

**ENVIRONMENTAL, NUTRITIONAL AND NEUROENDOCRINE
REGULATION OF SKIN COLORATION IN THE RED PORGY (*PAGRUS
PAGRUS*), TOWARDS THE DEVELOPMENT OF NATURAL HUE IN
CULTURED POPULATIONS**

FINAL REPORT



Acronym: COLORED

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Project Summary

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ENVIRONMENTAL, NUTRITIONAL AND NEUROENDOCRINE REGULATION OF SKIN COLORATION IN THE RED PORGY (<i>PAGRUS PAGRUS</i>), TOWARDS THE DEVELOPMENT OF NATURAL HUE IN CULTURED POPULATIONS		
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MATERIALS AND METHODS

EXPERIMENT 3. Manipulation of husbandry conditions towards the development of natural skin colour

The objectives of Experiment 3 were (a) to determine the effects of environmental and culture related factors (light, background colour, handling, and stocking density) on colour change, (b) to evaluate the relationship between pigmentation and stress, and (c) to identify the appropriate culture conditions for the development of natural hue. This study will allow to evaluate the relationship between pigmentation and stress and to identify the appropriate culture conditions for the development of natural hue. The experimental approach involved (i) the investigation of the effect of illumination and background colour on skin colour, (ii) the study of the changes in colour after acute capture and chronic crowding stress, and (iii) the study of the changes in colour after sacrifice in ice and post-mortem

3.1 Role of illumination and background colour

Experiment 3.1.1

This pilot experiment was performed at IMBC. Forty immature 11-month-old red porgies weighing ($\bar{x} \pm \text{S.D.}$) $101,5 \pm 15,8$ g, reared under intensive culture conditions were distributed randomly into four 500 l circular black background tanks (10 fish/tank) supplied with continuously aerated and running sea water (dissolved O_2 , 6,5 – 7,6 mg/l, water temperature, 14,5 – 17,3°C throughout the experimental period) and acclimated for one week before experiments started. Fish were fed by a self feeder system delivering commercial dry pellets (Biomar, Hellas). On the day of sampling no food was given. Black plastic curtains surrounded each set of tanks (**Figure 6**) and artificial illumination was achieved using fluorescent strip lights located 130 cm above the water surface (tank depth 1m), controlled by electric time clocks, set for a 12L:12D photoperiod regime (dawn at 6:00h and dusk at 18:00h). Two experimental groups were formed, according to the light spectrum: Group CON (control), fish exposed to full spectrum, and Group BLU: fish exposed to light with maximal absorbance in blue wave length (475 nm). Experiments were conducted in duplicate tanks.

Sampling took place on 0, 1, 3, 9 and 27 days after the start of the exposure of fish to the different spectra. Fish were lightly anaesthetised (0,25 ml/l Ethynelglycol-monophenylether, Merck, Hohenbrunn), their head was covered with a wet towel, skin colour measurements and blood sampling was performed. Plasma was aliquoted for the determination of glucose (IMBC), cortisol and MSH (KUN).

The last sampling date, 6 fish from each treatment were sacrificed for determination of melanin content in the skin.



Figure 6. Tank system and textile bags manufactured for the needs of the project and used as background colour in Experiment 3.

Experiment 3.1.2

This pilot experiment was performed at IMBC. A number of cultured fish was transferred to a 500 l circular polyester tank which had white nylon cloth perfectly fitting to the bottom and sides of the tanks to create a white background. The fish were illuminated by blue light (light intensity 148 lux); to eliminate the daylight the tank was placed within a black plastic “tent”.

After two weeks of acclimatisation, the fish had turned quite pale, and are therefore later on referred to as “pale fish”. Six fish were caught by net from the tank and placed into a bucket containing water with 2% phenoxy ethanol for anaesthesia. Notably, the fish started to show dark bars over their body while the experimenter was trying to catch them, and they turned quite dark after a short while in the bucket. At the same time, six fish were caught from one of the other tanks as controls (exposed to full spectrum light, with a similar intensity of 151 lux). After weighing, the blood was drawn as mentioned before to obtain plasma, which was subsequently frozen. The fish were then decapitated, the pituitary and the brain were dissected and fixed in Bouin’s fixative (8g of crystalline picric acid, 1ml acetic acid and 10ml formaldehyde, filled up to 100ml with demineralised water). A small piece of gills, taken from the second gill arch at the right hand side was also fixed together with a small piece of skin taken from the forehead of the fish, just above the eyes.

After two weeks of fixation, the samples were washed in demineralised water and subsequently dehydrated through an ethanol series and xylene before embedding into paraffin. Sections of 5µm thickness were cut with a microtome. For general overview, the sections were stained with Haemalum-Eosin. The pituitaries were stained with an α MSH antibody to locate the MSH cells in the pituitary and compare number and activity of these cells. SL-cells were localised by PAS-staining.

Plasma was analysed for MSH and cortisol levels through RIA and examined for lactate and glucose concentrations through commercial kits. The brains, skin and gills of this experiment were embedded for further analysis.

Experiment 3.1.3

The third pilot was performed at ICCM. While angling on the fishing boat, a small number of fish were sampled immediately after capture. This time point was designated as time zero. Length and weight were recorded, blood plasma was sampled as mentioned before, and pituitary, head skin and gills were taken and fixed in Bouin's fixative (picric acid : concentrated formaldehyde : acetic acid; 15/5/1). The other fish were kept in closed rectangular tanks on the deck of the vessel with a continuous flow of fresh seawater.

After returning to shore, the fish were transferred to four circular tanks, about 12m³ in size. Of these fish, 45 were distributed over 5 smaller tanks for the MSH release experiments as mentioned before. This left about 200 fish, 50 in each large tank. On day 3, day 7 and day 14, five fish were caught, each time from a different large tank, before feeding had occurred (fish were fed each morning around 10 a.m.). These five fish were anaesthetised in water containing 2% phenoxy ethanol, blood was sampled to obtain plasma and the pituitary, head skin and gills were taken and fixed in Bouin's fixative. Of one of the five fish, the pituitary was fixed for EM, and of this fish and three others of which the skin and gills had also been fixed in Bouin, a very small piece of skin and of the gills was fixed for electron microscopy (EM). EM fixation was performed as follows: pre-fixation, in a mixture of 25% EM standard glutaraldehyde, 0.4M cacodylate buffer and demiwater; 1/2/5. Prefixation lasted between 15 min and 1 hour, after which the samples were rinsed shortly in 0.1M cacodylate buffer. Fixation occurred with 2% osmiumtetroxide and 0.2M cacodylate buffer; 1/1 mixture. This step took approximately one hour. After fixation, the samples were thoroughly rinsed in demiwater.

The Bouin-fixed samples were dehydrated and embedded in paraffin, and cut into sections of 5µm thickness. Pituitaries were stained with an α -MSH antibody to locate the MSH cells in the pituitary and compare number and activity of these cells. SL-cells were localised by PAS-staining. The EM fixed pituitaries were dehydrated, embedded in resin, and cut into ultra-thin sections for micro-photographic analysis.

Plasma was analysed for MSH and cortisol levels through RIA and examined for lactate and glucose concentrations through commercial kits. Skin and gill pieces sampled were embedded in paraffin, for further analysis. This was also the case for the EM fixated pieces of gill and head skin.

Experiment 3.1.4

This pilot experiment was performed at the IMBC. 90 red porgies weighing ($x \pm$ S.D.) 350 ± 74 g, reared under intensive culture conditions were distributed randomly into 15 x 500 l circular tanks (6 fish/tank) and exposed for 1 week to different light spectra and background colour (**Table 3**). Rearing conditions were as in Experiment I, except of the photoperiod that was set at 24L:0D. At the end of the experimental period skin colour measurements were performed and blood was collected for glucose, lactate, epinephrine, norepinephrine (IMBC), cortisol and MSH determination (KUN) through RIA. Background colour was achieved by the use of special textile bags which manufactured at IMBC (**Figure 6**).

Table 3. Experimental groups and chromaticity parameters of the textile bags used as background colour.

Background colour	CIELAB color parameters			Light spectrum		
				Full	Blue (475 nm)	Red (650 nm)
	L*	a*	b*	Groups		
Black	11,54	2,87	4,41	BF	BB	BR
White	72,71	-1,45	-1,80	WF	WB	WR
Blue	25,14	-3,22	-3,75	BF	BB	BR
Red1	27,20	30,51	13,21	R ₁ F	R ₁ B	R ₁ R
Red2	47,38	64,11	39,49	R ₂ F	R ₂ B	R ₂ R

Experiment 3.1.5

Cultured fish, from IMBC (100g body weight), were kept in 10m³ circular polyester tanks filled with sea water, continuously replaced with a mixture of 1/3 fresh sea water and 2/3 recycled water. About 30 fish were present in each of eight tanks at the start of the experiments. Temperature of the water was measured daily, together with the oxygen levels of the water. Fish were fed with self-feeders containing INVE pagrus feed. Upon the start of the experiment, two fish per tank were sampled. To sample all the fish for colour, blood and weight took 1 hour. Colour measurements were taken immediately, after which the fish was placed in a water filled bucket containing phenoxyethanol (approx. 2 ml/l). Then blood samples were taken. After this the weight was recorded along with any external abnormalities and the fish was placed in a plastic bag with label. The blood was taken from the caudal vein of the fish, and was divided into two eppendorfs, each containing around 700µl of blood. One series of eppendorfs was used to obtain serum (UAB), the other series contained 10µl of Na-EDTA (to prevent clotting) and 50µl of aprotinin (to prevent proteolysis) and was used to obtain plasma. The plasma was spun immediately after finishing the sampling (4°C, 3000 RPM, 5 min), and osmolality was determined. The plasma was stored ($\leq -20^{\circ}\text{C}$) until analysed for glucose and lactate and for cortisol, MSH and MSH isoforms. After preparing the plasma, the fish were removed from the plastic bags and tissue samples were taken.

Pooled plasma samples (n=4 per group) were also used for catecholamines (NE, E) determination by commercial RIA kits (KatCombi RIA 100, IBL-Hamburg). Extraction efficiency was evaluated by the addition of unlabeled hormone to the plasma samples. The average recoveries for NE and E were 80.1 ± 5.3 and 82.1 ± 4.0 , respectively. The RIA procedure was validated by verifying that displacement of labeled hormone by serial dilutions of the extract was parallel to the standard curve. From the 16 control fish, we sampled 2 sets of pituitary, skin and gills for E.M., 3 sets of pituitary, skin and gills for LM, and 5 pituitaries for MSH contents. These were maintained in Teleost Physiological Saline, and homogenised using a Potter device in 200µl of 0.1M HCl. After homogenising, the samples were spun (6000 RPM, 5 min) and the supernatant was stored with the plasma samples.

After sampling of these control fish, nylon white and red covers that were made to fit the tanks were placed inside the tanks after which the full spectrum lights were replaced with blue TL-tubes (light intensity 148 lux).

For all the other sampling times (day 2, 8, 16 and 32), five fish per tank were sampled according to the same procedure. After sampling, the level of the water was lowered by replacing the central drain pipe with a pipe that was one-fifth shorter than the one before. In this way, a constant density was kept throughout the experiment. These pipes were painted in the same colour as the background of the tanks. After this, the fish were dissected. Of each tank (5 fish), one set of pituitary, skin and gills for EM was taken, together with one set of pituitary, skin and gills for LM, and of the two subsequent fish, the skin and gills were fixed for LM and the pituitary was placed in TPS to be homogenized for determination of MSH isoform contents. At the end of the experiment (Day 32) skin samples from D1 area (**Figure 4**) were taken for melanin determination (n=6-10 per group).

3.2 Changes in colour after acute stress

For this experiment, 400g fish, supplied by Interfish Inc., were used. Upon arrival of fish at IMBC, 10 fish per tank were transferred to 10m³ circular polyester tanks filled with sea water, continuously replaced with a mixture of 1/3 fresh sea water and 2/3 recycled water. Five tanks were covered with white nylon covers, four with red covers. All tanks received blue illumination and fish were fed as described before (*Experiment V*). After allowing the fish to adapt for two weeks, fish were stressed by applying confinement and air exposure for five minutes. From one white and one red tank, fish were sampled immediately after stressing. The other four white tanks were sampled 1 h, 2 h, 8 h or 24 h after applying the acute stress stimuli; the other three red tanks were sampled two, eight or 24 hours after stressing. Colour samples were taken at 0 h and 24 h after air exposure. Tissue samples (head skin, gills, pituitary glands for LM and EM analysis) were taken at 0, 2 and 24 h. Blood samples were taken at all time points. After spinning the plasma (4°C, 3000 RPM, 5 min), Na, Cl and K concentrations were determined. The plasma was stored ($\leq -20^{\circ}\text{C}$) until analysed for glucose and lactate (commercial kits; Sigma) and for cortisol, MSH and MSH isoforms (RIA).

3.3 Changes in colour after chronic crowding stress

For this experiment, 400g fish, supplied by Interfish Inc., were used. Upon arrival of fish at IMBC, 10 fish per tank were transferred to 10m³ circular polyester tanks filled with sea water, continuously replaced with a mixture of 1/3 fresh sea water and 2/3 recycled water. The set-up of this experiment was as follows: fish were subjected to a black, red or white background (red and white tanks were covered with nylon covers in these colours); to full spectrum or blue spectrum illumination (“white” light intensity 151 lux; blue light intensity 148 lux) and either a high density (25 kg fish/m³) or a low density (10 kg fish/m³) (**Table 4**).

17 control fish were sampled from black background, full spectrum tanks. Experimental fish were sampled 8, 16 and 30 days after transfer to the different tanks. Colour measurements were taken immediately, after which the fish was placed in a seawater filled bucket containing phenoxyethanol (approx. 2 ml/l). Then blood samples were taken. After this the weight was recorded (along with any external abnormalities) and the fish was placed in a plastic bag with label. The blood was

taken from the caudal vein of the fish, and was divided into two eppendorfs, each containing around 700µl of blood. One series of eppendorfs was used to obtain serum (UAB), the other series contained 10µl of Na-EDTA (to prevent clotting) and 50µl of aprotinin (to prevent proteolysis) and was used to obtain plasma. The plasma was spun immediately after finishing the sampling (4°C, 3000 RPM, 5 min), and osmolality was determined. The plasma was stored ($\leq -20^{\circ}\text{C}$) until analysed for glucose and lactate and for cortisol, NE, MSH and MSH isoforms.

Table 4: Experimental set up for chronic crowding stress experiment . 10 fish were used per treatment.

Background colour	Black				Red				white			
Lighting spectrum	Full		Blue		Full		Blue		Full		Blue	
Density (kg/ m ³)	10	25	10	25	10	25	10	25	10	25	10	25

After preparing the plasma, the fish were removed from the plastic bags and tissue samples were taken (head skin, gills for LM and/or EM stress parameters and pituitary glands for homogenisation and LM and EM). Plasma samples were analyzed for Na, Cl and K content (by auto analyzer) and for NE, MSH and cortisol content (by RIA) and for glucose and lactate content (by auto analyzer).

At the end of the experiment skin samples were also taken for the estimation of the number and density of melanophores and for the determination of melanin content.

3.4 Changes in colour after sacrifice in ice and post-mortem

The objective was to describe possible post-mortem changes in skin colour and to investigate the effect of diet and background colour on skin colour following harvesting.

Experiment 3.4.1

In the first experiment, conducted in ICCM, fish fed diets containing different carotenoid sources and concentrations were used. The experimental diets used were formulated in the subsequent manner (*for more details see EXPERIMENT 4.1*):

- **Diet 1** : Negative control (no carotenoid added)
- **Diet 2** : Astaxanthin (20 mg/kg diet)
- **Diet 3** : Astaxanthin (40 mg/kg diet)
- **Diet 4** : Astaxanthin (60 mg /kg diet)
- **Diet 5** : 15% krill meal
- **Diet 6** : 30% Krill meal
- **Diet 7** : 100% Fish meal with lipids from lobster (natural carotenoid)
- **Diet 8**: Japanese commercial feed for Japanese red sea bream (Otohim, Japan)

Fish fed the experimental diets for a period of 75 days. A week after the end of the feeding trial, 8 fish from each tank were sacrificed in a mix of ice and water and placed in polystyrene boxes at 4 °C (as in commercial preparation for the markets). Skin colour measurements in the dorsal part of the fish was determined 24 and 48 h post-mortem.

Experiment 3.4.2

In this experiment, conducted in ICCM, fish fed diets containing shrimp meal (*Sopropeche, France*), containing 40 mg/kg of esterified astaxanthin, for different time periods (**SM40 BI**: for 6 months; **SM 40 BII**: for 4 months; **SM 40 BIII**: for 2 months), were sacrificed in a mixture of ice and water and used to evaluate skin colour changes for 7 days (*for more details see EXPERIMENT 4.3*).

Experiment 3.4.3

In this experiment, conducted in IMBC, fish fed a non-carotenoid diet, were placed in black or white background 500 l circular tanks for a period of 4 weeks. At the end of the experiment fish (n=12 per group) were sacrificed in a mix of ice and water, placed in polystyrene boxes and then in a cold room (4 °C) for a period of 7 days. At regular intervals (0, 1, 3, 5 and 7 days) fish were sampled, rigor mortis was calculated and colour measurements at the dorsal and ventral body area were performed.

Experiment 3.4.4

This experiment was conducted at the Installations of Interfish S.A. Red porgies (n=15) reared in sea cages and fed a commercial diet (ProAqua, Dieta 1, Spain), prepared according to the results of *Experiment 4* (for details see *Experiment 5.2*), were harvested in a mix of ice and water and transferred within 10 min to the Laboratory unit of INTERFISH, where the following parameters were recorded (Day 0): body weight, rigor mortis, skin colour measurements, freshness and ocular refractometry. In addition, muscle samples were taken and place in perchloric acid for determination of the *K*value. Following this initial sampling fish were packaged in polystyrene boxes at 0-1 °C, transferred at the Fish Physiology Laboratory (Department of Biology, UoC), and placed in a cold room (4 °C). At regular intervals (Day 1, 3, 5, 7) fish were sampled and the same as in Day 0 measurements were performed.

Statistical analysis

Statistical analysis in *Experiment 3* was performed with SPSS 11.0 statistical software for Windows. Results were analyzed by non parametric ANOVA followed by Tukey HSD or Dunn's tests to test the difference between a value compared with the control or time zero value. For all statistically significant differences, the following marking pattern was used: columns sharing the same letter (black for wbg fish and red for rbg fish) are not significantly different from each other through time; a vs b / b vs c / c vs d → P<0.05; a vs c / b vs d → P<0.01 and a vs d → P<0.001. * indicates differences between treatments (*→ P<0.05 and ***→P<0.001).

Statistical analysis of the colour parameters was performed as described in *Experiment 1.1*.

RESULTS

EXPERIMENT 3. Manipulation of husbandry conditions towards the development of natural skin colour

3.1. Role of illumination and background colour

Experiment3.1.1

There was a significant effect of light spectrum on skin colour. In particular, fish exposed to blue spectrum became pale and showed statistical significant higher lightness value (L) than controls (**Figure 41 & 42**). There was no statistical significant difference in plasma glucose, cortisol and MSH between fish exposed to full (control) and blue light spectrum (**Figure 43**). Besides, there was no significant difference in skin melanin content at the end of the in the tested groups. Statistical higher glucose concentration was observed in both groups on day 1(D1). Statistical lower MSH concentration was observed in both groups on day 3 (D3).



Figure 41. Red porgies held in black background tanks under blue light spectrum, at the end of the experiment (day 27).

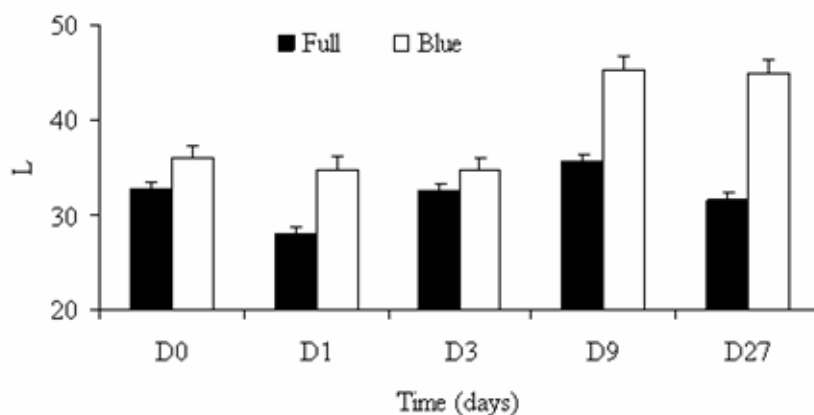


Figure 42. Lightness values (L) of the dorsal skin area in red porgies held in black background tanks under full (black columns) or blue (white columns) lighting spectrum.

