Abstract: The regulation of skin darkness in vertebrates is mediated by α-melanophore-stimulating-hormone (αMSH). For this action, αMSH binds to the melanocortin (MC)-1-receptor, a 7-transmembrane receptor located in melanophore cell membranes. The Mozambique tilapia, Oreochromis mossambicus, can change the hue of its body in response to a change in background, a process that may involve αMSH and the MC1R. Scale melanophores were isolated from tilapia that were acclimatised for 25 days to a black, control grey or white background and then tested for their sensitivity to des-, mono- and di-acetylated αMSH. On all backgrounds, mono-acetylated αMSH was the dominant isoform present in pituitary homogenates. Mono-acetylated αMSH also had the highest potency to disperse melanosomes. Black background adapted fish showed the highest dispersing response to αMSH, independent of the isoform applied. We elucidated the
nucleotide and amino acid sequence of the tilapia MC1R. We show that its expression in skin does not change when tilapia are acclimatised for 25 days to a black, grey or white background, while a clear change in hue is visible. This finding, combined with the absence of differential MC1R gene expression following background acclimation indicates that the increased sensitivity to αMSH is most likely a result of changes in the intracellular signalling system in melanophores of black background adapted fish, rather than up-regulation of the MC1R.
Nijmegen, 18/05/2005

Dear Sir/Madam,

Please find enclosed the revised manuscript GCE-05-26: “Alpha-MSH, the melanocortin-1 receptor and background adaptation in the Mozambique tilapia, Oreochromis mossambicus”. We have greatly appreciated the comments and suggestions of both reviewers, and, where applicable, have corrected the text accordingly.

We await your response,

Kind regards,

Gert Flik
Nijmegen, 18/05/2005

Comments addressing the revision by reviewer #1:

As suggested by this reviewer, we have added the grey background next to the black and white background in the abstract.

In the discussion, we wrote: “we present a complete deduced amino acid sequence and partion mRNA sequence for the tilapia melanocortin 1 receptor gene”. The use of the word “partial” here refers to the fact that a complete eukaryotic mRNA sequence finishes at the 3’terminus with a poly A tail. As the 3’UTR region we found does not end with this poly A tail, we cannot assume that we have uncovered the complete mRNA sequence. We did find an open reading frame for the MC1R peptide, from which we can present a complete deduced amino acid sequence.

We have extended the second paragraph of the discussion (Background adaptation) to describe some potential agents that may be more important in the physiological regulation of tilapia skin pigmentation.

Comments addressing the revision by reviewer #3:

We have added a figure showing that the αMSH content of the pituitary is not significantly different between fish from the three different experimental backgrounds. This again serves as evidence for adaptive melanophore responses via changes in intracellular signalling pathways.

Concerning the role of ACTH in the regulation of pigmentation, we are unfortunately unable to test our samples for the ACTH content in the plasma as an ACTH-RIA is currently unavailable. Furthermore, the in vitro response of melanophores to ACTH was lower than the response to αMSH, which reduces the likelihood of a physiological role for ACTH in the regulation of tilapia skin pigmentation. We agree however, that an indication of plasma ACTH levels in background adapted tilapia could increase the amount of evidence pointing to an adaptive change at the melanophore level.
Alpha-MSH, the melanocortin-1 receptor and background adaptation in the Mozambique tilapia, *Oreochromis mossambicus*

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Abstract

The regulation of skin darkness in vertebrates is mediated by α-melanophore-stimulating-hormone (αMSH). For this action, αMSH binds to the melanocortin (MC)-1-receptor, a 7-transmembrane receptor located in melanophore cell membranes. The Mozambique tilapia, Oreochromis mossambicus, can change the hue of its body in response to a change in background, a process that may involve αMSH and the MC1R. Scale melanophores were isolated from tilapia that were acclimatised for 25 days to a black, control grey or white background and then tested for their sensitivity to des-, mono- and di-acetylated αMSH. On all backgrounds, mono-acetylated αMSH was the dominant isoform present in pituitary homogenates. Mono-acetylated αMSH also had the highest potency to disperse melanosomes. Black background adapted fish showed the highest dispersing response to αMSH, independent of the isoform applied. We elucidated the nucleotide and amino acid sequence of the tilapia MC1R. We show that its expression in skin does not change when tilapia are acclimatised for 25 days to a black, grey or white background, while a clear change in hue is visible. This finding, combined with the absence of differential MC1R gene expression following background acclimation indicates that the increased sensitivity to αMSH is most likely a result of changes in the intracellular signalling system in melanophores of black background adapted fish, rather than up-regulation of the MC1R.
Introduction

