the second meal and 16.20 before the last meal). Using the focal sampling method, the following behaviours were counted in each tank: stereotypies (repetitive swim against the edge of the tank), jump, aggression, fast-start accelerations. In addition, scan samplings were performed to measure the time spent in activity (in % of time).

Emotional reactivity test: After focal observations, on D22 and D23,12 fish/group were analysed through an emotional reactivity test. Fish were individually transferred from their initial tank to another one (Ø=1m^2) and their immediate reaction was analysed using EthovisionXT software: Mean and maximum velocity, total distance travelled, time spent in thigmotaxis (close to the tank wall) and time spent immobile were recorded.

Learning paradigm: On D29, D30 and D31, 7 days after the end of exposure to low water quality, associative learning was assessed on each group previously exposed during 21 days to S or CTRL conditions (6 CTRL tanks, 6 S tanks). Acquisition phase consisted in exposing fish 6 times/day for 3 days to a neutral stimulus (Conditioned Stimulus, CS: water-flow stop) and to feed the fish 60 seconds later (Unconditioned Stimulus, US: Food-reward). After 17 trials, the conditioned response was evaluated by a learning index from 1 to 4 (1: no grouping in the feeder area; 4: all fish grouped in the feeder area). The index was given by scan samplings performed at 0, 55, 65, 115 and 120 seconds of the 2-minute-video-recording performed at trial 18.

Statistics: As data from focal observations and learning results had no normal distribution (Kolgomorov-Smirnof test), non-parametric tests were used (n = 6). Mann-Whitney and Wilcoxon tests were used to compare experimental groups (independent data) and days (dependent data), respectively. For the emotional reactivity data, t-Student test was applied (n =12).

2.3.3 Plasma biochemistry

Plasma cortisol: Steroids were extracted from 50 µl plasma with ethyl acetate/cyclohexane (v/v) and then dissolved in 300 µl assay buffer (0.01M NaH_2PO_4, 0.01M Na_2HPO_4, O.9% NaCl, 0.1% gelatine, pH 7.25). Cortisol was measured according to the method described in Auperin *et al.* (1997).

Ions and osmolality: Plasma sodium was analysed using flame atomic absorption spectrophotometry (Varian AA240FS, Agilent Technologies, Massy, France), chloride and calcium using colorimetric kits (chloride with a mercuric-thiocyanate method and calcium with Arsenazo III method (Biolabo, France). Osmotic pressure was analysed using a freeze-point osmometer.

2.3.4 Gene expression analysis

Real time PCR (RT-PCR): Total RNA from gill, brain, pituitary, head kidney and liver was extracted using TRIzol reagent, according to the manufacturer's instruction. RNA was quantified by measuring the optical density at 260nm. RNA integrity was checked using the Bioanalyser 2100 Agilent. cDNA synthesis and RT-PCR were performed as described in Lucas et al. (2014). Reverse transcription was applied to 1.5µg of RNA at 37°C for 1h using M-MLV reverse transcriptase (Promega). RT-PCR was carried out on a StepOnePlus system with SYBR-Green PCR master Mix (Applied Biosystem). Tissues from 9 fish of S and CTRL groups (trial 2) were used for carrying out gene expression analysis.

Microarray analysis: An Agilent-based microarray platform with 8X60K probes per slide (GEO platform record: GPL15840) was used. Gill, pituitary and head kidney RNA from 8 S fish and 8 CTRL fish from trial 2 were labelled and hybridized as indicated in Rescan *et al.* (2013). Hybridized slides were scanned using an Agilent microscanner. After data extraction, normalization and statistical analysis were performed using GeneSpring software (Agilent). A t-test with a Benjamini-Hochberg correction ($P < 0.05$), was used as the criteria for defining gene differentially expressed between control and stressed group for each organ. Gene ontology enrichment analysis was performed using the Functional Annotation Clustering tool within the DAVID Bioinformatics Database (Huang *et al.,* 2009).

3. Results

3.1. Gilthead sea bream (CSIC, IMR)

3.1.1. Fish performance: growth and feed intake

As shown in Table 3, fish exposed to thermal fluctuations (T-ST) showed an overall reduction of feed intake (nearly 40%) in comparison to CTRL fish and the other two stress groups, corresponding to changes in water levels and chasing (C-ST) and multiple sensory perceptions stressors (M-ST). This decrease in feed intake was accompanied by a statistically significant reduction ($P < 0.05$) of specific growth rates, but this detrimental effect was lower than expected due to a slight increase in feed conversion ratio (weight gain/feed intake) that was statistically significant ($P < 0.05$) for the paired stress comparisons between the T-ST and M-ST groups. C-ST fish showed similar growth, feed utilization and feed intake as CTRL fish. M-ST fish also showed a similar feed intake as CTRL fish, but growth rates achieved intermediate values between the two extreme groups (CTRL and T-ST) due to some detrimental effect of this type of stressor upon feed conversion. One fish died 2 days before start of the stress trial, and was not replaced. On day 1 of start-up one fish died in the M-ST group, and one fish died on day 20 in the C-ST group. No mortality was registered in the T-ST group.

When the daily feed intake was compared, there was a significant effect of treatment (Fig. 1). Fish in the T-ST group had appetite fluctuating with temperature dropping significantly compared to control fish ($P < 0.05$) when exposed to 12 °C (Fig. 1A). When water temperature was kept stable at 20 °C, feed in tank was similar between T-ST and CTRL fish with no compensatory appetite ($P > 0.05$, Figure 1A).

Table 3. Data on growth performance and plasma biochemistry and haematology of GSB exposed to stress stimuli or non-stressed (CTRL), thermal stress (T-ST), chasing stress (C-ST) and multiple sensory perception stress (M-ST). Data on growth performance are the mean ± SEM of triplicate tanks. Cortisol levels are the mean of 9 fish (3 fish per triplicated tank). Other systemic measurements are the mean of 20-24 animals (8-6 fish per triplicated tank).

	CTRL	T-ST	C-ST	M-ST	P^1
Initial body weight (g)	261.0 ± 1.6	252.1 ± 4.6	255.38 ± 3.9	259.4 ± 2.2	0.30
Final body weight (g)	329.1 ± 0.70^{a}	297.9 ± 7.1^{b}	319.79 ± 8.6^{ab}	316.47 ± 4.4^{ab}	0.03
Average feed intake (%)	0.56 ± 0.02^{a}	0.37 ± 0.01^{b}	0.55 ± 0.01^{a}	0.56 ± 0.02^{a}	<0.001
FCR	1.02 ± 0.04^{ab}	1.18 ± 0.02^{a}	1.00 ±0.04^{ab}	0.87 ±0.07^{b}	0.03
SGR (%)	0.58 ± 0.01^{a}	0.42 ± 0.03^{b}	0.56 ± 0.04^{ab}	0.49 ± 0.06^{ab}	0.05
Haematocrit (%)	35.3 ± 0.8^{a}	28.5 ± 1.2^{b}	37.5 ± 1.16^{a}	34.5 ± 0.81^{a}	<0.001
Haemoglobin (g/dL)	11.0 ± 0.27^{a}	9.5 ± 0.43^{b}	12.3 ± 1.2^{a}	10.6 ± 0.24^{ab}	0.005
Plasma cortisol (ng/mL)	10.4 ± 2.2	10.7 ± 1.9	17.2 ± 2.9	37.2 ± 19.7	0.24
Plasma glucose (mM/L)	3.8 ± 0.29	3.8 ± 0.25	3.9 ± 0.26	3.7 ± 0.3	0.93
Plasma lactate (mM/L)	2.78 ± 0.18^{b}	2.91 ± 0.34^{ab}	3.3 ± 0.18^{a}	2.0 ± 0.18^{c}	0.05

[1]*P* values result from analysis of variance. Different superscript letters in each row indicate significant differences among experimental groups (Student Newman-Keuls test, $P < 0.05$)

Both the C-ST and the M-ST treatments showed a similar feed intake pattern as the CTRL group (Fig.1B and Fig. 1C, respectively). However, a significant lower feed intake compared to CTRL was seen on Day 1 of the experiment ($P < 0.05$). The C-ST treatment also had a significant lower feed intake than CTRL on Day 11, Day 15 and Day 16, while on Day 10 there was a significant higher feed intake ($P < 0.05$). The M-ST treatment showed a significant higher and lower feed intake than the CTRL treatment on Day 6 and Day 14 respectively ($P < 0.05$). There were some notable drops in CTRL, M-ST and C-ST groups on Day 6 and Day 13 of the experiment (Fig. 1B and C). The causes for this are unknown, but it was assumed to be caused by external factors and were not further perused.

3.1.2. Oxygen consumption

The oxygen consumption data between treatments over the experimental period is given in Table 4. There was a tendency ($P < 0.1$) of reduced oxygen consumption of the TEMP group compared to the other test groups and CTRL when calculated over 24h and during daytime. There was however a significant treatment effect ($P < 0.05$) for the oxygen consumption rate when lights were off (dark period) (Table 4). This was due to reduced consumption for the TEMP treatment.

Accordingly, the daily dark period oxygen consumption of the TEMP group was consequently compared to CTRL over the entire experimental period (Fig. 2). On Day -1 and Day 0 there were no differences between the two groups ($P > 0.05$). However, after Day 0, and for nearly all of the experimental days (15 out of 21), the oxygen consumption rate for the T-ST treatment was significantly lower than the oxygen consumption rate of the CTRL treatment ($P < 0.05$). Furthermore, there was a noticeable drop in the TEMP group on days of 12 °C. No significant differences were found for day -1, 0, 4, 9, 15, 19 and 20 ($P > 0.05$). However, although water temperature was also 20 °C on days 3, 8 and 14, oxygen consumption was also lower compared to CTRL ($P < 0.05$). This shows that the oxygen consumption in the TEMP treatment did not reach comparable oxygen consumption rate as the CTRL treatment when the water temperature was restored to 20 °C.

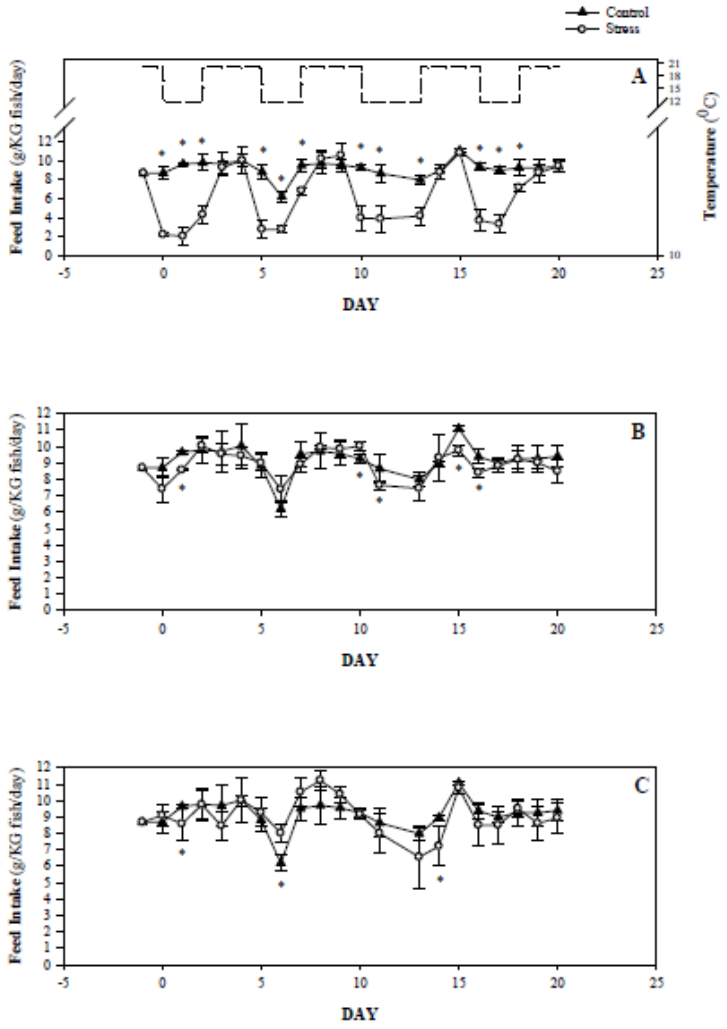


Figure 1. Average daily feed intake (g/kg/day), during the experimental period (21 days) of GSB exposed to three different stress treatments. (A), CTRL (triangles) and T-ST (open circles); (B) CTRL and C-ST and (C) CTRL and M-ST. Data points represent mean per day (*n* = 3 tanks/treatment). Error bars represent standard deviation of the mean of the model. Significant differences in means per day between a stress treatment and the CTRL treatment are indicated by * (*P* < 0.05).

Table 4. Oxygen consumption rates (mg O_2/kg fish/min)of GSB during different periods of the experimental phase (21 days), of gilthead sea bream exposed to four different treatments: CONTROL (CTRL), TEMPERATURE (T-ST), CHASE (C-ST) and MULTIPLE or AUTO (M-ST).

Time	CTRL	T-ST	C-ST	M-ST	*P*-value
Total (24 hours)	4.3 ± 0.18	3.1 ± 0.051	4.5 ± 0.15	4.5 ± 0.24	< 0.1
Dark period (12 hours)	3.7 ± 0.18	2.7 ± 0.055	4.0 ± 0.22	4.2 ± 0.20	< 0.05
Light period (12 hours)	5.0 ± 0.21	3.5 ± 0.057	5.0 ± 0.085	4.8 ± 0.27	< 0.1

Values are given as mean ± SE (*n* = 3 tanks/treatment). No significant differences were found after the post-hoc test. The mean oxygen consumptions were calculated over 15 min running averages. Total is the mean oxygen consumption calculated over the whole experimental period (includes both light and dark period of the days). Dark period is the mean oxygen consumption calculated over the period when lights where off during the experiment (from 20:00h till 8:00h). Light period is the mean oxygen consumption calculated over the period when lights were on during the experiment (from 8:00h -

20:00h).

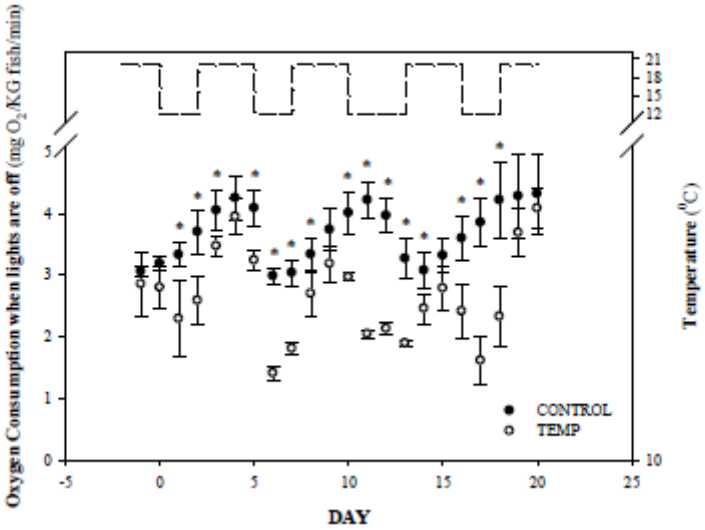


Figure 2. Mean oxygen consumption rate (mg O_2/kg fish/min) of GSB during the dark phase each day through the experiment (from 20:00h till 8:00h) in the TEMP and CONTROL groups. The mean oxygen consumptions were calculated over 15 min running averages. Data points represent mean per day (*n* = 3 tanks/treatment). Error bars represent standard deviation of the mean of the model. Significant differences in means per day between the TEMP treatment and the CONTROL treatment are indicated by '*'.

As both the AUTO and CHASE groups had defined stress moments, the effect of stress on immediate oxygen consumption was also compared. Fig. 3 compares the mean oxygen consumption over the entire experiment before and during stress. The mean oxygen consumption rate before the morning stress in the AUTO group (No Stress) was numerically higher than during stress (Stress) (Fig. 3A), but these values were not statistically significant (*P* = 0.109). In the CHASE group (Fig. 3B), mean oxygen consumption rate tended to increase following stress, but again values were not statistically different (*P* = 0.109).

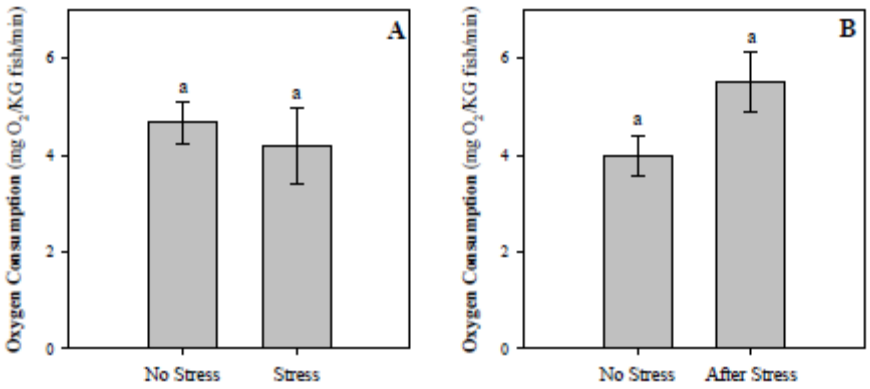


Figure 3. Mean oxygen consumption rate (mg O_2/kg/min), before and during stress in GSB in (A) the AUTO group and (B) the CHASE group. For the AUTO group, No Stress is the consumption in the morning before stress (from 8:30h till 9:30h), while Stress is the mean oxygen consumption during and just after the morning stress (from 9:30h till 10:30h). For the CHASE treatment No Stress is the mean oxygen consumption in the morning before stress (from 8:30h till 9:00h), while After Stress is the consumption after the morning stress (10:30h till 11:00h). Mean oxygen consumption rates were calculated over 15 min running averages. Bars are mean values (*n* = 3 tanks/treatment). Error bars represent standard deviation of the mean of the model.

The daily variation in oxygen consumption rate following stress is given in Fig. 4. On

day -1, 0 and 6, fish in the AUTO treatment used on average more oxygen during stress than they did before stress, while on all other days consumption values were either similar or the average oxygen consumption of No Stress was higher than the average consumption rate of stress (Fig. 4A). During all days of the experimental period, fish in the CHASE treatment used on average more oxygen after stress than before stress (Fig. 4B). However, for both treatments no significant differences were found for any of the experimental days (*P>0.05* for AUTO and CHASE).

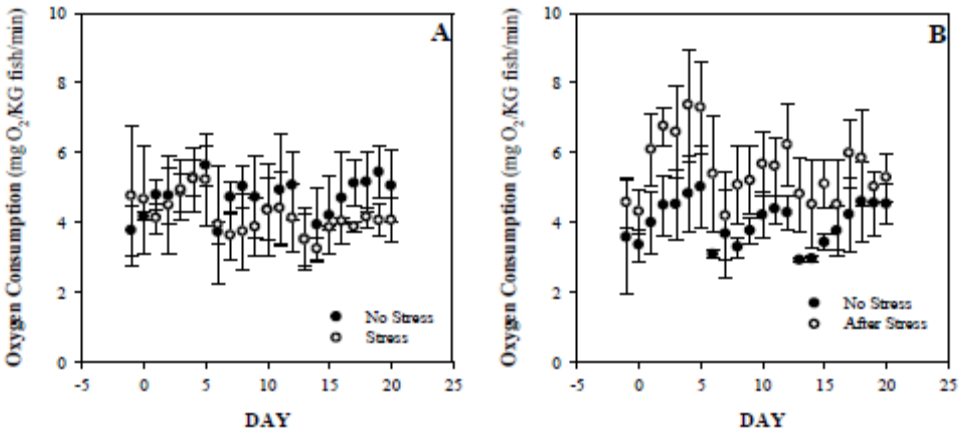


Figure 4. Mean oxygen consumption rate (mg O_2/kg/min) of GSB, before and during morning stress in *S. aurata* in (A) the AUTO group and (B) the CHASE group. For the AUTO treatment No Stress is the mean oxygen consumption before the morning stress (from 8:30h till 9:30h), while Stress is the oxygen consumption during and just after the morning stress (from 9:30h till 10:30h). For the CHASE treatment No Stress is the mean oxygen consumption before the morning stress (from 8:30h till 9:00h), while After Stress is the mean oxygen consumption after the morning stress (10:30h till 11:00h). Mean oxygen consumption rates were calculated over 15 min running averages. Data points represent mean per day (*n* = 3 tanks/treatment). Error bars represent standard deviation of the mean of the model.

3.1.3. Behavioural changes

Stress behaviour: For each treatment, the fish went to the bottom when the stress started. Data were the same during the morning and afternoon stress. Furthermore, within each treatment, data for the two top squares (square 1 & 2) and the two bottom squares (square 3 & 4) were identical. Therefore, for each treatment, only the results from one top square (top right square, square 2) and one bottom square (bottom right square, square 4) during the mornings stress will be presented.

The effect of stress on the tank distribution of fish is given in Fig. 5 (AUTO) and 6 (CHASE). In both cases stress caused the fish to descend to the bottom of the tank increasing their relative number at the lower square, and decreasing the number in the upper square. For both groups there was a clear and strong response over the first days, and a notable tendency of habituation throughout the trial. This was particularly noticeable for the bottom square as where it seemed like fewer fish dived upon stress. For the AUTO group, the differences between the control group were significant on days 0, 2 and 13 ($P < 0.05$), whereas no significant difference was found on the last day of the experiment (day 20, $P = 0.28$). The CHASE group (square 4), was generally similar to the AUTO group with significant differences between CHASE and CTRL on days 0, 2 and 13 ($P < 0.05$), and no significant differences by the end of experiment (day 20) ($P = 0.077$).

The results of TEMP are shown in Fig. 7. There were no effects of the treatment on the distribution of fish in the tank, and all values centred on zero ($P > 0.05$, Fig.7A-B).

