α-MSH acetylation in the pituitary gland of the sea bream (*Sparus aurata* L.) in response to different backgrounds, confinement and air exposure

R J Arends, J Rotllant[1], J R Metz, J M Mancera[2], S E Wendelaar Bonga and **G Flik**

Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

[1]Departamento Biologia Cellular i Fysiologia, Unitat de Fysiologia Animal, Universitat Autònoma de Barcelona, 08193-Bellaterra, Spain

[2]Departamento de Biologia Animal, Facultad de Ciencias del Mar, Universidad Cádiz, Puerto Real 11510, Cádiz, Spain

(Requests for offprints should be addressed to G Flik; Email: gertflik@sci.kun.nl)

Abstract

MSH is a pituitary hormone derived by post-translational processing from POMC and involved in stress and background adaptation. N-terminal acetylation of MSH to monoacetyl α-MSH or diacetyl α-MSH increases the bioactivity of the peptide. The aim of this study was to characterize α-MSH acetylation in the sea bream (*Sparus aurata* L.) pituitary gland in response to the stressors air exposure and confinement, as well as in fish adapted for 15 days to a white, gray or black background. Pituitary homogenates were purified by reversed-phase HPLC (RP-HPLC). The α-MSH content of fractions was measured by RIA. Immunoreactive RP-HPLC fractions were further analyzed by electrospray mass spectrometry and the peptide sequence determined as SYSMEHFRWGKPV-NH_2. In the pituitary gland of sea bream, des-, mono- and diacetyl α-MSH were identified. Then plasma α-MSH levels were measured in sea bream adapted to different backgrounds. Surprisingly, we found the highest plasma α-MSH levels in white-adapted as compared with black-adapted sea bream with intermediate values for gray-adapted fish. This observation is in contrast with results that have been obtained in eel, trout or terrestrial vertebrates. Next, des-, mono- and diacetyl α-MSH forms were measured in homogenates of the pituitary gland and in plasma of sea bream exposed to air, to confinement, or to different backgrounds. Monoacetyl α-MSH was the predominant form in all control and experimental groups. The lowest content of monoacetyl α-MSH relative to des- and diacetyl α-MSH was found in white-adapted fish. Levels of des- and diacetyl α-MSH forms were similar under all conditions. We observed that monoacetyl α-MSH is the most abundant isoform in the pituitary gland after background adaptation, confinement and air exposure, in sea bream. These data indicate that the physiologically most potent isoform of α-MSH may vary from species to species.

Journal of Endocrinology (2000) **166,** 427–435

Introduction

In fish, as well as in other vertebrates, the pituitary pars intermedia cells mainly synthesize pro-opiomelanocortin-derived peptides of which melanocyte-stimulating hormone (α-MSH) is considered a pivotal product. Post-translational processing of α-MSH such as C-terminal amidation and N-terminal acetylation results in three forms of α-MSH, viz. desacetyl, monoacetyl and diacetyl α-MSH, further referred to as α-MSH isoforms. Both amidation and acetylation are essential for optimal biological activity of the peptide (Castro & Morrison 1997). α-MSH has been chemically and biologically characterized in the pituitary gland of salmon (*Onchorhynchus keta*) (Kawauchi *et al.* 1984); goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*) (Follenius *et al.* 1985); tilapia (*Oreochromis mossambicus*) (Lamers *et al.* 1991); and gar (*Lepisosteus spatula* and *Lepisosteus osseus*) and bowfin (*Amia calva*) (Dores *et al.* 1994). Furthermore, α-MSH has been demonstrated in other parts of the fish brain (Kishida *et al.* 1988, Vallarino *et al.* 1989) and in ovary of the sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) (Mosconi *et al.* 1994).

In trout, as in the African clawed toad (*Xenopus laevis*), desacetyl α-MSH has been shown to be the most abundant isoform in the pituitary gland. Other studies in fish have reported different results regarding the acetylation of α-MSH in the pituitary gland. In carp and goldfish, diacetyl α-MSH has been detected predominately in the intracellular pool (Follenius *et al.* 1985). Similar results have been obtained in gar and bowfin (Dores *et al.* 1994).

0022–0795/00/0166–427 © 2000 Society for Endocrinology *Printed in Great Britain*

In lower vertebrates, α-MSH has been studied most intensively in the African clawed toad with respect to the regulation of melanophores of the skin during background adaptation (Roubos 1997). Transfer of white animals to a black background results in dispersion of melanophore pigment within a few hours and increases plasma α-MSH levels after 24 h (van Zoest *et al.* 1989). Desacetyl MSH has been determined as the major storage form of α-MSH in both white- and black-adapted toads, whereas monoacetyl α-MSH has been identified as the major secreted form (Maruthainar *et al.* 1992, van Strien *et al.* 1995). In fish, the involvement of α-MSH during background adaptation of the skin has been demonstrated in Mozambique tilapia (*Oreochromis mossambicus*) (van Eys & Peters 1981), and eel (*Anguilla anguilla)* and trout (*Salmo gairdneri*) (Baker *et al.* 1984, Gilham & Baker 1984).

Besides its role in dispersion of melanophore pigment in dermal melanophores, α-MSH is involved in the stress response in fish (Wendelaar Bonga 1997). Three minutes of air exposure induced an increase of plasma α-MSH levels in sea bream (Arends *et al.* 1999). When brown trout (*Salmo trutta*) were subjected to handling and confinement combined with a thermal shock, plasma α-MSH levels increased (Sumpter *et al.* 1985). In rainbow trout (*Onchorhynchus mykiss*), plasma α-MSH was elevated when fish were kept restrained out of water (Sumpter *et al.* 1986). Chronic exposure to acidified water activates the melanotropic cells of tilapia resulting in release of diacetyl α-MSH. In Mozambique tilapia, diacetyl α-MSH has been demonstrated to have corticotropic activity and stimulates cortisol release *in vitro* (Lamers *et al.* 1992) and the corticotropic activity of α-MSH is probably potentiated by acetylated β-endorphin (Balm *et al.* 1995). The latter study suggests that diacetyl α-MSH is the most important form of the hormone for stress adaptation.

