Title The effects of stress, background colour and steroid hormones on the lymphocytes of rainbow trout (Oncorhynchus Mykiss)

Name J A Cook

The Effects of Stress, Background Colour and Steroid Hormones on the Lymphocytes of Rainbow trout (*Oncorhynchus mykiss*)

By

Julie Cook

A thesis submitted in part for the degree of Doctor of Philosophy of the University of Sheffield

University of Luton,
Park Square
Luton
Bedfordshire
LU1 3JU

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Declaration

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Sheffield. It has not been submitted before for any degree or examination in any other University.

.....Julie Cook.....(name of candidate)

.....4th.....day of.....December.....19.....94

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Abstract

This study investigated the effect that adapting Rainbow trout to black or white backgrounds has on their stress and immune responses. Experiments *in vivo* showed that stressed fish, adapted to black backgrounds, had higher plasma cortisol levels and a suppressed immune system compared to white-adapted fish. Thus, stress reduced antibody production, induced lymphocytopenia and inhibited the ability of lymphocytes to grow *in vitro*. These effects were always more pronounced in black-adapted trout. It is argued that white-adapted fish are less susceptible to the effects of stress because of the neuromodulatory influence of the neuropeptide, melanin-concentrating hormone (MCH).

In fish reared from eggs on dark- or light-coloured backgrounds, differences between black and white groups were not so evident. In some cases, the effects seen in adapted fish became reversed when reared fish were used. It is suggested that homeostatic mechanisms counteract the modulatory actions of MCH in reared fish.

In experiments using radiolabelled thymidine to monitor lymphocyte growth *in vitro,* MCH enhanced both T and B cell-like proliferation. The peptides also modulated the action of corticosteroids on lymphocyte

growth and was found to reduce, but not prevent, the inhibitory influence of cortisol. Melanocyte stimulating hormone, an antagonist of MCH, had no effect on lymphocyte growth at concentrations normally found in fish plasma.

The major reproductive steroids of trout were tested for their ability to influence lymphocyte growth *in vitro*. The results were variable, some steroids were predominantly stimulatory (*e.g.* oestradiol), some inhibitory (*e.g.* ketotestosterone), while others had mixed actions (*e.g.* 17a-hydroxy 20b-dihydroxyprogesterone). These observations are discussed in relation to the normal plasma levels of reproductive steroids found at different stages in the life cycle of trout and the possible effects these steroids have on fish immunity.

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1 . The Endocrinology Of Stress

1.1. Introduction

The biological concept of "stress" in vertebrate animals is notoriously difficult to define precisely. Almost all physical and physiological disturbances may be stressful, but the intensity of response varies widely between different vertebrate classes and even between individuals of the same species. Seyle (1950; 1973; 1976) attempted to explain stress in terms of the General Adaptation Syndrome (GAS). This concept, modified by Wedemeyer and McLeay (1981), encompasses all the responses that occur to stabilise or reverse the effects of potentially harmful stimuli. Responses to stress are viewed as modifications to biological systems that allow independence from environmental perturbation and therefore help organisms maintain homeostasis.

In general terms, the "stress response" can be divided into three distinct phases. Each involves biochemical and/or immunological changes that are ultimately controlled by the nervous and endocrine systems. The primary phase involves alarm responses involving the activation of neuronal pathways that result in the secretion of catecholamines, glucocorticoids and neuropeptides (Seyle, 1950).

Plasma levels of catecholamines can rise dramatically within seconds of an acute stress, normally returning to base levels within minutes or a few hours (Mazeaud *et al.*, 1977; Mazeaud and Mazeaud, 1981; Barton and Iwama, 1991). The rise in glucocorticoid titres however, has a time course that is measured in minutes or hours and recovery to basal

levels can take several days (Pickering *et al.*, 1982; Pickering and Pottinger, 1989). Catecholamines have powerful effects on muscle contraction and are known to influence glucocorticoid secretion (White and Fletcher, 1985) while the glucocorticoids have prominent energy mobilising properties (Storer, 1967; Lidman *et al.*, 1979). These actions, amongst others, enhance the capacity of animals to escape a source of stress or overcome its effects (Pickering, 1989).

The secondary phase involves the physiological, biochemical and immunological consequences of the primary stress response, *i.e.* due to the effects of glucocorticoids and catecholamines (Mazeaud *et al.*, 1977). The organism adapts or compensates for the altered conditions caused by the stress in order to regain homeostatic balance, even if this means altering the previous resting state. The number and activity of circulating leukocytes is decreased (Weinreb, 1958; Mcleay, 1975) and there are many changes in blood chemistry and tissue metabolism.

Tertiary stress responses include behavioural changes (Wedemeyer and McLeay,1981) as well as decreased growth (Barton et al., 1987; Pickering, 1990), reproductive capacity (Carragher *et al.*, 1989) and further immunological effects (Mazeaud *et al.*, 1977; Wedemeyer and McLeay,1981). If the stress is severe or long-lasting, the organism enters the final phase of exhaustion and may be unable to maintain homeostasis with potentially fatal results.

Not all vertebrates always conform to the GAS pattern of response (Wedemeyer *et al.*, 1990). Schreck (1981) suggest that in fish for example, the GAS response is only evident when stressors cause fright, pain or discomfort. Other workers have questioned the adaptive role

suggested for glucocorticoids. Munck *et al.*, (1984) believe the suppressive effects of these steroids serve to protect the body from over-activity of the defence mechanisms that are the primary response to stressful stimuli.

Generally, the pattern of response that is observed as a result of infection, physical disturbance or detrimental changes in environmental conditions follows a similar pattern, and comes under the heading of 'stress response'. The magnitude of this response can be estimated by measuring the hormonal changes that form part of the primary response. For example, catecholamine secretion (Iwama *et al.*, 1989) has been used to measure stress, but quantification is difficult, since titres in the blood rapidly return to pre-stress levels (Mazeaud and Mazeaud, 1981). Advances in assay techniques now allow ACTH levels to be measured, but the rapid release of ACTH from the pituitary gland and its short half-life in the circulation present serious practical difficulties (Sumpter and Donaldson, 1986). By far the most commonly used hormonal indicator of stress is the measurement of plasma corticosteroids, a technique that is discussed in more detail in Section 1.3.

Measurement of secondary responses typically involve determination of changes in blood metabolites, often the result of the action of glucocorticoids. Plasma lactic acid and glucose both increase following stress and have been used to quantify the stress response. Other secondary responses include changes in haematocrit and leucocyte counts, but these parameters can be influenced by non stress factors such as nutritional status (Barton *et al.*, 1988). Tertiary stress responses that have been used include changes in growth and metabolic

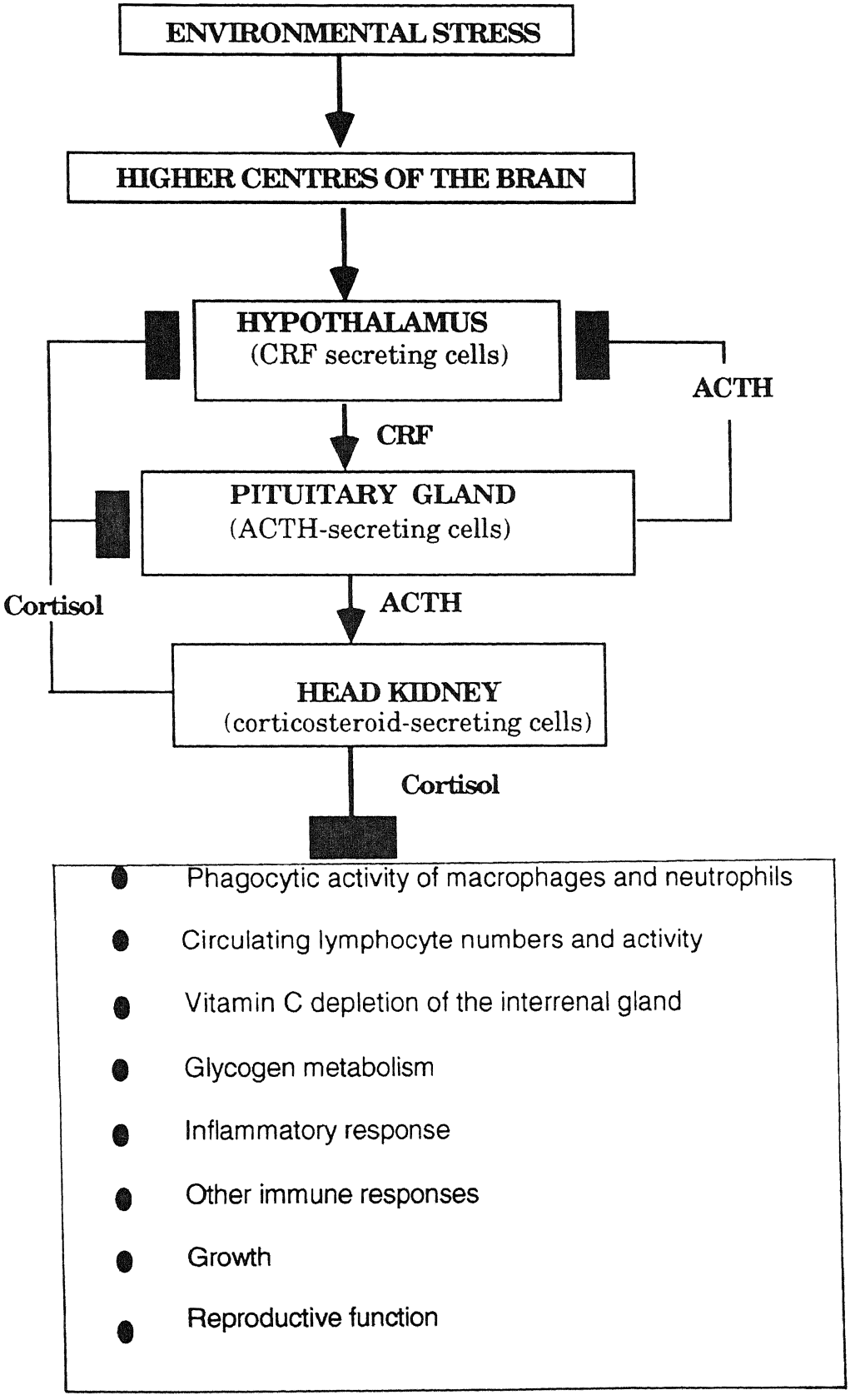
rate. Although these allow stress to be measured at the level of the population, interpretation can be difficult.

Thus the term 'stress response' covers a wide range of measurements and includes both short and long term effects. In this thesis, increases in plasma cortisol titres are used to measure the stress response that is observed following persistent chronic stress over a time period of days or weeks.

1.2. The Hypothalamo-Pituitary-Interrenal Axis of Fish

Almost all environmental and physiological stress in fish results in a cascade of hormone release. Although the intensity of this response depends on the nature of the stress and the species of fish (Pickering and Pottinger, 1989), and is dependent on age, strain and sex (Sumpter *et al.*, 1987), the sequence of events is invariably the same. Activation of neuronal pathways within the hypothalamus releases corticotrophin-releasing-factor (CRF;Hawkins *et al.*, 1970) from the hypothalamus which in turn stimulates the production of adrenocorticotrophic hormone (ACTH) from the pituitary gland (Sage and Purrot, 1969). ACTH reaches the interrenal via the blood, where it promotes the synthesis and release of corticosteroids (Donaldson *et al.*, 1968a; b). A negative feedback mechanism by corticosteroids regulates the secretion of CRF and ACTH (Sage, 1968; Sage and Purrot, 1969; Fryer and Peter, 1977b; c; Figure 1.1). This pattern of hormone secretion is often referred to as the hypothalamo-pituitary-interrenal (HPI) response (Donaldson, 1981).

FIGURE 1.1. Diagram illustrating the main features of the hypothalamo-pituitary-interrenal response in fish. Environmental stress leads to a cascade of hormonal release (→) ending in the release of cortisol. Cortisol inhibits (⊣) a wide range of cellular functions. Several feedback mechanisms are known to operate within the HPI axis.


ENVIRONMENTAL STRESS
HIGHER CENTRES OF THE BRAIN
HYPOTHALAMUS
(CRF secreting cells)
ACTH
CRF
PITUITARY GLAND
(ACTH-secreting cells)
Cortisol
ACTH
HEAD KIDNEY
(corticosteroid-secreting cells)
Cortisol
Phagocytic activity of macrophages and neutrophils
Circulating lymphocyte numbers and activity
Vitamin C depletion of the interrenal gland
Glycogen metabolism
Inflammatory response
Other immune responses
Growth
Reproductive function

1.2.1. The Hypothalamus and CRF

Hypothalamic control of ACTH release was first demonstrated in fish by Sage and Purrot (1969) who found that ACTH is released from goldfish (*Carassius auratus)* pituitary glands when they are incubated with a hypothalamic extract. Other work on betamethasone-blocked goldfish has shown that extracts of the hypothalamus and telecephalon injected *in vivo* raise plasma cortisol levels (Fryer and Peter, 1977a).

Although CRF was originally thought to be a single compound (Saffan and Schally, 1955; Saffan *et al*; 1955), work on mammals has suggested that CRF is a multifactorial complex and several other compounds also have ACTH-releasing activity (Figure 1.2). The structure of ovine CRF-41 was elucidated by Vale and co-workers (1981). Gillies *et al.* (1982) suggested that the CRF complex also contained another factor thought to be arginine vasopressin and two small synergising factors. Arginine vasopressin is replaced in pigs by lysine vasopressin. In rats, both arginine and lysine vasopressin (Junnilia and Sayers, 1977) can stimulate ACTH release for a short period. Rat hypothalamic extract causes prolonged ACTH release, as does vasotocin in teleosts (Buckingham and Hodges, 1977).

Evidence presently available for teleosts suggests that two related CRF peptides occur in the fish brain in addition to the neurohypophysial peptides vasotocin and isotocin. In fish, the nonapeptides vasotocin and isotocin, are produced in the nucleus lateralis tuberis (NLT) and have been shown to cause ACTH release from the pituitary gland either by stimulating CRF release, or by potentiating the effect of CRF on

FIGURE 1.2. Amino acid sequence for hormones with corticotrophic releasing properties. Amino acid sequence taken from Vale *et al.*, 1981 (1) , Lederis *et al.*, 1982 (2) and Heller, 1974 (3). Identical amino acids common to all forms are typed in bold.

Ovine CRF-41[1]

S **Q** E **P P** I S **L** D **L** T **F H L L** R **E V L E M** T **K A D Q L A Q Q A H S N R K L L D I A**

Teleost Urotensin I [2]

ND **D P P** I S I **D L** T **F H L L** R N M I **E M A** R I **E** N **E** R **E Q A** G **L** N **R K** Y **L D E V**

Arginine Vasopressin [3]

C Y F Q B **C P R** G

Lysine Vasopressin [3]

C Y F Q B **C P** K G

Isotocin [3]

C Y I S **B C P** I G

Vasotocin [3]

C Y I **Q** B **C P R** G

ACTH release (Fryer and Peter, 1977c). The other teleost peptides with CRF activity are urotensin I and a related peptide (Fryer *et al.*, 1983). Urotensin I, an important peptide for osmoregulation and smooth muscle contraction, has been found to be structurally similar to ovine CRF and cause cortisol-releasing activity in fish and mammals (Lederis *et al.*, 1982).

