Endocrine regulation of skin blanching in red porgy, *Pagrus pagrus*

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Previous research on red porgy, *Pagrus pagrus*, has shown that physiological colour change does not depend on changes in plasma cortisol or α -MSH. The purpose of this study was to identify the endocrine mechanism responsible for skin blanching in fish exposed to white background. There was no significant difference in serum cortisol, glucose, lactate, thyroid hormones, or plasma epinephrine concentration between black and white-background-adapted fish. Mean daily plasma melatonin concentration was significantly higher in black- (41.29 ± 2.46 pg ml⁻¹) than white-background-exposed fish (32.07 ± 1.26 pg ml⁻¹) while mean plasma norepinephrine was almost fivefold higher in white- (6.26 ± 1.57 ng ml⁻¹) than in black-background-adapted fish (1.01 ± 0.34 ng ml⁻¹). Skin melanin concentration did not differ significantly between the experimental groups. Our study indicates that skin blanching in red porgy is mediated through norepinephrine release.

Introduction

Skin coloration of fish is under multi-parametric control and a number of internal or external factors (physical, nutritional, neuro-hormonal) have been known to influence the chromatic state of fish (Fujii 2000). Some fish can alter their coloration in response to environmental conditions, physiological challenges and stressful stimuli. Changes in skin shade, hue and/or chroma patterns are due to changes in the motile activities of chromatosomes, to the increase or decrease in the number of chromatophores and/or to differences in the pigment quantity of the chromatosomes (Burton 1993). In teleosts, it is known that (depending on fish species) physiological colour changes are regulated by the nervous, or hormonal or, more commonly, by both systems (Fujii 1969, Fujii & Novales 1972). Endocrine control of physiological colour change is mediated mainly by α -melanophore-stimulating (α -MSH) and melanin-concentrating (MCH) hormones. Neural control involves the sympathetic nervous system with a catecholamine neurotransmitter, most likely noradrenaline which, when at physiological concentrations, causes a rapid aggregation of chromatophores (Fujii 1993). Slower chromatic reactions of fish are generally controlled by the endocrine system whereas rapid ones are mainly controlled by neural mechanisms

(Fujii 2000). The sophisticated chromatic properties observed in fish provide protection from predators, advertise territoriality and assist in both survival and intraspecific communication (Fujii 1961). Skin colour patterns are believed to be an effective compromise between the conspicuous coloration necessary for visual communication and the cryptic coloration essential for predators' avoidance. Background adaptation is useful in studying physiological colour changes in ectotherms. It is well known, that lower vertebrates adjust their skin colour in response to changes in background colour and/or reflectivity (Baker et al. 1985, Fernandez & Bagnara, 1991, Fujimoto et al. 1991, Kolk et al. 2002, Rotllant et al. 2003) through a combination of short term (physiological) and longer term (morphological) pigmentation changes (Bagnara & Hadley 1973, Fujii 2000). In higher vertebrates, the pigment cell system may have become simplified. For example, no definite role of the nervous system in regulating melanocyte function has been proven (Fujii 2000).

The pituitary gland has been related to pigmentation control in cyclostomes, elasmobranches, teleosts, amphibians and reptiles (Bagnara & Hadley 1973). Among the hormonal substances that affect chromatophore motility, α -melanophore-stimulating hormone (α -MSH), melanin-concentrating hormone (MCH) and melatonin have been investigated in several fish species. The main hormone causing dispersion of melanin granules in melanophores and thus the darkening of the skin is considered to be α -MSH. Plasma α -MSH levels have been found to be elevated in response to black background adaptation in the amphibian Xenopus laevis, dogfish (Scylliorhinus canicula), eel (Anguilla anguilla) and rainbow trout (Oncorhynchus mykiss) (Baker 1972, Wilson & Dodd 1973, Wilson & Morgan 1979, Bowley et al. 1983, Sumpter et al. 1984). Alternatively, in sailfin molly (Poecilia latipinna) and the red porgy

(*Pagrus pagrus*), physiological colour changes do not depend on changes in plasma α -MSH (Baker 1981, Ball & Baker 1981, Baker *et al.* 1984, Szisch *et al.* 2002, Rotlland *et al.* 2003, Van der Salm *et al.* 2004).