So far, little is known about the role of α-MSH isoforms in background and stress adaptation in sea bream. The aims of this study were: (i) to characterize α-MSH isoforms in the pituitary gland of sea bream (*Sparus aurata*) by means of HPLC combined with RIA; (ii) to isolate the isoforms of α-MSH and determine the molecular mass and peptide sequence by electrospray mass spectrometry (EMS); (iii) to study a possible involvement of α-MSH isoforms in background adaptation; and (iv) to investigate a possible involvement of α-MSH isoforms in the response to the stressors air exposure or confinement.

Materials and Methods

Fish

Immature male gilthead sea bream (*Sparus aurata* L., further called sea bream), weighing 60–200 g, were obtained from an experimental fish culture center (CICEM El Toruño, El Puerto de Santa Maria, Spain). During the experiments (May–July 1998), the fish were kept under natural photoperiod (14 h light:10 h darkness) and water temperature (22–24 °C). For each experiment, 200 fish were kept in well-aerated 5000 l stock-tanks at a density of 3–5 kg/m^3. The water was continuously refreshed (250 l/h) and supplied with air through air stones. Fish were fed twice a day (0900 and 0600 h) with 1% body weight commercial dry pellets (Dibaq-Diproteg SA, Segovia, Spain).

Experimental protocol

Fish, 12–16 per group and one group for each sample point, were transferred from their stock tank to a plastic-coated iron wire-net cage with a total volume of 250 l (inner diameter of cage 0·60 m) in an experimental tank. Three wire-net cages were placed in one white rectangular tank (volume=1600 l; length 2·50 m; height 0·85 m; width 0·75 m) for experiment I, or one wire-net cage was placed in either a gray cylindrical experimental tank (volume=500 l; tank diameter 0·85 m) for experiments I, II and III, or a black cylindrical experimental tank (volume=500 l; tank diameter 0·85 m) for experiment I. The fish density was 4 kg/m^3 in all cases. The fish were allowed to acclimate to the experimental tanks for 6 days. Feeding was stopped 24 h before the experiments. No mortality was observed in any group during the experiments. A single blood sample was taken from each fish at the end of experiments I, II and III.

Experiment I: background adaptation Fish were kept in white, gray or black tanks for 15 days. After the adaptation to the different backgrounds, fish were anesthetized for 1 min by taking the wire-net cage out of the tank and transferring the fish to a bucket containing 0·1% 2-phenoxyethanol (Sigma, St Louis, MO, USA). Once anesthetized (always within 1 min), the fish were taken out of the bucket. One milliliter of blood was taken from the caudal vessels using a syringe containing 35 µl 2% Na_2-EDTA (Sigma) and 25 µl Trasylol (equivalent to 250 kallikrein inhibiting units; Bayer, Leverkusen, Germany). Blood samples were taken within 5 min after capture (n=8 per group). Plasma was separated from cells by centrifugation for 20 min at 1500 ***g*** and was stored at − 80 °C until further analysis. After blood sampling, fish were killed and the pituitary glands were dissected and homogenized in ice-cold 0·1 M HCl.

Experiment II: air exposure Fish were exposed to air for 3 min, by lifting the wire-net cage out of the gray tank, after which the cages were put back into the tanks. Fish (n=8per group) were sampled at t=0 (without air exposure) and 20 or 60 min after air exposure, as described above.

Experiment III: 24 h confinement Fish were confined up to 24 h by lifting the wire-net cage in the gray tank

(water depth about 10 cm) to increase the stocking density from 5 to 70 kg/m^3. This treatment is further referred to as confinement. Fish (n=8 per group) were sampled as described under experiment I at t=0 (before confinement) and after 2, 5 or 26 h confinement.

Analyses

Hormone RIAs The plasma cortisol concentration was measured with a highly specific antibody for cortisol (Coat-A-Count Cortisol ^{125}I RIA kit; Diagnostics Product Corporation, LA, USA) in 25 µl plasma. The radioactivity was quantified using a Cobra II γ-counter (Packard Instruments Company, Meriden, CT, USA). Cross-reactivity with cortisone was less than 1·0%. The intra-assay variation was 5·1% (n=20), the inter-assay variation 6·4% (n=20). The RIA for α-MSH was based on an antibody described by Vaudry *et al.* (1978), and was used in a final dilution of 1:60 000. The cross-reactivity of this antiserum with des-, mono- and diacetyl α-MSH is 100%. Immune complexes were precipitated with 15% (w/v) polyethylene glycol and 2·4% (w/v) ovalbumin as described previously (van Zoest *et al.* 1989).

Reversed-phase HPLC (RP-HPLC) Pituitary glands were homogenized in 500 µl ice-cold 0·1 M HCl using a glass-to-glass Potter homogenizer device. Membranes and particulate material in the homogenate were removed by centrifugation (10 000 ***g***, 10 min). One hundred microliters of pituitary homogenate were separated on a Pharmacia µRPC C2/C18 sc 2·1/10 column (Roosendaal, The Netherlands) with ddH$_2$O/0·1% trifluoroacetic acid (TFA) as equilibration eluant and a gradient of acetonitrile/0·1% TFA from 0–100% as secondary eluant. The flow rate was 150 µl/min. One minute fractions were collected and tested for α-MSH immunoreactivity in an RIA. For calculation of the amount of des-, mono- or diacetyl α-MSH, the peak fraction and the two adjacent fractions were pooled. The sum of the amounts of des-, mono- and diacetyl α-MSH was set at 100% α-MSH. Synthetic human des-, mono- and diacetyl α-MSH (Sigma) were submitted to RP-HPLC as marker peptides.