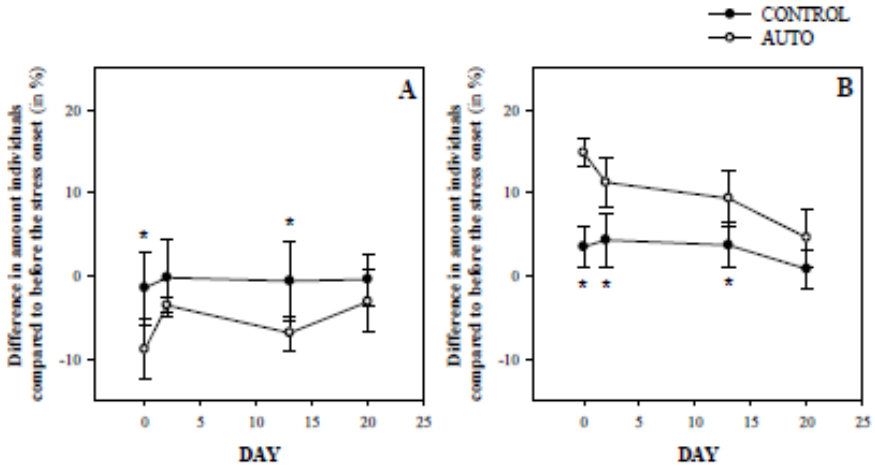


Figure 5. Stress response of GSB exposed to AUTO stress, measured as the mean difference between the percentage of fish present before the onset of the stress and the percentage of fish present after the onset of the stress for (A) the top right square (square 2) and (B) the bottom right square (square 4) of the morning stress. For comparison, the "stress response" of the CTRL treatment is plotted in the same graphs. Results of day 0, 2, 13 and 20 are shown. Data points represent mean per stress moment, per day (n = 3 tanks). Error bars represent standard deviation of the mean of the model. Significant differences in means per stress moment, per day between the AUTO treatment and the CTRL treatment are indicated by '*'.

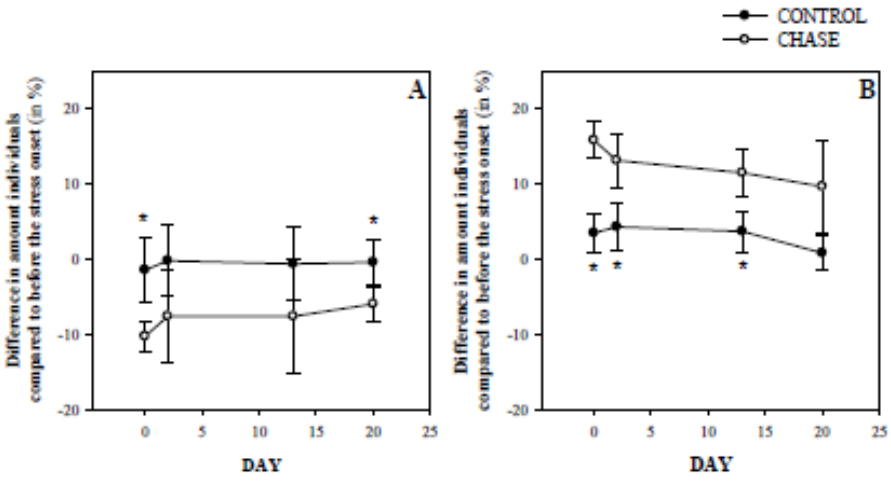


Figure 6. Stress response of GSB exposed to lowering of the water level and chasing (CHASE), measured as the mean difference between the percentage of fish present before the onset of the stress and the percentage of fish present after the onset of the stress for (A) the top right square (square 2) and (B) the bottom right square (square 4) of the morning stress moment. For comparison the "stress response" of the CONTROL treatment is plotted in the same graphs. Results are shown for day 0, 2, 13 and 20. Data points represent mean per stress moment, per day (n = 3 tanks). Error bars represent standard deviation of the mean of the model. Significant differences in means per stress moment, per day between the CHASE treatment and the CTRL treatment are indicated by *.

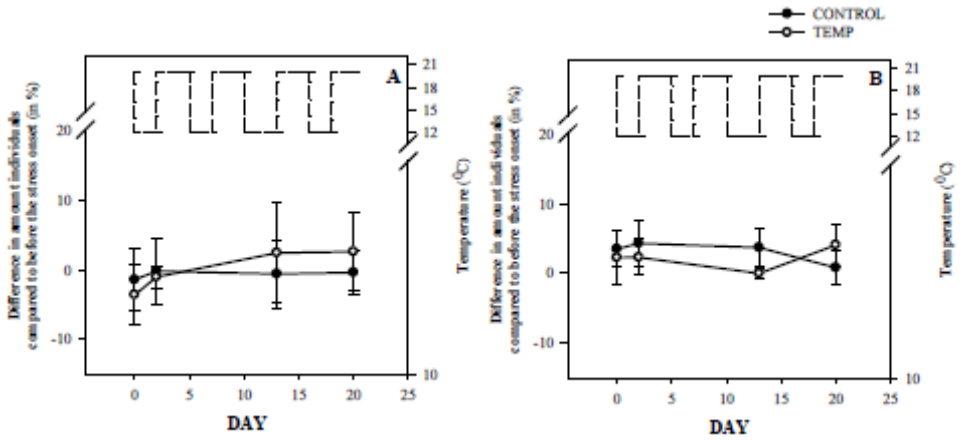


Figure 7. Stress response of GSB *aurata* exposed to TEMP stressor, measured as the mean difference between the percentage of fish present in each square before and after onset of stress. (A), top right square (square 2) and (B), bottom right square (square 4) of the camera field. Results are shown for day 0 (temperature increased during the morning), 2 (temperature increased during the morning), 13 (temperature decreased during the morning) and 20 (20 °C). Data points represent mean per stress moment, per day (n = 3 tanks). Error bars represent standard deviation of the mean of the model.

Feed behaviour: Feed behaviour was measured 1h post stress for both the AUTO and the CHASE groups. Fig. 8 shows the difference in the square directly underneath the feeder (Square 2). (No effect was observed for the other squares, data not shown). Except for a negative occurrence (-5) for the AUTO on day 1, all other data showed an increase in abundance of 10% or more following onset of feeding ($P > 0.05$).

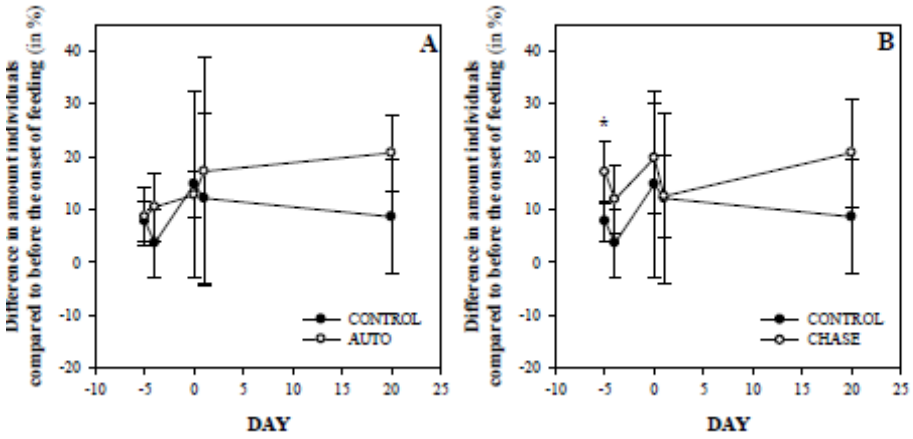


Figure 8 - Feed response of *GSB* of the (A) AUTO group and (B) CHASE group. The feeding was 1h post stress. The feed response was measured in the square directly underneath the feeder (Square 2) as the mean percentage difference of fish before and after the onset of feeding. The feed response of the CONTROL treatment is plotted in the same graphs. Results are shown for day -5, -4, 0, 1 and 20 of the feed moment during the morning (11:00h). Data points represent mean per feed moment, per day (*n* = 3 tanks). Error bars represent standard deviation of the mean of the model. Significant differences in the means per feed moment, per day between (A) the AUTO treatment and the CTRL treatment or between (B) the CHASE treatment and the CTRL treatment are indicated by *.

Feed behaviour data of the TEMP group is shown for video square 2 (underneath the feeder) and bottom left square 3. Remarkably the area just underneath the feeder had negative values on days -5, -4 and 1 (Fig. 9A). This implies that on these days, fewer fish migrated to this area during feeding than before. The area furthest away from the feeder (Square 3) had on the other hand more fish on days -5 and -4 (Fig. 9B). For both squares no significant

differences were found between the TEMP treatment and the CTRL treatment ($P > 0.05$).

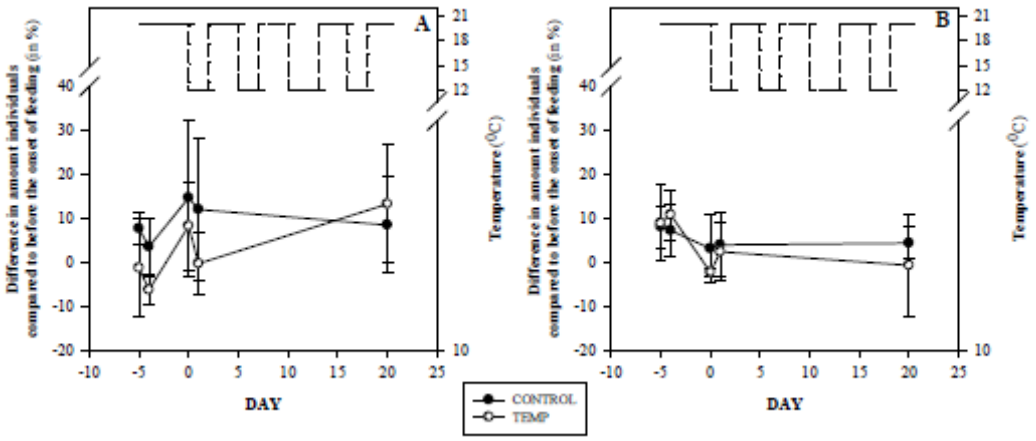


Figure 9. Feed response of GSB exposed to a temperature fluctuation (temperature was cycled from 2 days at 12 ºC and 3 days at 20 ºC) calculated for (A) Square 2 and (B) Square 3. Feed response was measured as the mean difference between the percentage of fish present before the onset of feeding and the percentage of fish present after the onset of feeding. For comparison the feed response of the CTRL treatment is plotted in the same graphs. Results are shown for day -5 (20 ºC), -4 (20 ºC), 0 (temperature decreased during the morning), 1 (12 ºC) and 20 (20 ºC) for the feed moment during the morning (11:00h). Data points represent mean per feed moment, per day (n = 3 tanks). Error bars represent standard deviation of the mean of the model.

3.1.4. Blood metabolic profiling

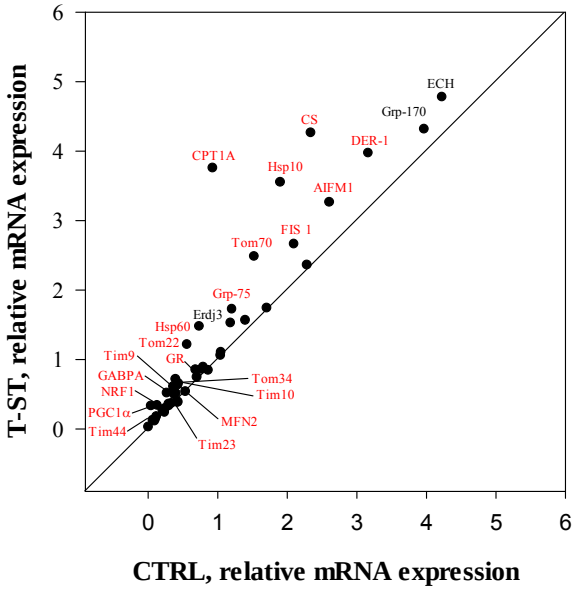
Table 3 also shows the haematological values of experimental fish. The average haematocrit value of T-ST fish was lower ($P < 0.001$) than in the other three experimental groups. A similar trend was found for the blood haemoglobin content, with the T-ST and C-ST groups becoming the two most extreme groups. No statistically significant changes were found in plasma cortisol levels, though a trend of increased cortisol titre in C-ST and M-ST fish was observed with the highest overall concentration in the latter group. No significant changes were found in plasma glucose levels regardless of the stress condition. Plasma lactate levels were statistically higher in C-ST fish than CTRL fish ($P < 0.05$). An opposite response was found in M-ST fish, and their plasma lactate values were significantly lower than CTRL and C-ST fish ($P < 0.05$). Plasma lactate levels in the T-ST group were not distinguishable from those of CTRL fish.

3.1.5. Mitochondrial gene expression profiling

The gene expression profile of liver mitochondria in response to intermittent and repetitive stress pulses is summarized in Supplementary Table 4 (Annex 1). As a general rule, repetitive thermal fluctuations triggered an up-regulated response that was statistically significant ($P < 0.05$) for one third of the genes present in the array (20 out of 60). In contrast, a slight or consistent down-regulated response affecting one or 11 genes was observed in the C-ST and M-ST groups, respectively. For a better understanding of the results, the gene expression pattern of a given group of stressed fish was plotted against the CTRL group in a scatter plot. In the T-ST group (Fig. 10), relatively low levels of expression were found for nuclear transcription factors but, at the same time, these molecular markers were strongly up-regulated with fold-change of 5.98 for the proliferator-activated receptor gamma coactivator 1 alpha (PGC1a), 2.32 for the nuclear respiratory factor 1 (NRF1) and 1.8 for the nuclear respiratory factor 2 (NRF2). Along with relatively high baseline levels of expression, carnitine palmitoyltransferase 1A (CPT1A) and citrate synthase (CS) were significantly up-regulated with fold changes of 4 and 1.8, respectively. Lower but statistically significant up-

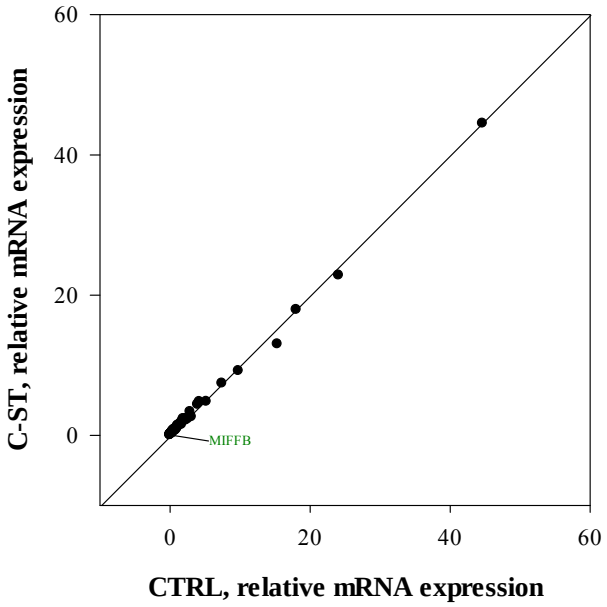
regulation (1.28) was observed for other related markers of oxidative metabolism, such as cytochrome C oxidase subunit IV isoform 1 (Cox4a). Interestingly, consistent up-regulation with fold changes ranging from 1.38 to 2.11 were also observed for most (9 out of 15) of the outer membrane translocases (TOM complex) and inner membrane translocases (TIM22 and TIM23 complexes). Mitochondrial molecular chaperones of the Hsp10, Hsp60 and Hsp70 families were also significantly up-regulated, ranging from 1.41 to 1.97. Transient fold changes (<1.45) were observed for markers of endoplasmic reticulum (ER) stress response (derlin 1, DER-1), mitochondrial dynamics (mitofusin 2, MFN2; mitochondrial fission 1 protein, FIS1), apoptosis (apoptosis-related protein 1, AIFM1) and antioxidant defence (GR).

Regarding husbandry stressors, only one gene (mitochondrial fission factor homolog B, MIFFB) of the PCR-array panel was significantly down-regulated in the C-ST group (Fig. 11). However, this down-regulated response was largely amplified in the M-ST group (Fig. 12), affecting primarily nuclear transcription factors (PGC1a, NRF1 and NRF2) and markers of oxidative metabolism (CPT1A, CS and 3-ketoacyl-CoA thiolase, ACAA2) with fold changes ranging from 0.43 to 0.52. Similarly, a down-regulated response was detected for markers of apoptosis (apoptosis regulator BAX, BAX; Bcl-2-like protein 1, BCLX), mitochondrial dynamics (MFN2; mitochondrial Rho GTPase 2 fission, MIRO2) and inner membrane translocation (mitochondrial import inner membrane translocase subunit Tim8A).



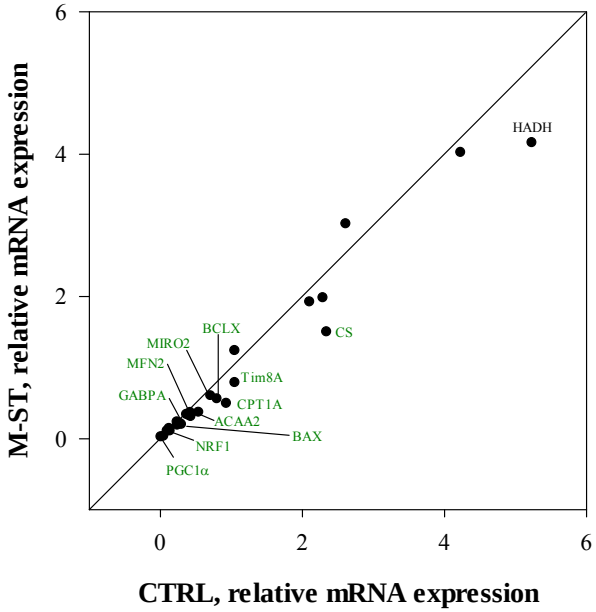
Genes	Fold change (T-ST/CTRL)
PGC1α	5.98
NRF1	2.32
NRF2	1.80
CPT1A	4.00
CS	1.81
COX4a	1.28
Tom22	2.11
Tim10	1.72
Tom70	1.61
Tim9	1.61
Tim44	1.45
Tom34	1.44
Tim23	1.38
mtHsp60	1.97
mtHsp10	1.85
mtHsp70	1.41
DER-1	1.35
MFN2	1.42
FIS1	1.32
AIFM1	1.24
GR	1.22

Figure 10. Mitochondria gene expression profile of GSB exposed to thermal fluctuations (T-ST group). Relative mRNA expression levels are plotted against the expression values from control fish (CTRL). Data (are the mean of 6–8 fish (for details see Supplementary Table 4, Annex 1). β-Actin was used as a housekeeping gene, and all data values in the scatterplot are relative to the expression level of PGC1β in CTRL fish. For differentially expressed genes, fold change calculations for a given gene were done using data from CTRL as arbitrary reference values (values >1 indicate stress up-regulated genes).



Genes	Fold change (C-ST/CTRL)
MIFFB	0.70

Figure 11. Mitochondria gene expression profile of GSB exposed to changes in water levels and chasing (C-ST group). Relative mRNA expression levels are plotted against the expression values from control fish (CTRL). Data are the mean values of 6–8 fish (for details see Supplementary Table 4, Annex 1). β-Actin was used as a housekeeping gene, and all data values in the scatterplot are relative to the expression level of PGC1β in CTRL fish. For differentially expressed genes, fold change calculations for a given gene were done using data from CTRL as arbitrary reference values (values <1 indicate stress down-regulated genes).



Genes	Fold change (M-ST/CTRL)
PGC1α	0.43
NRF1	0.71
NRF2	0.72
CPT1A	0.52
CS	0.64
ACAA2	0.66
BAX	0.62
BCLX	0.68
MFN2	0.69
MIRO2	0.83
Tim8A	0.73

Figure 12. Mitochondria gene expression profile of GSB exposed to multiple sensory perception stressors (M-ST group). Relative mRNA expression levels are plotted against the expression values from control fish (CTRL). Data are the mean values of 6–8 fish (for details see Supplementary Table 4, Annex 1). β-Actin was used as a housekeeping gene, and all data values in the scatterplot are relative to the expression level of PGC1β in CTRL fish. For differentially expressed genes, fold change calculations for a given gene were done using data from CTRL as arbitrary reference values (values <1 indicate stress down-regulated genes)

As a corollary of the mitochondria stress profiles, the fold changes of differentially expressed genes in at least one of the three stress conditions were compiled and represented in Fig. 13. The intensity of red (up-regulated genes) and green (down-regulated genes) colours indicates the magnitude of the change.

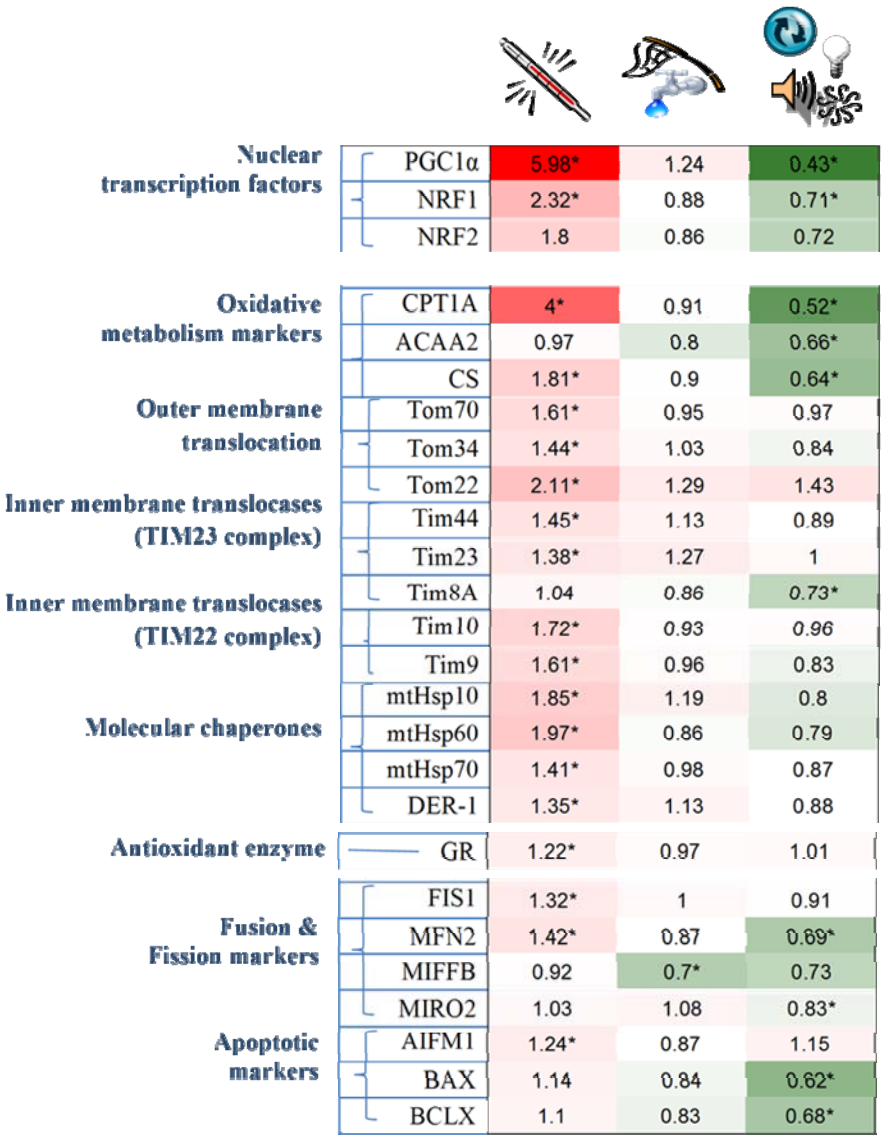


Figure 13. Corollary of mitochondria gene expression profiles of differentially expressed genes. GSB were exposed to intermittent and repetitive natural and husbandry stress stimuli: thermal stress (T-ST), chasing stress (C-ST) and multiple sensory perceptions stress (M-ST). Fold changes are relative to the control group (CTRL). Red tones correspond to up-regulated genes and green tones correspond to down-regulation. The intensity of the colour represents the degree of change. Statistically significant differences between CTRL and stressed groups are indicated ($^{*}P < 0.05$; Student t-test).