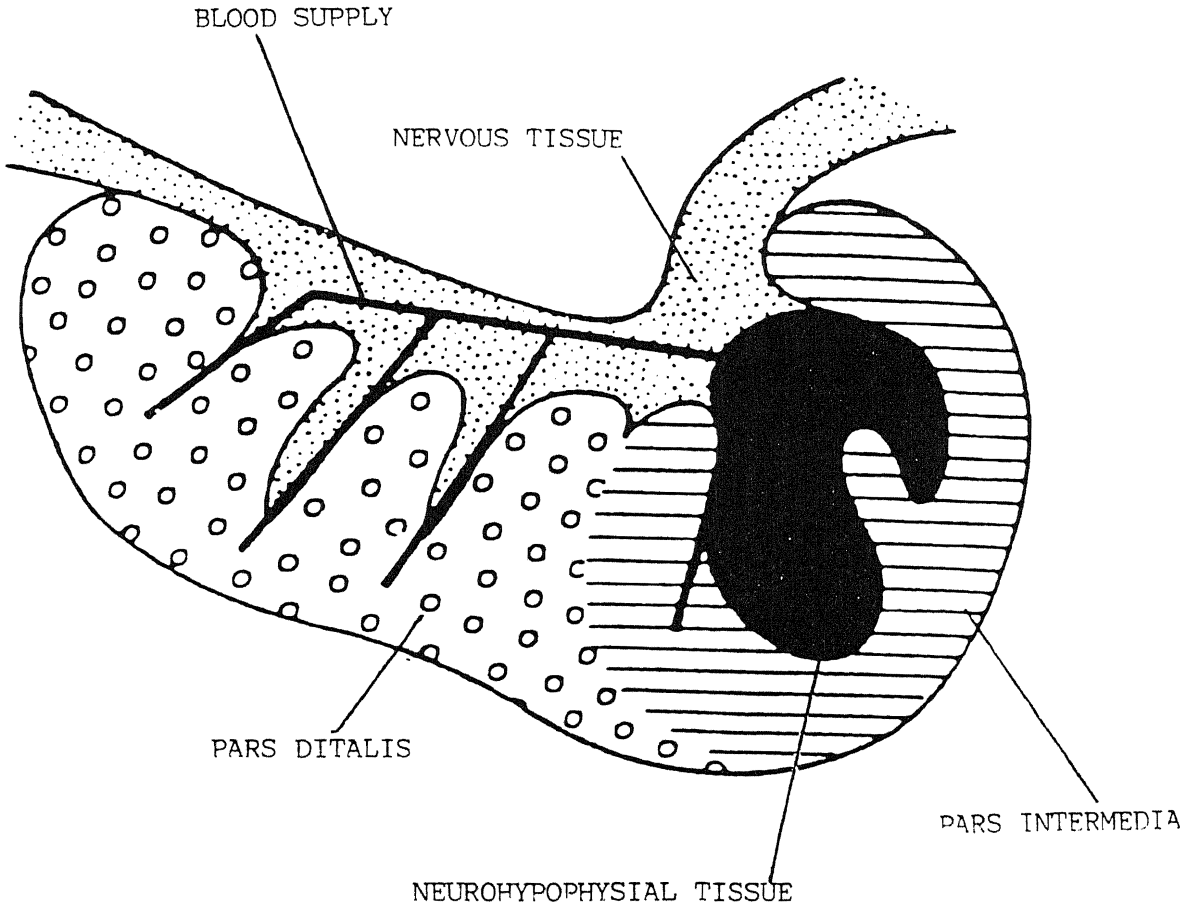
1.2.2. The Pituitary Gland and ACTH

The fish pituitary gland differs from its mammalian counterpart in two important respects. Firstly, the different types of secretory cell are arranged in discreet groups and are not scattered throughout the adenohypophysis and secondly, the CRF neurosecretory cells of the hypothalamus penetrate the pituitary gland to terminate near the corticotrophs. There is no median eminence as in mammals (Figure 1.3).

The activity of ACTH in the fish pituitary was first demonstrated by Ito *et al.*, (1952) and ACTH synthesis was later identified with "epsilon" cells in the adenohypophysis (Olivereaue and Ball, 1963; Olivereaue, 1964). Although fish ACTH had been widely known to cause corticosteroidogenesis in other animals, it was not demonstrated in fish until 1968 (Donaldson *et al.*, 1968a; b).

In vertebrates, ACTH is derived from a much larger precursor molecule, pro-opiomelanocortin (POMC; Mains and Eipper, 1976) which is produced by several types of cell including the corticotrophes of the pars distalis (Scott *et al.*, 1974). In all gnathostomes the prohormone consists

FIGURE 1.3. Diagrammatic representation of the pituitary gland of a teleost, midsagittal section. Note unlike the mammalian gland, there is no portal blood supply from the hypothalamus and the hypothalmic neurones penetrate the pituitary gland and terminate within it. (After Bentley, 1976).



of 260 amino acid residues, and contains within its structure the sequences of many hormones, each individual peptide being produced by cleavage from the parent molecule (Roberts and Herbert, 1977). Donaldson (1981) compared the structures of fish and human ACTH, and found strong sequence homology. Of the 39 amino acid residues in mammalian ACTH, the sequence of the first 24 residues are similar in all mammals, the dogfish differs by only three, and the salmon by just one amino acid from the mammalian form (Figure 1.4).

1.2.3. The Interrenal Tissue and Corticosteroids

The corticosteroid-secreting cells of teleost fish are located within a specialised area of head-kidney called the interrenal tissue (Nandi and Bern, 1960; 1965) other cell types such as chromaffin cells are also found within the pronephros or head kidney. When stimulated, the nuclear diameters of the interrenal cells increase markedly and they become hypertrophic and hyperplasic (Hanke and Chester Jones, 1966). The most abundant corticosteroids found in teleost fish are cortisol and cortisone (Idler and Truscott, 1972). Cortisol is a 21-carbon steroid hormone derived from the hydroxylation of cholesterol to progesterone which is in turn hydroxylated to cortisol (Figure 1.5) and is the major corticosteroid found in Rainbow trout (*Oncorhynchus mykiss;* Hane and Robertson, 1959). Cortisone is produced from cortisol by 11-b-hydroxysteroid dehydrogenation within the interrenal cells (Donaldson and Fungerland, 1972). There are also small amounts of other cortisol derivatives present in the plasma.

FIGURE 1.4. Amino acid sequence for adrenocorticotrophic hormone in mammals, teleosts and elasmobranchs. Amino acid sequences for ACTH from Riniker *et al.*, 1972 (1) Kawauchi, 1979 (2) and Lowry et al., 1974 (3). Identical amino acids common to all forms are typed in bold.

Human ACTH (1)

S Y S M E H F R W G K P V G K K R R P V K V Y P N G A E D E S A E A F P L E F

Salmonid ACTH (2)

S Y S M E H F R W G K P V **R P V K V Y T N G V E E E S S E S F P S E M**

Dogfish ACTH (3)

S Y S M E H F R W G K P M G R K R P I **K V Y P N S** F **E D E S** V **E N M G P E** L

1.3. Endocrine Regulation of the HPI Axis in Fish.

Plasma levels of corticosteroids are the most commonly used method of assessing the activity of the HPI axis and thus measuring the stress response, partly because steroids such as cortisol are easy to measure, originally by fluorometry (Donaldson *et al.*, 1968b) and now by radioimmunoassay (Hargreaves and Ball, 1977; Peter *et al.*, 1978; Rance and Baker, 1981) and partly because of their significance in processes affecting fish health (Barton and Iwama, 1991). Basal levels of plasma corticosteroids in teleosts are usually less than 30 ng ml^{-1} and may even fall below 5 ng ml^{-1} under ideal conditions (Pickering, 1989; Pickering and Pottinger, 1989; Wedemeyer *et al.*, 1990). In general, the magnitude of the corticosteroid response usually reflects the severity of the stress (Barton *et al.*, 1980; Pickering, 1989) and plasma cortisol titres of between 40 and 200 ng ml^{-1} post-stress are typical (Pickering and Pottinger, 1989), although even higher values are not unusual (Barton and Iwama, 1991).

Our present understanding of the mechanisms that control and regulate the HPI axis in fish is based mainly on experiments involving hypophysectomy and/or the administration of corticosteroids, their agonists and antagonists or of biochemical inhibitors of steroidiogenic pathways.

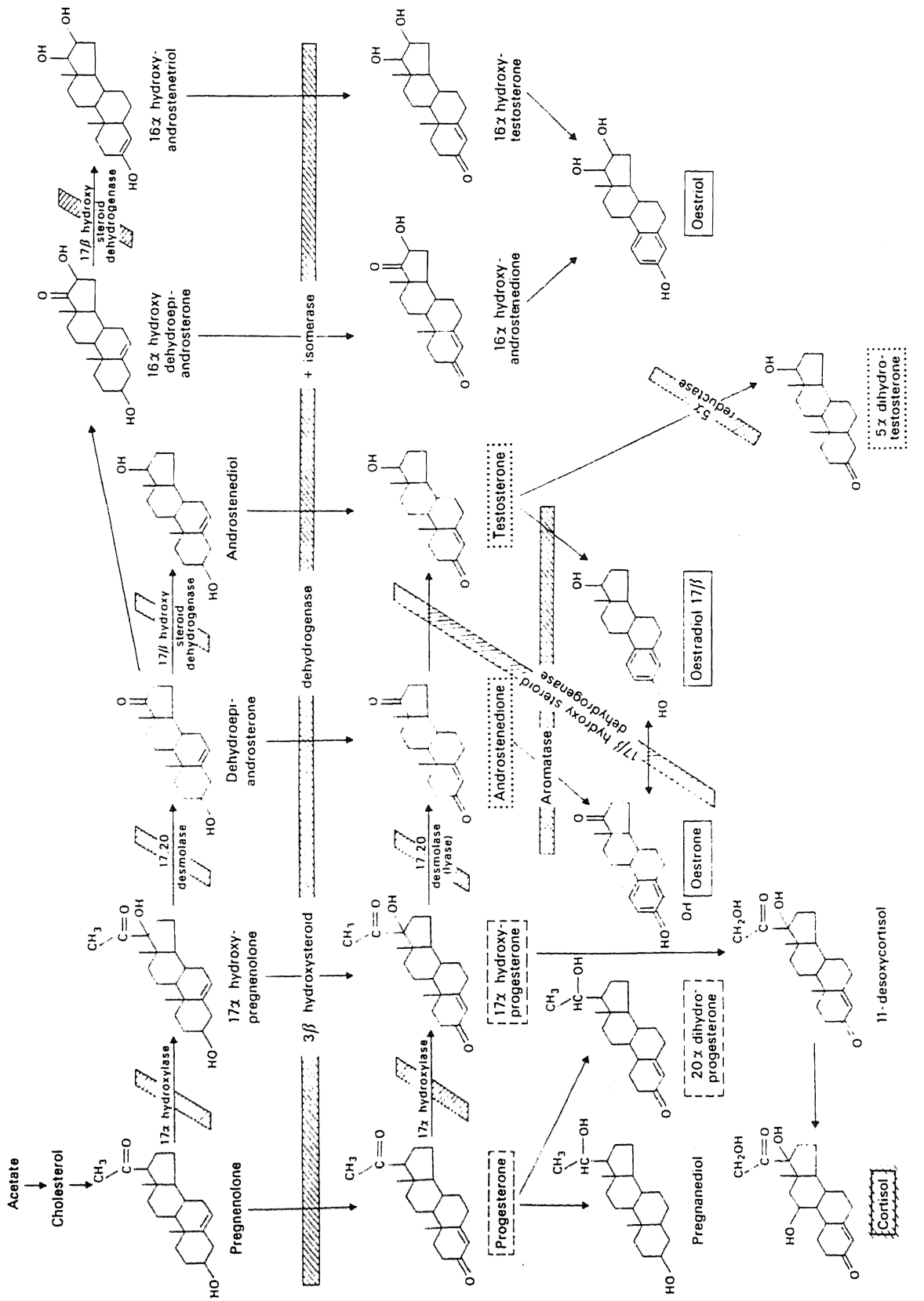
Early work on Rainbow trout has shown that hypophysectomy results in interrenal cell atrophy and a marked reduction in plasma cortisol levels which do not increase following stress (Donaldson and McBride, 1967). The interrenal cells of hypophysectomised Cyprinid *(Couesius*

plumbeus) can however be stimulated by pituitary extracts of Chinook salmon (*Oncorhynchus tshawytscha;* Van Overbeeke and Ahsan, 1966).

In intact fish, the stress response can be prevented by using the long-acting cortisol analogue, dexamethasone, a steroid which is believed to suppress both CRF and ACTH (Sumpter and Donaldson, 1986). This view is supported by the fact that dexamethasone-treated Rainbow trout are still capable of responding to injections of ACTH (Fagerlund, 1970). These, and many other experiments, strongly suggest that feedback by cortisol inhibits CRF and ACTH synthesis and release (Figure 1.1). The low levels of cortisol found in dexamethasone-treated salmon indicate that the feedback mechanism does not completely inhibit corticosteroid release, although when stressed, these fish show no significant increase in their plasma cortisol (Fagerlund and McBride, 1969). If diseased fish are treated with dexamethasone however, the plasma cortisol levels fall but a stress response still occurs indicating that the feedback mechanism can be impaired by disease. (Fagerlund and McBride, 1969).

Another approach to investigating the regulation of the HPI axis involves the use of metyrapone, an inhibitor of 11-b hydroxylation which therefore blocks the synthesis of cortisol (Figure 1.5). At low doses metyrapone is stressful and causes an increase in plasma cortisol. This is surprising since there is evidence that metyrapone is also toxic to corticotrophs, causing them to degenerate (Fagerlund *et al.,* 1968) and thus reducing the pituitary content of ACTH (Van Kemenode *et al.,* 1980). At higher doses however, given over a two, day period, cortisol production decreases (Fagerlund *et al.,* 1968).

Figure 1.5. The structure and biosynthesis of steroid hormones. The inter conversions of steroid hormones, with some of the principal enzymes shown by (▨). (Taken from Johnson and Everitt, 1993).



1.4. Factors Affecting the Stress Response.

The pattern of peptide and steroid hormone secretion elicited by stressful events depends not only on the intensity of the stress but also on the species involved, the nature of the stress and its duration.