EMS EMS was performed on a Q-Tof instrument (Micromass, Manchester, UK) using 6 kV fast xenon atoms according to the manufacture's guidelines. Approximately 2 pmol peptide (vacuum dried RP-HPLC fraction) were dissolved in 50% methanol/50% acetic acid and placed in the capillary probe. For peptide sequencing, collision-induced EMS spectra of the $[M+H]^+$ ion were obtained by scanning the second mass spectrometer at a 50% main beam using argon as collision gas.

Statistical analysis

In all experiments, differences among groups were assessed by means of one-way ANOVA. Subsequently, significance of differences between mean values was tested with the Dunett's multiple comparison test or the Kruskal–Wallis rank sum test. Where indicated by Bartlett's test the homogeneity of variance was improved and appropriate transformations of the data were carried out. Statistical significance was accepted at $P<0·05$. Values (n=8 for all groups) are means ± S.E.M.

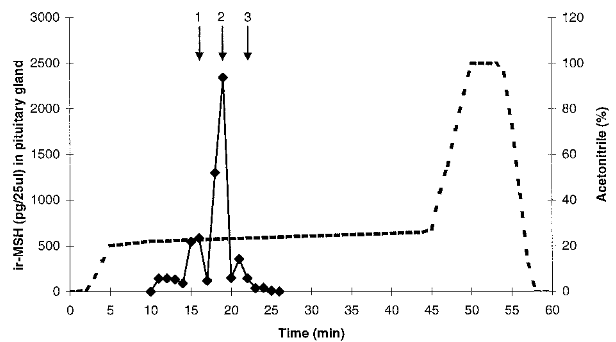
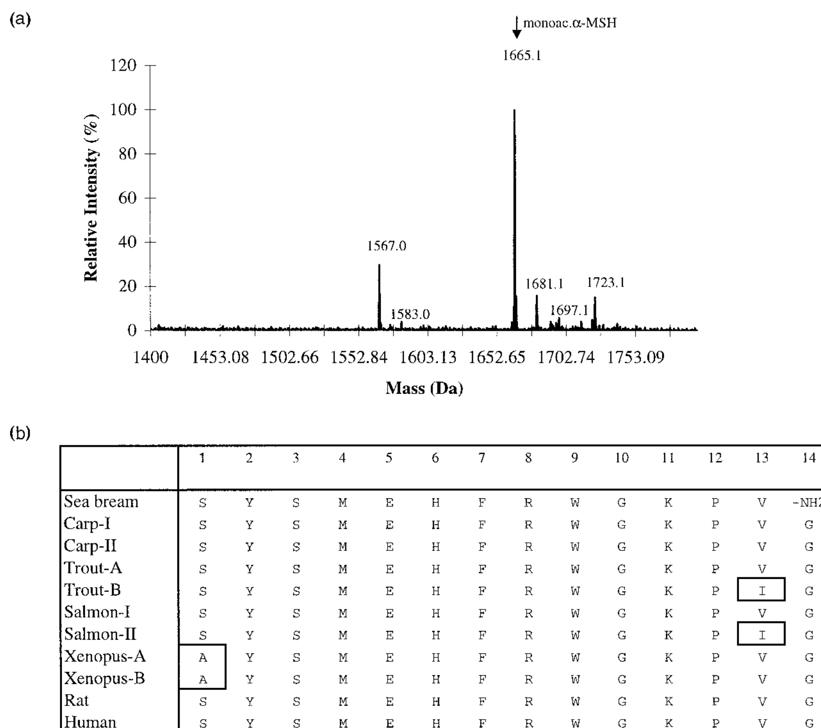


Figure 1 Representative profile of immunoreactive (ir) α-MSH in RP-HPLC fractions 5–30 of a homogenate of the pituitary gland. The acetonitrile gradient is shown by the dashed line. Indicated by arrows are the synthetic standards des- (1), mono- (2), and diacetyl (3) α-MSH, which were chromatographed separately and detected at an absorbance of 218 nm.

Results

Using the acetonitrile gradient depicted in Fig. 1, synthetic desacetyl MSH elutes after 16 min at 22·5% acetonitrile, monoacetyl α-MSH after 19 min at 22·8% acetonitrile, and diacetyl α-MSH after 21 min at 23·4% acetonitrile. The co-elution of sea bream pituitary gland immunoreactive α-MSH with synthetic α-MSH isoforms is consistent with the fact that the amino acid sequences of the MSH peptides are identical (Fig. 1). The immunoreactive α-MSH was analyzed next by EMS. To determine the molecular mass of immunoreactive α-MSH in sea bream, EMS spectra were checked for peptides with a molecular mass predicted on the basis of carp α-MSH (Arends *et al.* 1998), namely 1622·2 Da for desacetyl MSH, 1665·1 Da for monoacetyl α-MSH and 1706·8 Da for diacetyl α-MSH. A representative spectrogram of the 19 min peak is shown in Fig. 2a. The RP-HPLC fraction eluting after 16 min contained a peptide with a mass of 1622·4 Da, similar to that of carp desacetyl MSH. In the fraction eluting after 19 min a peptide with a mass of 1665·1 Da was found, similar to the mass of monoacetyl α-MSH, whereas in the fraction eluting after 21 min a peptide was identified with a mass of 1706·7 Da, which is in agreement with the mass of diacetyl α-MSH. In each of the analyzed fractions only one form of α-MSH was detected. Finally, to complete the characterization of α-MSH in sea bream, peptide sequence analysis was



	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sea bream	S	Y	S	M	E	H	F	R	W	G	K	P	V	-NH2
Carp-I	S	Y	S	M	E	H	F	R	W	G	K	P	V	G
Carp-II	S	Y	S	M	E	H	F	R	W	G	K	P	V	G
Trout-A	S	Y	S	M	E	H	F	R	W	G	K	P	V	G
Trout-B	S	Y	S	M	E	H	F	R	W	G	K	P	I	G
Salmon-I	S	Y	S	M	E	H	F	R	W	G	K	P	V	G
Salmon-II	S	Y	S	M	E	H	F	R	W	G	K	P	I	G
Xenopus-A	A	Y	S	M	E	H	F	R	W	G	K	P	V	G
Xenopus-B	A	Y	S	M	E	H	F	R	W	G	K	P	V	G
Rat	S	Y	S	M	E	H	F	R	W	G	K	P	V	G
Human	S	Y	S	M	E	H	F	R	W	G	K	P	V	G

