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The factors controlling the secretion of the melanin-concentrating hormone (MCH) in the rainbow trout

Green, Janice Ann

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THE FACTORS CONTROLLING THE SECRETION OF THE MELANIN-CONCENTRATING HORMONE (MCH) IN THE RAINBOW TROUT

Submitted by **Janice Ann Green**
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SUMMARY

The work reported in this thesis concerns the melanin-concentrating hormone (MCH), a vertebrate neuropeptide. MCH causes pallor in all teleosts studied so far. However, there is some evidence suggesting that MCH might modulate the response to stress. Both its pigmentary and extrapigmentary functions were investigated.

The effects of the administration of anaesthetics on plasma MCH titres in the trout were studied. Phenoxyethanol or benzocaine caused only minor changes in plasma MCH over a short period compared to death by stunning, MS222 and Urethane induced significant increases.

Studies into the response of trout melanophores to *in vitro* culture suggested that the resting state of teleost melanophores is punctate and not dispersed, as previously suggested, and that MCH synergises with noradrenaline to cause melanin concentration in melanophores. This explains the low plasma concentration of MCH in trout blood.

It was shown that plasma MCH concentrations were raised by stress in white- but not black-adapted fish and were reduced by dexamethasone. However, the stress-induced release of MCH and cortisol do not appear to be directly related. MCH can depress the sensitivity of fish to stress *in vivo*. Fish reared in white-tanks were much less easily stressed than black-reared fish. *In vitro* studies showed that MCH does not affect the response of the interrenal to adrenocorticotrophin but can inhibit the release of a corticotrophin-releasing factor from the hypothalamus.

The amount of MCH secreted by fish in the field was measured. Fish kept in earthen outdoor ponds contained and released similar amounts of MCH to fish reared in the University aquarium in black tanks. There was no detectable effect of the seasons on MCH secretion in farm-reared fish.

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
AVP	arginine vasopressin
B	label bound to antibody in presence of unlabelled peptide
B_o	label bound to antibody in absence of unlabelled peptide
B→W	Fish transferred from black to white tanks
BSA	bovine serum albumen
CLIP	corticotrophin-like intermediate lobe peptide
cpm	counts per minuite
CRF	corticotrophin-releasing factor
μCi	microCurie
D	dark
°C	degrees Celcius
≡	equivalent to
DAG	diacylglycerol
dpm	disintegrations per minuite
EDTA	ethylene-diamine-tetra-acetic acid
eg	for example
β-END	beta-endorphin
EtOH	ethanol
et al.	and others
Fig	figure
$\times g$	force due to gravity
kg	kilogrammes
g	grammes
mg	milligrammes
μg	microgrammes
ng	nanogrammes
pg	picogrammes
^{3}H	tritium
h	hours
HBSS	Hanks balanced salt solution
HCl	hydrochloric acid
HPA	hypothalamo-pituitary-adrenal
HPI	hypothalamo-pituitary-interrenal
I	iodine
IP	inositol phosphate
ir	immnunoreactive
IU	international units
KIU	Kallikrein inhibitor units
γLPH	gamma-lipotrophin
l	litres
ml	millilitres
μl	microlitres
L	light
LHRH	leutenising hormone releasing hormone
M	molar solution
m^3	cubic metres
mm^3	cubic millimetre
MCH	melanin-concentrating hormone
MEM	minimum essential medium
MI	melanophore index
min	minuite
MS222	ethyl *m*-aminobenzoate methanesulphonate)

αMSH	alpha-melanocyte-stimulating hormone
MW	molecular weight
n	number in sample
NA	noradrenaline
NIL	Neurointermediate lobe
NPP-1	N-terminal POMC peptide-1
NSB	nonspecific binding
%	percent
p	probability
PBSG	phosphate buffered saline with gelatine
PD	pars distalis
PEG	polyethylene glycol
PI	pars intermedia
POMC	pro-opiomelanocortin
RIA	radioimmunoassay
rpm	revolutions per minuite
s.e.m.	standard error of the mean
vs	versus, against
v/v	volume to volume ratio
w/v	wieght to volume ratio
W→W	Fish transferred from white to white tanks

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CHAPTER 1

INTRODUCTION

This thesis concerns the melanin-concentrating hormone (MCH), a vertebrate neuropeptide first characterised as recently as 1983 (Kawauchi *et al.*, 1983). The body of evidence providing information about its actions has grown rapidly, and it was the aim of the work reported in this thesis to contribute to the understanding of the effects of MCH on both colour change (the function in fish which led to its discovery) and its extra-pigmentary functions which are probably the most important in vertebrates as a whole. Each chapter will tackle a different aspect of the research topics undertaken over the last three years and will introduce each subject area in a more specific and relevant way than can be attempted in a general introductory chapter such as this. The function of this chapter is to outline the areas of investigation and provide a general understanding of the background of the subject as a whole.

1.1. THE DISCOVERY OF MCH

The topic of colour change has long been the subject of biological research. Many of the lower vertebrates exhibit variation in skin pigmentation in response to changes in background colour. Change in colour is achieved through altering the distribution of pigment granules in the skin pigment cells, the darkest and most noticeable pigment being melanin which is found in cells called melanophores. There are other types of pigmentary cells and they are named according to their colour or pigment types; xanthophores containing the yellow xanthin pigment, erythrophores containing pterins, leucophores (white) and irridophore (reflecting) both containing iridescent guanine derivatives. Animals can change their colour

by dispersing melanin in the dermal and epidermal melanophores on a black background and concentrating it on a white background. Dispersal of melanin can be achieved by the release of the melanocyte-stimulating hormone (αMSH) from the pituitary. This is a 13-residue peptide derived from the precursor molecule pro-opiomelanocortin (POMC) along with other molecules such as adrenocorticotropin (ACTH), β-endorphin, β-lipotrophin and other fragments. For a long time it was thought that background adaptation in amphibia and elasmobranchs could be explained exclusively in terms of the level of secretion of α-MSH produced in the pars intermedia of the pituitary gland since hypophysectomy lead to melanin concentration while injections of pituitary extracts caused melanin dispersion.

In 1931 it was suggested by Hogben and Slome that a melanin concentrating hormone existed in the amphibian pars tuberalis since lesions in this area caused very intense darkening. They proposed a bihumoral theory in the control of chromatic adaptation involving this "W" (whitening) hormone and αMSH. Later, Etkin (1962) showed that lesions in the pars tuberalis destroyed the hypothalamo-hypophysial nerve tracts which inhibit αMSH release; such lesions therefore caused darkening due to uninhibited secretion of αMSH.

The situation was very different in fish. Injection of teleost pituitary extracts frequently led to extreme pallor in some teleosts but which could also induce darkening in other vertebrates. Healey showed in 1948 that this melanin concentrating effect was not a pharmacological phenomenon. In 1955 Enami, working with the catfish *Parasilurus*, suggested a hypothalamic origin for MCH, analagous to other neurohypophysial hormones, but Kent

(1959) was unable to repeat Enami's finding in a different species, the minnow *Phoxinus laevis* and Enami's work was not subsequently cited. Interest in the existence of MCH waned as there was no economical bioassay for the substance. No MCH bioactivity was detectable in the pituitary gland of other vertebrate species and there were variable interspecific responses of teleost melanophores to pituitary extracts.

Interest was revived much later by Baker and Ball (1975) who provided some new observations. Denervated melanophores of the molly *Poecilia latipinna* show melanin concentration when fish are placed on white backgrounds but remain dispersed in fish placed in black tanks. Hypophysectomy significantly delayed the onset of pallor when fish were moved from a black to a white background. These workers also showed, using gel electrophoresis, that two colour change hormones were present in the pituitary and Baker and Rance (1979) subsequently showed that the hypothalamus does contain MCH, confirming Enami's proposal that MCH is a neurohypophysial hormone. Rance and Baker also showed that concentrations of MCH vary with changes in background colour. Later investigations by Baker and Rance (1983) mapped the distribution of MCH bioactivity in frozen brain sections. They showed that the majority of MCH was found in the ventral third of the hypothalamus with up to 30% in the dorsal hypothalamic region. The location of the cell bodies was uncertain but it was suggested that at least part of the ventral activity would be due to MCH axons converging towards the pituitary stalk. MCH-like bioactivity was demonstrated also in the hypothalamus, but not the pituitary, of *Lampetra, Rana, Xenopus* and the rat suggesting that the molecule might have a wide vertebrate distribution. The same authors showed that if the tissue was extracted in dilute acid the bioactivity was lost, and this could explain some negative results from earlier workers (Baker and Rance, 1983). Eels do

not respond to injections of teleost pituitary extract with pallor and for a long time it was thought that MCH might be important for colour change in only a few species and that chromatic adaptation in the eel was due (as in amphibia and elasmobranchs) exclusively to changes in the circulating titre of αMSH. Gilham and Baker (1984) showed that if black-adapted frogs were injected with αMSH antiserum they responded with marked pallor. If, however, black-adapted eels were injected with anti-αMSH serum sufficient to neutralise all the melanin dispersing activity in one pituitary they remained black but would then respond to injections of partially purified MCH with pallor suggesting that secretion of an MCH is indeed needed to achieve rapid pallor in the eel.

MCH concentrations in the pituitaries of fish change with adaptation to different backgrounds; thus, if long-term white-adapted trout (*Salmo gairdneri*), eels (*Anguilla anguilla*) or carp (*Ctenopharyngodon idellus*) are transferred to a black background for 4-6 days, the immunoreactive MCH (irMCH) content increases significantly, suggesting that MCH release is halted in black-adapted fish but synthesis continues, resulting in its storage in the pituitary (Baker, 1988b).

Recently, it has become possible to measure MCH directly in the plasma using a solid-phase radioimmunoassay (RIA) (Kishida *et al.*, 1989, Eberle *et al.*, 1989). MCH concentrations in the plasma change during background adaptation, being high in white-adapted fish (> 100 pg/ml) and low in black- or darkness-adapted fish (29 and 9 pg/ml respectively). These changes show an inverse relationship to plasma αMSH concentrations (Baker *et al.,*, 1984) suggesting that the two hormones have the opposite effects.

1.2. LOCALIZATION OF MCH

1.2.1. Localization in fish

Salmon MCH was isolated and characterised in 1983 by Kawauchi *et al.* and once the sequence (a cyclic heptadecapeptide) became known it was possible to raise antisera against the synthetic peptide and to map its distribution in the brain and pituitary. In the salmonids *Oncorhynchus keta* and *Salmo gairdneri*, MCH neuronal cell bodies are found in the nucleus lateralis tuberis (NLT) of the ventral hypothalamus (Naito *et al.*, 1985). Some immunoreactive MCH (irMCH) fibres from the NLT pass caudo-dorsally to the pre-tectal area where they are found lateral to the posterior commissure (Naito *et al.*, 1985), a distribution coinciding with MCH bioactivity (Baker and Rance, 1983). Naito *et al.* (1985) also point out that the pretectal region in the fish receives axonal projections not only from the MCH perikarya but also from the retina and the pineal and suggest the possibility of communication between these fibres, with potential regulation of MCH neurones. Some of the fibres arising from the NLT cell bodies project to the preoptic area of the anterior hypothalamus, but the majority of fibres enter the pituitary gland, mostly running near blood vessels in the pars nervosa (PN) and secretory cells of the pars intermedia (PI), although a few were seen among cells of the pars distalis (PD). The pituitary gland of the eel (*Anguilla anguilla*) has a similar distribution of irMCH in the PI. In the eel and the carp (*Ctenopharyngodon idellus*), ultrastructural studies show that MCH is contained within oval neurosecretory granules in fibres which terminate predominantly at the basement membrane separating the PN and PI (Powell and Baker, 1988). In the carp, some, but not all of the MCH neurones in the PN contain granules which interact with antisera against both MCH and αMSH (Powell and Baker, 1987). The significance of this will be discussed in the next section (1.3.)

but it is apparently due to cross-reactions between the αMSH antisera and another peptide cleaved from the MCH precursor molecule.

In the Molly (*Poecilia latipinna*), neuronal cell bodies were found in the tuberal hypothalamus, mostly within the NLT pars lateralis as in salmonids (Batten and Baker, 1988). Fibres were traced forwards through the pre-optic area as far forward as the olfactory bulb, and also through the posterior hypothalamus up into the pretectal thalamus and midbrain. Once again, in agreement with findings in other species, the majority of projections were to the neurohypophysis, mostly to the PI where they terminated on the basement membranes of blood vessels, on pituicytes and on endocrine cells. Occasionally terminals were seen near the corticotrophs of the PD.

In elasmobranchs, the distribution of irMCH has been investigated in the dogfish *Scyliorhinus canicula* by Vallarino and colleagues (1989). Using antisera raised against synthetic salmonid MCH (sMCH) they found three groups of cell bodies in the posterior hypothalamus, the most prominent group was detected in the nucleus sacci vasculosi. Scattered cells were seen in the nucleus tuberculi posterioris and in the nucleus lateralis tuberis. In the pituitary, the caudal median lobe of the pars distalis contained strongly MCH-positive perikarya, quite unlike the situation in teleosts. Also, no fibres were seen in the neurointermediate and rostral distal lobes of the pituitary which were devoid of immunoreactive material. Immunoreactive fibres originating in the hypothalamic perikarya projected throughout the dorsal wall of the posterior hypothalamus, positive fibres were also detected within the thalamus and the central grey of the mesencephalon. Some irMCH neurones of the nucleus sacci vasculosi also showed the presence of immunoreactive αMSH (irαMSH).

1.2.2. Localization in amphibia

The hypothalamus of the frog, *Rana ridibunda*, contains an irMCH that can be detected using an antiserum raised against synthetic sMCH. The highest concentration of MCH perikarya is found in the preoptic region where numerous other peptidergic neurones are located, such as αMSH, corticotrophin releasing factor (CRF), thyrotrophin releasing hormone (TRH), neuropeptide Y, leuteinising hormone releasing hormone (LHRH) and somatostatin. MCH neurones in this region also contain irαMSH although, as in the carp, this does not necessarily indicate the presence of true αMSH (see section 1.3.). A second group of MCH-containing cell bodies is observed in the dorsal infundibular nucleus which do not seem to react with αMSH antiserum (Andersen *et al.*, 1987). Nerve fibres extend to the PN but there are no projections into the PI nor into the PD. (Andersen *et al.*, 1986). In *Rana temporaria* and *Xenopus laevis*, neuronal MCH cell bodies are only found in the postero-lateral hypothalamus near the dorsal and ventral infundibular nuclei (Batten *at al.*, 1986).

1.2.3. Localization in rats

In the rat, an extensive system of MCH-immunoreactive neuronal cell bodies is located in the dorsolateral region of the hypothalamus, extending along the tract of the median forebrain bundle from the region of the paraventricular nucleus to the rostral tip of the submammillary body (Naito, *et al.*, 1985; Skofitsch *et al.*, 1985). Nearly all the cell bodies near the zona incerta co-localize with irαMSH, but no co-localization can be demonstrated with other POMC fragments (Naito *et al.*, 1986; Pelletier *et al.*, 1987). Fellman and others (1987) observed that irMCH cell bodies in the rat dorsal hypothalamus co-localize with immunoreactive growth hormone releasing factor (irGHRF) (see section 1.3.). Fibres extend to many

regions of the forebrain, midbrain, hindbrain and dorsal horn of the spinal cord; a few fibres are seen also in the median eminence and the PN. The distribution of MCH has been determined by RIA and the observations agree with those of the immunocytochemical studies (Zamir *et al.*, 1986). Sekiya *et al.*, 1988 have also detected irMCH in the brains of the guinea-pig and pig.

1.2.4. Localization in humans

Cell bodies staining for MCH are seen in the periventricular area and there is no apparent co-localization with αMSH (Pelletier *et al.*, 1987; Sekiya *et al.*, 1988).

1.2.5. Localization in insects

An irMCH has been observed in invertebrates such as the locust (*Locusta migratoria*) and the grey fleshfly (*Sarcophaga bullata*). In the locust, Schoofs *et al.*, (1988) found irMCH cell bodies in the optic lobes and some in the pars intercerebralis. Fibres were present in the optic lobes and in the proto- and deutero-cerebrum as well as the corpora cardiaca. The perikarya in the optic lobes also stain with αMSH antisera as in vertebrates. In the grey fleshfly, the same authors have observed irMCH cell bodies in the pars lateralis, some of which contain irαMSH.

1.3. CHARACTERISATION OF MCH AND ITS PRECURSOR

In 1983 MCH was characterised by Kawauchi and colleagues in the salmon and shown to be a cyclic heptadecapeptide closed by a disulphide bridge between residues 5 and 14. It has no similarity to any other known molecule except for a slight similarity to the C-terminal fragment of salmon prolactin (sPRL) which shows weak MCH-like activity (0.04%). The molecular structure of MCH has been determined for other teleost species.

The Bonito (*Katsuwonus pelamis*) has an MCH identical to salmonid MCH; the Japanese eel (*Anguilla japonica*) has a similar molecule with just one substitution of asparagine instead of aspartic acid at the N-terminal, suggesting that MCH is highly conserved in teleosts (Kawauchi, 1989). Recently Vaughan and colleagues have sequenced the mammalian peptide from the rat hypothalamus. Rat MCH (rMCH) differs from the salmon peptide (sMCH) by a two-amino acid N-terminal extension and four additional substitutions (fig 1.1.), Rat and human MCH are identical (Vaughan *et al.*, 1989; Nahon *et al.*, 1989).

In 1987, Kawazoe and co-workers reported observations which suggested that MCH might be cleaved from a large molecular weight precursor. They found that both MCH-immunoactivity and -bioactivity of hypothalamic extracts increased if the extracts were digested with lysylendopeptidase. Later, the cDNA sequence of a putative precursor molecule was reported for the Chum and Chinook salmon respectively (Ono *et al.*, 1988; Minth *et al.*, 1989). In fact, since salmonids are tetraploid, two MCH cDNA sequences were described, MCH-1 cDNA and MCH-2 cDNA, both consisting of 132 amino acid residues in which MCH was found at the C-terminus. In the Chum salmon sequence, Ono and colleagues reported that MCH was identical for both mRNAs, whereas the precursors showed only 80% homology in the amino acid sequence and 86% homology in the nucleotide sequence. Most differences occur at the N-terminus and involve single base changes. In the Chinook salmon, also, Minth and co-workers reported two MCH cDNA structures, their MCH-2 being very similar to the Chum MCH-1 described by Ono *et al.* with only 4 amino acid substitutions. The Chinook MCH-1 and Chum MCH-2 are even more alike with only 1 substitution. In both species at least one of the cDNA sequences reveals a putative 13 amino acid peptide which could be cleaved from the precursor molecule at the Arg-Arg residues at positions

99 and 100, termed the MCH gene related peptide (Mgrp) (Fig 1.1.). When RNA, isolated from a variety of Chinook salmon tissues, was hybridised with sequence-specific probes designed to distinguish MCH-1 and MCH-2, a positive signal was found only with extracts from hypothalamic tissue. *In situ* hybridization with several probes produced intense signals in the magnocellular neurones of the nucleus lateralis tuberis (NLT) of the hypothalamus, in the same area as immunocytochemical staining was found (Minth *et al.*, 1989). Recently the cDNA sequence of the rat MCH precursor has been reported by Nahon and colleagues (1989). Only one type of MCH-encoding mRNA was found in the rat but these authors suggest that there may be more than one gene present in mammals. The mRNA sequence consists of 165 residues with the 19-residue hormone at the C-terminus. Rat MCH and Salmon MCH precursors show only 28% homology (54% nucleotide similarities) as a whole but that the degree of homology varies at different regions of the precursor. The signal peptides and central sections of the molecules have diverged extensively showing only 17% homology while the C-terminus shows significant sequence conservation (42% amino acid homology). In the rat precursor there appear to be two putative peptides which could also be cleaved from the molecule, termed NGE (neuropeptide-glycine-glutamic acid) and NEI (neuropeptide-glutamic acid-isoleucine) between residues 110-128 and 132-144 respectively (see Fig 1.1.). These peptides share some sequence homology with αMSH, CRF and GRF and could explain some of the observations reported, using immunocytochemical methods, of co-localization of these peptides with MCH (see section 1.2.). Since such apparent co-localization is not observed in all MCH cell types it would appear that MCH precursor processing varies with cell type. The region termed NEI in the rat is equivalent to the

Fig 1.1. cDNA sequence of salmon and rat prepro-MCH and Aplysia prepro-Peptide A from Nahon *et al.*, 1989. ● repesents conservative substitutions

```
sMCH        ....... Met Arg Asp Ser Val Leu Ser Val Ile Phe Ala Leu Ala Leu Phe Leu Glu Cys
rMCH        Ile Leu Leu Ser Ala Ser Lys Ser Ile Arg Asn Val Glu Asp Asp Ile Val Phe Asn Thr
Peptide A   Cys Val Ser Ser Gln Ser Thr Ser Val His ............... Gly Lys Ile Phe Val Pro


sMCH        Tyr Thr Pro Ser Met ................................ Ala Ile Pro Met Gly Lys
rMCH        Phe Arg Met Gly Lys ................................ Ala Phe Gln Lys Glu Asp
Peptide A   Asn Arg Ala Val Lys Leu Ser Ser Asp Gly Asn Tyr Pro Phe Asp Leu Ser Lys Glu Asp


sMCH        Met Glu Asp Thr Ala Leu Glu Gln Asp Thr Leu Asp Ser Leu ........... Leu Asn Glu
rMCH        Thr Ala Glu Arg Ser Val Val Ala Pro Ser Leu Glu Gly Tyr ........... Lys Asn Asp
Peptide A   Gly Ala Gln Pro Tyr Phe Met Thr Pro Arg Leu Arg Phe Tyr Pro Ile Gly Lys Arg Ala


sMCH        Glu Val Ala ............... Asp Lys Asn Pro Asp Ser Val Arg Ser Gly Ser .......
rMCH        Glu Ser Gly Phe Met Lys Asp Asp Asp Asp Lys Thr Thr Lys Asn Thr Gly Ser .......
Peptide A   Ala Gly Glu Met Glu Gln Ser Glu Gly Gln Asn Pro Glu Thr Lys Ser His Ser Trp Arg


sMCH        Ser Lys Ile Ile Val Leu Ala Asp Ser Gly ........... Met Trp Lys Asn Leu Asn Arg
rMCH        Lys Gln Asn Leu Val Thr His Gly Leu Pro ........... Leu Ser Leu Ala Val Lys Pro
Peptide A   Lys Arg Ser Val Leu Thr Pro Ser Leu Ser Ser Leu Gly Glu Ser Leu Glu Ser Gly Ile


sMCH        Gly Leu Pro Leu ........... Tyr Lys Leu Lys Ala Ala Ala Ala Gly Leu Asp Arg Ala
                                                      └────────────── NGE ──────────────
rMCH        Tyr Leu Ala Leu Lys Gly Pro Ala Val Phe Pro Ala Glu Asn Gly Val Gln Asn Thr Glu
Peptide A   Ser Lys Arg Ile Ser Ile Asn Gln Asp Leu Lys Ala Ile Thr Asp Met Leu Leu Thr Glu
hGRF(30-37) ... ... ... ... ... ... ... ... ... ... ... ... ... ... Gln Asn Gly Glu
hCRF(24-41) ... ... ... ... ... ... ... ... ... ... ... Ala Glu Gln Gly Val Gln Gln Ala Met
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                                    ┌────────────── Mgrp ──────────────┐
sMCH        Leu Thr Leu Asp Arg Arg Glu ... Ala Asp Gln Asp Leu Ser Pro Ser Ile Ser Ile Val
            └──────────────────────────────┘                     └────── NEI ──────────────
rMCH        Ser Thr Gln Glu Lys Arg Glu Ile Gly Asp Glu Glu Asn Ser Ala Lys Phe Pro Ile Gly
Peptide A   Gln Ile Gln Ala Arg Arg Arg Cys Leu Asp Ala Leu Arg Gln Arg Leu Leu Asp Leu Gly
hGRF(30-37) Ser Asn Gln Glu
hCRF(24-41) Ser Asn Arg Lys Lys Met Ile Ile Ile
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                                       ┌────────────── MCH ──────────────
sMCH        Arg Arg ....... Asp ....... Thr Met Arg Cys Met Val Gly Arg Val Tyr Arg Pro Cys
                                                       └────── MCH ──────────────
rMCH        Arg Arg Asp Phe Asp ....... Met Leu Arg Cys Met Leu Gly Arg Val Tyr Arg Pro Cys
Aplysia PepA Lys Arg Asp Ser Asp Val Ser Leu Phe Asn Gly Asp Leu Leu Pro Asn Gly Arg Pro Gln
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            ┌───────┐
sMCH        Trp Glu Val

rMCH        Trp Gln Val *...... Tyr Leu Leu Val
Peptide A   Leu Lys Thr Ile Ser Asn Leu Leu Asp *
```

region termed Mgrp in the salmon. These putative peptides display only 30%

homologies. Nahon *et al.*, (1989) have proposed that it is the C-terminus

of NEI fragment which is amidated and responsible for the cross-reaction

with αMSH antisera. Based on the results of immunocytochemistry, NEI (or

Mgrp) are probably amidated in the rat, amphibia, carp, dogfish and

insects but not in the eel, trout or human.

1.4. ACTION OF MCH ON MELANOPHORES

MCH can be demonstrated to concentrate the melanophores of all teleost

species so far studied *in vitro*. Normally this effect is seen within the

range 10^{-9} to 10^{-12} M although the sensitivity of different species

varies, the chinese grass carp being extremely sensitive (EC_{50} 6×10^{-11} M)

but it is surpassed only by the eel *Synbranchus* at 10^{-11} M. Structure-

activity studies by various groups (Baker *et al.*, 1985; Hadley *et al.*, 1988;

Kawazoe *et al.*, 1987) demonstrate that the molecule has to retain its

disulphide bridge for it to remain highly bioactive. The Tyr^{11} as well as

the three $Arg^{(4,9,12)}$ residues are also required for full biological

activity to be retained. If the Trp^{15} is modified or the Met^3 and Met^4 are

oxidised, bioactivity is impaired to a much smaller degree (Kawazoe *et al.*,

1987). Recent work, however, by Matsunaga *et al.*, (1989) using *Synbranchus*

melanophores suggest that the Trp^{15} residue is the crucial one for

retaining full bioactivity and they claim that the fragment MCH_{5-15} is the

minimal fragment analogue with full potency. The same workers also claim

that the MCH_{5-14} was the minimal ring structure to retain full intrinsic

activity although it had only 0.1% potency. Contracted ring fragments

$[Cys^{10}]MCH_{10-17}$ and $[Cys^8]MCH_{8-17}$ retained weak but also full agonist

activity. This is contradictory to the results of some other workers (e.g.

Kawazoe *et al* 1987) but may be due to species sensitivity differences.

MCH is capable of antagonising the action of αMSH on melanophores *in vitro* but its ability to do so varies in different species (Baker 1988b). Of the three species studied, the chinese grass carp, the rainbow trout and the eel, the carp showed a steeper dose response curve to αMSH than MCH, but in the trout the opposite was true. In the eel, both curves were shallow, so the hormone secretion strategy required in order to achieve certain colour changes must vary with the species involved.

MCH also has a pigment-concentrating effect on other types of chromatophores which normally respond to αMSH with pigment dispersion, such as xanthophores (medaka) and erythrophores (platyfish, swordtail) (Fujii and Oshima, 1986). Leucophores, however, respond only with dispersion to either hormone, the high concentrations of MCH required to elicit a response indicating that it may be having its effect via the αMSH receptors (Oshima *et al.*, 1986).

The mode of action of MCH on melanophores involves interaction with its own specific receptors. In contrast to stimulation by αMSH , stimulation of these receptors is independent of external calcium (Oshima *et al.*, 1986). MCH does not act with the α- or β-adrenoreceptors, since phentolamine and propranolol have no antagonistic effect (Rance and Baker, 1979). There is some evidence that forskolin antagonises the response of melanophores to MCH (Baker, 1988a), αMSH causes melanin dispersion by stimulating cAMP production. However, recent work with *Synbranchus marmoratus* suggests that MCH is using diacylglycerol (DAG) as its intracellular second messenger (Abrão *et al.*, 1989). It is intriguing to note that the response of melanophores to MCH is seen more rapidly than the response to αMSH, suggesting that the second messenger system of MCH may be initiated more rapidly that of αMSH (Kawauchi *et al.*, 1983).