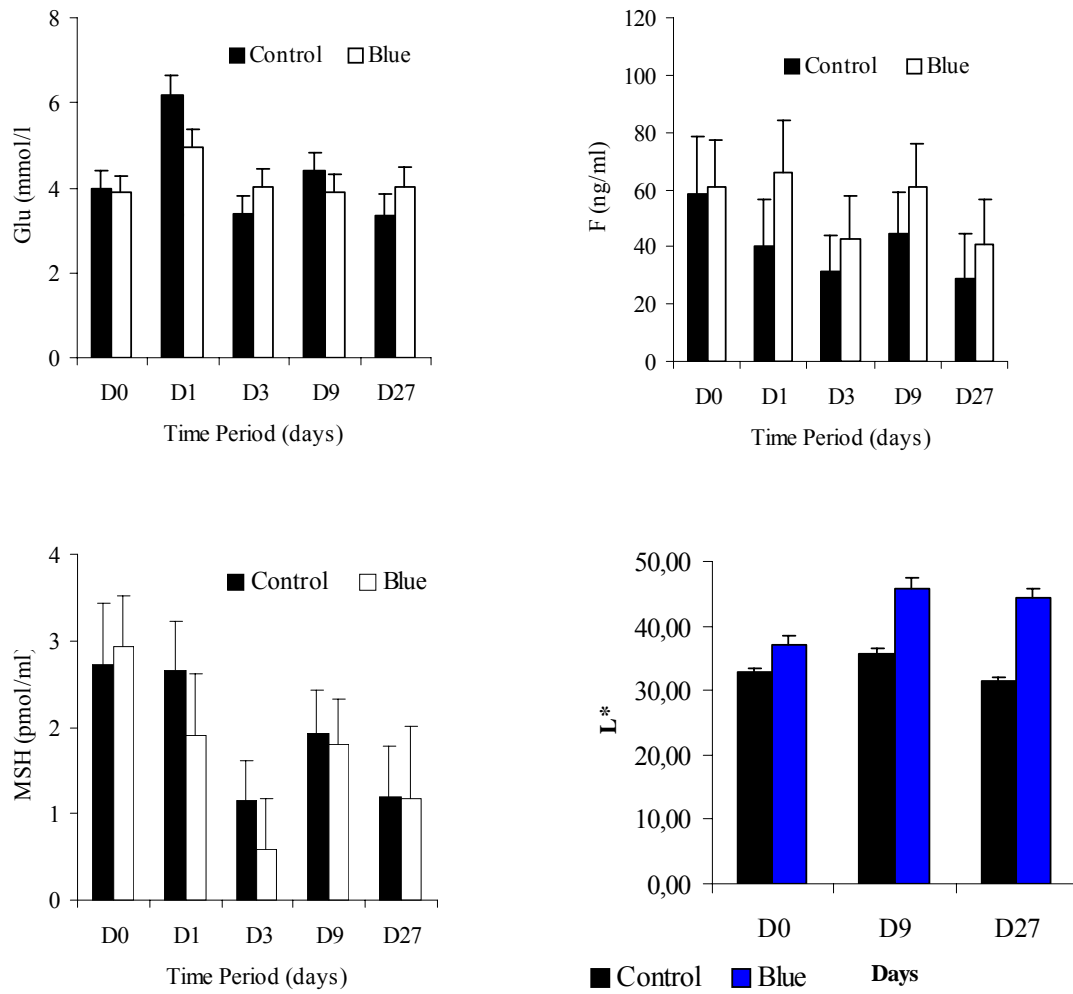


Figure 43. Plasma concentrations of glucose, cortisol and MSH and lightness parameter L*, in red porgy held in black background tanks and blue light spectrum for a period of 27 days.

Experiment 3.1.2

The fish that had been acclimatised to a white background under blue illumination, showed a much lighter colour than control fish that had been in black coated tanks under normal daylight. This colour rapidly darkened when the fish were caught and transferred to the anaesthetic. In the plasma, cortisol concentrations were about twice as high in pale fish compared to control fish (**Figure 44**, non significant). MSH levels were slightly higher in pale fish. For comparison, the average plasma concentration for cortisol in red porgy was 33.022 ng/ml and for MSH 0.762 pmol/ml (n=116 fish).

Glucose and lactate were also determined in the plasma as stress indicators. However, no significant difference was observed (control fish: glucose $12,1 \pm 0,88$ nmol/ μ l; lactate $0,53 \pm 0,08$ nmol/ μ l; pale fish: glucose $7,38 \pm 0,81$ nmol/ μ l; lactate $0,39 \pm 0,07$ nmol/ μ l; these values fall well within the range measured for untreated red porgy). Immunohistochemistry on the pituitary showed no difference in the amount of α -MSH cells. A small number of SL cells was visible in the pituitaries of both groups.

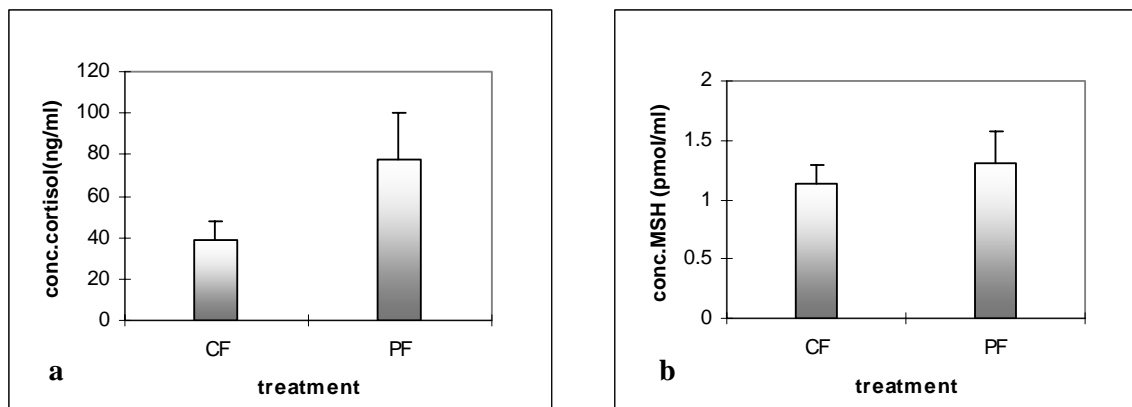


Figure 44. Differences in plasma cortisol levels (a) and MSH levels (b) between pale fish (PF), acclimatised to a white background for two weeks, and control fish (CF).

Experiment 3.1.3

Acclimation of wild fish to indoor tanks over two weeks showed an increase in plasma cortisol levels up to 7 days, after which a decline was observed (**Figure 45**). Plasma MSH levels stayed low in that first week and then increased five-fold at 14 days. These results indicate that the rise in cortisol is not mediated through the alternative stress axis in which MSH can release cortisol. Also, it seems that after one week of acclimatisation the fish are settling down and stress levels (as indicated by the cortisol concentration) decrease. Although the MSH levels were increased at 14 days, the fish had not yet developed a darker colour. Average values of cortisol and MSH in wild fish were 49.210 ng/ml and 0.9311 pmol/ml, respectively. Plasma glucose and lactate were also measured (**Table 15**). Glucose levels are in the lower part of the range measured for this species in wild fish, while lactate is low to average (mean value of glucose $5,13 \pm 0,62$ nmol/ μ l; lactate $0,33 \pm 0,06$ nmol/ μ l; n=21 wild fish)

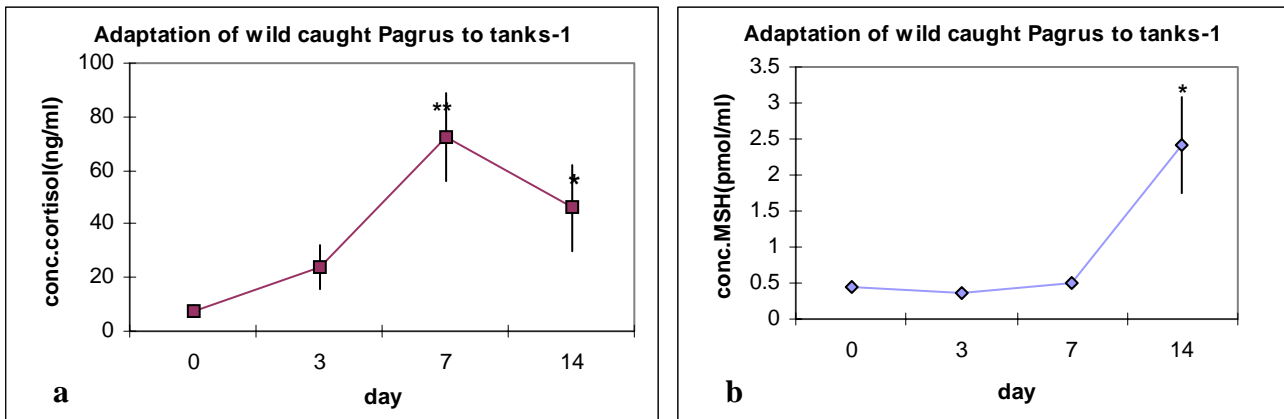


Figure 45. Plasma cortisol concentrations (a) and MSH levels (b) in wild caught fish acclimatised to indoor tanks for two weeks. (*, $p \leq 0.05$; **, $p \leq 0.01$).

Table 15. Plasma glucose and lactate concentrations in wild caught fish adapted to indoor tanks for two weeks

Time point (days)	Glucose (nmol/ μ l)	Lactate (nmol/ μ l)
0	3,72 \pm 0,83	0,54 \pm 0,13
3	6,35 \pm 0,53	0,19 \pm 0,03
7	5,32 \pm 0,32	0,14 \pm 0,02
14	5,92 \pm 1,99	0,30 \pm 0,06

In the pituitary, again a low number of SL cells was observed in all groups (no differences). Concerning the number of α -MSH however, a decrease in the number of immunoreactive cells was visible from day 0 up to day 14. This could indicate that the cells are releasing more MSH into the blood circulation, resulting in higher plasma MSH levels (as observed, **Figure 45**) and a lower pituitary MSH content.

Experiment 3.1.4

The effect of different background and lighting spectra on skin colour parameters is shown in **Figures 46 & 47**. There was not a statistically significant interaction between background and spectrum in light intensity (L) of the dorsal skin area of the fish (**Figure 46**). The highest light intensity value was observed in fish held under white background ($L = 49.34 \pm 1.65$) and the lowest under black background ($L = 38.45 \pm 1.34$). In addition, fish exposed to red and blue lighting spectra were brighter than those exposed to full spectrum, after allowing for effects of differences in background. Concerning the ventral area of the skin, there was a statistically significant interaction between background and lighting spectrum. In general, there was not any significant effect of background on L value in fish held under red lighting spectrum, while in fish exposed to full or blue spectra, the highest L value was observed under the white background.

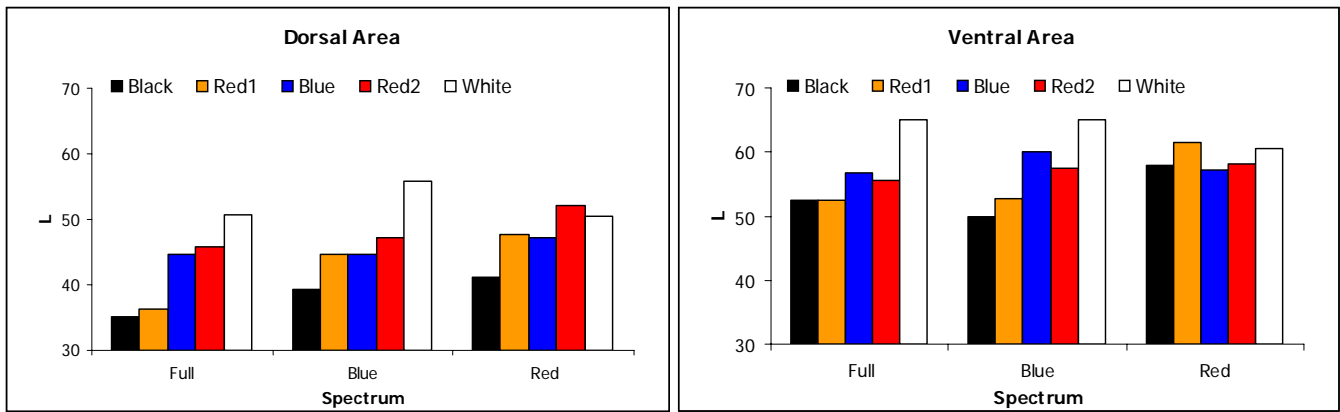
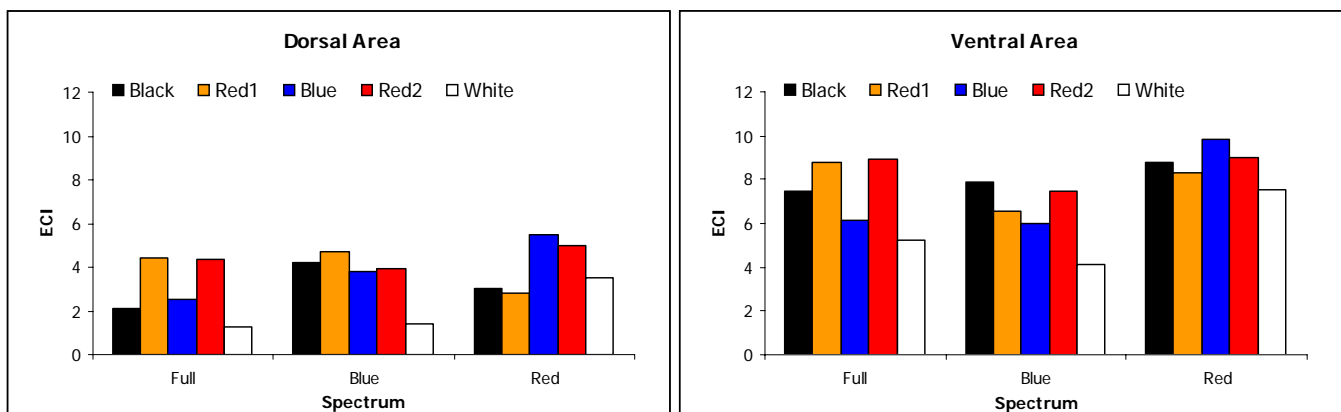


Figure 46. Effect of background and lighting spectra on the light intensity value (L) of the skin of reared red porgy.

Lower ECI was observed in fish held under white background, regardless of the lighting spectrum (**Figure 47**). This difference was more evident at the dorsal skin area. Within full spectrum, higher ECI was found in fish held under red backgrounds, while within black background higher ECI (dorsal skin area) was found in fish exposed to blue spectrum.

Figure 47. Effect of background and lighting spectra on the Entire Colour Index (ECI) of the skin of reared red porgy.



There was no statistically significant difference in plasma Cortisol, MSH, glucose and lactate between the experimental groups.

Experiment 3.1.5

Three Way Analysis of Variance showed that there was a statistically significant interaction between background, light intensity and time (day) in skin colour parameters L and ECI. This indicated that the effect of one factor was not consistent at all combinations of the two other factors, and therefore, an unambiguous interpretation of the main effects was not possible. There was not any statistically significant difference in L and ECI among the different levels of light intensity after allowing for the effects of differences in background and time.

Results were in accordance with those obtained in Experiment 4. L value was significantly increased following 2 days adaptation to white backgrounds and remains high till Day 32 (white background-low light intensity: 66.68 ± 0.91 ; red background-high light intensity: 55.96 ± 0.75) (**Figure 48**). On the contrary, maximum ECI was found in fish held under red background-high light intensity (10.54 ± 0.37), and the lowest in fish adapted to white background-low light intensity (3.90 ± 0.48) (**Figure 49**).

There was a statistically significant effect of background and light intensity on the skin melanin content of red porgy (**Figure 50**). Higher melanin content was observed in fish exposed to high light intensity, regardless of the background (Low intensity: $2.77 \pm 0.09 \mu\text{g}/\text{mm}^2$; High intensity: $4.00 \pm 0.07 \mu\text{g}/\text{mm}^2$). There was not a statistically significant difference in skin melanin among the red and white background adapted fish within low light intensity (Red background: $2.74 \pm 0.05 \mu\text{g}/\text{mm}^2$; White background: $2.80 \pm 0.12 \mu\text{g}/\text{mm}^2$), while a significant difference was observed among the two backgrounds within the high light intensity (Red background: $4.72 \pm 0.13 \mu\text{g}/\text{mm}^2$; White background: $3.28 \pm 0.09 \mu\text{g}/\text{mm}^2$).

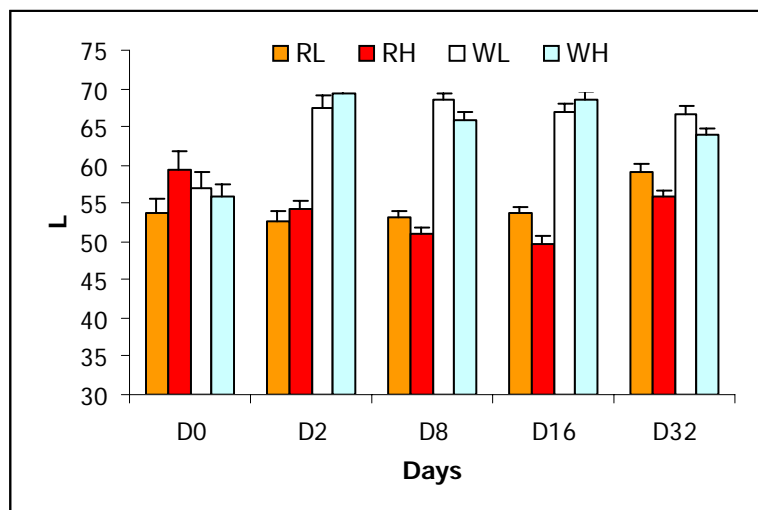


Figure 48. Effect of background and light intensity on skin lightness (L). R: red background, W: white background, L: low light intensity (130 Lux), H: high light intensity (2,000 Lux). All fish were exposed to blue lighting spectrum for 32 days.

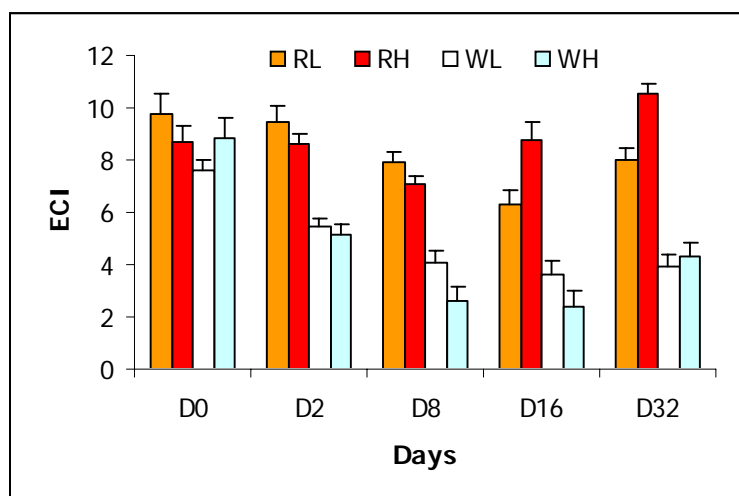


Figure 49. Effect of background and light intensity on skin colour parameter ECI. R: red background, W: white background, L: low light intensity (130 Lux), H: high light intensity (2,000 Lux). All fish were exposed to blue lighting spectrum for 32 days.

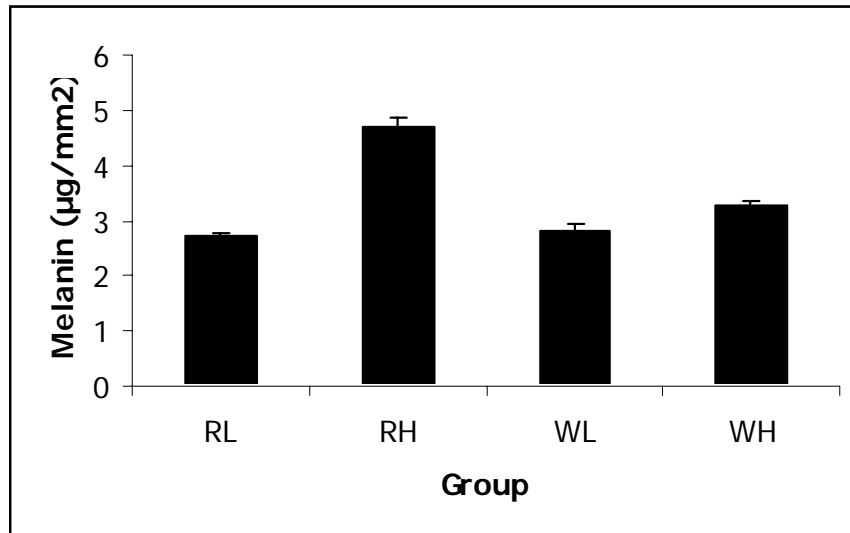


Figure 50. Effect of background and light intensity on melanin skin content of red porgy sampled on Day 32 following exposure to different backgrounds and light intensities. R: red background, W: white background, L: low light intensity (130 Lux), H: high light intensity (2,000 Lux).

There was no statistically significant difference in melanophore densities (number of cells per mm^2 of skin) between the different light intensities among each experimental background (**Figure 51**). However, for both light intensities, fish exposed to red background showed higher melanophore densities in the dorsal skin area than those exposed to white background. In general, melanophore densities in the dorsal body area ranged from 81.3 ± 20.7 (WL) to 90.9 ± 22.2 cells/ mm^2 (RL) and in the ventral area (K1), from 18.5 ± 12.1 (WH) to 23.4 ± 14.2 cells/ mm^2 (RH).