Alpha-Melanophore-stimulating-hormone (αMSH) is known for its role in the skin pigmentation of vertebrates (Bagnara and Hadley, 1973). Darkening of the skin and its derivatives (hair, fur, feathers) in mammals and birds is slow and can take up to weeks to fully develop. However, in lower vertebrates such as amphibians and fish, skin melanophores quickly change appearance due to fast movements of dark pigment (melanin) granules, melanosomes, within the melanophore. This enables these animals to show a rapid change of the hue (observable colour of the skin), in response to changes of the background (Healey, 1999; Roubos, 1997). Both the slow darkening process in mammals and birds, and the rapid responses in lower vertebrates can be stimulated by αMSH.

Peptides are often modified posttranslationally by glycosylation, amidation or acetylation. Alpha-MSH is found in three different N-terminal acetylation isoforms: des-, mono- and di-acetylated αMSH. In mammals, as in most other species where post-translational acetylation of αMSH occurs, the major form is diacetyl αMSH (Dores et al., 1993; Keller et al., 1994). In a number of fish species, however, including tilapia, the dominant form is mono-acetyl αMSH (Arends et al., 2000; Dores et al., 1993; Lamers et al., 1991). Acetylation modifies the bioactivity of the peptides (Keller et al., 1994).

The Mozambique tilapia, Oreochromis mossambicus, uses body pigmentation as a means to communicate with conspecifics. The social status of an individual is read from its darkness and pigmentation pattern of the skin. Next to that, tilapia is able to adjust the pigmentation of its skin to the background it is kept upon (Eys and Peters, 1981). A black skin pigmentation can be induced by in-vivo administration of mono-acetylated αMSH. This indicates that αMSH has melanotropic potency in tilapia and may be involved in the process of background adaptation. Interestingly, during prolonged acid water stress, a shift occurs in the ratio between di- and mono-acetylated αMSH in plasma, in favour of the di-acetylated isoform. This led Lamers and co-workers (1992) to propose a corticotrope role for di-acetylated αMSH (Lamers et al., 1992). In a study by (Rudman et al., 1983), it was shown that acetylation of the peptide increased the melanotropic potency in a frog skin bioassay (relative potency: di = mono > des-acetylated αMSH) and prevented the degradation of αMSH (di > mono > des-acetylated αMSH). In salmon, mono-acetylated αMSH was also more potent than des-acetylated αMSH to stimulate melanosome dispersion in a frog test (Kawauchi et al., 1984).
The bioactivity of a peptide is determined by the receptor(s) it binds to. In most vertebrates, the control of pigmentation of the skin by αMSH is regulated via the melanocortin (MC) 1-receptor that is localised in the membrane of melanocytes (Cone et al., 1996). This receptor was first designated the αMSH receptor, as binding of αMSH induced a darkening of the skin. In mammals, this MC1R has the highest affinity for αMSH of all five receptor subtypes (named MC1 to MC5 receptors; Schiøth et al., 1995). The MC2R is specific for ACTH and located mainly in the adrenal tissue. The MC3, MC4 and MC5 receptors can all be found in the brain, the MC3 (e.g. adrenal cortex, placenta) and MC5 (e.g. exocrine glands, muscle) receptors are also expressed in a multitude of peripheral organs (Cone et al., 1996). In fish, contradicting findings have been reported. Studies on Japanese pufferfish, Takifugu rubripes, and rainbow trout, Oncorhynchus mykiss, show that in both species most of the MC receptors have a higher affinity for ACTH than for αMSH (Haitina et al., 2004; Klovins et al., 2004).

In this paper, we compare the scale melanophores of fish adapted to three different backgrounds (black, white and grey background tanks) in their response to the three isoforms of αMSH. We present the cDNA and deduced amino acid sequence of the melanocortin-1-receptor of Mozambique tilapia and have quantitated expression of the MC1R in these background adapted fish.