It is now widely accepted that Rainbow trout are less susceptible to stress than Brown trout (Pickering *et al.,* 1989). In a series of experiments Wedemeyer (1971; 1972; 1973; 1976) investigated the effects of a wide range of stressors on fish which included handling, crowding, temperature change and formalin treatment. Differences in the sensitivity of the two species were reflected by differences in the circulating cortisol levels (Wedemeyer and Yasutake, 1974). The magnitude of the stress response can even differ between strains of the same species (Pickering and Pottinger, 1989) and in general, wild fish strains are more sensitive than domesticated varieties (Woodward and Strange, 1987).

Many common procedures employed in aquaculture induce a stress response. Thus handling (Pickering and Pottinger, 1989), netting (Wedemeyer, 1969), anaesthesia (Fagerlund,1967) and transfer from tanks to a bucket (Strange and Schreck, 1978) all induce a stress response. Other factors are confinement (Pickering and Pottinger, 1989), captivity (Miller and Tripp, 1982), temperature changes (Barton and Schreck, 1987), overcrowding (Pickering and Steward, 1984), transport (Barton and Peter, 1982), as well as more obvious stressors such as injection (Green *et al.*, 1991) and noxious chemicals (Pickering and Pottinger, 1984; 1985).

Stress is said to be acute if the time required for a fish to recover is much greater than the length of time the stress is applied. Examples of acute stress include short periods of handling or netting, predator avoidance and territorial disputes.

The time needed for recovery from acute stress also depends on the severity of the stressor (Pickering *et al.*, 1982). There is invariably a rapid increase in plasma levels of catecholamines followed by a corticosteroid increase which peaks approximately two hours after the stress and returns to basal levels within 24-48 hours (Pickering, 1984; Pickering and Pottinger, 1987d; Pickering and Pottinger, 1989). Other disturbances include increased epidermal mucification (Pickering and Macey, 1977), reduced glycogen synthesis in the liver, disturbed carbohydrate metabolism and depletion of lipids (especially steroids) and vitamin C from the adrenal cortex (Wedemeyer *et al.*, 1990). Feeding often stops until three days post-stress (Pickering *et al.*, 1982) although this effect varies between species (Wedemeyer, 1976).

The affects of stress are usually monitored by measuring cortisol and or blood glucose titres (Donaldson, 1981; Schreck, 1981). If the stress is short lived, e.g. handling for 5 seconds, fish may not evoke a response, but if the same stress is increased to 2 minutes a moderate response is evoked (Thomas and Robertson, 1991). Increasing the period of time fish are subjected to stress causes further increases in glucose and cortisol levels. Acute stress may also have long term effects on the metabolic and osmo-regulatory systems (Mazeaud and Mazeaud, 1981).

The corticosteroid response to handling can be eliminated by anaesthetising the fish beforehand (Iwama *et al.*, 1989). This

presumably eliminates the awareness of the stressor and therefore prevents any "fright" reaction (Schreck, 1981).

In contrast to acute stress, chronic stress generally lasts for days or weeks. Examples include overcrowding, low oxygen levels or poor water quality due to the accumulation of waste products of metabolism, or sub-lethal concentrations of pollutants. Stressors such as these tend to be persistent and inescapable, but in most cases, acclimation occurs over an extended period (Pickering and Steward, 1984; Pickering and Pottinger, 1989) with the time required depending on the species and life cycle stage (Wedemeyer, 1976). Blood cortisol leveles may be high initially, but these eventually return to normal levels by means of homeostatic mechanisms, even though the stress persists (Schreck, 1981).

Danielson and Stallcup (1984) have suggested that the sensitivity of cells to corticosteroids is controlled by varying the number of receptors present. There is a reduction in the number of cortisol binding sites in Rainbow trout liver cells (Pottinger, 1990) and Brook trout gill tissue (Weisbart *et al.,* 1987) following chronic stress and this reduction is believed to prevent the target tissues from over-responding (Pottinger, 1990).

Under some circumstances, plasma cortisol levels may not increase in response to stress (Schreck and Lorz, 1978; Pickering and Pottinger, 1987b; c;). For example, although a high stocking density is stressful, cortisol titres may either vary inversely with the number of fish per unit volume (Leatherland and Cho, 1985) or the fish may acclimatise (Pickering and Steward, 1984). These observations can be partly

explained by the increased metabolic clearance rate of the hormone that occurs under crowded conditions (Schreck *et al.*, 1985) or by changes in the number of interrenal ACTH receptors that make the steriodogenic tissue less responsive (Patino *et al.*, 1980). High stocking density is a good example of how an apparently straight forward explanation of observed cortisol titres is complicated by the fact that several stressors may be operating at the same time (Leatherland and Sonstegard, 1984). Fish held at high densities for prolonged periods experience both chronic stimulation and suppression of the HPI axis.

While acclimation to the stimulation of overcrowding occurs gradually over days or weeks (Schreck, 1981; Pickering and Steward, 1984), other inhibitory effects are caused by changes in water chemistry that are associated with high stocking densities (Pickering and Steward, 1984; Pickering and Pottinger, 1987c). Thus, a decrease in oxygen level causes mild suppression of the stress response and increases the toxicity of ammonia and carbon dioxide (Lloyd, 1961; Saunders, 1962). Ammonia decreases the oxygen carrying capacity of the blood but increases oxygen consumption (Brockway, 1950). Carbon dioxide decreases the oxygen carrying capacity of fish blood through the Root effect (Saunders, 1962). These combined chemical effects act synergistically to cause an overall suppression of the HPI axis so the chronic activation is effectively masked. Detoriating water quality may depress a fish's ability to trigger a HPI response (Schreck, 1981) and there is evidence that the level of consciousness of the fish may become impaired during confinement (Pickering and Pottinger, 1987c).

The fact that plasma cortisol and cortisone titres are higher in gonadectomised fish indicates that the HPI axis is influenced by the

reproductive system (Donaldson and McBride, 1974) and can also be modulated by gonadotrophins (Schreck *et al.*, 1989).

Several other factors are known to influence blood corticosteroids including smoltification (Barton *et al.*, 1985; Patino and Schreck, 1986), temperature (Barton and Schreck, 1987), and nutritional status (Barton *et al.*, 1988). There is also clear evidence for diurnal and seasonal cycles in corticosteroid synthesis and secretion (Van Kemenade *et al.*, 1980; Rance *et al.*, 1982).

2. Melanin-Concentrating Hormone

2.1. MCH and Colour Change in Fish.

The idea that two antagonistic hormones are involved in the control of skin colour in fish was first suggested by Hogben and Slome, (1931). These workers believed that the darkening effect of a melanocyte-stimulating hormone (αMSH) was opposed by a 'paling hormone' that we now know to be melanin-concentrating hormone (MCH). The assumptions supporting this hypothesis at the time however, were later shown to be invalid (Bradshaw and Waring, 1968).

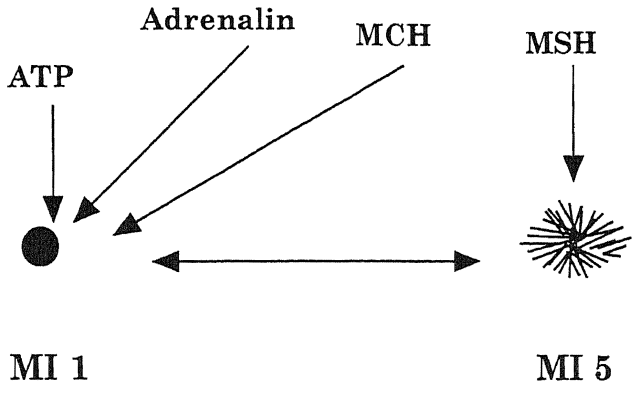
For many years it had been known that the injection of teleost pituitary gland extract causes skin pallor in bony fish (Hewer, 1926). A substance that induces melanin concentration was found in the ethanol soluble fraction of pituitary gland extracts of the catfish (*Parasilurus;* Enami, 1955), but in the ethanol insoluble fraction of killifish (*Fundus sp.*; Pickford and Atz, 1957). Apart from these observations, little progress was made until, almost 20 years later, interest in MCH revived when Baker and Ball (1975) noted that colour changes in the Guppy (*Poecilia sp.)* could only be explained by the existence of dual hormonal control. In this species the skin melanophores are controlled by both nerves and pituitary hormones. After hypophysectomy, the melanophores are still capable of background adaptation because of direct innervation. If the skin melanophores are denervated, then melanin concentration or dispersion only occurs if the pituitary gland is still present. While full melanin dispersion requires both nerves and pituitary hormones, full melanin concentration, only occurs following denervation. Thus, the denervation of melanophores causes initial

melanin dispersion, but if fish are transferred to a pale background, melanin dispersion is delayed. This indicates the existence of a factor released only when fish are placed on a white background.

Black-adapted fish are very dark and have fully dispersed melanin granules within their skin melanophores, while in white-adapted teleosts, the melanin granules are concentrated into the centre of the melanophores and the fish appear pale in colour. The melanophore index (MI) is used to quantify the degree of melanin dispersion on a scale from 1 (full concentration) to 5 (full dispersion) (Hogben and Slome, 1931; Figure 2.1). Melanophores settle at an intermediate MI value after hypophysectomy, and when black-adapted fish are hypophysectomized, the MI falls from 3.9 to 2.3, but in hypophysectomized white-adapted fish, the MI rises from 1.0 to 2.3. These results suggest that two hormones are responsible for melanin dispersion and concentration, both emanating from the pituitary gland (Baker and Ball, 1975).

Teleost pituitary hormones can be separated by polyacrylamide gel electrophoresis (PAGE). In the Rainbow trout, while melanin dispersing activity is found in three bands, with *rf* values of 0.55-0.65, 0.70-0.80 and 0.95-1.00, melanin-concentrating activity is located only between *rf* 0.60 and 0.70, thus indicating that melanin dispersing and concentrating factors are two distinct molecules, (Baker and Ball, 1975). Rance and Baker (1979) examined the PAGE bands from several teleost species. They found that, for all species, clear band separations were generally obtained with only one band exhibiting MCH activity and at least two bands showing MSH activity. The pituitary MSH/MCH ratio

FIGURE 2.1. Diagrammatic representation of the appearance of skin melanophores of Rainbow trout and the factors that influence the movement of melanin granules within them. The melanophore index ranges from 1 to 5 where full aggregation is scored as MI 1 and full dispersion as MI 5



varies widely between species and is more marked for MSH than for MCH (Rance and Baker, 1979).

2.2. The Site of MCH Production

It was initially proposed that MCH was a neurointermediate lobe hormone because of its abundance at this site in minnow (*Phoxinus phoxinus*) pituitary glands (Kent 1959). Removal of this lobe however, failed to prevent paling when fish were transferred to pale-coloured backgrounds and this led Healey (1948) to suggest that MCH was an anterior lobe hormone.

Enamis (1955) demonstrated that a melanin-concentrating substance was active in the hypothalamus of catfish (*Siluridae sp.*) and concluded it was a neurohypophysial hormone. Other workers could not repeat these observations, so they were dismissed until the hypothalamic location of MCH neurones was established by Rance and Baker (1979).

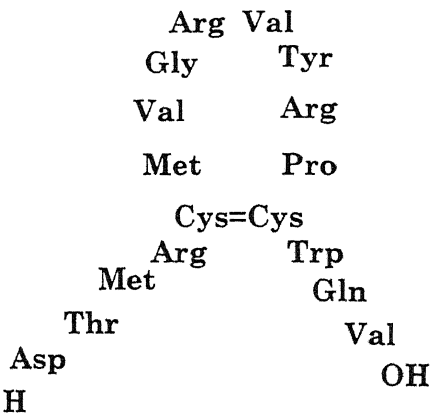
When antibodies to MCH became available, immunocytochemical studies confirmed that the precursor to MCH is produced by magnocellular neurones in the basal hypothalamus, and showed that axons from these perikarya project into the neurohypophysial lobe where the active hormone is stored before being released into the blood stream (Natio *et al.*, 1985; Bird *et al.*, 1989; Batten and Baker, 1988). Kent's (1959) inability to find MCH activity in the minnow was probably because the fish were adapted to a pale coloured background and under these conditions MCH is being actively released and levels in the hypothalamus will be consequently low.

Ultrastructural studies have shown that MCH is contained within membrane bound electron opaque secretory granules (Powell and Baker, 1987; 1988). In eels *(Anguilla anguilla)* adapted to a pale coloured background, there is increased secretory activity of MCH neurones and a decrease in the abundance of granules in the nerve terminals (Powell and Baker, 1988).

2.3. The Chemical Structure of MCH and its Precursor

The chemical structure of MCH was first determined from a purified extract of Chum salmon (*Oncorhynchus keta*) pituitary glands (Kawauchi *et al.*, 1983). The neurohormone is a cyclic heptadecapeptide with a disulphide bond between the two cysteine residues at positions five and 14 (Figure 2.2). The active site for melanin concentration is contained within the ring of the MCH molecule and most amino acid residues that form the ring are needed for full potency. The disulphide bond and tryptophan residue at position 15 are essential for MCH activity in eel (*Synbranchus marmoratus*; Matunaga *et al.*, 1989), however this differs in other species (Baker *et al.*, 1990, Hadley *et al.*, 1987). The side arms potentiate this activity and are important in the positioning and binding of the molecule to its receptor (Paul *et al.*, 1990).

FIGURE 2.2. The primary structure of salmonid MCH. After Eberle, 1988.



The structure of rat and human MCH are identical, and consist of 19 amino acid residues, differing from fish MCH by two extra amino acids at the N-terminal and four substitutions (Nahon *et al.*, 1989b; Vaughan *et al.*, 1989; Presse *et al.*, 1990).

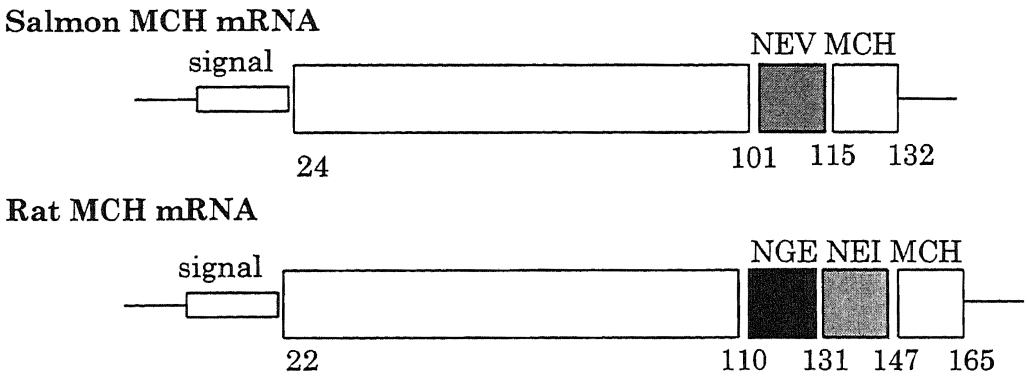
The nature of the MCH precursor within the hypothalamus was established by work on Rainbow trout by Bird and co-workers (1990). They measured immunoreactivity and bioactivity following gel filtration of hypothalamic and pituitary extracts, and found only 10% of the immuno-reactive (ir) MCH was due to the large molecular weight form in the hypothalamus but less than 1% of immunoreactive material in the neurointermediate lobe.