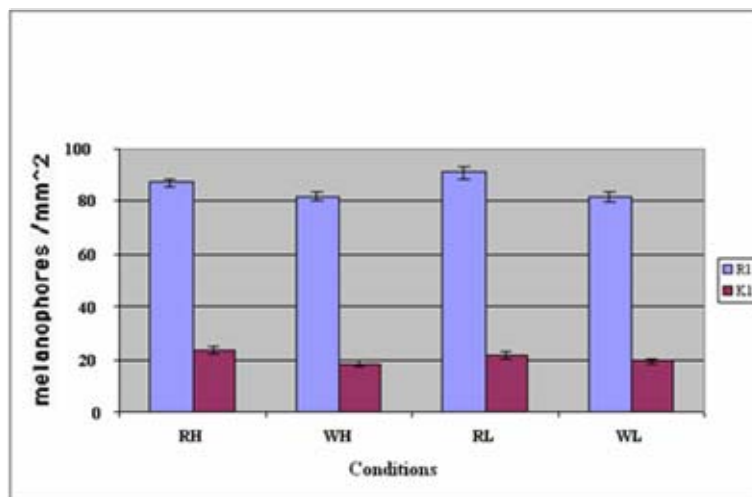


Figure 51. Number of melanophores per mm^2 of the dorsal (R1) and ventral (K1) skin area of red porgies exposed for 32 days to different backgrounds (R: red, W: white) and light intensities (L: low light intensity, 130 Lux; H: high light intensity, 2,000 Lux).

Plasma glucose and lactate were not statistically significant different between treatments or time points, although there is a steady, minor decrease visible through time (**Figure 52a**). Plasma lactate was not altered by time or by treatment (**Figure 52b**).

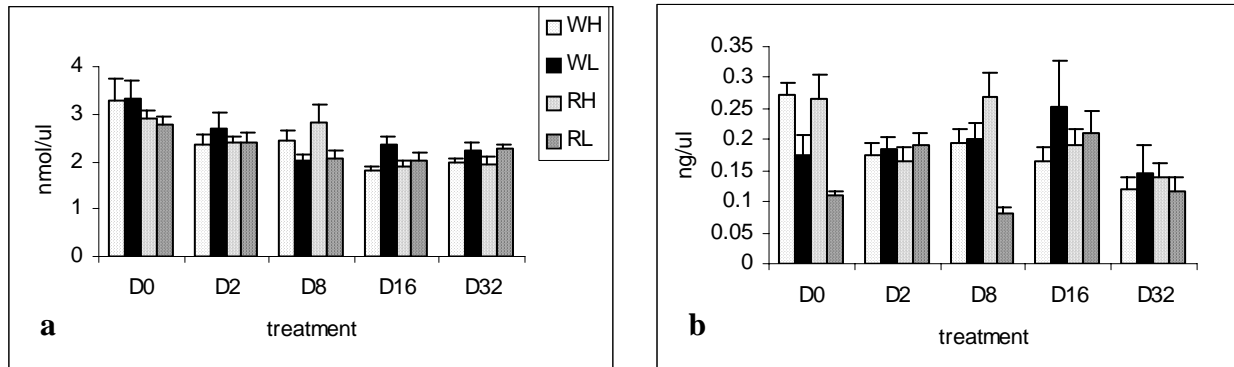


Figure 52. Plasma glucose (a) and lactate (b) from fish exposed to different background and light intensity. For details see Figure 48.

Plasma osmolality was not significantly different between time points or treatments (**Figure 53a**). Plasma cortisol was elevated at day 2 but levels returned to more normal values from day 8 onwards (**Figure 53b**). Individual variation in cortisol levels was quite high (visible in the large error bars).

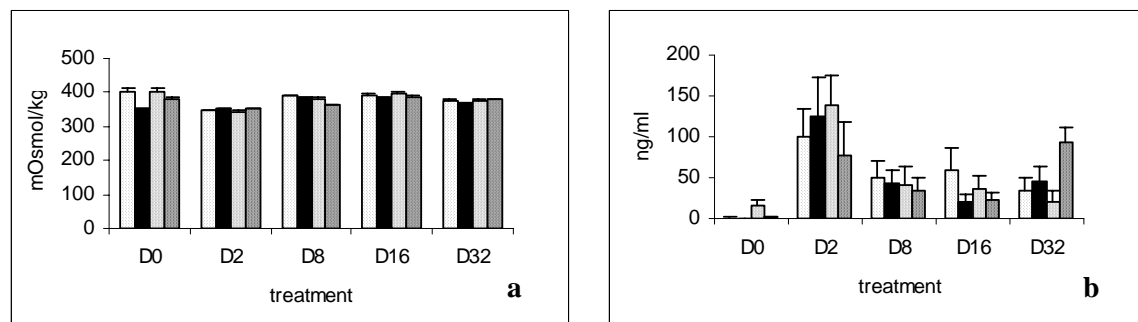


Figure 53. Plasma osmolality (a) and cortisol (b) from fish exposed to different background and light intensity. For details see Figure 48.

The total α -MSH concentration in the plasma was not significantly altered by treatment or time (despite the visible increase in concentration from day 0 to day 16, which was followed by a recovery at day 32; see **Figure 54a**). The α -MSH concentration stored in the pituitary was also not significantly different between groups or time points, although at most time points in the red background groups, the MSH content is slightly higher than in white background groups (**Figure 54b**).

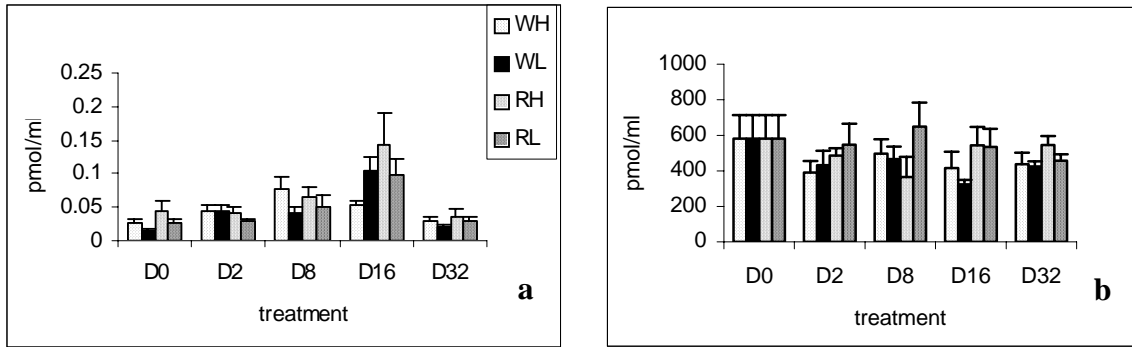


Figure 54. Concentration of α -MSH in the plasma (a) and pituitary glands (b) from fish exposed to different background and light intensity. For details see Figure 48.

There was a statistically significant effect of background and light intensity in plasma norepinephrine (NE) levels at the end of the treatment (Day 32). Higher plasma NE was observed in fish exposed to white background, regardless of light intensity (White background: 1.38 ± 0.16 ng/ml; Red: 0.71 ± 0.15 ng/ml), and in fish exposed to high light intensity, regardless of the background (High light intensity: 1.39 ± 0.16 ng/ml; Low: 0.70 ± 0.15 ng/ml) (**Figure 55**). There was no difference in plasma epinephrine (E) levels among the two backgrounds, while higher E was observed in fish exposed to high light intensity, regardless the background (**Figure 55**).

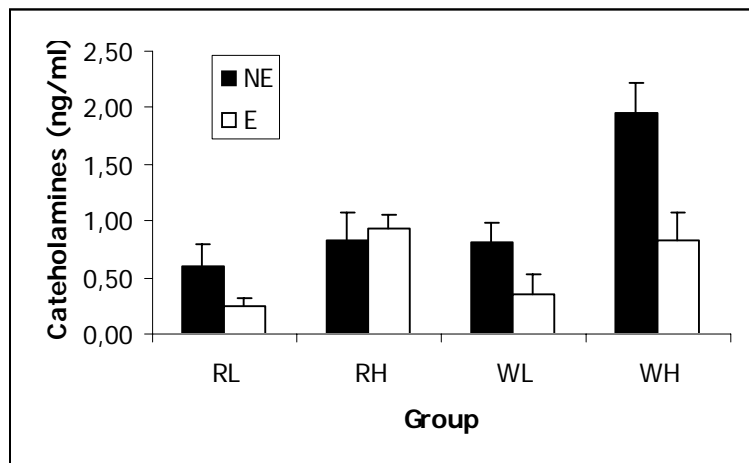


Figure 55. Effect of background and light intensity on plasma catecholamines of red porgy sampled on Day 32 following exposure to different backgrounds and light intensities. NE: norepinephrine; E: epinephrine. For details see Figure 48.

There were no statistically significant differences neither in the immunoglobulin nor the agglutination between experimental groups, but significant differences were observed in complement and lysozyme activities. **Figure 56** shows the values for complement, expressed as ACH50 units, at days 2 and 32 after exposing the fish to the specific combinations of light and background. No changes were found at 16 days. The results of this experiment concerning the complement values are that:

1. Dark background gives similar values to controls.
2. The best combination appears to be dark background and low intensity
3. Between groups, at 32 days there are no significant differences
4. Overall the complement values appear to be low

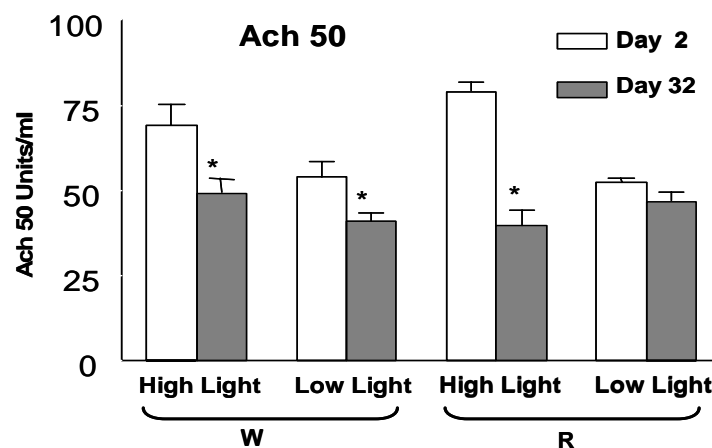


Figure 56. Changes in complement activity in fish exposed to different background and light intensity. For details see Figure 48.

Although no important changes are observed, the fish from a dark background show values more close to what has been described as basal or control values than light background. In this way, and taking into account both the values of day 2 and 32, it appears that the best combination may be dark background and low intensity, as it gives the most stable values. Nevertheless, no depression of complement can be derived from this experiment.

Regarding the results obtained at the end of the treatment it is interesting to notice that no changes are observed after 32 days. Thus, fish subjected to the different combinations of light and background show a similar complement activity. Therefore, although the levels appear to be in the low range, no effect can be attributed to either light intensity or background. Nevertheless, the fact that the values of complement are in the range of 50-80 ACH50 units could indicate a certain degree of suppression already in the stock group, perhaps related to the unfinished adaptive process to the culture conditions.

Regarding lysozyme values, **Figure 57** clearly shows that high intensity gives significant changes compared to low intensity and therefore that:

1. Light intensity induces bigger changes than background.
2. High intensity depressed lysozyme activity.
3. Best conditions are found at low intensity

The values found for lysozyme are in the normal range at the beginning of the treatments, but these values clearly are depressed after 32 days of high intensity, and not only for light backgrounds, but also for dark backgrounds. It is not the case for the low intensity groups, as no significant differences were observed between groups. A tendency to decrease (not confirmed by the statistical analysis) appears to take place also in low intensity. This could be related to certain effect of the experiment, but anyway, the comparison is clear between groups. The backgrounds do not affect the lysozyme activity.

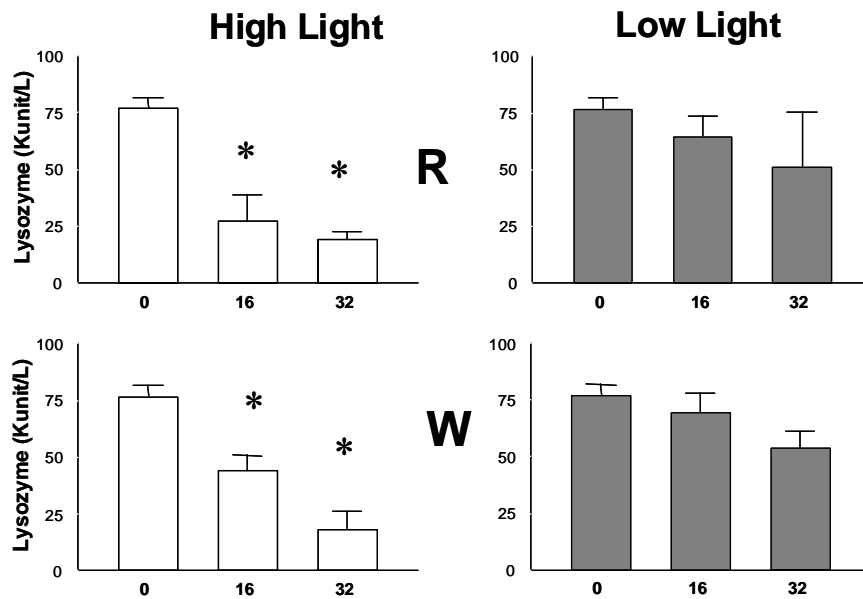


Figure 57. Changes in Lysozyme activity in fish exposed to different background and light intensity. For details see Figure 48.

3.2 Changes in colour after acute capture stress

Fish responded to 5 minutes of air exposure in a net by remaining motionless. After returning them to the water, it took between 5 and 20 minutes for individual fish to completely recover (fish were swimming upside down or seemed unconscious).

Skin colour parameters were determined immediately after air exposure and 24 h of recovery. The L-value was higher for fish kept on a white background, even immediately after stress (**Figure 58a**). The hue was higher for both groups at 0 h than at 24 h, while it was consistently higher in fish from a red background (**Figure 58b**). The chroma was more or less similar immediately after stress but was much higher for fish from a red background after 24 h (**Figure 58c**).

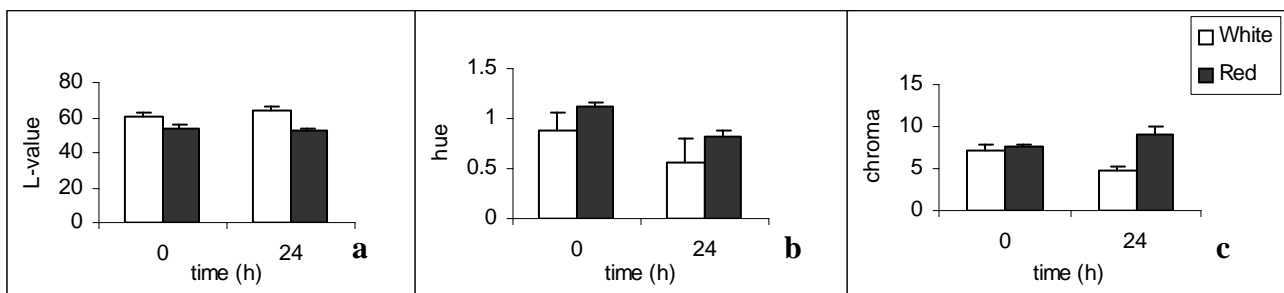


Figure 58. Skin colour parameters of fish kept on white or red background immediately after air exposure (time 0) and after 24 h. L-value; lightness (a), Hue (b) and Chroma (c).

The rise in cortisol levels that can be seen in both white and red background fish after applying the stressor is significantly different from control values at 2h post stress. At 8 and 24 h, the levels were comparable to the levels immediately after stress. Values are still quite high after 24 h when comparing these to control (unstressed) values (**Figure 59a**). Simultaneous with cortisol, lactate levels increased significantly (**Figure 59b**). Air exposure and netting stress can both lead to an increase in anaerobic metabolism, which is mirrored in the fast increase of lactate. For both parameters, there were no significant differences between treatments.

The plasma levels of glucose are also increased significantly by netting stress (**Figure 60a**). Lactate levels were restored to basal after 8h, while glucose levels did not decrease until 24h post stress. The anaerobic metabolism of glucose leads to increased lactate levels. Possibly, the fast increase in lactate levels may occur at the cost of glucose, explaining why an increase in glucose levels can not be seen until 8h post stress. Plasma electrolytes (Na, Cl and K) all showed a similar pattern. Plasma Na (**Figure 60b**) increased significantly from 0 to 2 h after air exposure, and was returned to control levels after 8 h. Plasma K was elevated in the first two hours after air exposure and was decreased to control values at 8 and 24 h (**Figure 60c**), and plasma Cl finally decreased a little from 0 to 2 h after air exposure and had returned to basal control levels at 8 h and 24 h (figure 8d). The stress caused by netting and air exposure often leads to a leakage of the gills, which in a hyperosmotic environment (sea water) will enable influx of ions into the body. This can explain the increase in Na, K and Cl in the first two hours after air exposure and netting stress. However, background colour again had no influence on the acute stress response.

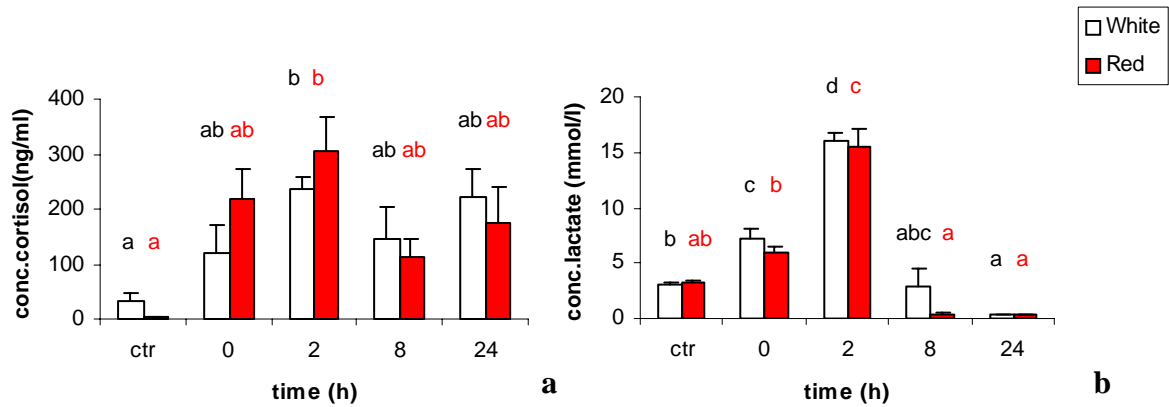


Figure 59. (a) plasma cortisol and (b) plasma lactate, in fish that were kept on a white or red background under blue illumination immediately after air exposure (time 0 h), and 2, 8 or 24 h after stress

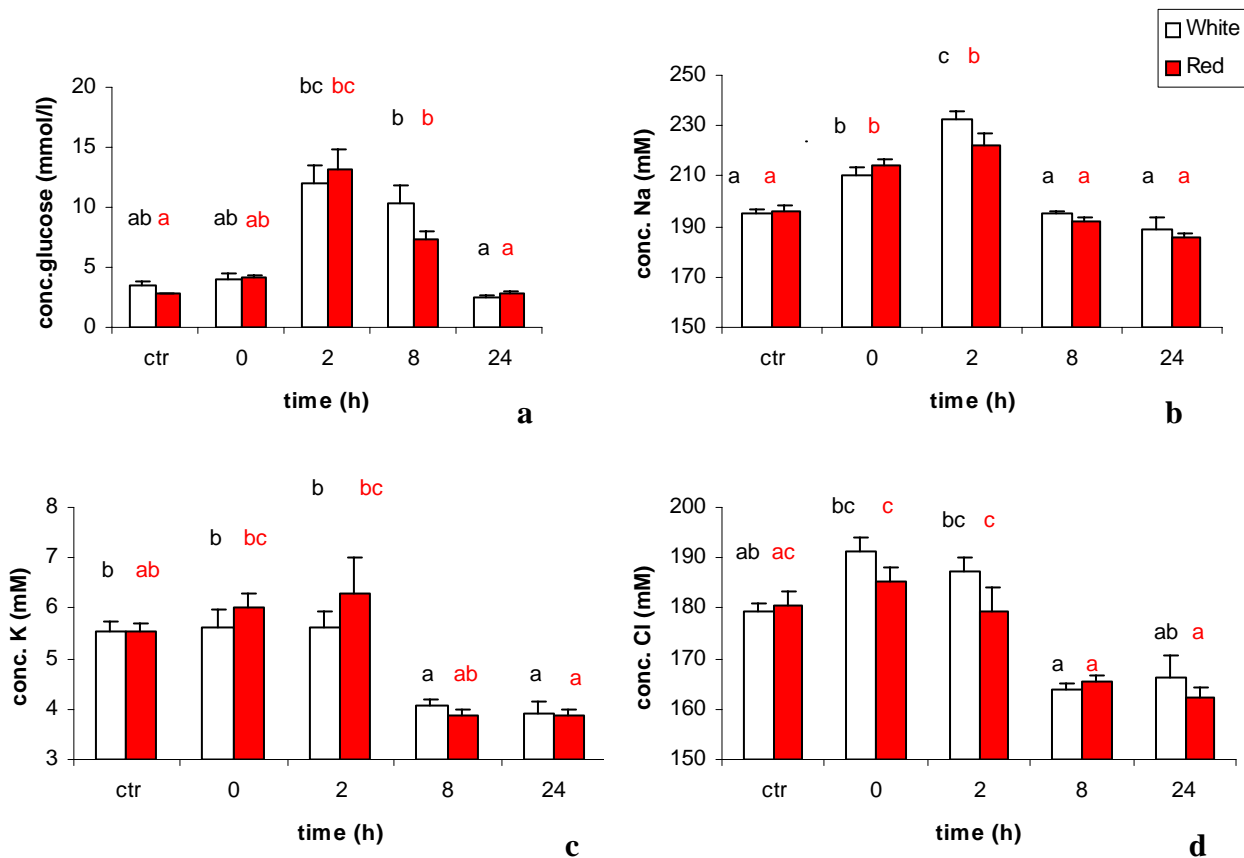


Figure 60. Plasma glucose (a), Na (b), K (c) and Cl (d) in fish from a white or red background 0, 2, 8 and 24 h after air exposure.

pH and Ca showed opposite effects to the stressor applied. The pH of the plasma decreased as a result of the acidosis induced by anaerobic metabolism (**Figure 61a**); which is also connected with increased lactate values. Ca levels showed an increase at 2h after stress, but at 8h the levels had returned to basal control levels (**Figure 61b**).