Many ectotherm vertebrates exhibit a circadian rhythm of color change, with nocturnal blanching usually associated with elevated melatonin levels (Filadelfi & Castrucci 1996). In amphibians, melatonin exerts a potent skin lightening activity. However, in teleosts and reptiles, melatonin effects may vary with species, developmental stage, and pigment cell location (Filadelfi & Castrucci 1996). Fujii (1961) first recorded that melatonin effectively aggregated melanophore inclusions in the goby, Chasmichthys gulosus. This action of melatonin was also found in other species like Scardinius erythrophtalmus (Mira 1962), Carassius auratus (Hu 1963), Phoxinus phoxinus (Healey & Ross 1966) and Salmo gairdneri (Hafeez 1970, Owens et al. 1978). However, in some species like Fundulus heteroclitus (Abbott, 1968), Lepidosiren paradoxa and Potamotrygon reticulatus (Visconti & Castrucci 1993) there was a weak or negative response.

Recent studies showed that red porgies subjected to either black, grey or white background, showed the classical dark-to-pale changes in skin colour but did not reveal any associated change in circulating α -MSH or cortisol (Rotllant *et al.* 2003, Van der Salm *et al.* 2004). The aims of the present study were to quantify skin colour changes and to identify the endocrine mechanism mediating the alternation of skin coloration in the red porgy following background adaptation.

Materials and methods

Fish and experimental design

Red porgies, weighing 403.8 ± 12.7 g (mean \pm SE), from the same broodstock reared under intensive culture conditions (National Centre for Marine Research, Institute of Aquaculture, Crete, Greece) and ambient photoperiod and temperature, were transferred to indoor laboratory facilities and randomly divided into four

500-1 circular black background polyester tanks (6 fish/tank, two tanks per treatment). Each tank was supplied with running, aerated seawater (dissolved $O_2 6.4 \pm 0.7$ mg l⁻¹; water temperature 18.3 \pm 0.7 °C, water flow: 8–10 1 min⁻¹) and acclimated for two weeks prior to exposure to the experimental backgrounds.

The experimental tanks were located in two light-proof enclosures (two tanks per enclosure), illuminated with fluorescent strip lights located 130 cm above the water surface (tank depth 1 m, light intensity $0.5 \pm 0.1 \ \mu \text{mol m}^{-2} \text{ s}^{-1}$ at the water surface) and controlled with electric timers, set for a 12L:12D photoperiod regime (dawn at 6:00 and dusk at 18:00). Fish were held for 21 days with black (Group B) or white (Group W) backgrounds in order to give time for possible longterm morphological colour changes. In Group W, special textile bags were attached to the bottom and sides of the tanks to create a white background. Fish were fed commercial dry pellets (Biomar, Hellas) ad libitum by means of selffeeders. Access to the self-feeder was blocked 15 h prior to blood sampling.

In order to minimize stress, 15 min prior to sampling the water level was lowered (by siphoning) and anesthetic (0.3 ml l⁻¹ ethylenglycol-monophenylether, Merck) was added to the tank water. While lightly anaesthetized, fish were netted, their heads were covered with a towel, blood was drawn from the caudal vessel, divided into aliquots of serum (for glucose, lactate and cortisol determination), Na heparin plasma (for melatonin determination) or EDTA plasma (for catecholamines determination), and skin colour measurements were performed. After centrifugation (2000 g, 4 °C), serum and plasma were stored at -20 °C until analysed.

Colour measurements

The CIELAB system proposed by the International Commission on Illumination (CIE 1978) was applied to measure the skin colour by means of a portable spectrophotometer (MiniScanTM XE, Hunter Assoc. Laboratory Inc., Va). Four measurements were taken per fish, two at the dorsal (D) and two at the ventral (V) body side of the fish. The three-dimensional characteristics of colour appearance, i.e. the brightness attribute (L^*) and the two chromatic attributes — hue (H^*_{ab}) and chroma (C^*_{ab}) — were calculated.