3.1.6. Mapping the skin mucus proteome

Proteinpilot comparison against Expasy protein database resulted in the identification of 1,595 MS/MS spectra when the protein score filter was set up at 1.3. Instead, the use of the GSB database allowed the identification of 2,466 spectra with a much higher protein score (>20.0). This number is reduced to 2,062 when a protein score filter higher than 30.0 was applied. Among them, 1,848 different proteins (89.6 %) were eligible for pathway analysis in IPA. These proteins were represented in 418 IPA canonical pathways mapped from a total of 644. To identify relevant pathways and processes for proteins present in skin mucus, an

overlapping analysis was made in IPA with a filter of three or more common proteins among related pathways. From this analysis, seventeen canonical pathways with *P*-values lower than 1E-08 were found to be associated in three distinct nodes (Fig. 14).

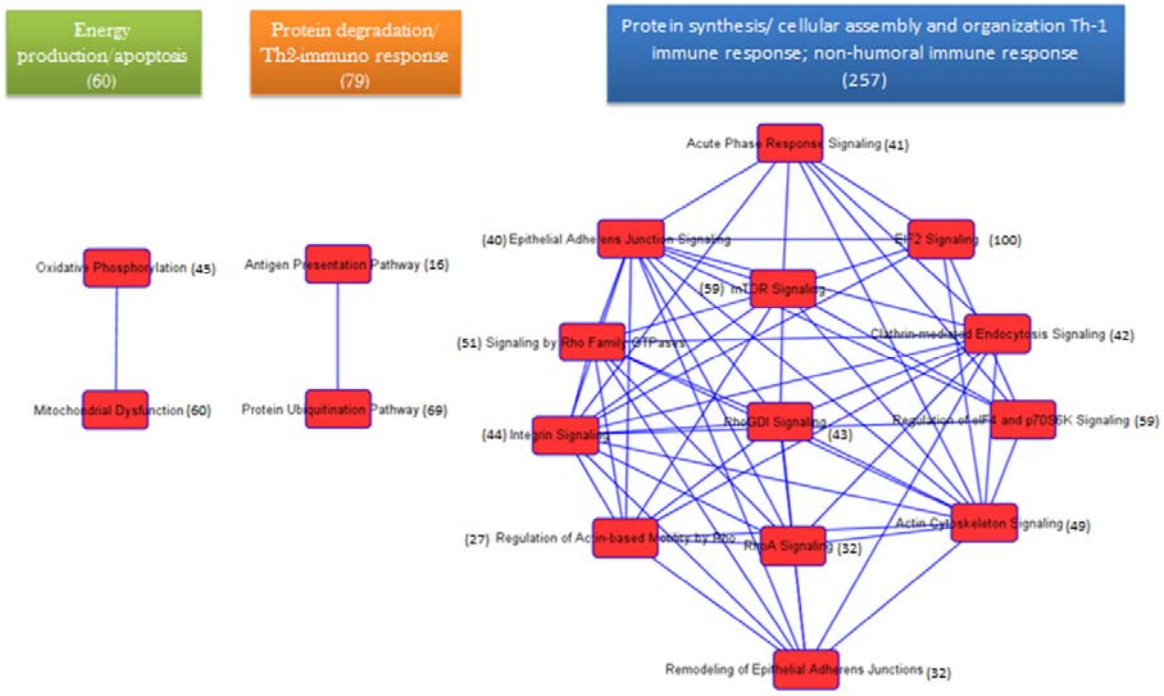


Figure 14. Schematic representation of the three main nodes of overlapping pathways of proteins detected in the skin mucus of gilthead sea bream. Each canonical pathway is represented in a box, and numbers in parenthesis indicate the number of detected proteins. Connection lines between two boxes indicate that they share three or more common proteins.

The first node was composed of 60 proteins comprising the pathways “oxidative phosphorylation” and “mitochondrial dysfunction” with a vast majority (58 out of 60) of cytoplasmic proteins, mainly mitochondrial, involved in processes of energy production and apoptosis (data not shown due to the vast extension). The second node comprised 79 proteins involved in processes of Th2 immune system (“antigen presentation pathway”) or protein degradation (“protein ubiquitination pathway”), that are mainly present in the cell cytoplasm (64.5 %), nucleus (19.0 %) and plasma membrane (11.4 %) (data not shown due to the vast extension). The third node was the most populated one, with 257 proteins in 13 interconnected canonical pathways (data not shown due to its vast extension). Many of them are involved in protein synthesis (“EIF2 signalling”, “mTOR signalling”…) and the maintenance of epithelium integrity (“remodelling of epithelial adherens junctions”, “regulation of actin-based motility by Rho”, “epithelial adherens junction signalling”, etc.). It is also noteworthy the presence of 41 identified proteins that are part of the non-humoral immune response (“acute response signalling” canonical pathway), with 22 of them characteristically located in the extracellular space. As supplemental data, graphical representations of a canonical pathway for each of the three identified nodes are provided (Supplementary Fig. 1 to 3, Annex 1).

3.1.7. Stress effects on the skin mucus proteome

A representative image of a two-dimensional gel electrophoresis analysis of the mucus proteins profile from CTRL *vs* M-ST fish is shown in Fig. 15. Each protein spot was assigned

an average ratio (i.e. change in expression level due to M-ST treatment) and *P*-value (t-student) to indicate the level of significance. The abundance of six spots was significantly different (*P*-value < 0.03) in stressed fish. Among these protein spots, three were up-regulated (fold-change 1.6-2.7) and three were down-regulated (0.6-0.7) by the multiple sensorial stress treatment. All six proteins spots were unequivocally identified by comparison of the LC-MS/MS data with the GSB transcriptome database (Table 5). Down-regulated spots were identified as elongation factor 2 (spot 743) and cytoplasmic actin (spots 1549 and 1816). Spot 2181 (fold-change 1.64) was identified as the mitochondrial protein cytochrome c1 heme. The two most up-regulated spots were both recognized as keratin type II cytoskeletal 8.

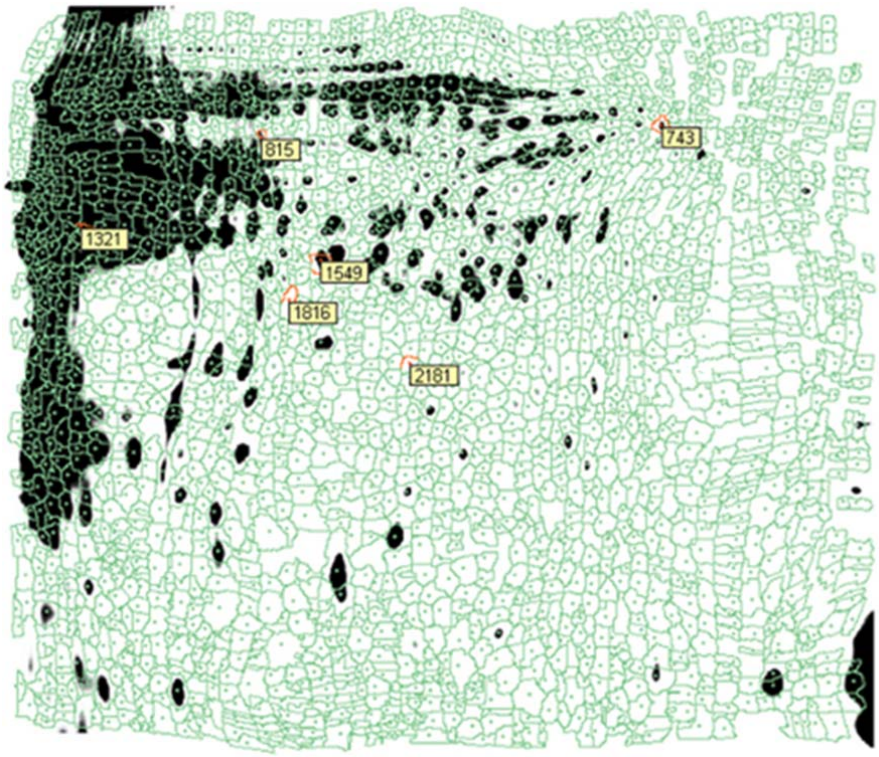


Figure 15. Digital image of a representative two-dimensional gel electrophoresis analysis of the different proteins found in the skin mucus of gilthead sea bream of M-ST *vs* CTRL fish.

Table 5. Protein spots identified as differentially expressed in gilthead sea bream skin mucus after multiple sensorial stress (M-ST).

Spot number	Clone accession	Protein name	*P*-value	Average ratio (M-ST *vs* CTRL)
743	C2_534	Elongation factor 2	0.026	0.71
815	C2_1442	Keratin type II cytoskeletal 8	0.014	2.71
1321	C2_1442	Keratin type II cytoskeletal 8	0.024	1.78
1549	C2_2	Actin, cytoplasmic 1	0.019	0.71
1816	C2_2	Actin, cytoplasmic 1	0.026	0.63
218	C2_785	Cytochrome c1, heme protein mitochondrial	0.018	1.64

3.2. Atlantic salmon (IMR, UoS)

3.2.1. Growth

Final weight, k factor and SGR are given in Fig. 16A and B. Over the three weeks of the trial, the fish grew an average of 200 g. The control and the AUTO group showed similar weight development and SGRs of 0.69 and 0.70 respectively. The CHASE group had a significantly lower growth development and also a tendency to a lower k-factor.

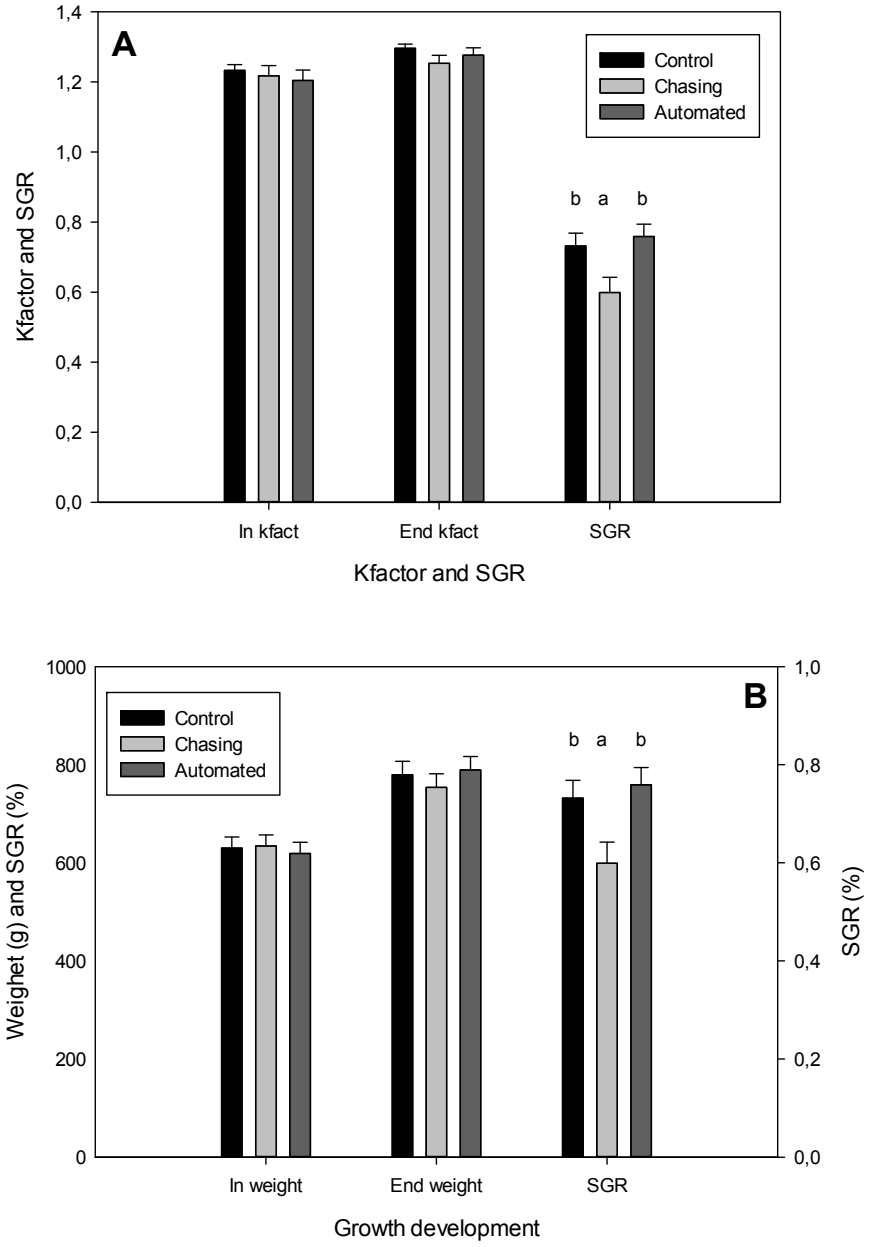


Figure 16. Growth development of Atlantic salmon subjected to CHASE and AUTO stress over 21 days. Data within each group not sharing the same letter are significantly different at $P< 0.05$.

3.2.2. Feed intake

Fig. 17 shows the daily feed intake from 3 days before the stress trial and towards the end using samples over 3 days per week. Stress significantly reduced feed intake over the first 2 weeks of trial. However, a significant recovery was seen in fish in the AUTO group with clear indications of a compensatory increased feed intake over the last week. The feed intake in the CHASE was also fully recovered by the last week, but there were no clear indications of compensatory increase in feed intake.

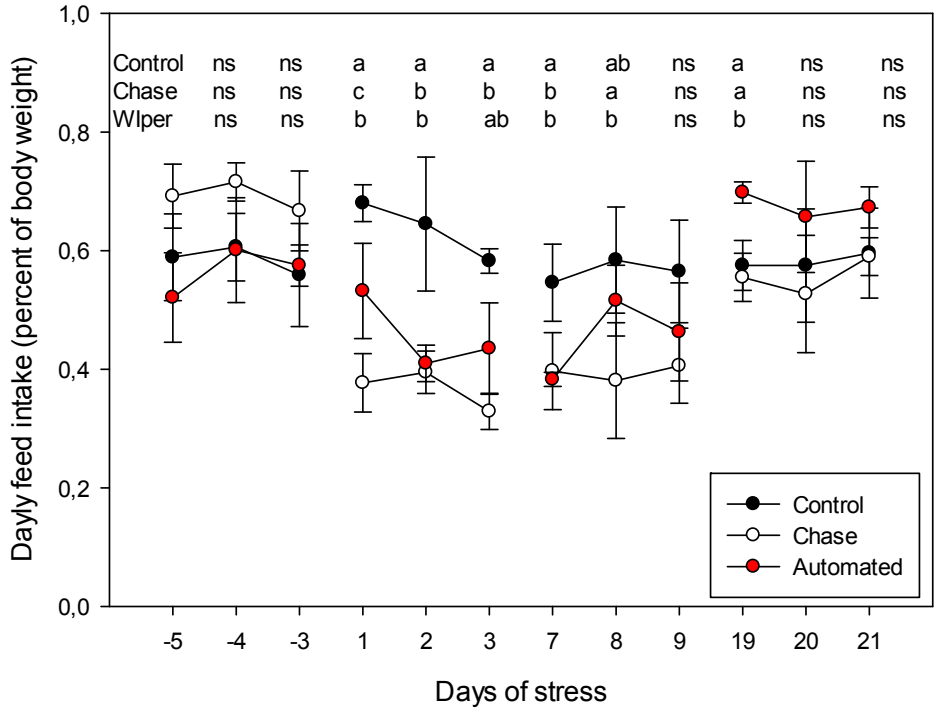


Figure 17. Feed intake (percent of body weight) in Atlantic salmon subjected to chronic stressors. Surplus feed was collected 3 days prior to start of the experiment, then 3 days every week until conclusion of the trial. For each day, groups not sharing a common letter are significantly different.

3.2.3. Oxygen consumption

As the application of stressors occurred during daytime, we were not able to obtain good oxygen consumption data. It was therefore decided to use logging data from the nigh period that was outside both of feeding and stress. There were no differences between control and AUTO groups. Accordingly, Fig. 18 only compares the oxygen consumption of the CHASE and control groups. Throughout the trial, the oxygen consumption was reduced during the night period in the CHASE group compared to the control group. Note that the oxygen consumption did not appear to recover despite the fact that appetite did.

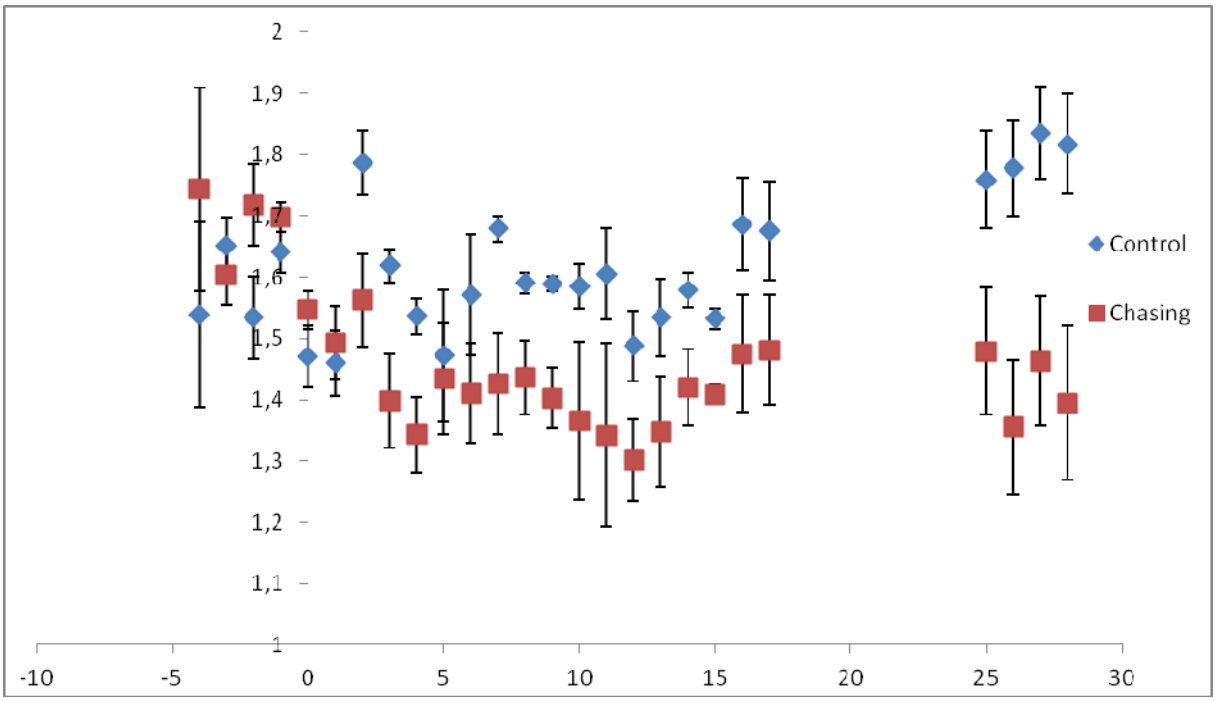
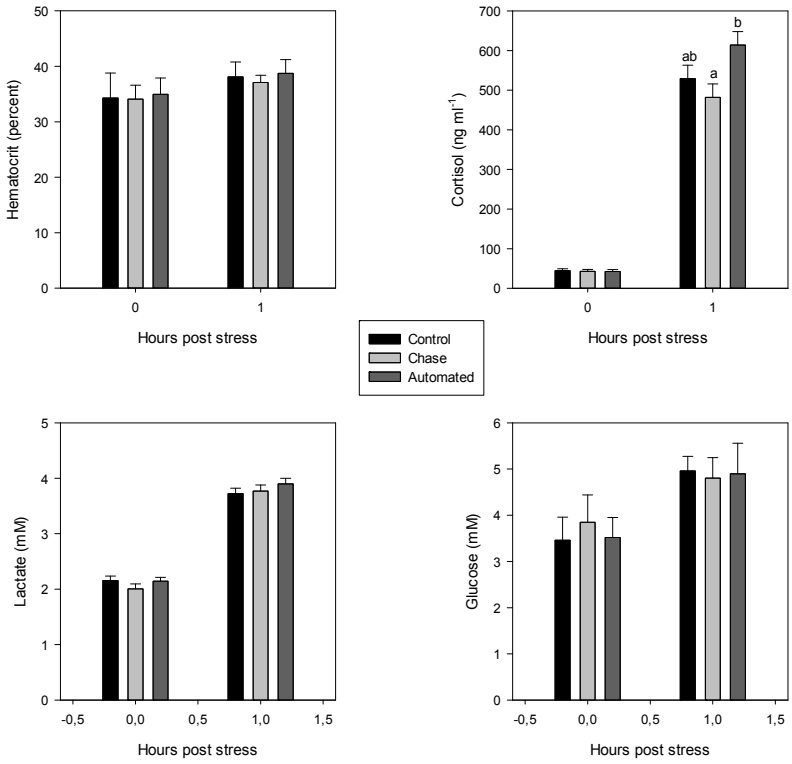


Figure 18. Oxygen consumption in (mg/kg/min) during night time in control group of Atlantic salmon and fish subjected to CHASE stress over 21 days.

3.2.4. Clinical chemistry

The clinical test data before and after 1h of stress at termination of study, is given in Fig. 19 and 20.