At pharmacological doses it seems that MCH may stimulate the αMSH-receptor on the fish melanophore, which has been termed auto-antagonism (Hadley et al., 1988). In tetrapods MCH has no concentrating effect but at high doses MCH will apparently bind non-specifically to the αMSH-receptor in amphibian and reptile melanophores causing melanin dispersion (Baker, Eberle et al., 1985; Wilkes et al., 1984; Oshima et al., 1986; Hadley et al 1988). The same may be said for the MCH-stimulated tyrosinase activity reported in B16 mouse melanoma cells (Baker et al., 1985)

1.5. EFFECT OF MCH ON THE PITUITARY

MCH acts not only at the melanophore level but also at the pituitary level. Baker et al (1986) implanted trout with alzet minipumps containing MCH which were then kept on a black background. The implanted fish in black tanks became pale, their skin melanin content failed to increase, and their plasma αMSH concentration was low compared with black-adapted controls, suggesting a reduction of αMSH release. The reduced skin melanin content may not be due to a direct action of MCH on melanogenesis or of melanin degradation but could be an indirect result of the depression of αMSH secretion to levels found in white-adapted fish. The inhibitory effect of MCH on αMSH release was confirmed by Barber et al. (1987), who showed that after the addition of MCH antiserum to incubation medium, pituitaries were able to release more αMSH than those incubated without anti-MCH.

The hormone has other effects on the pituitary apart from its action on αMSH release. It seems to be involved in the release of another POMC peptide, namely bioactive ACTH. In 1981 Baker and Rance observed that the plasma cortisol levels of white adapted trout were lower than those of black adapted fish. Further experimentation showed that although basal cortisol titres were the same in fish on black or white backgrounds, black-

At pharmacological doses it seems that MCH may stimulate the αMSH-receptor on the fish melanophore, which has been termed auto-antagonism (Hadley *et al.*, 1988). In tetrapods MCH has no concentrating effect but at high doses MCH will apparently bind non-specifically to the αMSH-receptor in amphibian and reptile melanophores causing melanin dispersion (Baker, Eberle *et al.*, 1985; Wilkes *et al.*, 1984; Oshima *et al.*, 1986; Hadley *et al* 1988). The same may be said for the MCH-stimulated tyrosinase activity reported in B16 mouse melanoma cells (Baker, Eberle *et al.*, 1985)

1.5. EFFECT OF MCH ON THE PITUITARY

MCH acts not only at the melanophore level but also at the pituitary level. Baker *et al* (1986) implanted trout with alzet minipumps containing MCH which were then kept on a black background. The implanted fish in black tanks became pale, their skin melanin content failed to increase, and their plasma αMSH concentration was low compared with black-adapted controls, suggesting a reduction of αMSH release. The reduced skin melanin content may not be due to a direct action of MCH on melanogenesis or of melanin degradation but could be an indirect result of the depression of αMSH secretion to levels found in white-adapted fish. The inhibitory effect of MCH on αMSH release was confirmed by Barber *et al.* (1987), who showed that after the addition of MCH antiserum to incubation medium, pituitaries were able to release more αMSH than those incubated without anti-MCH.

The hormone has other effects on the pituitary apart from its action on αMSH release. It seems to be involved in the release of another POMC peptide, namely bioactive ACTH. In 1981 Baker and Rance observed that the plasma cortisol levels of white adapted trout were lower than those of black adapted fish. Further experimentation showed that although basal cortisol titres were the same in fish on black or white backgrounds, black-

adapted fish have higher plasma cortisol levels under conditions of moderate stress. Moreover, cortisol titres were less readily blocked by dexamethasone in stressed, black-adapted fish than those of white adapted fish (Gilham and Baker, 1985). On the other hand, Pickering et al. (1986) were unable to repeat these observations. This may reflect a difference in the levels of stress used in the two laboratories. Further investigations by Baker et al. (1985) revealed that the the spontaneous in vitro release of ACTH from pituitaries taken from black-adapted fish was higher that that from white-adapted ones. The question then arose: was it MCH that was inhibiting the release of ACTH in the white-adapted fish, thus causing the difference in ACTH secretion? If the pituitaries were incubated in vitro with different concentrations of MCH, it could be shown to inhibit ACTH release in a dose-dependent manner (Baker et al., 1985) in the range 10^{-10} to 10^{-7} M. MCH was also shown to inhibit CRF-stimulated ACTH release from PDs in vitro in the concentration range 10^{-10} to 10^{-11} M. Similar effects were seen with rat pituitaries although the concentration of MCH required was very much higher (10^{-8} M), possibly because rat MCH receptors may not recognise salmonid MCH very well.

Kawauchi et al., (1986), have shown that intravenous injections of natural or synthetic salmonid MCH (440µg/kg) will stimulate the release of growth hormone in 10 week old male Wistar rats. The response became apparent after one hour and persisted for several hours, thus acting via a different mechanism to GHRF which has a very rapid effect. There is practically no evidence for the effect of MCH on the CNS in mammals because very little work has been done in this area. One relevant observation by Zamir et al. (1986) showed that the MCH concentration in the lateral hypothalamus is increased in rats given 2% saline as drinking water over 5 days. These workers point out that lesions in the areas known

to be rich in MCH cell bodies have been associated with disrupted eating and drinking behaviour, but direct evidence of a role for MCH in this activity has not been provided. De Graan *et al.*, (see Eberle, 1988) reported that MCH will partially antagonise the grooming response to αMSH in rats, if injected icv. Since the MCH fibres extend to many regions of the brain it may have a neuromodulatory role in many different functions as yet undetermined.

CHAPTER 2

MATERIALS AND METHODS

This chapter deals with materials and methods which are common to all chapters. Treatments applicable to specific sections of the work are described under the appropriate chapter.

2.1. FISH

Fingerlings (about 6 months, 10-50g) and adult (1+) trout (150-250g) were obtained from Alderley Trout Farm, Wotton-Under-Edge, Gloucestershire. Some trout were also obtained from Rainbow Trout Farm, Longbridge Deverill, Warminster, Wiltshire. At the Alderley site fish were kept outdoors in large tanks at a stocking density of 1.5 kg fish/m^3. At Warminster, fish were kept indoors under a variable photoperiod of dim artificial light which was approximately 9h L: 15h D. They were kept in tanks of 5 m^3 containing 200 kg fish ($\equiv$ 40 kg fish/m^3). These fish were consistently darker than the trout from Alderley.

2.2. UNIVERSITY AQUARIUM CONDITIONS

Fish were maintained in black or white 250l fibreglass tanks in continuous flowing tap water, (100ml/min), at 11° under a photoperiod of 16h L : 8h D unless otherwise stated. All fish were adapted to aquarium conditions for at least a week before use. Adult fish were not fed, but fingerlings were maintained on a diet of commercial trout pellets at the manufacturers recommended rate.

2.3. COLLECTION OF BLOOD AND STORAGE OF PLASMA

Fish were anaesthetised in phenoxyethanol (diluted 1 : 1667 v/v) or benzocaine (diluted 1 : 1200 w/v, see Chapter 3) or stunned with a blow to the head before bleeding. They were bled from the caudal vein into ice cold 4 ml polypropylene tubes containing 50μl 6% EDTA (w/v) and 2500 KIU Aprotinin as inhibitor. The blood samples were then centrifuged at 2000×g for 15 min, and the plasma was stored at -20° in the appropriate volumes according to type of RIA.

2.4. PLASMA EXTRACTIONS

Plasma NPP-1 measurements did not require any extraction of the plasma prior to assay. MCH concentrations in the plasma were determined using the solid phase method which does not require extraction steps.

2.4.1. αMSH

Measurements did require extraction of plasma samples before RIA. The method used was that of Wilson and Morgan (1979). Briefly, the procedure was as follows:

(a)   Plasma samples of 500 μl were acidified with 200 μl 0.4 M HCl before the addition of 1 ml distilled water containing 50 mg Florisil (100 mesh, Sigma).

(b)   Tubes were tumble-mixed on a stuart rotator (30 rpm, 4°, 30 min)

(c)   Tubes were centrifuged (1500×g, 4°, 15 min) and the supernatant discarded.

(d)   Pellet was resuspended and washed twice with 2 ml 0.4 M HCl and the supernatant discarded each time.

(e)   The pellet was washed twice with 2 ml distilled water and the supernatant discarded.

(f)   the pellet was resuspended in 1 ml of the elution solvent

        prepared as follows: 100ml EtOH

                  150ml Ammonia 88

                  47.8mg EDTA

                  5.86ml Mercaptoethanol

        The elution solvent was made up fresh for each extraction.

        the tubes were tumble mixed for 30 min at 4° and 30 rpm.

(g)   The tubes were centrifuged (3000×g, 4°, 15 min) and the

        supernatant transferred to fresh tubes containing 20 µg polypep

        (Sigma).

(h)   Tube contents were dried under vacuum overnight.

(i)   Dried samples were resuspended in 250 µl 0.05 M phosphate buffer,

        pH 7.5 containing 0.8 % NaCl and 0.1 % gelatine (PBSG) prior to

        assay and left to stand at 4° for 30 min before use in RIA, using

        100µl per assay tube.

2.4.2. Cortisol

Measurement in plasma required extraction in ethanol to precipitate the

carrier proteins and liberate the steroid before assay.

(a)   5 -10 µl of plasma was used per tube in duplicate

(b)   250 µl EtOH was added to each tube which was then vortexed.

(c)   Repeat (b)

(d)   Tube contents were dried under vacuum.

(e)   Contents were resuspended in 100 µl PBSG for RIA.

2.5. RADIOIMMUNOASSAYS

Since most of the data reported in this thesis involve measurement by

radioimmunoassay, a brief consideration of the technique and its

limitations is appropriate. The use of saturation analysis techniques,

which include radioimmunoassay (RIA), protein binding assays and

radioreceptor assays has had a marked impact on endocrinology over the last 20 years. The method allows the specific measurement of biologically active compounds even when present in extremely low concentrations. Saturation analysis techniques, though far more sensitive than the bioassays which preceeded their development, possess the major disadvantage that they measure immunoactivity, not bioactivity. This becomes important in the presence of abundant biologically inactive hormone fragments (both precursor and breakdown products) in the plasma and tissue extracts. By measuring biologically inert fragments, RIA may give misleading results. Therefore, the epitope against which the antibody is directed is of prime importance.

The general principle of RIA is that an unknown concentration of the substance under investigation (the sample) is mixed with a known concentration of the synthetic compound. The synthetic compound is distinguishable from the natural compound by labelling with a radioactive isotope. This may be achieved by addition to or incorporation into the molecule. A fixed amount of antibody is added to this mixture. The amount of antibody added should be sufficient to bind to only a fraction of the substances in the assay. It is very important that the antibody recognises both the natural substance and the labelled synthetic compound equally well. The mixture is then left so that the molecules can compete equally for the antibody binding sites. Once this is done the molecules that have bound to the antibody are separated from the unbound molecules. The concentration of the radioactively labelled compound in either antibody bound or free fraction can be measured by counting. If there is a high concentration of the natural substance in the sample there will be proportionally less radioactively labelled compound bound to the antibody. This can be quantified by setting up a standard curve. A series of

increasing concentrations of unlabelled compound are allowed to compete for antibody sites with radioactively labelled synthetic compound. In this way, since all the concentrations are known, the decrease in binding of the radioactive substance can be plotted. If it is assumed that the natural substance behaves in the same way as the synthetic non-radioactive compound the concentration of the sample can be read off the standard curve.

The antibody is characterised by its titre and its specificity. The titre of an antibody is the amount of antibody that will bind to 50% of the tracer, (the labelled compound). In effect, the titre of the antibody is the dilution at which it is used in the assay tube. The specificity of the antibody is determined by displacement studies, where its cross reaction with other substances is measured. The less crossreactivity there is between the antibody and other substances, the higher the specificity the antibody is said to have, and the more valuable it is for use in RIA.

The validity of the RIA depends on the assumption that the standard and the sample compound are identical and that they compete equally for the binding sites on the antibody. To test this assumption a standard curve is compared to a the dilution curve dilution of the test substance. If the two curves are parallel, the assay is valid.

In choosing an isotope for the labelled compound, the suitable choice should comply with several requirements :

1.   The concentration of label should be easily detected by a suitable counting system.

2.   The half-life of the label should not be so short that it reduces
the storage time of the labelled material to an uneconomical and
unmanageable time.

3.   The labelled product should not be damaged in the radiolabelling
procedure such that it is unable to compete adequately with the
sample for antibody binding sites.

After equilibration the bound and unbound fractions must be separated.
Several different separation methods are available. A cheap and easy
method is that using dextran-coated charcoal which selectively binds the
free fraction, ie those molecules not attached to the antibody. The
dextran component reduces the overall avidity of the charcoal. This
method does not work well if the assay buffer contains the detergent
Triton X-100. An alternative method of separation involves the selective
precipitation of the bound fraction which may be achieved by several
different means. If the conditions in the incubation mixture are changed
such that a large immunoglobulin (the antibody) will be likely to
precipitate out, the bound fraction will be effectively isolated. One
substance used in the present work which will induce the precipitation of
the antibody complex is Polyethylene Glycol (PEG). Other substances that
in the right conditions will cause the precipitation of the bound fraction
are ethanol, dioxan, sodium sulphate, ammonium sulphate and
trichloroacetic acid. The most widely used separation method involves the
use of a second antibody which selectively binds to the first antibody
and causes precipitation of the antigen-antibody complex. This method
involves a second incubation step and is expensive although it is highly
sensitive. A fourth method is to bind the antibody beforehand to an
insoluble matrix (eg Sepharose, cellulose). Assays using this technique
are known as Solid Phase RIA. In this method no steps are required to

separate the bound and unbound fractions apart from centrifugation and decantation. A further advantage is that the antigen may be measured directly in the plasma without extraction steps. The solid phase antibody effectively extracts the substance from the plasma which is then removed by washing. The label is added after this washing step and binds to the unoccupied sites of the immunobeads.

2.5.1. Preparation and storage of standards

2.5.1.1. MCH was obtained from Peninsula Laboratories (Richmond, Calif.) and a 1×10^{-5} M solution prepared in 0.1 M HCl with 1% BSA and stored at -40°. This solution was then diluted $\times 100$ fold with 0.05 M phosphate buffered saline (PBSG) containing 1 % BSA (pH 7.4) to give a 10^{-7} M solution. A second dilution step produced the final concentration of 4 ng/ml which was then aliquotted out in 500 μl volumes and stored at -20°. The diluent solution (10^{-7} M) was also frozen and new batches of standards prepared as required. This procedure gave a high degree of reproducibility in standard preparation.

2.5.1.2. αMSH was obtained from Sigma and a 10^{-5} M concentrate prepared, diluted and stored as in (1.) above.

2.5.1.3. NPP-1 Purified salmonid NPP-1 was a gift from Prof H Kawauchi. It was diluted in assay buffer A (0.05 M PBS, 1% BSA, 0.1% Triton X-100, 0.1% NaN₃, pH 7.3) in 500 μl aliquots in the concentration 4 ng/ 100 μl. Standards were stored at -20°.

2.5.1.4. Cortisol was obtained from Sigma and a concentrated stock of 12 ng/ 10 μl ethanol was prepared. This was diluted $\times 7.5$ to give the highest standard of 1600 pg/ 10 μl which was in turn serially diluted to give the range 12.5 - 1600 pg/ 10μl. Standards were stored at -20°.

2.5.2. Iodination of peptides for RIA

Two different methods were employed. That used in the preparation of labelled MCH and αMSH, involves the oxidation of tyrosine residues in the peptide by reacting it with Chloramine-T for a short time thereby attaching ^{125}I to the molecule by an oxidation reaction. If the reaction is allowed to proceed for too long, the peptide may be damaged by oxidation and will not compete effectively with unlabelled hormone for the binding sites on the antibody; the assay efficiency will then be affected. The method is described in the Appendix.

The iodination method used for the iodination of NPP-1 (N-terminal peptide of pro-opiomelanocortin) was that of Takahashi *et al.* (1987) using a slightly modified version of the lactoperoxidase method of Thorell and Johansson (1971). Relevant details are also given in the Appendix.

2.5.3. RIA involving charcoal separation of free/bound antigen

This RIA method was used to measure αMSH in plasma extracts as well as MCH in tissue extracts and incubation media. The buffer used throughout was 0.05 M phosphate buffered saline with 0.1% gelatine (PBSG). All solutions and samples were kept on ice during preparation of the RIA.

The typical assay procedure was as follows:

Day 1

1.   Test samples were added to assay tubes (4 ml, polypropylene) in 100 μl volumes, with dilutions made as required with PBSG

2.   The stored standard (400 pg/ 100 μl) was thawed, serially diluted and 100 μl added to the appropriate tubes in the range 3.1 - 400 pg/ 100 μl.

3.   An internal standard was prepared from an acid extracted trout pituitary which was diluted to approximately 50 pg/ 100 μl, stored in aliquots and used as 100 μl/ tube. The intra-assay variations were: (a) MCH 6 %, (b) αMSH 6.9 %; inter-assay variations were (a) MCH 5.5 %, (b) αMSH 10.1 %

4.   The antibodies used in the assay were:

A. Anti-MCH: a gift from Dr A N Eberle, used at a dilution of 1 : 60 000 in assay buffer.

B. Anti-αMSH: the Bath University antibody designated R$_6$FB (raised by Dr T Bowley), used at the same dilution as for anti-MCH.

A 50 μl volume of antibody was added to all tubes except the HOT and non specific binding tubes (NSB). These dilutions of antibody bound about 50 % of the added labelled hormone.

5.   The labelled hormone solution was prepared by diluting the stored concentrated label with PBSG, sufficient to give about 5000 cpm in 50 μl. This volume was then added to all the tubes.

6.   The tube contents were whirlimixed and centrifuged briefly (300$\times$g) to ensure that all droplets were at the bottom of the tubes which were then incubated at 4° for 24h.

TABLE 2.1. Summary of tube contents for Charcoal-separated RIA.

Volume (μl)

Tube Title	Antibody	Label	Buffer	Standard	Sample	Total
HOT	--	50	--	--	--	50
NSB	--	50	150	--	--	200
0%	50	50	100	--	--	200
Standards	50	50	--	100	--	200
Samples	50	50	--	--	100	200

Day 2

7. About half an hour before it was required, the charcoal-dextran mixture was prepared (0.5 % activated charcoal, 0.125 % Dextran-70 in PBSG). This was kept chilled in an ice bath and stirred vigorously. Once prepared, the charcoal mixture remained useable for up to a week when kept at 4°. 500 μl of this mixture was added to each tube (except the HOT tubes). Using an Eppendorf Multi-pipette, the charcoal mixture could be added to 240 tubes in less than 3 min. The tubes were incubated for 15 min exactly, timed after addition of charcoal to the first tube, then centrifuged at 2000×g for a further 15 min. No assay drift was noted when standard curves were placed at the start and end of an assay.

8. After centrifugation the charcoal pellet was aspirated to dryness, and all the tubes were counted on a LKB minigamma counter for 5 min or until a total count of 10 000 was reached.

9. Counts due to non-specific binding were deducted from all values and the standard curve was plotted as % B/ B_0, where B = label bound in presence of unlabelled peptide and B_0 = label bound in absence of unlabelled peptide.

Fig. 2.1.

A typical standard curve for the measurement of MCH in the charcoal-separated RIA.
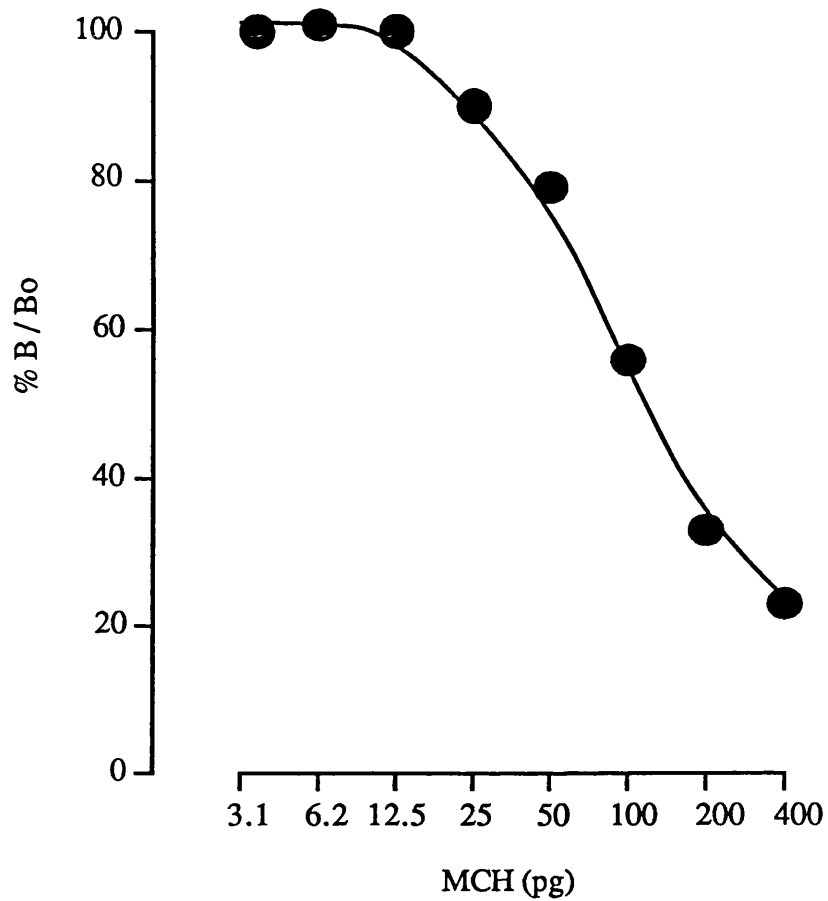
Fig. 2.2.

A typical standard curve for the measurement of MSH in the charcoal-separated RIA.
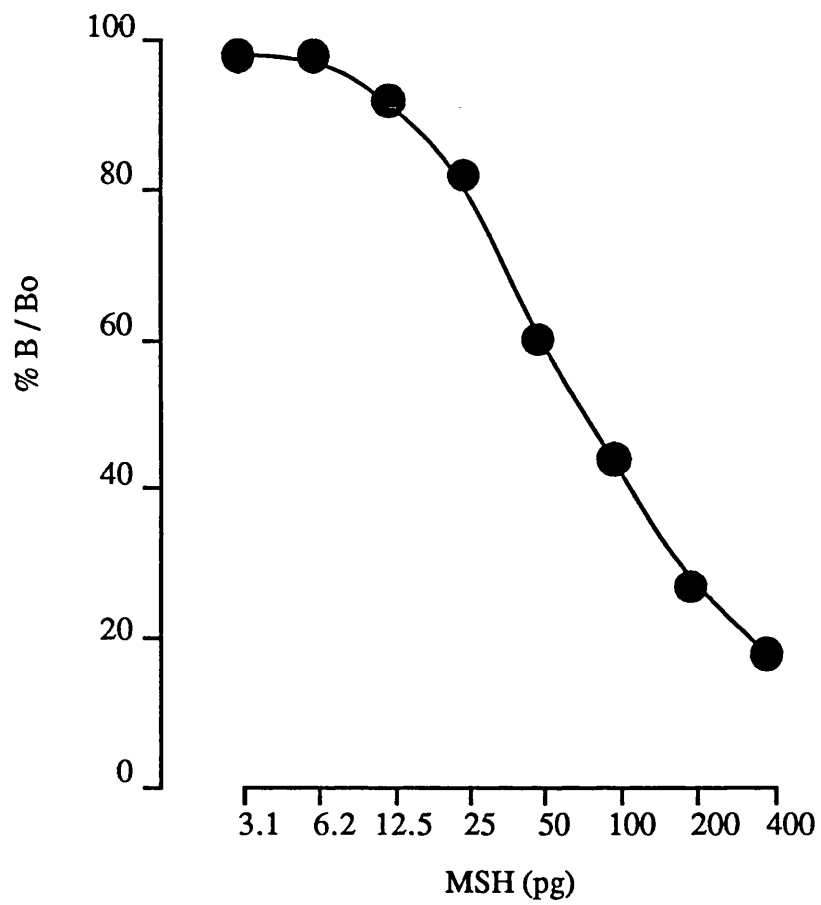
2.5.4. Solid Phase RIA for MCH Protocol

A typical assay procedure follows, (all standards were measured in duplicate and samples singly as large volumes of plasma were required, 500 µl/single sample). The buffer used throughout was "Triton X Buffer": 0.05 M phosphate buffer, pH 7.6, containing 1 % BSA, 0.1 % Triton X-100 and 0.01 % NaN$_3$.

Day 1

1. Standards were prepared from the frozen stock, as described in the section 2.5.1.1. They were serially diluted with assay buffer (Triton-X Buffer) to give the range 3.1 - 400 pg/ 100 µl.

2. The immunobeads were prepared as described previously (Kishida *et al*, 1989) and used at a concentration suitable to give about 30 % maximum binding of label as this gave the best standard curve over the range of standards used. They were diluted in assay buffer just before use.

3. An internal standard was prepared from an acid-extracted trout pituitary which was diluted as required, to give 50 pg/ 100 µl/ tube. Intra-assay variation was 8.7 %, interassay variation was 10.2 %

4. A pool of plasma was aliquotted out in 500 µl volumes and to half of these tubes a "spike" of 50 pg MCH was added. The concentrations of measured hormone were compared in the two pools of plasma, and the difference calculated to show the % recovery of the hormone in the assay. This could be calculated and the results of the assay corrected as appropriate. The recoveries measured in this assay were 113.1 ± 6.8 (n=13).

5. Only when all the solutions were ready were the samples thawed and the appropriate volumes added to each tube (see the summary of tube contents below, table 2.2.)

TABLE 2.2. Summary of tube contents for the first stage of the solid phase assay for MCH in plasma.

Volume (μl)

Tube Title	ImmunoBeads	Standard	Plasma	Buffer	Spike	Total
HOT	--	--	--	--	--	--
NSB	--	--	--	1200	--	1200
0%	100	--	--	1100	--	1200
Standards	100	100	--	1000	--	1200
Unknowns	100	--	500	600	--	1200
Recoveries	100	--	500	500	100	1200
Spike	100	--	--	1000	100	1200

6.   The tubes were whirlimixed and briefly centrifuged (800×g) to ensure that all droplets and beads were mixed and none were stuck to the walls of the tubes. The assay was left to incubate for 24h at 4° with gentle rotation.

Day 2

7.   The tubes were placed into specially adapted centrifuge carriers and centrifuged at 2000×g for 15 min. The supernatants were then decanted and the tubes blotted by inversion onto blotting paper to remove excess liquid. Using the adapted carriers it was possible to decant and blot 37 tubes together which greatly enhanced the reproducibility of the results as well as speeding up the assay.

8.   The bead pellet was washed with 1 ml assay buffer, the tubes were again centrifuged, the supernatant decanted and 1 ml buffer added.

9.   50 μl ^{125}I-labelled MCH, diluted to contain about 5000 cpm, was added to each tube followed by 500 μl assay buffer. Finally, the tube contents were whirlimixed and briefly centrifuged again, then left to incubate for a further 24h with gentle rotation at 4°.

Day 3

10.   The HOT tubes were set aside and 1 ml of assay buffer was added to all the remaining tubes. They were then centrifuged at 2000×g for 15 min, decanted and blotted. The HOT tubes and the rest of the assay tubes were counted for 5 min or until 10 000 counts were reached on an LKB minigamma counter.

11.   The standard curve was expressed as bound labelled MCH ($\% B/B_o$) vs log standard, after subtracting the non-specific binding.

Fig. 2.3.