Analytical procedures

Serum metabolites were measured using enzymatic colorimetric procedures (glucose GOD/ PAP; lactate: PAP) with commercial kits (Biosis, Greece and BioMerieux, France, respectively). Serum cortisol measurements were based on the radioimmunoassay method described by WHO (Sufi et al. 1994), with the following modifications. The cortisol antibody was purchased from Chemicon International, (262MDL) and was diluted 1:1600 before use. Tritiated cortisol was purchased from Amersham International (TRK4O7). Samples for cortisol determination were diluted ten times to eliminate interference from plasma proteins and enhance cortisol-antibody binding. Aliquots of 50 μ l of diluted plasma samples were used for the radioimmunoassay. Displacement curves (three different pools of red porgy plasma, diluted 1:2, 1:4, 1:6, 1:8 and 1:10) demonstrated linear parallelism after logit-log transformation ($r^2 = 0.971$, slope = -2.205 vs. standard curve: $r^2 = 0.992$, slope = -2.120). The recovery of cortisol added to plasma was 103% $\pm 2.5\%$ (mean \pm SE, n = 4). Thyroid hormones (T_2, T_4) were analyzed using coated-tubes radioimmunoassay commercial kits (Orion Diagnostica, Finland), with the following modifications: T₂ unknown samples were diluted 1:4 with saline and T₄ sample volume was increased by a factor of two, in order to have the unknown sample value within the optimal portion of the standard curve. Melatonin was analyzed from chloroform-extracted plasma samples using a specific RIA (Fraser et al. 1983, Valtonen et al. 1993). Displacement of radioligand with three different volumes (25, 50, and 100 μ l) of red porgy plasma pool taken at night demonstrated acceptable parallelism after logit-log transformation (plasma pool: $r^2 = 0.998$, slope = -0.952 vs. standard curve: $r^2 = 0.998$, slope = -0.990). The mean $(\pm$ SE, n = 6) recovery of melatonin (10, 20, or 40 pg) added to the plasma pool was 97.1% \pm 3.9%. Catecholamines were analysed using commercial coated-tube radioimmunoassay kits (KatCombi RIA, Immuno Biological Laboratories). Displacement curves (a pool of plasma, with dilutions of 1:2, 1:4, 1:8) demonstrated linear parallelism after logit-log transformation for epinephrine and norepinephrine ($r^2 = 0.999$, slope = -2.612 vs. standard curve $r^2 = 0.994$, slope = -2.258 and $r^2 = 0.996$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994$, slope = -2.132 vs. standard curve $r^2 = 0.994 \text{ vs.}$ standard stand vs.}

Melanin determination

Skin was dissected from two different parts of the dorsal body area at the positions where the line perpendicular to the longitudinal body axis pass through (i) the anterior margin of the dorsal fin and (ii) through the anus. Each skin sample was measured by employing a video-image processing apparatus adapted to a stereoscope. Skin melanin concentration was determined according to Wilson and Dodd (1973) and Sugimoto (1993), with several modifications. Briefly, samples were fixed in 95% ethanol for 24 h and then incubated with 1% HCl (at 60 °C for one hour) for decalcification. The decalcified pieces were washed several times with distilled water and then boiled in 0.2% NaOH for 1 hour in order to extract melanin. The melanin concentration was determined by measuring the absorbance of the supernatant at 340 nm against a sepia melanin synthetic standard (Sigma, M-2669). The melanin standard was solubilized in 1 ml 1 M NaOH and 10 μ l 3% H₂O₂ by heating in a boiling water bath for 30 min.

Statistical analysis

The statistical errors are expressed as the standard error (SE). Data were analysed for normality (Kolomogorov-Smirnov test) and homoscedacity of variance (Bartlett's test) and, when necessary, log-transformed before being treated statistically. Student's *t*-test was applied to check for significant changes between experimental groups within a single plasma component.

Statistical differences in the colour parameters brightness or value (L^*) and chroma (C^*_{ab}) were tested by ANOVA. For statistical comparison of the hue variable (H^*_{ab}) among experimental groups, the following approach was selected. Hue, being an angle, is a circular variable where 0° indicates a red hue and 90° denotes a yellow hue; therefore analysis for the estimation of mean value and standard deviation was performed according to Zar (1996). Differences between groups were tested with the Watson-Williams test.

Results

In all fish, there was a significant dorsoventral gradient in the estimated colour parameters (Table 1), with the ventral area having higher L^* , H^*_{ab} and C^*_{ab} values than the dorsal one (p < 0.001). Background colour did not affect skin hue (H^*_{ab}) but had a significant effect on skin brightness (L^*) and chroma (C^*_{ab}) (Table 1).