Figures 19. Clinical chemistry (Hc, cortisol, lactate, glucose) data from Atlantic salmon from control, AUTO and CHASE groups before and after being subjected to acute stress.

Previous treatment had no significant effect on any parameter tested. Subjecting the fish to acute stress increased haematocrit, lactate, glucose, osmolality and the main ions sodium and chloride 1h post stress while potassium was reduced. There were no treatment effects on the responses. Plasma cortisol increased to more than 450 ng/ml, with the lowest response in the CHASE group being significantly lower than the AUTO group. However, neither CHASE nor AUTO groups showed cortisol levels significantly different from control.

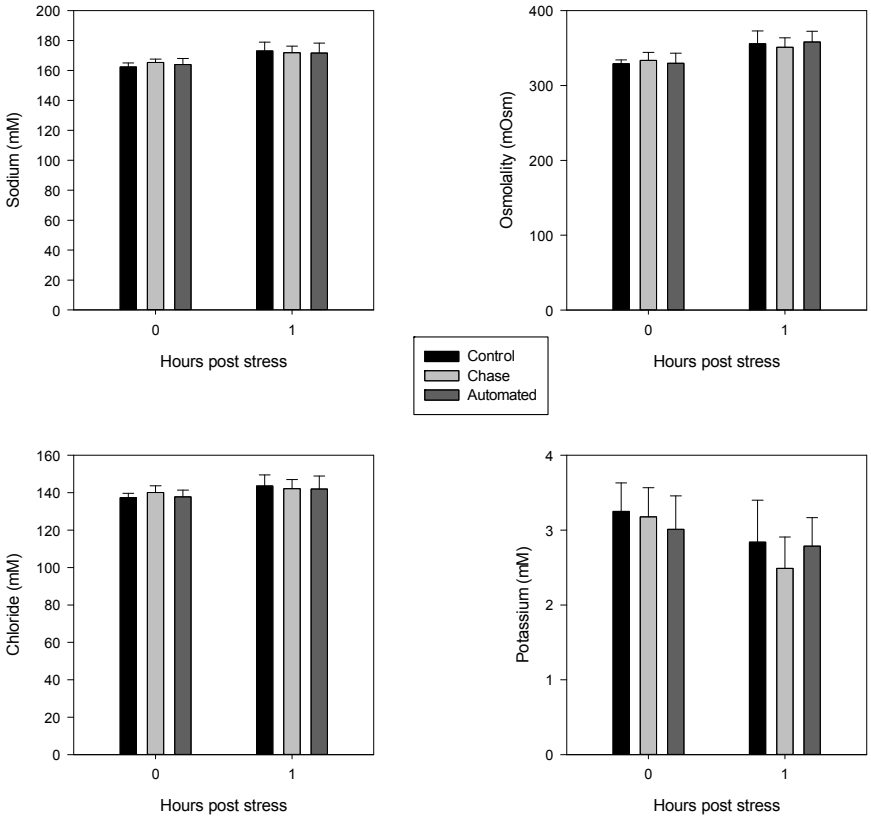


Figure 20. Clinical chemistry (Na, K, Cl^-, osmolality) data from Atlantic salmon from control, AUTO and CHASE groups before and after being subjected to acute stress.

3.2.5. Transcriptomic analysis

When using a stringent *P* value (Benjamini-Hochberg corrected *P* <0.01), analysis of transcriptomic data indicated that no genes showed significant differential expression in CHASE and AUTO stressed fish. This result is in agreement with that observed in trout exposed to chronic poor water quality stress (see below). However, when using non-corrected p value (p<0.01), 106 and 148 genes were differentially expressed in CHASE and AUTO stressed fish, respectively, compared with control. In that context, we still decided all the same to carry out a more refined analysis of these gene lists, albeit that such information are only indicative and any hypothesis should be validated by qPCR measurements. Thus, a Venn diagram analysis indicated that a total of 14 genes were affected in both treatments

and of these 6 were up-regulated and 8 were down-regulated (Fig. 21). The genes commonly affected in both treatments are shown in Table 6.

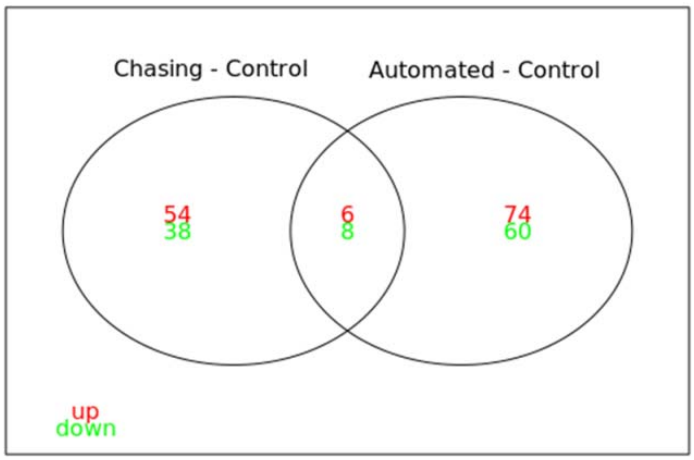


Figure 21. Venn diagram showing the relation between differentially expressed genes (*P* < 0.01). Numbers in green indicate down-regulated genes, in red up-regulated genes.

Table 6. Genes affected (*P* < 0.01) in Atlantic salmon under CHASE and AUTO stressors compared with control fish. Only genes that were changing in the same direction (i.e. both up-regulated or down-regulated) are displayed.

		Chasing		*Automated*	
KEGG ID	**KEGG Definition**	**LogFC**	**P Value**	**LogFC**	**P Value**
K00710	polypeptide N-acetylgalactosaminyltransferase	0,55	0,000	0,51	0,000
K00944	nucleoside-triphosphate--adenylate kinase	0,29	0,001	0,25	0,005
K01367	calpain-1	-0,21	0,007	-0,23	0,003
K02896	large subunit ribosomal protein L24e	-0,18	0,008	-0,23	0,001
K03231	elongation factor 1-alpha	-0,22	0,001	-0,18	0,003
K04439	beta-arrestin	0,29	0,004	0,26	0,008
K05095	fibroblast growth factor receptor 4	-0,35	0,002	-0,29	0,007
K05148	tumor necrosis factor receptor superfamily member 11B	-0,27	0,003	-0,29	0,002
K05853	Ca2+ transporting ATPase, sarcoplasmic/endoplasmic reticulum	0,24	0,004	0,27	0,002
K06087	claudin	-0,52	0,007	-0,69	0,001
K06556	antigen identified by monoclonal antibody MRC OX-2	0,25	0,001	0,21	0,004
K10083	lectin, mannose-binding 2-like	0,25	0,004	0,22	0,008
K11322	enhancer of polycomb-like protein	-0,32	0,000	-0,22	0,010
K16533	centrosomal protein CEP290	-0,33	0,001	-0,29	0,003

In addition, we carried out a gene-set enrichment analysis using the package GAGE (Luo *et al.*, 2009). This GAGE analysis revealed the presence of coordinated changes of gene expression. In particular, a set of genes involved in the regulation of the phagosome was affected in CHASE fish (q < 0.1) resulting in significant results in both *1d* and *2d* tests. This gene set was also affected in fish exposed to the AUTO stressor. However, it was also evident that the genes regulating this process in fish that were subjected to CHASE stressor were not necessarily the same genes as affected by fish subjected to AUTO stressor (Fig. 22).

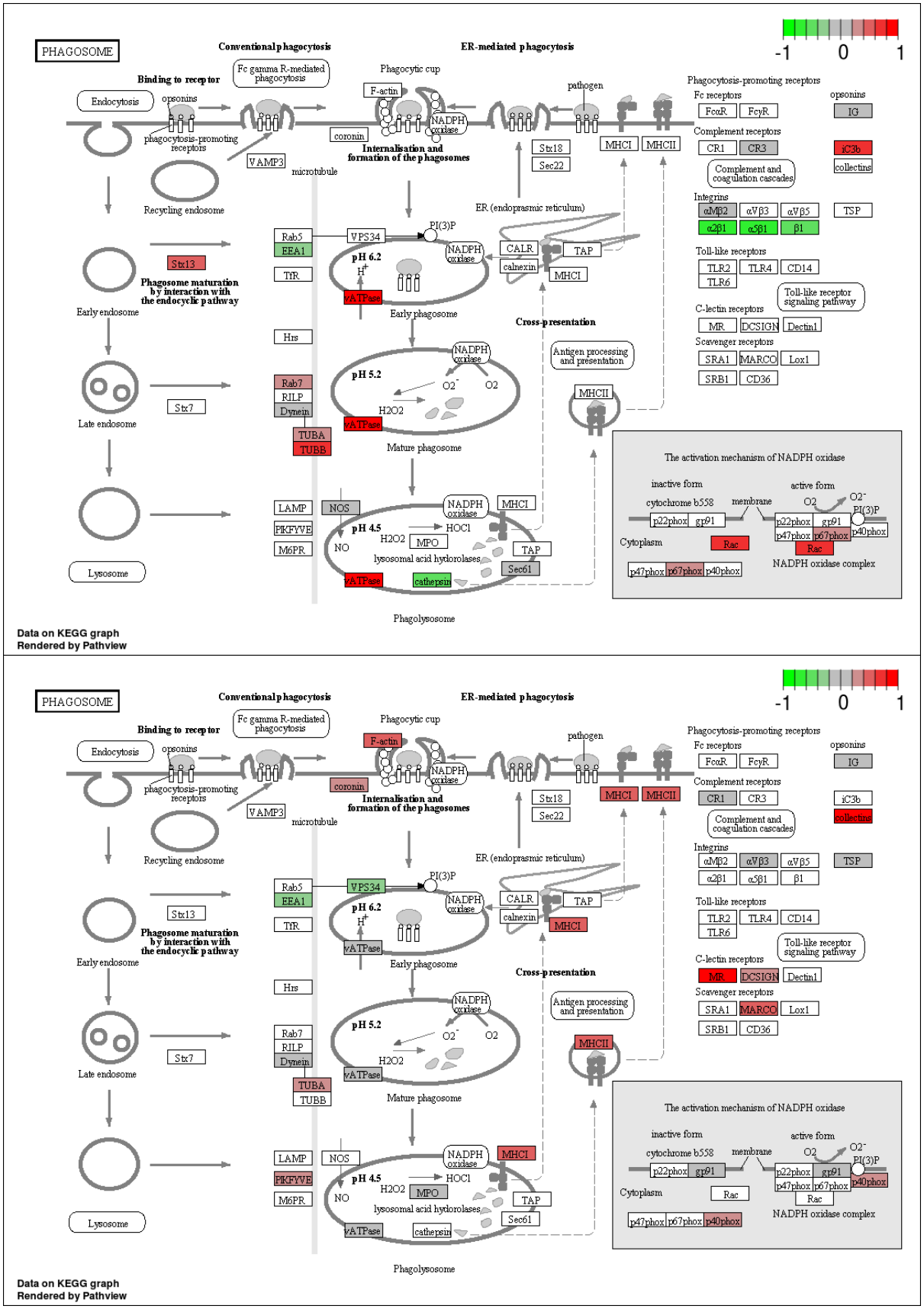


Figure 22. Comparison of the genes regulating the phagosome gene set (ko04145) in Atlantic salmon exposed to CHASE (panel in upper position) and AUTO (panel in lower position) stressors. Figures were generated using the R package *pathview* (Luo and Brouwer, 2013).

The GAGE analysis also indicated gene sets involved in other biological functions such as oxidative phosphorylation, GABAergic synapse, synaptic vesicle cycle, retrograde endocannabinoid signalling and circadian entrainment that tested significant in the *1d* test. A further two gene sets tested significantly affected in the 2d test and included neuroactive ligand-receptor interaction and phagosome.

Table 7. Gene set significantly affected ($q < 0.1$) in Atlantic salmon exposed to the automated stressor compared with control.

Gene Set	Stat mean	p value	q value	Set size	Test
ko00190 Oxidative phosphorylation	2.51	0.000	0.000	107	*1d*
ko04727 GABAergic synapse	1.45	0.000	0.023	48	*1d*
ko04721 Synaptic vesicle cycle	1.37	0.001	0.043	38	*1d*
ko04723 Retrograde endocannabinoid signaling	1.30	0.001	0.043	58	*1d*
ko04713 Circadian entrainment	1.25	0.001	0.046	57	*1d*
ko00190 Oxidative phosphorylation	1.37	0.001	0.039	107	*2d*
ko04080 Neuroactive ligand-receptor interaction	1.34	0.001	0.039	160	*2d*
ko04721 Synaptic vesicle cycle	1.37	0.001	0.039	38	*2d*
ko04145 Phagosome	1.26	0.001	0.051	79	*2d*

3.3. Rainbow trout (INRA)

3.3.1. Trial 1

Plasma cortisol levels after 1 h of ACTH injection were measured in the 3 experimental groups (Fig. 23) and they differed significantly from those in saline injected fish. However, within ACTH injected animals, there were no significant differences between control fish and fish exposed to a mixture of acute stressors, which suggests that fish were not in a chronic stress status. By contrast, fish exposed to chronic confinement stress had a significantly higher level of cortisol than the two other groups.

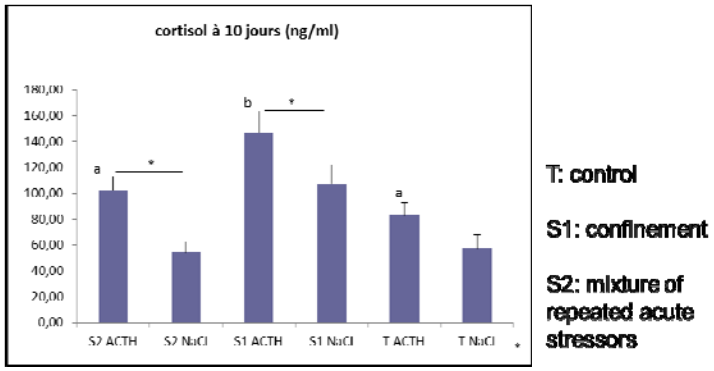


Figure 23. Plasma cortisol levels in rainbow trout 1h after acute injection of ACTH or saline solution. Fish were exposed to control conditions (T), high density confinement (S1) (200 kg/m3) or a mixture of repeated acute stressors (S2) applied daily during 10 days.

Therefore, exposure to a mixture of repeated acute stressors is not adequate to induce

chronic stress in the rainbow trout line used in this trial. As this is a domesticated trout line we hypothesized that fish get quickly adapted/used to the applied acute stressors. Thus, another trial (2) was performed with another chronic stress model.

3.3.2. Water quality and growth performance (trial 2)

Water renewal decrease in the tanks from 3 to 0.8-1 renewal/h induced an important decrease in oxygen (Fig. 24, red line). Oxygen levels ranged between 8-11mg/l and 3-6 mg/l in CTRL and S, respectively. During the day, 3 acute and abrupt drops in oxygen levels CTRL and S tanks were observed, which correlated with the 3 feeding times per day, due to the higher oxygen consummation during food ingestion and digestion. Two others peaks were also observed after on and off of lights, which must correspond to a higher fish oxygen demand induced by light stress situation. During the night, oxygen level increased significantly in S.

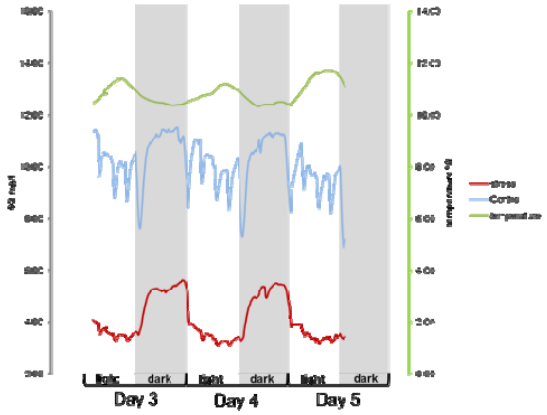


Figure 24. Example of the evolution of oxygen in control (CTRL) (blue line), stress (S) (red line) and temperature (green line) in tanks used in rainbow trout trial 2.

Ammonia concentration was around 0.3 and 0.6 mg/l in CTRL and S tanks, respectively. Mean temperature levels increased from 10.5 to 12.5 °C during the 3 weeks of the experiment. At the end of the trial, growth was significantly higher in CTRL fish (180.5 g ± 6.1, n = 27) than in S fish (152.5 g ± 3.8, n = 30), $P < 0.01$.

3.3.3. Behavioural studies (trial 2)

Focal observations: Stereotypies were more frequent in the CTRL than in S group at all the observation days except D-1 and D11 ($P < 0.05$) (Fig. 25, left). Within S group, the observations at D-1 differed significantly from all subsequent days ($P < 0.05$). Within CTRL group, D1 observations differed significantly from all subsequent days (except D16) ($P < 0.05$). All activity-related behaviours (stereotypies, jumps, aggression, accelerations) had the same profile: higher frequency in CTRL than in S group and decreasing with time from D-1 in S and from D1 in CTRL. Time spent immobile was higher in S than in CTRL group at D11, D16 and D18 ($P < 0.05$, Fig. 1, right) and increasing from D1 in S group ($P < 0.05$) (Fig. 3, right).

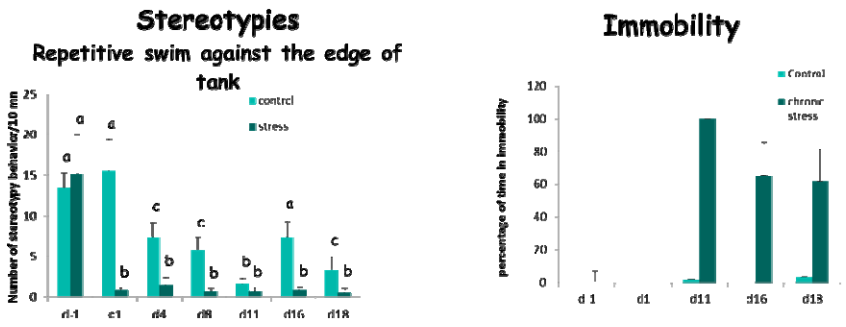


Figure 25. Focal observations of control and stressed rainbow trout along the different sampling points in trial 2. Bars with no letters in common are significantly different ($P < 0.05$).

Emotional reactivity: No significant difference was observed between the two groups in any behavioural parameters recorded during the emotional reactivity tests (Fig. 26).

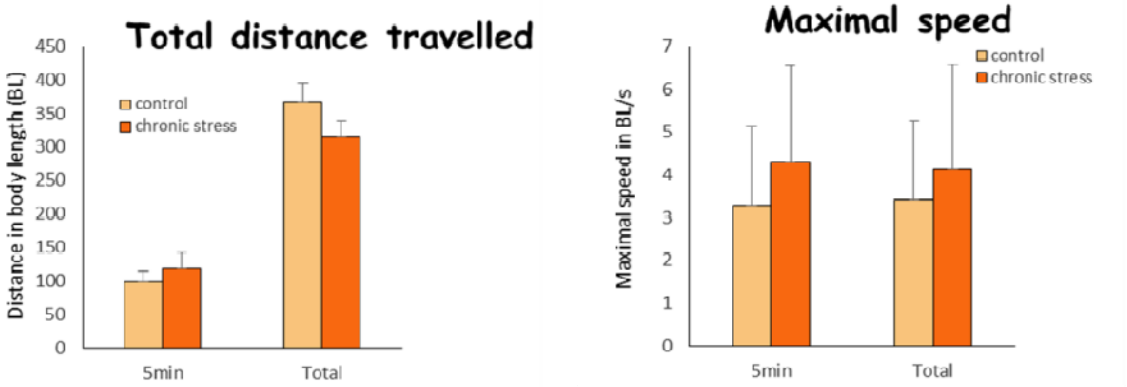


Figure 26. Emotional reactivity of control and stressed rainbow trout in trial 2.

Learning performance: At the 3rd day of test, CTRL fish had a higher learning index that S fish when the conditioned stimulus (CS) occurred ($P < 0.05$), even in the absence of food. When the food (unconditioned stimulus, US) was given at 120s, fish from both groups were all grouped in the feeder area (Fig. 27).

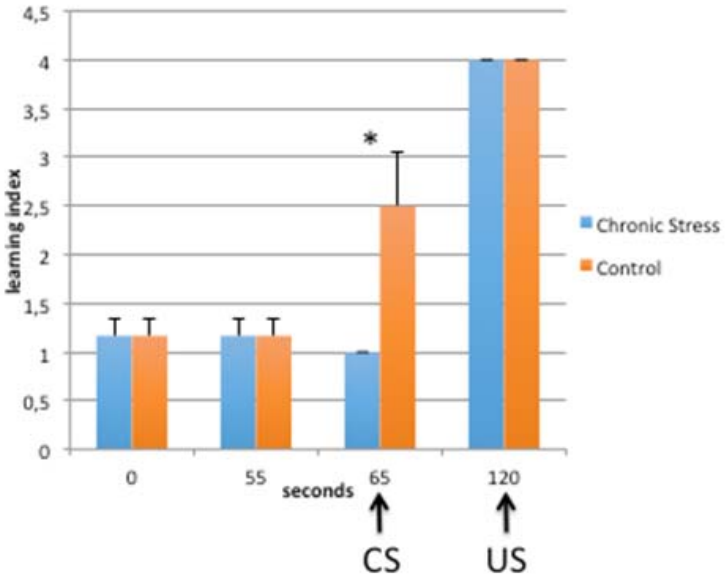


Figure 27. Learning performance of rainbow trout 7 days after the end of the trial 2. CS= conditioned

stimulus, U = unconditioned stimulus.* $P < 0.05$.

3.3.4. HPI axis responsiveness (trial 2)

Acute stress (confinement, 2 min) after the 21 days in poor water quality conditions (S), led to a significant increase in plasma cortisol both at 1h and 3h. Interestingly, this increase was significantly higher in S than in CTRL (Fig. 28). Such deregulation of the HPI axis responsiveness to an acute stress indicated us that low water quality exposure led to a chronic stress status.