Materials and Methods

Animals

Male and female tilapia were obtained from laboratory stock (n=24 per background). Fish weighed around 70 g and were kept in 50L tanks containing tap water of pH 7.8. Water temperature was 24° C and fish were kept at a day/night rhythm of 12 L:12 D. Fish were fed commercial tilapia food (Tilapia 3.0, Trouw, Putten, The Netherlands). The walls of each experimental tank were covered with self-adhesive black (black background; B) or white foil (white background; W). The control tanks (control full-glass, grey background; G) were fitted with light-permeable one-sided see-through foil. In this way, disturbance of the fish by external movements or other stimuli was kept at a minimum and was similar for all groups, and the fish kept on the full-glass, grey underground served as extra controls, comparable to the background situation experienced in the rearing tanks.

Sampling
For scale melanophore studies, three fish from every background were lightly anaesthetised in 0.1% (w/v) 2-phenoxyethanol (Sigma). Scales were taken from the left-hand side of the fish and placed in a physiological salt solution (169 mM NaCl; 5.4 mM KCl; 1.8 mM CaCl₂; 1.3 mM MgCl₂; 5 mM Tris and 5.6 mM D-glucose) during transfer to the microscope. Fish were returned to the experimental tanks and not sampled again for a week to enable regrowth of scales.

For MC1R expression studies, six fish from every background were euthanized in 0.2% (w/v) 2-phenoxyethanol. Blood was drawn from the caudal vessels, with syringes containing Na₂EDTA as anticoagulant, and transferred to ice-cold eppendorfs containing 1 TIU of aprotinin, a serine-protease inhibitor. The blood was spun at 4 °C for 10 minutes at 13,500 rpm, after which the supernatant plasma was stored in Eppendorf vials and quickly frozen. Fish were subsequently placed on ice and a piece of head skin and several scales from the left-hand side of the fish were transferred to sterilised Eppendorfs and immediately frozen in dry ice. Samples were stored at -80 °C until RNA isolation.

αMSH determination

The αMSH concentration in the plasma and in the pituitary gland was determined as described by Arends et al. (1999). The antiserum used for the αMSH radio immunoassay cross-reacts for 100% with des-, mono- and di-acetyl αMSH, and was used in a final dilution of 1:60,000. Immunocomplexes were precipitated by 7.5 % (w/v) polyethylene glycol and 2.5 % (w/v) bovine serum albumin. The detection limit was 25.2 pg/ml of sample. Pituitary glands were homogenized mechanically on ice in 100 μl of 0.1M HCl. After centrifugation (13,600 rpm, 4 °C) for removing membranes and cellular debris, the supernatant was used to assess the total amount of αMSH in the pituitary glands. To separate the different isoforms of αMSH, reversed-phase HPLC was used as described by Arends et al. (2000). First, a mixture of synthetic des-, mono- and diacetyl αMSH (Sigma) was separated on a Pharmacia μRPC C2/C18 sc 2.1/10 column with ddH₂O/0.1% trifluoracetic acid (TFA) as equilibration eluent and a gradient of acetonitril/0.1% TFA from 0-100% as secondary eluent to determine the elution time of each isoform, and subsequently, the superfusates were fractionated following this protocol. One-minute fractions were collected and the αMSH concentration in these fractions was determined by RIA. To calculate the amount of des-, mono- and diacetyl αMSH, the area under the curve was measured for each form. The sum of the amounts of des-, mono- and diacetyl αMSH was set at 100%. The amount of each individual form was expressed as a part of this 100%.
Melanophore responses

Micrographs were taken from scales of fish from the three different backgrounds prior to the start of the experiments and the degree to which the skin was covered by dark melanophores was assessed. These values indicate the initial scale coverage values for each background.