In salmonids, which are believed to be tetraploid, two MCH genes are expressed in the hypothalamus. The structural organisation of the MCH genes in fish and mammals show several differences . In fish there is one exon (Takayama *et al.*, 1989) but in mammals there are three, with the sequence for MCH being dissected by the second intron (Thompson and Watson, 1990). The splitting of the MCH sequence by an intron is found throughout the mammalian orders that have been investigated.

In salmonids, the MCH mRNAs derived from each gene both code for a pre-prohormone 132 amino acids long (Figure 2.3). Cleavage of the signal peptide at alanine 24 gives rise to 108 amino acid prohormone in chum salmon (*Oncorhynchus keta*; Ono *et al.*, 1988; Minth *et al.*, 1989; Takayama *et al.*, 1989). In higher vertebrates, the prohormone contains 165 amino acid residues (Nahon *et al.*, 1989b). The MCH neuropeptide is located at the C-terminal in all vertebrates, cleavage of the region

between proMCH and the MCH neuropeptide generates a second 13 amino acid peptide in fish named MCH Gene Related Peptide (MGRP; Bird *et al.*, 1990) or NEV (Nahon *et al.*, 1991). In higher vertebrates, a 13 amino acid and a 19 amino acid peptide are released by cleavage, these are called NEI and NGE respectively (Parkes and Vale, 1992). A comparison of the structure of proMCH in different mammals reveals a sequence homology of 80-90% but between salmonid and mammalian proMCH less than 20% of the amino acid sequence are identical (Nahon *et al.*, 1989a; b).

FIGURE 2.3. The structural organisation of the pre-pro MCH in salmonids and the rat. After Nahon *et al.*, 1989a; b.



The process by which MCH is derived from its precursor was studied *in vivo* in Rainbow trout hypothalami using radioisotope labelling (Bird *et al.*, 1990). The prohormone generates an unstable intermediate complex of NEV-MCH, and this is converted by several small steps to produce mature MCH.

2.4. MCH Synthesis and Secretion

2.4.1. Hormonal Aspects

MCH release from the pituitary gland occurs most actively when fish are kept on a white background. (Baker and Ball, 1975;Rance and Baker, 1979; Barber *et al.*, 1987). Circulating MCH titres are raised in white-adapted fish (>50 pmol l^{-1}) and fall when they are transferred to black tanks or to darkness (5-10 pmol l^{-1}) (Kishida *et al.*, 1989). In the blood, the level of circulating MCH is at a lower concentration than its antagonist αMSH (150-300 pmol l^{-1}). In white-adapted fish, the molar concentration of αMSH is 3 fold higher than that of MCH but in black-adapted fish the molar concentration of αMSH is 80 fold higher than that of MCH (Kishida *et al.*, 1989).

The plasma levels of MCH observed in trout are inadequate to achieve maximum pallor in this species (Baker *et al.*, 1986; Green and Baker, 1989) yet in other fish such as the Grass carp (*Ctenopharyngodon idellus*) , circulating hormones are high enough to cause colour change after denervation (Baker and Ball, 1975; Pickfold and Atz, 1957). Trout depend on the synergy between the action of the autonomic nervous system and MCH to produce maximum pallor. Further evidence for direct innervation of the melanophores comes from the speed with which fish can change colour when transferred between black and white tanks (Rodrigues and Sumpter, 1984). Thus MCH does not act alone, noradrenalin markedly potentiates the affect of MCH on trout melanophores. This explains why denervated trout caudal fins remain dispersed despite high MCH titres since noradrenalin is no longer able

to enhance the melanin concentrating properties of MCH (Baker *et al.*, 1986). Noradrenalin causes melanin aggregation by inhibiting adenylcyclase (Eberle, 1988) whereas MCH acts via the phosphoinositol pathway and diacylgycerol (Abrao *et al.*, 1991).

It is also believed that MCH may indirectly suppress the release of αMSH from the pars intermedia (Baker *et al.*, 1986) since αMSH release from cultured trout neural intermediate lobes is enhanced when MCH is removed by immunoabsorbtion (Barber *et al.*,1987).

MCH titres can change rapidly compared to the relatively slow change in blood MSH titres (Rodrigues and Sumpter, 1984; Baker 1988) The intermediate colour that trout exhibit when kept in the dark cannot be explained by plasma MSH/MCH titres alone, because dark adapted fish have lower MCH titres than are found in black-adapted. This effect could be due to a change in the neural input to the melanophores (Kishida *et al.*, 1989) or to the effects of another circulating melanin aggregating factor such as melatonin (Hafeez, 1970).

All teleost melanophores respond to MCH but species sensitivity to the hormone varies *in vitro* (Hadley *et al.*, 1987). MCH can cause melanin dispersion in amphibians and reptiles, and high concentrations will have the same effect in fish (Wilkes *et al.*, 1984; Baker *et al.*, 1985b; Ide *et al.*, 1985). The αMSH -like activity of MCH is due to sequences outside the ring structure which are able to bind to the αMSH receptor (Matsunaga *et al.*, 1989). Other factors apart from MSH and MCH play a role in adaptation to colour change. Neurotransmitters from the autonomic nervous system induce pigment aggregation as discussed earlier, and ATP can also induce melanin dispersion (Fujii and Oshima,

1986; Figure 2.1). Second messenger system activators are potent stimulators of MCH and its precursors in hypothalamic cell culture. Both cAMP and cGMP pathways regulate MCH peptides (Parkes and Vale, 1992).

As well as causing the concentration of pigment granules in skin melanophores (Rance and Baker, 1979; Baker, 1988) MCH will also elicit pigmentary effects on other skin chromatophores (Oshima *et al.*, 1986).

In summary, in all teleosts MSH and MCH have antagonistic effects on melanin aggregation and the combined effect depends on the relative concentrations of both hormones. Different species have different sensitivities to each hormone and their mode of action also varies between species. Thus in trout, melanin concentration is caused by MCH overriding the effect of MSH, but in Grass carp melanin concentration is caused by the withdrawal of MSH (Baker, 1991).

2.4.2. Anatomical Aspects

The background conditions fish are exposed to influences the structure and activity of the MCH neurones. When Grass carp are kept on illuminated black backgrounds the synthetic activity is depressed, secretory granules are sparse in the cell bodies and the nuclei are small with indiscernible nucleoli and the production of irMCH is reduced. By contrast, Grass carp kept on white backgrounds for 6 months show enhanced synthetic activity, with enlarged strongly granulated neuronal cell bodies and nuclei and distinct nucleoli indicating increased production of MCH (Bird and Baker, 1989).

Immunohistochemical studies have shown that MCH neurones do not form a homogeneous population (Bird and Baker 1989). The secretory granules of some neurones contain only MCH while others apparently contain MCH and its antagonist αMSH (Powell and Baker, 1987). The highest concentration of ir αMSH co-localised with irMCH is found in the basal hypothalamus (Kishida *et al.*, 1988). When fish are moved from pale to dark backgrounds the hypothalamic ir αMSH content remains unchanged unlike the irMCH content which increases (Kishida *et al.*, 1988). This difference suggests that either the two products are independently regulated in the same neurone or that the MSH-like activity in the MCH neurones may not be authentic but due to antibody cross reactivity (Kishida *et al.*, 1988). Recent evidence in rat shows that irMCH coexists with unrelated peptides such as growth hormone-releasing factor (GRF), CRF and αMSH in neurones within the hypothalamus. It has been suggested that the product of MCH precursors i.e. NEI and NGE are the peptides responsible for immunoreactivity previously attributed to αMSH, GRF and CRF (Watson and Akil, 1980; Fellman *et al.*, 1986; Merchenthaler *et al.*, 1986; Naito *et al.*, 1986; Cardot *et al.*, 1994).

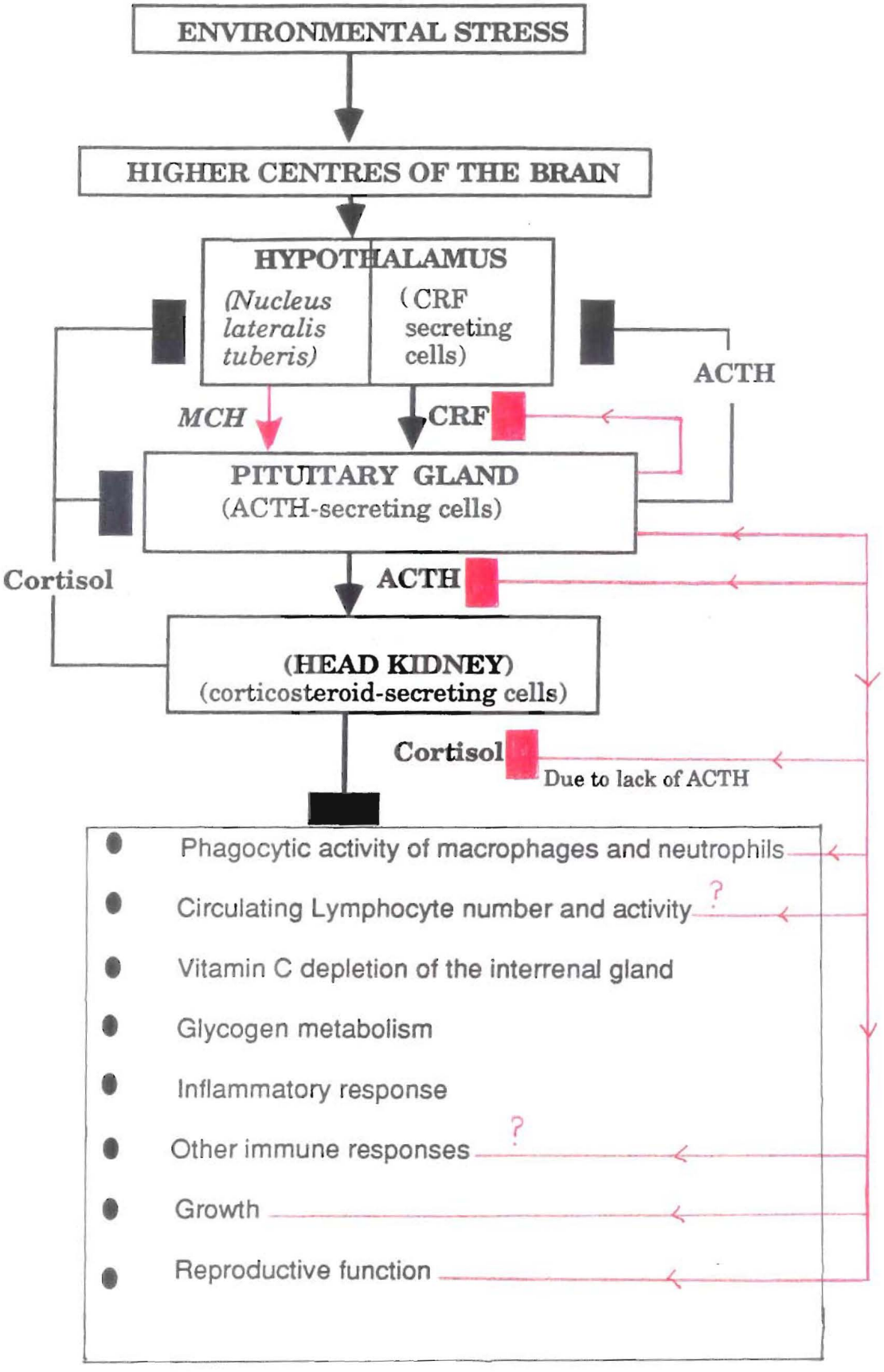
MCH neurones also have different axonal projections. Some axons extend into the brain while others project only into the pituitary gland (Kishida *et al.*, 1988). There is also evidence that some neurones are much more responsive to background colour than others. Whether the degree of responsiveness is due to functional differences between the neurones or to different granular structures within the neuron is unclear (Bird and Baker, 1989).

2.5. MCH and the Stress Response

The idea that the hypothalamo-pituitary-interrenal axis might be influenced by MCH was first suggested in the early 1980s (Figure 2.4). Trout adapted to black tanks are more responsive to moderate stress and show higher plasma ACTH and cortisol concentrations than fish kept in white tanks (Baker and Rance, 1981; Gilham and Baker, 1985). Cortisol secretion was originally thought to be stimulated by melanotrophic cells from the pars intermedia and in Rainbow trout and eels the melanotrophic cells are very active in black-adapted fish, but this is not true of other teleosts (Scott and Baker, 1975). In view of the fact that MSH and ACTH are both derived from the same precursor molecule, the possibility that MSH could stimulate cortisol secretion was considered, but eliminated since isolated trout interrenal tissue cannot be stimulated by MSH to release cortisol. (Rance and Baker, 1981).

The ACTH released spontaneously from cultured partes distales of stressed trout is significantly higher when they are taken from black- rather than white-adapted fish (Baker *et al.*, 1985a). The enhanced secretion of MCH in white-adapted fish inhibits ACTH release and when MCH is added to cultured black-adapted pituitaries, ACTH release is inhibited. These initial results suggested that MCH could act as a corticotrophin-release inhibiting factor in trout, preventing the stress-elevated release of ACTH and resulting in the lower plasma cortisol titres observed in white-adapted fish (Baker *et al.*, 1985a). Later work by this author suggested MCH did not effect ACTH secretion, but the MCH preparations used in earlier experiments were altered in such

FIGURE 2.4. Diagram illustrating how MCH effects the HPI axis. The secretion of MCH (→) and its inhibitory effects on the HPI axis (⊣). Environmental stress leads to a cascade of hormonal release (→). Cortisol inhibits (⊣)a wide range of cellular functions. Several negative feedback mechanisms are known to operate within the HPI axis.


ENVIRONMENTAL STRESS
HIGHER CENTRES OF THE BRAIN
HYPOTHALAMUS
(Nucleus lateralis tuberis)
(CRF secreting cells)
ACTH
MCH
CRF
PITUITARY GLAND
(ACTH-secreting cells)
Cortisol
ACTH
(HEAD KIDNEY)
(corticosteroid-secreting cells)
Cortisol
Due to lack of ACTH
Phagocytic activity of macrophages and neutrophils
Circulating Lymphocyte number and activity ?
Vitamin C depletion of the interrenal gland
Glycogen metabolism
Inflammatory response
Other immune responses ?
Growth
Reproductive function

a way as to endow them with corticotrophin inhibiting properties (Baker, 1994).

Other work on rats failed to demonstrate that MCH inhibits the release of ACTH. Navarra (1990) and Jezova *et al.* (1992) have shown that the introduction of rat MCH into rat brain stimulates the release of ACTH. Clearly, the effect of MCH on the secretion of ACTH and cortisol still needs to be clarified.