A typical standard curve for the measurement of MCH in the solid-phase RIA.
The open symbols show a dilution curve for plasma.
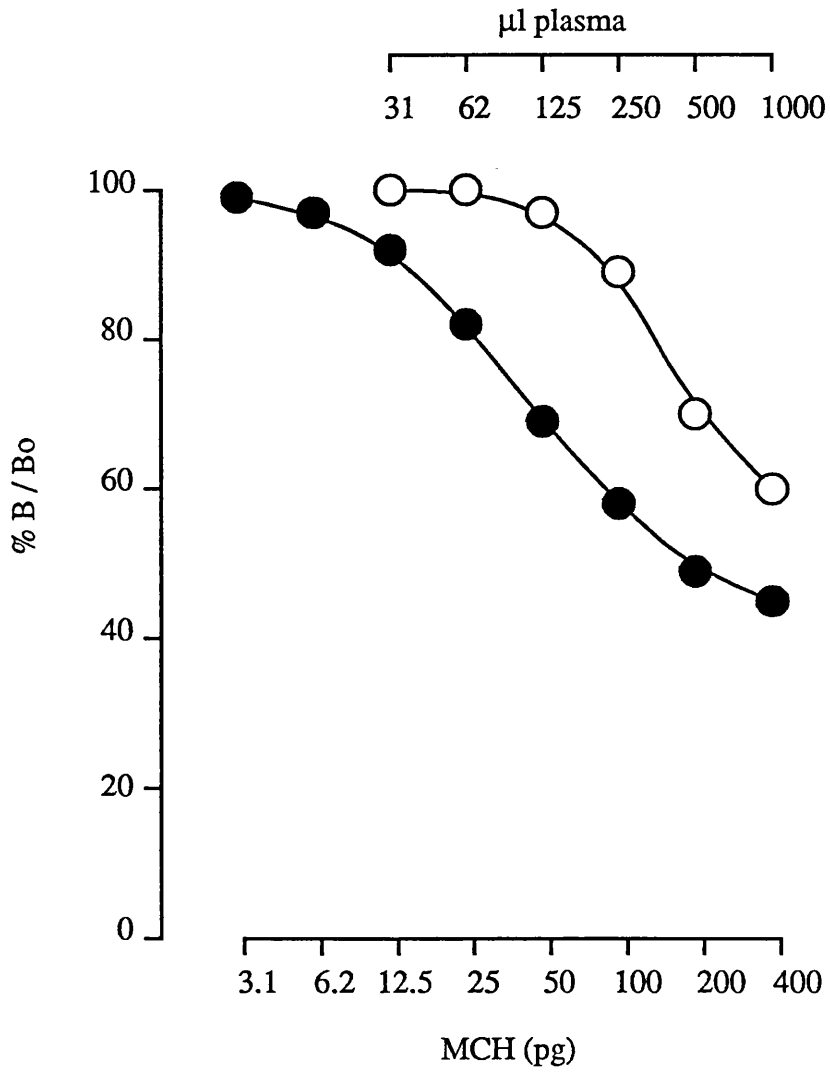
2.5.5. Radioimmunoassay for NPP-1

The method used was broadly that of Takahashi *et al.* (1987) summarised as follows:

Day 1

1. Incubation media was measured without extraction, diluted in 300 μl of assay buffer A (see section 2.5.1.3.).

2. The standards (purified NPP-1, a gift from Prof. Kawauchi) were diluted in the range 15.6 - 4000 pg in buffer and 100 μl added per tube. The tube contents were made up to 300 μl with buffer A.

3. The antibody (from Prof. Kawauchi) was used at a final dilution of 1:10 000 and was prepared in a separate buffer (Buffer B, 0.05 M phosphate buffed saline with 0.01 M EDTA, 0.1 % NaN_3); 100 μl antibody solution was added was per tube. The assay was left to incubate for 24h.

Day 2

4. Label was added to each tube (100 μl per tube giving approx 6000 cpm), and then left for a further 24h

Day 3

5. Bound and unbound fractions were separated with 500 μl 20 % PEG 8000 (in distilled water). The tubes were mixed and then 200 μl 50 % Human serum (in distilled water) was added. The tubes were then mixed again and then centrifuged at 2000×g for 15 min. The supernatant was decanted and the pellets counted on an LKB minigamma counter. Intra-assay variation was 8.1 %, inter-assay variation was 7.2 %, recovery 88 ± 5.5 (n=6).

6. The standard curve was expressed as bound labelled NPP-1 (% B/ B_o) vs log standard, after subtracting the non-specific binding.

Fig 2.4.

A typical standard curve for the measurement of NPP-1 in the PEG-separated RIA.
The open symbols show a dilution curve for incubation medium.
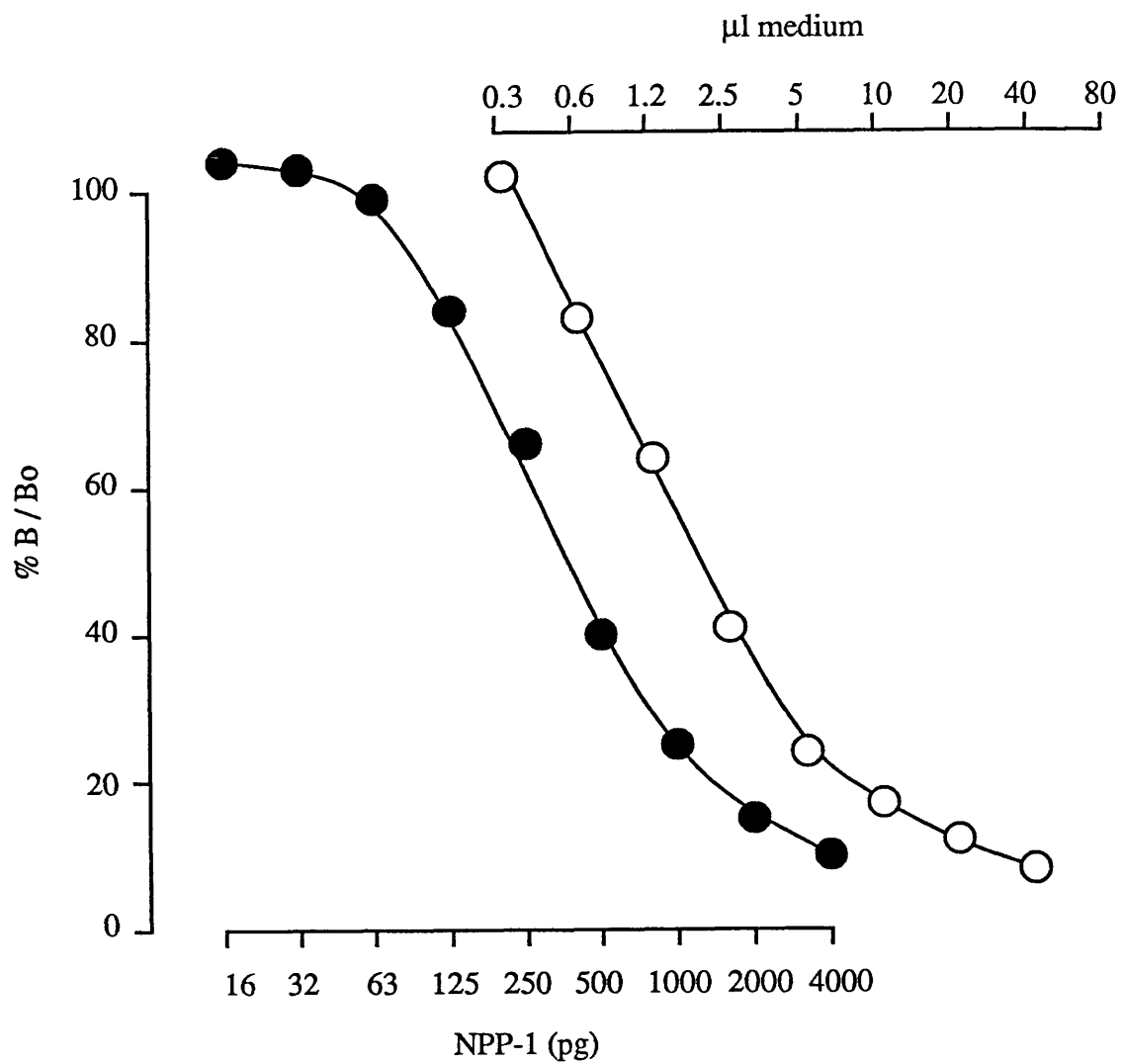
2.5.6. Cortisol RIA Protocol

The method used is that of Rance and Baker (1981) and a brief account of the method is given below:

Day 1

1. Cortisol titres were measured in plasma samples (in duplicate) after extraction in ethanol See section 2.4.2. Recoveries were calculated by measuring aliquots of pooled plasma cortisol, and aliquots to which 200 pg of steroid had been added and assessing the difference. The results could be corrected by the appropriate factor. Recoveries averaged at 92.8 ± 4.6 % (n=5).

2. The standards were prepared as described in section 2.6.1. and measured into the bottom of the tubes (in triplicate) and dried in the vacuum rotary drier. Once all traces of alcohol had been removed, 100 μl assay buffer (PBSG) was added to all tubes and they were left to stand for 30 min at 4°

3. ^{3}H-[1,2,6,7]-Cortisol (Amersham, 250 μCi in 250 μl toluene/ ethanol solution) was diluted to 25 ml with ethanol and stored at -40°. 100 μl of this stock were dried down in a small glass beaker and resuspended in 10 ml of PBSG.

4. The antibody (Cortisol R5) was raised by Dr I Gilham. It was stored at -40° as a 10-fold dilution in PBSG, 50 μl of the stock diluent were added to 10 ml PBSG and allowed to mix for a few minutes.

5. 100 μl of ^{3}H-cortisol solution and 100 μl antibody solution were added to standard and sample tubes. In addition, two tubes contained 100 μl buffer instead of antiserum to determine non-specific binding (NSB). Aliquots of ^{3}H-cortisol solution were added to 6 ml scintillant as a measure of label added to all tubes. The tubes were left to equilibrate at 4° overnight.

Day 2

6.   Half an hour before it was required, the dextran-charcoal was made up according to the same recipe used in the peptide assay (see section 2.2.2.). 500 μl was dispensed to each tube which were left to incubate for 15 min.

7.   The tubes were centrifuged at 2000×g for 15 min and the supernatants decanted into scintillation vials containing 6 ml of scintillant.

8.   The vials were shaken vigorously and left to stand in the dark for 2h to reduce background resulting from "unsettled" scintillant, before counting.

9.   The vials were counted for 10 min or 10 000 counts on a LKB Rack-Beta scintillation counter. The results were converted by computer into dpm using the appropriate quench correction curve.

2.6. STATISTICS

All statistical comparisons of means were made using the pooled students t-test, the appropriate transformations (square root, logarithmic) were performed on the data before testing.

Fig 2.5.

A typical standard curve for the measurement of Cortisol in the charcoal-separated RIA. The open symbols show a dilution curve for plasma.
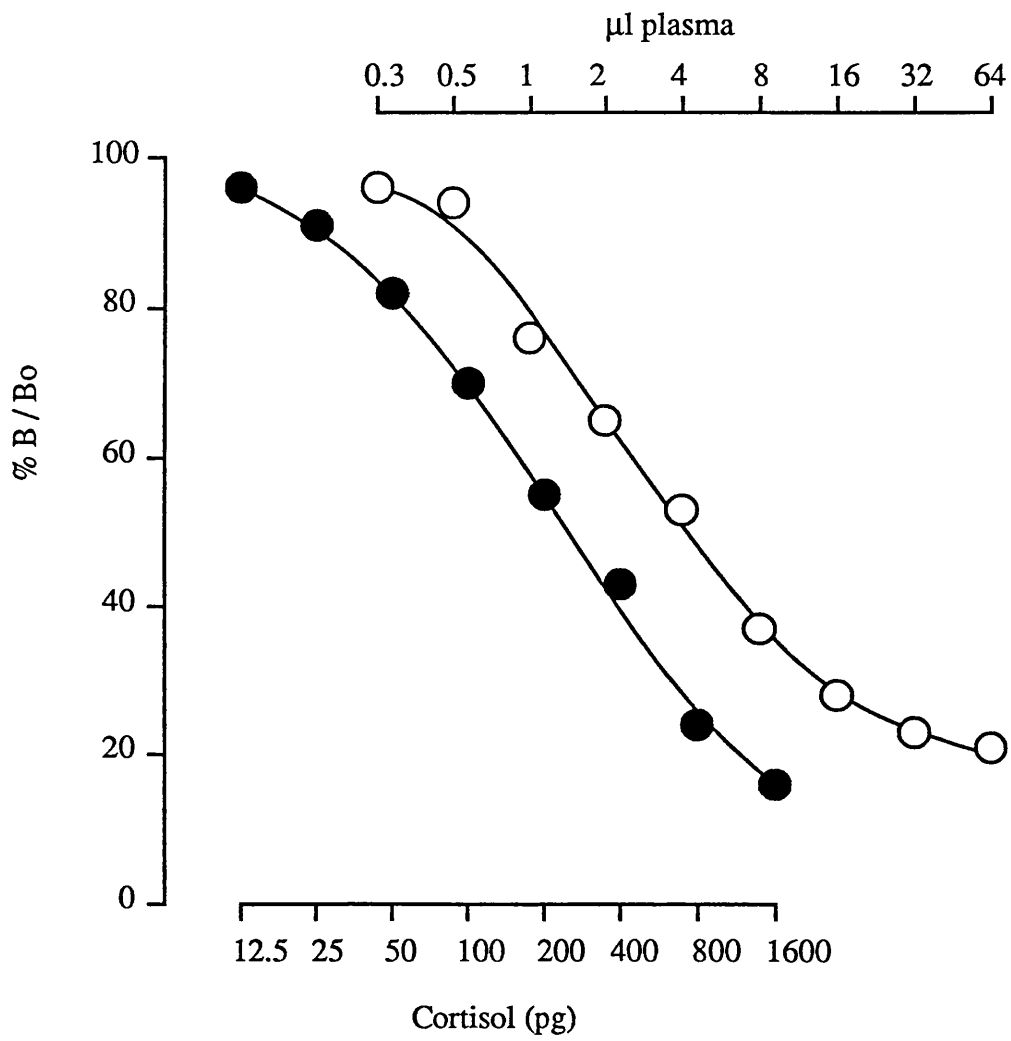
CHAPTER 3

THE EFFECT OF ANAESTHETICS ON THE RELEASE OF MCH AND CORTISOL IN THE RAINBOW TROUT *IN VIVO*

3.1. INTRODUCTION

Trout are very easily stressed and it is therefore necessary to anaesthetise them when collecting blood samples for hormone measurement. Several studies have shown that anaesthetics may artificially raise or lower the plasma concentrations of some hormones. Pentobarbitone anaesthesia in rats decreases the rate of firing of hypothalamic neurones, when tested by Dyball and McPhail (1974). These workers also found that different cell types responded differently to different anaesthetics. Urethane is known to increase the concentrations of vasopressin and ACTH in the rat plasma (Dyball, 1971; Dyball and McPhail 1974). MS222 at sublethal doses increases the release of cortisol, and presumably ACTH, into the plasma of trout (Strange and Schreck, 1978).

Since the plasma concentration of MCH has not been studied before, it was considered important to investigate thoroughly a method by which blood samples could be obtained quickly and efficiently from a group of experimental animals.

The main aim of this study was therefore to identify an anaesthetic which would not grossly alter the plasma concentrations of MCH, αMSH or cortisol in the few minutes between anaesthesia and bleeding the fish. Normally a high, lethal concentration of anaesthetic is used in such

terminal experiments, but because we also envisaged examining the hormonal response to various drugs, which might be injected under anaesthesia, the present work also examined the effects of low doses of anaesthetics over a time course of 40 min.

3.2. MATERIALS AND METHODS

All fish used in this series of experiments were obtained from Alderley fish farm and maintained under the conditions stated in Chapter 2. All were adapted to a white background. The effects of 5 different anaesthetics were tested on plasma MCH, cortisol and αMSH titres: Phenoxyethanol (1 : 1666, 5000, or 10000 v/v), MS222 (1 : 24000 w/v), Urethane (1 : 200 w/v) or Benzocaine (1 : 1200, 2000 w/v) were added to the tank water; the fifth anaesthetic, Saffan (0.6 mg in 50μl) was injected subcutaneously into the lateral line. The fish in this last group were tagged and killed in order of injection. The other groups were placed in aerated anaesthetic in small (40l) tanks and killed at intervals up to 40 min. The animals were caught and transferred from the holding tanks (in which they had been adapting since arrival from the farm) into the tank of anaesthetic. Depending on the experiment, groups of up to 50 fish were transferred in this way. Animals were kept in the aerated anaesthetic for various times (5-40 min) before killing. Control (T_o) fish were not anaesthetised but killed by stunning. Blood samples were collected as described in chapter 2. In order for the fish to survive for 40 min, the doses of anaesthetic used were not lethal. Fish responded by losing orientation or rolling on their backs, but continued to show opercular respiratory movements.

3.3. RESULTS

The plasma concentrations of various hormones are shown in Figure 3.1. and Table 3.1. (A), (B) and (C).

3.3.1. Phenoxyethanol

At a high (lethal) dose (1 : 1667) for 5 min this anaesthetic did not significantly affect the measured plasma concentrations of MCH, cortisol or αMSH when compared with the stunned fish. At lower doses, MCH titres were progressively reduced after 10 - 20 min but this was only statistically significant in experiment 3. αMSH titres were not significantly altered either but cortisol secretion was significantly elevated.

3.3.2. MS222

The use of this anaesthetic at the dose tested induced high plasma MCH titres after 5 min, which persisted for 40 min.

3.3.3. Urethane

This anaesthetic at a dose of 1 : 200 had a profound effect on plasma MCH concentration, double the control value at 5 min and which was very significantly raised, over 6-fold, after 30 min. αMSH titres were not affected.

3.3.4. Benzocaine

Plasma MCH values were not significantly changed. A high dose slightly increased the secretion of αMSH compared to stunned fish after 5 min exposure, but a low dose did not significantly affect measured plasma αMSH titres.

3.3.5. Saffan

Did not significantly alter the measured plasma MCH or αMSH titres compared with stunned fish.

Table 3.1. The effect of different anaesthetics on plasma (A) MCH, (B) cortisol and (C) αMSH titres. Plasma was collected from white-adapted trout exposed to various doses of anaesthetic for various times. Control fish (T_o) were killed by stunning. All data are form 5 different experiments and are given as mean ± s.e.m., n ⩾ 6.

(A)

MCH (pg/ml)

Time in anaesthetic (min)

Anaesthetic	Dose	0	5	10	20	30	40
1. Phenoxyethanol	1 : 5000	134 ± 24	155 ± 39	155 ± 60	103 ± 31	93 ± 11	82 ± 19
2. "	1 : 10000	74 ± 29	132 ± 11	---	79 ± 18	---	39 ± 8
3. "	1 : 1666	134 ± 41	146 ± 22	---	---	---	---
"	1 : 5000	*	134 ± 32	---	98 ± 35	28 ± 4	32 ± 9
*	1 : 10000	*	74 ± 18	---	170 ± 49	55 ± 26	47 ± 10
4. MS222	1 : 24000	83 ± 13	233 ± 66	---	---	163 ± 59	203 ± 50
Urethane	1 : 200	*	181 ± 80	---	---	662 ± 77	605 ± 69
5. Benzocaine	1 : 1200	115 ± 16	83 ± 11	---	---	---	---
*	1 : 2000	*	---	---	135 ± 28	---	---
Saffan	0.6 mg	*	---	175 ± 36	---	162 ± 43	---

(B)

Cortisol ng/ml

Time (min)

Anaesthetic	Dose	0	5	10	20	30	40
3. Phenoxyethanol	1 : 1666	16 ± 6	18 ± 6	---	---	---	---
"	1 : 5000	*	105 ± 5	---	87 ± 10	88 ± 6	107 ± 5
"	1 : 10000	*	52 ± 12	---	87 ± 11	99 ± 6	109 ± 4
4. MS222	1 : 24000	9 ± 1	19 ± 8	---	---	105 ± 5	115 ± 4
Urethane	1 : 200	*	63 ± 14	---	---	67 ± 13	55 ± 12

(C)

αMSH pg/ml

Time (min)

Anaesthetic	Dose	0	5	10	20	30	40
3. Phenoxyethanol	1 : 1666	161 ± 10	166 ± 20	---	---	---	---
"	1 : 5000	*	171 ± 16	---	---	---	143 ± 23
"	1 : 10000	*	211 ± 49	---	---	---	136 ± 14
4. MS222	1 : 24000	84 ± 14	110 ± 14	---	---	102 ± 18	105 ± 22
Urethane	1 : 200	*	145 ± 19	---	---	115 ± 13	113 ± 13
5. Benzocaine	1 : 1200	96 ± 11	157 ± 12	---	---	---	---
*	1 : 2000	*	94 ± 12	---	---	76 ± 9	---
Saffan	0.6 mg	*	---	99 ± 14	---	93 ± 8	---

Fig 3.1.

The effect of different anaesthetics on MCH and Cortisol release in white-adapted trout. Results are expressed as relative change from control (stunned) fish (t = 0, Controls = 100 %). Standard errors are not shown to avoid obscuring the data. Statistical analysis was performed on raw data.

★ $p \leq 0.05$ ★★ $p \leq 0.01$ ★★★ $p \leq 0.001$

● Phenoxyethanol 1 : 5 000   ○ Phenoxyethanol 1 : 10 000

☐ MS222   ■ Urethane
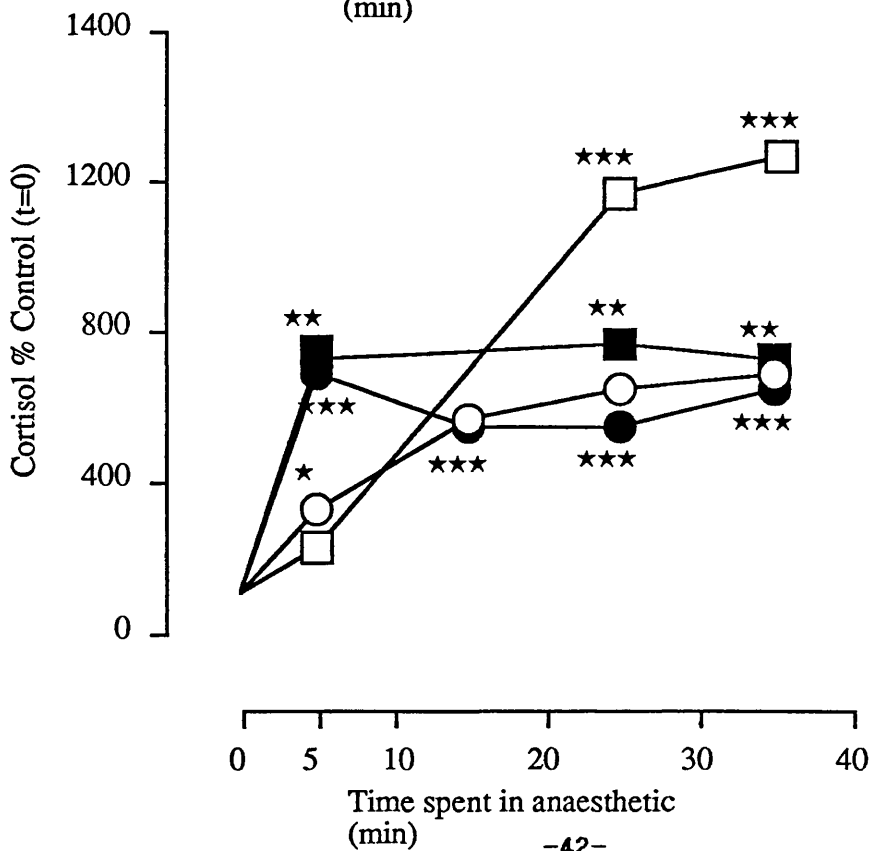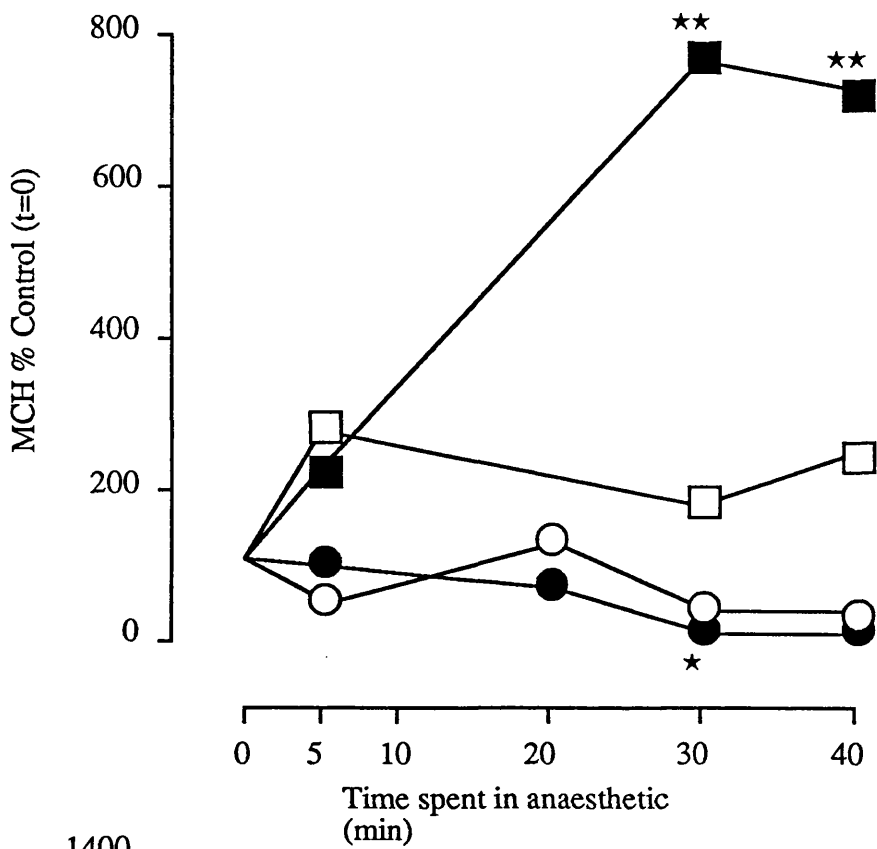
3.4. DISCUSSION

It is apparent from the results that different anaesthetics would suit different purposes. It is assumed that plasma hormone titres are not changed in fish killed by stunning and so this method was used for killing the control fish, even though, for some hormones eg ACTH we know this is untrue (Gilham and Baker, 1985). For work on plasma MCH, however, stunning is not the best method for killing fish before bleeding. As described in Chapter 2 the plasma MCH assay requires the use of relatively large volumes of plasma (500 µl), this is also true for the detection of αMSH. When fish are stunned in order to obtain a blood sample, haemorrhaging occurs around the brain and a smaller blood sample is obtained compared with anaesthetised fish. To permit duplication of assays it was necessary either to use larger fish or anaesthetics, and the less expensive option was to use anaesthesia. The treatment that gave values least different to stunned fish was a high dose (1 : 1666) of phenoxyethanol or benzocaine provided that animals were bled within 5 min of exposure to the anaesthetic. However in this and other experiments benzocaine did elevate αMSH titres and should therefore be a second choice. At lower doses phenoxyethanol depressed plasma MCH titres, αMSH titres were not affected. Lower doses of phenoxyethanol (1 : 5000, 10000) progressively decreased the amount of MCH in the plasma. This suggests that it depresses the release from the nerve terminals. Thus, in experiment 3, plasma MCH concentrations in fish placed in 1 : 5000 phenoxyethanol was only 21 % that of control fish, suggesting that this anaesthetic depresses MCH release and that the hormone may have a half life of about 20 min or less. An alternative interpretation might be that the anaesthetic increases clearance of MCH by increasing urine flow.

Saffan also did not appear to modify plasma titres of MCH, but it is an impractical choice for use with large groups as it had to be administered individually by injection into the lateral line. This is difficult in itself as only small volumes can be used and have to be injected very precisely and slowly to avoid it leaking out again through the puncture in the skin. It is a good anaesthetic to use for surgery because if applied in this way the animal becomes unconscious very quickly (since the lateral line overlies the highly vascular red muscle). The effects are long lasting and the fish is not prone to the hypoxia or oedema that often results from the use of other anaesthetics; recovery is consequently better (Hawkins, 1981).