Mean serum glucose concentration did not show any significant difference between the two groups (black background: 3.67 ± 0.41 mmol l⁻¹; white background: 4.28 ± 0.37 mmol l⁻¹). Similarly, there was no significant difference in mean serum lactate concentration (black background: 0.93 ± 0.14 mmol l⁻¹; white background: 0.64

Table 1. Chromaticity parameters (SE, n = 22) of the dorsal and ventral skin area of red porgy adapted to black- or white-background tanks. Means (within each respective colour parameter) with different letters differ significantly from one another (p < 0.05).

	Brightness (<i>L</i> *)		Hue (H^*_{ab}) (degrees)		Chroma (C^*_{ab})	
	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral
Black White	49.1 (1.5)ª 55.1 (1.4)°	60.5 (1.4) ^b 65.1 (1.0) ^d	56.3 (5.2) ^a 42.1 (5.4) ^a	70.9 (1.1)⁵ 66.3 (1.3)⁵	6.03 (0.65)ª 3.75 (0.41)°	9.83 (0.42) ^b 7.41 (0.29) ^d



Fig. 1. Plasma concentrations of melatonin (+ SE, n = 12) in red porgy held against black and white backgrounds. Means with different letters differ significantly from one another (p < 0.05).

 \pm 0.08 mmol l⁻¹). The background colour did not affect the mean (SE) serum thyroid hormones concentrations; T₃ concentrations were 11.0 (1.2) ng ml⁻¹ in black- and 10.1 (0.9) ng ml⁻¹ in white-background-adapted fish while T₄ concentrations 30.2 (4.1) ng ml⁻¹ in black- and 24.7 (2.7) ng ml⁻¹ in white-background-adapted fish. Low mean cortisol concentrations were found in both groups (black background: 7.95 \pm 2.6 ng ml⁻¹; white background: 5.77 \pm 1.4 ng ml⁻¹).

Plasma melatonin concentrations displayed statistically significant differences between the two experimental groups, with a mean (SE) of 41.29 (2.46) pg ml⁻¹ in black- and 32.07 (1.26) pg ml⁻¹ in white-background-adapted fish (Fig. 1). There was no significant difference in mean plasma epinephrine concentrations between black- (2.77 ± 0.47 ng ml⁻¹) and white-background-adapted (3.69 ± 1.03 ng ml⁻¹) red porgies. Fish exposed to the white background showed an almost 6 times higher mean norepine-phrine concentration (6.26 ± 1.57 ng ml⁻¹) than those exposed to the black background (1.01 ± 0.34 ng ml⁻¹) (Fig. 2).

There was no statistical difference in the two skin samples of the dorsal skin area of each fish, therefore results were grouped together. Skin melanin concentration (mean \pm SE) did not show significant differences between red porgies adapted to black (4.32 \pm 0.4 mg mm⁻²) or white (4.46 \pm 0.5 mg mm⁻²) background.

Discussion

The purpose of the present study was to investigate the endocrine mechanism responsible for



Fig. 2. Plasma concentrations (+ SE, n = 6) of norepinephrine in red porgies held against black and white backgrounds. Means with different letters differ significantly from one another (p < 0.05).

the regulation of skin colour change in red porgy, *Pagrus pagrus*, held against black and white backgrounds. The white background, as expected, resulted in skin blanching. However, the objective analysis of skin colour performed in our study showed that pale fish have a similar skin hue to dark-skinned fish. Thus, background colour affects only skin brightness (L^*) and chroma (C^*_{ab}). The observed changes are not due to the increase of skin melanin concentration but because of changes in the motility of the melanosomes (aggregation vs. dispersion), as the skin of reared red porgy fed non-carotenoid diet contains only melanophores and iridophores (Sterioti 2004, Pavlidis 2004).

Skin colour change was not related to the stress response since (a) there was no significant effect of background colour on plasma concentrations of cortisol, glucose or lactate and (b) all the determined stress indicators (including catecholamines) were within the range of baseline values for red porgy (Fanouraki et al. 2007). These results are in accordance with previous studies on red porgy (Rotllant et al. 2003, Van der Salm et al. 2004), blenny, Blennius pholis, and molly, Poecilia latipinna, exposed to black or white backgrounds (Baker 1963, Baker & Ball 1970, Ball & Baker 1981) but contrast with studies on the rainbow trout, Oncorhynchus mykiss, and eel, Anguilla anguilla, where differences in plasma cortisol concentrations following adaptation to black or white backgrounds were found (Baker & Rance 1981).