CRF stimulates the release of ACTH from cultured pituitary glands taken from stressed fish, but the addition of MCH blocks this effect or renders the corticotropes less sensitive to CRF (Green and Baker, 1991). Whether MCH acts via the general circulation or by direct innervation of the corticotrophs is unclear. MCH probably also acts by blocking the release of CRF from the CRF neurones. The idea of neuronal centres inhibiting ACTH secretion is common to other teleosts. For example when the habenular nuclei are destroyed in goldfish, corticosteroid secretion is increased (Fryer and Peter, 1977b).

Plasma levels of MCH increase following repeated stress of white-adapted fish, and this response is antagonised by negative feedback of corticosteroids (Green and Baker, 1991). There is an inverse relationship between plasma MCH and cortisol titres. Green and Baker (1991) investigated the affect of MCH directly on interrenal cells and found that MCH had no affect on cortisol release but could inhibit CRF release from the hypothalamus.

Using Rainbow trout that had been reared from eggs on black or white backgrounds, Green *et al.* (1991) attempted to produce fish with marked differences in MCH synthesis and secretion. However, cortisol release was found to be similar for black- and white-reared fish after stimulation with synthetic ACTH and the release of CRF was found to be the same for both groups. If the fish were slightly stressed, there was a slight increase in the CRF release, particularly from black-reared fish hypothalami. When endogenous MCH was removed by immunoabsorbtion, CRF release was found to be significantly raised in the white-reared fish. This indicates that the hypothalami of white-reared fish contain more releasable CRF than black-reared fish (Green *et al.,* 1991) and implies that under stressful rearing conditions, white-reared fish have reduced cortisol negative feedback, causing an accumulation of CRF. Thus MCH does not inhibit CRF synthesis, only its release (Green *et al.,* 1991). Although it is still unclear whether the HPI axis is affected by the circulating or centrally released MCH when under stressful conditions, it is clear that MCH depresses the release of CRF and not its synthesis. Which CRF is affected by MCH has not been established.

Since MCH has been indicated in the control of cortisol secretion in fish, Nahon and co-workers (1991) examined the effect of adrenalectomy on the production of MCH mRNA in rats using the northern blotting technique. They found there was a 2.5 fold decrease in the production of MCH mRNA in adrenalectomised rats, but treatment with dexamethasone fully restored the MCH mRNA levels. Further support for the idea that glucocorticoids may positively control the expression of MCH *in vivo* has been suggested recently by Presse *et al.* (1992). In rat hypothalamic cell culture, NEI and MCH content are both enhanced by

treatment with dexamethasone. A 60% reduction in MCH mRNA levels was evoked after one day of chronic stress in rats, but by seven days the levels of MCH mRNA were the same for control and stressed groups. Presse and co-workers explained these results in terms of a positive glucocorticoid feedback mechanism counteracting the inhibitory effect of a neurogenic shock on MCH gene activity. CRF was also found to suppress the secretion of NEI and NGE in hypothalamic cells, further support for the theory that the stress response reflects the interactions between CRF and MCH neuronal networks, with a feedback loop of glucocorticoids inducing opposite effects on synthesis and secretion of MCH and CRF.

3. Fish Immunology

3.1. Introduction

Compared with mammals, the fish immune system is poorly understood. For example, the presence of immunoglobulins (Ig) on the surface membranes of fish lymphocytes has been the subject of much controversy (Etlinger *et al.*, 1977; Chilmonczyk, 1982; Clem *et al.*, 1985). Distinct T and B cell populations have not been convincingly demonstrated, due to the difficulty in thymectomizing young fish and the lack of inbred strains, and only an analogy can be drawn with the mammalian immune system. The separation of two functionally distinct cell types on nylon wool columns has suggested the possibility of two discreet lymphoid cell populations (Ruben *et al.*, 1977). Monoclonal antibodies to serum IgM have been raised, but these only react with about 30-40% of blood lymphocytes. (Deluca *et al.*, 1983), presumably recognising B cell-like lymphocytes which also respond to lipopolysaccharide (LPS). Presumptive T cells lack surface immunoglobulins but respond to concanavalin A (Con A) and also slightly to LPS (Sizemore *et al.*, 1984; Clem *et al.*, 1985).

3.2. The Lymphatic System

3.2.1. The Haematopoietic Organs

Lymphoid organs are classified as either primary or secondary, depending on whether they are major sites of lymphopoiesis. In mammals, T cells mature in the thymus, B cells in the fetal liver and bone marrow. In birds, where the bursa of Fabricius is a specialised

region for B cell maturation (Lydyard and Grossi, 1993). In these primary lymphoid organs, stem cells are present as well as differentiated lymphocytes and other mature white blood cells (WBC). After maturation mammalian lymphocytes circulate to secondary lymphoid organs (Brahim and Osmond, 1970). In fish there is tentative evidence that T cells mature in the thymus and B cells in the head kidney (Etlinger *et al.*, 1976). In mammals it is believed that the lymphocytes acquire their specific antigen receptors in the primary lymphoid organs, but, there is no equivalent information available for fish.

Secondary lymphoid tissues in mammals include the spleen, lymph nodes and tonsils where lymphocytes interact with each other and with antigens (Lydyard and Grossi, 1993). The corresponding organs in fish are the spleen and head kidney.

In fish the importance of the various lymphoid organs is influenced by the age of the fish. The kidney and blood are both important in fry and adults but in the spleen and thymus the number of lymphoid cells gradually declines with age. Blood leucocyte levels vary enormously between fish, an observation thought to be due to a range of genetic, physiological and immunological factors (Chilmonczyk, 1982).

3.2.2. Primary Lymphoid Organs

The Thymus Gland

Whereas the thymus gland in mammals is located in the thorax, in fish it is a bilateral gland that lies in the gill chamber in the angle between the operculum and the head wall (Chilmonczyk, 1982). The mammalian gland is bilobed and within each lobe, the thymocytes are arranged into a inner medulla, containing mature cells, and an outer cortex of mainly immature cells. The fish thymus is enclosed in a thin epithelial capsule, which is perforated in Rainbow trout, a feature that allows intimate contact between the thymus and the outside environment (Chilmonczyk, 1985). It has been postulated that the exposed position of the thymus in fish means that it is always exposed to pathogens (Grace and Manning, 1980) and can therefore rapidly activate defence mechanisms (Tatner and Manning, 1982). Beneath the epithelial capsule is an outer zone of small lymphocytes with poorly developed epithelial support and a inner zone of lymphocytes with a developed framework of epithelial cells, although neither zone is clearly delineated (Chilmonczyk, 1985). No definitive conclusions can be drawn as to whether the inner and outer zones of the fish thymus are equivalent to the cortex and medulla of the mammalian gland (Zapata, 1981).

There is some debate as to whether the thymus is a site for development of both T and B cells. Ellis and Parkhouse (1975) found up to 80% of lymphocytes in the skate (*Raja naevus*) thymus bear M-type immunoglobulins, a feature of mammalian B cells. Other workers have also noted the B cell-like nature of some teleost thymic cells, such as their ability to form plaques (Sailendri and Muthukkaruppan, 1975;

Kaattari and Irwin, 1985) and the presence of plasma cells (Zapata, 1981).

The mammalian thymus degenerates with age, beginning at puberty in man and continuing throughout life. Chilmonczyk (1985) observed no marked degeneration of the thymus in Rainbow trout even after 5 years, but observed it was better developed in young rather than adult trout. As a proportion of total body weight, the thymus gland reaches its maximum size in fish at 2-3 months but its maximum absolute weight is achieved at 5-12 months (Tatner, 1985). Thymocyte numbers decrease steadily with age, but the proportion of epithelial tissue increases so that the weight of the thymus remains more or less constant in adult fish.

The Head Kidney

Many stem cells as well as immature and mature erythrocytes and leucocytes are present within the head kidney, supported by a reticular cell stroma (Yasutake and Wales, 1983). Of the leukocytes in the head kidney about 50% are lymphocytes, 35% neutrophils and 11% macrophages (Congelton *et al.,* 1990). Blast cells, large undifferated cells, characterised by prominent euchromatic nuclei with distinct nucleoli, have also been reported in the head kidney (Cenini, 1984) although no clear lineage between these blast cells and mature leukocytes has been suggested. Macrophages are more numerous in the head kidney than in the circulation or in other organs, and they usually occur in aggregates with other lymphoid cells (Ellis and de Sousa, 1974). Melanomacrophages, cells that contain melanin granules within

their cytoplasm, can also be identified within the head kidney (Anderson, 1974).

The anterior kidney is considered the most important lymphoid organ in fish and has the largest lymphoid population (Chilmonczyk, 1982). There is evidence that the anterior kidney is the primary site of B cell development (Chiller *et al.*, 1969b; Etlinger *et al.*, 1976; Kaattari and Irwin, 1985; Irwin and Kaattari, 1986) and it is also important in producing erythroid, lymphoid and myeloid cells (Temmink and Bayne, 1987).

In mammals, in addition to being the site of B cell differentiation, the adult bone marrow is also an important secondary lymphoid organ containing mature T cells and numerous plasma cells. Based on ultrastructural (Zapata, 1979) and histoenzymic similarities (Castillo *et al.*, 1990) there is growing evidence that the anterior kidney is homologous to the bone marrow of higher vertebrates. Chilmonczky (1982) has shown that 60% of spleen and kidney lymphocytes in trout are of thymic origin, thus supporting the theory that the anterior kidney also acts as a secondary lymphoid organ. Temmink and Bayne (1987) reported ultrastructural observations suggesting fish T cell-like and B cell-like populations are present in the head kidney. Razquin and co-workers (1990) reported IgM-negative cells in the kidney of trout, and similar results have been reported for carp (Secombes *et al.*, 1983). The presence of IgM-negative cells could be due to the early development of cellular immunity in teleosts (Botham *et al.*, 1980; Tatner and Manning, 1983) or to the presence of pre-B cells that do not stain for the immunoglobulin marker.

3.2.3. Secondary Lymphoid Organs

During their life lymphocytes migrate from the primary lymphoid organs to the secondary peripheral tissues. In mammals, the secondary sites are of two forms, well ordered encapsulated organs such as the spleen and lymph nodes and non-encapsulated accumulations associated with mucosal surfaces (Lydyard and Grossi, 1993). In fish, the homologous structures of mammalian lymph nodes have not been identified (Corbel, 1975), although the spleen and head kidney of fish are considered secondary lymphoid organs.

The Spleen

The mammalian spleen consists of a collagenous capsule which, together with a reticular framework, aids cell support. There are two types of tissue within the mammalian spleen, the red pulp which is mainly concerned with red blood cell (RBC) destruction, as well as being a reservoir of RBCs, platlets and granulocytes. The white pulp consists of discreet lymphoid areas arranged around a central artery were antigen presentation to B cells occurs (Lydyard and Grossi, 1993) The fish spleen serves as an accessory haematopoietic organ and, as in mammals, a site for blood cell destruction and erythrocyte storage. It differs from the mammalian spleen because the red and white pulps are not markedly distinct from one another (Anderson, 1974). The connective tissue framework is poorly organised (Robertson and Wexler, 1960) and the spleen capsule is made up of only a thin connective tissue layer, which is not as thick or as prominent as in the mammalian organ.

Spleen imprints reveal the presence of lymphocytes, neutrophils, granulocytes as well as erythrocytes at various stages of development. The fish spleen contains both T and B cell-like lymphocytes (Razquin *et al.*, 1990) and is the last organ where B cell-like lymphocytes appear during the development of the Rainbow trout, suggesting the spleen is not important during the development of the immune system (Ellis, 1977; Rijkers and Muiswinkel, 1977; Grace and Manning, 1980).

In order to correlate with mammalian terminology, the criteria used in fish for the identification of blood cells tends to be morphological, ontogenic and functional (Ellis, 1976).

3.2.4. Erythrocytes

The majority of cells in vertebrate blood are erythrocytes. The number of circulating RBCs in fish usually ranges between 0.77 and 1.58×10^6 cells ml^{-1} (McCarthy *et al.*, 1973; Wedemeyer and Yatasuke, 1977) and this is low compared with mammals (Mott, 1957) which have from 3.9 to 6.5×10^6 cells ml^{-1} (Richmond and Parker, 1985). Their morphological characteristics are shown in Plate 3.1.

The phenomenon of increased erythrocyte counts have been reported previously. In freshwater fish raised haematocrit values following stress are common (Housten *et al.*, 1971).

3.2.5. Leucocytes

The white blood cell population in fish lies between 7.8 and 80 $x10^3$ cells ml^{-1} The actual number is influenced by factors such as, sex, age, diet, temperature, season, health and water quality (Ellis, 1976; Pickering, 1986; Pickering and Pottinger, 1987c; Congelton *et al.*, 1990). The leucocyte count in fish is considerably higher than that of man and most other vertebrates (4 to 11 $x10^3$ ml^{-1}; Andrews, 1965; Richmond and Parker, 1985).

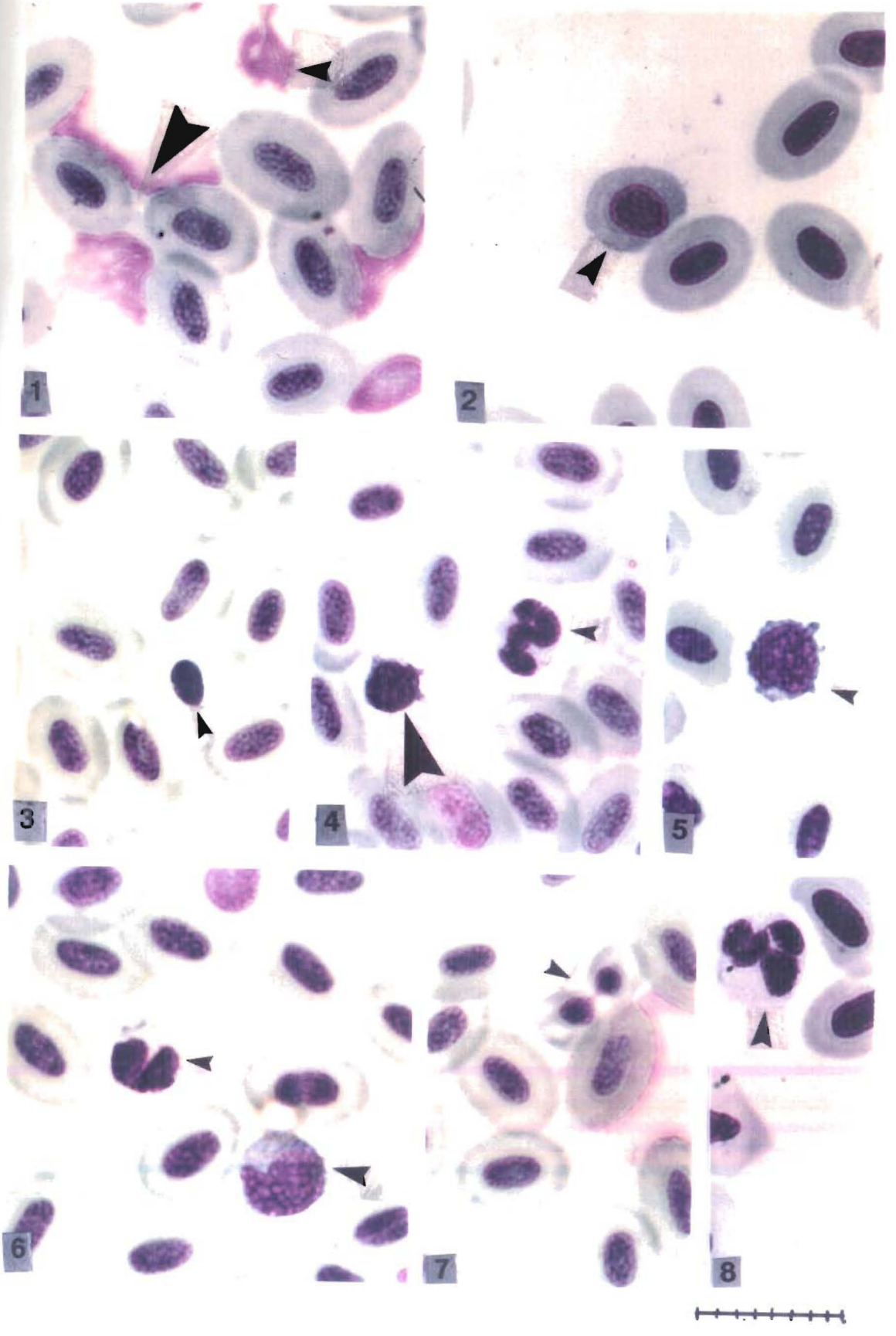
Lymphocytes.