The effects of the other anaesthetics (MS222 and Urethane) were interesting in that they provoked MCH release, either by acting directly at the pituitary nerve terminals, or by affecting the firing rate of the whole neurone, or acting indirectly by inhibiting some inhibitory input on MCH. It has been suggested for mammals that urethane exerts its anaesthetic effects at the spinal level whilst its effect on neurohypophysial hormones like vasopressin and oxytocin, whose release is also stimulated (Dyball 1971), occurs at the site of the membrane in the neurohypophysis and not at the hypothalamus (Dyball and McPhail, 1974). Urethane also affects hypophysial hormones, causing increased ACTH release (Spriggs and Stockham, 1964) probably by stimulating CRF release. It is not clear how MS222 exerts its effect; this too can cause ACTH release (Strange and Schreck, 1978) and may act in a similar manner.

As a result of these investigations it was determined that if used in the described manner, phenoxyethanol and benzocaine were to be the chosen

anaesthetics for all subsequent practical work involving the measurement of MCH and αMSH in the plasma.

The effect of anaesthetics on ACTH and cortisol secretion in the present study are not altogether clear. Since, in some experiments, the cortisol titres were already raised 5 min after anaesthesia, it would seem that the fish must have been disturbed immediately previously, while they were distributed to the different tanks of anaesthetics, MS222 clearly stimulated cortisol secretion, but the other 5 results can be interpreted with less confidence.

CHAPTER 4

THE INFLUENCE OF NERVES AND HORMONES ON THE CONTROL OF TROUT MELANOPHORES

4.1. INTRODUCTION

Teleost fish are capable of altering their colour to match that of their environment. Such changes occur over a span of minutes or hours and are due to the movement of melanin granules within integumentary melanophores. Both hormonal and neuronal factors control such movements in teleost melanophores. Thus melanin aggregation, resulting in pallor, can be induced by the melanin concentrating hormone (MCH) acting on its own specific receptor (Oshima *et al.*, 1985), by melatonin in some species and by noradrenaline (NA) from sympathetic neurones which innervate the melanophores (for a review see Fujii and Oshima, 1986). Melanin dispersion which causes darkening, occurs in response to the melanocyte stimulating hormone (αMSH) and possibly also to neuronal agents, eg adenosine or ATP (Miyashita *et al.*, 1984).

The plasma titres of both αMSH and MCH have been measured in trout kept on different coloured backgrounds (Kishida *et al.*, 1989). Not only is the concentration of MCH always lower than αMSH by 3-80 fold, depending on the background and illumination but its titre in white-adapted fish is at least 20-fold less than that required to cause maximum melanin aggregation in trout scales *in vitro* (>1nM) (Kishida *et al.*, 1989; Baker, 1988a). It may be that the difference arises partly because of diffusional barriers in the *in vitro* assay system. Additionally, however it is possible that circulating titres of MCH in the trout may not be

sufficient alone to induce full melanin aggregation, pallor being achieved *in vivo* by the concerted action of both hormonal and neuronal factors.

The present work was done to investigate the potential importance of neuronal agents in the control of trout melanophores, and the interaction between NA and MCH.

4.2. MATERIALS AND METHODS

4.2.1. Fish

Rainbow trout (200g) *Salmo gairdneri*, were obtained from a local fish farm, and 30g chinese grass carp, *Ctenopharyngodon idellus*, obtained from the Wessex Water authority.

4.2.2. Preparation of Scales

Fish were killed by a blow to the head and the dorsal scales removed by scraping from tail to head. Scales were separated and washed in two changes of sterile culture medium containing double strength antibiotics. They were then transferred to normal sterile culture medium in polystyrene well-plates (Corning Ltd). The culture medium was Eagles Minimum Essential Medium (MEM) with Hanks salts and 20mM Hepes buffer to which was added 10% foetal calf serum, 200 IU/ml penicillin, 0.2 mg/ml streptomycin (Sigma), 5 μg/ml fungisone (all from Flow laboratories). The pH of the medium was adjusted to 7.8 with Tris.

4.2.3. Test agents

Synthetic salmonid MCH (Peninsula Laboratories) was stored as a concentrated solution in 0.01M HCl containing 1% BSA and diluted in culture medium as required. αMSH (Sigma) was prepared similarly.

Phentolamine (Rogitine, Ciba Ltd) was used at a final concentration of 10^{-4}M. A concentrated solution (10^{-3}M) of noradrenaline was prepared in 0.001M HCl containing 1mg/ml ascorbic acid and diluted in culture medium as required. To prepare hypothalamic extract, tissues from two fish were sonicated in 200μl 5M acetic acid.

4.2.4. Determination of melanophore state

The extent of melanin dispersion or concentration of the melanophores was scored using Hogbens Melanophore Index (MI) in which full melanin dispersion is scored as MI 5.0 and full melanin aggregation as MI 1.0.

4.3. RESULTS

4.3.1. The effect of culture on the melanophore index

Immediately after removal from the fish, scale melanophores showed full melanin dispersion. During the following 6 hours they displayed varying degrees of melanin aggregation but re-dispersed overnight to MI >4.75 (Fig 4.1.). Such initial aggregation was prevented by the addition of the α-adrenergic blocker phentolamine to the medium. Over the next few days the melanophores underwent a gradual progressive melanin concentration, reaching an MI of about 2-2.5 by day 4. The rate at which this second phase of melanin aggregation occurred varied between experiments. It was not significantly affected by the presence of phentolamine but could be

prevented by the inclusion of αMSH $(10^{-7}M)$ in the medium. (Fig 1).

Melanophore indices of less than 2.0 were never observed in control

cultures maintained up to 6 days. Melanophores remained fully viable when

maintained *in vitro* and were able to respond appropriately and rapidly

when either trout hypothalamic extract or αMSH were added at the end of

a 6 day culture period, most of them attaining a fully aggregated or

dispersed state (Fig 4.1.).

Fig 4.1. The response of trout scales to culture over a period of 3 or 6 days in the absence or presence of αMSH $(10^{-7}M)$ or phentolamine $(10^{-4}M)$. The response of the melanophores to hypothalamic MCH after 6 days of culture is also shown. Values are means ± s.e.m., n = 20. Where s.e.m.s are not shown they fall within the area of the symbol.

○ Control
■ Phentolamine
● MSH
☆ Hypothalamic extract
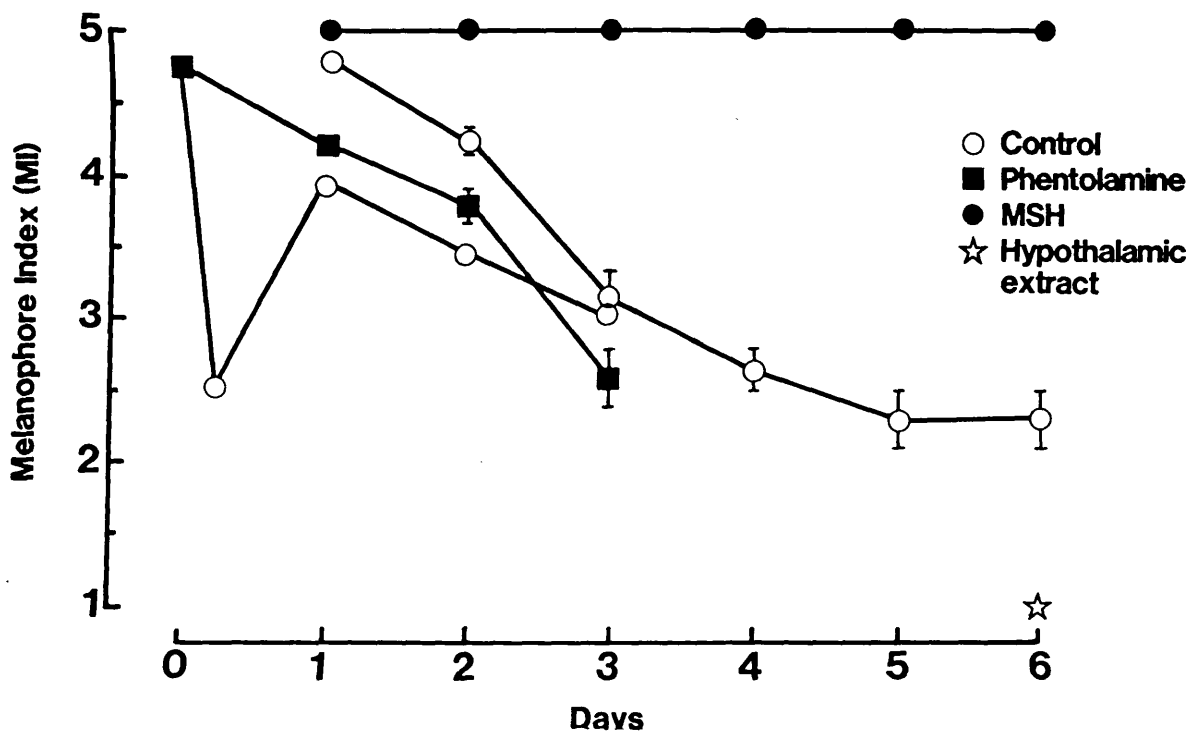
4.3.2. Synergy between MCH and NA

Synergism may be defined as an enhanced response of a tissue to two or more hormones which is greater in magnitude to the sum of the individual actions of the hormones (Hadley 1988). Before investigating any interaction between NA and MCH, scale melanophores were first incubated for 24h in culture medium to remove the interfering effect of endogenous NA. Scales with the MI of >4.5 were then incubated in medium containing either MCH or NA over a range of concentrations (Fig 4.2.). Both MCH and NA caused full melanin concentration with an EC_{50} of about 3×10^{-10}M and 3×10^{-8}M respectively, the precise dose varying with scales from different trout. When MCH and NA were added to the medium together at doses which alone had minimal effect, significant melanin concentration was observed (Fig 4.2.). These agents were therefore acting synergistically to cause melanin aggregation. The experiment was repeated four times with similar results although the extent of synergy varied in different experiments (Figs 4.3., 4.4.) and with the doses used.

The experiment was repeated using scale melanophores from the grass carp. No evidence for synergy between MCH and NA was found in this species (data not shown). This observation may be related to the fact that the melanophores of this species are much more sensitive *in vitro* to MCH (EC_{50} 3×10^{-11}M) than the trout, although the plasma MCH titre has not yet been determined.

Fig 4.2. Dose response curves of trout melanophores to NA (10^{-7}M to 10^{-9}M) and MCH (10^{-9}M to 10^{-11}M) alone or to NA (10^{-7}M to 10^{-9}M) with MCH (3×10^{-11}M). Experiment 1. Values are means $\pm$ s.e.m. (n = 5). Where s.e.m.s are not shown they fall within the area of the symbol.
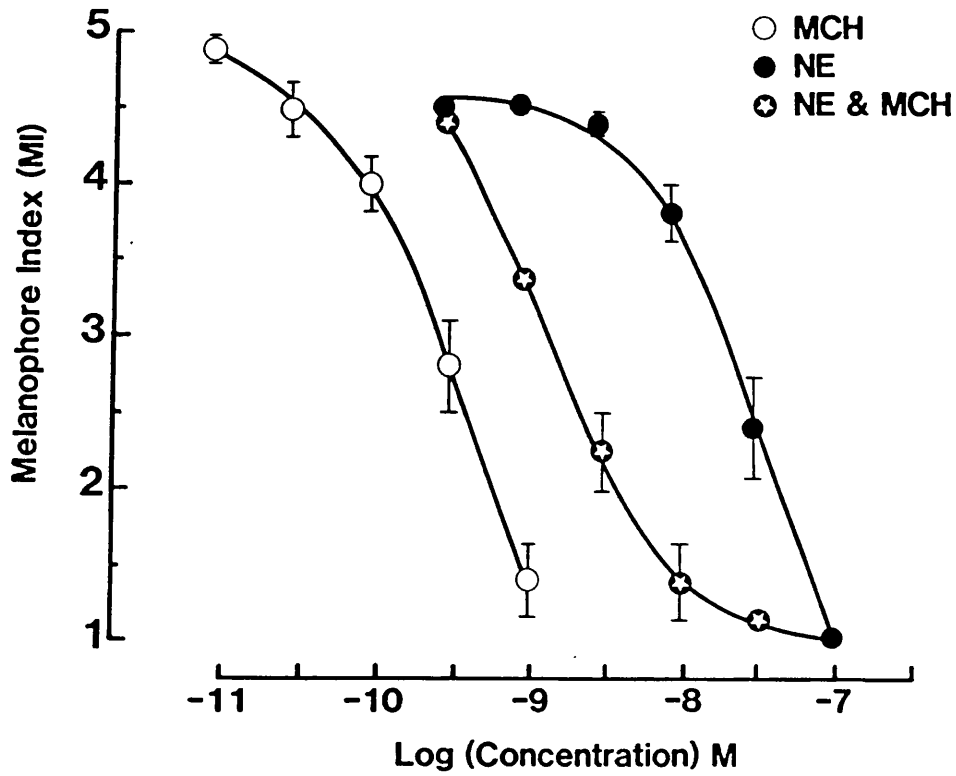
Fig 4.3. Histogram showing varying extent of synergy between NA and MCH. Dose of NA in all cases was 3×10^{-9}M. Dose of MCH was 3×10^{-11}M in Experiments 1 and 3, and 10^{-11}M in Experiment 2 . Values are mean ± s.e.m. (n = 5).
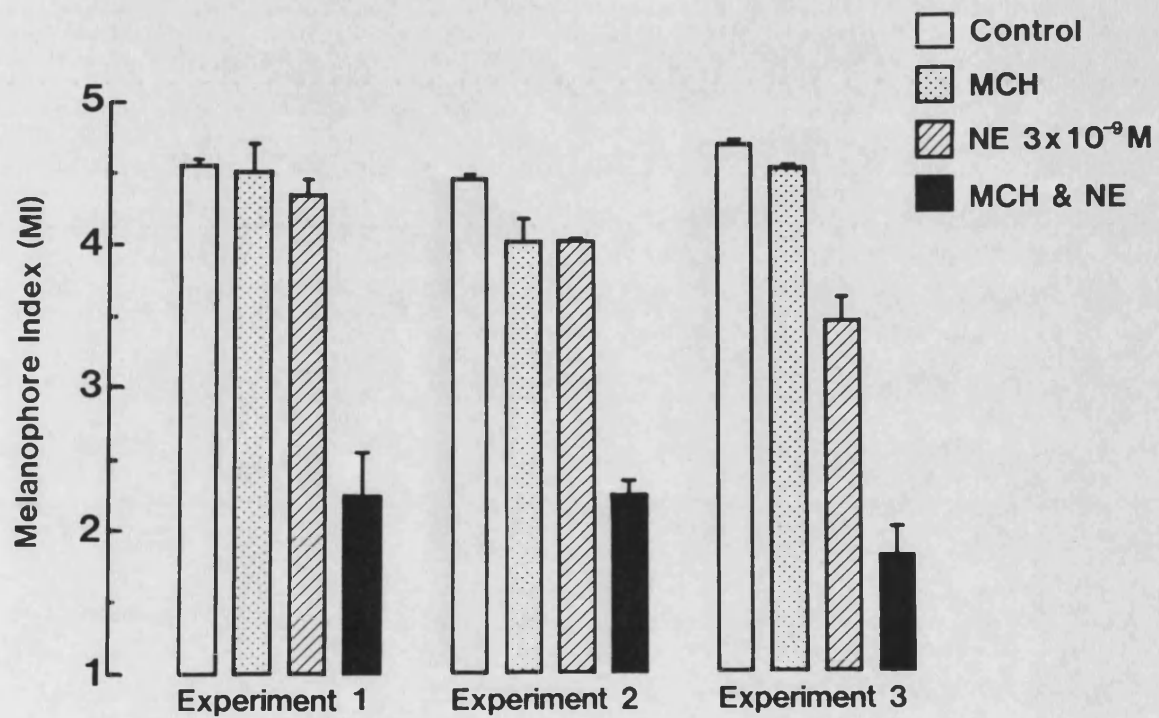
Fig 4.4. Experiment 4. Trout melanophores treated with (a) NA $(3\times10^{-9}M)$, (b) MCH $(3\times10^{-11}M)$, (c) NA and MCH together.

(a)
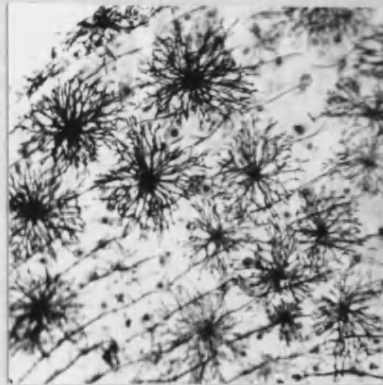
(b)

(c)
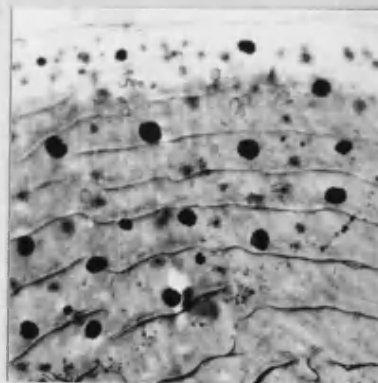
4.4. DISCUSSION

These results suggest that the initial, phentolamine-reversible, melanin concentration is attributable to the release of NA from degenerating or depolarised local nerve terminals while the subsequent state of melanin dispersion may be due to release of a relatively long-lasting agent,such as adenosine or ATP. With prolonged culture these neuronal factors gradually degrade or diffuse away and the melanophores attain their more concentrated resting state. A fully aggregated state of MI 1.0, seen in isolated amphibian melanophores, was not observed in trout scale melanophores but it remains possible that this might be achieved if the medium were changed regularly or the culture period extended. These results provide evidence that teleost melanophores may also have a punctate resting state for their melanophores similar to all other vertebrates studied so far. Some workers have claimed that the resting state of teleost and holostean melanophores is stellate (Sherbrooke *et al.*, 1988a). Such a 'resting' state may actually be the product of action of spontaneously released adenosine or other neurotransmitter. Such spontaneous release is known to take place in tilapia melanophores in split fin preparations (Kumazawa and Fujii, 1984). These workers reported that adenosine was released concomittantly with NA, the spontaneous release was presumed to be due to leakage of the transmitters from the disintegrating terminals.

Hormones that act synergistically are generally found to be acting via different second messengers; if they were both acting via the same messenger, their combined actions would not be expected to be anything other than additive (Hadley 1988). It has long been known that αMSH

exerts its action by a Gs protein, stimulating the adenylate cyclase pathway, thus stimulating the formation of cAMP and hence melanin dispersion. The mechanism of action of NA on pigment cells is via the α_2 receptor. Eberle and Girard (1985), working with *Anolis* melanophores, have speculated that the signal is transduced via the action of a Gi-cAMP second messenger system although there is no firm evidence for this suggestion, and it might act via the IP_3 pathway, as discussed below. Recently Abrão et al., (1989) have suggested that MCH may exert its effects through the phosphoinositide system, specifically through the action of diacylglycerol (DAG). The phorbol ester TPA was shown to mimic the effects of MCH, subthreshold doses of this substance also acted synergistically with MCH. Phorbol esters are known to mimic DAG in activating protein kinsase C. Dibucaine, H-7, 4-bromo-phenacyl-bromide and neomicin displaced the dose response curve of MCH to the right, showing inhibitory actions. None of these substances were effective on NA action. It is well documented in many cell types (see Sekar and Hokin, 1986 for review) that the two bifurcating pathways of the phosphoinositide pathway, viz the production of IP_3 and DAG, are capable of synergising with each other and it is suggested that on the basis of this data that this is the mode of action for MCH reported here.

Lithium treatment of melanophores results in aggregation of melanosomes in a dose dependent manner, and its action is presumed to be of an intracellular nature since its action was most rapid if LiCl is injected into the cell (Namoto and Yamada, 1983). Li^+ is known to inhibit the enzymes responsible (inositol phosphatases) for the degradation of the phosphoinositides IP_3, IP_2 and IP_1. IP_3 is known to cause an increase in intracellular Ca^{2+} concentrations by releasing Ca^{2+} ions from intracellular stores. In turn this causes protein phosphorylation by a

containing EDTA for several days show extreme dispersion (Challinor, 1985). It is possible that intracellular Ca^{2+} stores are depleted under these conditions and the aggregating mechanisms normally resulting in a punctate resting state are unable to function

These results show that the two major melanin aggregating agents in trout - MCH from the neurointermediate lobe and NA from sympathetic nerve terminals - can act synergistically on trout melanophores to cause pallor. When trout are placed on a pale coloured background they show very rapid chromatic adaptation (Rodrigues and Sumpter, 1984). This is almost certainly neuronally induced. Although plasma MCH titres are significantly raised within 2h after transfer from a black to a white background (Kishida et al., 1989), plasma αMSH titres fall only slowly (Baker, 1988a; Rodrigues and Sumpter, 1984) remaining for many hours at concentrations which MCH alone would be unable to override (Baker, 1988a). This highlights the predominant role of the nervous system in achieving rapid pallor. Even after long-term adaptation to a white background when the hormonal status has stabilized, denervated melanophores in the trout remain fully dispersed (Baker et al., 1986) in contrast to those of many other species e.g. Poecilia latipinna (Baker and Ball, 1975). This suggests that the circulating level of MCH in white adapted trout remains inadequate to override circulating αMSH. The use of MCH as a colour-controlling hormone appears to be an evolutionary novelty in teleost fish. It does not have this effect in other vertebrates (Baker et al., 1985; Wilkes et al., 1984a) although an MCH homologue occurs in the brains of all vertebrate classes so far examined (Baker, 1988b). The fact that MCH secretion in the trout is rapidly influenced by the colour of the background speaks for its active participation in colour adaptation in the trout. Nevertheless, the present results suggest that, at least in this teleost species, plasma MCH alone may be inadequate to achieve maximum

vertebrate classes so far examined (Baker, 1988b). The fact that MCH secretion in the trout is rapidly influenced by the colour of the background speaks for its active participation in colour adaptation in the trout. Nevertheless, the present results suggest that, at least in this teleost species, plasma MCH alone may be inadequate to achieve maximum pallor. The relative importance of different agents for colour change may vary between species.

CHAPTER 5

THE INFLUENCE OF REPEATED STRESS ON THE SECRETION OF MCH IN THE TROUT

5.1. INTRODUCTION

The response of vertebrates, including fish, to stressful conditions has been extensively studied because persistent or excessive stress has very deleterious effects on the animal causing, among other responses, depression of the immune system, impaired growth and increased mortality. The responses of the animal to stress can be divided into primary and secondary responses (Mazeaud, 1977). The primary phase is that in which there is stimulation of the nervous and endocrine systems and this leads to the secondary phase consisting of osmoregulatory, metabolic and blood cell population changes. One major component of the hormonal response to stress is the hypothalamo-pituitary-adrenal (HPA) system. Hypothalamic neurosecretory agents, including CRF and its homologues such as the urotensins; vasopressin (or vasotocin); and probably other peptides and amino acids, are released and stimulate the corticotrophic cells of the pituitary to release ACTH. This in turn stimulates the synthesis and release of cortisol or other corticosteroids from the adrenal gland (or interrenal gland of fishes). It is these steroids that influence the inflammatory and immune systems, and affect metabolism and ionic balance in fish.

The hormone ACTH is derived from a much larger precursor molecule, pro-opiomelanocortin (POMC) which is cleaved to form other peptides such as γ-lipotrophin (γLPH) and β-endorphin as well as an N-terminal fragment called the N-terminal POMC peptide (NPP). The pro-hormone is further

processed in other cell types such as the melanotrophs and nerve cells, to give more fragments including the melanocyte stimulating hormone (αMSH), corticotrophin-like intermediate lobe peptide (CLIP) and β-MSH. There is good evidence to suggest that the melanotrophs can also be stimulated under certain stressful conditions. In mammals the function of the melanotrophs is not known but increased activity has been seen in response to stressors such as stroboscopic light (Moriarty et al., 1975). In fish it was observed that conditions of other mild chronic stress, eg noise, fungal infection and thermal shock cause increased αMSH plasma titres (Rance and Baker, 1981; Sumpter et al., 1985; Sumpter et al., 1986) but not under all conditions tested (Sumpter et al., 1985; Pickering et al., 1986). Although αMSH was the melanotrophic peptide measured in these studies the other POMC products are expected to be released simultaneously in equimolar concentrations (Eipper and Mains, 1980). One proposed role of these secretions is to enhance the classical HPI axis by potentiating the response of steroidogenic cells to ACTH (Baker and Rance, 1981; Gilham and Baker, 1985; Takahashi et al., 1985).

An additional hypothalamic factor which could modulate the response of the corticotrophs to CRFs and also influence the melanotrophs is the melanin-concentrating hormone (MCH). Previous studies suggest that this can depress the secretion of αMSH (and presumably all associated POMC peptides) from the melanotrophs both in vivo (Baker et al., 1986) and in vitro (Barber et al., 1987). MCH also appears to depress the secretory action of the corticotrophs. Thus MCH will reduce the release of CRF-stimulated ACTH from the PD in vitro (Baker et al., 1985). This could be a physiologically relevant effect since fish maintained under conditions of mild chronic stress on a white background (when plasma MCH titres are

high) have lower plasma cortisol titres than fish kept on a black background (Baker and Rance, 1981; Gilham and Baker, 1985).

In view of these effects of MCH on the response to stress it was also of interest to determine if stress can influence the release of MCH, and whether the release of MCH is susceptible to plasma corticosteroid feedback.

5.2. MATERIALS AND METHODS

5.2.1. Fish

Adult, immature fish were obtained from a commercial fish farm and were adapted to either white or black tanks under a photoperiod of 18h light : 6h dark at 11°C for at least 2 weeks before use. They were not fed during this time.

5.2.2. Administration of Stress

Control fish remained undisturbed throughout each experiment. The injection of 1ml saline (0.8% NaCl) into the peritoneal cavity without anaesthesia was used as a stress. Animals were usually given 1 injection per day for one or several days (see individual experimental details) and killed 1h after the final injection.

5.2.3. Killing methods

As in all experiments involving the measurement of stress, great care was taken to disturb the fish as little as possible before or during killing. When blood samples were to be taken, fish were caught in a single group and anaesthetised in phenoxyethanol (1 : 1667 v/v). Blood was collected

according to the procedure described in Chapter 2. All fish within each group were bled within 5 min of catching.

5.2.4. Measurement of hormones

All measurements were done by RIA methods as described in chapter 2. Plasma MCH was determined using the solid phase method, plasma αMSH was extracted (using the method of Wilson and Morgan, 1979) and measured using the charcoal separated RIA. Plasma cortisol titres were measured using the method of Baker and Rance (1981).

5.3. RESULTS

5.3.1. Acute stress and MCH secretion

A single injection of saline did not affect the plasma MCH concentration of white-adapted fish 1 or 2h after injection but raised cortisol titres at both periods. See table 5.1.

Table 5.1. The effect of acute injection stress on plasma MCH and cortisol concentrations.

Time after injection	n	MCH pg/ml	Cortisol ng/ml	
1 HOUR				
Control	7	93.4 ± 25.3	1.3 ± 0.02	
Injected	6	128.5 ± 16.8	28.4 ± 6.9	***
2 HOURS				
Control	7	101.0 ± 17.8	9.8 ± 1.7	
Injected	7	126.3 ± 21.0	171.0 ± 29.0	***

Results are means ± s.e.m.   *** p < 0.001 (on untransformed data) compared with uninjected controls
(a) Fish were adapted to a white background for 18 days and then were injected at 13:30h with 1 ml saline (0.8 % NaCl) without anaesthesia and killed 1h later.
(b) Fish were adapted to a white background for 2 weeks then were injected at 12:30h with 1 ml saline (0.8 % NaCl) without anaesthesia and killed 2h later.

5.3.2. Repeated stress and MCH secretion

The effect of repeated stress and dexamethasone on white-adapted fish:

Since acute injection stress had no effect on plasma MCH titres, fish were injected daily over a longer period (4 days) and killed 1h after the last injection. A second group of fish was given an injection of dexamethasone (0.5 mg/kg) instead of saline alone. The results are shown in table 5.2. and fig 5.1. After 2 injections on the first day followed by several daily injections, plasma MCH titres were very significantly affected, rising to levels nearly 9 times the control values. Cortisol titres were also raised but αMSH titres were unaffected. Dexamethasone administration severely depressed the stress-induced rise in all hormone titres, although at the dose given (0.5 mg/kg) the titres were not lowered to control levels.