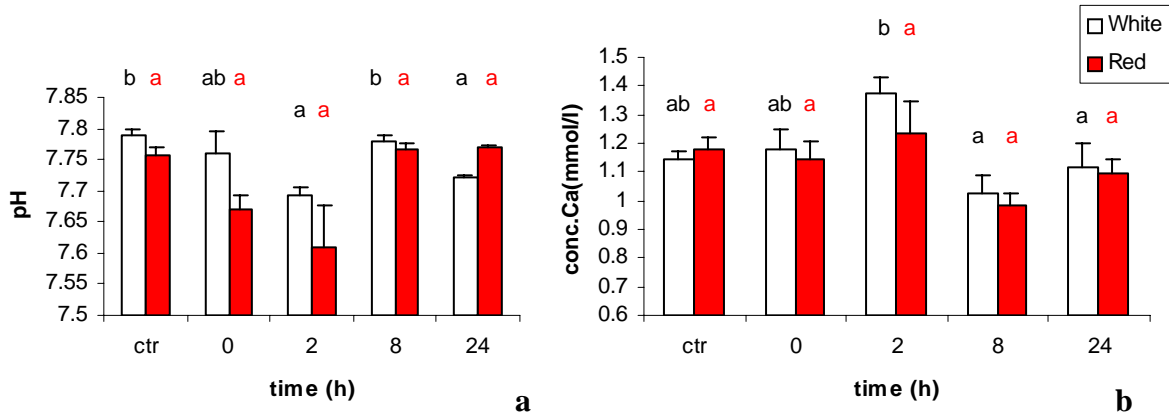


Figure 61. Plasma pH (a) and Ca (b) in fish from a white or red background 0, 2, 8 and 24h after air exposure.

MSH levels also showed an increase after stress compared to control values and a slow recovery after 8h (**Figure 62a**). The difference between control and elevated levels was significant only in the white background group at 2h post stress. In general, levels were higher in fish kept on a red background, but this difference was not significant. Colour parameters were determined immediately after air exposure and after 24 h of recovery. The L^* was higher for fish kept on a white background at all timepoints (**Figure 62b**). This difference was most significant at 24h post stress. Applying the stressor did not darken the fish, since the L^* was a little higher immediately after stress than in control fish. This difference was not statistically significant, however. The hue remained between 0.55 and 1.1 throughout the experiment (**Figure 62c**). There were no statistically significant differences between treatments nor through time. Chroma, on the other hand, decreased 24h after stress in white background fish (**Figure 62d**). In red background fish, chroma did not change through time. Both hue and chroma were generally higher in fish kept on a red background compared to the fish that were kept on a white background.

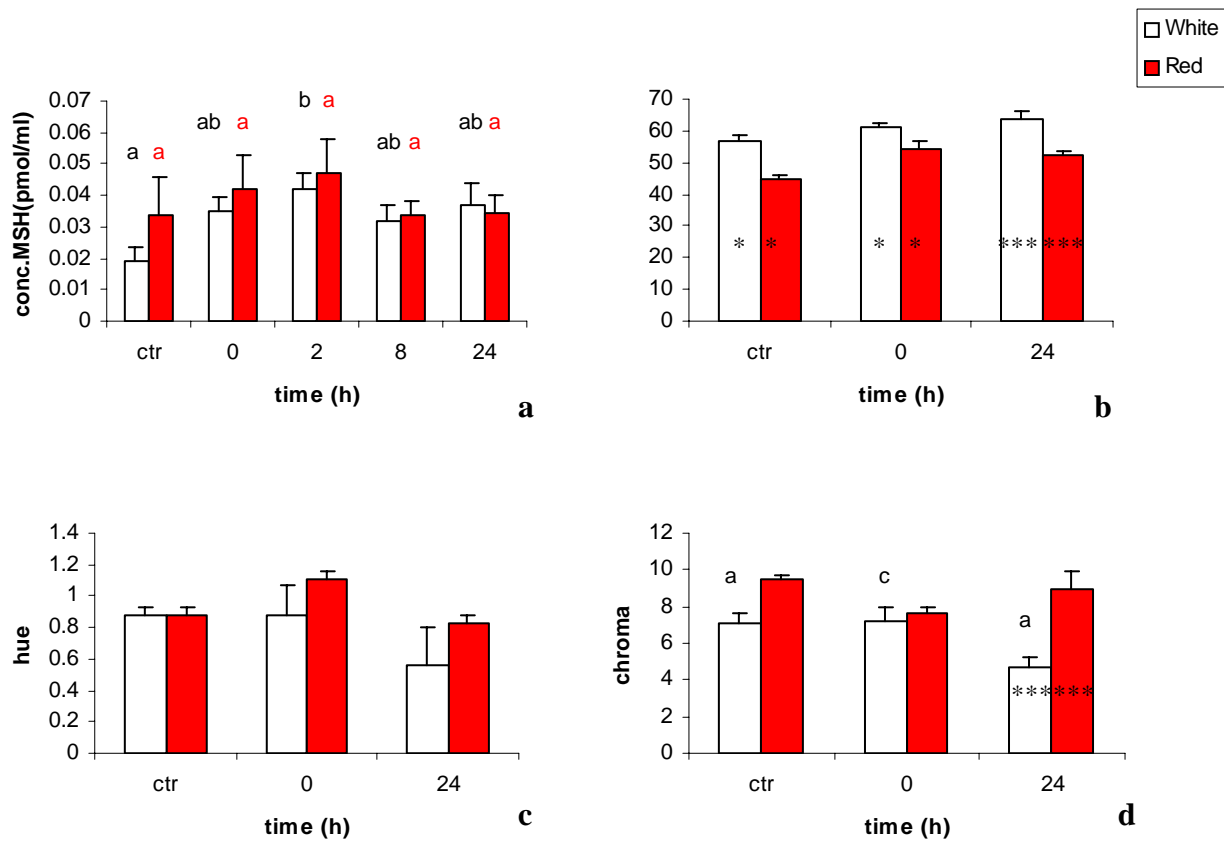


Figure 62. Plasma MSH (a) and colour parameters of fish kept on white or red background immediately after air exposure (time 0) and after 24 h. L*; lightness (b), Hue; observed colour (c) and Chroma; saturation of the colour (d).

Correlation analysis showed positive correlations between almost all parameters, with the exception of pH (Table 16), as could be also seen in Figure 61. The only value that correlated significantly with L* and chroma was the plasma lactate level. Hue (possibly due to a smaller range of values) was also correlated with Na, K and glucose levels.

With the exception of L* and chroma, no parameters were different between treatments. It therefore seems that the observed differences in colour can not be attributed to any of the plasma parameters described above.

These observations have led us to limit histological analysis to pituitary MSH cells and gill Na-K ATPase determinations. Staining of the pituitary gland with α MSH antibody showed that the intensity of the staining immediately after applying stress is higher than at 2h post stress in both treatments (Figure 63). At 24h post stress the intensity of the staining is again as high as immediately after the application of air exposure. There were no differences visible between pituitary glands from fish on a white background and those of fish on a red background.

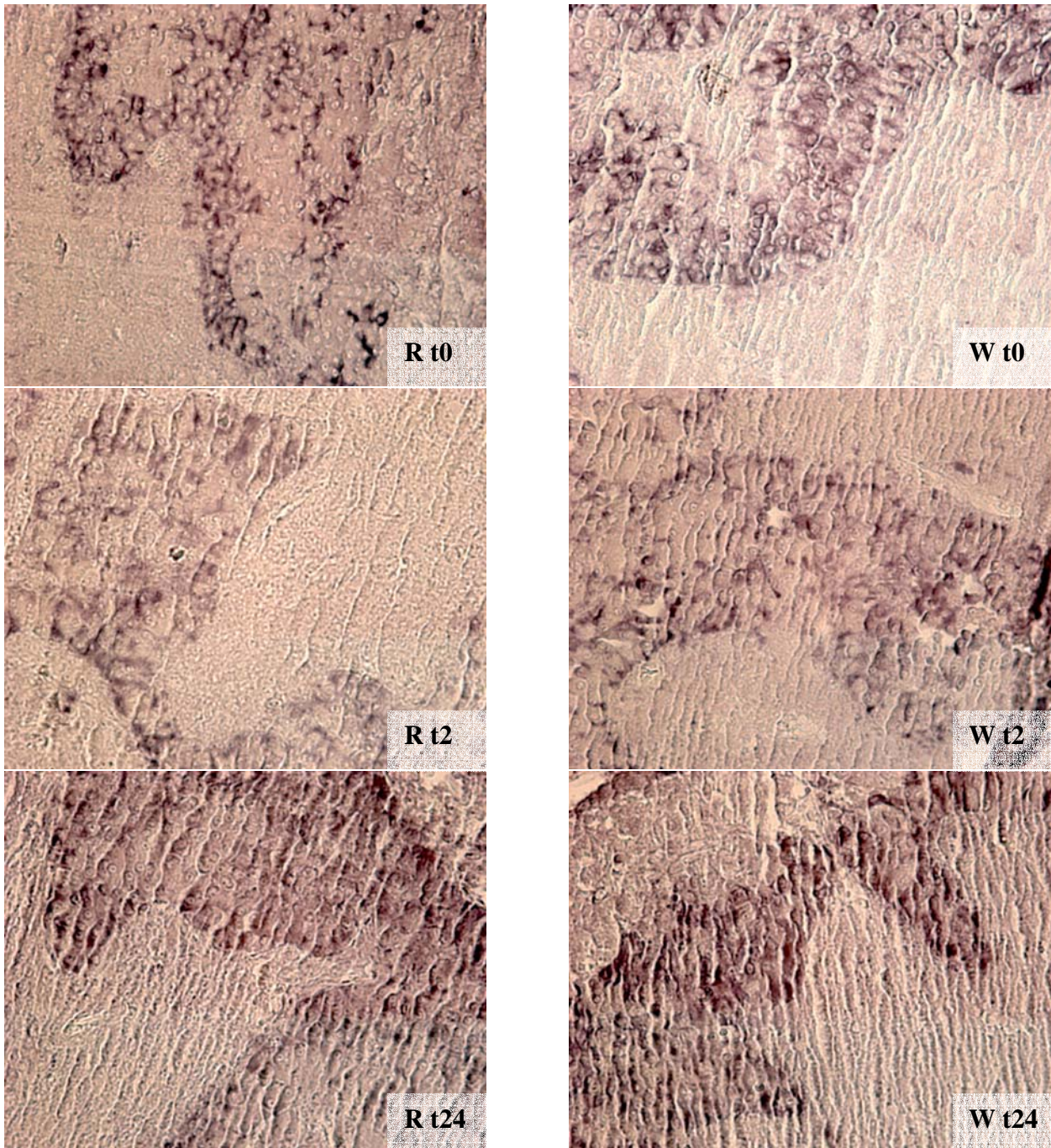


Figure 63. Pituitary glands stained for MSH content of the cells. Pi=pars intermedia, pd=pars distalis. MSH containing melanotrope cells are generally localized in the pi near the border with the pd. R=red background, W=white background and pictures are from 0, 2 and 24h post air exposure.

3.3 Changes in colour after chronic crowding stress

Colour parameters measured for the fish are shown in **Figure 64**. The lightness of the skin is highest in fish kept on WBG (**Figure 64a**). For these fish, BS gives an additional increase of the L^* . In general, excluding the fish kept in BS and/or on WBG, LD gives a higher L^* than HD. Contradictory to the L^* , the chroma (colour saturation of the skin; **Figure 64b**) and hue (visually observed colour; **Figure 64c**) are generally lowest in WBG fish and peak in RBG/BBG full spectrum fish.

For chroma, at day 8 and 16 the highest colour saturation can be found in fish kept on RBG or BBG, in FS and at LD. Through time, it seems that chroma slowly decreases below control levels. For hue, this effect was not present.

The crowding stress (high density versus the low density at which WP 3.1 was performed) had no strong effect on the skin colour of the fish, as was also found for the acute stress in WP 3.2.

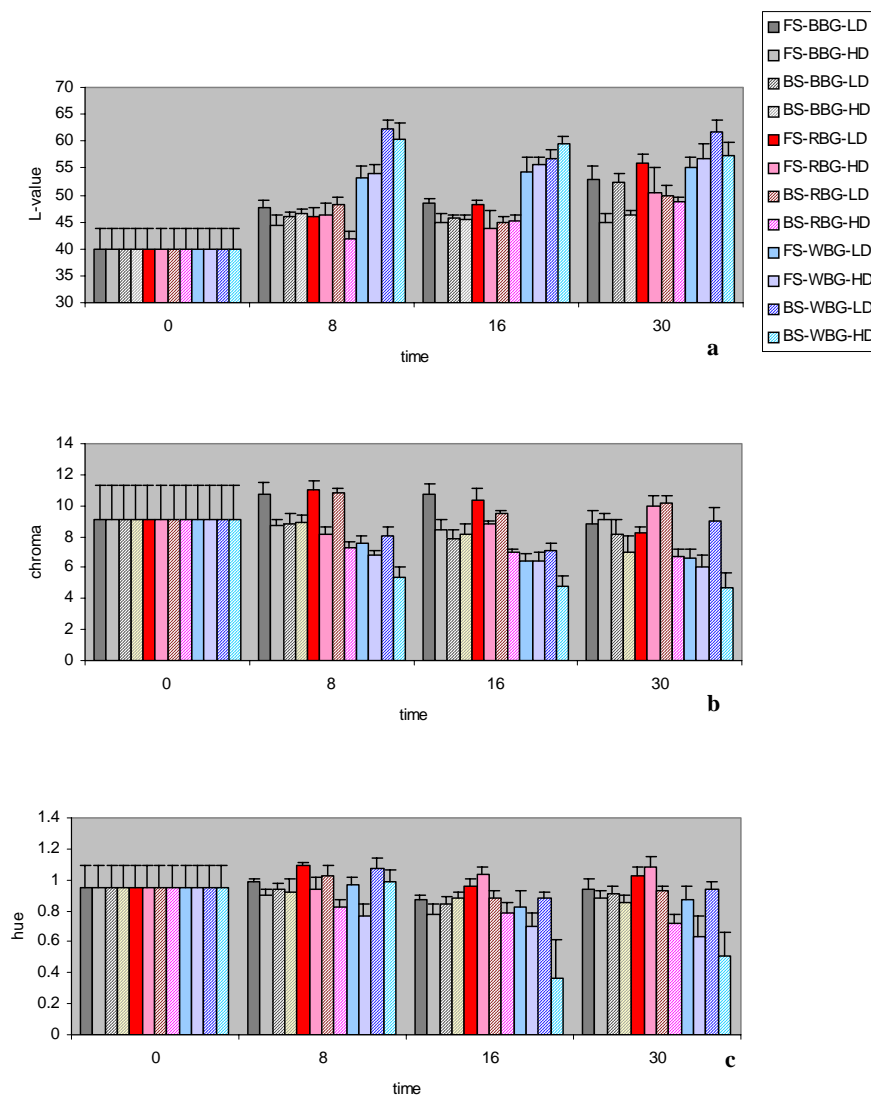


Figure 64. Lightness (L^* ; a), saturation of colour (chroma; b) and visually observed colour (hue; c) of skin of red porgy kept in full or blue spectrum (FS/BS), on a black, red or white background (BBG/RBG/WBG) and high or low density (HD/LD).

Fish held under blue lighting spectrum, showed higher skin melanin content when kept under high ($7.32 \pm 0.55 \mu\text{g}/\text{mm}^2$) than low density ($6.18 \pm 0.32 \mu\text{g}/\text{mm}^2$), while there was no statistically significant effect of background (Figure 45). In fish held under full spectrum, the lowest skin melanin content was observed under the red background ($4.82 \pm 0.29 \mu\text{g}/\text{mm}^2$) than the black ($5.91 \pm 0.41 \mu\text{g}/\text{mm}^2$) and white backgrounds ($6.07 \pm 0.36 \mu\text{g}/\text{mm}^2$), while density did not show any significant effect.

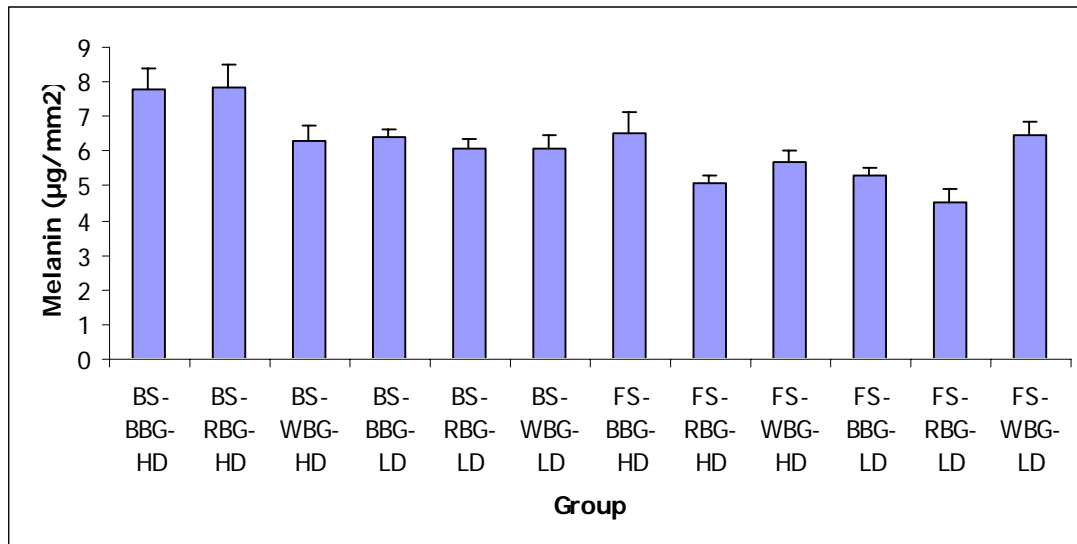


Figure 65. Skin melanin content of red porgy kept in full or blue spectrum (FS/BS), on a black, red or white background (BBG/RBG/WBG) and high or low density (HD/LD) for a period of 30 days.

Despite the fact that some minor differences among groups in skin melanophore density of the dorsal body area were observed, there was no clear effect of background, lighting spectrum and stocking density on the number of melanophores per mm^2 of skin (**Figures 66a**). On the ventral skin area there was no difference in skin melanophore density among groups (**Figure 66b**). In general, melanophores in the dorsal area ranged from 79 ± 3 (FS-RBF-HD) to 101 ± 3 cells/ mm^2 (BS-RBG-HD) and in the ventral area from 18 ± 3 (FS-RBG-HD) to 26 ± 4 cells/ mm^2 (BS-BBG-HD). Mean skin melanophore density was 58 ± 3 and 56 ± 3 cells/ mm^2 for Blue and Full spectrum respectively; 59 ± 2 , 59 ± 2 and 54 ± 2 cells/ mm^2 for black, red and white background respectively; 57 ± 2 cells/ mm^2 for both high ($25 \text{ kg}/\text{m}^3$) and low ($10 \text{ kg}/\text{m}^3$) density.