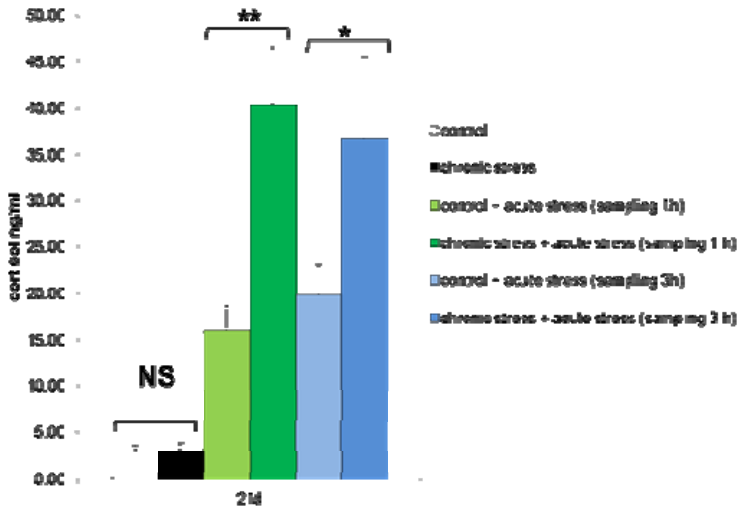


Figure 28. Plasmatic cortisol levels (mean ± SEM of 8-10 fish/group). * $P < 0.05$ and ** $P < 0.01$ statistical significance with Mann-Whitney test. NS = non-significant.

3.3.5. Ions and osmolality (trial 2)

Chronic exposure to poor-quality water did not modify plasma ionic homeostasis: chloride, sodium and calcium concentrations were similar in CTRL and S fish in most sampling points. A higher concentration of calcium was only measured at D2 in S fish (Fig. 29). Plasma osmolality measured at the end of the trial (D21) did not differed between CTRL (307.9 ± 2.3, n = 9) and CTRL (308.2 ± 0.9, n = 10) groups.

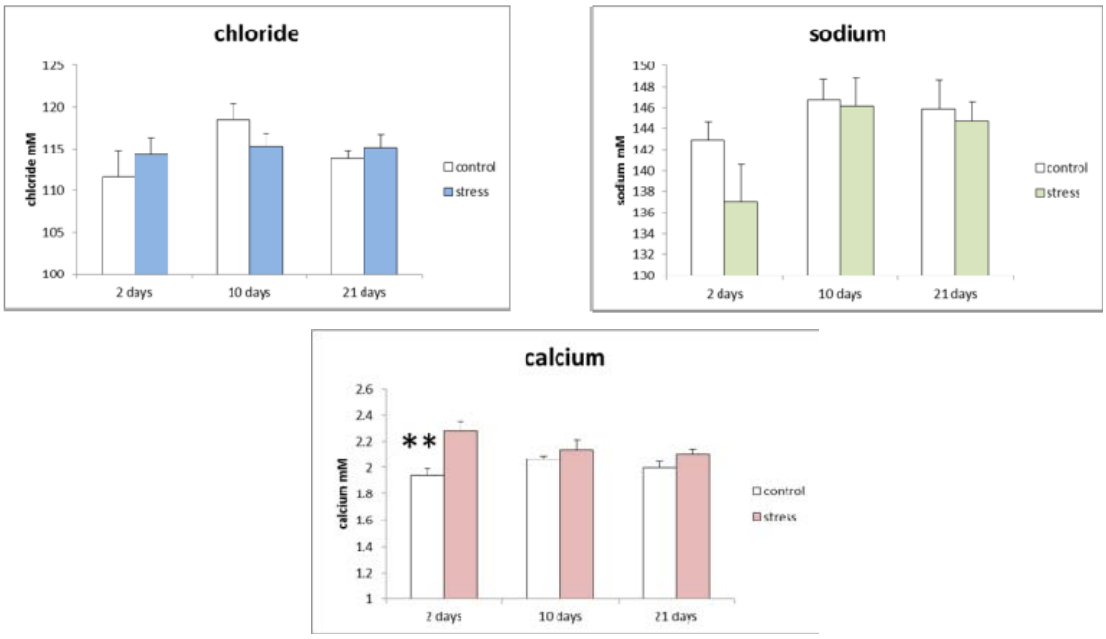


Figure 29. Plasmatic ions concentration of rainbow trout during trial 2. Values are mean ± SEM of 8-10 fishes. Mann-Whitney test was used for statistical analysis, ** *P* <0.01.

3.3.6. Gene expression analysis (trial 2)

The microarray analysis of the **pituitary and head kidney** samples taken at D21 showed unexpectedly that no gene was differentially expressed in the head kidney (corrected p value = 0.05), whereas only 2 genes were in the pituitary gland (corrected p value<0.05). Thus, it was not possible to draw any conclusion from these data.

Therefore, the expression of some selected candidate genes, known to be the main actors of the HPI axis in the **brain,** which included forebrain (telencephalon, preoptic area, and hypothalamus) and midbrain, such as CRF, CRF-BP, corticosteroid receptors, AVT and urotensin 1 was measured by RT-PCR. Only corticosteroid receptors (GR1, GR2 and MR) show a tendency to be higher ($p < 0.1$) in the S group (Fig. 30), whereas no differences were detected in any corticosteroid receptors in the pituitary gland (Fig. 31). By contrast, two major genes involved in cortisol synthesis, StAR and P450, were significantly up-regulated in the S head kidney samples at D21 (Fig. 32).

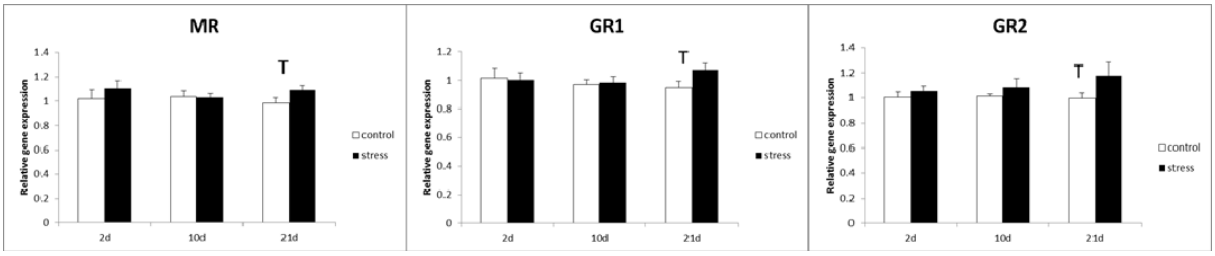


Figure 30. Brain gene expression of rainbow trout in trial 2.Values are mean ± SEM of 8-10 fishes. T is a tendency *(P* = 0.07-0.1).

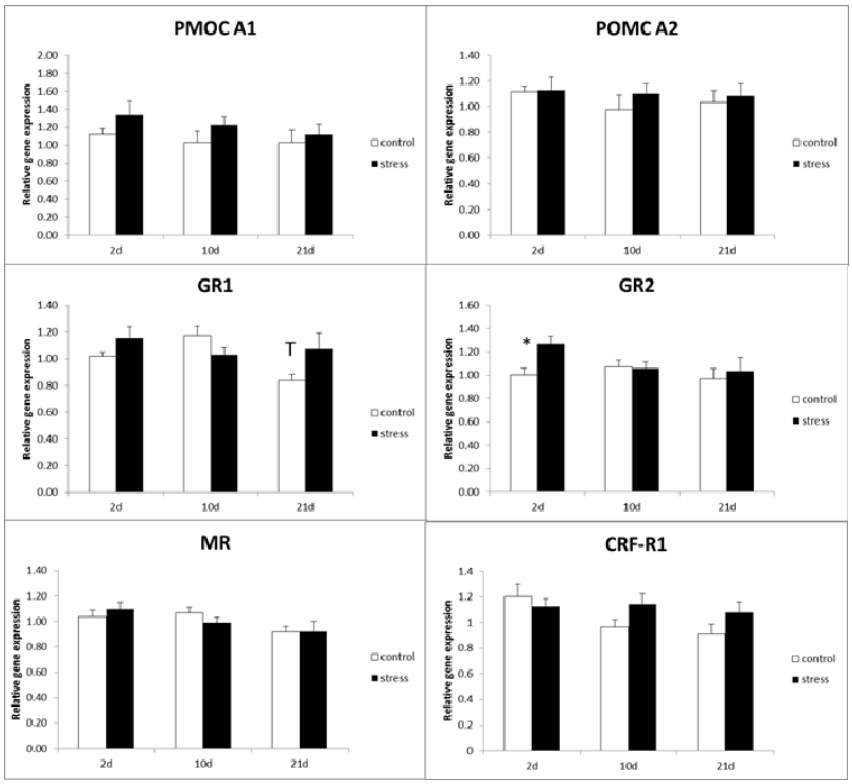


Figure 31. Pituitary gene expression of corticosteroid receptors of rainbow trout in trial 2. Values are mean ± SEM of 8-10 fishes. T is a tendency (*P* = 0.06-0.1)

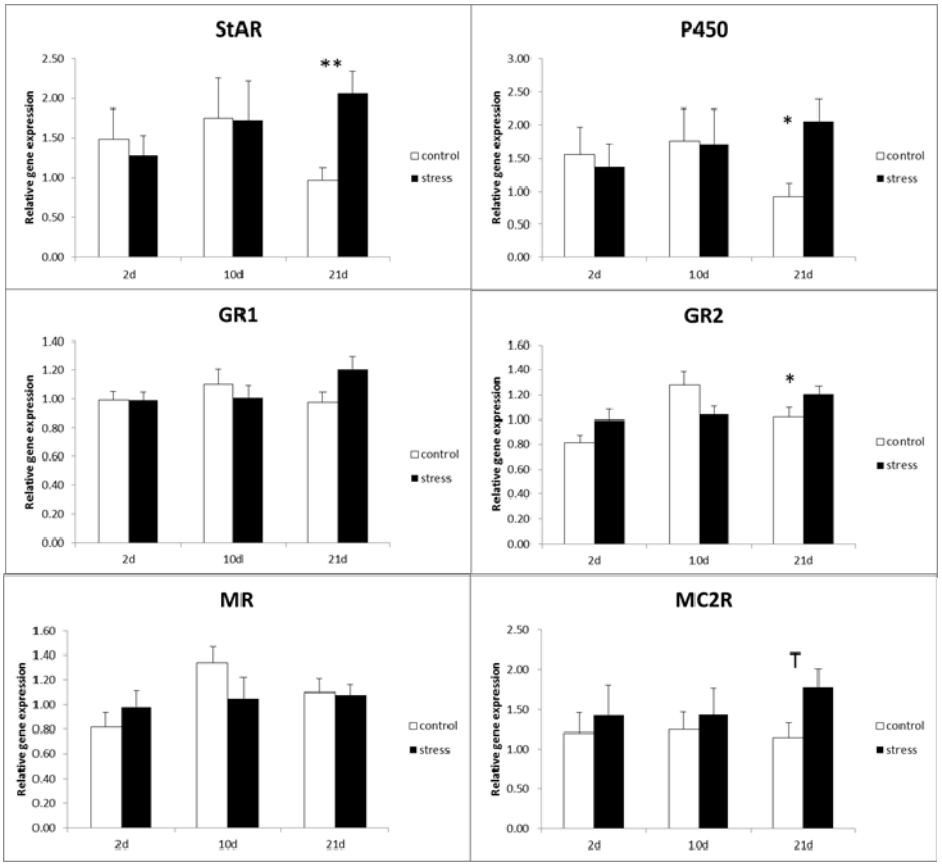


Figure 32. Head kidney gene expression of corticosteroid receptors at D21. Values are mean ± SEM of 8-10 fishes. Mann-Whitney test was used for statistical analysis. T is a tendency (*P* = 0.08-0.1), * *P* < 0.05, ** < 0.01.

The gene expression of **gills** was first studied focusing on several selected candidate genes that could be involved the adaptation of the gill epithelium to poor water conditions (low oxygen and high ammonia), such as apoptosis or cell proliferation. The gene expression of the anti-apoptotic protein bcl2 decreased progressively during the experimental period in S fish, until being significantly different from that in CTRL fish at D21 (Fig. 33). The expression of the hypoxia inducible factor (HIF-1α) gene was significantly increased in S fish at D21 also (Fig. 33) as expected, since this is an important gene induced when fish are exposed to low oxygen levels. Several genes related to ammonia transporters (rhbg and rhcg2) were down-regulated in S fish, but the differences were not statistically significant in comparison with CTRL (Fig. 33).

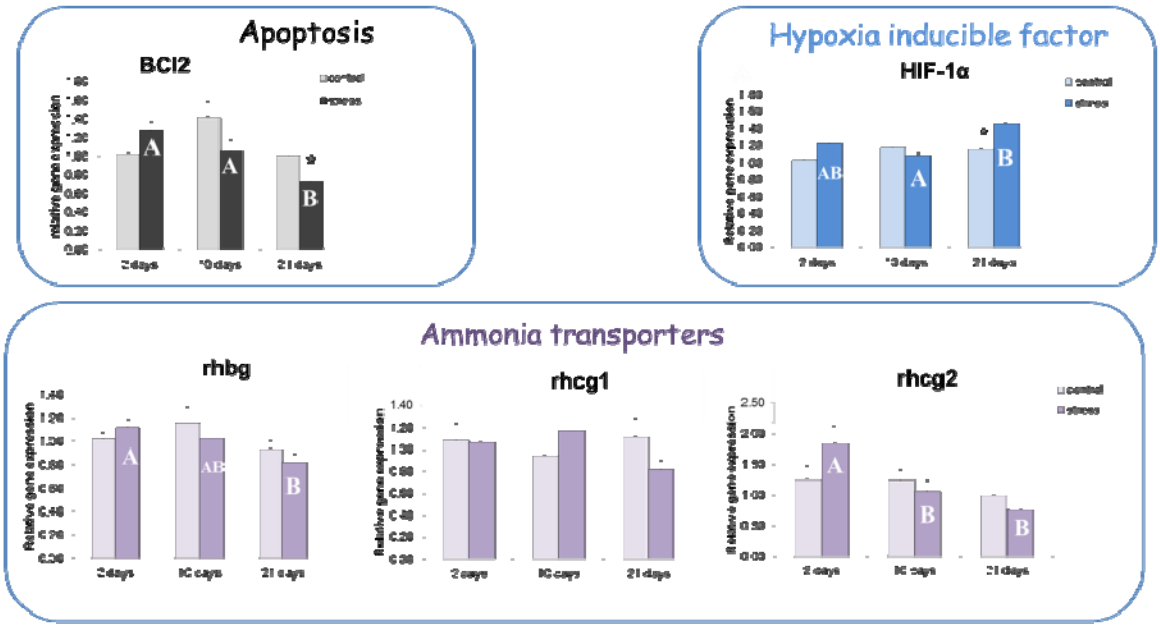


Figure 33. Time-lapse gene expression of some candidate gene in gills during trial 2. Values are mean ± SEM of 8-10 fishes. Mann-Whitney and Kruskall-Wallis tests were used for statistical analysis. * $P < 0.05$.

Since, most of the observed changes were observed in the last sampling point, gills samples were studied more in depth by microarray analysis only at D21. A t-test with FDR 0.05 allowed to identify 1668 oligos differentially expressed between CTRL (n = 8) and S (n = 8) trout: 1098 down-regulated and 570 up-regulated in S fish. When selecting oligos with a fold change higher than 2, then only 245 oligos appeared differentially expressed: 112 down-regulated and 133 up-regulated in S fish. Gene ontology grouping showed that down-regulated genes were involved cytoskeleton organization, muscle contraction and lactate dehydrogenase activity. By contrast, up- regulated genes played roles in immune and inflammatory responses, regulation of oxygen and ROS and cell proliferation (Fig. 34).

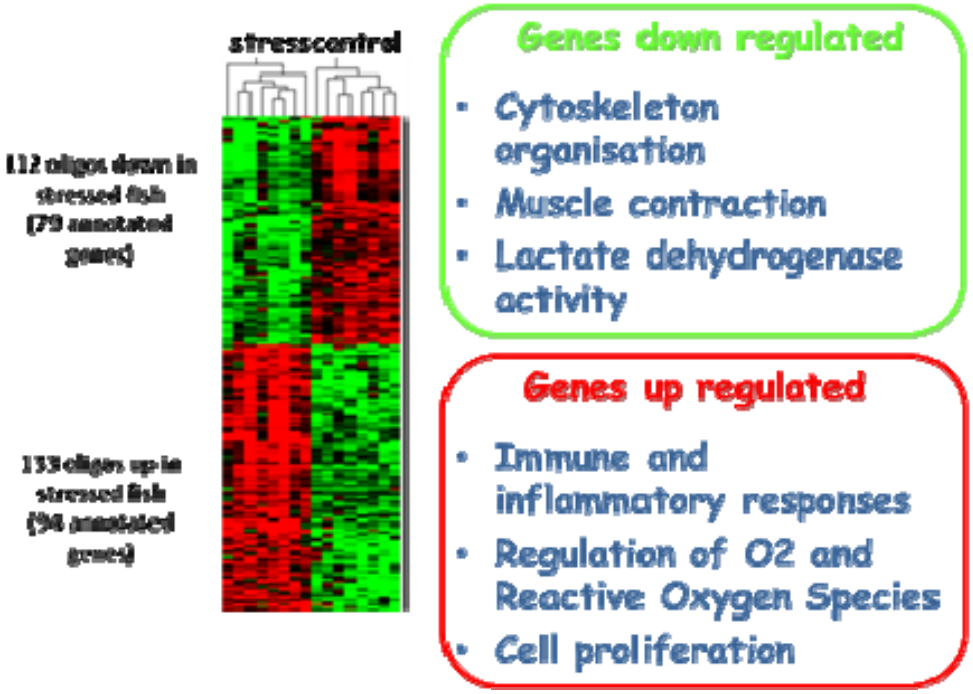


Figure 34. Summary of the microarray results of gills from rainbow trout in trial 2.

4. Discussion

One of the main aims of the current study was to compare and adapt methodology to study the individual responses to chronic stress mechanisms that fish can experience in normal aquaculture settings. Chronic stress approaches in salmon and many other fish have proven rather difficult to accomplish. The main reason is a great capacity to habituate to most stressors within a week or so (Folkedal *et al.* 2010, Schreck 2000, Pottinger *et al.* 1992). As a consequence, the unpredictable chronic stress paradigm was established in mammals (Grissom and Bhatnagar, 2009). The paradigm states that when an animal is exposed to a series of unpredictable stressors, it eventually fails to habituate and enter a state of chronic stress. The paradigm is now entering the fish science (Piato *et al.* 2011).

The current study clearly showed species differences in front of similar stressors and the need of a multi-methodological approach to better define chronic stress.

Rainbow trout does not enter into a chronic stress status easily, as deduced from the first preliminary trial 1, since the multiple stressor model (similar to the AUTO applied to salmon and sea bream) did not induce a deregulation of the HPI axis responsiveness after 10 days of applying the stressors. These results would be explained by the fact that the trout population used in the trial has been reared for many generations and probably is domesticated and therefore capable to cope with or get used to common stressors under culture conditions. Chronic stress was only induced when a model of poor water quality was applied for 3 weeks. The main water factor involved in this model was dissolved oxygen (2-5 mg/l), since ammonia values were very low and not toxic (EFSA, 2009), which is in agreement with the absence of significant changes of transcript values of ammonia receptors in gills of S fish. In any case, oxygen levels in S tanks did not reach extreme hypoxia (below 2 mg/l), which induces metabolic acidosis and mortality (EFSA, 2009).

The induced hypoxia produced changes at the different studied levels in rainbow trout. At *the organism level*, the main effect was the decrease in growth performance in S fish. *At the behavioural level*, only some tests correlated well with other physiological and transcriptomic changes. This was the case of the focal observations and the learning capacity test. S fish showed a dramatic decrease in their group activity, which is in accordance with previous studies in Atlantic cod (Herbert and Steffensen, 2005) and a reduction in activity represents an adaptive trait for the survival of fish at low oxygen levels. A reduced learning capacity was also observed in S fish. This capacity was measured as the anticipatory response of fish to feeding, which is considered a common indicator of good health and coping ability (Martins *et al.,* 2012). NS fish correctly learned to associate a neutral stimulus with a food reward, but not S fish. However, fish from both groups were grouped around the feeder area when food was provided at the end of the 18th trial. This indicates that S fish were still able to swim and orientate towards the food when the distribution really occurred, despite the lack of response when the conditioned stimulus was activated alone. We can therefore establish that a hypoxic chronic stress can inhibit fish food-anticipatory behaviour. This reduction in appetitive conditioned response has also been demonstrated in Atlantic salmon after different acute stressors (Folkedal *et al.,* 2012a, b). However, this is the first time that cognitive performances are shown to be decreased after a hypoxic chronic stress. The impaired responsiveness to rewarding or aversive stimuli could be adaptive compared to maintaining useless effort. Interestingly, this effect is observed even 7 days after the end of the chronic poor water quality exposure which suggests that such chronic stressor has profound and long-lasting effect on learning capacity. By contrast, the emotional reactivity test did not give any information about the welfare or sensitive status of the fish. In accordance with some authors (Destrez *et al.,* 2013), we consider that increased fearfulness observed in animals may be the consequence of a previous chronic stress. In the present study, no significant difference appeared between control and chronic stress groups. This indicates that emotional reactivity tests, which usually induce fast-start reactions and therefore mobilize energy, are not good indicators in the case of a previous

chronic lack of oxygen. Whether this absence of effect is associated with too mild hypoxia or whether oxygen level is not a major regulator of emotional reactivity still needs to be clarified.

At the *endocrine level*, plasma cortisol levels after an acute stressor (2 min) were significantly higher in fish exposed previously to chronic stress than in control ones, which corroborates that S fish were really in a stressed status. Thus, our experimental model overcame the fact showed by other authors that cortisol may not be elevated in chronically stressed fish due to a negative feedback mechanism of cortisol that causes a down-regulation of the HPI axis (Pickering and Stewart, 1984; Procarione *et al.,* 1999). When analysing at the *transcriptomic level* the effects in the HPI axis, no gene expression changes (either by microarray or candidate gene approach) were found at the first point of the axis (pituitary), and at the following level of the axis (brain) only a non-significant increasing pattern was found in some corticosteroid receptors. At the subsequent point of the axis (interrenal cells within head kidney), a significant increase was detected in two major genes involved in cortisol synthesis (P450 and StAR) in S fish. This suggests that the dysregulation effects of hypoxia may be linked to cortisol synthesis in the interrenal cells. Further experimental studies are needed to confirm these mechanisms.