Figure 2 (a) Partial mass spectrum (from 1400 to 1800 Da) from an RP-HPLC fraction of a pituitary gland homogenate eluting at 19 min. The arrow indicates the position of monoacetyl α-MSH. (b) Alignment of α-MSH sequences. Amino acid differences from sea bream MSH are boxed. The sequences are from the EMNEW database (accession number for carp-I, Y14618 and for carp-II, Y14617) (Arends *et al.* 1998), and from the SWISS-PROT database (accession number for trout-A, Q04617; for trout-B, Q04618; for salmon-II, P10000; for xenopus-A, P06298; for xenopus-B, P06299; for rat, P01194; and for human P01189). The amino acid sequence of salmon-I has been taken from Kawauchi (1983).

performed on the peptides with a mass corresponding to the predicted mass of α-MSH. In Fig. 2b the amino acid sequence of sea bream α-MSH obtained by EMS is given and compared with α-MSH of other species elucidated by molecular biological techniques. The α-MSH peptide sequence of sea bream measured by EMS was identical to the nucleotide-deduced amino acid sequences of carp, salmon-I, trout-A and mammalian α-MSH.

Next the functional role of α-MSH acetylation was studied. In fish adapted for 15 days to different backgrounds, plasma α-MSH levels were significantly higher in white-adapted fish than in gray- or black-adapted fish (Fig. 3a). In the pituitary gland of white-adapted fish the total amount of α-MSH was significantly lower than in gray- or black-adapted fish (Fig. 3b), indicating an increased release or a decreased synthesis of α-MSH in white-adapted sea bream. To investigate whether, in addition to the changes in absolute levels of plasma α-MSH, changes in isoforms of α-MSH in the pituitary gland occurred, percentages of des-, mono- and diacetyl α-MSH were determined. In fish adapted to gray and black backgrounds, 70 and 74% respectively of total α-MSH in the pituitary gland was monoacetylated (Fig. 3c), whereas des- and diacetyl α-MSH represented 20 and 14% respectively. In fish adapted to a white background, 53% of the total amount α-MSH consisted of monoacetyl α-MSH. Des- and diacetyl α-MSH were not significantly higher in white- compared with gray- or black-adapted sea bream.

The measured values of plasma α-MSH were decreased in fish confined for 26 h (Fig. 4a); however, 2 h confinement did not alter the absolute values of plasma α-MSH. Except for changes in absolute plasma α-MSH levels no differences could be detected in relative amounts of α-MSH isoforms in the pituitary glands of fish subjected to confinement (Fig. 4b). The plasma α-MSH levels in air-exposed sea bream are shown in Fig. 5a. Twenty minutes after air exposure, plasma α-MSH levels were over three times higher than in control fish, whereas 60 min following air exposure the plasma α-MSH levels had returned to control levels. No changes in the relative amounts of α-MSH isoforms were detected in the pituitary gland (Fig. 5b). Both the confinement and the air exposure experiment were performed in gray tanks. The

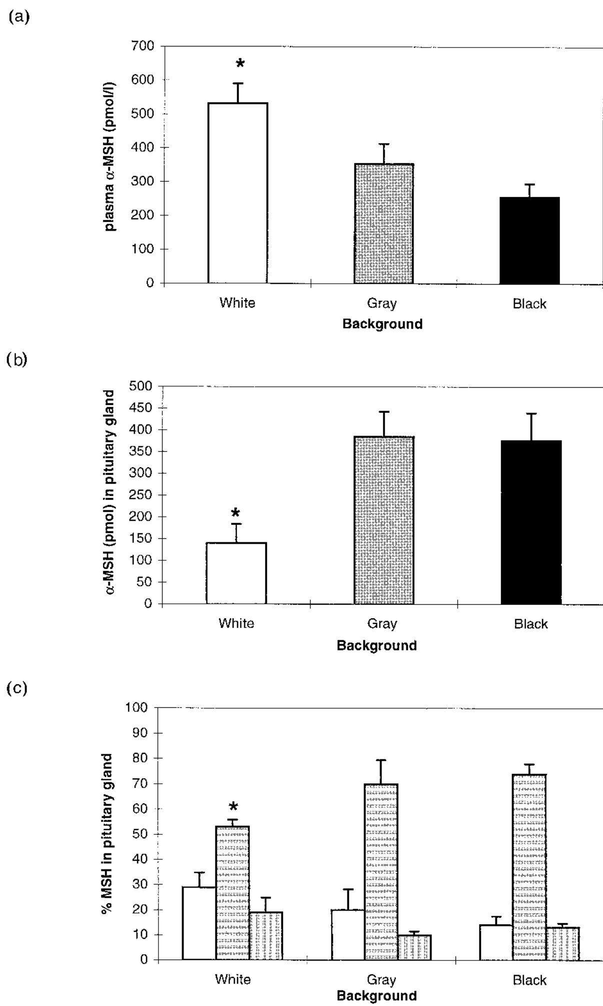


Figure 3 (a) Plasma levels of α-MSH (n=8), (b) total α-MSH in the pituitary gland (n=8), and (c) relative contents of des- (open bars), mono- (horizontally dashed bars) and diacetyl (vertically dashed bars) α-MSH in the pituitary gland (n=5) of sea bream adapted to different backgrounds. *P<0·05 significant difference between white- and gray- or black-adapted fish, analyzed separately for each isoform; means ± S.E.M.

relative amounts of des-, mono and diacetyl α-MSH in these experiments were comparable with the percentages measured in the background adaptation and our exposure experiments.