Fifteen minutes prior to the experiment, scales were immersed in physiological saline containing 60 mM KCl. The high concentration of K⁺ depolarized the melanophores causing aggregation of the melanosomes. Scales were photographed immediately prior to the start of the experiment, designated the “time 0 melanophore status”. Subsequently, scales were immersed in a solution containing αMSH for 30 min after which another micrograph was taken. Des, mono and di-acetylated αMSH (Sigma) were tested at concentrations ranging from 10⁻¹¹ to 10⁻⁷ M. The melanophore responsiveness was determined by the changes in dispersion state after 30 min of immersion in test solution. We used Adobe Photoshop 7.0 to measure the area (pixels) of the scales covered by epidermis and then measured the area (pixels) covered by melanin using the “Magic Wand” selection tool. By dividing the two values, a melanophore coverage value expressed as a percentage of the scale surface (epidermis) could be obtained. Changes were initially quantified by assessing the percentage of scale covered by melanophores at time 0 and subtracting these values from the percentage of scale melanophore coverage after 30 min of immersion.

Cloning and sequencing

To obtain the nucleotide sequence of the melanocortin-1-receptor in tilapia, two oligonucleotide primers were designed based on the Fugu MC1R sequence: F1fw, 5’- GGT GGA GAA CAT CCT GGT GAT TCT GG-3’; F3rv, 3’- CGC AGC TCC TGG CTC CGG TAC GCG-3’. Head skin tissue was homogenized in TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA) and RNA was isolated according to the manufacturer’s instructions. 1μg of RNA was reverse-transcribed with 300 ng of random primers, 10nmol dNTPs, 200 nmol, 10 U RNase inhibitor and 200 units of RT Superscript II (Gibco BRL) for 50 min at 37 °C. PCR of the above mentioned primers on the obtained cDNA yielded a partial MC1R sequence. The remainder of the sequence was obtained by RACE (rapid amplification of cDNA ends)-PCR (GeneRacer, Invitrogen) according to the manufacturer’s protocol, including the use of nested PCR. Gene specific primers used for the RACE-PCR were: MC1RC3fw, 5’- AAC AGG CGC CAG TCC ACA AGT ATG A-3’; MC1RC3nestedfw, 5’-
CTG TAA CTC CCT CAT CGA CCC GCT TA-3'; RACE5'fw, 5'- GAT GCT GTG ATA CCT CAG CGC GT-3'; RACE5’nestedfw, 5'- TAG TAC ATG GGC GAG TGG AGG TT-3'.

PCR products were ligated into pCR4-TOPO plasmid vector and transformed into chemically competent TOP 10 Escherichia coli cells (TOPO TA cloning kit, Invitrogen). After selection on LB-kanamycin agar, transformed cells were screened for appropriate size inserts with T3 and T7 primers. Sequence determination was carried out with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Phylogenetic analysis

Sequences for other fish melanocortin-1 receptors were retrieved from the NCBI site (www.ncbi.nih.gov), using the Genbank database. Multiple-sequence alignment was carried out with ClustalW at the BioAsp website (www.bioasp.nl) from the Centre of Molecular and Biomolecular Informatics. A phylogenetic tree was constructed on the basis of amino acid difference (p-distance) with a neighbour-joining method with 1000 bootstrap replications, with MEGA version 2.1 (Kumar et al., 2001; Saitou and Nei, 1987).

MC1R expression

Relative expression of the melanocortin-1-receptor was assessed by quantitative RT-PCR. Head skin or scale tissue was homogenized in TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA). Total RNA was extracted according to the manufacturer’s instructions and reverse transcribed. On the basis of the tilapia MC1R sequence, the following quantitative PCR primers were designed: MC1fw, 5’- GGA GAC CAT ATT CAT GCT TCT CAA-3'; MC1rv, 5’- ATC ATC ACG TCG ATG ACG TTG T-3'. Reference housekeeping genes used were β-actin and 40S, of which the following primer sets were constructed: BACTfw, 5’- GCC CCA CCT GAG CGT AAA TA-3'; BACTrv, 5’- CCT GCT TGC TGA TCC ACA TCT-3'; 40Sfw, 5’- GAG ATG CTT ACA GGC GAT CTG-3'; 40Srv, 5’- GCC ACC TCT GAA CTG GAA CT-3'.

Five μl of 50 times diluted RT-mix was used as a template in an amplification mixture containing 12.5 μl SYBR Green Master Mix (Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands) and 3.75 μl of each primer (final concentration 300 ng). Real-time quantitative PCR was performed on a GeneAmp 5700 (Applied Biosystems). Ct-values were determined and expression of MC1R was calculated as a percentage of β-actin or 40S.
All results presented here are expressed relative to 40S, as expression patterns did not vary between 40S and β-actin.