As a proportion of leucocytes, fish blood contains a far higher percentage of lymphocytes (70-98%; Weinreb and Weinreb, 1969; Yatasuke and Wales, 1983) than mammalian blood, where the lymphocytes only account for 20-40% (Richmond and Parker, 1985).

Fish lymphocytes are mostly relatively small cells 7-10μm in diameter (see Plate3.1) but large lymphocytes 10-15μm are occasionally seen in blood smears, and are obvious in kidney and spleen imprints. These large cells are thought to be immature lymphocytes (Yasatake and Wales, 1983). Some workers have also reported the presence of plasma cells, that is B cells that have developed into antibody producing cells (Ellis, 1976).

PLATE 3.1. Rainbow trout *(Oncorhynchus mykiss)* peripheral blood smears, cells stained with the Leishman-Giemsa stain. Figure scale 20μm. Photomicrographs were taken by Olympus microscope X 1000.

Fig 1 Arrows show erythrocytes in various stages of degeneration.
Fig 2 Arrow highlights an immature erythrocyte.
Fig 3 Arrow indicates a thrombocyte.
Fig 4 Large arrow shows a lymphocyte and small arrow a neutrophil.
Fig 5 Arrow points to a lymphocyte with pseudopia.
Fig 6 Large arrow indicates a possible monocyte and the smaller arrow a neutrophil.
Fig 7 Arrow points to an erythrocyte undergoing mitosis.
Fig 8 Arrow indicates a neutrophil.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Polymorphonucleocytes

In Rainbow trout blood neutrophils are the only polymorphonuclear (PMN) cells present (Klontz, 1972; Yasutake and Wales, 1983) although other workers disagree with this conclusion (Chiller *et al.*, 1969b; Ellis, 1977). In other species of fish eosinophils and basophils have been reported in blood (Weinreb, 1963a; b). Neutrophils account for between 1 and 9% of total leucocytes in juvenile Rainbow trout (Yasutake and Wales, 1983), much lower than in mammals where PMNs account for 40-75% of the WBC population. Numerous factors affect PMN cells, including age and health of the fish (Pickering, 1986).

Neutrophils are important in the inflammatory response to microbial agents. They have phagocytic ability (Finn, 1970; Finn and Nielson,1971) although this may not be their primary function (Ellis *et al.*, 1976).

Other granulocytes, apart from the neutrophils, are evident in the anterior kidney and spleen, including basophils and occasional eosinophils (Temmink and Bayne, 1987; Yatasuke and Wales, 1983).

Monocytes/Macrophages

Monocytes account for between 2 and 10% of the leukocyte population in mammals, but only for 0.1 to 0.2% of fish WBCs (Weinreb, 1963b). Macrophages are not usually seen in the circulation but develop from monocyte precursors following migration into tissues including the anterior kidney, spleen and gut lining. In fish, it is well documented that macrophages have phagocytic ability (Anderson, 1974; Ellis, 1976;

1977) as well as being necessary as accessory cells for mitogenic responses of T cells and for antibody responses (Clem *et al.*, 1985). In higher vertebrates the role of macrophages as accessory cells is also well established (Unanve *et al.*, 1984).

Thrombocytes

Thrombocytes account for between 1 to 6% of the total leukocyte population (Wedemeyer and Yasutake, 1977). They are believed to play a role in blood clotting (Srivastava, 1969; Wardle, 1971) and there is some debate over their phagocytic ability (Fange, 1968; Ellis, 1977). Strong debate surrounds the fish thrombocyte, its structure, function and ontogeny have been the subject of much contention.

3.3. Factors Influencing the Immune System

3.3.1. Biological Rhythms

The immune system of mammals is influenced by their circadian rhythms (Haus *et al.*, 1983) and other biological and seasonal rhythms (Laerum and Aardal, 1981; Shifrine *et al.*, 1982). Careful consideration needs to be given to the time of day at which immuno-active substances are administered (Hrushesky, 1984) as well as other conditions such as the lighting regime and length of time animals have been acclimatised. The light-dark cycle can influence the resultant humoral and cell-mediated immune responses (Hayashi and Kikuchi, 1982) and the time of day that antigenic exposure occurs can significantly influence the magnitude of the resulting immune response (Pownall *et al.*, 1979).

Fish are also influenced by similar rhythms (Schwassmann, 1971; Thorpe, 1978) that affect the immune system (Lehmann, 1976; Matty, 1978) and there are seasonal variations in the relative weights of the kidney and spleen (White and Fletcher, 1985).

3.3.2. Glucocorticoids

Glucocorticoids usually cause depression of the immune response whether applied *in vivo* or *in vitro*. However, glucocorticoid treatment may enhance the immune response depending on the steroid concentration, the physical state of the animal (Cupps *et al.*, 1982), the timing of treatment and the immune assay employed (Cupps and Fauci, 1982).

In mammals, glucocorticoids generally have suppressive effects and are believed to interact with a single population of glucocorticoid receptors (Munck and Leung, 1977), although the receptors in different organs have different sensitivities to cortisol in fish (Maule *et al.*, 1993). In humans, treatment with cortisol causes the redistribution of lymphocytes, T-cells are most sensitive, from the blood to the bone marrow (Cupps and Fauci, 1982). In mice and rabbits corticosteroids induce lysis of lymphocytes (Dougherty and White, 1945).

The suppressive effects of corticosteroids on the immune system of teleosts are well established. Stress causes the release of glucocorticoids that reduce the number of white blood cells, particularly lymphocytes in fish (Mcleay, 1973a; b; Pickering, 1984; Pickering and Pottinger, 1987a; d; Maule *et al.*, 1987) and suppresses their activity

(Ellsaesser and Clem, 1986). Cortisol treatment also reduces thrombocyte numbers (Wiik *et al.*, 1989) but its affect on neutrophils is unclear. Some workers have observed neutrophil degeneration (Ellsaesser and Clem, 1986) but others have found no effect (Pickering *et al.*, 1982; Pickering, 1984).

Effects of Glucocorticoids on T cells

In recent years mammalian T cells have been shown to secrete a wide range of chemical messengers generally referred to as cytokines which modulate and control the activity of the immune system. Glucocorticoids have far reaching effects on the activity of these and other cells and some relevant examples of their action are shown in Table 3.1.

Compared with mammals information on fish immune cells is very limited but it is well established that fish lymphocytes can secrete interferon molecules (De Sena and Rio, 1975; Okamoto *et al.*, 1983; Graham and Secombes, 1988; 1990). Moreover, Carp (*Cyprinus carpio L.*) and Atlantic salmon (*Salmo salar*) leucocytes have been shown to produce and release growth factors in response to mitogen stimulation that are believed to be analogous with mammalian interleukins (Grondel and Harmsen, 1984; 1985; Smith and Braun-Nesje, 1982). Two actions of mammalian lymphokines have also been demonstrated in fish; (1) the inhibition of leukocyte migration by migration-inhibition factor and (2) mixed leucocyte reactions mediated by a mitogenic factor (Ellis, 1986).

TABLE 3.1. Some effects of glucocorticoids on immune cell types and on cytokine production in mammals

Cytokine	Abbreviation	Site of production	Main Actions	Effect of Glucocorticoids	References
Macrophage activating factor	MAF	T-cells	activates macrophages	suppresses action and production of MAF	Kelso & Munck (1984) Schultz *et al.* (1979)
Gamma Interferons	δIFN	T cells Natural Killer cells	activates macrophages, stimulates natural killer cells, regulates IgG receptors	suppresses action and production of δIFN and blocks macrophage IgG receptors	Stebbing & Weck (1983) Guyre *et al.* (1981) Rytel & Kilbourne(1966).
Interleukin 1	IL-1 (LAF)	many cell types	regulates T-cell pfoliferation, stimulates Il-2 production	inhibits production of IL1 and therefore suppresses synthesis of IL-2	Snyder & Unanue (1982)
Interleukin 2	IL-2 (TCGF)	T-cells	stimulates T and B-cells, natural killer cells, macrophages and monocytes.	inhibits production of Il-2	Gillis *et al.* (1979 a,b) Oppenheim *et al.*, (1982)
Colony Stimulating Factor	CSF including IL-3	T-cells	stimulates maturation of macrophages and granulocytes	inhibits development of cells, and production of CSF.	Kelso & Munck (1984)

Effects of Glucocorticoids on B cells

The actions of glucocorticoids on B cells in mammals is variable and depends on the species involved, mice being particularly sensitive (Clamen, 1972). There is also evidence that different B cells show different sensitivities (Clamen, 1972; Roess *et al., 1982).* Virgin and immature B cells are steroid sensitive before exposure to antigens (Clamen, 1972) but once they develop into memory or plasma cells they become unresponsive (Clamen, 1972).

Antibody production is diminished by high doses of glucocorticoids, yet after primary immunisation, IgG production is enhanced (Tuchindaa *et al.*, 1972; Rusu and Cooper, 1975). Cupps *et al.,* (1984) postulated that this increase in antibody production is due to altered T cell function rather than to a direct action on B cell function, other workers suggested similar mechanisms (Saxon *et al.,* 1977).

The mechanism that causes cortisol-induced suppression of the fish immune system is not yet clearly understood, but there is some evidence to suggest that cortisol acts by suppressing the production or activity of interleukin-like factors necessary for the differentiation of lymphocytes from their precursors (Tripp *et al.,* 1987; Kaattari and Tripp, 1987; Table 3.2). Thus, inhibition by cortisol can be reversed by the addition of supernatant from antigen-stimulated cultures (Tripp *et al.*, 1987) or by the addition of interleukin 1 (IL-1; Kaattari and Tripp, 1987).

TABLE 3.2. Effect of Glucocorticoids on the Immune Systems of Fish.

Function	Action	Reference
macrophage secretion of IL-1 (LAF)	unaffected	Ellsaesser & Clem, 1986
T cell-like proliferation	suppressed	Grimm, 1985
Circulating antibody titres	suppressed	Anderson *et al.*, 1982
Plaque formation	suppressed	Tripp *et al.*, 1987 Bennett & Wolke, 1987
B cell-like proliferation	suppressed	Ellsaesser & Clem, 1986

Recent work has revealed the presence of high-affinity, low capacity glucocorticoid receptors on pronephric and spleen leukocytes (Maule and Schreck, 1990; 1991), but their distribution on different types of lymphocytes is not established.

3.3.3. Stress

Following stress there is a increase in the levels of glucocorticoids, catecholamines and neuropeptides, as previously discussed (see 1. The Endocrinology of Stress). These hormones have wide-ranging effects and can impair immune function and lead to decreased disease resistance (Monjan and Collector, 1977; Riley, 1981; Yu and Clements, 1976; Munck *et al.*, 1984; Spangelo *et al.*, 1985). Leucocytes seem particularly sensitive to stress (Mcleay, 1973; Pickford *et al.*, 1971) and increases in plasma cortisol titres are associated with leucopenia (Pickering, 1981). Maule *et al.* (1989) working on Chinook salmon (*Oncorhynchus tshawytscha*) have shown that one day after stress,

disease resistance is actually enhanced due to non specific immune mechanisms and even seven days later, disease resistance may not necessarily be affected even though antibody production by lymphocytes has been reduced.

Dupont and co workers (1983) believe T suppresser cells are resistant to cortisol and T helper cells are glucocorticoid sensitive (Bradley and Mishell, 1982; Dupont *et al.,* 1983). Okimura *et al.* (1986a,b) suggested that stress-induced increases in the levels of corticosteroids and catecholamines act to suppress the function of T helper and T suppresser cells , thus reducing the response of T-dependent (TD) B cells whereas T-independent (TI) B cells were augmented by stress (Okimura *et al.,* 1986b).

In fish although T and B cells have not been confirmed, Tripp *et al.* (1987) has demonstrated that cortisol suppression of B-like cells in Chinook salmon is due to the inhibition of an interleukin-like substance necessary for activating antigen specific B-like cell precursors. Ellsaesser and Clem (1986) found that stress acts directly on circulating lymphocytes but does not affect the ability of macrophages to secrete lymphocyte growth factors.

3.3.4. Reproductive Hormones

It has been suggested that gonadal steroids may play an important role in the immune response of mammals. This view is based on evidence of sexual dimorphism in the immune response, changes in immunity during pregnancy (Anderson and Monroe, 1962; Finn *et al.*, 1972), changes following gonadectomy and sex hormone replacement (Sthoeger *et al.*, 1988; Castro *et al.*, 1973) and the presence of gonadal hormone receptors in lymphoid organs (Cohen *et al.*, 1983).

Oestrogens are natural modulators of the immune system (Calzolari, 1898) and females of many mammalian species produce higher antibody titres (Batchelor and Chapman, 1965; Eidinger and Garrett, 1972; Krzych *et al.*,1981), but have a less responsive cellular immune system than males (Santoni *et al.*, 1976; Inman, 1978; Kalland, 1980). Progesterone has also been shown to inhibit cellular immunity (Hulka *et al.*, 1965).

Sex steroids are believed to enter the target cells, where they bind to a specific receptor which then interacts with specific regions of DNA (Ringold, 1985; Barrack, 1987). Oestrogen receptors have been found in the mammalian thymus (Grossman and Natham, 1977; Grossman *et al.*, 1979) and spleen (Detlefsen *et al.*, 1979; Danel *et al.*, 1983), but testosterone receptors have not been observed (Rife *et al.*, 1990; Cohen *et al.*, 1983).