Discussion

Characterization of α-MSH in sea bream

In this study, sea bream α-MSH was characterized by RP-HPLC and EMS. The amino acid sequence of sea bream α-MSH is identical to that of α-MSH of other freshwater fish and mammals (Arends *et al.* 1998). The

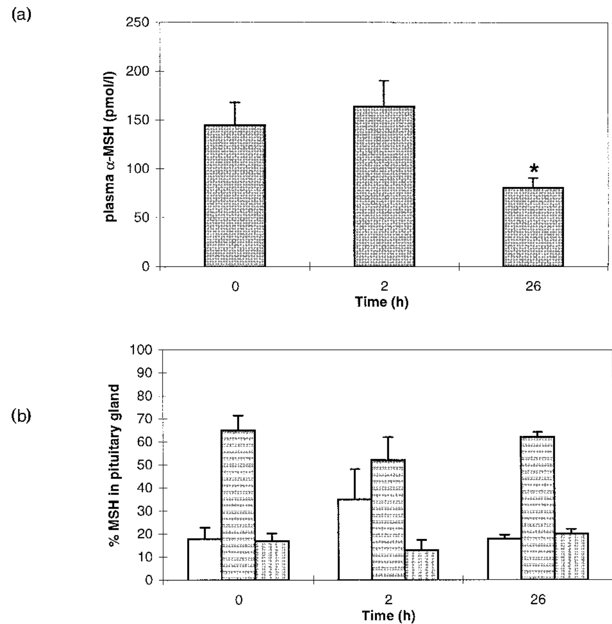


Figure 4 (a) Plasma levels of α-MSH (n=8), and (b) relative contents of des- (open bars), mono- (horizontally dashed bars) and diacetyl (vertically dashed bars) α-MSH in the pituitary gland (n=5) of confined sea bream. *P<0·05 significant difference compared with t=0 min; means ± S.E.M.

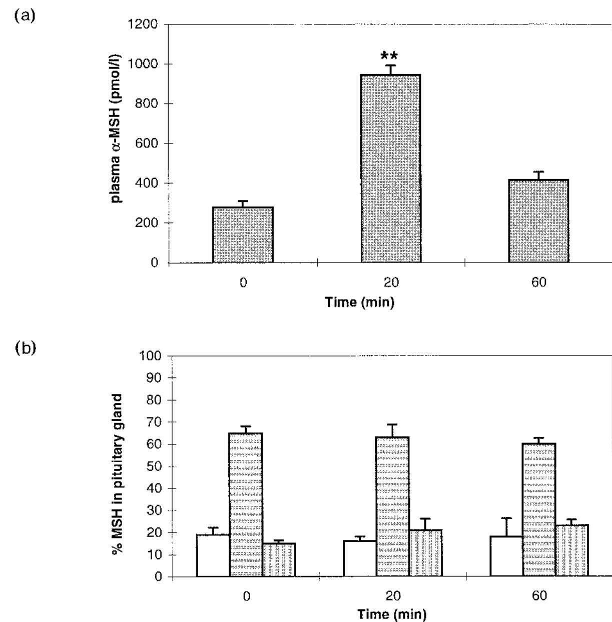


Figure 5 (a) Plasma levels of α-MSH (n=8; note the y-axis scale compared with Fig. 4a), and (b) relative contents of des- (open bars), mono- (horizontally dashed bars) and diacetyl (vertically dashed bars) α-MSH in the pituitary gland (n=5) of air-exposed sea bream. **P<0·01 significant difference compared with t=0 min; means ± S.E.M.

sequence of sea bream α-MSH contains an N-terminal serine, which can be N- and O-acetylated (Driessen *et al.* 1982) by post-translational modification. C-terminal glycine may serve as amide donor for the potential amidation site Pro-X-Gly which is present in α-MSH of sea bream. Indeed, EMS measurements showed that immunoreactive α-MSH fractions of RP-HPLC-purified pituitary homogenates contained all predicted forms of N-terminally acetylated and C-terminally amidated α-MSH, e.g. des-, mono- and diacetyl α-MSH. These data are in line with results obtained for salmon (Kawauchi *et al.* 1984), goldfish and carp (Follenius *et al.* 1985), trout (Vallarino *et al.* 1989), tilapia (Lamers *et al.* 1991), and gar and bowfin (Dores *et al.* 1994). It is concluded that both N-terminal N- and O-acetylation and C-terminal amidation occur *in vivo* in sea bream pituitary gland. Although in previous studies immunoreactive α-MSH has been found in the pars intermedia of sea bream pituitary gland (Quesada *et al.* 1988, Mancera & Fernandez-Llebrez 1995*b*), the primary structure was still unknown. Immunoreactive des- and monoacetyl α-MSH have been demonstrated before in sea bream ovary, but diacetyl α-MSH was not detected (Mosconi *et al.* 1994).

A role for α-MSH in background adaptation in sea bream?

This is the first study to show that plasma α-MSH levels of a fish species adapted to a white background are elevated compared with these in dark background-adapted animals. To further substantiate this observation, the total amount of α-MSH was measured in the pituitary gland. The hypophysis of white-adapted sea bream contained significantly less α-MSH compared with gray- and black-adapted animals, indicating an increased release, or a decreased synthesis of α-MSH. Furthermore α-MSH levels were significantly higher in 15 independent experiments performed in white tanks. Interestingly, in another seawater species, the red porgy (*Pagrus pagrus*), similar observations on plasma α-MSH levels were made in fish adapted for 1 month to a white or black background (J Rotllant, unpublished observations). In the African clawed toad it was demonstrated that α-MSH antiserum injection caused pallor of the skin (Gilham & Baker 1984) and plasma α-MSH levels were high in black-adapted and low in white-adapted toads (van Zoest *et al.* 1989). The role of α-MSH in fish background adaptation has been studied for several species, but sometimes unexpected results were presented, which suggested a multiple endocrine control, i.e. α-MSH may not necessarily be the dominant melanophore-stimulating hormone. For instance, injection of antiserum against α-MSH caused melanin concentration in the black-adapted hypophysectomized eel (Gilham & Baker 1984), but not in intact animals. In tilapia, infusion of α-MSH stimulated pigmentation in the dermis (van Eys & Peters 1981). Furthermore, increased plasma α-MSH levels have been detected in black-adapted eel, trout and salmon (Baker *et al.* 1984, 1986). In flounder, however, no changes in plasma α-MSH levels were detected during background adaptation (Baker *et al.* 1984), whereas melanophore dispersion was higher in the skin of the black-adapted compared with the white-adapted flounder (Burton & Snow 1993). Experiments with the hypophysectomized flounder demonstrated that the pituitary gland has no influence on melanophore responses in this species (Burton 1981). Thus, the role of α-MSH during background adaptation in fish may vary from species to species.