Statistical analyses

Physiological parameters between fish from different backgrounds and differences between the groups in expression of MC1R were assessed by ANOVA, followed by Dunnet’s C post-testing when significance was indicated. The differences in percentage of scale coverage by melanophores was arcsine transformed (Sokal and Rohlf, 1995) and then tested with the method described above. Correlations between background and experimental parameters were assessed using Spearman’s rho correlation testing. All parameters are expressed as means ± sd. Statistical analyses were performed with SPSS 12.0.1 statistical software.

Results

Background adaptation

After 25 days of adaptation to the different backgrounds, a clear difference in skin darkness was visible (Figure 1). Fish on a black background (BBG) had the highest melanophore coverage level (43%), and white background (WBG) fish had the lowest coverage values (10%; P<0.01). WBG scale coverage values were also significantly lower than the corresponding values of grey background (GBG) scales (P<0.05). The skin darkness of GBG and BBG fish did not differ significantly. In both groups individual variation was quite high. There was a strong positive correlation between the darkness of the background and the scale coverage level (P<0.01, r =0.763). There was no significant difference in the plasma concentrations of aMSH between the fish from the different backgrounds (data not shown). The total amount of aMSH present in the pituitary gland was also not different in fish from either background and varied around 0.11μg (Figure 2A). The ratio of aMSH isoforms in pituitary gland homogenates had not changed following adaptation to the backgrounds (Figure 2B). More than half of the aMSH present was mono-acetylated; des and di-acetylated aMSH were present in similar amounts.

Response to aMSH isoforms

Overall, scale melanophores responded strongest to mono-acetylated aMSH (P<0.05, Figure 3). Scales from fish from a black background showed the strongest dispersion
response, followed by control fish whereas white background fish had the least responsive melanophores. There were no significant differences between the different concentrations of the hormones tested. A significant correlation was found between the intensity of the dispersing response and the darkness of the background (P<0.01, r =0.159).

**Melanocortin-1Receptor**

Cloning and sequencing of the tilapia MC1R revealed a 1959 bp cDNA sequence with an open reading frame of 325 amino acids (Figure 4, EMBL accession number AJ871147). Amino acid identity is highest to various puffer fish species (74-78%) and zebrafish (68%) and around 50% to various mammalian and avian species (human, 47%; mouse, 50%; dog, 50%; chicken, 55%).

Two potential sites for N-linked glycosylation are present in the N-terminal extracellular domain (Asn³ and Asn²⁶) and there are two potential sites for protein kinase C phosphorylation in the intracellular domain between transmembrane regions 5 and 6 (Ser²²³ and Ser²³⁸). In this domain a potential cAMP phosphorylation site is also present (Arg²³³). Figure 5 shows a multiple alignment of the tilapia MC1R with MC1R amino acid sequences of various other species. The seven predicted transmembrane regions are indicated and these stretches show a high degree of conservation between piscine, avian and mammalian sequences.

In figure 6, a phylogenetic tree is shown for the five different melanocortin receptor genes in various species. This tree was constructed with the neighbour-joining method with 1000 bootstrap replications. The tilapia MC1R clusters together with the fugu MC1R with a reliability of 100%, and together with the zebrafish MC1R these form a distinct cluster within the group of MC1R. This tree also confirms that the obtained sequence is indeed an MC1R and not one of the other four melanocortin receptors.

Based on the nucleotide sequence as shown in figure 4, we constructed primers for determination of the expression of the MC1R in skin relative to the expression of the 40S housekeeping gene. There were no significant differences in MC1R expression in the skin between fish adapted to a black or white background compared to control glass background (Figure 7).

**Discussion**
In this study we present a complete deduced amino acid sequence and partial mRNA sequence for the tilapia melanocortin 1 receptor gene. The expression of this receptor in skin is not influenced by background adaptation to a black or white background and the resulting significant changes in hue. The dispersion of melanosomes within melanophores upon stimulation with αMSH isoforms is significantly higher in black background acclimatised fish than in fish from white or grey backgrounds, indicating that the melanophores have become sensitised to αMSH. Mono-acetylated αMSH is the dominant isoform present, and also the most bioactive isoform to stimulate melanosome dispersion, independent of the background.