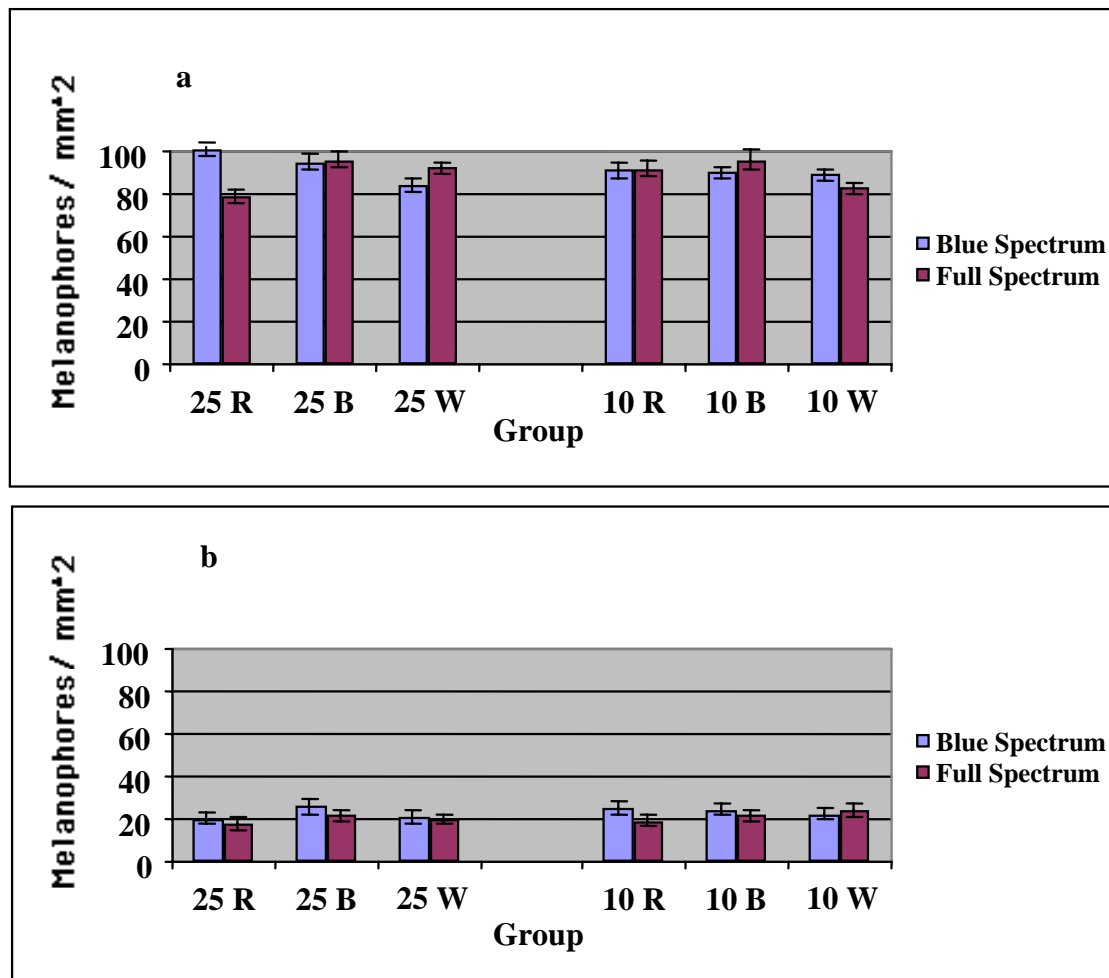


Figure 66. Melanophore densities (mean, SEM) in the dorsal (a) and ventral (b) skin area of reared *P. pagrus* held under different backgrounds (R: red, B: black, W: white), lighting spectra (Blue, Full) and stocking densities (25 kg/m³, 10 kg/m³) conditions.

The cortisol values determined for these fish again showed very high individual variation (**Figure 67a**). Control values (n=17) are in the expected range of 0-20 ng/ml. These values could be found throughout the experiment in the full spectrum (FS) red and black background (RBG, BBG) low density (LD) groups. At day 8, it seems that in blue spectrum (dashed bars) the level of cortisol in the plasma increased compared to the full spectrum (same background, same density). This can not be seen in the white background (WBG) groups. At day 16 and 30 however, this effect of the blue spectrum is lacking for most groups. There is no clear effect of density, nor of background, except for the aforementioned red and black low density groups kept in full spectrum. MSH levels (**Figure 67b**) showed an increase from day 8 to day 30, with a strange sudden increase in red and white background, full spectrum groups at day 16. In the other treatments, this effect is lacking. Since we only found this unexpected result at day 16 we consider this to be an artifact and focus on day 8 and day 30 for these groups. In general, we can say that levels are higher at day 30 than at day 8 for all treatments. This effect was however not significant.

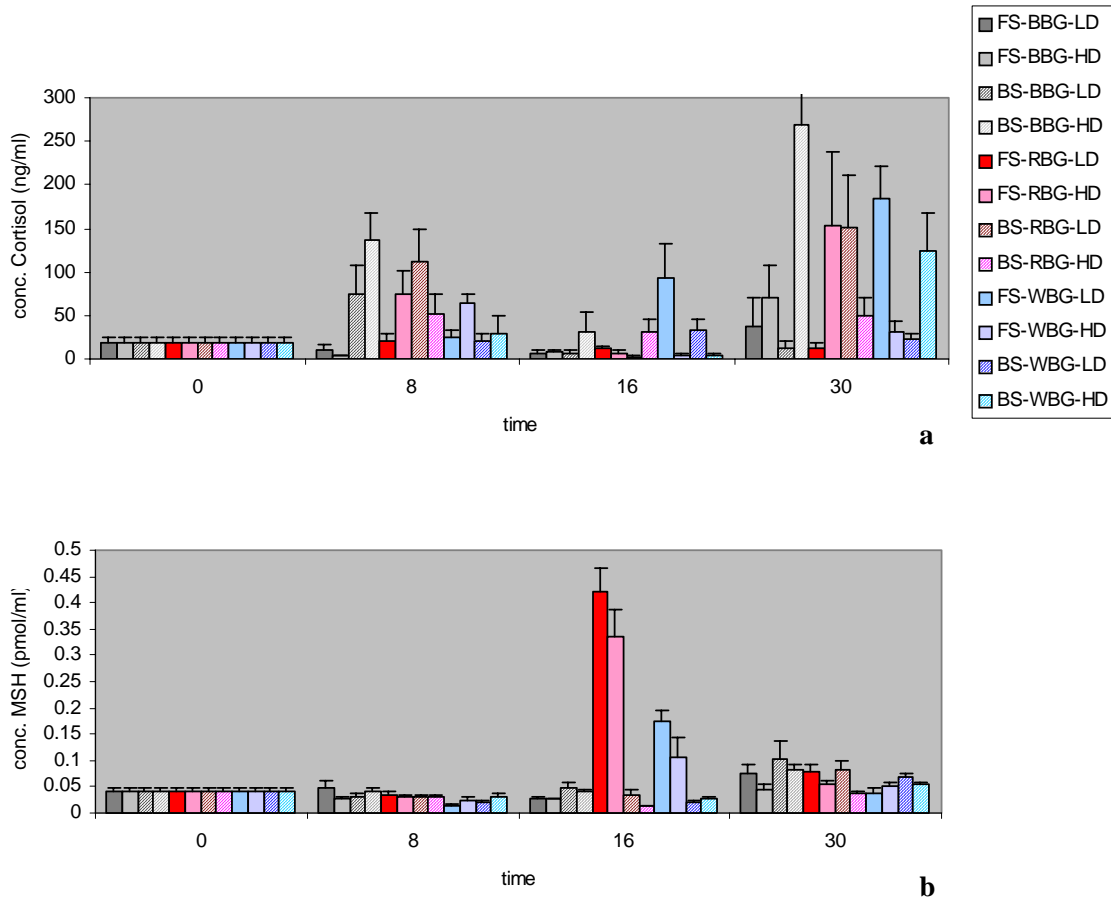


Figure 67. Plasma cortisol (a) and MSH (b) in fish that were exposed to blue (dashed bars) or full (filled bars) spectrum illumination (BS/FS); to a black, red or white background (BBG/RBG/WBG) and / or to a high density (HD; 25 kg/m³) or low density (LD; 10 kg/m³) during 30 days.

Glucose levels (**Figure 68a**) did not change compared to control values at any time throughout the experiment. Variation between treatments is high again and there are no clear effects of separate treatment conditions on plasma glucose. Plasma lactate levels on the other hand show an increase compared to control levels in some groups (**Figure 68b**) again however no consistent effect of one of the treatment conditions can be elucidated. From day 8 to day 30, lactate levels seem to decline in all groups.

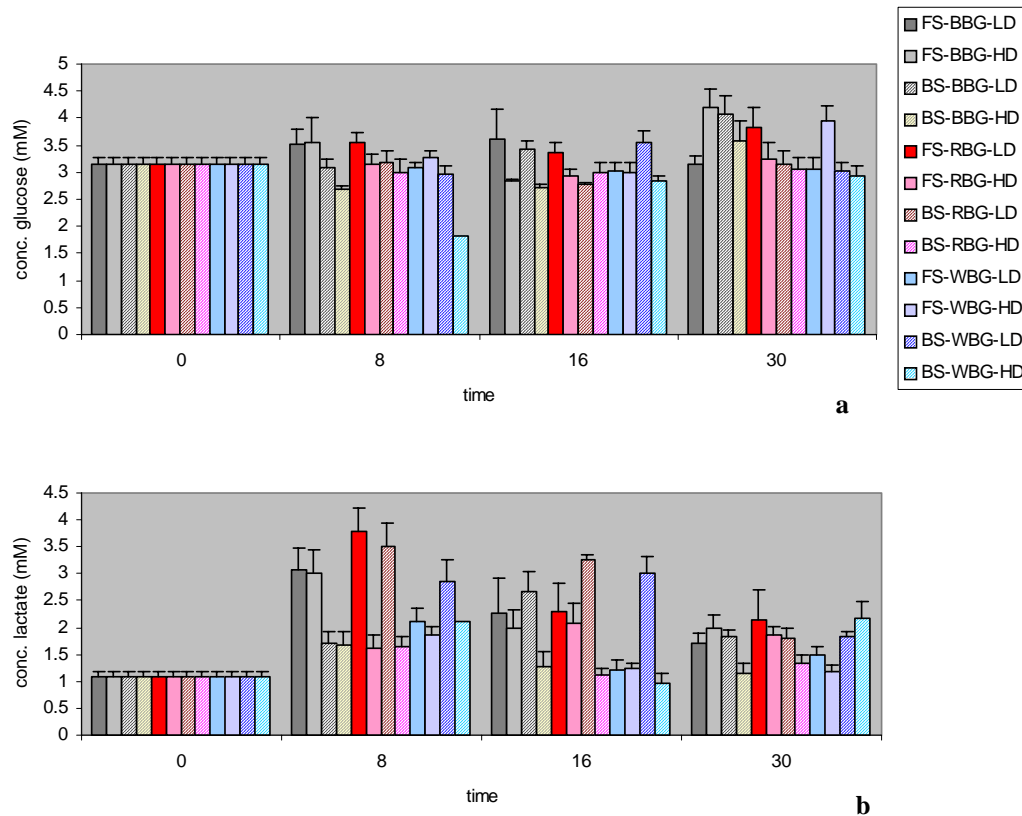


Figure 68. Plasma glucose (a) and lactate (b) in fish that were exposed to blue (dashed bars) or full (filled bars) spectrum illumination (BS/FS); to a black, red or white background (BBG/RBG/WBG) and / or to a high density (HD; 25 kg/m³) or low density (LD; 10 kg/m³) during 30 days.

Plasma electrolytes are shown in **Figure 69**. Na remains elevated compared to control values throughout the experiment (**Figure 69a**). There is no general increase or decrease in any of the treatment groups. However, at all time points Na levels are lowest in the fish kept in Blue spectrum (BS), RBG and BBG at High Density (HD). Next to that, at D30 for almost all backgrounds and densities, the Na levels are higher in FS compared to BS. Throughout the experiment, Na levels remain elevated above control values. Plasma K levels are shown in **Figure 69b**. The concentration of K in the plasma is highly variable between the different treatments. There is a shift visible in higher levels of K that at day 8 can be found in the WBG groups but at day 30 are present more in the BBG groups. Next to this, it can be seen that at day 30 the concentration of K is a little elevated in most BS groups compared to FS groups. Other than this, no decisive conclusion can be drawn from these results. Cl levels in the plasma are shown in figure 14c. The Cl concentration is lowest after 30 days, and is lower in BS groups compared to FS treated fish. There is no difference in BS versus FS at the other time points.

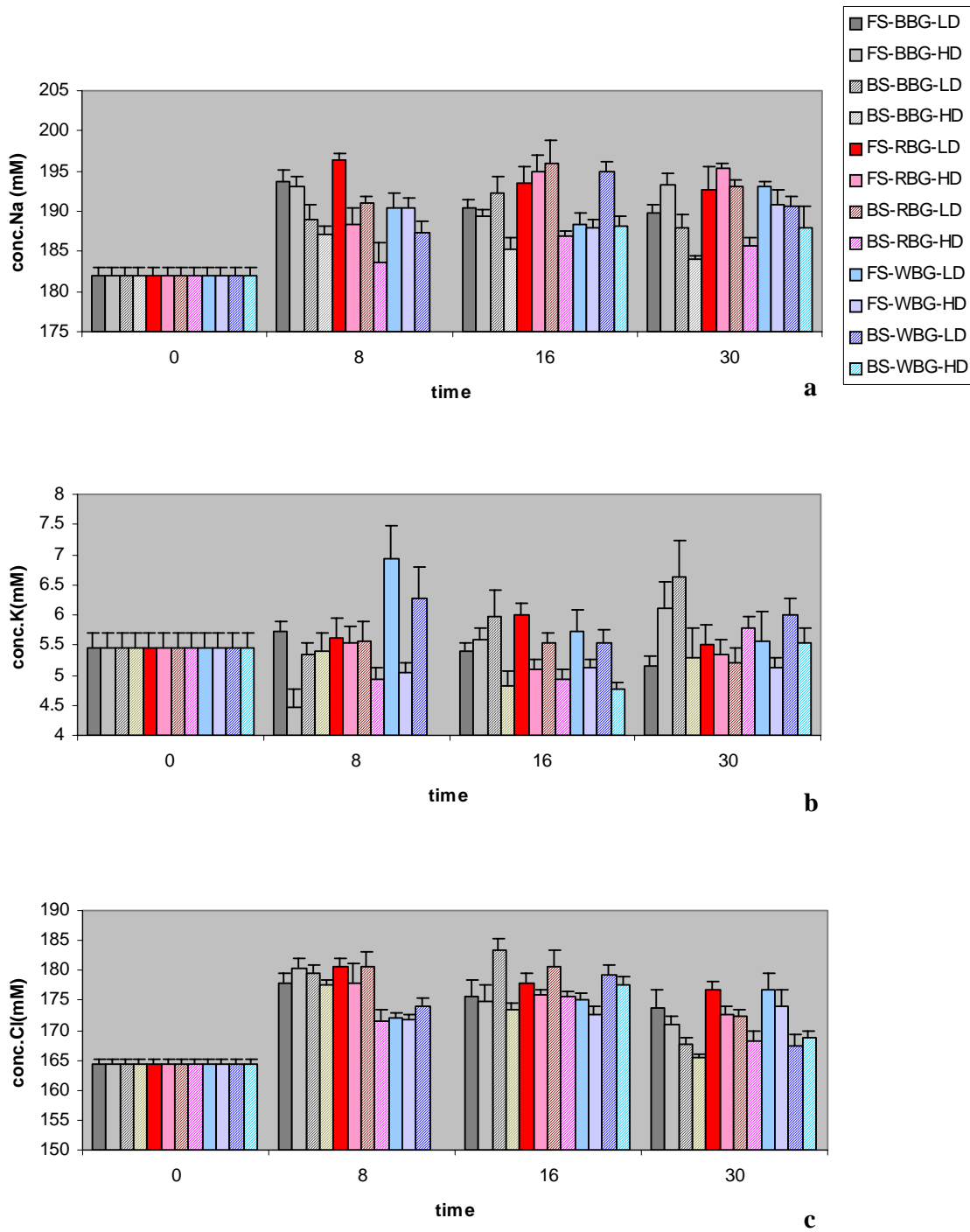


Figure 69. Plasma Na (a), K (b) and Cl (c) in fish exposed to blue or full spectrum (BS/FS); black, red or white background (BBG/RBG/WBG) or high or low density (HD/LD) during 30 days.

Plasma pH (**Figure 70a**) and Ca levels (**Figure 70b**) again support the previous results in that no clear effect of any of the treatment parameters can be found and that the values do not deviate from control levels.

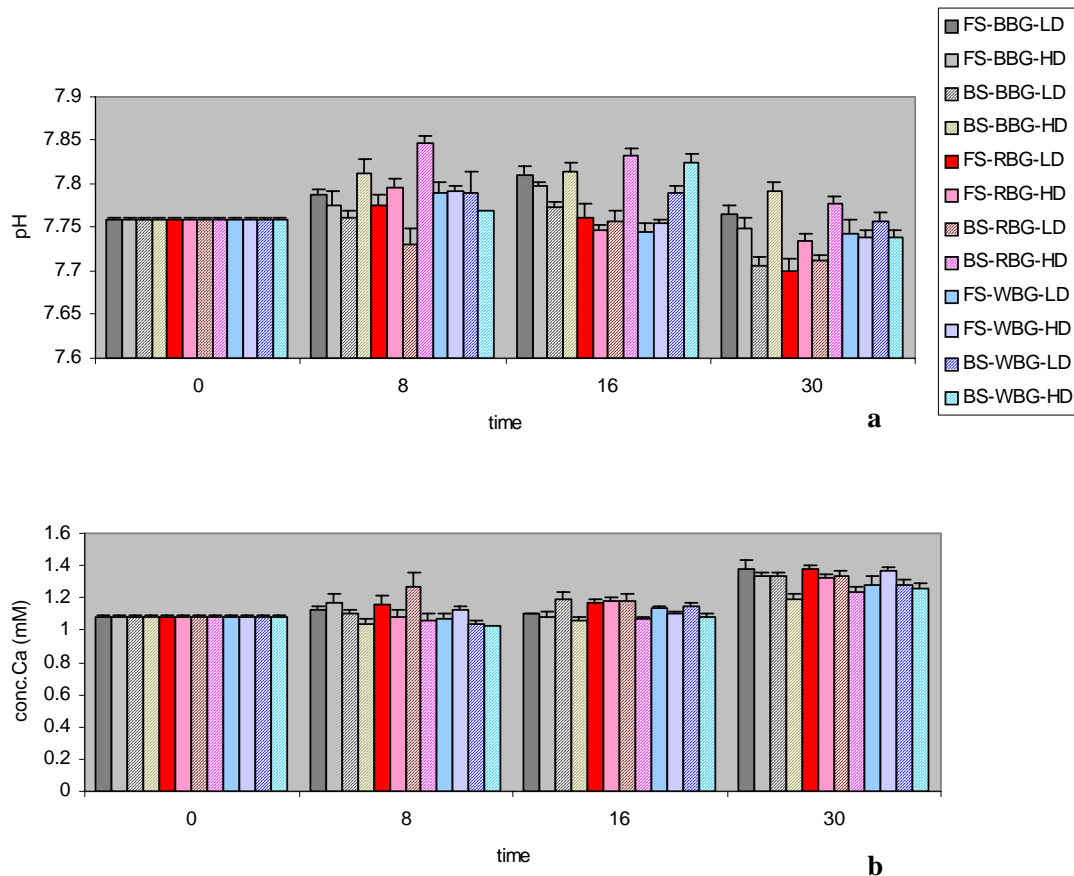


Figure 70. Plasma pH (a) and Ca (b) in fish exposed to blue or full spectrum (BS/FS); black, red or white background (BBG/RBG/WBG) or high or low density (HD/LD) during 30 days.

There was a statistically significant interaction between spectrum, background and density on plasma NE concentrations (**Figure 71**). The effect of spectrum and background depends on what level of density is present. In fish held at a density of 25 kg/m³, there were statistically significant higher NE levels under the full (7.44±1.34 ng/ml) than the blue spectrum (3.88±1.39 ng/ml), and in the white (6.98±1.13 ng/ml) and red background (6.69±2.46 ng/ml) compared to the black (3.38±1.59 ng/ml) background.

In fish held under a low density (10 kg/m³), there were statistically significant higher NE levels under the full (14.38±4.49 ng/ml) than the blue spectrum (4.74±2.41 ng/ml) only in black and white background adapted fish. Higher NE levels were also observed in fish adapted to red background tanks compared to those under black and white tanks, only in the blue spectrum.

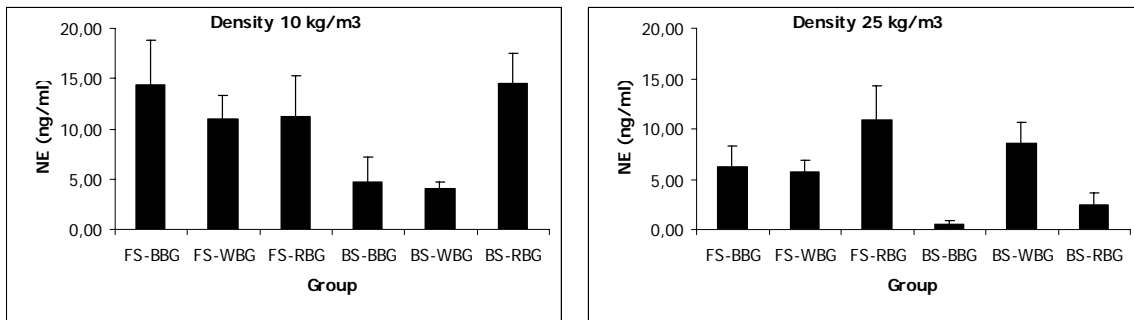


Figure 71. Plasma norepinephrine (NE) concentrations at the end of the experiment in fish exposed to blue (BS) or full (FS) lighting spectrum under black (BBG), red (RBG) or white (WBG) background and high (HD; 25 kg/m³) or low density (LD; 10 kg/m³) during 30 days.

No differences were found in immunoglobulin or agglutination levels and the results for complement and lysozyme activity are shown in Figures 72 & 73.