Other hormones that may be involved in melanophore control are melanin-concentrating hormone (MCH) and somatolactin. MCH is a neurohypophyseal peptide that opposes the actions of α-MSH on skin coloration in teleost fish (Baker *et al.* 1986). Also in sea bream, immunoreactive MCH positive neurons have been demonstrated in the pars intermedia (Mancera & Fernandez-Llebrez 1995*a*) and thus the classical endocrine tools for background adaptation appear to be present. In trout, the amount of MCH in the pituitary gland increased when fish were adapted to a black background (Barber *et al.* 1987). In that study it was demonstrated that the *in vitro* release of α-MSH from the neurointermediate lobe is enhanced by immunoabsorption of endogenous MCH, indicating that MCH has a paracrine inhibitory effect on α-MSH release. In tilapia, an inhibitory effect of MCH on α-MSH secretion was found, but only at low concentrations of MCH (Gröneveld *et al.* 1995). On the other hand, injection of homologous MCH in the African clawed toad had no effect on melanin concentration in the skin (Gilham & Baker 1984). Somatolactin is a pituitary-derived hormone that might also be involved in fish background adaptation. Increased plasma somatolactin levels have been found in red drum (*Sciaenops ocellatus)* and Atlantic croaker (*Micropogonias undulatus*) following transfer from a light- to a dark-colored background (Zhu & Thomas 1996). Furthermore, somatolactin mRNA and plasma levels were higher in blind red drum and intact fish kept under constant darkness for 1 week, indicating that illumination levels and somatolactin are related in this species (Zhu *et al.* 1999). However, unexpectedly, somatolactin was shown to stimulate melanosome aggregation *in vitro* (Zhu & Thomas 1997).

In this study, no somatolactin or MCH levels were measured and thus statements on the involvement of these hormones in the outcome of the results of this study are merely speculative. However, an inhibition of MCH activity could underlie darkening of the skin during background adaptation in sea bream. It should be noted that the sea bream possesses a light-colored skin on a light background, but dark vertical bands appear immediately when the animal is stressed. Similar bands have been associated with morphologically distinct dermal and epidermal melanophores in flounder (Burton 1980). The nervous system may be involved in the control of these stress-induced bands using norepinephrine as a

neurotransmitter (Kumazawa & Fuijii 1984). Thus, in sea bream neural rather than hormonal mechanisms may be pivotal in the rapid control of melanin dispersion in melanophores.

α-MSH acetylation

We studied the question whether a white background had an effect on α-MSH acetylation in the pituitary gland of sea bream after prolonged adaptation, because the results of analysis of the acetylation mechanisms in teleost fish and mammals suggest that α-MSH undergoes acetylation prior to secretion (Dores *et al.* 1993). This is in contrast with amphibian species, where acetylation is delayed until exocytosis (van Strien *et al.* 1995). In sea bream, monoacetyl α-MSH appeared to be the most abundant form in white- as well as gray- or black-adapted animals, whereas des- and diacetyl α-MSH levels were low in all groups. Significantly increased levels of monoacetyl α-MSH could be detected in gray- and black-adapted sea bream. Our data indicate that in sea bream, regulation of α-MSH acetylation is an important mechanism for background adaptation, more so than the total amounts of α-MSH released into the blood. This is in line with results obtained in salmon where monoacetyl α-MSH was found to be more active than desacetyl MSH in stimulating melanophore dispersion in a frog skin test (Kawauchi *et al.* 1984).

In order to investigate the role of α-MSH acetylation during long-term stress, sea bream were confined for 26 h. Long-term confinement significantly decreased plasma α-MSH levels. Previously, similar results have been reported for rainbow trout (Balm & Pottinger 1995). However, the ratios of the α-MSH isoforms were unaffected in long-term confined sea bream. Monoacetyl α-MSH was predominantly present in control as well as in confined sea bream, suggesting no prominent role for acetylation of α-MSH during this stressful event.

In air-exposed fish, the increased plasma α-MSH levels may be a response to plasma acidosis. Plasma acidosis was indicated by a rise in plasma lactate in this experiment (from 0·9 mM for controls to 2·7 mM 30 min after air exposure) (Arends *et al.* 1999). Previously, elevation of plasma α-MSH levels has been correlated with plasma acidosis in tilapia exposed to a low water pH (Lamers *et al.* 1994). In tilapia exposed to acidified water, diacetyl α-MSH is predominantly synthesized and released, whereas in control fish higher levels of monoacetyl α-MSH were found (Lamers *et al.* 1992). Although plasma α-MSH levels rose in air-exposed sea bream, no differences in α-MSH acetylation ratios were found in the pituitary glands of these fish. This experiment was performed in gray tanks, and therefore controls could be compared with gray-adapted fish of the background experiment. In the fish of both groups α-MSH ratios were similar, indicating no specific role for MSH acetylation during the plasma acidification that may be expected to occur during and after air exposure (van Raaij *et al.* 1996).