Thyroid hormones are known to affect the integument and visual pigments in teleosts. Thy-

roxine (T_{A}) and TSH are known to affect the purine synthesis in skin as well as the melanophore production. A decrease in the number of melanophores was reported in Channa punctatus and Oncorhynchus keta treated with T_{A} , whereas radiothyroidectomy caused an increase in the number of melanophores in Oncorhynchus mykiss (Leatherland 1982). In the moor variety of Carassius auratus, TSH stimulated melanogenesis and melanophore expansion (in contrast with T_{A}) which had no effect, suggesting that TSH acts directly on the melanophores rather than operating via the thyroid gland (Leatherland 1982). In our study, background colour had no significant effect on circulating T₄ and T₃, suggesting that there is no relationship between the circulating thyroid hormones and skin colour changes in red porgy. These findings are in accordance with the results of Szisch et al. (2002) on red porgy.

As early as in 1961, Fujii reported for the first time that melatonin effectively aggregated melanophore inclusions in the goby, Chasmichthys gulosus. This in vitro action of melatonin was also found in other species (Mira 1962, Hu 1963, Healey & Ross 1966, Hafeez 1970, Ohta 1976, Fujii & Miyashita 1978). In contrast, melatonin had no significant effect on the melanophore motility of the killifish, Fundulus heteroclitus, the crucian carp, Carassius carassius, the elasmobranch P. reticulate, and the lungfish, Lepidosiren paradoxa (Abbott 1968, Fujii & Miyashita 1978, Visconti & Castrucci 1993), and the red porgy, Pagrus pagrus (Sterioti et al. 2004). Other studies did not reveal any relationship between plasma melatonin and background adaptation in rainbow trout and medaka (Owens et al. 1978, Sugimoto 1993). In our study, there was a significant increase in plasma melatonin concentration in black-background-adapted fish. Unpublished data from our laboratory also show that the skin of red porgy darkens at night (L^* = 54.87 ± 1.1 and 49.35 ± 1.2 (mean \pm SE), day and night, respectively, p > 0.001). These results may be explained by the assumption that red porgy melanophores possess MT receptors that mediate melanosome dispersion rather than aggregation. Alternatively, it is also possible that the decrease of melatonin plasma concentration in white-background-adapted fish is not related to melanosome motility but rather to the increased light intensity

that the retina receives due to reflection from the background. On a dark background, the ventral retina receives a considerable amount of light as compared with the dorsal retina (Fujii 2000). Thus, neurons of the ventral retina are activated resulting in the suppression of the spontaneous discharge of motoneurons in the medulla that belong to the inhibitory system, and so the fish darkens. On a white background, both ventral and dorsal retinas are stimulated. From the latter, the excitatory system originates and spontaneous discharge of motoneurons is augmented, leading to skin blanching (Fujii 2000).

There was a statistically significant difference in norepinephrine between fish adapted to black and white backgrounds indicating that the mechanism controlling skin colour in red porgy is neural. Thus, norepinephrine (NE), the most important neurotransmitter regulating chromatophore motility, was elevated in red porgies adapted to white background. These data are in accordance with previous studies indicating that in vitro administration of norepinephrine, at a concentration of 10⁻⁶ M, had a significant (approximately 80%) aggregating effect on red porgy melanosomes (Pavlidis 2004, Sterioti 2004). Therefore, it is suggested that skin blanching in red porgy is mediated through an increase in plasma NE as a result of the activation of the hypothalamic-sympathetic-chromaffin-cell (HSC) axis. NE acts to induce a rapid aggregation of melanosomes, via mainly α -adrenoreseptors on the melanophore membrane, as is the case in other species (Kamada & Kinosita 1944, Yamada et al. 1984, Morishita 1987). Epinephrine, which is known to disperse chromatosomes via β -adrenoreseptors at physiological concentrations (Fujii 2000), did not differ significantly between fish from the two colour treatments. Iwata and Fukuda (1973) also reported that the adrenergic neurotransmitter responsible for pigment aggregation is released during adaptation to a white background but not during adaptation to a black background.

Previous studies on red porgy did not reveal any significant effect of circulating α -MSH and cortisol on the control of skin colour change (Rotllant *et al.* 2003, Van der Salm *et al.* 2004). The results of the present study show that skin colour change in red porgy (*Pagrus pagrus*) acclimated to white background is due to alternations in skin brightness (L^*) and chroma (C^*_{ab}) but not in hue, with plasma norepinephrine being the mediator for the observed skin blanching.

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