In **Figure 72** lysozyme activity during chronic stress shows a dynamic activation and a later recovery. Therefore, as in other species, red porgy shows lysozyme sensitivity to chronic stress. Black and red backgrounds show the expected patterns, but this cannot be seen for the white background. Thus, whereas dark backgrounds show an activation at about 2 weeks and later on a recovery to basal values, white background groups show an early activation at the second sampling point (8 days) and return to basal levels also earlier than fish from a dark background. Full spectrum illumination shows a similar type of response, although delayed, as blue light. Fish kept at both 10 and 25 kg/m³ densities show a pattern of stress, with recovery after 32 days in most cases. The data on lysozyme activity show changes related to the conditions of the experiment. However, as low density fish show chronic stress, it is not clear which is the relevant factor producing the significant lysozyme changes and probably all factors induce a period of adaptation with significant changes at the beginning and further recovery. Two factors appear to have a significant effect:

- 1) Full spectrum seems to induce higher levels than blue light.
- 2) White background shows earlier changes than the other backgrounds, but later showing the same type of recovery dynamics.

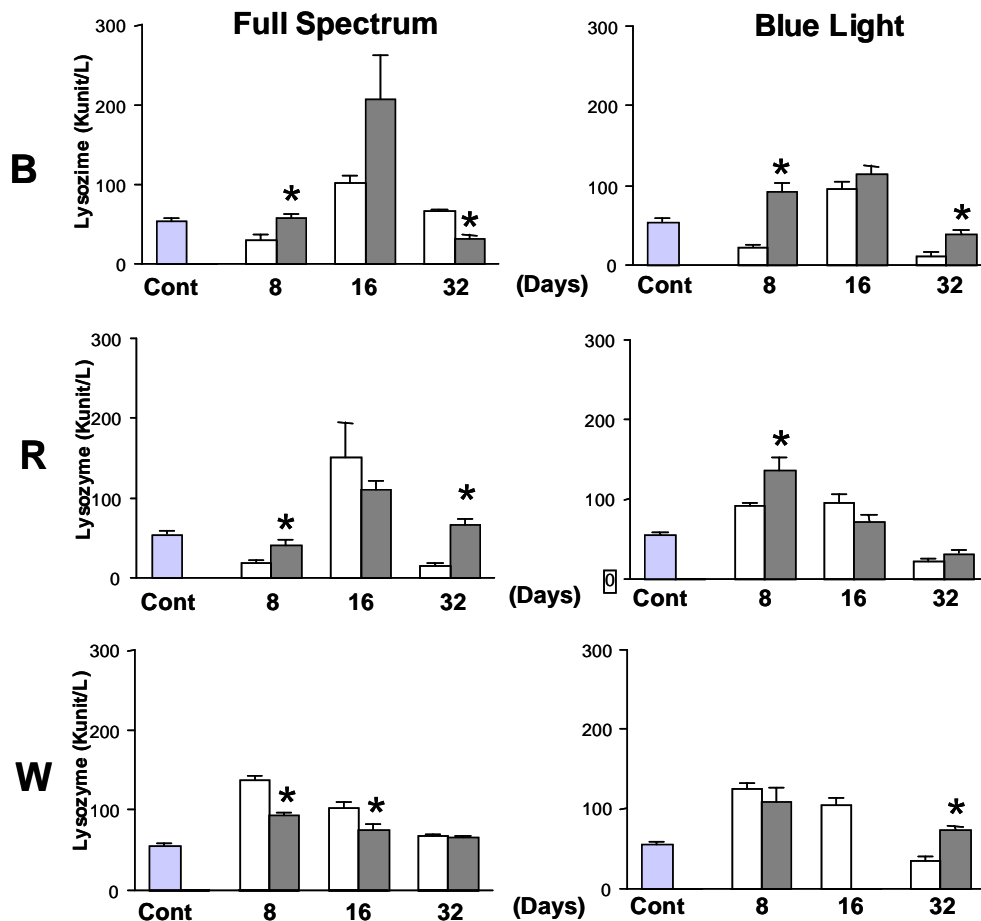


Figure 72. Changes in lysozyme activity following changes in light intensity and background colour (W, white background; R dark background; dark bars 25kg/m³ density, white bars 10).

Figure 73 shows the results for the complement. Two conclusions can be drawn from this figure:

- 1) No significant changes are observed between control levels and levels at the end of experiment (both at higher and lower density).
- 2) The complement levels are consistently low.

Therefore in this experiment, no effects on complement levels were observed of the different experimental conditions. Again, one possibility is that the stock fish are incompletely adapted in terms of the immune response, leading to lower complement levels, as has been demonstrated in previous studies. If so, a second possibility is that the experimental changes applied do not induce further depression of this variable. This might have been caused by chronic stress of the initial stock.

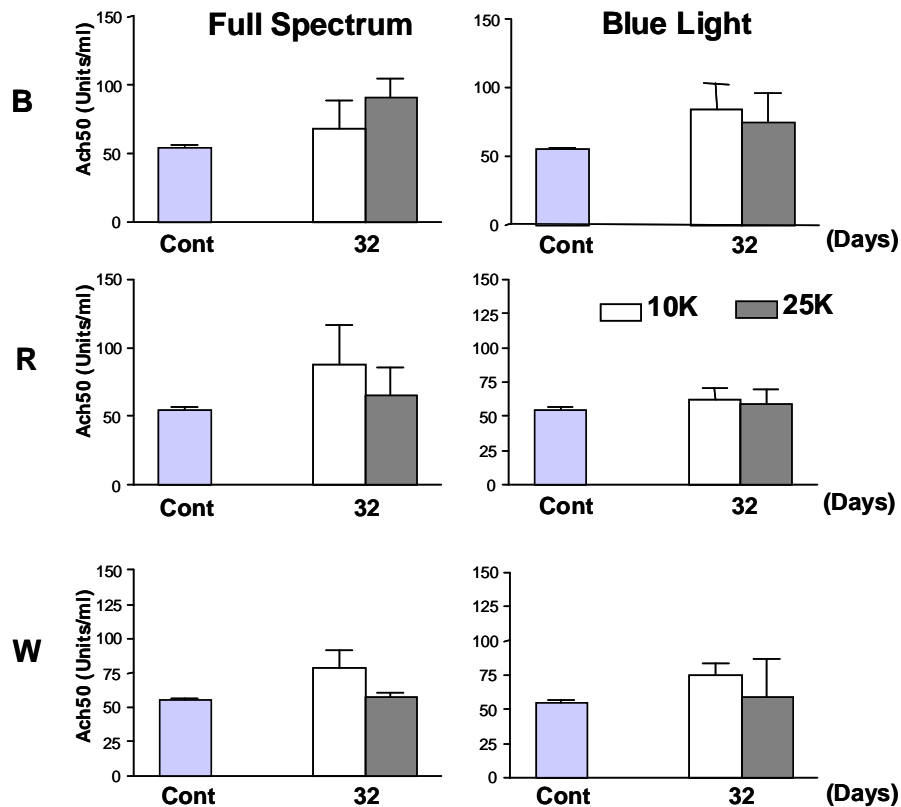


Figure 73. Changes in lysozyme activity following changes in light intensity and background colour (W, white background; R dark background; dark bars 25kg/m³ density, white bars 10).

The effect of carotenoids on indicators of immune response (*additional work (not included in the work package)*)

An experiment was carried out in which the effects of carotenoids on selected immune responses were assessed. Thus, the objective was to test whether carotenoids induce significant changes on these indicators and therefore to know and prevent unwanted effects when carotenoids may be included in the diets.

The preliminary results are shown in **Figure 74** where the carotenoid concentration in plasma is plotted and the phagocytic activity through the reduction of NitroBlue Tetrazolium (NBT) is also shown. It appears that the NBT values are depressed following higher concentrations of carotenoids in plasma. Further work will be performed with more time points.

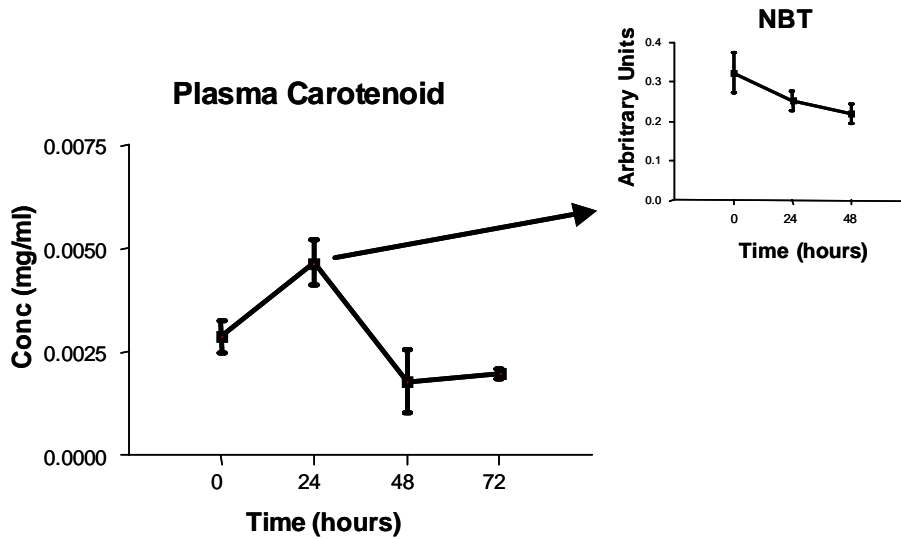
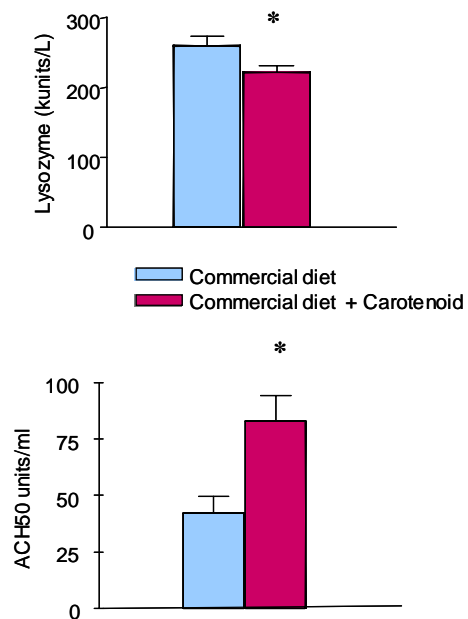


Figure 74. Changes in phagocytic activity (measured through the reduction of NBT) following intake of carotenoids in the red porgy

Figure 75 shows the levels of complement and lysozyme after 90 days of feeding a commercial diet with or without carotenoid supplement. The preliminary results appear to indicate two types of response. Regarding lysozyme, very high values are observed. This could be due to the effect of carotenoids but also to other factors, possibly the higher load of pathogens/parasites carried out by the wild fish. Regarding complement a low level is detected for non-treated fish, which would indicate some sort of suppression.

Figure 75. Levels of lysozyme and complement after 90 days of feeding with a commercial diet or a diet supplemented with carotenoid.



3.4 Changes in colour after sacrifice in ice and post-mortem

Experiment 3.4.1

In this experiment fish fed diets containing different carotenoid sources and concentrations were sacrificed in a mix of ice and water, as for commercial purposes, and the changes in skin colour for a period of 48 h was evaluated.

For all the diets in general, the lightness in skin showed a tendency for significantly lower values between 0 h and 24 h after fish were sacrificed in ice. During the last 24 to 48h period values for lightness continue lowering but not as pronounced than in the previous 24 h. The red hue showed in the same manner lower values after 24 and 48 h post-mortem. The lowering in the red hue was low in those fish with higher red hue at 0 h post (D5, D6, D7 y D8). Regarding to the yellow hue, all fish showed some higher values a 0 h post-mortem compared with those fish fed with the negative control diet. After 24 h higher values were observed in the yellowness being significantly different only in the case of some of the diets (D1, D2 y D4). This increase was continued for diminished values during the last 24 h to 48 h period with similar values than those measured at 0h post mortem (**Figures 76, 77 & 78**).

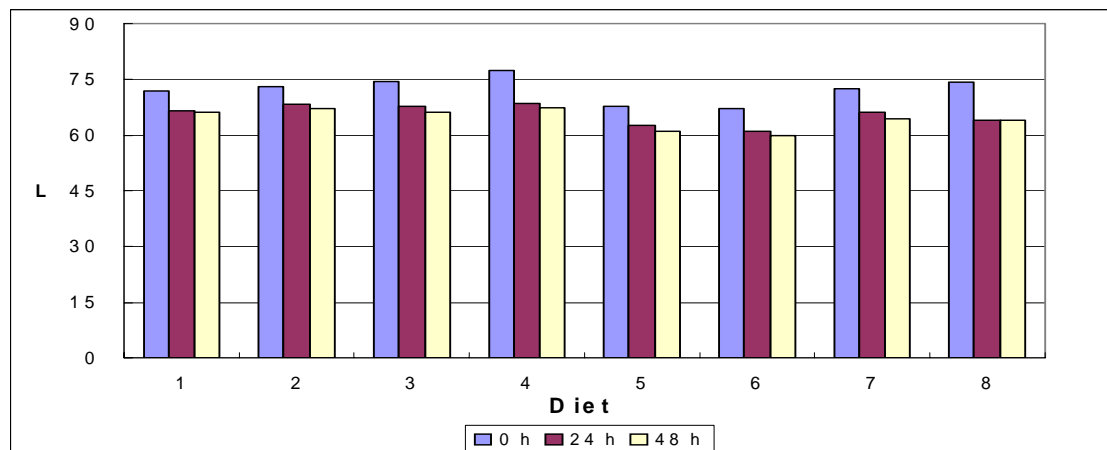


Figure 76. Skin lightness evolution (L) after 0, 24 & 48 hours post mortem of fish on ice. The diets used were:

- **Diet 1** : Negative control (no carotenoid added)
- **Diet 2** : Astaxanthin (20 mg/kg diet)
- **Diet 3** : Astaxanthin (40 mg/kg diet)
- **Diet 4** : Astaxanthin (60 mg /kg diet)
- **Diet 5** : 15% krill meal
- **Diet 6** : 30% Krill meal
- **Diet 7** : 100% Fish meal with lipids from lobster (natural carotenoid)
- **Diet 8**: Japanese commercial feed for Japanese red sea bream (Otohim, Japan)

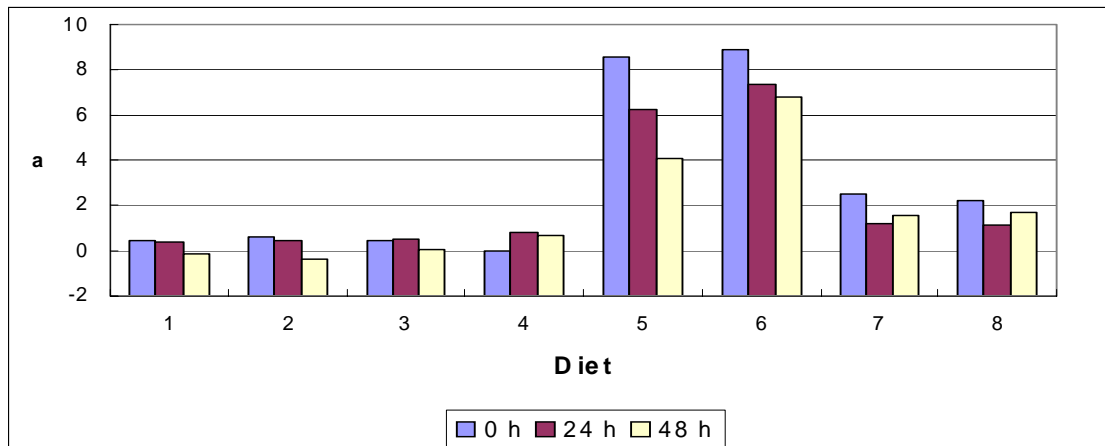


Figure 77. Skin evolution in parameter a (redness) after 0, 24 & 48 hours post mortem of fish on ice.

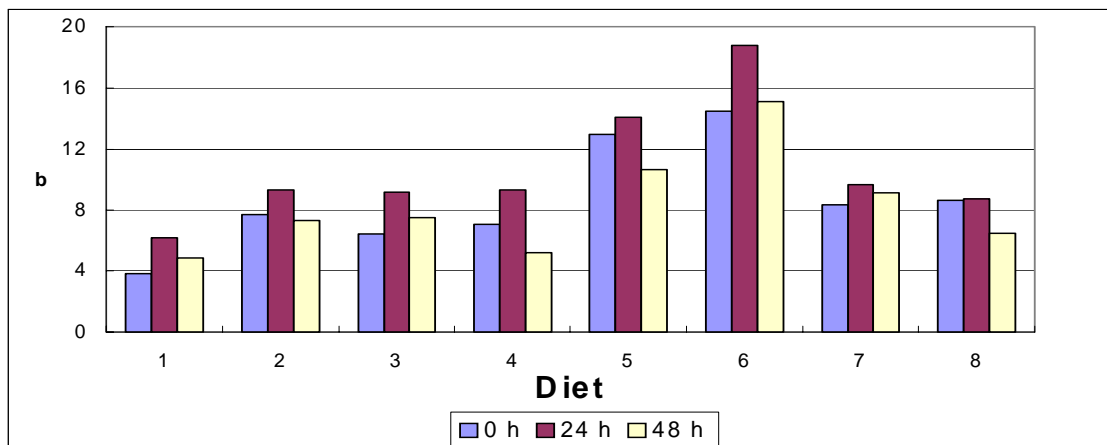


Figure 78. Skin evolution in parameter b (yellowness) after 0, 24 & 48 hours post mortem of fish on ice.

Experiment 3.4.2

In this experiment fish fed diets containing shrimp shell meal (Sopropêche, France) containing 40 mg/kg of esterified astaxanthin for different time periods (6, 4 or 2 months) were sacrificed in a mix of ice and water, as for commercial purposes, and the changes in skin colour for a period of 7 days post mortem was evaluated. For this propose fish were ice packed in polystyrene boxes and stored at 10 degrees simulating as much as possible extended commercial procedures. For this trial, the different colour parameters where evaluated only in the ventral skin area.

Results are shown in two ways: Comparisons between diets at the same sampling point, *i.e* hours after sacrifice (days 0 to 7 days or 0 to 168h), and also within the same diet for the whole experimental period (7 days).

There was an increase in Hue values at 24 h between diets (**Figure 79**), with fish fed the SM40 BIII showing a in significantly different Hue value to those fed the control diet. From the 24h till the 168h no differences were found between diets. In general, increased Hue values were found in all fish for longer storage periods. The hue

evolution (**Table 16**) showed only differences with time for those fish fed the SM 40 BII and SM 40 BIII, with lower values in both cases at the moment of the sacrifice (higher skin redness) and higher at 24 h post-mortem, which was more noticeable for fish fed the SM 40 BIII.

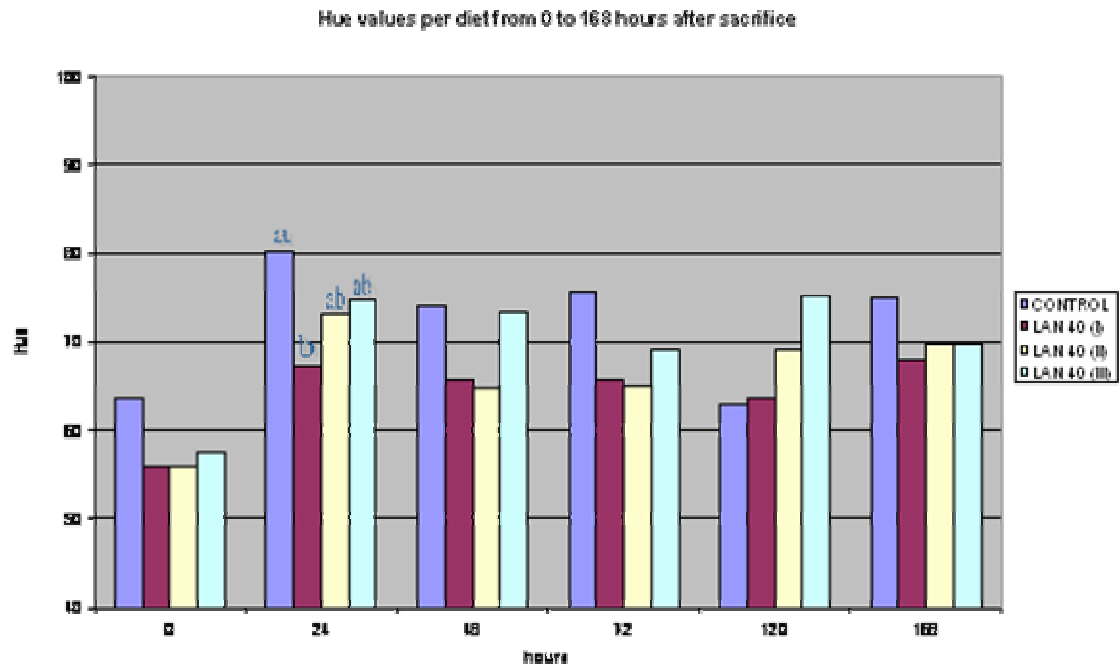


Figure 79. Post-mortem changes in Hue value in fish fed a shrimp shell based diet for a period of 6 [LAN 40 (I)], 4 [LAN 40 (II)], or 2 [LAN 40 (III)] months.

Table 16. Statistical differences in Hue post-mortem changes in fish fed a shrimp shell based diet for a period of 6 (SM 40 BI), 4 (SM 40 BII), or 2 (SM 40 BIII) months. Different letters differ significantly each other ($p < 0.05$).