The analysis of the gill physiology revealed different information depending on the tools applied. At the *metabolic level*, all plasma parameters related to gill function were similar in control and stressed trout. These results could initially suggest that fish were adapted to poor water quality and reached a compromise between respiration and osmoregulation. However, this does not exclude the fact that this trade-off was obtained at a high energetic cost and that energy-demanding process may be activated at the level of gill, a major organ for osmoregulation and respiration. Gill epithelium has an important plasticity to cope with external modifications. For example gill remodelling occurred after seawater transfer of euryhaline fish (Pisam and Rambourg, 1991) or acute hypoxia (Matey *et al.,* 2011). This apparent lack of effect on gills function was not paralleled at the *transcriptomic level*, as a large number of genes appeared significantly up- or down-regulated after the microarray analysis. Interestingly, these differentially expressed genes were not directly involved in gill osmoregulatory functions (ion transporters). By contrast, genes involved in defence (against pathogen or oxidative stress) were increased, and genes related to muscle contraction and cytoskeleton organization were down regulated. The expression analysis of candidate genes also indicated a higher cell renewal in S fish (genes involved in apoptosis and proliferation). However, further morphological observations will be necessary to determine which cell types are affected. In addition, some important biological pathways have to be studied more in detail (validation by qPCR, immunology and morphological analysis) for further conclusions on their respective roles. Finally, comparison with others gill transcriptomic studies using different acute/chronic stressors could be explored using the Fish and Chip tool developed also in WP7 (D7.1). Fig. 35 summarizes the outcomes of the different methodologies applied for rainbow trout.

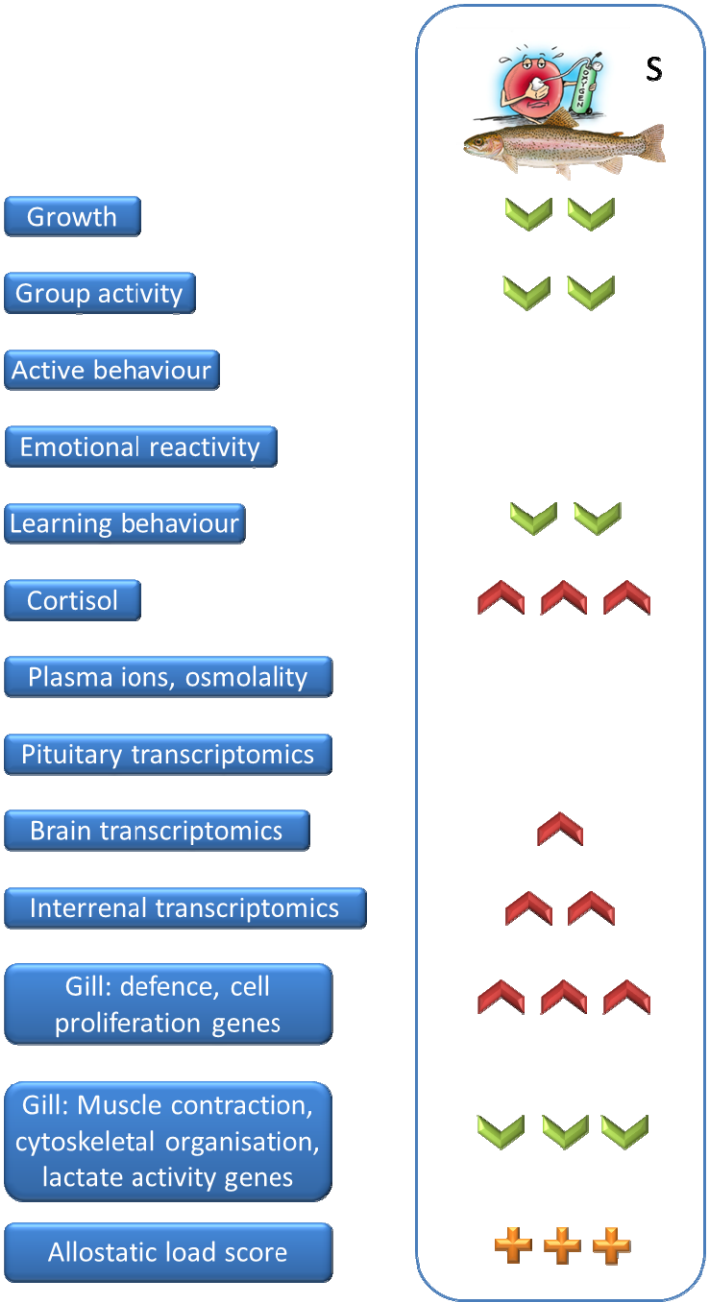


Figure 35. Integrative physiological response of rainbow trout. This response includes growth performance, behaviour, plasma biochemistry, transcriptomics of different tissues of fish exposed to chronic stress (poor water quality). The number of chevrons indicates the intensity of the response in comparison with undisturbed animals.

Atlantic salmon also appeared to be very robust towards the applied stressors, as can be seen in Fig. 36, which summarizes the outcomes of the different methodologies applied. Although AUTO stressors were applied in a random fashion, there were only few signs of chronic stress, and adaptation was complete within a few days. Even for the CHASE stressor, the depression in feed intake only lasted for 1-2 weeks before recovering. However, CHASE is a stressor that has the capacity to damage the fish in addition to being a stressor. From the stress test at the end, the data did not show any difference between the control and the stressed groups although there was a tendency of a down-regulation of the stress

response in the CHASE fish indicating a down-regulation of the HPI axis, a common feature in chronic stress (Barton *et al.,* 1987; Vijayan and Leatherland, 1990; Barton, 2002). This argument is strengthened by the clear down-regulation of oxygen consumption in the CHASE fish compared to the AUTO and CTRL groups throughout the trial.

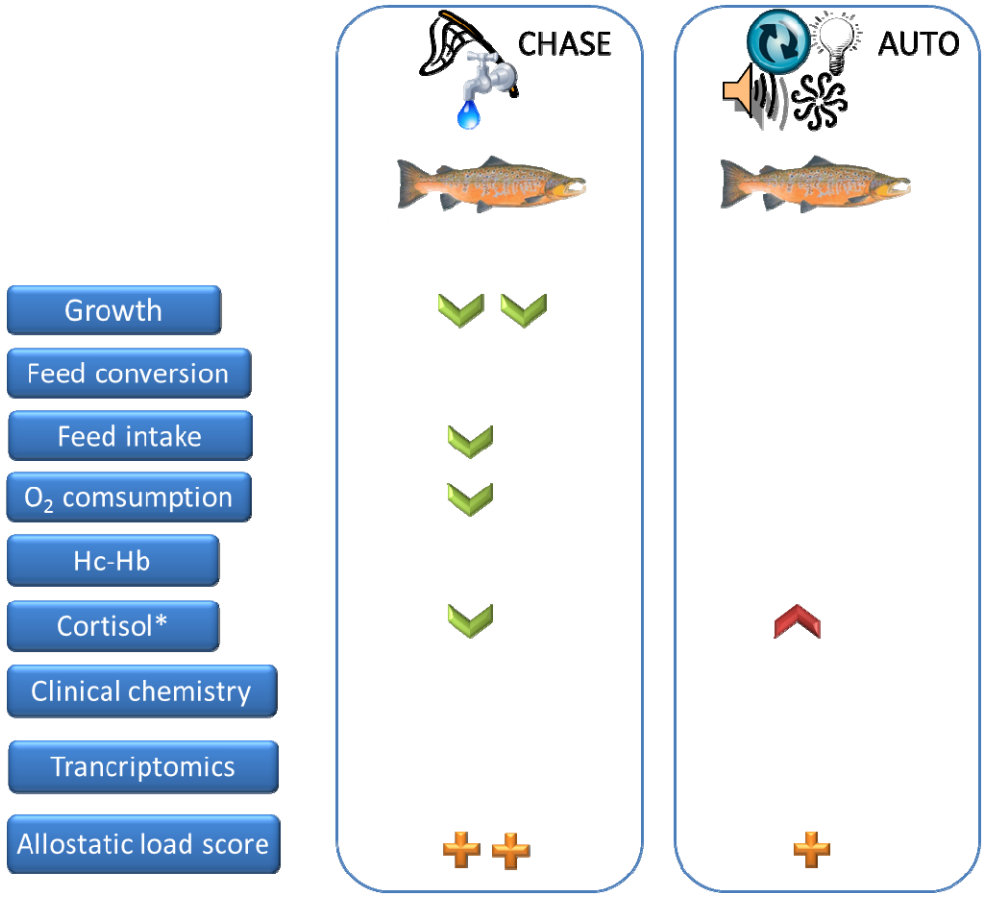


Figure 36. Integrative physiological response of Atlantic salmon to chronic stress. This response includes growth performance, plasma biochemistry, transcriptomics of pituitary of fish exposed to chasing (CHASE) and multiple sensory perception stress (AUTO). The number of chevrons indicates the intensity of the response in comparison with undisturbed animals, except for plasma cortisol (*) for which levels after an additional acute stressor indicate differences between AUTO and CHASE.

When the same stressors were applied to **Gilthead sea bream (GSB)**, the M-ST stressors appeared more detrimental than the C-ST. Furthermore, the changes in feed intake and oxygen consumption rates, which were observed during the whole experimental period in the T-ST group, demonstrate that juvenile GSB did not fully acclimate to the thermal shifts. According to Ibarz *et al.,* (2007), thermal shifts can be considered a natural stressor for GSB and this stressor may be responsible for the problems often observed during the cold seasons for GSB cultured in sea cages. In the current study, the reduced feed intake was observed when temperature decreased, increased or maintained at 12 ˚C. This indicates that the fish were stressed, although it was not possible to determine if the stress was caused by the drop in temperature and/or the low temperature itself. In any case, it is noticeable that thermal stress did not modify significantly the food anticipatory behaviour and the reduced growth of GSB exposed to repetitive thermal fluctuations (T-ST) compared to fish maintained at 20 ˚C (CTRL) was mainly a consequence of the lower feed intake during the days when the temperature was dropped.

Apart from a brief reduction of feed intake following increase in temperature, feeding and oxygen consumption was generally similar between the T-ST and the CTRL groups at 20 ˚C. This might be indicative of a good ability to recover from thermal stress, which is supported at the *endocrine level* by a lack of a plasma cortisol rise after prolonged exposure to cyclic thermal stress. This was further substantiate at the *molecular level* by a consistent up-regulation of: i) master regulators of mitochondria respiration and biogenesis (PGC1α, NRF1), ii) key enzymes of β-fatty acid oxidation and tricarboxylic acid cycle (CPT1A, CS), iii) outer-inner mitochondrial membrane translocases (TIM/TOM complex), molecular chaperones of mitochondria (mtHsp10, mtHsp60, mtHsp70) and endoplasmic reticulum (DER-1), and iv) key elements of glutathione metabolism (GR), and mitochondria dynamics (FIS1, MFN2) and apoptosis (AIFM1), which was related to improvement of FCR despite of the impaired growth performance due to overall reduction of feed intake. This might be of interest for fish farmers and could be important for the management and farming of GSB in order to produce and culture more robust fish before to the entry to the cold season, which might involve some kind of epigenetic regulation. Strong support for this notion comes from inbreeding selection of rat strains with “low power” versus “high power” mitochondria, which demonstrates that most stressful and health risk factors segregate with low expression levels of genes required for mitochondrial biogenesis and oxidative phosphorylation (Wisløff *et al.,* 2005).

GSB showed a similar behavioural response when exposed to M-ST or C-ST. Both groups attempted to avoid the stressors by moving down to the bottom part of the tank. Avoidance behaviour can result in reduced or even no exposure to the stress agent and is thus important for stress coping (Schreck *et al.,* 1997). In the current study daily acute stress in the form of lowering the water level in the tank combined with chasing did not affect the general performances (i.e. growth, total feed intake), while daily acute stress in the form of a set of automatic stressors did affect growth and feed utilization. These results demonstrate that in the current study GSB were able to cope with the C-ST, but not with M-ST, which suggests that the actual given stressors play a role in stress coping. Nevertheless, the stress frequency might also have had an influence; fish under the C-ST were only twice a day subjected to the stress, while fish under M-ST were three times a day subjected to the stress. Indeed, at the *hormonal and metabolic level*, the response achieved by the two groups of fish was different. Thus, the C-ST fish showed an increased Hc and blood Hb concentration in combination with the rise of plasma lactate concentration in comparison to CTRL fish, which suggests that juvenile GSB experienced this treatment as light stressful condition with a shift to anaerobic metabolism to cope at long-term with this type of stressor. By contrast, daily exposure to a set of automatic stressors is experienced by fish as a strong stressor condition with a persistent increase in plasma cortisol levels and a lower FCR, which might be the result of a different reallocation and utilization of energy metabolic substrates as often observed during stress adaptation (Wendelaar Bonga, 1997). At the *molecular level*, the overall down-regulation of mitochondria-related genes, especially evident for master regulators of mitochondria function and biogenesis, highly contributed to this suggestion. As stated before for T-ST fish, these observations are of interest for GSB farmers, because holistic approaches including fish behaviour, general performance and metabolic phenotyping at the metabolite, hormonal and molecular levels are emerging as a highly promising set of methodologies for the accurate assessment of welfare status in farmed GSB.

Furthermore, since distress can lead to exhaustion and deprived welfare (Moberg, 2000; Schreck, 2000; Folkedal, 2010), for the aquaculture industry it is important to know how fish with a different genetic and epigenetic background are indeed able to both behaviourally and physiologically habituate to different daily acute stressors. Further research on this field is therefore recommended without ruling out the study of the breakdown of social hierarchies in fish exposed to natural and husbandry related stressors, which seems to be highly related to skin coloration on the basis of the experience achieved during the course of the trials conducted in this deliverable. In any case, the procedure for husbandry stressor is labour intensive, varies with operator, and has the capacity to harm the

skin/mucus. Therefore, the procedures will be designed to cause minimum harm to fish, replicating what would be recognized as normal handling routines and to make use of the unpredictable chronic stress paradigm. This consideration reinforces, thereby, the interest of mucus protein profiling as a complementary tool to assess fish welfare as an example of a non-invasive sampling in the absence of apparent skin lesions. Importantly, the approach used herein was able to unequivocally annotate more than 2,000 proteins that were positively matched in the protein database derived from the GSB transcriptomic database hosted by the CSIC partner (www.nutrigroup-iats.org/seabreamdb). It is noteworthy a high overrepresentation of mitochondria, but more importantly, of cytoskeletal-related proteins. Furthermore, the comparison of the mucus protein content of M-ST fish with CTRL fish, showed that well-known markers of mitochondrial apoptosis and epithelial cell desquamation were more abundant in M-ST. Attempts to characterise protein skin mucus in fish are mostly reduced to antimicrobial peptides in fish challenged with pathogens (Provan *et al.,* 2013; Rajan *et al.,* 2013), and to our knowledge this is the first report showing that skin mucus is a highly informative tool to assess chronic stress. Further work on these candidate mucus proteins could allow in the future developing quick, easy and rapid dip-stick tests to be used for the routine and clinical diagnosis of welfare condition without disturbing farmed fish.

In conclusion, our results highlight for the first time in fish the transcriptional plasticity of most nuclear-encoded mitochondria proteins that affect a vast array of processes, including mitochondrial biogenesis and oxidative metabolism, mitochondrial protein import/folding/assembly, as well as mitochondrial dynamics and apoptosis. Importantly, most of the genes on the array were differentially regulated by repetitive exposure to natural and husbandry stressors, and the magnitude of the mitochondrial transcriptionally-mediated changes reflects the intensity and severity of the stressor. Thus, the present study revealed new insights on the capacity of fish to efficiently manage allostatic load, defined as the process that maintains stability through change of a number of stress mediators. The ultimate physiological consequences are still under investigation but, as summarized in Fig. 37, the gene expression profile of fish exposed to the repetitive cycling of water temperature indicates that a reactive mitochondrial phenotype helps to increase the aerobic oxidative capacity of fish. In contrast, in the C-ST group, an apparent lack of mitochondria response in combination with increased lactate production is indicative of some kind of metabolic switch that primes the anaerobic metabolism in response to short periods of increased energy demand that do not have a major impact on fish performance. The third response pattern, with the highest theoretical allostatic load score, is represented by the M-ST group, in which the overall down-regulation of mitochondrial-related genes in combination with decreased lactate production is indicative of reduced energy demand and oxidative metabolic capacity, leading to the impairment of feed conversion in a changing and poor predictive milieu.

The described species differences in front of environmental stress wait to be further studied in the future. We plan to go further in the transcriptomic approach, by analysing other samples taken during this collaborative project, and also to enter more transcriptomic data in the Fish and Chips tool developed in D7.1. This would allow more inter-specific comparisons and to obtain more clear-cut possible biomarkers.

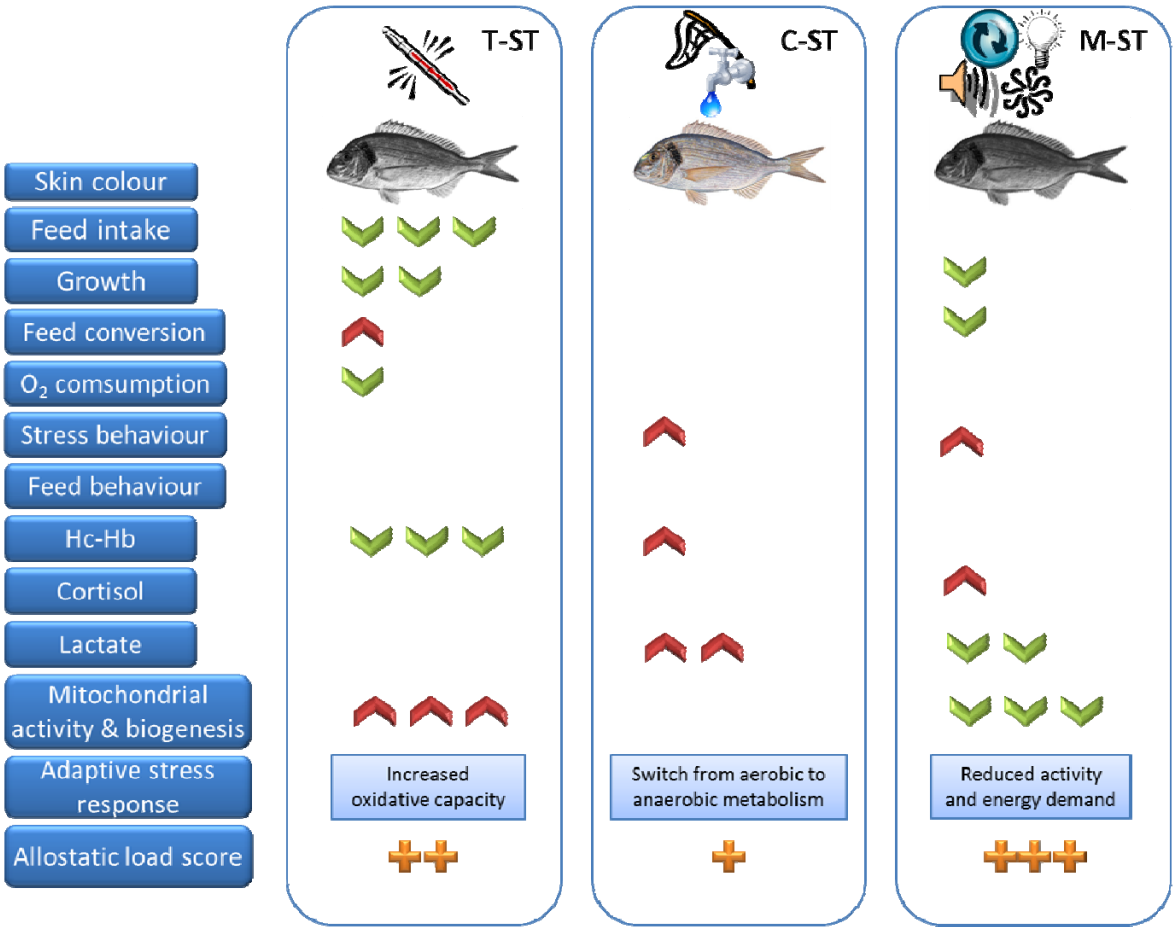


Fig. 37. Integrative physiological response of gilthead sea bream. This response includes growth performance, plasma biochemistry, haematology, behavioural changes, mitochondrial activity and biogenesis with a given allostatic load score of fish exposed to natural and husbandry stress stimuli, including thermal stress (T-ST), chasing stress (C-ST) and multiple sensory perception stress (M-ST). The number of chevrons indicates the intensity of the response in comparison with undisturbed animals.

5. Conclusions

- The integration of data of growth performance, plasma biochemistry, haematology and mitochondrial activity and biogenesis gives a global physiological view of the allostatic load score of gilthead sea bream exposed to environmental chronic stressors.
- Liver mitochondrial-related genes are good biomarkers to assess the type of stressor and the true level of chronic stress in gilthead sea bream.
- Skin mucus is a promising not-lethal sample for the identification of protein biomarkers of chronic stress in gilthead sea bream.
- Mitochondrial apoptosis and epithelial desquamation proteins appear as highly promising skin mucus biomarkers of chronic stress in gilthead sea bream.
- Colour skin changes of gilthead sea bream are easy and rapid biomarkers of hierarchies and stress within a tank of gilthead sea bream exposed to stressors.
- Atlantic salmon adapt to repeated stressors within 2-3 weeks.
- Although feed intake is restored in salmon exposed to repeated stressors, depressed oxygen consumption is not.
- Rainbow trout is resistant to repeated acute stressors and gets used in 10 days.
- A model for chronic stress, based on exposure to poor water quality, has been proposed for rainbow trout.
- Gills are the best tissue for detecting transcriptomic changes in chronically stressed rainbow trout exposed to poor water quality.

Conclusions driven from the multi-species studies:

- The multiple-parameters approach is necessary to get a better picture of chronic stress and allostatic load.
- The usefulness of cortisol responsiveness as a marker of chronic stress is variable and relies on intensity/quality of the stressor. It cannot be used alone as a biomarker of chronic stress.
- Some behavioural tests could also be complementary non-invasive tools for detecting chronic stress.
- Specific biomarkers for any type of chronic stressors probably do not exist, although the simultaneous monitoring of a set of mitochondria-related genes is a promising highly informative tool of the allostatic load score.
- Pituitary is not a responsive tissue in terms of transcriptomic changes to study environmental stress in salmonids
- Limits between adaptation or stress responses still need to be further clarified for each fish species and physiological condition.