Table 5.2. The effect of repeated stress and dexamethasone administration (0.5mg/kg) in white adapted fish on plasma MCH, αMSH and cortisol.

Treatment	n	MCH pg/ml	αMSH pg/ml	Cortisol ng/ml
Control	8	83.6 ± 12.6	86.7 ± 10.3	7.0 ± 1.3
Saline	8	748.1 ± 142.1 ***	83.0 ± 18.7	111.5 ± 21.1 ***
Dexamethasone	8	152.9 ± 20.5 * s	74.4 ± 9.7	14.8 ± 1.1 ** s

Results are means ± s.e.m. Statistical analysis was performed on log-transformed data. * p < 0.05, ** p < 0.01, *** p < 0.001, compared with uninjected controls. 's' p < 0.001 compared with saline injected fish.
Fish were adapted to a white background for two weeks and then injected with 1 ml saline (0.8 % NaCl) without anaesthesia twice on the first day (at 11:30 and 17:30) and then once a day (11:30) for a further 3 days. They were killed 1h after the last injection.

Fig 5.1.

The effect of repeated stress and dexamethasone administration (0.5mg/kg) in white adapted fish on plasma MCH, MSH and Cortisol. Fish were adapted to a white background for 2 weeks and then injected with 1 ml saline (0.8 % NaCl) without anaesthesia twice on the first day (at 11:30 and 17:30) and then once a day (11:30) for a further 3 days. They were killed 1h after the last injection. Results are means and standard errors, statistical analysis was performed on log-transformed data.

☐ Control ⬚ Saline ⬚ Dexamethasone

★ p ≤ 0.05 ★★★ p ≤ 0.001 compared to controls.
ooo p ≤ 0.001 compared to saline injected fish.
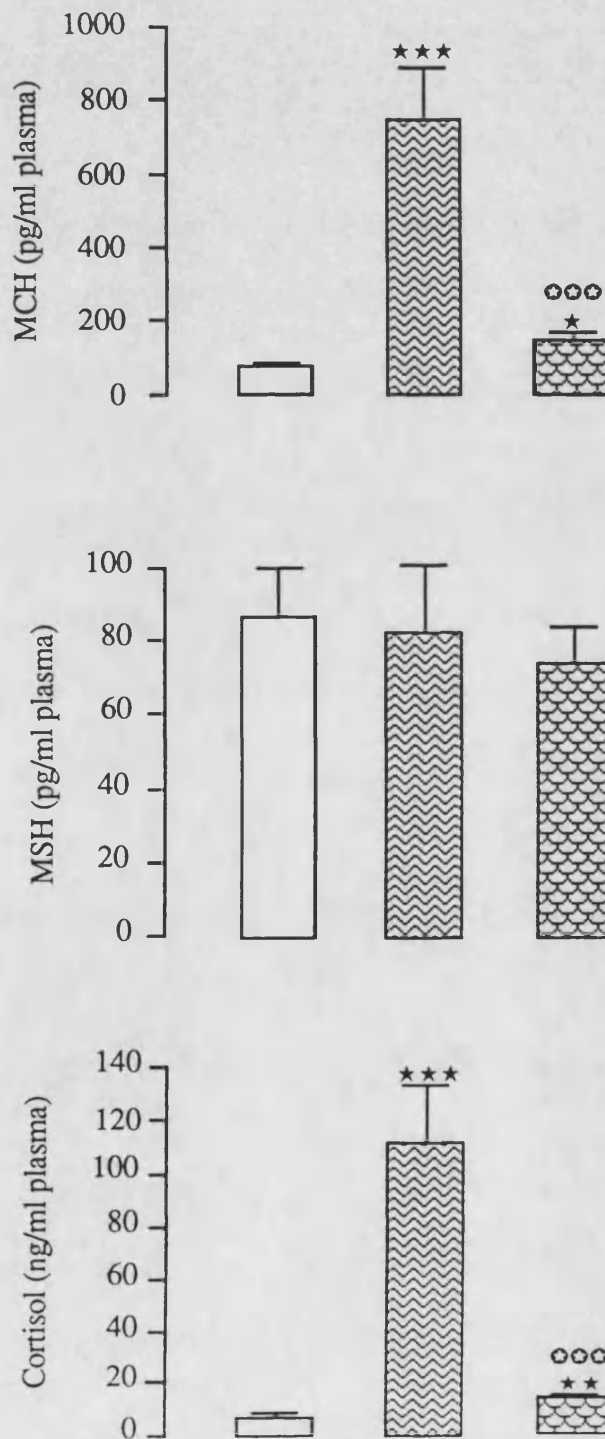
5.3.3. Time of day of administration of stress and MCH secretion

A study of the diurnal changes in MCH secretion show that plasma titres are low early in the morning then rise steadily, reaching a peak at mid-photophase and decline thereafter (V Lyon, personal communication). It is therefore possible that the time of day could influence the stress-induced rise in MCH secretion. White-adapted fish (lights on 4:30am, lights off 10:30pm) were injected without anaesthesia at either 10:30 or 18:00 once a day for 3 days without anaesthesia and killed 1h after the final injection. The results are summarised in Table 5.3. The results show that repeated injection-stress stimulates the HPI axis and raises MCH titres independently of the time of day that the stress is applied.

Table 5.3. The effect of time of day when stress is administered on plasma hormone titres in white adapted trout.

Time of injection	n	MCH pg/ml		Cortisol ng/ml	
		Control	Injected	Control	Injected
10:30	8	106.1 ± 14.1	174.9 ± 18.8 *	19.1 ± 4.4	83.7 ± 11.7 ***
18:00	8	67.2 ± 4.1	149.6 ± 19.6 ***	3.3 ± 1.4 a	73.8 ± 7.2 ***

Results are means ± s.e.m. Statistical analysis was performed on log-transformed data. * p (0.05, *** p (0.001 compared with uninjected controls. 'a' p (0.01 compared with fish sampled at 10:30.
Fish were adapted to a white background for 14 days before use. They were injected without anaesthesia once a day at 10:30 or 18:00 with 1ml saline (0.8% NaCl) for 3 days and killed 1h after the last injection.

Fig 5.2.

The effect of time of day when stress is administered on plasma hormone titres in white adapted trout. Fish were injected without anaesthesia once a day at 10:30 or 18:00 with 1 ml saline (0.8 % NaCl) for 3 days and killed 1 h after the last injection

★ $p \leq 0.05$,   ★★★ $p \leq 0.001$ compared to uninjected controls

✪✪ $p \leq 0.001$ compared to fish sampled at 10:30

☐   Control   ▨   Saline
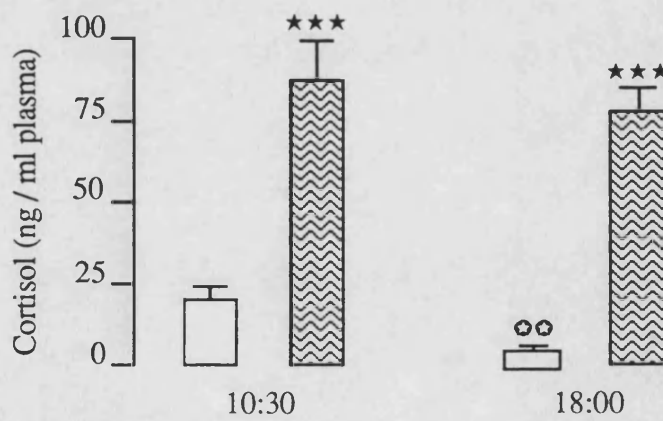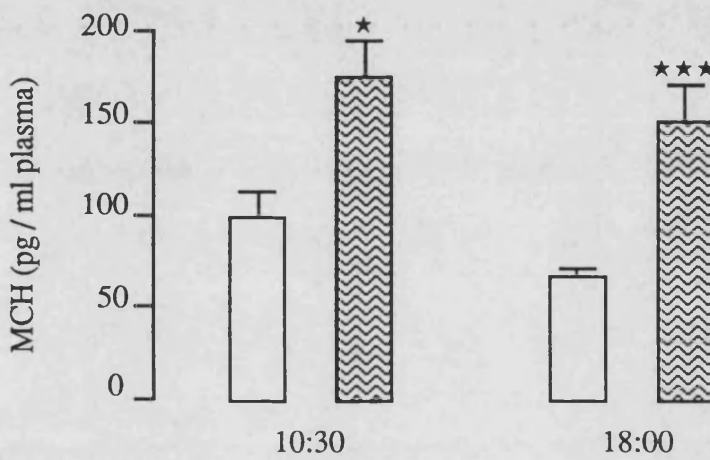
5.3.4. The effect of background colour, repeated stress and dexamethasone administration on MCH secretion

It was investigated whether MCH titres could be raised in response to a repeated stress irrespective of background colouration. As can be seen from the results in table 5.4. and fig 5.3., injection of saline without anaesthesia stresses the animal, thus stimulating the HPI axis, resulting in elevated cortisol titres. Additionally, the colour-change-hormone titres are raised following this repeated stress, but the response was severely modulated by the background colour. In a white-adapted animal, in which the plasma MCH titres are already high, only the MCH levels are further raised with repeated stress; the αMSH titres are not significantly raised. Conversely, in black-adapted fish MCH values are unaffected by stress but the plasma αMSH values are significantly increased. Dexamethasone markedly depressed the stress-induced rise of all hormones.

Table 5.4. The effect of background colour, repeated stress and dexamethasone administration on plasma cortisol, MCH, and αMSH.

	n	MCH pg/ml	αMSH pg/ml	Cortisol ng/ml
WHITE ADAPTED				
Control	9	90.4 ± 9.8	131.1 ± 21.5	15.1 ± 4.3
Saline	7	373.3 ± 102.3 **	211.1 ± 47.6	296.2 ± 56.4 ***
Dexamethasone	8	108.7 ± 16.7 ss	135.4 ± 22.0	30.4 ± 14.4 sss
BLACK ADAPTED				
Control	8	50.3 ± 7.2 ww	148.8 ± 18.5	32.7 ± 8.8
Saline	6	35.1 ± 1.8 www	330.0 ± 70.6 **	333.2 ± 46.4 ***
Dexamethasone	9	45.9 ± 5.9 ww	107.0 ± 15.6 ss	52.4 ± 22.3 sss

Results are means ± s.e.m. Statistical analysis was performed on log-transformed data. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with uninjected controls. 'ss' $p < 0.01$, 'sss' $p < 0.001$ compared with saline injected fish. 'ww' $p < 0.01$, 'www' $p < 0.001$ compared with white-adapted counterparts.
Fish were adapted to either white or black backgrounds for 2 weeks, then injected without anaesthesia once a day (10:30) for 4 days with 1 ml saline (0.8 % NaCl) or dexamethasone (0.5 mg/kg). They were killed 1h after the final injection.

Fig 5.3.
The effect of background colour, repeated stress and dexamethasone administration on plasma hormone titres. Fish were adapted to either white or black tanks for 2 weeks then injected without anaesthesia once a day (10:30) for 4 days with 1 ml saline (0.8 % NaCl) or dexamethasone (0.5 mg/ kg) and killed 1 h after the last injection. Results are arithmetical means and standard errors, statistical analysis was performed on log-transformed data.
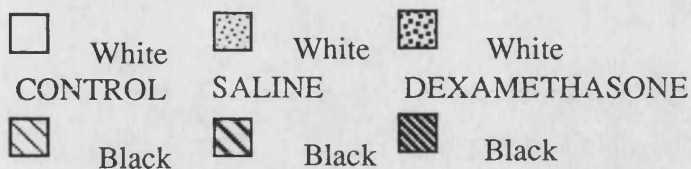
★★ p ≤ 0.01, ★★★ p ≤ 0.001 compared to uninjected controls.
◐◐ p ≤ 0.01, ◐◐◐ p ≤ 0.001 compared to saline injected fish.
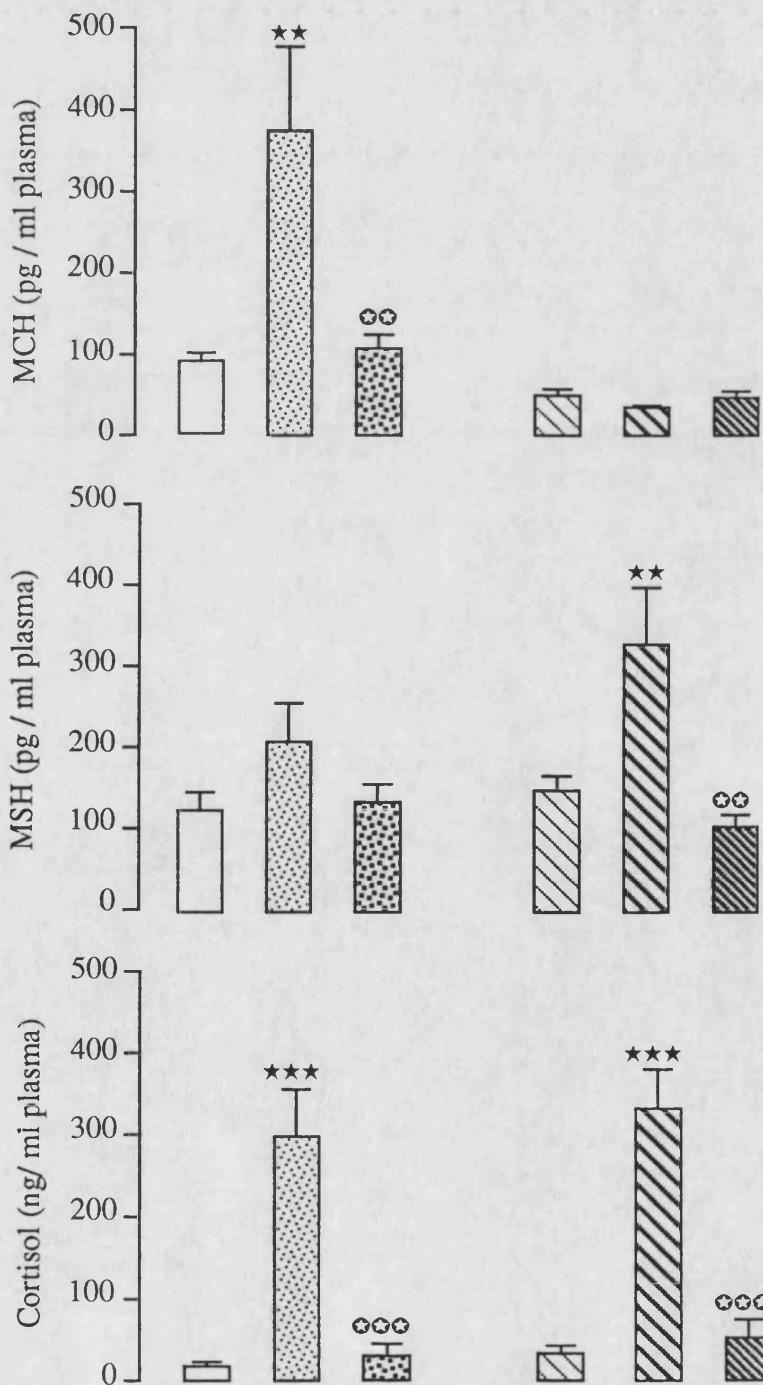
5.3.5. The effect of stress over time on MCH and cortisol secretion

In order to determine how many times it was necessary to repeat a stress in order to elicit an increase in plasma MCH levels, fish were injected daily over several different periods of time. White-adapted trout were given one injection of saline a day for 2, 4, 6 or 8 days, and usually killed 1h after the final injection. Control fish were killed on the first day without any stress. The resultant hormone values are summarised in Table 5.5. and Fig 5.3. In this experiment, an increase in MCH titres was observed by the second day but then showed a gradual decrease over successive days. The concentration of MCH measured in fish injected for 2 days was significantly higher than MCH titres in any of the other injected groups, although their values usually remained significantly higher than those of uninjected controls. Plasma cortisol titres peaked at 4 days stress and then declined gradually. All groups subjected to injection stress (even those showing apparent habituation) have plasma cortisol titres that are significantly higher than the control group.

Table 5.5. The effect of stress over time on cortisol and MCH secretion.

Treatment		n	MCH pg/ml	Cortisol ng/ml
Control		7	159.3 ± 12.2	4.8 ± 1.3
Injected	2 days	11	378.9 ± 47.2 ***	45.6 ± 7.3 ***
"	4 days (30 min)	11	222.9 ± 24.7 aa	60.1 ± 9.5 ***
"	4 days (1 hr)	11	249.0 ± 20.1 ** a	79.6 ± 6.7 ***
"	6 days	11	189.4 ± 27.5 a	38.2 ± 10.3 ***
"	8 days	11	242.8 ± 22.9 ** a	20.4 ± 3.4 ***

Results are means ± s.e.m. Statistical analysis was performed on log-transformed data. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with uninjected controls. 'a' p < 0.05, 'aa' p < 0.01 compared to 2 day injected fish. Fish were adapted to a white background for 14 days before use. Controls were killed on the first day, and the remaining fish were injected once a day for 2, 4, 6 or 8 days between 10:30 and 11:00 with 1ml saline (0.8% NaCl). They were killed 1h after the final injection (unless indicated).

Fig 5.4.
The effect of stress over time on cortisol and MCH secretion. Fish were adapted
to white tanks for 2 weeks before use. Controls were killed on the first day of the
experiment and the remaining fish were injected once a day between 10:30 and
11:00 for 2, 4, 6, or 8 days with 1 ml saline (0.8 % NaCl). They were killed 1 h
after the final injection. Results are arithmetic means and standard errors,
statistical analysis was performed on log-transformed data.

☐ Control   ▨ Injected

★★ $p \leq 0.01$, ★★★ $p \leq 0.001$ compared to uninjected controls.
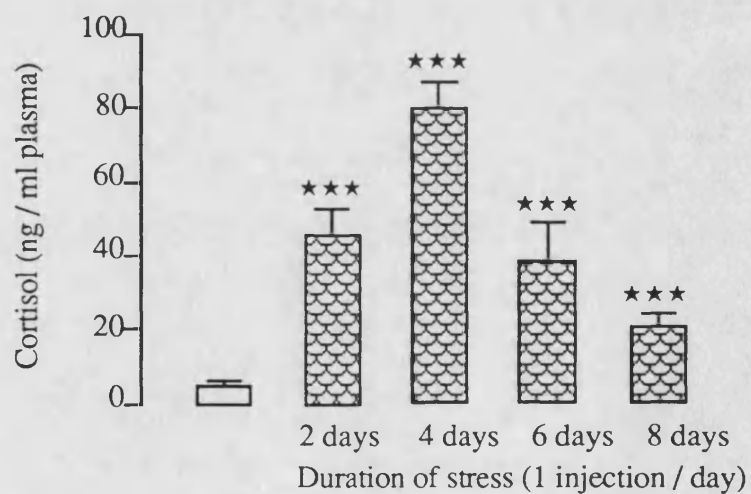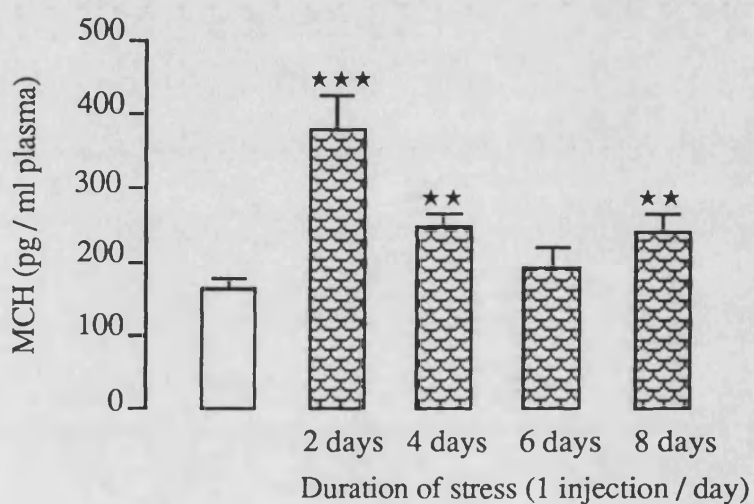
5.3.6. The recovery of plasma MCH and cortisol titres after repeated stress

White-adapted fish were divided into 7 groups held in separate tanks. Three groups were undisturbed for the duration of the experiment and served as controls. Four groups of fish were injected daily at 10 minute intervals between 10:30 and 11:00 over 3 days and were killed 1, 3, 8, and 23h after the final injection. The control groups were killed at 10:30, 13:30 and 18:00 of the third day. The results are shown in table 5.6. and fig 5.5. Plasma MCH titres were increased 1h after the last injection. After 3 hours values had fallen to pre-injection values but after 8 hours values were significantly higher in injected groups compared to the 18:00h controls. Control fish killed at 18:00 showed MCH levels significantly lower than the animals killed at 10:00 whereas the injected fish show MCH plasma titres similar to those uninjected fish killed at 10:00. Therefore repeated injection-stress has the effect of initially raising the MCH plasma titre but may also result in prolonged elevated titres at the nadir phase of the diurnal rhythm. This pattern is seen also in the cortisol profile for injected and control fish.

Table 5.6. The recovery of plasma MCH and cortisol titres after repeated stress.

Hours (after last injection)	n	MCH pg/ml Control	MCH pg/ml Injected	Cortisol ng/ml Control	Cortisol ng/ml Injected
0	8	106.1 ± 14.1		19.1 ± 4.4	
1	8		174.9 ± 18.8 *		83.7 ± 11.7 ***
3	8	80.4 ± 12.5	102.7 ± 15.3 a	23.3 ± 5.9	18.3 ± 6.2 a
8	8	67.2 ± 4.1	122.4 ± 5.2 *** a	3.3 ± 1.4 bb	22.4 ± 6.1 * a
23	8		83.6 ± 10.9 aaa		13.6 ± 3.6 aaa

Results are means ± s.e.m. Statistical analysis was performed on log-transformed data. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with uninjected controls, 'a' p < 0.05, 'aaa' p < 0.001 compared to fish sampled 1 hour after injection, 'bb' p < 0.01 compared to fish sampled at t=0 (10:30).
Fish were adapted to a white background for 14 days before use. They were injected without anaesthesia once a day at 10:30 for 3 days and killed 1h after the last injection.

Fig 5.5.

The effect of repeated stress and recovery on plasma hormone titres. Fish were adapted to white tanks for 2 weeks then injected without anaesthesia once a day for 3 days and killed 1, 3, 8 or 23 h after the last injection. Results are arithmetic means and standard errors, statistical analysis was performed on log-transformed data.

☐ Control   ⊠ Injected

★ $p \leq 0.05$,   ★★★ $p \leq 0.001$ compared to uninjected fish
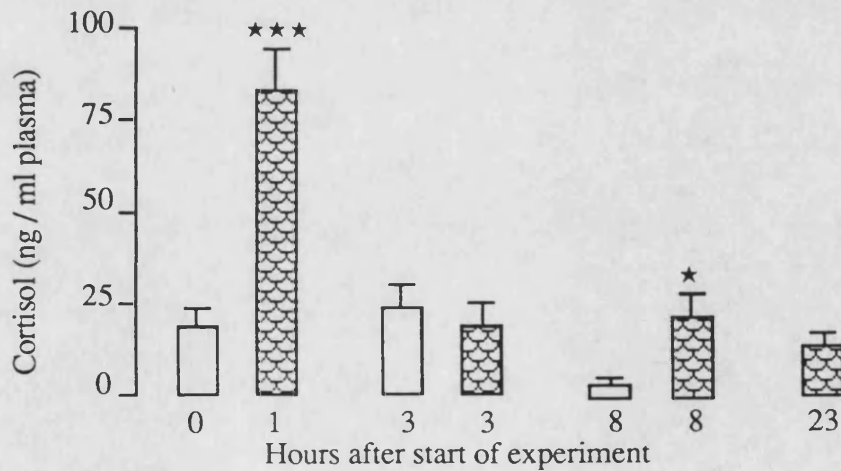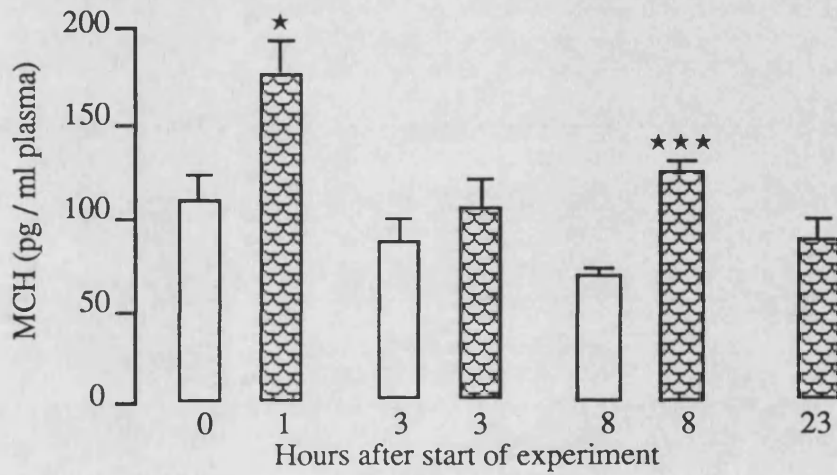
The data from the preceding experiments were collated in a single table to see if there was any apparent relationship between the stress-induced MCH and cortisol responses. The data shows that repeated stress causes an increase in both plasma MCH and cortisol values. The relative increase in plasma MCH, following stress, varied in different experiments between 1.2 and 8.9 (table 5.7., Fig 5.6.). There was no strict relationship between the two; in other words, a high increase in plasma cortisol was not necessarily linked to equally high response in MCH secretion or vice-versa.

Table 5.7. Summary of all experimental data showing variation in plasma titres of MCH and cortisol in fish killed 1 h after the last injection. Results are means ± s.e.m.

Expt	Days/ injections	MCH (pg/ml)			Cortisol (ng/ml)		
		Control	Injected	Factor of increase	Control	Injected	Factor of increase
5.3.2.[1]	4/5	83.6 ± 12.6	748.1 ± 142.1	8.9	7.0 ± 1.3	111.5 ± 21.1	15.9
5.3.3.	3/3	106.1 ± 14.1	174.9 ± 18.8	1.6	19.1 ± 4.4	83.7 ± 11.7	4.3
" [2]	3/3	67.2 ± 4.1	149.6 ± 19.6	2.2	3.3 ± 1.4	73.8 ± 7.2	22.3
5.3.4.	4/4	90.4 ± 9.8	373.3 ± 102.3	4.1	15.1 ± 4.3	296.2 ± 56.4	19.6
5.3.5.	2/2	159.3 ± 12.2	378.9 ± 47.2	2.3	4.8 ± 1.3	45.6 ± 7.3	9.5
" [3]	4/4		222.9 ± 24.7	1.4		60.1 ± 9.5	12.5
"	4/4		248.0 ± 20.1	1.6		79.6 ± 6.7	16.5
"	6/6		189.4 ± 27.5	1.2		38.2 ± 10.3	8.0
"	8/8		242.8 ± 22.9	1.5		20.4 ± 3.4	4.3

In all experiments, fish were injected at 10:30 and killed 1h after the final injection with the following exceptions:

1 Fish injected at 11:30 and 17:30 on the first day, then at 11:30 for remaining days.

2 Fish injected at 18:00.

3 Fish killed 30 min after final injection.

5.4. DISCUSSION

The results reported here show that MCH plasma concentrations can be increased by repeated stress. This response is subject to negative feedback by corticosteroids.

The effect is seen only in white-adapted fish in which plasma MCH titres are already high. It seems that the control of MCH release is subject to many inputs both stimulatory and inhibitory. The pathway controlling MCH in response to colour change appears to be dominant, so that in black-adapted fish the net inhibitory effect on MCH release prevents the release of the hormone in response to stress. An interesting parallel here is the response of αMSH to stress seen in the present work and also previously reported by Gilham and Baker (1985). Repeated stress induced increases in the release of αMSH which were sensitive to a negative feedback by dexamethasone. This phenomenon was observed only in black-adapted animals where plasma αMSH titres were already high, not in white-adapted animals in which the secretory activity of the melanotrophs is inhibited as a result of visual information.