		Hue (Ventral body area)					
Diet	Time (h)	0	24	48	72	120	168
	CONTROL		63,66 ± 7,37 ^a	80,14 ± 7,96 ^a	74,11 ± 3,94 ^a	75,56 ± 6,12 ^a	62,98 ± 14,49 ^a
SM 40 BI		56,10 ± 3,25 ^a	67,27 ± 3,28 ^a	65,78 ± 3,76 ^a	65,72 ± 3,27 ^a	63,67 ± 5,79 ^a	68,06 ± 5,91 ^a
SM 40 BII		55,97 ± 2,55 ^b	73,21 ± 3,94 ^a	64,86 ± 4,06 ^{ab}	64,94 ± 3,98 ^{ab}	69,01 ± 4,47 ^a	69,70 ± 4,13 ^a
SM 40 BIII		57,54 ± 1,36 ^b	74,84 ± 0,12 ^a	73,28 ± 2,76 ^a	69,12 ± 1,85 ^a	75,25 ± 2,35 ^a	69,83 ± 5,76 ^a

Chroma showed higher values for all fish fed the SM 40 diet in comparison to control; the highest differences between the control fish and the others were found at 72 h and 120 h post mortem. 168 h post mortem chroma showed some lower values in all treatments but without significant differences between them (**Figure 80**). Chroma was decreasing with increasing time after dead of the fish but without significant differences between 0 h and 168 h (**Table 17**).

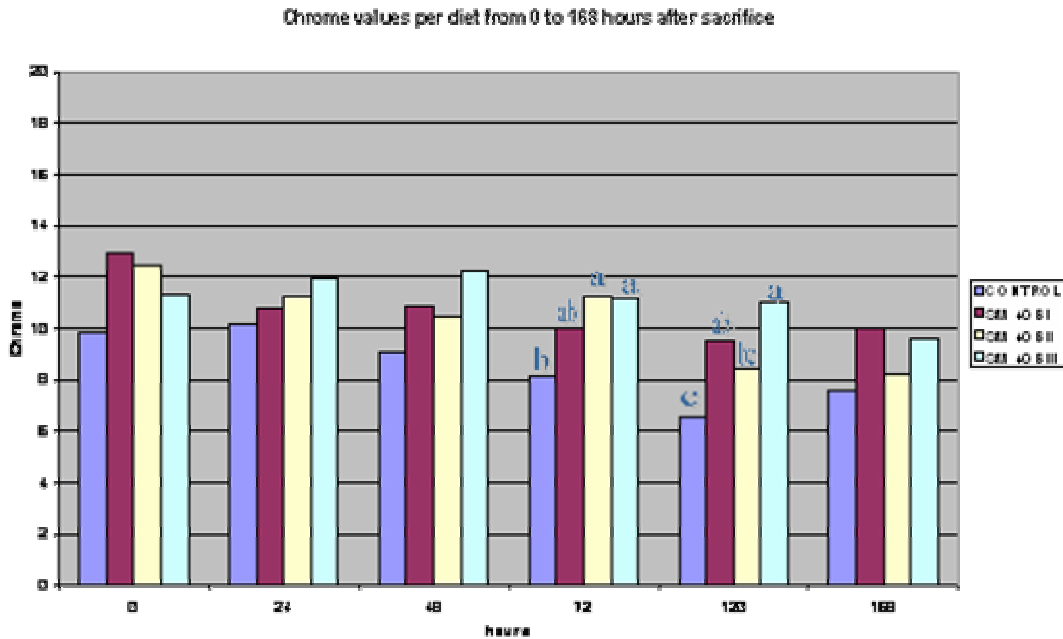


Figure 80. Post-mortem changes in Chroma value in fish fed a shrimp shell based diet for a period of 6 (SM 40 BI), 4 (SM 40 BII), or 2 (SM 40 BIII) months.

Table 17. Statistical differences in Chroma post-mortem changes in fish fed a shrimp shell based diet for a period of 6 (SM 40 BI), 4 (SM 40 BII), or 2 (SM 40 BIII) months. Different letters differ significantly each other ($p < 0.05$).

		Chrome (Ventral body area)					
Time (h)	Diet	0	24	48	72	120	168
		CONTROL	9,86 ± 0,52 ^{ab}	10,16 ± 1,31 ^a	9,05 ± 0,85 ^{ab}	8,13 ± 0,25 ^{abc}	6,59 ± 0,53 ^c
SM 40 BI	12,94 ± 1,52 ^a	10,77 ± 2,04 ^a	10,86 ± 2,38 ^a	10,00 ± 0,59 ^a	9,53 ± 0,68 ^a	9,97 ± 0,74 ^a	
SM 40 BII	12,53 ± 2,96 ^a	11,22 ± 0,77 ^a	10,49 ± 0,67 ^a	11,22 ± 1,62 ^a	8,41 ± 1,03 ^a	8,23 ± 1,13 ^a	
SM 40 BIII	11,33 ± 1,09 ^a	11,91 ± 0,42 ^a	12,28 ± 1,44 ^a	11,14 ± 0,81 ^a	11,03 ± 1,40 ^a	9,63 ± 0,91 ^a	

The Lightness parameter showed decreasing values from 0 to 168 h post mortem but without significant differences between diets (**Figure 81, Table 18**).

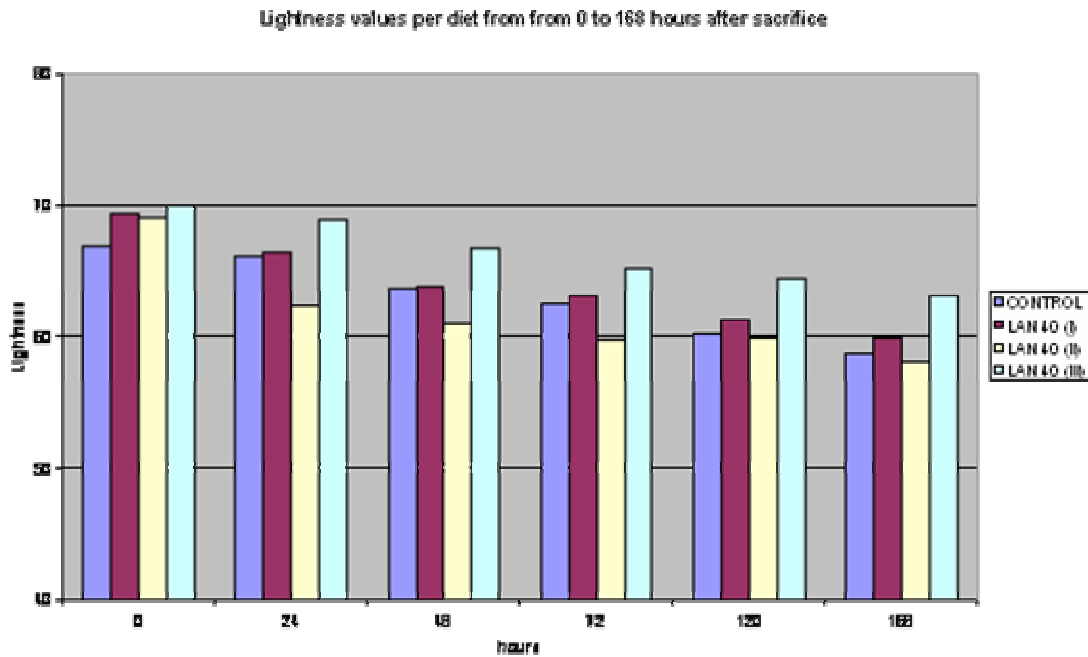


Figure 81. Post-mortem changes in Lightness parameter in fish fed a shrimp shell based diet for a period of 6 [LAN 40 (I)], 4 [LAN 40 (II)], or 2 [LAN 40 (III)] months.

Table 18. Statistical differences in Lightness post-mortem changes in fish fed a shrimp shell based diet for a period of 6 (SM 40 BI), 4 (SM 40 BII), or 2 (SM 40 BIII) months. Different letters differ significantly each other ($p < 0.05$).

Lightness (Ventral body area)						
Time (h)	0	24	48	72	120	168
Diet						
CONTROL	66,98 ± 2,03 ^a	66,13 ± 1,84 ^a	63,75 ± 2,53 ^{ab}	62,54 ± 3,86 ^{ab}	60,25 ± 1,8 ^{ab}	58,71 ± 2,51 ^b
SM 40 BI	69,28 ± 3,49 ^a	66,44 ± 4,23 ^{ab}	63,89 ± 1,87 ^{ab}	63,05 ± 2,54 ^{ab}	61,28 ± 1,40 ^{ab}	59,86 ± 1,07 ^b
SM 40 BII	69,07 ± 2,153 ^a	62,38 ± 3,90 ^{ab}	60,91 ± 3,88 ^{ab}	59,743 ± 2,94 ^b	59,903 ± 3,30 ^b	58,043 ± 3,37 ^b
SM 40 BIII	70,00 ± 2,88 ^a	68,94 ± 0,48 ^{ab}	66,76 ± 1,26 ^{abc}	65,23 ± 1,49 ^{bc}	64,43 ± 0,73 ^c	63,11 ± 1,15 ^c

Experiment 3.4.3

In black background adapted fish there was a significant increase in mean lightness (L) of the dorsal skin area from 46.57 ± 1.43 on day 0 to 54.10 ± 1.11 on day 7 of storing in ice (0-2 °C) (**Figure 82**). In white background adapted fish there was no effect of storage on skin lightness and in general higher mean L values were found in comparison to black background adapted fish (**Figure 82**).

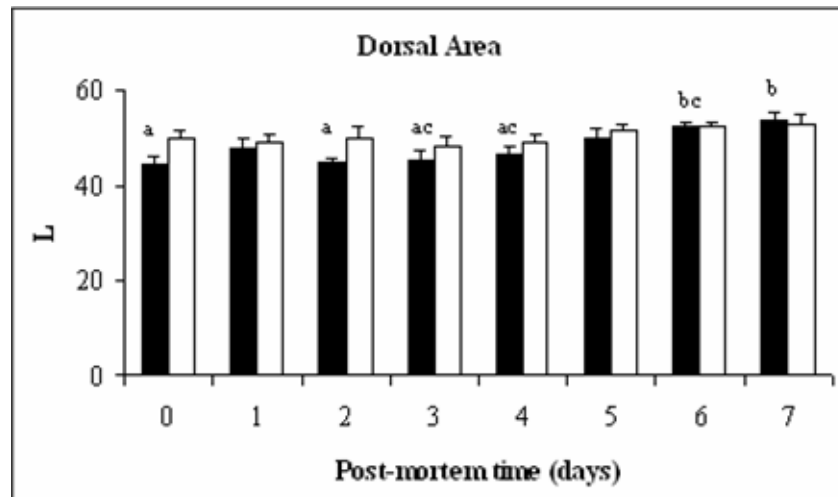


Figure 82. Mean (SEM) lightness values of the dorsal skin area of fish adapted to black (black bars) or white (white bars) background, sacrificed in a mix of ice and water and stored in ice for a period of 7 days. Different letters indicate statistically significant differences between the different sampling points (days) within each group.

In the ventral body area there was no effect of storage time on skin lightness in both groups (**Figure 83**).

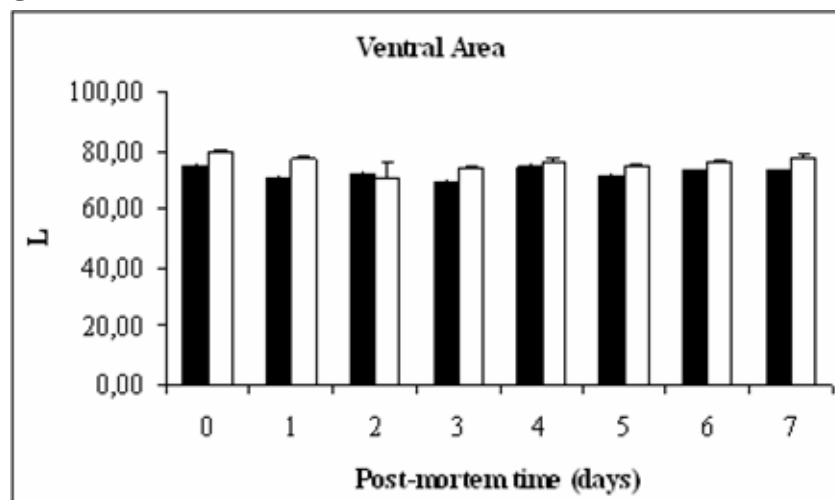


Figure 83. Mean (SEM) lightness values of the ventral skin area of fish adapted to black (black bars) or white (white bars) background, sacrificed in a mix of ice and water and stored in ice for a period of 7 days.

Results for Hue are illustrated in **Figures 84 & 85**. In both groups there was a significant decrease in Hue of the dorsal body area from Day 0 to Day 7 of ice storage moving skin from a pinkish to a reddish hue. There was no significant effect of time storage on hue values of the ventral skin area in both groups.

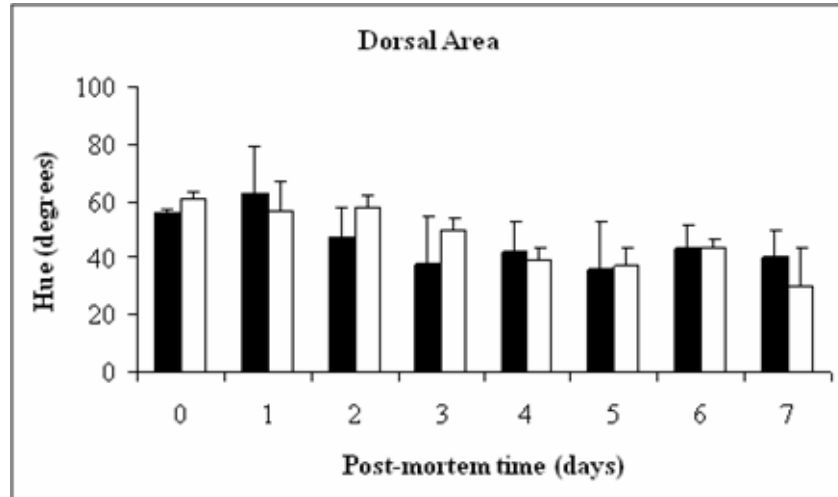


Figure 84. Mean (SEM) hue values of the dorsal skin area of fish adapted to black (black bars) or white (white bars) background, sacrificed in a mix of ice and water and stored in ice for a period of 7 days.

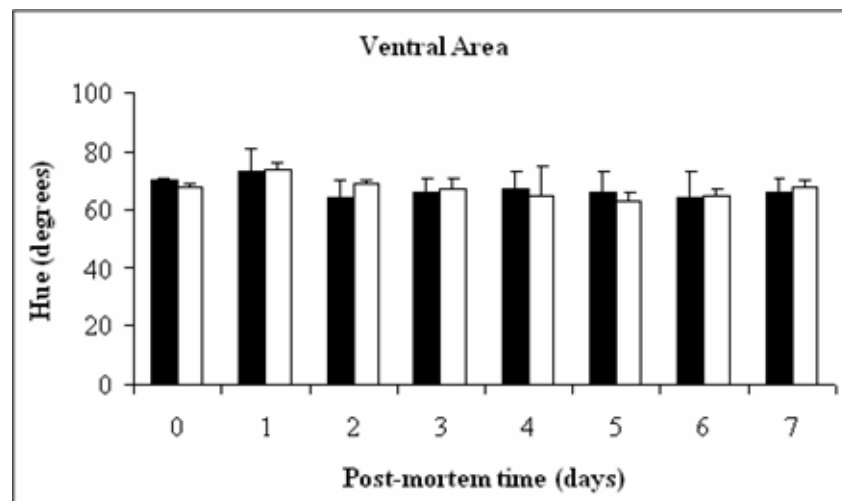


Figure 85. Mean (SEM) hue values of the ventral skin area of fish adapted to black (black bars) or white (white bars) background, sacrificed in a mix of ice and water and stored in ice for a period of 7 days.

The effect of storage time on skin colour was better reflected in the Entire Colour Index (ECI). There was a marked decrease in both groups and in both the dorsal and ventral skin area from day 1 or 2 to day 7 of storage (**Figures 86 & 87**). In the dorsal skin area of black adapted fish there was a significant ECI decrease from an initial value of 9.51 ± 0.51 to 2.84 ± 0.19 on Day 7. A similar pattern was observed in white adapted fish (Day 0: 9.47 ± 0.51 ; Day 7: 2.19 ± 0.40) (**Figure 86**).

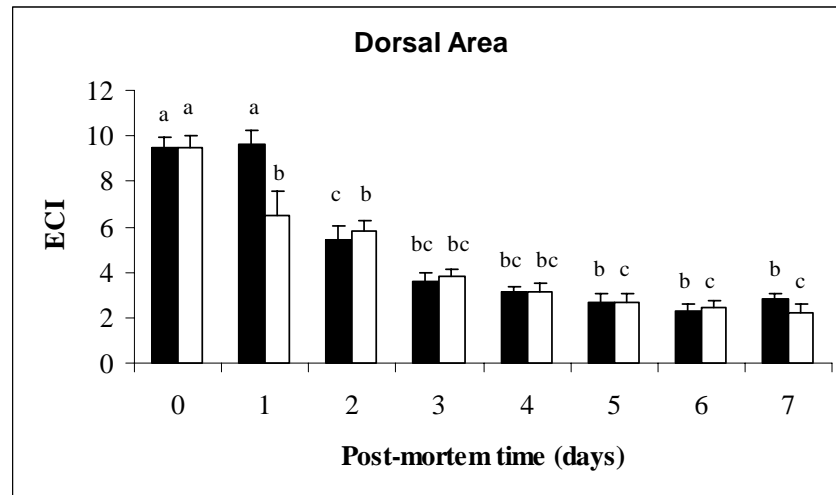


Figure 86. Mean (SEM) ECI values of the dorsal skin area of fish adapted to black (black bars) or white (white bars) background, sacrificed in a mix of ice and water and stored in ice for a period of 7 days. Different letters indicate statistically significant differences between the different sampling points (days) within each group.

A similar pattern was observed in the ECI values of the ventral skin area (**Figure 87**).

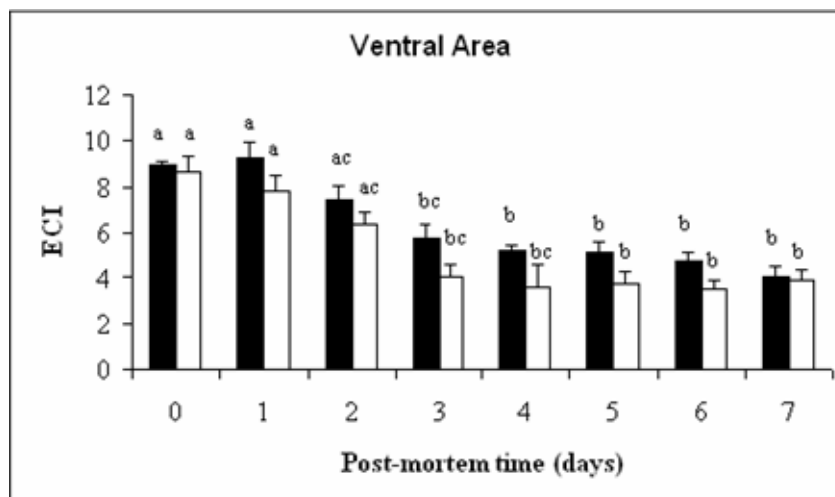


Figure 87. Mean (SEM) ECI values of the ventral skin area of fish adapted to black (black bars) or white (white bars) background, sacrificed in a mix of ice and water and stored in ice for a period of 7 days. Different letters indicate statistically significant differences between the different sampling points (days) within each group.

Experiment 3.4.4

There were no significant changes in lightness of the dorsal and ventral skin area in red porgies stored at 0-2 °C for up to 7 days (**Figure 88 & 89**).

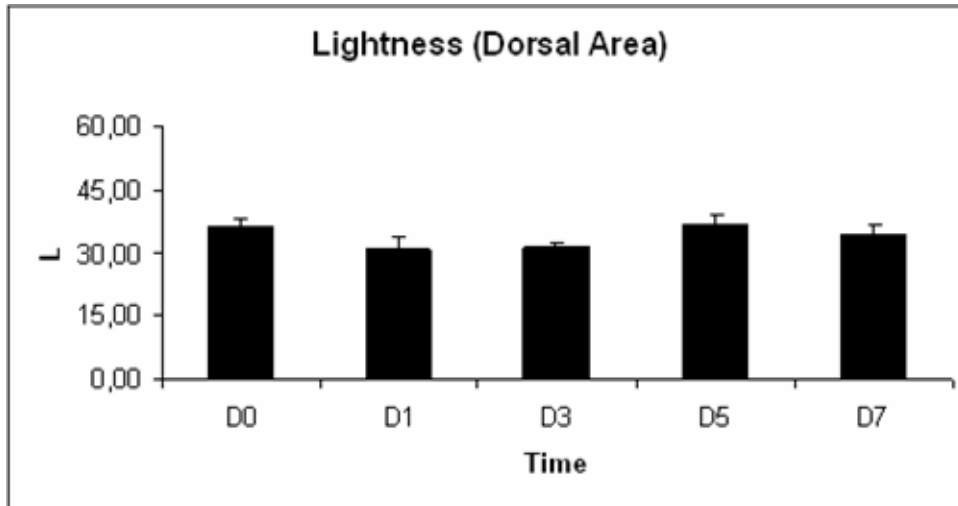


Figure 88. Mean (SEM) L values of the dorsal skin area of red porgies sacrificed in a mix of ice and water and stored in ice for a period of 7 days.