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Annex 1- Supplementary tables and figures

Supplementary Table 1. PCR-array layout of 60 mitochondrial related genes with extra-wells for housekeeping genes and general controls of PCR performance used in gilthead sea bream transcriptomic analyses.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hsp10	CAT	CPT1A	COX4a	Tom5	Tim22	MIRO2	NRF1	ACTB	ACTB	PPC1	PPC1
B	DnaJA3A	GPX4	CPT1B	UCP1	Tim44	Tim10	AIFM1	NRF2	EF-1	EF-1	PPC2	PPC2
C	DnaJC20	GR	ECH	UCP2	Tim23	Tim9	AIFM3	PGC1α	α-tubulin	α-tubulin	PPC3	PPC3
D	mtHsp60	GST3	HADH	UCP3	Tim17A	FIS1	BAX	PGC1β	18S rRNA	18S rRNA	PPC4	PPC4
E	mtHsp70	PRDX3	CS	Tom70	Tim16	MIFFB	BCL2				NPC	NPC
F	DER-1	PRDX5	IDH3A	Tom34	Tim14	MFN1	BCLX					
G	ERdj3	SOD2	IDH3B	Tom22	Tim13	MFN2	PERP					
H	Grp170	ACAA2	IDH3G	Tom7	Tim8A	MIRO1A	mtTFA					

Position	Symbol	Description	Accession No.
A1	mtHsp10	10 kDa heat shock protein, mitochondrial	JX975224
B1	DnaJA3A	40 kDa heat shock protein DnaJ (Hsp40) homolog, member 3A	JX975225
C1	DnaJC20	Iron-sulfur cluster co-chaperone protein HscB, mitochondrial	JX975226
D1	mtHsp60	60 kDa heat shock protein, mitochondrial	JX975227
E1	mtHsp70	70 kDa heat shock protein, mitochondrial	DQ524993
F1	DER-1	Derlin-1	JQ308825
G1	ERdj3	ER-associated Hsp40 co-chaperone	JQ308827
H1	Grp170	170 kDa glucose-regulated protein	JQ308821
A2	CAT	Catalase	JQ308823
B2	GPX4	Glutathione peroxidase 4	AM977818
C2	GR	Glutathione reductase	AJ937873
D2	GST3	Glutathione S-transferase 3	JQ308828
E2	PRDX3	Peroxiredoxin 3	GQ252681
F2	PRDX5	Peroxiredoxin 5	GQ252683
G2	SOD2	Superoxide dismutase [Mn]	JQ308833
H2	ACAA2	3-ketoacyl-CoA thiolase, mitochondrial	JX975228
A3	CPT1A	Carnitine palmitoyltransferase 1A	JQ308822
B3	CPT1B	Carnitine palmitoyltransferase 1B	DQ866821
C3	ECH	Enoyl-CoA hydratase, mitochondrial	JQ308826
D3	HADH	Hydroxyacyl-CoA dehydrogenase	JQ308829
E3	CS	Citrate synthase	JX975229
F3	IDH3A	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	JX975231
G3	IDH3B	Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	JX975232
H3	IDH3G	Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial	JX975233
A4	Cox4a	Cytochrome C oxidase subunit IV isoform 1	JQ308835
B4	UCP1	Uncoupling protein 1	FJ710211
C4	UCP2	Uncoupling protein 2	JQ859959
D4	UCP3	Uncoupling protein 3	EU555336
E4	Tom70	Mitochondrial import receptor subunit Tom70	JX975234
F4	Tom34	Mitochondrial import receptor subunit Tom34	JX975235
G4	Tom22	Mitochondrial import receptor subunit Tom22	JX975236
H4	Tom7	Mitochondrial import receptor subunit Tom7 homolog	JX975237
A5	Tom5	Mitochondrial import receptor subunit Tom5 homolog	JX975238
B5	Tim44	Mitochondrial import inner membrane translocase subunit 44	JX975239
C5	Tim23	Mitochondrial import inner membrane translocase subunit 23	JX975240
D5	Tim17A	Mitochondrial import inner membrane translocase subunit 17A	JX975241

Suppl. Table 1. Continued.

Position	Symbol	Description	Accession No.
E5	Tim16	Mitochondrial import inner membrane translocase subunit 16	JX975242
F5	Tim14	Mitochondrial import inner membrane translocase subunit Tim14	JX975243
G5	Tim13	Mitochondrial import inner membrane translocase subunit Tim13	JX975244
H5	Tim8A	Mitochondrial import inner membrane translocase subunit Tim8A	JX975245
A6	Tim22	Mitochondrial import inner membrane translocase subunit Tim22	JX975246
B6	Tim10	Mitochondrial import inner membrane translocase subunit Tim10	JX975247
C6	Tim9	Mitochondrial import inner membrane translocase subunit Tim9	JX975248
D6	FIS1	Mitochondrial fission 1 protein	JX975249
E6	MIFFB	Mitochondrial fission factor homolog B	JX975252
F6	MFN1	Mitofusin 1	JX975250
G6	MFN2	Mitofusin 2	JX975251
H6	MIRO1A	Mitochondrial Rho GTPase 1	JX975253
A7	MIRO2	Mitochondrial Rho GTPase 2	JX975254
B7	AIFM1	Apoptosis-related protein 1	JX975255
C7	AIFM3	Apoptosis-related protein 3	JX975256
D7	BAX	Apoptosis regulator BAX	JX975257
E7	BCL2	Apoptosis regulator Bcl-2	JX975258
F7	BCLX	Bcl-2-like protein 1	JX975259
G7	PERP	p53 apoptosis effector related to PMP-22	JX975260
H7	mtTFA	Mitochondrial transcription factor A	JX975262
A8	NRF1	Nuclear respiratory factor 1	JX975263
B8	NRF2	Nuclear respiratory factor 2	JX975261
C8	PGC1α	Proliferator-activated receptor gamma coactivator 1 alpha	JX975264
D8	PGC1β	Proliferator-activated receptor gamma coactivator 1 beta	JX975265
A9, A10	ACTB	ß-actin	X89920
B9, B10	EF-1	Elongation factor 1	AF184170
C9, C10	α-tubulin	α-tubulin	AY326430
D9, D10	18S rRNA	18S ribosomal RNA	AY993930
A11-D11	PPC1/PPC4	Positive PCR control (serial dilutions of standard gene)	AY590304
A12-D12	PPC1/PPC4	Positive PCR control (serial dilutions of standard gene)	AY590304
E11, E12	NPC	Negative PCR control	

Mitochondrial chaperones: mtHsp10, DnaJA3A, DnaJC20, mtHsp60, mtHsp70
Endoplasmic reticulum stress response: DER-1, ERdj, GRP-170
Antioxidant defense: CAT, GPX4, GR, GST3, PRDX3, PRDX5, SOD2
Oxidative metabolism: ACAA2, CPT1A, CPT1B, ECH, HADH, CS, IDH3A, IDH3B, IDH3G, Cox4a
Mitochondrial respiration uncoupling: UCP1, UCP2, UCP3
Outer membrane translocases (TOM complex): Tom70, Tom34, Tom22, Tom7, Tom5
Inner membrane translocases (TIM23 complex): Tim44, Tim23, Tim17A, Tim16, Tim14, Tim13, Tim8A
Inner membrane translocases (TIM22 complex): Tim22, Tim10, Tim9
Mitochondrial dynamics: FIS1, MIFFB, MFN1, MFN2, MIRO1A, MIRO2
Apoptosis: AIFM1, AIFM3, BAX, BCL2, BCLX
Nuclear transcription factors: mtTFA, NRF1, NRF2, PGC1α, PGC1β
Housekeeping genes: ACTB, EF-1, α-tubulin, 18S rRNA

Supplementary Table 2. Characteristics of new assembled sequences of mitochondria-related genes of gilthead seabream according to BLAST searches.

Contigs	F[a]	Size (nt)	Annotation[b]	Best match[c]	E[d]	CDS[e]
C2_4023	95	570	mtHsp10	ACQ58985	2e-55	98-397
C2_6932	96	2165	DnaJA3A	XP_003450123	0	33-1397
C2_4907	110	1192	DnaJC20	XP_003454205	9e-111	243-968
C2_5222	145	3195	mtHsp60	ADM73510	0	123-1862
C2_1174	472	1864	ACAA2	XP_003451083	0	131-1321
C2_2740	171	3386	CS	Q6S9V7	0	124-1533
C2_5093	53	1542	IDH3A	XP_003440485	0	55-1212
C2_1275	295	1650	IDH3B	CBN81104	0	53-1201
C2_3444	204	2138	IDH3G	XP_003448211	0	43-1233
C2_22819	13	976	Tom70	XP_003452621	0	<1->976
C2_17904	30	1398	Tom34	ACI33761	8e-123	140-1072
C2_3036	199	1588	Tom22	ACO09752	6e-36	209-610
C2_4825	51	565	Tom7	CAF89564	1e-30	70-237
C2_5265	74	736	Tom5	ACQ58779	5e-13	113-268
C2_15041	40	777	Tim44	XP_003439971	5e-120	<1->777
C2_4029	124	1606	Tim23	CBN81624	5e-104	308-943
C2_138	777	1364	Tim17A	CBN80814	1e-82	45-551
C2_15083	34	556	Tim16	NP_957098	2e-40	105-476
C2_5885	44	611	Tim14	ACO07830	3e-41	121-471
C2_2920	189	1609	Tim13	ACQ58319	7e-46	112-399
C2_1464	245	891	Tim8A	XP_003457647	8e-49	190-459
C2_12934	24	1018	Tim22	XP003456212	5e-99	20-625
C2_8198	87	515	Tim10	XP_003448035	1e-48	140-406
C2_7203	64	890	Tim9	NP_001153383	3e-51	75-344
C2_198	1062	1200	FIS1	XP_003449392	5e-68	33-497
C2_1143	198	1369	MIFFB	XP_003441668	9e-106	217-909
C2_7180	62	2455	MFN1	CAG08068	0	<1-1355
C2_11777	44	2215	MFN2	XP_003459870	0	482->2215
C2_9297	66	2398	MIRO1A	XP_003452865	0	362-2221
C2_3084	160	3257	MIRO2	CBN81307	0	162-2018
C2_6260	91	1715	AIFM1	XP_003456194	0	<1-1328
C2_358	651	1386	AIFM3	ACQ58260	7e-139	41-736
C2_2702	178	2082	BAX	CAG02784	1e-109	206-784
C2_23085	12	1056	BCL-2	XP_003437950	6e-100	548->1056
C2_7453	45	1321	BCL2L	CBN81010	3e-96	243-890
C2_2885	200	1139	PERP	NP_001135192	3e-70	227-784
C2_1902	207	2381	mtTFA	ACQ58415	1e-104	180-1058
C2_23517	16	1297	NRF1	XP_003445010	1e-31	<1-267
C2_11237	36	1092	NRF2	XP_003452933	0	145->1092
C2_43962	5	252	PGC1α	CAG02304	1e-20	25->252
C2_65322	7	770	PGC1β	XP_003447675	2e-88	<1-500

[a] Number of reads composing the assembled sequences.
[b] Gene identity determined through BLAST searches, for abbreviations see table
[c] Best BLAST-X protein sequence match (lowest E value).
[d] Expectation value.
[e] Codifying domain sequence.

Supplementary Table 3. Forward and reverse primers for real-time PCR for mitochondrial-related genes of gilthead sea bream

Gene name	Symbol	Primer sequence
β-actin	ACTB	F TCC TGC GGA ATC CAT GAG A
		R GAC GTC GCA CTT CAT GAT GCT
Elongation factor 1	EF-1	F CCC GCC TCT GTT GCC TTC G
		R CAG CAG TGT GGT TCC GTT AGC
α-tubulin	α-tubulin	F GAC ATC ACC AAT GCC TGC TTC
		R GTG GCG ATG GCG GAG TTC
18S ribosomal RNA	18S r RNA	F GCA TTT ATC AGA CCC AAA ACC
		R AGT TGA TAG GGC AGA CAT TCG
10 kDa heat shock protein, mitochondrial	mtHsp10	F CAT GCT GCC AGA GAA GTC TCA AGG
		R AGG TCC CAC TGC CAC TAC TGT
40 KDa heat shock protein DnaJ (Hsp40) homolog, subfamily A, member 3A	DnaJA3A	F CCA AAT GCT GTC TCC TCA CTG TCC TTT C
		R ACC TGA TAG AAG TCC TGC TTG CTG CTA
Iron-sulfur cluster co-chaperone protein HscB	DnaJC20	F GCC AGA AGC AGC CAA TAG GAT
		R CTT TGA GCA GGG CAG CGT CTA
60 kDa heat shock protein, mitochondrial	Hsp60	F TGT GGC TGA GGA TGT GGA TGG AGA G
		R GCC TGT TGA GAA CCA AGG TGC TGA G
70 kDa heat shock protein, mitochondrial	mtHsp70	F TCC GGT GTG GAT CTG ACC AAA GAC
		R TGT TTA GGC CCA GAA GCA TCC ATG
Derlin-1	DER-1	F ACT GCC TCG GTT GCC TTT CC
		R TGG CTG TCA CAA GTC TCC AGA TAT G
ER-associated Hsp40 co-chaperone	ERdj3	F AAC CGA CAG CAG CAG GAC AG
		R ACT TCT TCA AGC GTG ACC TCC AG
170 kDa glucose-regulated protein	Grp-170	F CAG AGG AGG CAG ACA GCA AGA C
		R TTC TCA GAC TCA GCA TTT CCA GAT TTC
Catalase	CAT	F TGG TCG AGA ACT TGA AGG CTG TC
		R AGG ACG CAG AAA TGG CAG AGG
Glutathione peroxidase 4	GPX4	F TGC GTC TGA TAG GGT CCA CTG TC
		R GTC TGC CAG TCC TCT GTC GG
Glutathione reductase	GR	F TGT TCA GCC ACC CAC CCA TCG G
		R GCG TGA TAC ATC GGA GTG AAT GAA GTC TTG
Glutathione S-transferase 3	GST3	F CCA GAT GAT CAG TAC GTG AAG ACC GTC
		R CTG CTG ATG TGA GGA ATG TAC CGT AAC
Peroxiredoxin 3	PRDX3	F ATC AAC ACC CCA CGC AAG ACT G
		R ACC GTT TGG ATC AAT GAG GAA CAG ACC
Peroxiredoxin 5	PRDX5	F GAG CAC GGA ACA GAT GGC AAG G
		R TCC ACA TTG ATC TTC TTC ACG ACT CC
Superoxide dismutase [Mn]	SOD2	F CCT GAC CTG ACC TAC GAC TAT GG
		R AGT GCC TCC TGA TAT TTC TCC TCT G
3-ketoacyl-CoA thiolase, mitochondrial	ACAA2	F CAT CAC TGC CCA CCT GGT TCA T
		R CCA ACA GCG TAC TTG CCT CCT
Carnitine palmitoyltransferase 1A	CPT1A	F GTG CCT TCG TTC GTT CCA TGA TC
		R TGA TGC TTA TCT GCT GCC TGT TTG
Carnitine palmitoyltransferase 1B	CPT1B	F CCA CCA GCC AGA CTC CAC AG
		R CAC CAC CAG CAC CCA CAT ATT TAG
Enoyl-CoA hydratase, mitochondrial	ECH	F GCC CAA GAA GCC AAG CAA TCA G
		R CTT TAG CCA TAG CAG AGA CCA GTT TG

Supplementary table 3. Continued I.

Gene name	Symbol	Primer sequence
Hydroxyacyl-CoA dehydrogenase	HADH	F GAA CCT CAG CAA CAA GCC AAG AG R CTA AGA GGC GGT TGA CAA TGA ATC C
Citrate synthase	CS	F TCC AGG AGG TGA CGA GCC R GTG ACC AGC AGC CAG AAG AG
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	IDH3A	F CCA CCC ATC TAT GAA CCT GCT GCT GAG R CAC ACA CGG ACG CAC ATT GGC ATA
Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	IDH3B	F CCT CGG TCT GTT CAC GGA TGA TGA R CAG CAC TCG CCA CAA CAA CCT
Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial	IDH3G	F GCT TAG ACC TCT ATG CGA ATG TGA TG R TGT CAA TGT TCT TGT GGC GAG TC
Cytochrome C oxidase subunit IV isoform 1	Cox4a	F ACC CTG AGT CCA GAG CAG AAG TCC R AGC CAG TGA AGC CGA TGA GAA AGA AC
Uncoupling protein 1	UCP1	F GCA CAC TAC CCA ACA TCA CAA G R CGC CGA ACG CAG AAA CAA AG
Uncoupling protein 2	UCP2	F CGG CGG CGT CCT CAG TTG R AAG CAA GTG GTC CCT CTT TGG TCA T
Uncoupling protein 3	UCP3	F AGG TGC GAC TGG CTG ACG R TTC GGC ATA CAA CCT CTC CAA AG
Mitochondrial import receptor subunit Tom70	Tom70	F GAG TCA GGT GGT CGA TAC A R CCA ATG AGC AGG TAG AAT GTG
Mitochondrial import receptor subunit Tom34	Tom34	F GCT ACC GCC ACT TCT CCA CAA R TCT GTT TGG TGC CGT TCT GCT
Mitochondrial import receptor subunit Tom22	Tom22	F CGC TCT GGG TGG GTA CTA CCT CCT T R CGA ACA CAA CAG GCA GCA CCA GGA T
Mitochondrial import receptor subunit Tom7 homolog	Tom7	F CGT GCT GTA CCT CGG TTT CAA A R ACT CAA GAC CGT GGG CTC AG
Mitochondrial import receptor subunit Tom5 homolog	Tom5	F GGA GGA GAT GAA GAA GAA GAT GCG TCA AGA R CTC TGA GAA GGG CGA CGT AAA GAA GAA AGT
Mitochondrial import inner membrane translocase subunit Tim44	Tim44	F GAT GAC CTG GGA CAC ACT GG R TCA CTC CTC TTC CTG AGT CTG G
Mitochondrial import inner membrane translocase subunit 23	Tim23	F CAA GTC AGG AAG TGG CGT AA R AGA GCG TAG GCA CCA GAT A
Mitochondrial import inner membrane translocase subunit Tim17-A	Tim17A	F GGC GGT ATC CTT CTG GCA TTG R GGG AAC TGT GAG GAG GCA AAC
Mitochondrial import inner membrane translocase subunit 16	Tim16	F CGT GCC TTT GCT CGT GCC TTA R GCC TTC GCT GCT GCT TGA CT
Mitochondrial import inner membrane translocase subunit 14	Tim14	F AAT GAT CCT GAA CCA TCC AGA CAG AGG R GCC GTC CAT CAA ATC CTT CGC TTC
Mitochondrial import inner membrane translocase subunit 13	Tim13	F GGT TCG GTT CAG ACT TCT CA R GAC CTT GAC CTG CTC CAT
Mitochondrial import inner membrane translocase subunit Tim8 A	Tim8A	F CGA CAC CAC CCT GAC CAT CAC R CGC CCT TCT GCA CCA TCT GT

Supplementary Table 3. Continued II.

Gene name	Symbol	Primer sequence
Mitochondrial import inner membrane translocase subunit Tim22	Tim22	F TCC GAC AGC ACG AGA AGT R AGA ACA TGG CAC CGA CGA T
Translocase of inner mitochondrial membrane 10 homolog	Tim10	F TAC CGC CAC ATT ACA AGG AGC R ATC CAG GCA CAC CGA CTC
Mitochondrial import inner membrane translocase subunit Tim9	Tim9	F CGT CAA AGA TTT CAC CAC CAG AGA G R GGA GAC ACG ACT CGG AGC A
Mitochondrial fission 1 protein	FIS1	F TCT CAG GAA CGA GCC AGG GAA CA R CCT TGT CGA TGA GTT TCT CCA GGT CCA G
Mitochondrial fission factor homolog B	MIFFB	F CGC AGC AGC ATT CCC TTC R CTC GTA CTG GAT TCG GTT CAT CT
Mitofusin 1	MFN1	F CAT CGT TGG AGG AGT GGT GTA R CCG TAC AGT GAG GCT GAG AG
Mitofusin 2	MFN2	F GGG ATG CCT CAG CCT CAG AAC CT R CTG CCT GCG GAC CTC TTC CAT GTA TT
Mitochondrial Rho GTPase 1	MIRO1A	F CAG GAC TTC TGC CGT AAG C R TAA GTG CAT CGG TCG TGT TG
Mitochondrial Rho GTPase 2	MIRO2	F TGA GGT GGA TGT GGA GGT GGA GTT R CAA GCA ACA TCA CAG GAG GCG TCT
Apoptosis-related protein 1	AIFM1	F ACA GAG GAG TCA GGA ACC R GGA GCA GGC AAT GAA GAG
Apoptosis-related protein 3	AIFM3	F GCA GCG GTA CAG TCT TGA ATG G R CCA GCG GAC GAG GAG CAA
Apoptosis regulator BAX	BAX	F GTG GCA GAC GGT GGG TGT TT R GCG AAT GAC GAG AAC AGT GGT GAG
Apoptosis regulator Bcl-2	BCL2	F GCT GTA TCT CAC CTC CAC CAC GG R TCT ATC ACC TCG GCG AAC CTC CT
Bcl-2-like protein 1	BCLX	F CGA CAT CAC TCC TGA CAC AGC CTA C R CCG TCC TTG AAC ACC TCG TCC ATC
p53 apoptosis effector related to PMP-22	PERP	F GGA GCA ACC ATC CTC AGC AT R GCG AGG CAG ACA GCA GAA
Transcription factor A, mitochondrial	mtTFA	F GAG CCC GCA ACA GAA ACA GCC ATT R ACT GCT CCC TGT CCC GCT GAT AG
Nuclear respiratory factor 1	NRF1	F CAG ATA GTC CTG GCA GAG A R GAC CTG TGG CAT CTT GAA
GA-binding protein alpha chain	NRF2	F CAT TGC CGT GGA CCG ATC TG R GCG TGT GAC CTG CTC TGA C
Proliferator-activated receptor gamma coactivator 1 alpha	PGC1α	F CGT GGG ACA GGT GTA ACC AGG ACT C R ACC AAC CAA GGC AGC ACA CTC TAA TTC T
Proliferator-activated receptor gamma coactivator 1 beta	PGC1β	F TCA GAG GAA GAG GCG GAT R GAC ACA GGT GGA GGA TGG

Supplementary Table 4. Effect of three challenging stressors on liver mRNA gene expression of gilthead sea bream. CTRL, control group; T-ST, thermal stress group; C-ST, chasing stress group; M-ST, multiple sensory perception stress group. Values are the mean ± SEM (n = 6-8). Rows with unlike superscript letters were significantly different ($P < 0.05$; Student-Newman-Keuls). All data values were in reference to the expression level of proliferator-activated receptor gamma coactivator 1 β (PGC1β) in CTRL fish, which was arbitrarily assigned a value of 1.