A single stress, in the form of an injection without anaesthesia, is ineffective in raising the MCH plasma titre of white-adapted trout, although plasma cortisol titres are raised, as would be expected, one or two hours after the stress. However, when the same stress is administered on two or more consecutive days, MCH plasma titres are increased. This suggests that the initial stress must have altered the neurological and/or endocrinological state of the fish such that it now responds to the repeated stimulus with raised MCH release. It is not clear what factors are involved in this change of responsiveness but it could be due, for instance, to a change in the number of neurotransmitter

receptors on the MCH neurones after exposure of the fish to a single stress. A similar increase in responsiveness is shown by fish gonadotrophs to LHRH after a priming injection of LHRH (Crim et al., 1981). In rats, investigations into the changes in the HPA axis with a similar regime of repeated stress has been reported (Young and Akil, 1985). The neurological state of rats was demonstrated to alter with different forms of stress and was postulated to involve receptor changes. Such mechanisms may be operating in the fish HPI axis in the adaptation of cortisol secretion to chronic or repeated stress and such changes may be related to the MCH response described here. There may be innervation of the MCH neurones by CRF or other neurones and changes in the receptor levels or sensitivities on these cells may be involved in the triggering of the MCH response.

Dexamethasone and presumably other corticosteroids can feedback and reduce the response of the MCH cells to stress, possibly acting directly on the MCH neurones or at the MCH nerve terminals in the NIL, although other sites of action are also possible. For instance, it is known that dexamethasone will depress CRF secretion (Suda et al., 1984; Jingami et al., 1985) although the effect is not as marked as that on the pituitary. If CRF were involved in the stress-activated pathway which enhances the release of MCH, then dexamethasone will inevitably suppress the MCH response as well as the corticotroph response to stress.

The degree to which the animals respond to the stress of injection varied in different experiments, not only in the MCH response but also in the cortisol response (see table 5.7.). The factor of increase in MCH secretion

varies between 1.2 and 4.0 (mean 2.0 ± 0.3, n=8) with one exception, experiment 5.3.3. when the fish were injected twice on the first day. When plotted against the changes in plasma cortisol titres, there is apparently no precise relationship between the intensity of the two responses (see fig 5.6.). Even uninjected control fish show variation in their plasma MCH titres. This variability in hormone values is not due to assay artefact, since measurements of MCH by two different methods yielded similar values (r=0.938, Kishida et al., 1989). Many hormones show a diurnal rhythm in secretion, for example cortisol (Rance and Baker, 1981; Pickering and Pottinger, 1983), and MCH is no exception. Lights-onset is the trigger for MCH secretion and titres peak at mid photophase before declining through the rest of the day and on into the night (V Lyon, personal communication). In mammals there is evidence that it is more difficult to elicit a steroid response during the nadir of corticosterone secretion (Gibbs, 1970; Dunn et al., 1972). There is no evidence to suggest such a refractory period for the stress-related response of either MCH and cortisol in trout. Fish injected at 18:30 showed the same incremental rise in plasma cortisol and MCH as fish injected at 10:30. It is clear that factors other than the daily injection must modulate the sensitivity of MCH neurones to stress. Cortisol feedback is a possible factor, a history of chronic stress with raised cortisol titres would be likely to alter the sensitivity to a subsequent stress.

In summary, basal stress was variable in different experiments as indicated by control basal cortisol values. The responsiveness of MCH neurones also varied and this could be due to several different factors (a) the response may have been depressed by chronically elevated cortisol titres before the start of the experiment (b) the response may be increased due to repeated prior stress, which current experiments have

shown may enhance the response of the MCH neurones. Cortisol titres also vary suggesting differences in basal levels of stress and this probably reflects the conditions prevalent in the aquarium. It proved impossible to control certain factors, such as the quality of the running tap water or interaction between fish within groups such as bullying by a dominant trout, which could contribute to mild chronic stress.

It is clear that there could be interaction between MCH and the HPI axis. MCH is released during chronic stress and then may act to modulate and depress the HPI axis to further stress as suggested by Baker *et al.*, (1985; 1986). The site of action of this depressive effect of MCH will be discussed further in the next chapter.

CHAPTER 6

THE EFFECT OF REARING ON BLACK OR WHITE BACKGROUNDS ON MCH AND THE SENSITIVITY OF RAINBOW TROUT TO STRESS

6.1. INTRODUCTION

Previous work has shown that fish maintained on a black background secrete more cortisol in response to moderate stress than white-adapted fish (Rance and Baker, 1981; Gilham and Baker, 1985). This difference in responsiveness could be due to either the actions of MCH in white-adapted fish, the effect of αMSH on black-adapted fish or other factors, possibly psychological, not involving these two hormones.

The work described in this chapter examines how prolonged adaptation to black or white background colour affects the HPI axis and investigates possible sites in the HPI axis - hypothalamic, pituitary and interrenal - which are sensitive to modulation by MCH.

6.2. MATERIALS AND METHODS

6.2.1. Fish

Eyed eggs were obtained from Bibury Hatchery and the fry were reared in either white or black tanks for 15 months, after which time they weighed 150-200g. During the first 3 months of rearing they were kept under continuous light and thereafter on 18h L : 6h D. Fish were fed commercial trout food according to manufacturers instructions. Some immature fish of comparable size (~200g) were also obtained from a local fish farm.

6.2.2. Administration of Stress

Control fish remained undisturbed throughout each experiment. Some fish were mildly disturbed by daily activity in the aquarium. The injection of 1ml saline (0.8% NaCl) without anaesthesia into the peritoneal cavity was used as an intense stress. Animals were given 1 injection per day for four days and killed 1h after the final injection.

6.2.3. Killing methods

To avoid additional stress when blood samples were to be taken, fish were caught in a single group and anaesthetised together in phenoxyethanol (1:1667, v/v). Blood was collected according to the procedure described in Chapter 2. All fish within each group were bled within 1-5 min of catching. Fish used for *in vitro* incubations were decapitated without anaesthesia.

6.2.4. Extraction of hypothalami and pituitaries

Hypothalami and thalami were removed rapidly and separated into the ventral part containing the MCH perikarya, and the dorsal part including the posterior commissure. Pituitaries were separated into NILs and PDs. Each fragment was individually extracted in 500 µl 0.1 M HCl (on ice), and sonicated for 30 sec. Extracts were centrifuged and the supernatant stored in aliquots at -20° until assay. Extracts were diluted before use and the pH was adjusted to about 7.6 with 10 % Tris where necessary.

6.2.5. Dissection of brain and pituitary tissue for *in vitro* incubations

Hypothalami were removed immediately after decapitation, taking the entire hypothalamic/ thalamic region between the lateral sulci, and from the mid saccus vasculosus to the optic chiasma. The pituitary pars distalis (PD)

was cut from the rest of the gland, removing all pars intermedia (PI) cells as far as possible. Hypothalami were individually pre-incubated in 400 μl of Eagles' minimum essential medium (MEM) containing Hank's salts and Hepes Buffer (Flow Laboratories). PDs were incubated in 300 μl of the same medium. Incubations were done at 11° in polystyrene 24-well plates (Sterilin Ltd) placed in sealable plastic bags, gassed with oxygen and agitated gently. After the hypothalami had pre-incubated for 1h, the medium was renewed and the tissue was left to incubate for a further hour. MCH was immunoabsorbed from the medium to prevent its subsequent inhibitory effect on the PD. Neat antiserum (30 μl/ 300 μl medium) was added either at the beginning of the hypothalamic incubation period or at the the end, when the hypothalamic fragment was removed and the medium left for 30 min before its addition to the pituitary tissue. The PDs were pre-incubated for 2-2.5h and their medium was then replaced with 300 μl fresh MEM (controls) or with 300 μl hypothalamic incubation medium. After a further 30 min incubation the medium was collected and stored at -20° to await assay. (See fig 6.1.)

6.2.6. Dissection of interrenal tissue and *in vitro* incubations

Interrenal tissue in trout is not a discrete organ but occurs within the matrix of the pronephric or 'head' kidney. This tissue from white-adapted farm fish was separated sagittally into right and left halves, freed from connective tissue and diced into cubes <1mm³. The macerated tissue was then transferred to a polypropylene tube containing 3 ml of incubation medium. The medium was Hanks' balanced salt solution (HBSS) with 0.5 % BSA, pH 8. Tubes were tumble-mixed on a Stuart rotator at ≈30 rpm. The tissue was given two pre-incubations of 1½h each, the medium changed at

Fig 6.1.

Diagrammatic representation of the *in vitro* incubation protocol

Hypothalamus   **Pars Distalis**

A. Pre-incubation

1 Hour   2 Hours

Expt 1   Expt 2

B. Hypothalamic
   incubation
   1 Hour

C. Medium
   incubation
   30 min

Transfer medium

D. PD incubation 30 min. Store medium.

MCH antiserum added to medium
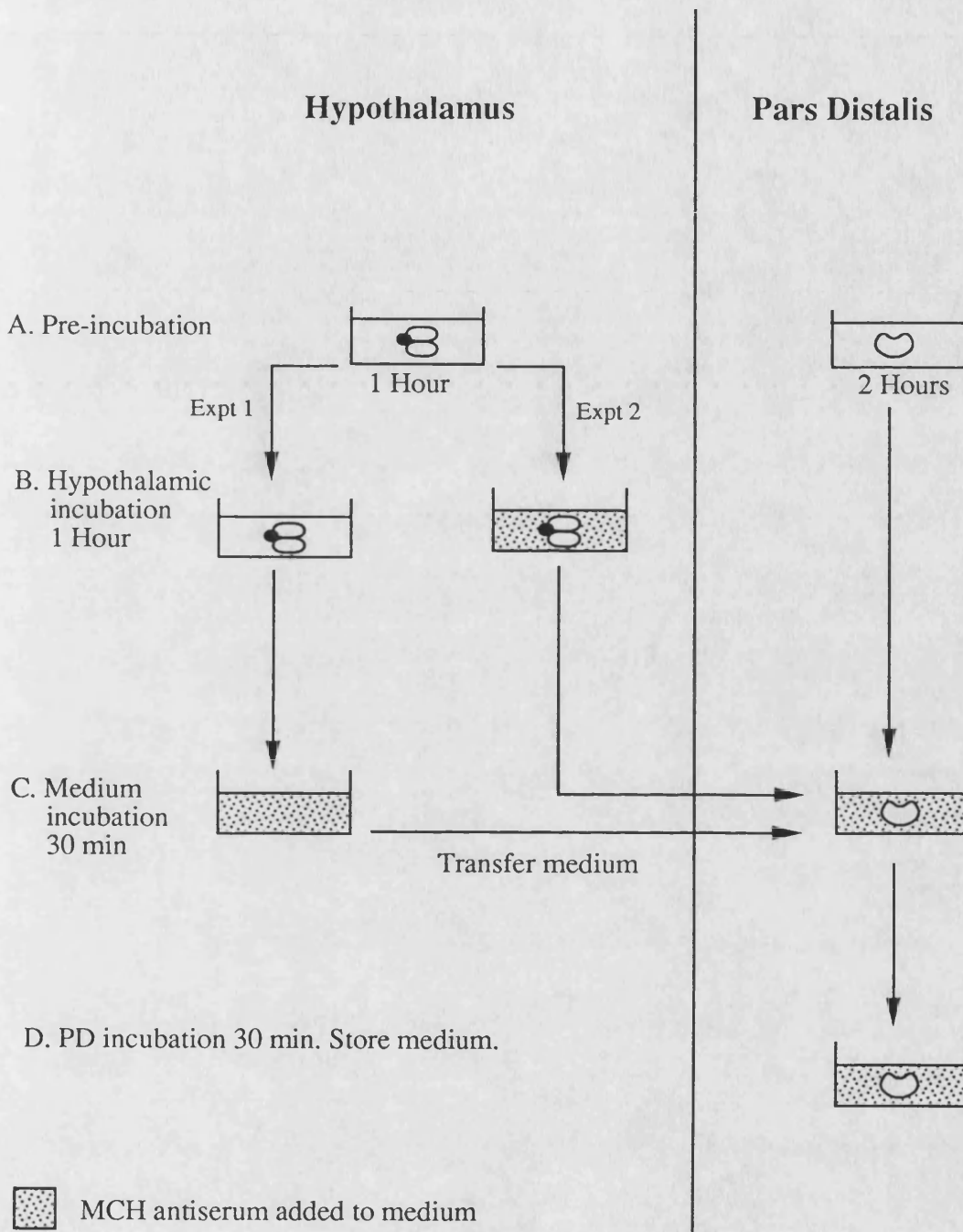
the end of each period. At the end of this time, basal cortisol release was attained. Tissue was then rinsed briefly in 2 ml medium and given a pre-stimulatory incubation for 1h in 3 ml medium, to measure basal cortisol release. After the pre-stimulatory incubation, the medium was replaced again and the tissue was stimulated with $ACTH_{1-24}$ for 1h. All samples were stored at $-20°$ until assay.

6.2.7. Extraction of cortisol from incubation medium

The cortisol from an appropriate volume of incubate (usually 10 μl) was extracted into 2 ml of distilled diethyl ether (stored over sodium wire). The tubes were vortexed, then placed in a freezing mixture of acetone and cardice. The unfrozen solvent was decanted into a fresh tube and dried under vacuum.

6.2.8. Measurement of hormones

All measurements were done by RIA methods as described in chapter 2. Plasma MCH was determined using the solid phase method, tissue MCH concentrations were measured by conventional RIA with charcoal separation. Plasma cortisol titres were measured using the method of Baker and Rance (1981). NPP-1 was measured using the RIA as described by Takahashi *et al.* (1987).

6.3. RESULTS

6.3.1. *In vivo* studies of the HPI axis

6.3.1.1. The influence of background colour and rearing conditions on the plasma and tissue MCH titres and responsiveness of the HPI axis to repeated stress Fish, reared for 15 months in either white or black tanks, were divided into 3 groups from either background colour

comprising one control and two experimental groups. The control black-
and white-reared fish (Group A) were sampled from the stock tanks in
which they had been reared (White, Black, unstressed). White-reared fish
had higher plasma MCH titres than black-reared fish and lower basal
cortisol levels (Table 6.1., Fig 6.2.). Black- and white-reared fish in
Group B were all transferred to clean white tanks and left for 11 days
(W→W and B→W, mild noise disturbance). Both groups show raised plasma
MCH titres. This anticipated increase in the black-reared trout is an
adaptation to background colour; the increase to much higher values in
the white-adapted fish may have been due either to the tanks being a
cleaner, brighter white compared to the stock tank or because the fish
were slightly stressed by transfer to their new environment, as indicated
by the rise in plasma cortisol titres. There was no statistical difference
between the plasma cortisol values of transferred black- or white-reared
fish in group B although values were nearly twice as high in the black-
reared fish. Black- and white-reared fish in Group C were also all
transferred to clean white tanks adjacent to Group B. After 7 days they
were injected with 1 ml saline once a day for 4 days and killed 1h after
the final injection (W→W, B→W, injection stress). The plasma cortisol
titres were elevated significantly in both W→W and B→W transferred,
injected fish; but the cortisol titres in the W→W fish increased less than
in the B→W fish. There was also a significant difference between the
plasma MCH levels measured in these two groups of fish. The plasma MCH
values in white-reared fish of Group C were significantly raised by the
injection stress compared with values in both Group A and Group B fish.
The MCH values of the black-reared fish, however, were significantly lower
than those of mildly stressed fish in group B, and not significantly
different from unstressed black-reared values in Group A. The MCH and
cortisol titres are inversely related, as can be seen in Fig 6.2. The

results show that the white-reared trout with high plasma MCH titres are

less easily stressed than their black-reared counterparts.

Table 6.1. The influence of background colour and rearing conditions on plasma MCH and cortisol titres.

Group/ Treatment	n	Plasma MCH pg/mℓ	Plasma Cortisol ng/mℓ
A. Controls			
White	6	101.9 ± 21.6 *	2.4 ± 0.4 **
Black	6	27.4 ± 7.2	5.9 ± 1.1
B. Transfer/Mild stress			
W→W	5	269.8 ± 53.8 *	24.7 ± 9.7
B→W	6	113.9 ± 13.4	45.5 ± 20.9
C. Transfer/Injection			
W→W	8	603.0 ± 94.6 ***	79.1 ± 15.7 ***
B→W	8	54.3 ± 12.2	204.3 ± 11.1

Results are means ± s.e.m. Statistical analysis was performed on log-transformed data.
* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ compared to black adapted or B→W fish.
Fish were reared on either a white or black background (group A), transferred to white tanks and subjected to either mild (group B) or severe stress (group C, injected once a day for 4 days).

Fig 6.2.
The influence of background colour and rearing conditions on plasma MCH and
Cortisol titres. Fish were reared in either black or white tanks (Group A, Controls),
transferred to white tanks and subjected to either mild noise stress (Group B,
Mild stress & Transfer), or severe stress (Group C, Injection & Transfer, injected
once a day for 4 days, killed 1 h after final injection). Results are arithmetic means
and standard errors, statistical analysis was performed on log-transformed data.

☐ White-reared stocks   ■ Black-reared stocks

▨ White to white   ▨ Black to white

★ p ≤ 0.05, ★★★ p ≤ 0.001 compared to black stocks or black to white transferred
          fish

✪ p ≤ 0.05, ✪✪ p ≤ 0.01, ✪✪✪ p ≤ 0.001 compared to Group A fish

☆ p ≤ 0.05, ☆☆☆ p ≤ 0.001 compared to Group B fish
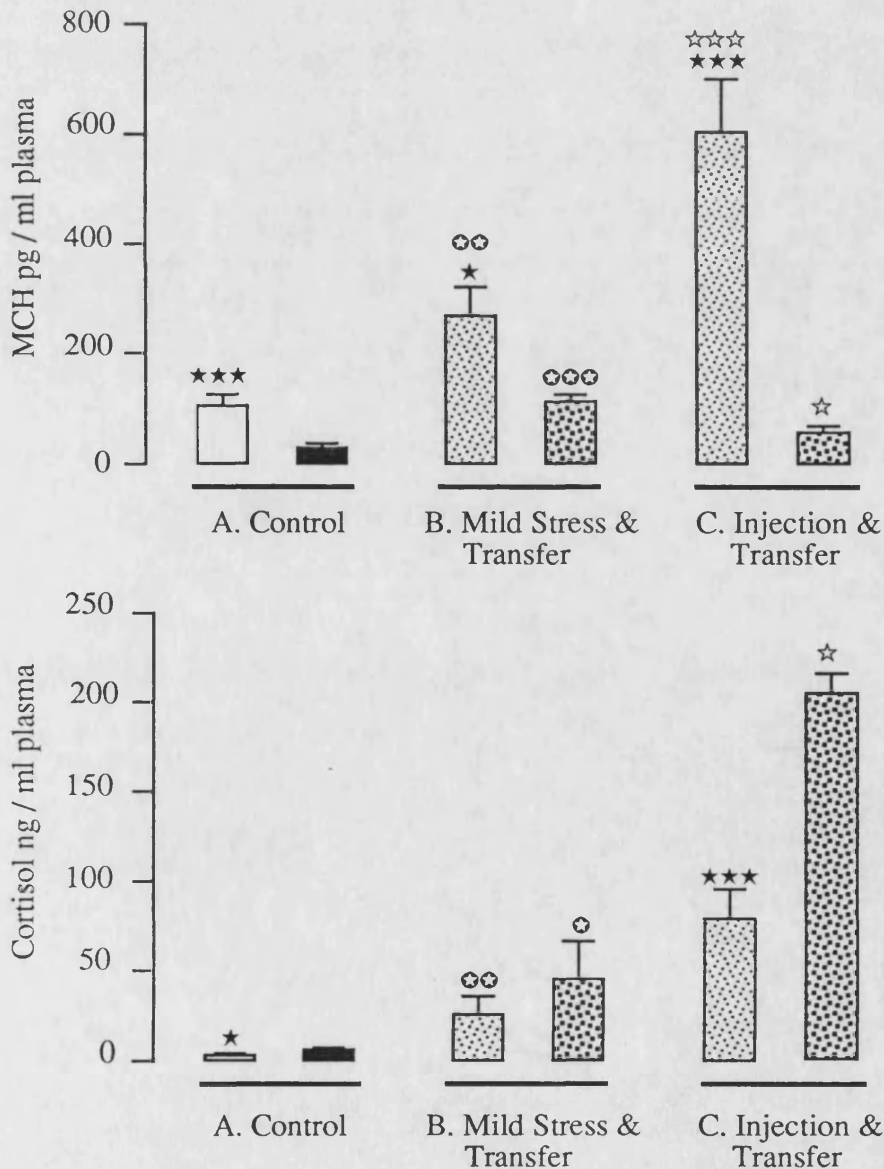
The tissue MCH contents of fish reared in black or white tanks and given different degrees of stress were examined. When white-reared fish which possessed abundant stores of MCH in their hypothalami and pituitaries were transferred to clean white tanks, the MCH content of the ventral hypothalamic region, containing the MCH perikarya, was significantly decreased, but the dorsal area, containing axons and nerve terminals, was unaffected (Table 6.2a.). Chronic stress caused a further slight but statistically insignificant depletion of MCH in the ventral region, but halved the MCH content of the dorsal hypothalamus. In black-reared fish, which contained much less tissue MCH, the response was more striking. Transfer to white tanks caused an absolutely less but proportionately greater loss of MCH from both dorsal and ventral hypothalamic regions (Table 6.2a, b.). Chronic stress did not further deplete the very low concentrations in the dorsal hypothalamus but caused a statistically significant loss of MCH from the ventral hypothalamus. The difference in the response of white- and black-reared fish is seen more clearly by expressing the contents as a percentage of control values (Table 6.2b.).

Table 6.2a. MCH content of hypothalami taken from white- and black-reared fish subjected to transfer, mild noise stress or injection stress.

Condition	n	MCH (ng/Hyp) Dorsal	Ventral	Total
A. Controls				
White	6	9.6 ± 0.1 *	309.0 ± 39.1 ***	318.8 ± 40.3 ***
Black	6	3.2 ± 0.1	8.3 ± 1.1	11.5 ± 1.2
B. Transfer/Mild stress				
W→W	5	8.1 ± 2.2 ***	159.0 ± 26.1 *** a	167.1 ± 27.6 *** a
B→W	6	0.8 ± 0.1 aa	2.1 ± 0.2 aaa	2.9 ± 0.2 aaa
C. Transfer/Injection				
W→W	8	3.8 ± 0.4 ***,bbb	135.9 ± 16.0 ***	139.7 ± 16.1 *** aa
B→W	8	0.7 ± 0.1 aaa	1.0 ± 0.1 bbb	1.8 ± 0.2 bb

Results are means ± s.e.m. Statistical analysis was performed on log-transformed data. * p < 0.05, *** p < 0.001 compared to black adapted or B→W fish. 'a' p < 0.05, 'aa' p < 0.01, 'aaa' p < 0.001 compared to Group A counterparts, 'bb' p < 0.01, p < 0.001 compared to Group B counterparts.
Fish were reared on either a white or black background (group A), transferred to white tanks and subjected to either mild (group B) or severe stress (group C, injected once a day for 4 days).

Table 6.2b. MCH content (expressed as a % of control values) of hypothalami taken from white- and black-reared fish subjected to transfer, mild noise stress or injection stress.

Condition	n	MCH content as % of control values Dorsal	Ventral	Total
B. Transfer/Mild stress				
W→W	5	84	51	53
B→W	6	25	25	26
C. Transfer/injection				
W→W	8	39	43	43
B→W	8	21	12	15

Results are a % value of control levels (group A = 100%).
Fish were reared on either a white or black background (group A), transferred to white tanks and subjected to either mild (group B) or severe stress (group C, injected once a day for 4 days).

The changes in MCH content of the NILs taken from fish subjected to transfer with or without injection stress follow the trend seen in total hypothalamic content. That is, white-reared fish lose less MCH upon transfer with or without injection stress than black-reared fish when measured as a proportion of control levels, but in absolute terms white-reared fish lose much more MCH than black-reared fish (see table 6.3.). The gross decline in the pituitary MCH content in the stressed, black-reared fish (Group C, B→W), to 1 % of control values, is relevant when interpreting the fall in plasma MCH content of those fish.

In conclusion, stress stimulates MCH release from the pituitary (as indicated by the increased plasma levels) and this may be responsible for most of the peptide depletion from the ventral perikaryal region. MCH stores in the dorsal hypothalamic region can also be affected by both mild and intense stress, but whether this is apparent depends on the amount of MCH available for release.

Table 6.3. MCH content of pituitary neurointermediate lobes (NIL) taken from white- and black-reared fish subjected to transfer, mild noise stress or injection stress.

Condition	n	MCH ng/NIL		% of control
A. Controls				
White	6	1050.0 ± 151.8 ***		
Black	6	236.3 ± 50.0		
B. Transfer/Mild stress				
W→W	5	549.0 ± 30.3 ***	aa	52
B→W	6	20.0 ± 3.5	aaa	8
C. Transfer/Injection				
W→W	8	580.0 ± 77.6 ***	a	55
B→W	8	3.4 ± 0.6	bbb	1

Results are means ± s.e.m. Statistical analysis was performed on log-transformed data. *** p<0.001 compared to black adapted or B→W fish. 'aa' p ‹ 0.01, 'aaa' p ‹ 0.001 compared to Group A counterparts. 'bbb' p ‹ 0.001 compared to Group B counterparts.
Fish were reared on either a white or black background (group A), transferred to white tanks and subjected to either mild (group B) or severe stress (group C, injected once a day for 4 days).

6.3.2. *In vitro* studies of the HPI axis

6.3.2.1. The effect of MCH on cortisol secretion from the interrenal *in vitro* The possibility that MCH acts directly on the interrenal to depress the release of cortisol was investigated. Interrenals, from farm fish, were dissected into right and left paired halves, one half of each pair serving as a control while MCH was added to the medium of the opposite half. The medium of each pair was MEM alone or contained added $ACTH_{1-24}$ $(5 \times 10^{-10} M)$ as shown in table 6.4. The addition of MCH had no effect on basal or ACTH stimulated cortisol release by interrenal tissue.

Table 6.4. The effect of MCH $(10^{-8} M)$ on cortisol secretion from the interrenal *in vitro*.

Conditions	n	Cortisol ng/½interrenal/h
BASAL		
Control	6	16.4 ± 7.2
MCH $(10^{-8} M)$	6	17.9 ± 7.8
ACTH-STIMULATED		
Control	10	64.9 ± 9.8
MCH $(10^{-8} M)$	10	58.9 ± 8.3

Results are means ± s.e.m.
Basal release and $ACTH_{1-24}(5 \times 10^{-11} M)$-stimulated experiments were done using paired interrenal halves.

6.3.2.2. the effect of rearing conditions and background colour on cortisol secretion by trout interrenals *in vitro* Since MCH had no direct effect on cortisol release from interrenals taken from farm-reared fish, the effect on adrenal responsiveness of rearing fish in black or white-coloured tanks was investigated. Basal plasma cortisol levels in such aquarium-reared fish suggest they may be chronically, if mildly, stressed compared with farm-reared fish. Fish reared on either a white or a black

background, or fish from a local fish farm were decapitated and half of each interrenal was removed. After a 3hr pre-incubation each half was given two successive incubations; first without, and then with $ACTH_{1-24}$ ($5 \times 10^{-9}M$). Interrenals of white- and black-reared fish released similar basal amounts of cortisol which was significantly greater than the levels secreted by tissue from farm-reared fish (table 6.5.). After stimulation with ACTH, tissue from black- and white-reared fish both secreted similar amounts of cortisol and this was significantly more than that secreted by the interrenals of farm-reared fish. However when the data are expressed as a ratio of stimulated/unstimulated release, it is seen that all groups respond to a similar degree (Table 6.5., Fig 6.3.). Hence the apparently greater response to ACTH of aquarium-reared fish compared with farm-reared fish suggests the former had more interrenal tissue in their head kidneys, but that the sensitivity to ACTH was the same in all groups.