Background adaptation

Twenty-five days of adaptation to a black or white background resulted in marked and significant changes in the hue of tilapia. In white background tilapia, the lightness of the skin as appreciated from the melanophore coverage of scales was significantly lower than in black and grey background fish. It is generally believed that the regulatory factors involved in initial rapid pigmentation pattern changes (physiological colour change) are also of importance on the longer term (morphological) changes in pigmentation (Fujii, 2000; Sugimoto, 2002). The significant decrease in scale coverage by melanosomes is likely to be the result of degeneration by apoptosis of melanophores. Norepinephrin is a likely candidate to induce this apoptosis; in this context also melanin concentrating hormone (MCH) is a potential apoptosis inducing factor (Sugimoto et al., 2000; Sugimoto, 2002). The increase of melanophore cell numbers observed on a black background in fish and amphibians is classically linked to αMSH (Bagnara and Hadley, 1973; Fujii, 2000). However, in the present study no differences were found in plasma αMSH levels between fish from the three different backgrounds. This suggests that at this stage of the background adaptation process, the in-vivo regulation of skin pigmentation in tilapia is under the control of physiological factors other than αMSH. A likely candidate is melanophore concentrating hormone (MCH), which is elevated in the hypothalamus of white background adapted tilapia (Groneveld et al., 1995) and in plasma and hypothalamus of pale-background adapted trout (Baker, 1993). Moreover, MCH is a very potent melanophore aggregating agent that is able to antagonise αMSH in an in-vitro set-up in various fish species (Baker, 1993; Burton and Vokey, 2000b) and even directly inhibits the release of αMSH from the pituitary gland (Baker, 1994; Gröneveld et al., 1995). Catecholamines (particularly noradrenaline) may also be involved in more rapid, nervously regulated colour changes (Burton and Vokey, 2000a; Filadelfi et al., 2002; Fujii, 2000).
Bioactivity of αMSH isoforms

While there is apparently no in-vivo relation between plasma αMSH levels and the darkness of the skin of tilapia, in-vitro application of αMSH to scales has a clear dispersing effect on the melanin granules within the melanophores. This suggests once more that in tilapia the endocrine regulation of skin pigmentation includes αMSH, but only as a minor factor. The post-translational acetylation of αMSH indeed seems to affect bioactivity as mono-acetylated αMSH had a stronger dispersing effect on the melanosomes of scale melanophores than desacetyl αMSH. In mammals, des-acetylated αMSH blocks opiate receptor binding stronger than monoacetyl αMSH, whereas monoacetyl αMSH was 10-100 fold more effective in increasing pigmentation, arousal, memory, attention and excessive grooming (see Mountjoy et al., 2003). Rudman et al., (1983) have shown that di- and monoacetyl αMSH have similar melanotropic potency on frog melanophores, whereas desacetyl αMSH had the lowest melanotropic potency. We now find the same for tilapia melanophores.

The bioactivity of αMSH isoforms is determined by the receptor profile of the cell and the combined second messenger pathways. In scales of black background fish the response to each individual isoform was highest. In medaka, Oryzias latipes, enzymes that are part of the intracellular signalling pathway are more active in black background melanophores than in melanophores of white background fish. These protein phosphatases decrease the sensitivity of the melanophores to externally administered cAMP and black background adapted medaka required more cAMP than white background fish (Sugimoto, 1993; Sugimoto et al., 1997). The higher sensitivity to cAMP observed in melanophores of white background adapted medaka facilitates a quick adaptive response should the background darken (Sugimoto et al., 1997). A similar situation may be found in background acclimatised tilapia as well. Circulating levels of αMSH show no differences between black, grey and white background adapted fish, while the black background fish show a higher responsiveness to αMSH than grey and white background fish. The possibility exists that αMSH turnover could be higher at constant plasma levels, but this was not further analysed. Rather than changes in plasma αMSH levels upon background adaptation, the sensitivity of the αMSH receptor in melanophores may be increased on dark backgrounds and reduced on light backgrounds. Upon binding of αMSH to its receptors, intracellular cAMP levels rise. Enhanced αMSH binding may therefore stimulate higher intracellular levels of cAMP and in this way compensate for the reduced sensitivity to cAMP as described by Sugimoto and co-workers (1997).
Mono-acetylation apparently stimulates the potency of αMSH whereas di-acetylation may reduce the activity. According to (Mountjoy et al., 1999; Mountjoy et al., 2003) the acetylation state of αMSH is the key factor for its bioactivity: for some functions acetylation of αMSH inhibits its bioactivity, and for other functions monoacetyl αMSH is more potent than the des-acetylated form. Di-acetylated αMSH has been indicated to be the isoform with corticotropic potency (Balm et al., 1995; Lamers et al., 1992), and (Rudman et al., 1983) have shown that the higher the acetylation-state, the longer it takes for the peptide to be degraded. The prolonged presence of the di-acetylated αMSH isoform may compensate for its reduced melanotropic bioactivity.