Because in sea bream monoacetyl α-MSH is the most abundant isoform, the question arises whether it possesses corticotropic activity. Superfusion of sea bream head kidney with concentrations ranging from 10 to 500 nM monoacetyl α-MSH did not evoke any cortisol release, whereas a pulse of 5 nM adrenocorticotropin (ACTH) (the EC_{50}) given after the α-MSH pulse clearly stimulated cortisol release (data not shown). Further studies in sea bream are required to investigate the potentiating effect of co-factors on the corticotropic activity of α-MSH. Balm *et al.* (1995) have demonstrated that a pituitary co-factor identified as β-endorphin was essential for obtaining a corticotropic response of α-MSH comparable with that of ACTH.

The results obtained in this study indicate that α-MSH can be N- and O-terminally acetylated and C-terminally amidated in sea bream. Background adaptation in sea bream is probably not directly related to plasma total α-MSH levels, although endocrine control by acetylated α-MSH forms can not be excluded. Control by other hormones and/or neural control is well possible. In sea bream, monoacetyl α-MSH is the most abundant isoform found in the pituitary gland after background adaptation, confinement and air exposure, indicating that the physiologically most potent isoform of α-MSH may vary from species to species.

Acknowledgements

The authors gratefully acknowledge J M Naranjo, director of CICEM El Toruño, Junta Andalucia, El Puerto de Santa Maria 11510, Cádiz, Spain for hospitality and the generous supply of experimental fish. The authors would like to thank Dr K W Li and Mr R van der Schors (Research Institute Neurosciences, Free University, Amsterdam, The Netherlands) for EMS measurements, Dr M A Rodriguez Gomez (Central Research Services, University of Cádiz, Spain) for support in the isotope laboratory, and Mr F Repiso, Mr J L Morales and Mr L M Torres for technical assistance and maintenance of the experimental fish. This work has been partially supported by the Royal Dutch Academy of Science AFD/CZ/8763 to R A, CYTMAR (97–402-C02–02) to J R and by DGES PB96–1511 and APC1998–0024 to J M M.

References

Arends RJ, Vermeer H, Martens GJM, Leunissen JAM, Wendelaar Bonga SE & Flik G 1998 Cloning and expression of two proopiomelanocortin mRNAs in the common carp (*Cyprinus carpio* L.). *Molecular and Cellular Endocrinology* **143** 23–31.

Arends RJ, Mancera JM, Wendelaar Bonga SE & Flik G 1999 The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *Journal of Endocrinology* **163** 149–157.

Baker BI, Wilson JF & Bowley TJ 1984 Changes in pituitary and plasma levels of MSH in teleosts during physiological colour change. *General and Comparative Endocrinology* **55** 142–149.

Baker BI, Bird DJ & Buckingham JC 1986 Effects of chronic administration of melanin-concentrating hormone on corticotrophin, melanotrophin, and pigmentation in the trout. *General and Comparative Endocrinology* **63** 62–69.

Balm PHM & Pottinger TG 1995 Corticotrope and melanotrope POMC-derived peptides in relation to interrenal function during stress in rainbow trout (*Onchorhynchus mykiss*). *General and Comparative Endocrinology* **98** 279–288.

Balm PHM, Hovens MLM & Wendelaar Bonga SE 1995 Endorphin and MSH in concert form the corticotropic principle released by tilapia (*Oreochromis mossambicus*, Teleostei) melanotropes. *Peptides* **16** 463–469.

Barber LD, Baker BI, Penny JC & Eberle AN 1987 Melanin concentrating hormone inhibits the release of α-MSH from teleost pituitary glands. *General and Comparative Endocrinology* **65** 79–86.

Burton D 1980 A cellular analysis of chromatophore patterning in winter flounder (*Pseudopleuronectes americanus*, Walbaum). *Comparative Biochemistry and Physiology* **67A** 453–457.

Burton D 1981 Physiological responses of melanophores and xanthophores of hypophysectomised and spinal winter flounder (*Pseudopleuronectes americanus*, Walbaum). *Proceedings of the Royal Society of London* **213B** 217–231.

Burton D & Snow A 1993 An *in vivo* and *in vitro* comparison of differential melanophore responsiveness in flatfish integumentary color patterns. *Zoological Science* **10** 577–585.

Castro MG & Morrison E 1997 Post-translational processing of proopiomelanocortin in the pituitary and the brain. *Critical Reviews in Neurobiology* **11** 35–57.

Dores RM, Steveson TC & Price ML 1993 A view of the N-acetylation of α-melanocyte-stimulating hormone and β-endorphin from a phylogenetic perspective. *Annals of the New York Academy of Sciences* **680** 161–174.

Dores RM, Keller H, White Y, Marra LE & Youson JH 1994 Detection of N-acetylated forms of α-MSH and β-endorphin in the intermediate pituitary of the holostean fishes, *Lepisosteus spatula*, *Lepisosteus osseus*, and *Amia calva*. *Peptides* **15** 483–487.

Driessen HPC, de Jong WW, Tesser GI & Bloemendal H 1982 The mechanism of N-terminal acetylation of proteins. *CRC Critical Reviews in Biochemistry* **18** 281–325.

Eys van GJJM & Peters PTW 1981 Evidence for a direct role of α-MSH in morphological background adaption of the skin in tilapia *Sarotherodon mossambicus*. *Cell and Tissue Research* **217** 361–372.

Follenius E, van Dorsselaer A & Meunier A 1985 Separation and partial characterization by high-performance liquid chromatography and radioimmunoassay of different forms of melanocyte-stimulating hormone from fish (Cyprinidae) neurointermediate lobes. *General and Comparative Endocrinology* **57** 198–207.

Gilham ID & Baker BI 1984 Evidence for the participation of a melanin-concentrating hormone in physiological colour change in the eel. *Journal of Endocrinology* **102** 237–243.

Gröneveld D, Balm PHM & Wendelaar Bonga SE 1995 Biphasic effect of MCH on α-MSH release from the tilapia (*Oreochromis mossambicus*) pituitary. *Peptides* **16** 945–949.