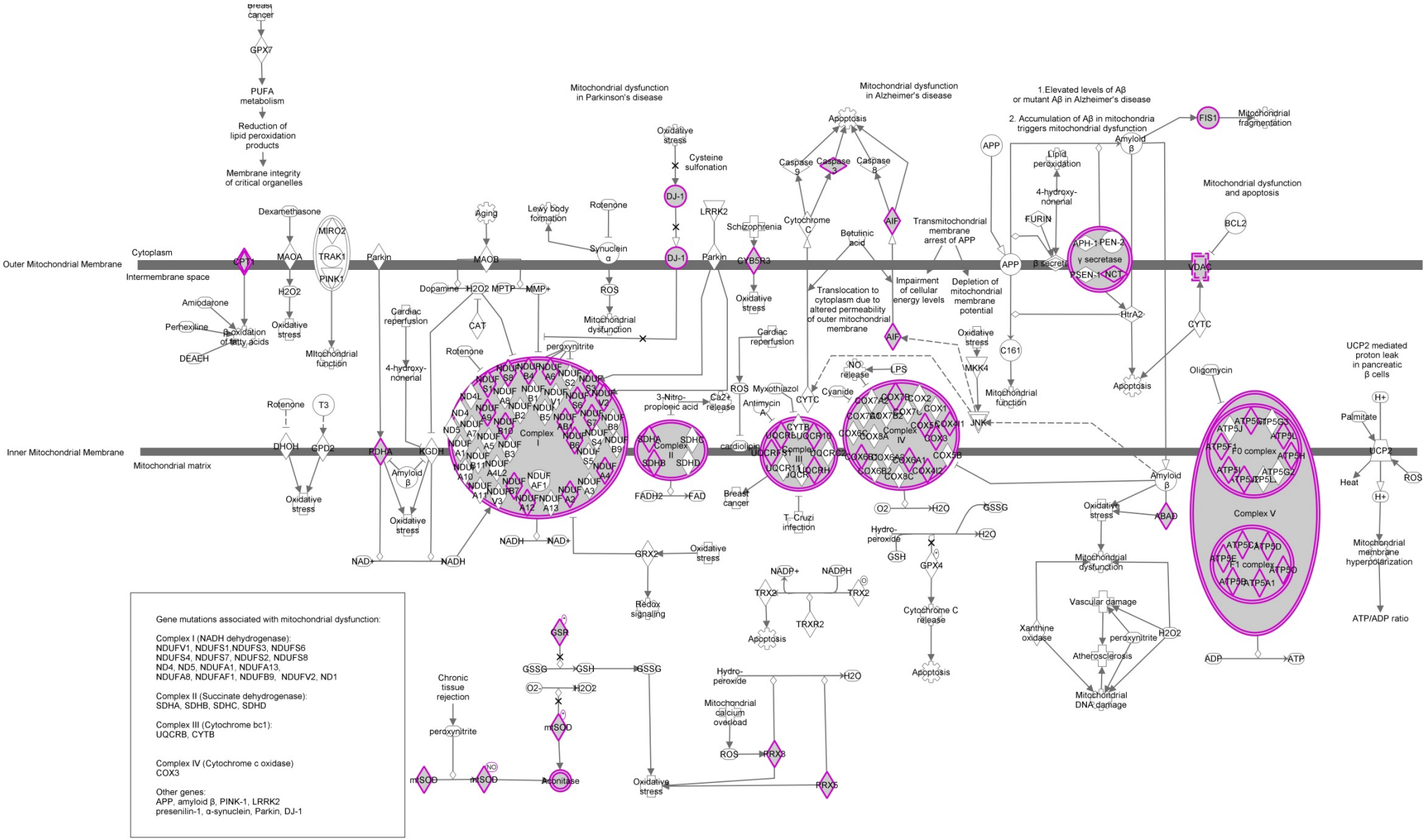
Gene name*	CTRL	T-ST	C-ST	M-ST
mtHsp10	1.91 ± 0.33^{a}	3.54 ± 0.59^{b}	2.29 ± 0.38^{ab}	1.53 ± 0.14^{a}
DnaJA3A	0.35 ± 0.02	0.36 ± 0.06	0.38 ± 0.07	0.33 ± 0.05
DnaJC20	0.26 ± 0.02	0.29 ± 0.03	0.22 ± 0.02	0.23 ± 0.01
mtHsp60	0.75 ± 0.08^{a}	1.47 ± 0.23^{b}	0.64 ± 0.11^{a}	0.59^{a} ± 0.09
mtHsp70	1.22 ± 0.15	1.72 ± 0.16	1.19 ± 0.24	1.06 ± 0.18
DER-1	2.93 ± 0.20	3.96 ± 0.32	3.32 ± 0.38	2.57 ± 0.20
ERdj3	1.20 ± 0.20	1.52 ± 0.24	1.38 ± 0.27	0.93 ± 0.25
Grp-170	3.98 ± 0.45	4.31 ± 0.58	4.35 ± 0.59	2.83 ± 0.47
CAT	44.71 ± 5.80	54.80 ± 2.97	44.43 ± 5.36	47.64 ± 7.96
GPX4	24.14 ± 3.73	29.81 ± 1.57	22.78 ± 2.55	21.95 ± 2.27
GR	0.69 ± 0.04	0.84 ± 0.04	0.67 ± 0.07	0.70 ± 0.14
GST3	9.80 ± 1.78	11.56 ± 0.79	9.15 ± 1.56	13.75 ± 2.84
PRDX3	1.63 ± 0.20	1.72 ± 0.21	1.69 ± 0.17	1.42 ± 0.28
PRDX5	1.81 ± 0.19	2.06 ± 0.18	1.96 ± 0.20	2.06 ± 0.37
SOD2	3.12 ± 0.41	2.41 ± 0.30	2.58 ± 0.26	2.86 ± 0.44
ACAA2	0.55 ± 0.07	0.53 ± 0.08	0.44 ± 0.08	0.36 ± 0.03
CPT1A	0.94 ± 0.13^{b}	3.75 ± 0.26^{c}	0.86 ± 0.21^{b}	0.49± 0.12^{a}
ECH	4.23 ± 0.19	4.77 ± 0.63	4.72 ± 0.58	4.01 ± 0.61
HADH	5.24 ± 0.44	6.81 ± 0.59	4.76 ± 0.42	4.15 ± 0.97
CS	2.35 ± 0.09^{b}	4.26 ± 0.32 ^{c}	2.11 ± 0.23 ^{b}	1.49 ± 0.12 ^{a}
IDH3A	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
IDH3B	0.24 ± 0.03	0.29 ± 0.03	0.23 ± 0.03	0.23 ± 0.04
IDH3G	0.38 ± 0.01	0.40 ± 0.04	0.30 ± 0.04	0.33 ± 0.04
Cox4a	0.13 ± 0.01	0.17 ± 0.01	0.12 ± 0.01	0.14 ± 0.01
UCP1	18.07 ± 4.17	20.71 ± 1.85	17.86 ± 4.30	18.78 ± 3.35
UCP2	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
UCP3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Tom70	1.53 ± 0.11^{a}	2.47 ± 0.17^{b}	1.46 ± 0.11^{a}	1.49 ± 0.22^{a}
Tom34	0.45 ± 0.03^{a}	0.64 ± 0.05^{b}	0.46 ± 0.06^{a}	0.38 ± 0.03 ^{a}
Tom22	0.57 ± 0.04^{a}	1.20 ± 0.17^{b}	0.74 ± 0.12^{a}	0.82 ± 0.07^{a}
Tom7	0.88 ± 0.12	0.83 ± 0.09	0.66 ± 0.03	0.69 ± 0.12
Tom5	0.78 ± 0.09	0.85 ± 0.07	0.87 ± 0.08	0.88 ± 0.19

Supplementary table 4. Continued.

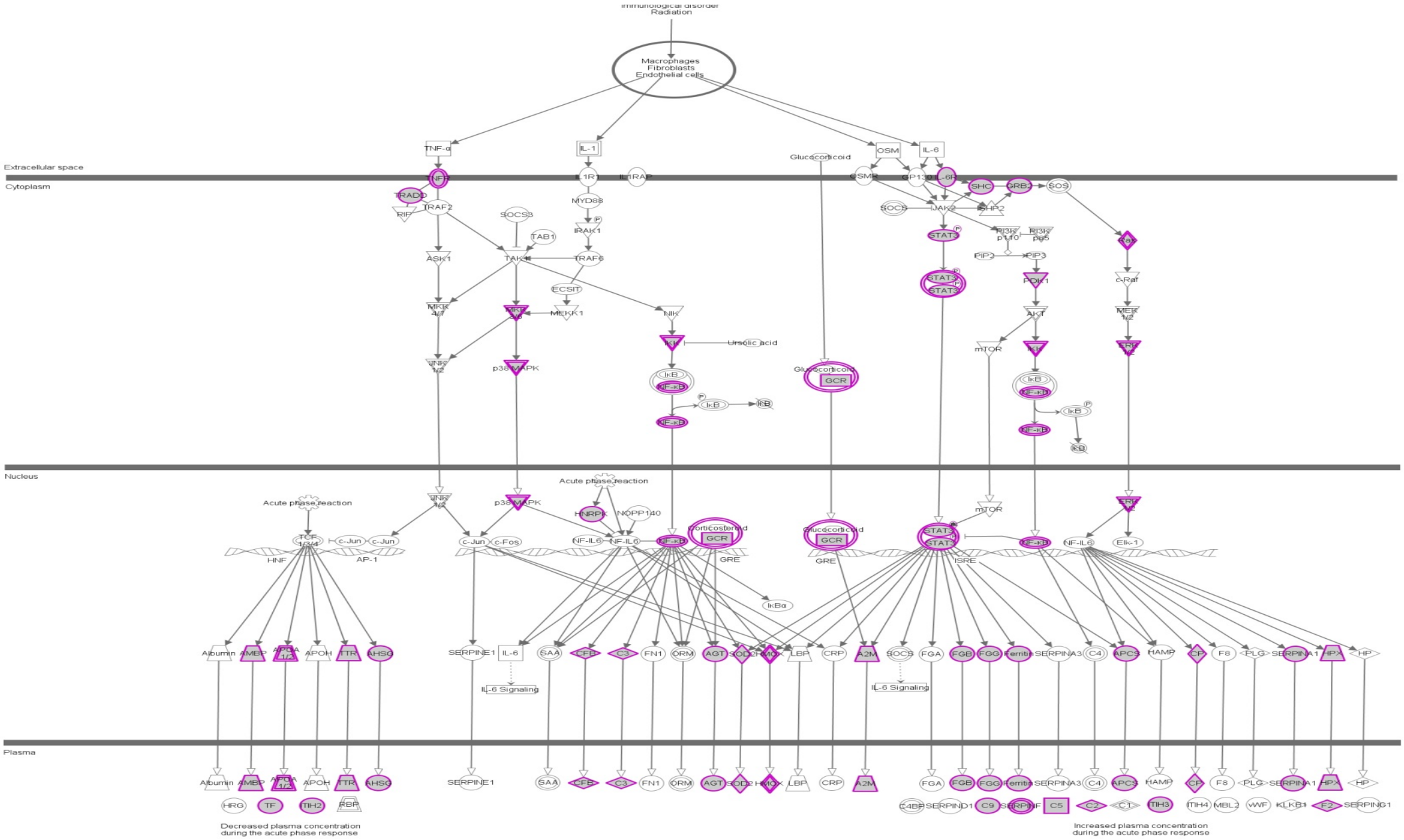
Gene name*	CTRL	T-ST	C-ST	M-ST
Tim44	0.08 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.07 ± 0.01
Tim23	0.38 ± 0.02	0.53 ± 0.05	0.48 ± 0.08	0.38 ± 0.05
Tim17A	1.41 ± 0.14	1.56 ± 0.09	1.48 ± 0.14	1.22 ± 0.17
Tim16	0.31 ± 0.02	0.32 ± 0.02	0.27 ± 0.02	0.26 ± 0.04
Tim14	1.72 ± 0.21	1.73 ± 0.06	1.51 ± 0.11	1.49 ± 0.11
Tim13	0.39 ± 0.03	0.48 ± 0.07	0.34 ± 0.06	0.34 ± 0.05
Tim8A	1.06 ± 0.06	1.09 ± 0.15	0.91 ± 0.09	0.78 ± 0.08
Tim22	0.22 ± 0.03	0.27 ± 0.03	0.22 ± 0.05	0.20 ± 0.04
Tim10	0.41 ± 0.08^{a}	0.70 ± 0.05^{b}	0.38 ± 0.10^{a}	0.39 ± 0.08^{a}
Tim9	0.37 ± 0.04^{a}	0.60 ± 0.06^{b}	0.36 ± 0.06^{a}	0.31 ± 0.03^{a}
FIS1	2.11 ± 0.15	2.79 ± 0.18	2.11 ± 0.15	1.91 ± 0.16
MIFFB	0.25 ± 0.02^{b}	0.23 ± 0.02^{ab}	0.17 ± 0.01^{a}	0.18 ± 0.03^{a}
MFN1	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
MFN2	0.44 ± 0.03^{a}	0.62 ± 0.07^{b}	0.38 ± 0.04^{a}	0.30 ± 0.04^{a}
MIRO1A	0.11 ± 0.01	0.13 ± 0.01	0.11 ± 0.02	0.10 ± 0.02
MIRO2	0.71 ± 0.03	0.74 ± 0.04	0.77 ± 0.10	0.59 ± 0.04
AIFM1	2.62 ± 0.39	3.26 ± 0.55	2.28 ± 0.19	3.01 ± 0.98
AIFM3	0.41 ± 0.03^{ab}	0.50 ± 0.04^{b}	0.36 ± 0.02^{ab}	0.32 ± 0.06^{a}
BAX	0.30 ± 0.03^{b}	0.35 ± 0.03^{b}	0.26 ± 0.02^{ab}	0.19 ± 0.02^{a}
Bcl-2	0.44 ± 0.08	0.38 ± 0.01	0.34 ± 0.03	0.34 ± 0.04
BCLX	0.81 ± 0.05^{b}	0.88 ± 0.08^{b}	0.67 ± 0.05^{ab}	0.55 ± 0.04^{a}
PERP	2.30 ± 0.15	2.35 ± 0.11	2.31 ± 0.17	1.97 ± 0.13
mtTFA	0.43 ± 0.03	0.37 ± 0.04	0.38 ± 0.04	0.36 ± 0.02
NRF1	0.14 ± 0.01^{a}	0.33 ± 0.04^{b}	0.13 ± 0.01^{a}	0.10 ± 0.01^{a}
NRF2	0.28 ± 0.02^{a}	0.51 ± 0.04^{b}	0.24 ± 0.01^{a}	0.20 ± 0.01^{a}
PGC1α	0.05 ± 0.00^{b}	0.32 ± 0.06^{c}	0.07 ± 0.02^{ab}	0.02 ± 0.01^{a}
PGC1β	1.05 ± 0.11	1.05 ± 0.13	1.21 ± 0.43	1.23 ± 0.16

* Gene identity determined through BLAST searches, for abbreviations of the genes, see supplementary table 1.

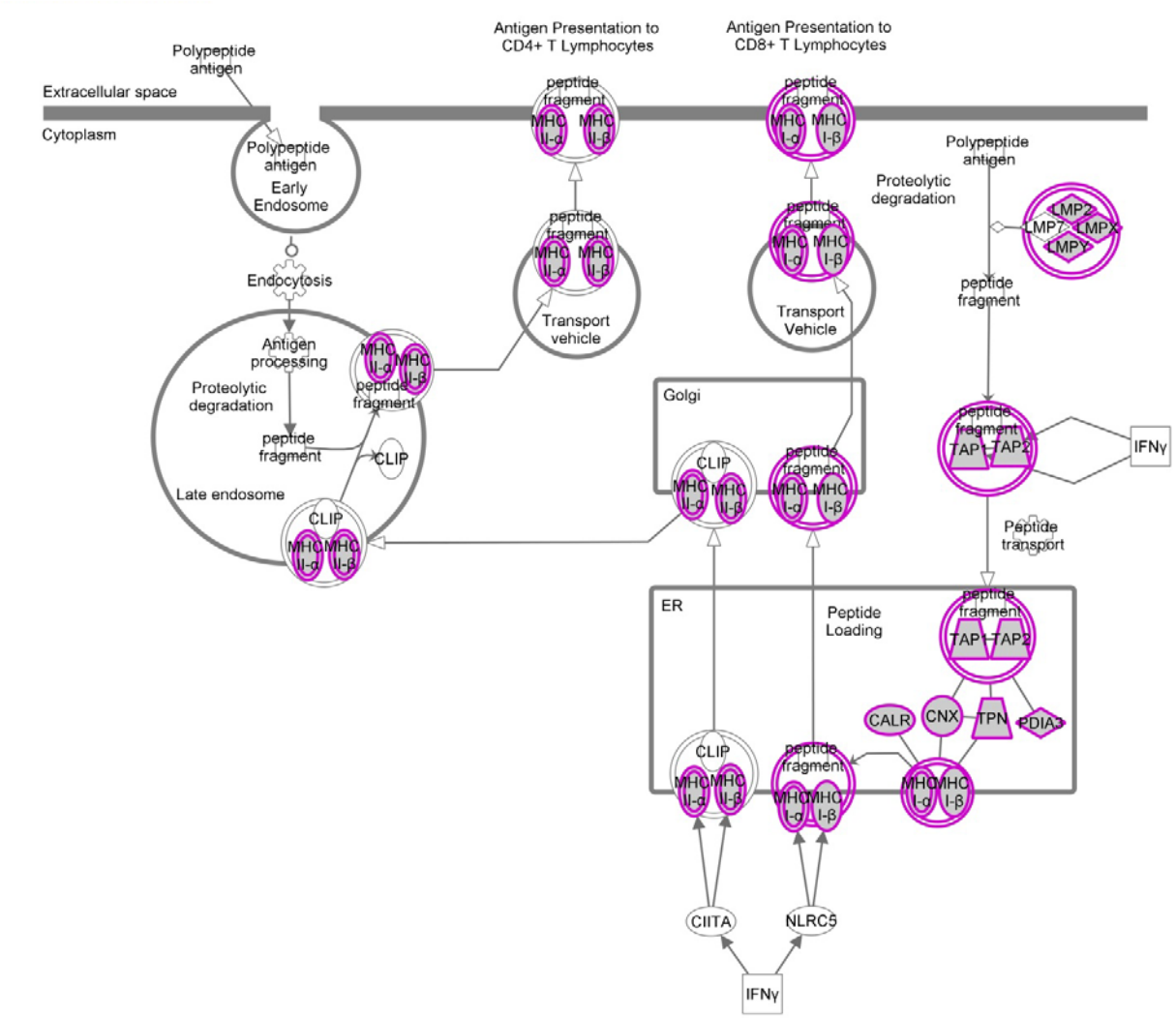
Supplementary Figure 1. Biological network of proteins linked to the canonical "mitochondrial dysfunction" pathway according to IPA. Proteins detected in the skin mucus of gilthead sea bream are coloured in magenta.



Supplementary Figure 2. Biological network of proteins linked to the canonical "acute phase responses" pathway according to IPA. Proteins detected in the skin mucus of gilthead sea bream are coloured in magenta.



Supplementary Figure 3. Biological network of proteins linked to the canonical “antigen presentation” pathway according to IPA. Proteins detected in the skin mucus of gilthead sea bream are coloured in magenta.



Annex 2- Publications related to D7.3

Bermejo-Nogales, A., Nederlof, M., Benedito-Palos, L., Ballester-Lozano, G.F., Folkedal, O., Olsen, R.E., Sitjà-Bobadilla, A., Pérez-Sánchez, J. (2014). Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata* L.) to environmental stress: New insights in fish mitochondrial phenotyping. General and Comparative Endocrinology 205: 305-315.

Calduch-Giner, J.A., Bermejo-Nogales, A., Benedito-Palos, L., Estensoro, I., Ballester-Lozano, G., Sitjà-Bobadilla, A., Pérez-Sánchez, J. (2013). Deep sequencing for *de novo* construction of a marine fish (*Sparus aurata*) transcriptome database with a large coverage of protein-coding transcripts. BMC Genomics 14: 178.

Colson, V., et al. Effect of chronic poor water quality on behavioural responses in rainbow trout (in preparation).

Pérez-Sánchez, J., et al. Protein profiling of skin mucus of gilthead sea bream in chronically stressed fish (in preparation).

Remen, M., Nederlof, M., Folkedal, O., Thorsheim, G., Sitjà-Bobadilla, A., Pérez-Sánchez, J., Oppedal, F., Olsen, R.E. (2015). The effect of temperature on metabolism and oxygen requirement of gilthead sea bream (*Sparus aurata*) and the behavioural response to a progressive decline in oxygen. Aquaculture Environment Interactions (under revision, 2nd version).

Deliverable Check list (to be completed by Deliverable leader)

	Check list		Comments
BEFORE	I have checked the due date and have planned completion in due time	☺	*Please inform Management Team of any foreseen delays*
	The title corresponds to the title in the DOW	☺	*If not please inform the Management Team with justification*
	The dissemination level corresponds to that indicated in the DOW	☺	
	The contributors (authors) correspond to those indicated in the DOW	☺	
	The Table of Contents has been validated with the Activity Leader	☺	*Please validate the Table of Content with your Activity Leader before drafting the deliverable*
	I am using the AQUAEXCEL deliverable template (title page, styles etc)	☺	*Available in "Useful Documents" on the collaborative workspace*
The draft is ready			
AFTER	I have written a good summary at the beginning of the Deliverable	☺	*A 1-2 pages maximum summary is mandatory (not formal but really informative on the content of the Deliverable)*
	The deliverable has been reviewed by all contributors (authors)	☺	*Make sure all contributors have reviewed and approved the final version of the deliverable. You should leave sufficient time for this validation.*
	I have done a spell check and had the English verified	☺	*Ask a colleague with a good level of English to review the language of the text and do a spell-check too.*
	I have sent the final version to the Activity Leader and to the 2nd Reviewer for approval	☺	*Send the final draft to your Activity Leader and the 2nd Reviewer and leave 2 weeks for feedback and final changes before the due date. Once validated by the 2 reviewers, the draft is ready to be sent to the Management Team that will ask for the Coordinator validation and then transfer it to the EC.*