**Melanocortin-1-receptor**

The MC1R is known from mammalian literature to be involved in the regulation of skin pigmentation (Ha et al., 2003; Kijas et al., 1998) and is considered the main receptor for melanotrophic actions of αMSH. The high degree of homology between the piscine MC1R sequences and those in amphibians, birds and mammals indicates that this function has been conserved throughout the evolution of the vertebrates. We therefore assume that the MC1R is also the main receptor for αMSH binding in tilapia to serve melanotropic actions. The expression of the tilapia MC1R does not differ between tilapia adapted to different backgrounds. We have postulated earlier that a change occurs in the intracellular signalling system following adaptation to a black background, rather than an increase in receptor expression. An affinity change in the receptor seems unlikely as our nucleotide sequence was derived from mRNA originally obtained from head skin of black and white as well as glass background adapted fish. The cDNA was identical for the clones we obtained from each background.

Interestingly, affinity studies on the MC1R in fugu show that, unlike in mammals, the fugu MC1 receptor does not have the highest affinity for αMSH. The affinity of the MC1R for ACTH appeared to be 10 times higher than for αMSH (Klovins et al., 2004). These authors postulate the hypothesis that the MC1R has evolved from a receptor with preference for ACTH in early vertebrates to one that has specific affinity for αMSH in mammals. These findings also point to a possible role for ACTH in the regulation of pigment dispersion in fish as has been demonstrated by (Fujii, 1993; Fujii, 2000). Indeed, initial tests in our laboratory with ACTH have shown that this peptide also has melanotrophic bioactivity on scale melanophores of tilapia (unpublished results), albeit to a lesser extent than αMSH.
The main findings of our study are that the MC1R receptor is present in tilapia skin and shows high homology with various other vertebrate species and that this receptor is not differentially up-regulated in fish adapted to different backgrounds. We hypothesize that αMSH binds to this receptor to invoke melanosome dispersion. Acetylation of des-acetylated αMSH stimulates the melanotropic potency of the peptide and fish on a black background show the highest response to all αMSH isoforms. The increased potency of αMSH either by acetylation or by previous background adaptation is not mediated by increased receptor expression, but may result from receptor affinity changes or from adaptive changes in the intracellular signalling system. Indeed, while plasma and pituitary αMSH levels are unchanged between fish from the three different backgrounds used in this study, the response to αMSH is different. This indicates an increased sensitivity to αMSH in black background acclimatised fish.

Acknowledgements

The authors wish to thank Rianne van den Broek and Klaas Medendorp for lab assistance and Tom Spanings for perfect fish husbandry. This study has been carried out with financial support from the Commission of the European Communities, DG Fisheries, QLRT programme, QLK5-2000-031629, “Environmental, nutritional and neuroendocrine regulation of skin coloration in the red porgy (Pagrus pagrus), towards the development of natural hue in cultured populations”. This paper does not necessarily reflect its views and in no way anticipates the Commission’s future policy in this area.

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Legends to the figures

Figure 1- Differences in hue between tilapia from a control grey, black or white background, based on scale coverage by melanophores. Significant differences are indicated by * (P<0.05; n=12 per background). A significant correlation between the darkness of the background and the scale melanophore coverage is indicated by ▼▼ (P<0.01).