Kawauchi H 1983 Chemistry of proopiocortin-related peptides in the salmon pituitary. *Archives of Biochemistry and Biophysiology* **227** 343–350.

Kawauchi H, Kawazoe I, Adachi Y, Buckley DI & Ramachandran J 1984 Chemical and biological characterization of salmon melanocyte-stimulating hormone. *General and Comparative Endocrinology* **53** 37–48.

Kishida M, Baker BI & Bird DJ 1998 Localisation and identification of melanocyte-stimulating hormones in the fish brain. *General and Comparative Endocrinology* **71** 229–242.

Kumazawa T & Fujii R 1984 Concurrent releases of norepinephrine and purines by potassium from adrenergic melanosome-aggregating nerve in tilapia. *Comparative Biochemistry and Physiology* **78C** 263–266.

Lamers AE, Balm PHM, Haenen HE, Jenks BG & Wendelaar Bonga SE 1991 Regulation of differential release of α-melanocyte-stimulating hormone forms from the pituitary of a teleost fish, *Oreochromis mossambicus*. *Journal of Endocrinology* **129** 179–187.

Lamers AE, Flik G, Atsma W & Wendelaar Bonga SE 1992 A role for di-acetyl α-melanocyte-stimulating hormone in the control of cortisol release in the teleost *Oreochromis mossambicus*. *Journal of Endocrinology* **135** 285–292.

Lamers AE, Flik G & Wendelaar Bonga SE 1994 A specific role for TRH in release of diacetyl α-MSH in tilapia stressed by acid water. *American Journal of Physiology* **267** R1302–R1308.

Mancera JM & Fernandez-Llebrez P 1995*a* Development of melanin-concentrating hormone-immunoreactive elements in the brain of gilthead seabream (*Sparus auratus*). *Cell and Tissue Research* **282** 523–526.

Mancera JM & Fernandez-Llebrez P 1995*b* Localization of corticotropin-releasing factor immunoreactivity in the brain of the teleost *Sparus aurata*. *Cell and Tissue Research* **281** 569–572.

Maruthainar K, Peng LY & Smyth DG 1992 The processing of β-endorphin and α-melanotrophin in the pars intermedia of *Xenopus laevis* is influenced by background adaptation. *Journal of Endocrinology* **135** 469–478.

Mosconi G, Carnevali O, Facchinetti F, Radi D, Pestarino M, Vallarino M & Polzonetti MAM 1994 Ovarian melanotropic peptides and adaptation in two teleostean species: *Sparus aurata* L. and *Dicentrarchus labrax* L. *Peptides* **15** 927–931.

Quesada J, Lozano MT, Ortega A & Agullairo B 1988 Immuno-cytochemical and ultrastructural characterization of the cell types in the adenohypophysis of *Sparus aurata* L. (teleost). *General and Comparative Endocrinology* **72** 209–225.

Raaij van MTM, Thillart van den GEEJM, Vianen GJ, Pit DSS, Balm PHM & Steffens B 1996 Substrate mobilization and hormonal changes in rainbow trout (*Onchorhynchus mykiss* L) and common carp (*Cyprinus carpio* L) during deep hypoxia and subsequent recovery. *Journal of Comparative Physiology* **166B** 443–452.

Roubos EW 1997 Background adaptation by *Xenopus laevis*: A model for studying neuronal information processing in the pituitary pars intermedia. *Comparative Biochemistry and Physiology* **118A** 533–550.

Strien van FJC, Galas L, Jenks BG & Roubos EW 1995 Differential acetylation of pro-opiomelanocortin-derived peptides in the pituitary gland of *Xenopus laevis* in relation to background adaptation. *Journal of Endocrinology* **146** 159–167.

Sumpter JP, Pickering AD & Pottinger TG 1985 Stress-induced elevation of plasma α-MSH and endorphin in brown trout, *Salmo trutta* L. *General and Comparative Endocrinology* **59** 257–265.

Sumpter JP, Dye HM & Benfey TJ 1986 The effects of stress on plasma ACTH, α-MSH, and cortisol levels in salmonid fishes. *General and Comparative Endocrinology* **62** 377–385.

Vallarino M, Delbende C, Ottonello I, Tranchand-Bunel D, Jegou S & Vaundry H 1989 Immunocytochemical localization and biochemical characterization of melanocyte-stimulating hormone in the brain of the rainbow trout, *Salmo gairdneri*. *Journal of Neuroendocrinology* **1** 53–60.

Vaudry H, Tonon MC, Delarue C, Vaillant R & Kraicer J 1978 Biological and radioimmunological evidence for melanocyte stimulating hormones (MSH) of extrapituitary origin in the rat brain. *Neuroendocrinology* **27** 9–24.

Wendelaar Bonga SE 1997 The stress response in fish. *Physiological Reviews* **77** 591–625.

Zhu Y & Thomas P 1996 Elevations of somatolactin in plasma and pituitaries and increased α-MSH cell activity in red drum exposed to black background and decreased illumination. *General and Comparative Endocrinology* **101** 21–31.

Zhu Y & Thomas P 1997 Effects of somatolactin on melanosome aggregation in the melanophores of red drum (*Sciaenops ocellatus*) scales. *General and Comparative Endocrinology* **105** 127–133.

Zhu Y, Yoshiura Y, Kikuchi K, Aida K & Thomas P 1999 Cloning and phylogenetic relationship of red drum somatolactin cDNA and effects of light on pituitary somatolactin mRNA expression. *General and Comparative Endocrinology* **113** 69–79.

Zoest van ID, Heijmen PS, Cruijsen PMJ & Jenks BG 1989 Dynamics of background adaptation in *Xenopus laevis*: role of catacholamines and melanophore-stimulating hormone. *General and Comparative Endocrinology* **76** 19–28.

Received 15 November 1999
Accepted 21 March 2000