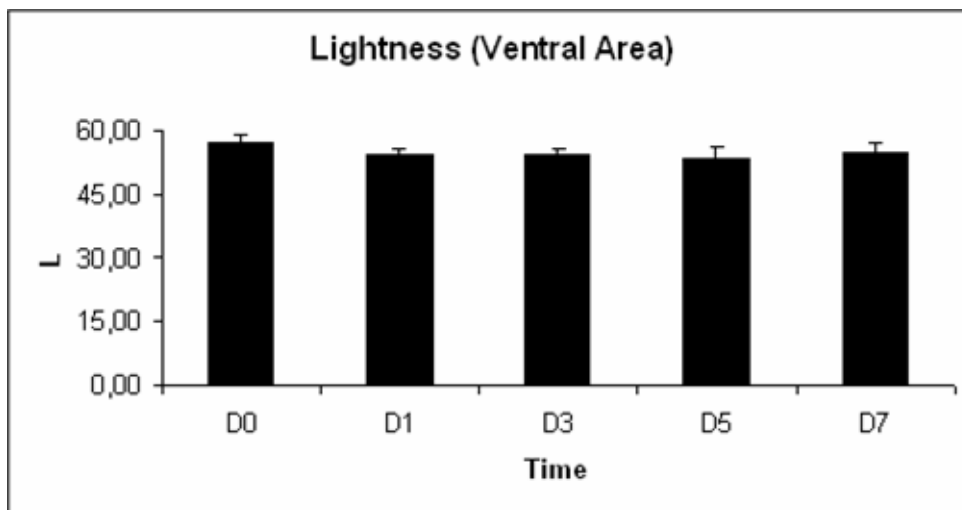


Figure 89. Mean (SEM) L values of the ventral skin area of red porgies sacrificed in a mix of ice and water and stored in ice for a period of 7 days.

Results for Hue are illustrated in **Figures 90 & 91**. There was a significant decrease in Hue of the dorsal body area in Days D1 and D3 and then an increase in Days D5 and D7 to values similar to those of the initial sampling D0 (**Figure 90**). There was no significant effect of time storage on hue values of the ventral skin area (**Figure 91**).

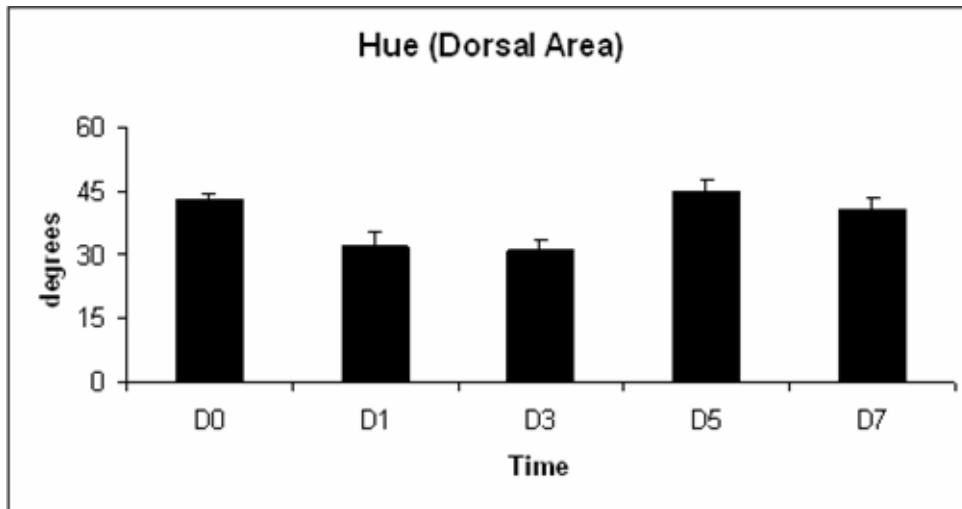


Figure 90. Mean (SEM) Hue values of the dorsal skin area of red porgies sacrificed in a mix of ice and water and stored in ice for a period of 7 days.

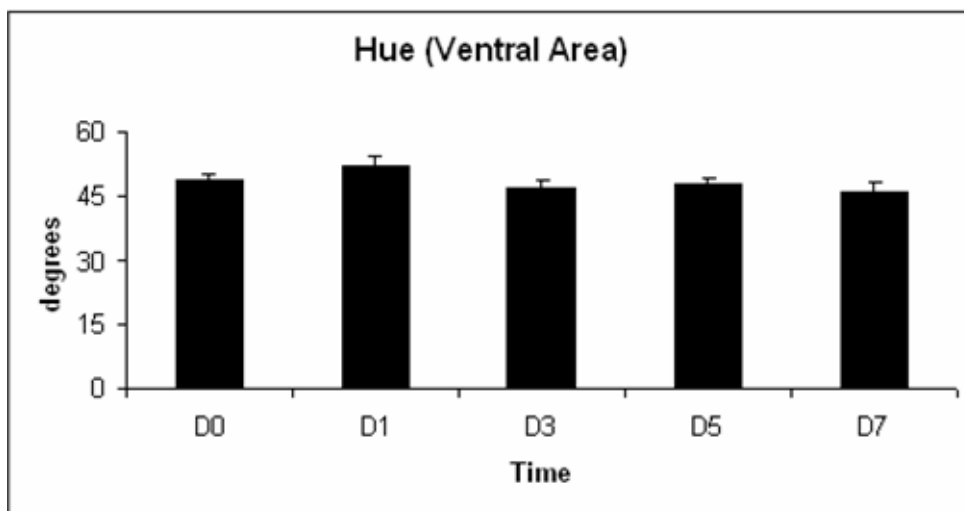


Figure 91. Mean (SEM) Hue values of the ventral skin area of red porgies sacrificed in a mix of ice and water and stored in ice for a period of 7 days.

The effect of storage time on skin colour was better reflected in the Entire Colour Index (ECI). There was a marked decrease in ECI of both the dorsal and ventral skin area in Day 3 onward to Day 7 (**Figures 92 & 93**). In the dorsal skin area, ECI decreased from an initial value of 11.00 ± 1.03 to 6.85 ± 0.61 on Day 5 and to 6.03 ± 0.49 on Day 7. In the ventral skin area, ECI decreased from an initial value of 15.01 ± 1.78 to 10.65 ± 0.95 on Day 5 and to 7.56 ± 0.83 on Day 7.

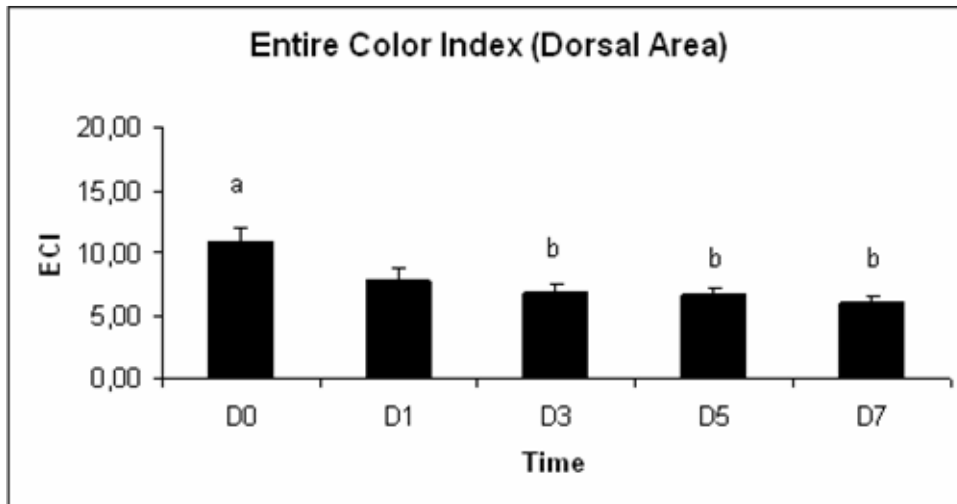


Figure 92. Mean (SEM) ECI values of the dorsal skin area of red porgies sacrificed in a mix of ice and water and stored in ice for a period of 7 days. Different letters indicate statistically significant differences between the different sampling points (days).

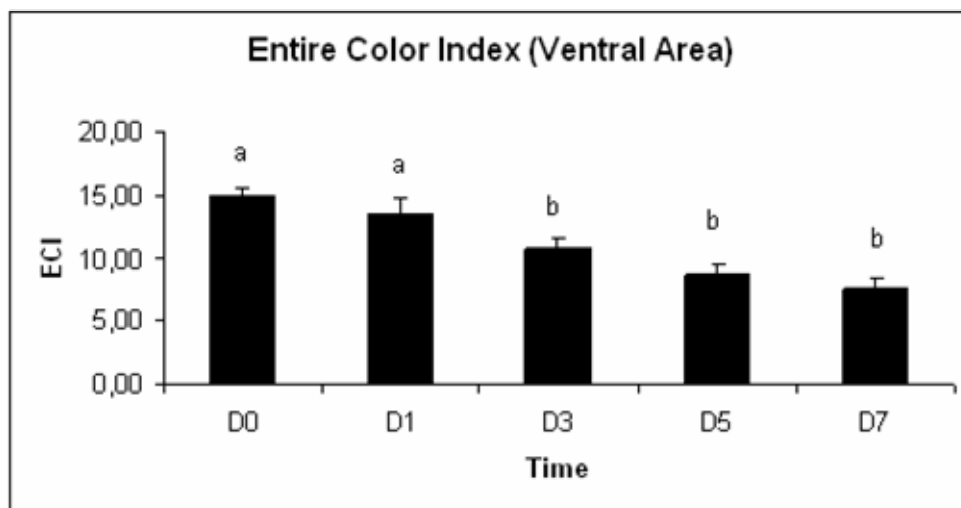


Figure 93. Mean (SEM) ECI values of the ventral skin area of red porgies sacrificed in a mix of ice and water and stored in ice for a period of 7 days. Different letters indicate statistically significant differences between the different sampling points (days).

Statistically significant differences from the initial values of D0 were observed from Day 3 onwards to all the freshness quality indexes used. Freshness ranged from an initial value of 12 ± 0 (Day 0) to a minimum of 7.85 ± 0.31 on Day 7 (**Figure 94**).

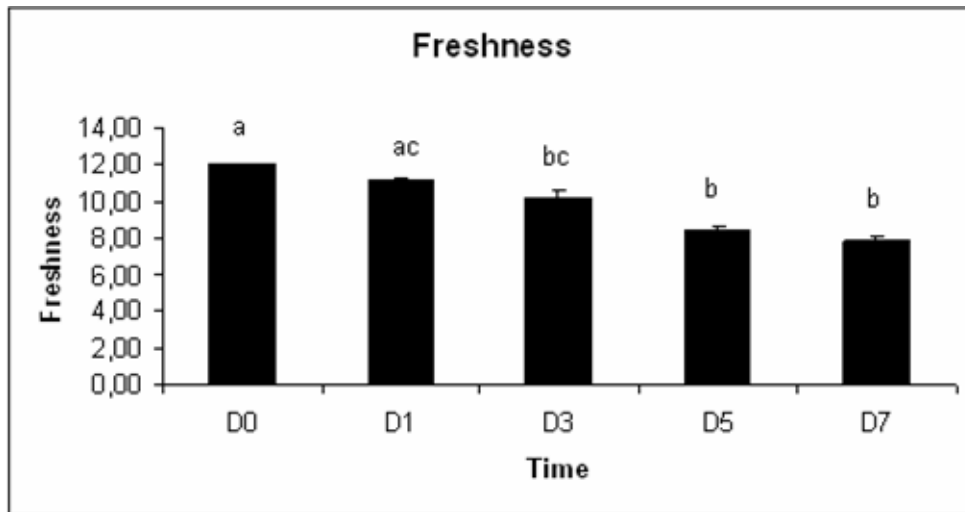


Figure 94. Freshness (mean \pm SEM) values of red porgies sacrificed in a mix of ice and water and stored in ice for a period of 7 days. Different letters indicate statistically significant differences between the different sampling points (days).

Kvalue decreased from an initial value of 97.59 ± 1.08 % on Day 0 to 92.71 ± 0.19 % on Day 7 (**Figure 95**). In a similar way sensorial analysis score showed a significant decrease from an initial value of 30 ± 0 to 4.33 ± 0.21 on Day 7 (**Figure 96**).

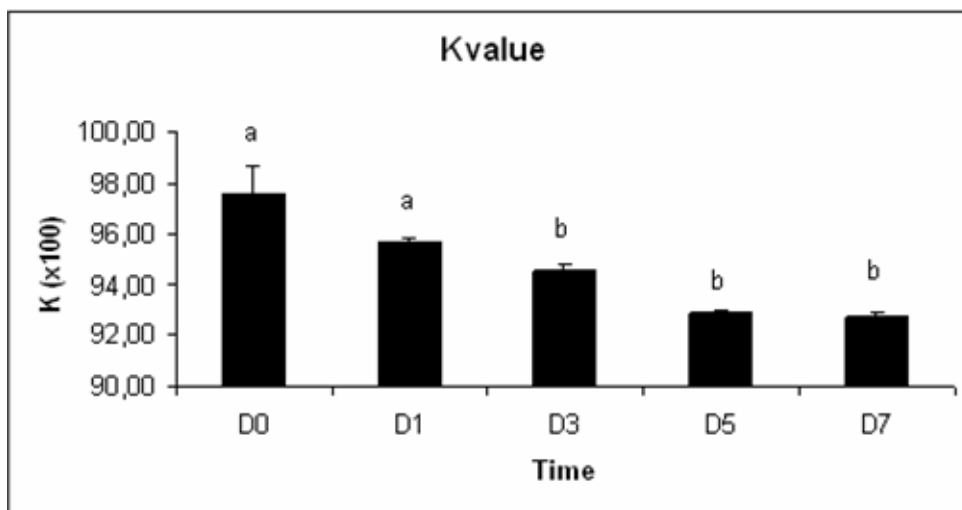


Figure 95. Kvalue (mean \pm SEM) of red porgies sacrificed in a mix of ice and water and stored in ice for a period of 7 days. Different letters indicate statistically significant differences between the different sampling points (days).

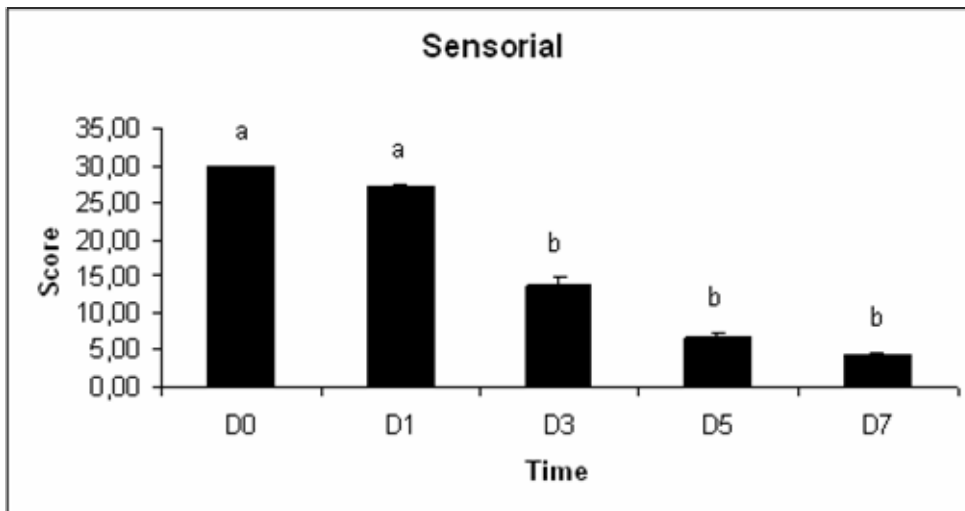


Figure 96. Sensorial analysis of red porgies sacrificed in a mix of ice and water and stored in ice for a period of 7 days. Different letters indicate statistically significant differences between the different sampling points (days).

Finally, eye refractometry showed a significant increase from 11.17 ± 0.17 on Day 0 to 17.25 ± 0.25 on Day (Figure 96).

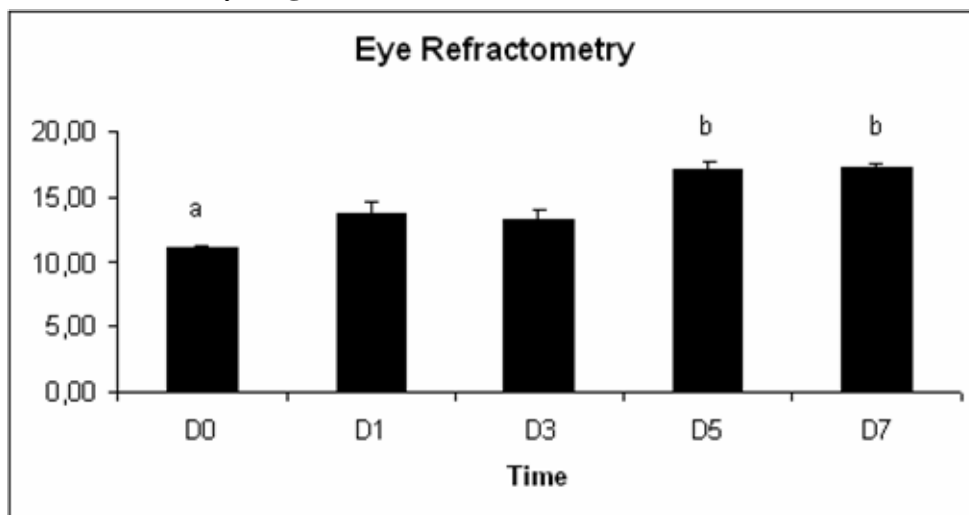


Figure 97. Eye refractometry values (mean \pm SEM) of red porgies sacrificed in a mix of ice and water and stored in ice for a period of 7 days. Different letters indicate statistically significant differences between the different sampling points (days).

There were statistically significant correlations between all the estimated freshness indicators (Table 19). There was no significant correlation between these quality parameters and skin lightness (L). However, there were significant correlations between estimated freshness indicators and the Entire Color Index.

Table 19. Correlation coefficient values, as defined by Pearson product moment correlation analysis, of freshness (quality) indicators and colour parameters in red porgy sacrificed in a mix of ice and water and stored in ice for a period of 7 days ($P < 0.001$).

	EyeR	Freshness	Sensorial	L-dorsal	L-ventral	ECI-dorsal	ECI-ventral
Kvalue	-0.699	0.787	0.823	NS	NS	0.551	0.682
EyeR		-0.837	-0.749	NS	NS	NS	-0.696
Freshness			0.908	NS	NS	0.543	0.786
Sensorial				NS	NS	0.628	0.813
L-dorsal					NS	NS	NS
L-ventral						NS	NS
ECI-dorsal							0.471

DISCUSSION

4. Role of illumination and background color

Colour changes in fish are often stress-induced. The main pigmentation controlling hormones α -Melanocyte Stimulating Hormone (α MSH) and Melanin-Concentrating Hormone (MCH) are pleiotropic and not only control skin pigmentation but also regulate the response to stressors. In vertebrates in which the pigmentation of the skin can be changed by hormonal stimulation, the colour of the background and the illumination are determining factors for the intensity and / or the pattern of skin pigmentation.

The aim of the present task was to identify the major environmental and husbandry factors implicated in skin colour regulation and to evaluate the stress response of red porgy in relation to changes in the chromaticity parameters.

Results clearly showed that background, lighting spectrum, light intensity and stocking density have an important effect on skin chromaticity parameters. White background increases skin brightness in levels similar to that of wild fish but reduces skin chroma. Blue lighting spectrum increases skin brightness in dark background (black and red) held fish but did not show any significant effect on hue or chroma. Low light intensity resulted also in higher L in fish held under dark backgrounds. Stocking density affected significantly L, Hue and ECI. In particular, fish held under dark backgrounds and high stocking density showed lower L and ECI values than that held at low stocking density.

There was no effect of background, lighting spectra, light intensity and stocking density on the number of melanophores per mm² of skin. Higher skin melanin content was observed in red porgies held under dark backgrounds and high light intensity and stocking density compared to that in fish held under low light intensity and stocking density. In all experiments, there was no significant effect of background colour, lighting spectrum & intensity and stocking density on the circulating stress indicators determined (cortisol, α MSH, glucose, lactate, osmolality, electrolytes). In addition, there was no correlation between chromaticity parameters and the used stress indexes. However, significant differences were observed in circulating catecholamines and in the complement and lysozyme activities.

In all experiments skin paling was corelated with increased norepinephrine plasma concentrations. High light intensity induced a significant decrease in lysozyme activity. Full lighting spectrum seems to induce higher levels of lysozyme than the blue spectrum and white background induces earlier changes than the black and red background, but later on (Day 32) fish in all groups showed the same type of recovery dynamics.

The results altogether indicate that the observed differences in skin lightness are related to differences in the motility of skin chromatophores; melanophores in pale fish are aggregated while in control fish dispersed. Concerning the neuroendocrine regulation of this motility, melanophore aggregation is not mediated through circulating cortisol or MSH, but through changes in plasma catecholamines.

CONCLUSIONS