Table 6.5. The effect of rearing conditions and background colour on cortisol secretion by interrenals *in vitro*. Statistical analysis was performed on log-transformed data.

| | n | Cortisol (ng/½interrenal/hr) | | Ratio |
		Basal	Stimulated	Stim/Unstim
Farm	6	19.4 ± 5.1	464.6 ± 47.1	28.2 ± 4.0
White	6	32.8 ± 4.2 *	1148.1 ± 115.7 ***	37.6 ± 5.4
Black	6	33.2 ± 5.1 *	1106.1 ± 97.0 ***	38.2 ± 7.8

Results are means ± s.e.m. Statistical analysis was performed on log-transformed data. * p < 0.05 *** p < 0.001 compared to farm fish.
Stimulation was achieved by $ACTH_{1-24}$ ($5 \times 10^{-9}M$) at a concentration of 5×10^{-9} M. White and black reared fish were maintained under conditions of mild chronic stress.

Fig 6.3.

The effect of rearing conditions and background colour on the responsiveness of interrenals to ACTH$_{1-24}$ (5×10^{-9} M) *in vitro* . White- and Black-reared fish were maintainedunder conditions of mild chronic stress. Results are arithmetic means and standard errors, statistical analysis was performed on log-transformed data.

[legend] Farm-reared   □ White-reared   ■ Black-reared

★ $p \leq 0.05$,   ★★★ $p \leq 0.001$ compared to Farm-reared fish
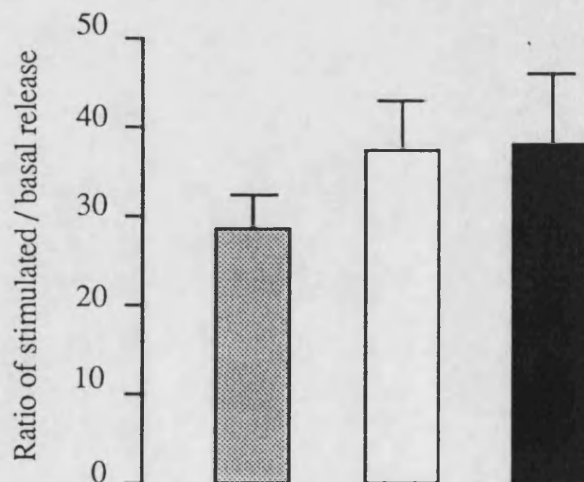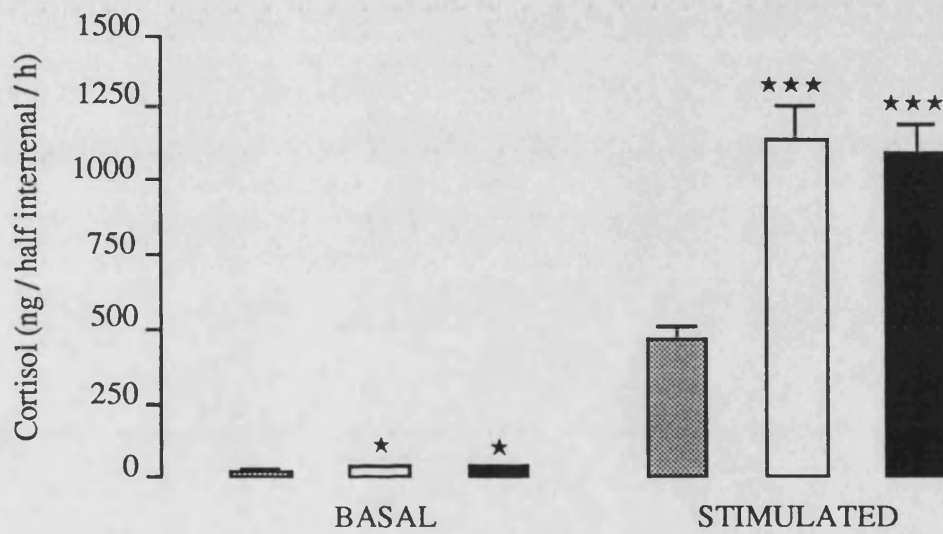
6.3.2.3. Corticotrophic activity and rearing conditions Since MCH had no

effect on the interrenal, its influence on the pituitary and hypothalamus

was investigated. The fish pars distalis (PD) secretes NPP-1 into the

incubation medium, and measurement of this POMC fragment can be used as

an indicator of corticotrophic activity provided the pars intermedia is

removed. Tissue was used from fish reared in the aquarium in white or

black tanks and from farm-reared fish. There was no difference in the

basal release of NPP-1 by PDs from white- or black-reared fish, but the

tissue from white-reared fish released significantly more NPP-1 than PDs

from farm reared fish (table 6.6., fig 6.4.). The amount of NPP-1 released

by PDs from black-reared fish was apparently higher than that released by

farm fish but the difference was not statistically significant due to

larger standard errors. These data support the results of the previous

experiment (6.3.3) suggesting that fish reared under conditions of mild

chronic stress have a hyperactive HPI axis.

Table 6.6. Basal corticotrophic cell activity in PDs of fish reared in different conditions.

Condition	n	Corticotrophic secretion (as NPP-1 release) ng/PD/30 min
Farm	6	2.5 ± 0.6
White	6	11.1 ± 3.1 *
Black	6	11.4 ± 3.4

Results are means $\pm$ s.e.m. Statistical analysis was performed on log-transformed data. * p<0.05 compared to farm fish, statistical analysis was performed on log-transformed data.
White and black reared fish were maintained under conditions of mild chronic stress.

Fig 6.4.

The ability of pars distales (PDs) from fish reared on different backgrounds and conditions to release ACTH as measured by NPP-1 release *in vitro*. White- and Black-reared fish were maintained under conditions of mild chronic stress. Results are arithmetic means and standard errors, statistical analysis was performed on log-transformed data.

★ p ≤ 0.05 compared to Farm-reared fish
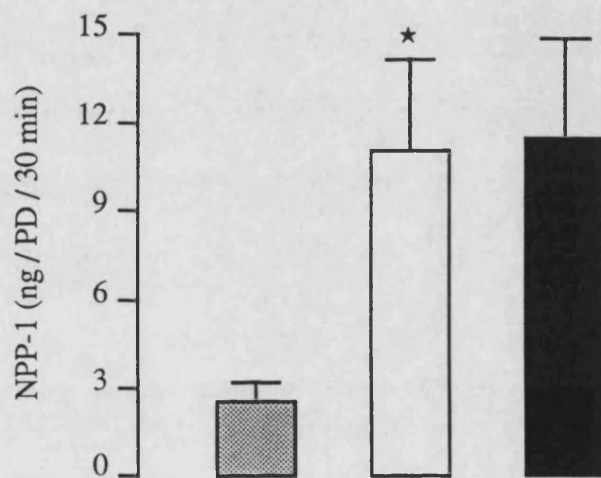
6.3.2.4. The effect of MCH on CRF release *in vitro* Trout hypothalami

incubated *in vitro* have previously been shown to release 'CRF' into the

medium as assessed by the ability of the hypothalamic medium to

stimulate the release of POMC-derived fragments from control PDs (Baker

et al., 1985). Two experiments were done to test whether the hypothalami

from black- or white-(aquarium)-reared fish secrete similar amounts of

CRF when incubated *in vitro*. In a first experiment, hypothalami were

obtained from fish that were unstressed (decapitated) or stressed (killed

by a blow to the head and left for 5 min before decapitation).

Hypothalami were incubated individually for 1h in 400 μl medium, after

which PDs from farm-reared fish were incubated in 300 μl of this

hypothalamic medium for 30 min. The amount of NPP-1 released by these

PDs should reflect the amount of bioactive CRF in the medium (Bird *et al.*,

1987). The MCH released from the hypothalami during incubation was

immunoabsorbed from the medium for 30 min before its addition to the

PDs, since MCH has depressive effects on corticotrophic activity (Baker *et

al.*, 1985). In this first experiment, hypothalamic medium from black or

white unstressed fish stimulated NPP secretion from PDs of farm fish to a

similar extent (table 6.7.). A severe stress caused apparently increased

secretion of CRF from the hypothalamus but the difference was not

statistically significant (Table 6.7.). In a second experiment MCH

antiserum was added to the hypothalami during their incubation period,

thus removing the influence of MCH from the neurones secreting 'CRF'. PDs

from farm-reared fish were then incubated in this medium or in control

medium as before. Under these conditions, hypothalami from white-reared

fish secreted significantly more 'CRF' than did hypothalami from black-

reared fish.

Table 6.7. The effect of MCH on CRF release *in vitro*.

Origin of		n	Medium added to Hypothalami	Pituitaries	NPP release (ng/PD/30min)
Experiment 1					
--		6	---	MEM+MCH a/s	3.0 ± 0.4
White reared	unstressed	6	MEM	Hth med+MCH a/s	5.9 ± 0.5 f
	stressed	6	MEM	Hth med+MCH a/s	8.2 ± 1.2 ff
Black reared	unstressed	6	MEM	Hth med+MCH a/s	6.2 ± 0.3 ff
	stressed	6	MEM	Hth med+MCH a/s	11.0 ± 3.3 f
Experiment 2					
--		6	---	MEM + MCH a/s	2.5 ± 0.6 ww
White reared		12	MCH a/s	Hth med	11.1 ± 1.7 bbb
Black reared		12	MCH a/s	Hth med	4.4 ± 0.5

Results are means ± s.e.m. Statistical analysis was performed on log-transformed data. bbb p < 0.001 compared to PDs treated with hypothalamic medium from black adapted fish. 'ww' p < 0.01 compared to PDs treated with hypothalamic medium from white-reared fish. , 'f' p < 0.05, 'ff' p < 0.01 compared to PDs without hypothalamic medium.

Hypothalami from white- or black- reared trout were incubated *in vitro* with or without MCH anti-serum (a/s). The incubation medium was then added to pars distales from farm-reared fish; control PDs did not receive hypothalamic medium. Groups where MCH had not been immunoabsorbed in the first (hypothalamic) incubation period received antiserum during the pituitary incubation medium.

Fig 6.5.
The effect of MCH on CRF release *in vitro* .
Experiment 1. Fish were killed by decapitation and their tissues removed (Controls),
or stunned by a blow to the head and left for 5 min before removal of tissues for
incubation (Stressed). MCH antiserum was added to the medium after removal of
the hypothalami prior to the pituitary incubation.
Experiment 2. Hypothalami from white- or black-reared trout were incubated
with or without MCH antiserum (a/s). The incubation medium was then added to
pars distales from farm-reared fish; controls did not receive hypothalamic medium.
Groups where MCH had not been immunoabsorbed in the first (hypothalamic)
incubation period received antiserum during the pituitary incubation period. Results
are arithmetic means and standard errors, statistical analysis was performed on
log-transformed data.

▨ Farm PD only   ☐ Farm PD treated with hypothalamic medium from
                              white-reared fish

■ Farm PD treated with hypothalamic medium from black-reared fish.

★ p ≤ 0.05, ★★ p≤ 0.01 compared to farm PDs alone

☆☆☆ p ≤ 0.001 compared to farm PDs treated with hypothalamic medium from
black-reared fish

Experiment 1

MCH a/s added post   MCH a/s added during
hypothalamic incubation   hypothalamic incubation

Experiment 1   Experiment 2
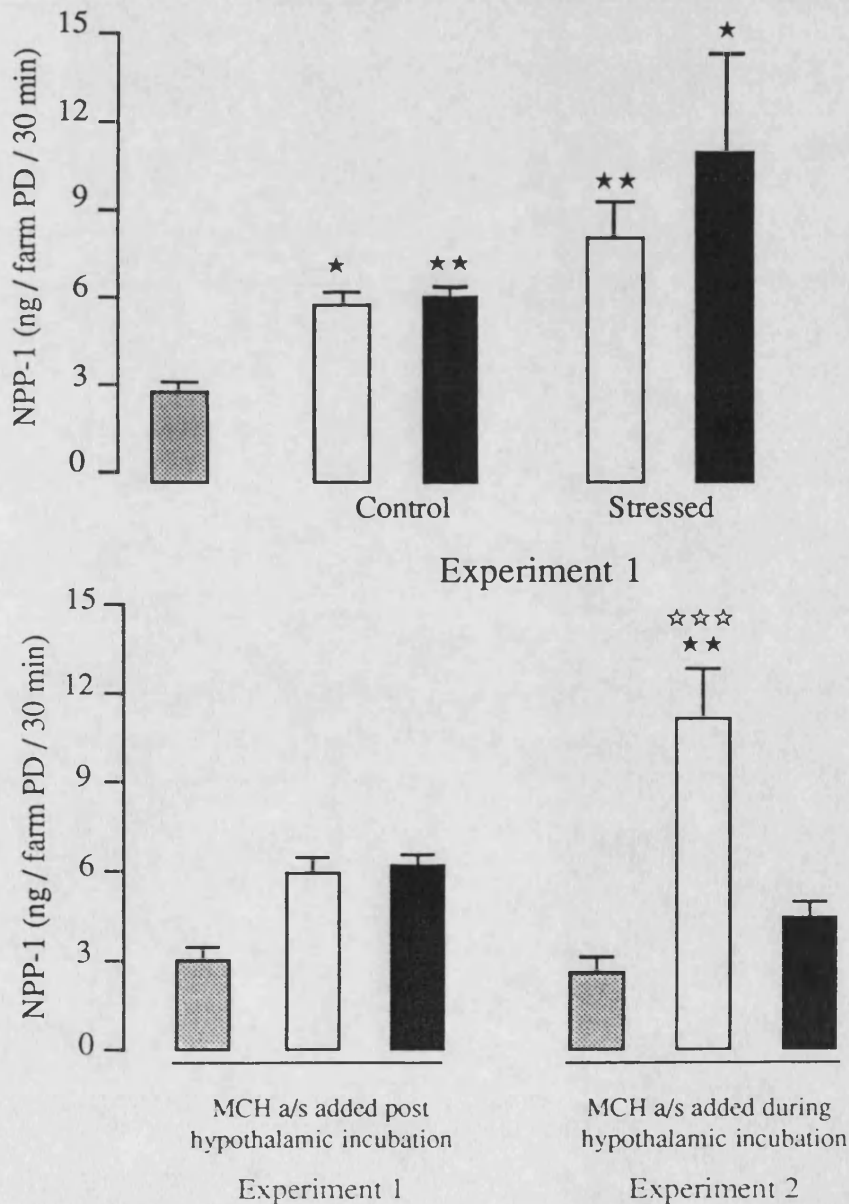
6.4. DISCUSSION

The previous chapter discussed the response of MCH secretion to repeated stress and the effects on this of dexamethasone but left unanswered the converse question whether MCH influences the HPI axis and if so its site of action. Apart from work reported by Baker and co-workers (1985, 1986) nothing is known about the action of MCH on the HPI axis. Their data showed that MCH can reduce the CRF-induced release of ACTH from the trout PD *in vitro*. They also showed that PDs from white-adapted fish, or from black-adapted fish implanted with minipumps containing MCH, released less ACTH *in vitro* than PDs from control black-adapted fish. The present work has provided more information about the activity of MCH on the HPI axis. When fish were reared in either black or white tanks their plasma MCH titres were significantly different. This is not surprising since MCH is released as a paling hormone. Additionally, their levels of plasma cortisol were different, the white-reared fish showing slightly but significantly lower concentrations than the black-adapted fish. This confirms earlier reports by other workers (Baker and Rance, 1981; Gilham and Baker, 1985). The plasma titres of MCH and cortisol are inversely related. When white- or black-reared fish were transferred to clean white tanks, and subjected to mild disturbance, MCH plasma titres rose in both B→W and W→W transferred fish. This rise is not surprising in the case of the B→W fish; that in W→W fish may be due to the tanks being a cleaner white than the stock tanks, or to stress of transfer and disturbance causing increased MCH release, since their circulating cortisol levels were higher than in the stock fish. B→W fish had apparently higher cortisol plasma titres than W→W fish but this was not significantly different, although once again the plasma MCH and cortisol titres are inversely related. If the transfer to white tanks is followed by repeated

injection stress, the difference between the plasma MCH titres of B→W and W→W fish is now very marked. The plasma MCH titre of B→W stressed fish appears to be decreased and is not significantly different to that in the black-reared stock fish. Conversely, plasma MCH in the W→W stressed fish is significantly higher than either the stock white-reared fish or the W→W mildly stressed fish. The plasma concentrations of cortisol are once more inversely related to those of plasma MCH, the B→W stressed fish having significantly higher plasma cortisol than the W→W stressed fish. It is apparent, therefore that trout with high plasma MCH titres release less cortisol than those in which plasma MCH is low. MCH seems to be modulating the activity of the HPI axis in these fish.

The concentrations of MCH in the ventral hypothalamus and pituitary NIL were found to decline following transfer to white tanks. This is in agreement with the increase in plasma MCH titre; ie the loss of MCH from these tissues is associated with the increased secretion of hormone as expected. Stress results in proportionately similar losses of MCH stores from both the dorsal and ventral hypothalamus which could reflect an increase in MCH release and a failure of synthetic activity to replace the depleting stores. The role of central MCH is uncertain but it might influence the secretion of CRF.

In summary, it is possible to interpret the results of the first part of this section as follows: the white-reared fish release more MCH under stress, which modulates (depresses) the activity of the HPI axis. Black-reared fish subjected to transfer to a white background are able to release MCH and show plasma MCH titres comparable to white-reared stock fish. However, when severely stressed they are unable to increase MCH release further due to low reserves of the peptide and presumably a low

synthetic capacity: the available MCH becomes depleted and is insufficient to modulate the activity of the HPI axis. Plasma cortisol therefore rises.

The site of modulation of the HPI axis by MCH was investigated. The effects of MCH *in vitro* on basal and ACTH-stimulated cortisol release from the interrenal were demonstrated to be negligible. Additionally, long-term administration of MCH (in the form of rearing on a white background) could not be shown to have had any influence on the responsiveness of the interrenal to ACTH *in vitro.* However, both black- and white-reared fish were shown to have hypertrophied interrenals suggesting that they had been exposed to slight but chronic stress.

Since MCH did not affect the interrenal by direct hormonal action, the other possible target organs through which MCH might modulate the HPI pathway were the brain and the pituitary. Other workers have already shown that MCH can reduce CRF-stimulated ACTH release from the PD *in vitro* (Baker *et al.,* 1985). In the present study, PDs from white- and black-reared fish showed similar levels of corticotrophic activity *in vitro* . These results apparently contrast with the findings of Baker *et al.* (1985, 1986), who reported that PDs from short-term white-adapted fish showed depressed ACTH secretion *in vitro* compared to their black-adapted counterparts. The difference between our and their observations is probably due to the fact that Baker *et al.* acutely stressed their fish immediately before the experiment; the difference they observed might therefore be attributable to different levels of CRF secretion between fish from black and white backgrounds, rather than a difference in the basal activity of the corticotrophs. Our observations showed that glands from fish reared in the University aquarium released significantly more POMC product than PDs from farm fish. This, together with the greater basal release of cortisol by their interrenals, shows that the University

fish were probably reared under conditions of slight but chronic stress, resulting in hypertrophy of both corticotrophs and interrenal tissue.

Baker and co-workers (1985) did not examine CRF secretion but allowed the possibility that MCH could have a modulatory influence on the HPI axis via the brain. While it is possible that circulating MCH could affect the brain, it is not known whether it can cross the blood-brain barrier. Additionally MCH fibres are distributed in various regions of the brain (Bird et al. 1989) and the peptide could therefore be released locally and exert its neuromodulatory effects. Experimental results from incubated hypothalami suggest that MCH released in the dorsal hypothalamus during stress might influence CRF secretion. Hypothalami from black- or white-reared fish released similar amounts of CRF-bioactivity when they were incubated in vitro. The interpretation of this observation is not simple, however, because tissue from the two groups of fish contain very different amounts of MCH, which is also released into the medium and could potentially influence the 'CRF' neurones. The results of experiments in which MCH antiserum was added to the incubated hypothalami suggest that this does in fact occur: immunoneutralization of MCH in and around the hypothalamus significantly enhanced the release of 'CRF' from brain tissue of white-reared fish, indicating that MCH normally inhibits the release of 'CRF'. Since CRF release by hypothalami from the black-reared fish was significantly lower and not affected by immunoneutralization, the results suggest that the 'CRF' content must be greater in white-reared fish, although its release is depressed in vivo. A possible interpretation is suggested in Fig 6.6. If MCH depresses CRF and ACTH secretion, cortisol titres will also be reduced; low cortisol, like adrenalectomy, will then result in an enhanced CRF synthesis and release until plasma cortisol titres attain "set point" basal levels. The hypothalami of white-reared

trout may have more CRF cells or more CRF production per cell than those of black-reared fish. Whether MCH affects the CRF neurones directly or acts indirectly via other inter-neuronal pathways is not known. Fryer and Peter (1977) found that lesions in the habenular region led to hypersecretion of ACTH and postulated an inhibitory centre , possibly controlling the release of CRF. Since MCH fibres project to the habenular region they may also influence the HPI axis at this site.

In summary, the data reported in this chapter show:

1. Differences in MCH production and release are associated with differences in the HPI axis *in vivo*.

2. MCH has no effect at the interrenal level *in vitro*.

3. MCH can inhibit 'CRF' release from the hypothalamus *in vitro*.

Fig 6.6.

A. Postulated mechanisms for the interaction of MCH with the HPI axis in white-reared trout.
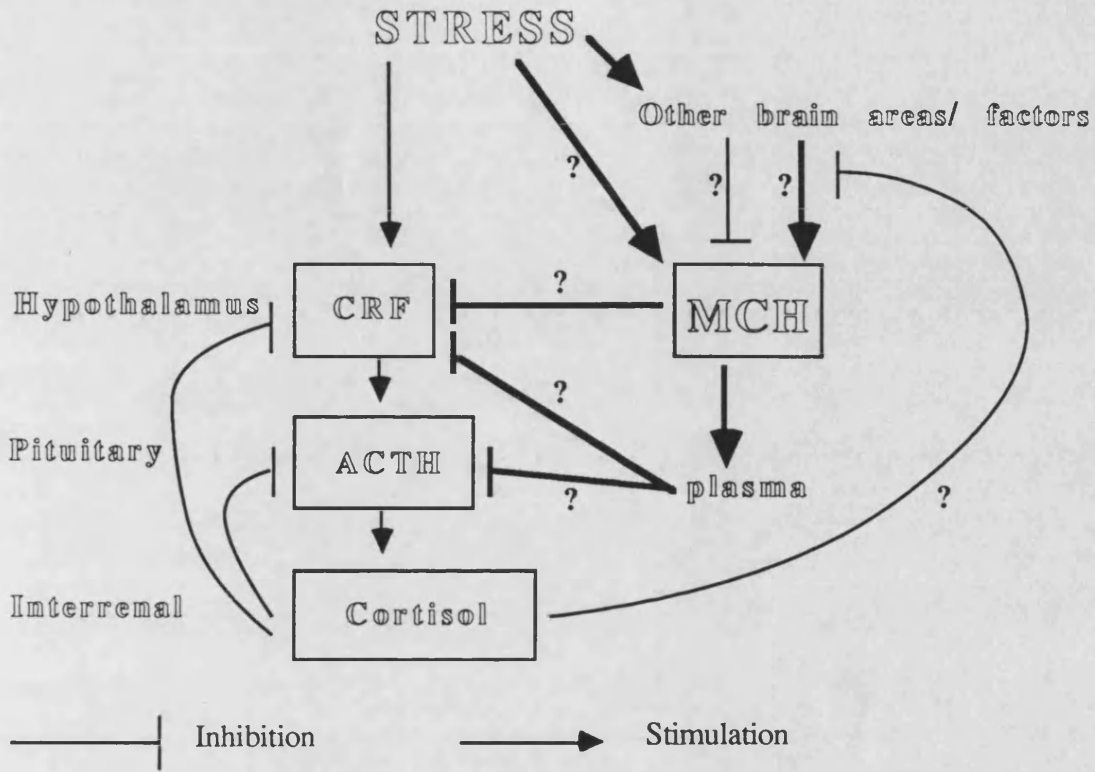
B. Postulated mechanisms for the interaction of MCH with the HPI axis in black-reared trout.

Table 7.1. The effect of background colour and rearing on MCH synthesis and storage.

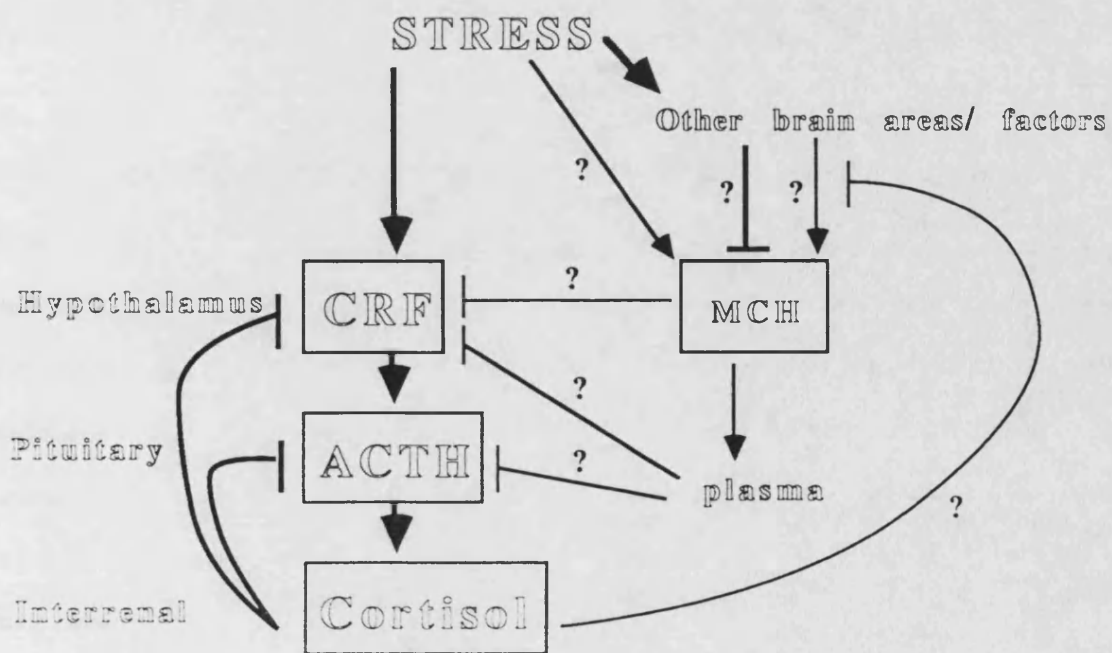| Conditions	n	Weight (g)	Concentration of MCH (ng)		Pit/Hyp	(pg/ml)
			Hypothalamus	Pituitary		Plasma
BLACK	6	2.2 ± 0.2	0.9 ± 0.1	4.7 ± 0.6	6	---
	12	134.3 ± 2.7	9.2 ± 1.1	231.3 ± 25.4	25	27.4 ± 7.2
WHITE	11	2.8 ± 0.2	9.7 ± 1.5	25.4 ± 4.3	26	---
	12	183.8 ± 17.8	249.5 ± 29.2	1089.4 ± 93.4	4	101.9 ± 21.6

Fish were reared in either black or white tanks under a long photoperiod and sampled at 4 or 15 months.

Looked at in a different way, the pituitary: hypothalamus ratio in adults is 25 for B adults but only 4.3 for W adults. It is possible that the relatively lower pituitary content in W fish reflects their more rapid rate of hormone release into the blood: why MCH should accumulate so much more extensively in the perikarya is not clear. It could be anticipated *a priori,* that hormone granules would be translocated to their site of release (terminals in the pituitary or brain) as soon as they are released from the golgi; clearly this does not occur, and some are stored within the cell bodies. The nature and routes of communication between perikaryon and nerve terminals regulating granule transport are not known, but possibly include retrograde transport mechanisms.

It is not known whether under constant background colour conditions the accumulation of MCH increases in a linear relationship with body weight; the two points on each line in fig 7.1. were the only two measured and are not intended to imply such a relationship but to illustrate the differences between the two groups of fish. The other points in fig 7.1 show the relative MCH contents of fish obtained from Alderley and Warminster sampled as fingerlings and adults and tend to suggest that the increase in tissue MCH concentrations relative to body weight may not be linear but may start to plateau below 50 g body weight. Comparison of the values

from farm-reared fish relative to Bath-reared fish shows that their capacity to synthesise MCH is very similar to the black-reared fish, i.e. it is low. Alderley fish contain slightly more MCH than Warminster fish, and this presumably reflects the the differences in lighting and stocking density although other factors eg. genetic strain may be involved.