Figure 2- A) the amount of αMSH present in the pituitary gland of tilapia from a grey, black or white background (μg), B) the ratio of αMSH isoforms in pituitary homogenates of background adapted tilapia (n=12 per background).

Figure 3- Average response after 25 days of adaptation to a grey, black or white background of scale melanophores to des-, mono- and di-acetylated αMSH (n=8 per background, data ± SEM). The significant correlation between the darkness of the background and the responsiveness is indicated by ▼▼ (P<0.01). The correlation between responsiveness and isoforms was significant for mono-acetylated αMSH (P<0.05; *).

Figure 4- Full-length nucleotide and deduced amino acid sequence of tilapia MC1R cDNA. The deduced amino acid sequence is displayed above the nucleotide sequence. The start codon is boxed in black, the stop codon is indicated by an asterisk (*). Two potential glycosylation sites are boxed in white and two potential PKC phosphorylation sites are boxed in grey. A potential cAMP phosphorylation site is indicated in bold. Two potential adenylation sites are underlined in the 3'-UTR. The EMBL accession number is AJ871147.

Figure 5- Multiple alignment of MC1Rs of eleven different vertebrate species. Hyphens indicate gaps introduced for maximum identity. Identical amino acids are indicated in black boxes, conservative constitutions in grey boxes. The seven predicted transmembrane regions (TM 1 to 7) are indicated. Accession numbers: human (Homo sapiens), Q01726; chimpanzee (Pan troglodytes), Q9TUK4; mouse (Mus musculus), Q01727; dog (Canis familiaris), O77616; chicken (Gallus gallus), P55167; zebrafish (Danio rerio), Q7ZTA3; fugu (Takifugu rubripes), Q7ZSY9 and tilapia (Oreochromis mossambicus), AJ871147.
Figure 6- Neighbour joining tree of melanocortin-receptor amino acid sequences. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. The starting point for cluster formation is indicated by an open circle. Light grey indicates the different MC-receptor clusters and fish species are indicated in dark grey within these clusters. Accession numbers: tilapiaMC1R (*Oreochromis mossambicus*), AJ871147; fuguMC1R (*Takifugu rubripes*), Q7ZSY9; zebrafishMC1R (*Danio rerio*), Q7ZTA3; chickenMC1R (*Gallus gallus*), P55167; mouseMC1R (*Mus musculus*), Q01727; dogMC1R (*Canis familiaris*), O77616; chimpanzeeMC1R (*Pan troglodytes*), Q9TUK4; humanMC1R (*Homo sapiens*), Q01726; humanMC2R (*Homo sapiens*), Q01718; mouseMC2R (*Mus musculus*), Q64326; cowMC2R (*Bos Taurus*), P34974; fuguMC2R (*Takifugu rubripes*), Q7ZSX7; zebrafishMC2R (*Danio rerio*), Q7ZTA2; carpMC2R (*Cyprinus carpio*), Q6EWJ2; humanMC5R (*Homo sapiens*), P33032; chimpanzeeMC5R (*Pan troglodytes*), Q9TT23; cowMC5R (*Bos Taurus*), P56451; sheepMC5R (*Ovis aries*), P41983; mouseMC5R (*Mus musculus*), P41149; chickenMC5R (*Gallus gallus*), O73671; fuguMC5R (*Takifugu rubripes*), Q7ZT40; zebrafishMC5R (*Danio rerio*), Q8JGW1; zebrafishMC4R (*Danio rerio*), Q8JGW3; goldfishMC4R (*Carassius auratus*), CAD58853; fuguMC4R (*Takifugu rubripes*), Q90VY0; chickenMC4R (*Gallus gallus*), Q6E6M6; ratMC4R (*Rattus norvegicus*), P70596; humanMC4R (*Homo sapiens*), P32245; pigMC4R (*Sus scrofa*), O97504; zebrafishMC3R (*Danio rerio*), Q7ZTA1; chickenMC3R (*Gallus gallus*), O93259; humanMC3R (*Homo sapiens*), P41968; mouseMC3R (*Mus musculus*), P33033; ratMC3R (*Rattus norvegicus*), P32244.

Figure 7- Expression of MC1R in skin tissue of tilapia from a control glass, black or white background relative to 40S expression.
Figure

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Expression of MCIR relative to 40S

- black
- grey
- white