Androgens and oestrogens have been reported to induce atrophy of the thymus and peripheral immune organs (Dougherty, 1952) and to effect the number and function of thymocytes (Luster *et al.*, 1984; Ahmed *et*

al., 1985) and thymic factors (Stimson and Crilly, 1981; Grossman *et al.*, 1982). The mechanisms involved are unclear, but are thought to be due to the direct effect of ostrogen on thymocytes (Barr *et al.*, 1982) or by action on the hypothalamo-pituitary-gonadal-thymic (HPGT) axis (Beredovsky and Solkin, 1977). Receptors for other androgens and corticosteroids are present in thymic medullary tissue (Brodie *et al.*, 1980) and thymic factors can be regulated by oestrogens, androgens and corticosteroids (Stimson and Crilly, 1981).

Oestradiol exerts stimulatory effects principally on IgM production without increasing the number of antibody producing cells, possibly by a direct action on B cells (Myers and Petersen, 1985). Oestradiol only affects IgM antibodies, and it is believed that different steroids control the synthesis of different immunoglobulin classes (Borgatze and Katz, 1980). It is well documented that oestradiol increases protein synthesis (Jensen and De Sombre, 1972) and the increase in the plaque area seen after oestradiol is added *in vitro*, supports the idea that this steroid has the ability to enhance protein (antibody) synthesis by B cells (Sthoeger *et al.*, 1988).

Testosterone causes a marked reduction in the number of PFCs (Fujii *et al.*, 1975; Sthoeger *et al.*, 1988) in the spleen, probably by causing the inhibition of the differentiation of certain B lymphocyte stem cells.

In mammals the relationship between gonadal steroids and the immune system has been well documented (Table 3.3), however possible immune gonadal interactions in fish have not been widely investigated although seasonal changes in the immune function of fish have been related to reproductive cycles (Fletcher, 1986; Pickering, 1986; Pickering and

Table 3.3. Some effects of sex steroids on the immune system of mammals and fish

Effect of steroid	Mammals	Fish	Reference
Oestradiol on antibody production	enhances	no effect	Erbach & Bahr, (1988) Slater &Schreck, (1993)
Oestradiol on T cell number and activity	no effect	unknown	Myers *et al.* (1986) Holdstock *et al.* (1982)
Oestradiol on IL-1 production	stimulates	unknown	Hu *et al.*, (1988)
Testosterone on antibody production	suppresses	suppresses	Fujii *et al.*, (1975) Slater & Schreck, (1993)
Testosterone on T-cell proliferation	suppresses	unknown	Wyle & Kent, (1977)
Progesterone on T-cell proliferation	suppresses/ stimulates	unknown	Holdstock, (1982) Wyle & Kent, (1977)
Progesterone on IL-1 production	stimulates	unknown	Polan *et al.*, (1988)

Pottinger, 1987a). Slater and Schreck (1993) demonstrated the inhibitory effect of testosterone *in vitro*, the immunosuppressive effect was comparable to an equivalent dose of cortisol, but the effect of cortisol and testosterone together was greater than either alone. The authors believe the additive effect was due to each steroid acting on different cells. In Chinook salmon, testosterone causes immunosuppression of B-like cells (Slater and Schreck, 1993) but oestrogen has no significant inhibitory effect on antibody secretion (Slater and Schreck, 1993).

3.3.5. Temperature

Low environmental temperatures can be immunosuppressive in ectothermic vertebrates including fish (Avtalion, 1981). Temperatures of less than 17°C impairs T cell-like function, but other populations of lymphocytes are insensitive to temperature (Clem *et al.*, 1984, Miller and Clem, 1984; Miller *et al.*, 1986; Ellsaesser *et al.*, 1988). Changing the water temperature from 23 to 11°C over a 24 hour period however, suppresses both T and B cell responses (Bly and Clem, 1991).

3.3.6. Genetic Differences

The increased responsiveness to stress means that Brown trout have a greater predisposition to disease (Pickering and Pottinger, 1989; Pickering *et al.*, 1989). The mortality rates of Brown trout were increased more than 15 times compared to Rainbow trout subjected to similar stress, and it may be relevant that Brown trout lymphocytes are more responsive to cortisol than those of Rainbow trout.

The differences in stress susceptibility and disease resistance in different strains are genetically based and affect survival (Pickering *et al.*, 1989; Fevolden *et al.*, 1991). Recent work has suggested that genetic manipulation of sensitivity to stress may be feasible (Pottinger *et al.*, 1994).

3.3.7. Dominance hierarchy

When exposed to the pathogen *Aeromonas hydrophilia,* subordinate fish are more frequently infected with the bacteria than dominant fish (Peters *et al.*, 1988). Only dominant fish within a single population produce antibodies to infection (Barrow, 1955) and subordinate fish have more active interrenals (Scott and Currie, 1980) and produce more cortisol, so reducing their immune response.

3.4. Aims of this Investigation

In view of the well established links between the HPI axis and the immune system and the emerging role of MCH as a modulator of the stress response, the primary aim of this study was to explore the possibility that MCH could also modulate the immune system of Rainbow trout *(Oncorhynchus mykiss).*

Plasma levels of MCH can be manipulated by adapting fish to black or white backgrounds. By measuring plasma cortisol levels in these fish and by monitoring the number of leucocytes in the blood and major lymphatic organs, any action of MCH in modulating the stress response and /or immune function would become apparent. The results of these experiments could be further investigated by using fish reared on black

or white backgrounds, conditions that might be expected to produce maximum differences between experimental groups.

It was believed that this *in vivo* approach, while demonstrating MCH might, under certain circumstances, modulate the stress and/or immune response, interpretation of how this was achieved would require more detailed experiments under controlled conditions. Thus, a second aim of this work was to develop a culture system for spleen and kidney lymphocytes that would enable the effects of cortisol and MCH to be studied *in vitro*. The uptake of radioisotopes would provide simple methods of measuring cell division and protein synthesis while the development of assays to measure key immune responses would provide additional information about the possible involvement of MCH as a modulating hormone.

While cortisol is the primary steroid hormone involved in the stress response in fish, all glucocorticoids including cortisol, are known to have a powerful immunosuppressive actions. A review of the literature revealed strong circumstantial evidence that the structurally related reproductive steroids can also have immunological actions. A third aim of this work was to use lymphocyte culture to investigate the role of reproductive steroids on immunological function.

4. Materials and Methods

4.1. Experimental Rationale

Two approaches were made to assess the effects of MCH and stress on the immune system. Whole animal were used to study the effects of black and white backgrounds on stress responses and immunological functions. Studies *in vitro* were also developed to try and elucidate the actions of these hormones without the interactions that occur in intact animals which complicate interpretation of *in vivo* results. Cultured lymphocytes were stimulated with both T and B-cell mitogens to investigate whether MCH and steroid hormones had different actions on these two populations.

To assess the effects of background colour *in vivo*, Rainbow trout (*Oncorhynchus mykiss*) were adapted to black and white tanks for a 10 and 14 day period. Some parameters known to be influenced by stress (e.g. cortisol levels, erythrocyte and leucocyte counts) were studied to see if adaptation to different backgrounds had any effect on the stress response. As a control for this experiment, fish were killed at a local fish farm, and these were used as a baseline to compare with the results obtained in adapted fish.

In the second experiment, the effects of injection stress on black and white-adapted fish were examined. Although a fish farm group would have provided useful controls for this and later experiments the number of fish required would have made the experimental protocol too demanding and could not be carried out quickly enough to prevent the

experiment itself affecting plasma cortisol levels. Six fish were used in each experimental group, and because of the time involved in processing the fish spleen and kidney lymphocyte counts were not performed on black- and white-adapted controls. The results from Experiment 1 however, provide a useful reference for comparison with the results from a later experiment.

In Experiment 3, two assays were employed to assess the immunological status of white-adapted fish. This experiment used only white fish as a trial to verify the effectiveness of the haemagglutination and haemolytic plaque assays. Uninjected fish were the controls for both the saline-injected group (exposed to the stress of injection, but without antigenic stimulation) and SRBC-injected fish (exposed to injection stress and antigenic challenge). The next logical step was to assess the effect of background colour on the result of immunological stimulation with SRBC. The number of fish, and number of assays required in this experiment (Experiment 4). precluded the use of uninjected fish as controls.

The results of these experiments revealed that the differences between black- and white-adapted groups were not as marked as had been expected. It is possibly that the white colour of the tanks was not sufficient to produce maximal stimulation of MCH release and when fish that had been reared all their lives on black and white backgrounds became available a further series of experiments were carried out using these reared-fish, to try and maximize the action that MCH could have on stress and immune responses.

In the next series of experiments the effect of T-cell stimulated lymphocytes taken from black- and white-adapted (Experiment 6) and

reared fish (Experiments 7 and 8) and cultured *in vitro* were examined. This approach revealed how previous background exposure, and therefore differences in plasma MCH titres, could affect the cellular responses of fish lymphocytes. In the early experiments, the uptake of two radioisotopes was used to monitor cell growth, ^{3}H-thymidine was used to measure cell division and ^{14}C-leucine to assess protein synthesis. In later experiments, only ^{3}H-thymidine was used to measure cell division, since ^{14}C-leucine uptake gave less consistent results and the additional work required to set up parallel cultures for each isotope limited the scope and number of factors that could be investigated in each experiment.

Once the cell culture technique had been developed and refined and had shown that background colour could indeed affect lymphocyte growth, it was used to explore the influence that a range of steroid and colour change hormones have on T-cell stimulated lymphocyte division. The decision to expand the scope of the work to look at the effects of reproductive steroids on T-cell proliferation was prompted by the findings of Slater and Schreck (1993) that reproductive steroids could influence B-cell responses in culture. In retrospect it might have been wiser to consolidate the data on MCH, but at the time, it was difficult not to become interested in how reproductive hormones would influence cell growth using the culture system that had been developed.

Since the response to MCH using T-cell mitogens caused enhanced lymphocyte growth, the effects of MCH on B cell lymphocyte growth was also assessed, and although these results opened up possibilities for many further experiments, it was decided that enough experimental evidence was now available to draw conclusions about the role of MCH in immunological function in trout.

4.2. General Techniques

4.2.1. Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) obtained from Weirhouse Trout Farm, Chesham, Buckinghamshire, The Berkshire Trout Farm Ltd, Hungerford, Berkshire or Gade Water Nurseries, Hemel Hemstead, Hertfordshire were held in black or white tanks for a minimum period of two weeks before use. All fish used in adapted experiments were fingerling trout to try and minimise the differences between different batches of fish. A few Brown trout (*Salmo trutta)* were also obtained from The Berkshire Trout Farm Ltd. The fish were maintained in as near stress-free conditions as possible, in 230 litre tanks interlinked to allow a slow flow of tap water (250ml min^{-1}) that replaced the aquarium water every 2-3 days. Two biological/charcoal filters (Ehiem Ltd, Berlin, Germany), were used to maintain the water quality.

The fish were held under a lighting regime of 12 hours light/12 hours dark at a temperature of 12 ± 2 °C and fed on alternate days on commercial trout pellets. Up to thirty 250g fish could be held in each tank. Some experimental work was carried out on fish reared from eggs on black or white backgrounds and held in similar aquaria at Bath University, Bath, Avon.

4.2.2. Collection of Blood Samples

Fish were caught quickly using a net and rapidly anaesthetised in benzocaine (Sigma; 5 ml l^{-1} of a stock solution of 10g l^{-1} benzocaine dissolved in ethanol). This procedure minimised stress-induced increases in plasma cortisol (Strange and Schreck, 1978). After the fish

became unconscious, blood was obtained from the severed caudal vein and collected into ice-cold 4ml polypropylene tubes containing 3mg of disodium ethylenediamintetra-acetate (EDTA; BDH, Poole, Dorset). Sub samples of blood were used to prepare blood smears (2µl) and blood cell counts (10 µl).

4.2.3. Blood Cell Counts

The number of red and white blood cells per ml of blood were calculated using an improved Neubauer haemocytometer. For each fish, 10µl of blood were diluted 250 fold with Dacies fluid for total WBC counts and 700 fold for total RBC counts. For each fish a minimum of 2 counts per sample were made. The possibility of using a Coulter counter for the blood cell counts was investigated, but this gave unreliable results due to the nuclear debris that remains when fish RBCs are haemolysed.

4.2.4. Identification of Cells in Blood Smears

Blood smears were air-dried then fixed with methanol for 5 minutes and stained with Leishman-Giemsa Stain. The filtered Leishman stain (BDH) was applied to the slides for 2 minutes, then gently flooded-off with a solution of the Giemsa stain (BDH), made from one part filtered Giemsa stain and seven parts sodium phosphate buffer (0.1M, pH 6.0). This solution was left on the slides for 9 minutes then washed off, first with phosphate buffer and then with tap water. The slides were then gently heat dried overnight in the dark. Five hundred blood cells were counted from each smear in randomly selected areas of the slide and for each fish two slides were counted. Cell identification was made by reference to an atlas of salmonid microscopic anatomy (Yasutake and Wales, 1983) and the relative proportions of each cell type calculated.

4.2.5. Identification of Cells from Spleen and Kidney

Whole spleens and the kidney were dissected from the body cavity. The spleen weights were expressed as a percentage of the wet body weight. Identification of cells from wax sections of the head kidney and spleen proved difficult, greater clarity was obtained using whole tissue imprints and smears of lymphoid organ preparations which enabled individual cell types to be identified. Although several staining techniques were investigated the best results were obtained using Haematoxylin and Eosin, Leishman-Giemsa or Periodic Acid Schiffs.

4.2.6. Cortisol Radioimmunoassay

Plasma was separated from whole blood samples by microfugation at 8800*g* for 5 minutes, and aliquots of plasma were stored at -20°C before being assayed. Duplicate 10µl aliquots of plasma were used for each determination.

The concentration of cortisol in plasma samples was measured using the method described by Rance and Baker (1981). Briefly, 100µl of 100% ethanol were added to 10µl plasma samples, to precipitate plasma proteins. The ethanol was then evaporated from the samples and from a set of cortisol standards (Sigma Chemicals Ltd, Poole, Dorset) in a centrifugal freeze drier. Neat serum containing a polyclonal antibody raised in rabbits to cortisol (Sigma)(courtesy of Dr. B. Baker, Bath University) was diluted 10 fold with phosphate buffered saline (0.1M, 0.85% NaCl, pH 7.4). This antiserum also cross reactions with 11-deoxycortisol (3.3%), corticosterone (2.1%), 17α-hydroxyprogesterone (1.3%).