Table 7.1. The effect of background colour and rearing on MCH synthesis and storage.

Conditions	n	Weight (g)	Concentration of MCH (ng)		Pit/Hyp	(pg/ml) Plasma
			Hypothalamus	Pituitary		
BLACK	6	2.2 ± 0.2	0.8 ± 0.1	4.7 ± 0.6	6	---
	12	134.3 ± 2.7	9.2 ± 1.1	231.3 ± 25.4	25	27.4 ± 7.2
WHITE	11	2.8 ± 0.2	9.7 ± 1.5	25.4 ± 4.3	26	---
	12	133.8 ± 17.8	249.5 ± 29.2	1033.4 ± 93.4	4	101.9 ± 21.6

Fish were reared in either black or white tanks under a long photoperiod and sampled at 4 or 15 months.

Looked at in a different way, the pituitary: hypothalamus ratio in adults is 25 for B adults but only 4.3 for W adults. It is possible that the relatively lower pituitary content in W fish reflects their more rapid rate of hormone release into the blood: why MCH should accumulate so much more extensively in the perikarya is not clear. It could be anticipated *a priori*, that hormone granules would be translocated to their site of release (terminals in the pituitary or brain) as soon as they are released from the golgi; clearly this does not occur, and some are stored within the cell bodies. The nature and routes of communication between perikaryon and nerve terminals regulating granule transport are not known, but possibly include retrograde transport mechanisms.

It is not known whether under constant background colour conditions the accumulation of MCH increases in a linear relationship with body weight; the two points on each line in fig 7.1. were the only two measured and are not intended to imply such a relationship but to illustrate the differences between the two groups of fish. The other points in fig 7.1 show the relative MCH contents of fish obtained from Alderley and Warminster sampled as fingerlings and adults and tend to suggest that the increase in tissue MCH concentrations relative to body weight may not be linear but may reach a plateau below 50 g body weight. Comparison of the values from

farm-reared fish relative to Bath-reared fish shows that their capacity to synthesise MCH is very similar to the black-reared fish, i.e. it is low. Alderley fish contain slightly more MCH than Warminster fish, and this presumably reflects the the differences in lighting and stocking density although other factors eg. genetic strain may be involved.

Fig 7.1.

The effect of rearing conditions on MCH content in hypothalami and pituitaries from fish reared at two different farms and fish reared in either white or black tanks in the University aquarium.

☐   Fish reared in white tanks under a long photoperiod

■   Fish reared in black tanks under a long photoperiod.

○   Fish reared at Alderley   ●   Fish reared at Warminster
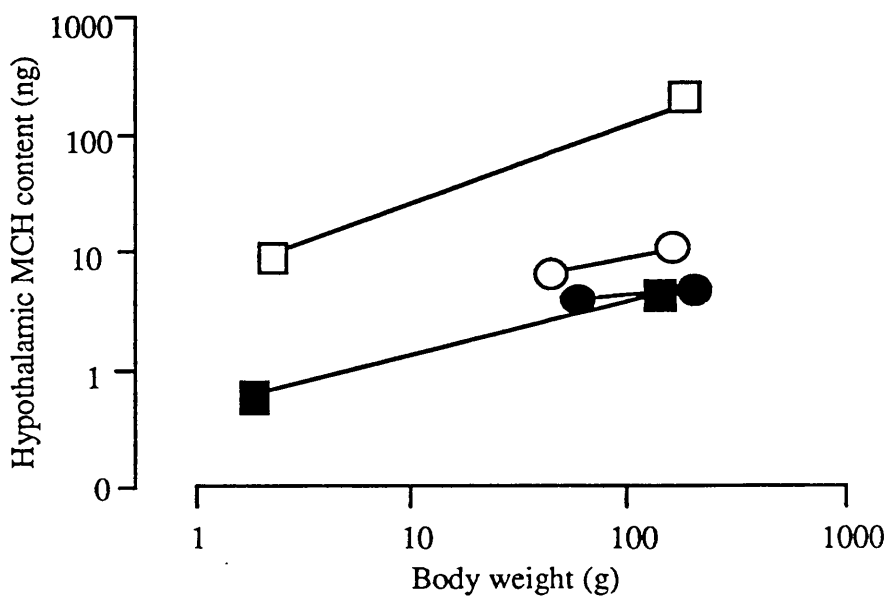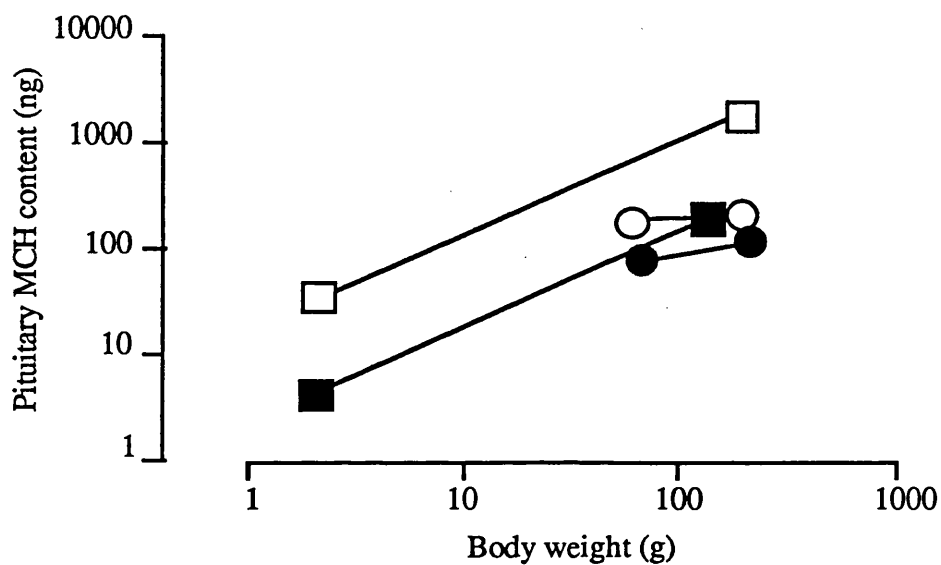
7.3.2. Seasonal changes in MCH synthesis and content

When adult fish were sampled at Alderley trout farm throughout the year (tables 7.2-3., Fig 7.2.), no clear seasonal changes were detected in the MCH content of hypothalami and pituitaries, neither did plasma concentrations show any seasonally related pattern. Plasma values were low at all times, similar to black adapted fish. Fish were brought back to the University aquarium and adapted to Black or White backgrounds for 3 weeks. The rationale behind this protocol was that the rate of hormone accumulation in fish placed in black tanks, or hormone loss in fish placed in white tanks might reflect the initial synthetic activity of the MCH neurones when they came from the farm. In support of this idea it has been shown that fish transferred from white to black tanks can show a 3-fold accumulation of MCH in their pituitaries within 6-13 days (Barber et al., 1987) suggesting that hormone synthesis continues after release has stopped. In Alderley adults placed in black tanks, MCH accumulation in the hypothalamus and pituitary (compared with farm fish) was evident only in October (fig 7.2.). The fingerlings showed a similar trend to the adults (table 7.2.). In trout placed in W tanks, hormone loss was least marked again in October adults and fingerlings. It may be, therefore, that the synthetic capacity of the MCH neurones is highest at this time in fish held under natural photoperiod conditions. On the other hand, the tissue contents in the farm were lowest at this time and measurements of plasma MCH titres in white-adapted fish also show apparently lowest concentrations in October, so any conclusion based changes in tissue contents alone must be treated with caution. In retrospect, it is possible that an adaptation period of 3 weeks was too long: this long period of adaptation to black or white tanks may have led to a change in the rate of MCH synthesis, which would mask the difference between groups.

Fig 7.2.

Variation in MCH concentration in Farm reared fish (a) and the difference in
response to white- or black-adaptation (b) over a period of 1 year. Measurements
from farm-reared fish are in arithmetic means and standard errors, Changes with
white- or black adaptation are represented as a % of farm content, farm = 100 %.

J = January, Ap = April, Au = August, O = October.

O Farm-reared fish   ☐ Farm-reared fish adapted to white tanks for 3 weeks

                             ■ Farm-reared fish adapted to black tanks for 3 weeks
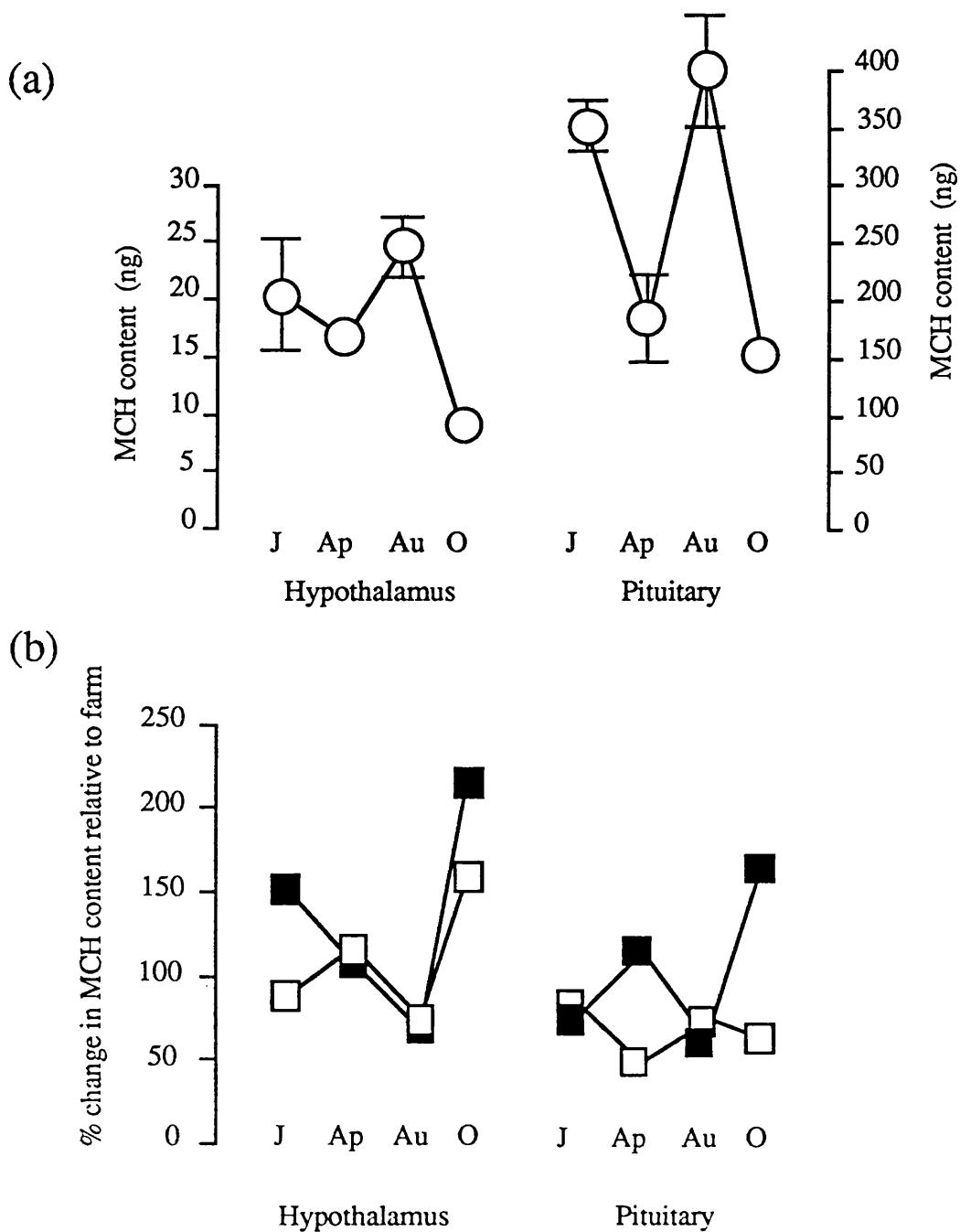
Table 7.2. Variation in tissue and plasma MCH concentration in adult fish sampled at Alderley and Warminster farms and in their adaptation to black and white backgrounds.

HYPOTHALAMIC MCH (ng)

Source	January	April	August	October
ALDERLEY				
Farm	21.4 ± 4.8 (8)	17.2 ± 1.2 (8)	24.9 ± 3.0 (8)	8.9 ± 1.3 (8)
White	20.2 ± 5.0 (8)	21.1 ± 2.9 (8)	19.4 ± 2.1 (8)	15.7 ± 2.0 (7)
Black	34.2 ± 3.4 (6)	20.4 ± 2.8 (8)	19.9 ± 2.3 (7)	19.9 ± 2.5 (6)
WARMINSTER				
Farm	10.6 ± 2.3 (8)	10.4 ± 0.5 (8)	10.8 ± 4.1 (8)	9.3 ± 1.5 (6)
White	6.1 ± 2.1 (7)	8.9 ± 0.4 (8)	10.2 ± 1.6 (6)	5.4 ± 0.5 (11)
Black	11.3 ± 3.9 (6)	5.1 ± 0.2 (7)	12.3 ± 1.7 (7)	5.7 ± 1.4 (7)

PITUITARY MCH (ng)

Source	January	April	August	October
ALDERLEY				
Farm	349.3 ± 22.1 (8)	191.0 ± 36.3 (8)	400.8 ± 47.0 (8)	148.8 ± 12.5 (8)
White	306.9 ± 32.4 (8)	99.0 ± 27.0 (8)	259.0 ± 33.7 (8)	125.5 ± 18.1 (7)
Black	270.6 ± 9.9 (6)	220.0 ± 37.0 (8)	264.8 ± 50.7 (7)	253.1 ± 31.3 (6)
WARMINSTER				
Farm	162.2 ± 33.5 (8)	104.0 ± 3.9 (8)	135.9 ± 11.0 (8)	145.4 ± 16.8 (6)
White	140.3 ± 2.7 (7)	55.0 ± 18.0 (8)	58.9 ± 16.0 (6)	120.2 ± 22.3 (11)
Black	137.0 ± 14.3 (6)	136.0 ± 7.0 (7)	190.7 ± 19.1 (7)	125.0 ± 15.0 (7)

PLASMA MCH (pg/ml)

Source	January	April	August	October
ALDERLEY				
Farm	15.5 ± 3.0 (8)	28.9 ± 4.0 (8)	8.7 ± 1.3 (8)	14.0 ± 1.6 (6)
White	155.0 ± 20 (6)	143.1 ± 28 (8)	108.6 ± 18.9(7)	79.6 ± 8.2 (7)
Black	19.0 ± 12 (3)	24.4 ± 6.0 (8)	7.6 ± 1.2 (8)	12.0 ± 0.4 (6)
WARMINSTER				
Farm	11.2 ± 2.0 (8)	21.1 ± 9.0 (8)	6.2 ± 1.0 (8)	12.4 ± 0.1 (6)
White	80.2 ± 18 (6)	55.6 ± 10 (8)	185.0 ± 80.3 (6)	27.2 ± 5.2 (11)
Black	13.0 ± 2.0 (6)	17.7 ± 3.0 (7)	8.0 ± 1.8 (7)	13.8 ± 1.5 (7)

Table 7.3. Variation in tissue and plasma MCH concentration in fingerling fish sampled at Alderley and Warminster farms and in their adaptation to black and white backgrounds.

HYPOTHALAMIC MCH (ng)

Source	January	April	August	October
ALDERLEY				
Farm	10.2 ± 1.1 (7)	10.6 ± 1.0 (8)	11.2 ± 2.0 (8)	9.4 ± 2.0 (6)
White	5.6 ± 0.8 (7)	6.1 ± 1.8 (9)	11.1 ± 4.3 (3)	5.6 ± 1.8 (7)
Black	10.2 ± 1.0 (7)	8.1 ± 0.9 (9)	14.1 ± 1.8 (3)	11.2 ± 1.7 (6)
WARMINSTER				
Farm	4.0 ± 0.4 (8)	13.0 ± 1.5 (8)	10.1 ± 2.3 (8)	7.6 ± 0.8 (6)
White	3.0 ± 0.4 (7)	2.6 ± 0.2 (8)	10.9 ± 1.9 (6)	2.1 ± 0.7 (7)
Black	6.4 ± 0.6 (6)	12.2 ± 3.2 (4)	4.9 ± 0.5 (8)	6.1 ± 1.1 (7)

PITUITARY MCH (ng)

Source	January	April	August	October
ALDERLEY				
Farm	90.3 ± 10.0 (7)	154.5 ± 26.0 (8)	103.7 ± 4.3 (8)	130.7 ± 16.0 (6)
White	45.5 ± 11.4 (7)	68.7 ± 21.0 (9)	49.9 ± 26.2 (3)	131.0 ± 5.0 (7)
Black	92.2 ± 7.6 (7)	134.8 ± 19.4 (9)	113.5 ± 16.7 (3)	198.0 ± 14.0 (12)
WARMINSTER				
Farm	48.9 ± 4.9 (8)	124.8 ± 16.5 (8)	155.1 ± 14.4 (8)	80.9 ± 10.5 (6)
White	23.9 ± 7.5 (7)	16.7 ± 2.0 (8)	26.5 ± 3.2 (6)	11.0 ± 1.0 (7)
Black	64.4 ± 6.6 (6)	105.8 ± 4.1 (4)	167.9 ± 6.5 (8)	116.0 ± 12.0 (6)

Fish reared at Warminster were not kept under natural photoperiod and again showed no seasonal variations in tissue contents. Their tissue contents were lower than in Alderley fish and the marked decline in tissue concentration when they were placed on white tanks suggest a relatively low level of MCH synthesis. Their plasma hormone concentrations on a white background were also significantly lower than Alderley fish in October and April although they showed surprisingly high values in August white-adapted fish. These values merit confirmation, in view of the observations of Bowley et al., 1983) that plasma αMSH titres tend to be higher in

Warminster than Alderley fish; suggesting a reversal of the melanotrophic balance between fish from these two sources.

In conclusion:

1.   Rearing conditions can affect MCH activity, rearing fish in white tanks stimulates the MCH system resulting in higher secretory activity than in fish reared in black tanks.

2.   These studies have failed to detect any clear seasonal changes in MCH synthesis and content in fish reared under farm conditions , with the possible exception of a slight apparent increase in the synthetic capacity of the MCH neurones in October. The physiological control and adaptive advantage of such a change is unknown.

CHAPTER 8

FUTURE INVESTIGATIONS

Now that the sequence of mammalian MCH is known, future work is likely to focus on its mammalian functions as well as the role of the other putative neuropeptides found within the mRNA sequence (eg NGE and NEI, Nahon *et al.*, 1989). There is a high degree of homology in the sequence of MCH found in fish and rats. This would imply selection pressures to preserve its structure because of an important physiological function, but the nature of that function is as yet unknown as far as mammals are concerned.

Until now nearly all work has focussed on the role of MCH in fish. As a colour-change hormone it induces pallor, but only in teleost and holostean fish (Baker 1988a, 1988b). This suggests that the melanin-concentrating ability of MCH is an evolutionary peculiarity of the neopterygians. It is released into the blood (Kishida *et al.*, 1989; Eberle *et al.*, 1989) and interacts with specific receptors on the melanophore (Oshima *et al.*, 1985) causing melanin concentration and counteracting the effect of αMSH (Baker 1988a). Additionally MCH release at terminals in the neural lobe (Powell and Baker, 1987) depresses αMSH secretion from the melanotrophs in the NIL (Barber *et al.*,1987). There is also evidence that MCH can depress ACTH release (Baker *et al.*, 1985; 1986) and observations described in chapter 6 of this thesis suggest that MCH can also modulate CRF release under certain conditions.

In all groups examined so far, other than the neopterygians, MCH distribution is concentrated primarily in the brain (see chapter 1 and

Eberle, 1988 for review). A hormonal function for MCH in tetrapods seems unlikely in view of the small amounts of MCH detected in the neural lobe; it appears, rather, that MCH is acting as a neuromodulator of brain function.

Since the structure of MCH has been highly conserved, it seems plausible that its physiological effects in fish could provide a clue to its function in mammals. The reasoning behind this statement is as follows: It is likely that fish and rat MCH in their present forms evolved from an ancestral molecule (MCH is found in lampreys also and the structure of MCH in this species may be close to that of the ancestral molecule). At some point the role of MCH in these groups diverged. In fish, MCH became involved in chromatic adaptation. Thus, evolution of a colour change function for MCH must have followed gradually from the ancestral function which presumably had features which pre-disposed the subsequent development of pigmentary control. If the colour change function of MCH is regarded as the 'new' function, the following question arises: Are the non-colour-change functions of MCH in fish relics of the function of the ancestral molecule? Also, by examining the mechanisms by which it exerts its effects in fish, is it possible to extrapolate backwards to the ancestral role of MCH and thereby deduce possibilities for its present function in mammals? For example, MCH can inhibit αMSH action on melanophores in is 'new' capacity as a colour change hormone; but in its ancestral form it may have been able to inhibit αMSH effects on a different target, and today it may inhibit the effects of αMSH in the brain in mammals.

It is striking that the known functions of MCH can be taken together and used to describe a possible role for MCH as a modulator for POMC-HPA. It may be able to do this by antagonising the effects of αMSH. The release

of αMSH from the pituitary has been shown to increase with stress although different types of stress do not cause parallel increases in the release of all POMC peptides from the corticotrophs and melanotrophs. For example, in fish, Gilham and Baker found that αMSH secretion increased with repeated injection stress (Gilham and Baker, 1985). Sumpter and co-workers stressed fish by different methods and found that an acute confinement stress did not affect plasma αMSH titres, but confinement stress coupled with restraint or thermal shock did result in increased plasma αMSH and β-endorphin titres (Sumpter et al., 1985, 1986). In rats, some types of stress-induced secretion in αMSH are thought to be mediated by β-adrenergic receptors, for example blockage of these receptors with propranolol reduces or prevents the secretion of αMSH or β-endorphin from the PI of rats exposed to emotional stressors (Berkenbosch et al., 1981; Tilders et al., 1985). The purpose of these secretions is not known, but they may have an enhancing effect on the classical HPI axis in fish (Baker and Rance, 1981; Takahashi et al., 1985; Gilham and Baker, 1985). However, the mode of action of αMSH in this respect is unclear, it may be acting on a low-affinity ACTH receptor rather than on a specific αMSH receptor, although αMSH has been shown to stimulate ACTH release especially if AVP is present (Lis et al., 1982). It is possible that MCH could modulate the HPA-enhancing effects of αMSH. MCH may also be able to modulate the stress response by depressing the release of ACTH from the other POMC-secreting cells; the corticotrophs. Stress-induced rises in ACTH release are under inhibitory control by α-adrenergic receptors, and since MCH can synergise with noradrenaline in fish, it may be able to synergise with noradrenaline in rats and thus modulate the activity of the HPA axis. Additionally, it is interesting to note that the MCH perikarya of the subzona incerta receive ACh input, itself a stimulator of ACTH release (Köhler and Swanson, 1984; Fellman et

al., 1989). However, hypothalamic αMSH has been implicated in the inhibition of CRF release: perfused rat brain slices showed reduced CRF release when treated with αMSH at nano molar concentrations (Suda et al., 1986).

MCH could antagonise other effects of centrally-derived αMSH. Not only is αMSH produced by the pituitary, but both des-acetylated and acetylated forms are produced by neurones in the brain which could have neuromodulatory effects. For instance, αMSH in the CNS is thought to be a physiological regulator of prolactin (PRL) release in the rat because stimuli known to alter plasma PRL levels simultaneously change hypothalamic αMSH: centrally administered desacetyl-αMSH inhibits both basal and stimulated release of PRL in the male and ovariectomised female rat (Khorram et al., 1982; Newman et al., 1985). Injection of αMSH antibodies into the third ventricle increases the basal PRL levels and prevents the decline in plasma PRL that occurs 60 min after the onset of stress in controls (Khorram et al., 1984; 1985). This PRL-inhibiting action of αMSH is dopamine mediated. αMSH will also block the opiate-induced rise in PRL release if it is injected together with β-endorphin (Khorram and McCann, 1986; Wardlaw et al., 1986).

There is evidence that MCH may enhance GH release in rats. Kawauchi et al., (1986) showed that injection with 440 μg MCH/ kg caused plasma levels of GH to rise after 1h and remain high for several hours. The long latent period before a rise in plasma GH is observed suggests that MCH was having some indirect effect in order to cause GH release, but whether this effect is due to physiological or pharmacological action on the brain is unknown. It is interesting to note however, that in a paper published in 1935, Mottley and Deno reported that brown trout reared in a tank subdivided into black or white halves showed different growth rates,

those in the white half growing faster than their black-adpted tank-mates. It is possible that they grew faster because they were less stressed due to high MCH production (see Chapter 6) rather than any GH-releasing effect.

Finally, Zamir and co-workers (1985) suggested that there might be a link between MCH function and the regulation of food and water intake in the rat, since rats given 2% NaCl to drink for 5 days showed increased MCH concentrations in the lateral hypothalamus and the pituitary neural lobe.

Methods for investigating the involvement of MCH with any of these actions of αMSH, other POMC peptides, catecholamines and ACh should initially include mapping of the distribution of MCH receptors in the brain in conjunction with the occurrence of ir-MCH, in order to be able to correlate the site of action of MCH with its sites of release. Similarly, the distribution of MCH receptors could be compared to brain areas known to receive catecholamine and POMC input. Results from such experiments would give a clearer idea about the direction research into mammalian MCH should follow.

APPENDIX

1. Iodination procedure for MCH and αMSH.

Solutions required:

0.25 M Phosphate buffer pH 7.4

0.05 M Phosphate buffer pH 7.4

0.05 M Phosphate buffer + 0.25% BSA + 1% Mercaptoethanol (MEth)

1% Trifluoroacetic acid (TFA)

80% Methanol (MeOH)

Polypep (20mg in 2ml 0.05 M Phosphate buffer)

Graded MeOH + 1% TFA (40% - 62.5%)

Also required are tubes containing the following:

4ml 50% MeOH

4ml 0.05 M Phosphate buffer + 0.25% BSA + 1% MEth

Procedure

1.   The SEP-PAK (C-18, Waters Associates, Milford, MA) was washed slowly

with 2ml of each of the following solutions in the order:

1% TFA

80% MeOH

Polypep

80% MeOH

1% TFA

2.   In an eppendorf, the following reagents were assembled: 1mCi ^{125}I

(10μl, from Amersham, in the form of NaI) was placed in the bottom of

the tube along with 20μl 0.25 M Phosphate buffer. Then 10μl MCH (2μg

in 0.001% HCl) was placed in the tube such that it stuck to the tube

wall, the same for 15μl Chloramine T (5mg in 10ml 0.25M Phosphate

buffer). This last component was prepared at the last minute.

3. The tube lid was replaced securely and its contents vortexed and allowed to react for 30 sec exactly.

4. The reaction was stopped with the addition of 600μl 0.05 M Phosphate buffer + 0.25% BSA + 1% MEth.

5. The contents of the eppendorf are then applied to the SEP-PAK twice and then washed twice with 600μl 0.25 M Phosphate buffer. The labelled peptide was eluted with graded MeOH solutions (+1% TFA) three washes for each MeOH fraction.

6. The peaks were placed in the storage tubes and kept at -20°.

2. Iodination procedure for NPP-1

Briefly, to 0.4mCi [125]I (5μl) were added 25μl 0.05 M phosphate buffer (pH 7) containing 5μg NPP-1, and 5μl 0.05% H_2O_2 in 0.2 M phosphate buffer (PB). The reaction was started by adding 5μl 10% lactoperoxidase (90 U/mg in distilled water) and stopped after 3 min by the addition of 75μl chilled 0.05 M PB. The labelled peptide was separated from free iodine and unlabelled peptide on a sephadex G-75 column (primed with 1% BSA) with 0.02 M PB at a flow rate of 6-10 ml/ h. Volumes of 0.5 ml/ tube were collected, the radioactive peak was diluted with an equal volume of buffer A and stored at -40°.

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