Two hundred and fifty microlitres of 9.25Bq ml^{-1} , of ^{3}H-cortisol with an activity of 3.0TBq $mmol^{-1}$ (Amersham International, Aylesbury, Buckinghamshire) was diluted to 100ml with 100% ethanol and stored at -20°C. To assay up to 100 plasma samples, 100µl of the tritiated cortisol solution were dried down with gentle heat, then resuspended in 20ml of phosphate buffered saline gelatine (PBSG, 0.1M, pH7.4) and mixed with 100 µl of stock cortisol antibody. Two hundred microlitres of this working solution were added to each sample and thoroughly mixed to resuspend the dried plasma before being left overnight at 4 °C. Unbound cortisol was absorbed onto dextran-coated charcoal which was separated from antibody-bound cortisol by centrifugation at 3000rpm (1500*g*). The supernatant was mixed with 5ml of scintillation fluid (Emulsifier Safe, Packard Ltd., Cambridge, Cambridgeshire) and counted on an LKB liquid scintillation counter. The standard curve was linearon a semilog dose/response plot (Figure 4.1).

Using 10µl of plasma, the assay detected 5ng cortisol/ml plasma. Intraassay variation was 2.5% (n=9) and interassay variation, 9.6% (n=7).

4.3. Immunological Techniques

In addition to assessing the numbers and types of immune cells, immunological assays were developed to monitor the functional activity of the immune system.

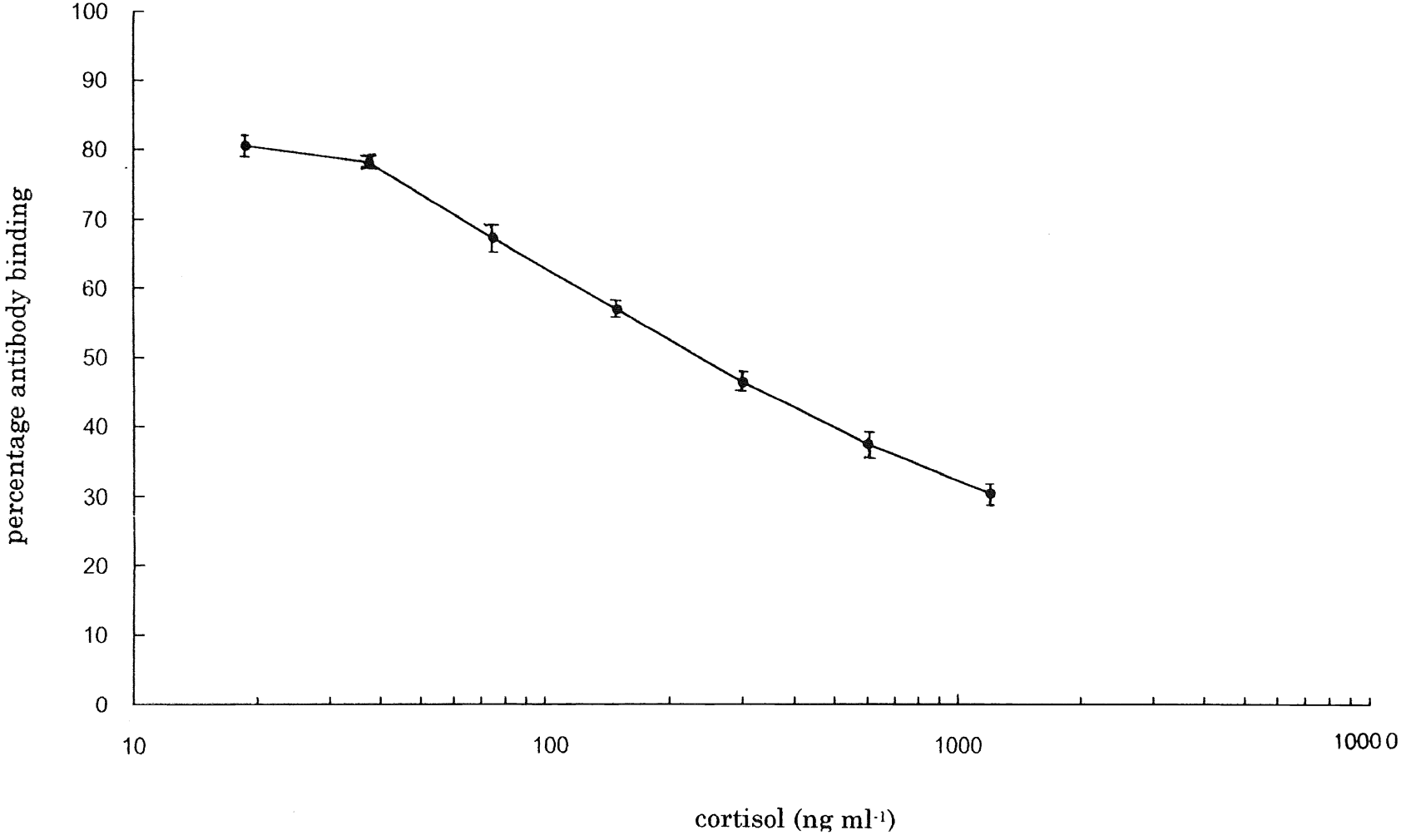


FIGURE 4.1. Standard curve for the radioimmunoassay of plasma cortisol.

4.3.1. Immunisation Regime.

Fish were anaesthetised in benzocaine, ($4ml^{-1}$ of a stock solution of $10g\ l^{-1}$ of benzocaine dissolved in ethanol) and injected with sheep red blood cells (SRBCs). The SRBCs were supplied suspended in Alsever's solution (Unipath Ltd, Basingstoke, Hampshire) and remained usable for up to four weeks when stored at 4°C . Before use the cells were washed three times by centrifugation (3000rpm, 1000*g*) in sterile phosphate buffered saline (PBS, 0.1M, pH 7.1), and re-suspended in PBS. Fish were injected interperitoneally with 200 µl of washed 10% SRBC or 200µl of PBS. Upto three experimental groups were used; SRBC-injected, PBS-injected and uninjected controls. The fish were injected three times on alternate days, and killed ten or eleven days after the final injection.

4.3.2. Lymphocyte Cell Isolation.

The head kidney and spleen were removed aseptically from recently killed fish under a lamina flow hood and held on ice in Leibovitz-15 medium (L-15; Gibco Ltd, Paisley, Strathclyde). The tissue was transferred to fresh medium supplemented with 10% foetal calf serum (FCS; Gibco Ltd) and single cell suspensions obtained by repeated aspiration and expulsion of the tissue through a 1ml syringe fitted with a 21 gauge needle. This suspension was then allowed to stand in a 4 ml polypropylene tube on ice for 10-20 minutes until the larger cell fragments had settled. The supernatant was removed and centrifuged at 3000rpm (1500*g*) for 15 minutes. The supernatant was discarded and the cell pellet resuspended in 1ml of FCS-supplemented medium. One hundred microlitres of this single cell solution were added to

200µlof filtered 0.4% trypan blue (BDH) and, after gentle mixing, the solution was loaded onto a haemocytometer and viable and non-viable leucocytes were counted to give total lymphocytes present in the kidney and spleen respectively, duplicate counts were made for each sample.

4.3.3. Passive haemagglutination test.

This procedure was used to assess the amount of specific antibody present in fish serum. (Ruglys, 1985). Blood collected by caudal puncture was left overnight to coagulate . The coagulated blood was then microfuged (8800*g*) for 5 minutes to obtain the blood serum. One hundred microlitres of serum was serially diluted with L-15 medium using doubling dilution's in a microtitre plate and 25µl of 20% washed SRBCs were added to each well. After a two hour incubation at 4°C, the weakest dilution at which the SRBCs showed agglutination was recorded as a arbitrary score. While SRBCs in wells which contained antibodies are held in suspension to form a 'carpet', those in wells without sufficient antibody, collect on the bottom of the wells as tight 'buttons'

4.3.4. Passive Haemolytic Plaque Assays

Passive haemolytic plaque assays are used to measure the number of antibody producing cells in lymphoid tissue. After immunisation against SRBCs, the number of cells secreting SRBCs antibodies can provide an estimate of immunological competence. Two types of plaque assays techniques were employed.

Cunningham Plaque Assay.

Following the technique developed by Cunningham and Szenberg (1968), 100µl of lymphocyte suspension was added to 10µl of FCS supplemented L-15 and 10µl Brown trout serum (to provide a source of complement) on ice. Thirty microlitres of a 20% solution of washed SRBCs in PBS were added to the suspended lymphocyte solution which was agitated gently before being loaded into a Cunningham plaque chamber. Cunningham plaque chambers are constructed from two clean microscope slides held together with double sided tape to sandwich cells in a monolayer. The sides of the chambers are sealed with wax and incubated for 8 hours at 14°C in a humid environment. After incubation, the number of antibody producing cells were identified by the holes or plaques that form in the lawn of SRBCs due to cell lysis . The assay failed to give distinct plaques however, due to movement of the cells within the monolayer.

Jerne Passive Haemolytic Plaque Assay

An alternative technique, the Jerne passive haemolytic plaque assay (Jerne and Nordin, 1968; Chiller *et al.*, 1968) was found to give superior results and was easier to carry out on a large scale. In 4.5cm diameter petri dishes, a 1ml layer of 1.4% w/v technical grade agar solution (Difco Ltd, West Mosley, Surrey) was prepared in L-15 medium. When solidified, 250µl of lymphocyte suspension was layered onto the agar. Finally, 1ml of a 0.7% w/v solution of technical grade agar in L-15 at 45°C mixed with 200µl of washed 20% SRBCs was poured onto the set agar in each petri dish. The agar was allowed to solidify, before the addition of 1.5ml of 20% rainbow trout complement solution in L-15.

Duplicate plates were prepared for each sample, before incubation overnight at 22°C. The number of plaques formed was recorded the following day.

Since plaque size varied enormously, plaques greater than 3mm in diameter were counted as large (L) and all others as small (S).

4.3.5. Rosette Assay

The rosette assay (Zaalberg, 1964) was also investigated as a means of assessing the immunocompetence of kidney and spleen lymphocytes by measuring their ability to bind antigens.

A mixture of 200µl of lymphocyte suspension and 200µl of 1% washed SRBCs were added to a test tube, mixed gently then incubated overnight at 4°C. The following day, after diluting the suspension in Dacies fluid, the number of rosettes (lymphocytes clustered around SRBCs) were counted. To be scored as a rosette a minimum of three lymphocytes clustered around a SRBC was necessary.

4.4. *In Vitro* Methods

4.4.1. Cell culture Development

The ability of lymphocytes to proliferate *in vitro* from the anterior head kidney and spleen was assessed using a mitogen stimulation assay (Etlinger *et al.*, 1976; Spitsberg *et al.*, 1986).

Two media, Leibovitz 15 (L-15) and RMPI-1640, (Gibco Ltd), both routinely used in fish cell culture, were tested to see which best supported lymphocyte cell growth. Leibovitz 15 was chosen because it did not require gasing with 10% carbon dioxide. The Leibovitz 15 medium, which already contained L-glutamine, was supplemented with 10mg l^{-1} each of adenosine, cytosine, guanosine and uracil (Sigma Chemicals Ltd) and is subsequently referred to as "tissue culture medium". Initially, cultured cells were counted after four days of growth using a haemocytometer. Trial experiments were also carried out with lymphocytes grown on 70% methanol-washed circular coverslips. After fixation, the cells were stained and counted on the coverslip, but with this technique cell growth was often uneven. The uptake of isotopes by the cells was developed as a method of estimating cell growth. The incorporation of the radioactive labels was low when cells were grown on cover slips. More consistent results were obtained by growing the cells directly on the bottom of the culture well plates. In early experiments the tissue culture medium was further supplemented with FCS, but this was later replaced with the synthetic serum Ultroser (Gibco Ltd) which had a longer shelf life and was more cost effective.

4.4.2. Final Cell Culture Technique

The final method used for all *in vitro* experiments was as follows: Organs were dissected from the body and placed in ice-cold tissue culture medium supplemented with 0.5% Ultroser solution. The organs were disrupted into a test tube by being forced through a 1ml syringe fitted with a 21 gauge needle. The resulting solution was then allowed to settle on ice for 30 minutes and the supernatant was then centrifuged at 3000rpm (1000*g*) for 10-15 minutes at 4°C. The resulting pellet of cells was resuspended in 1ml of Ultroser-supplemented tissue culture medium, with the addition of an antibiotic mixture (100mg ml^{-1} of streptomycin-penicillin, tissue culture grade; Sigma). Cell density and viability were determined using 0.4% trypan blue. Single cell lymphocyte suspensions were diluted to within the range 0.5-5.0 x 10^6 cells ml^{-1} using antibiotic supplemented medium. Using Nuncon 24-well tissue culture plates (Philip Harris Scientific, Lichfield, Staffordshire), 100ml of cell suspension and 100ml of the growth lectin were added to each well.

In experiments investigating T like-cell growth, the T cell stimulator Concanavalin A (Con A; from *Canavalia ensiformis* type IV-S; tissue culture grade; Sigma) was the growth mitogen selected. The concentration of Con A, added to the cultures, was optimised in preliminary experiments (see Section 6.1). In experiments investigating B cell mitogenesis, the mitogen used was Lipopolysaccharide (LPS; from *E. coli* 026:B6; tissue culture grade; Sigma). One hundred microlitres of steroid or peptide hormones of predetermined concentration were added at this point bringing the final volume in each well to 300ml. If a

second steroid was also being investigated it was added with the mitogen.

The plates were then incubated sealed in a plastic bags at 20°C for two or three days (see Section 4.3.3 for exact timing of each experiment). The cells were then pulsed with 18.5kBq of ^{3}H-thymidine (185GBq mmol^{-1}; Sigma) to estimate cell division or 7.4kBq of ^{14}C-leucine (11GBq mmol^{-1}, Sigma) to monitor protein synthesis during cell growth. After up to four days of growth, the cells were scraped from the wells using a spatula, and washed twice by centrifugation (8800*g*) in 0.85% w/v physiological saline. The washed cells were added to 1ml of scintillation fluid (Emulsifier Safe, Packard Ltd, Cambridge, Cambridgeshire) and the radiolabel incorporated into the cells determined in a LKB-Wallace RackBeta Scintillation counter. The results were expressed as the percentage change from the control values. In this way, the degree of inhibition or stimulation by various test substances was assessed. All treatments were carried out in triplicate for each organ of each fish.

4.4.3. Hormones Investigated in Culture

Cortisol

Cortisol (tissue culture grade; Sigma) was initially dissolved in 100% ethanol then dried down and resuspended to the appropriate concentration using tissue culture medium. One hundred microlitres of this solution was added to each well to give a final concentration of 10, 30, 100 and 300ng ml^{-1} of cortisol. The wells were then incubated for seven days.

MCH

The MCH (Novabiochem UK Ltd, Nottingham, Nottinghamshire) was made up to 0.5mg ml^{-1} in 1% Bovine Serum Albumin (BSA) in L-15 medium (Baker *et al.,* 1985a). A 100ml aliquot of this solution was then diluted in tissue culture medium to give final concentrations of 10, 100, 1000 or 10,000pg ml^{-1} in the wells. The wells were then incubated for seven days, with the addition of ^{3}H-thymidine on day three.

Cortisol and MCH

In an experiment to assess whether MCH could influence the action of cortisol on lymphocytes in culture, MCH used at a fixed final concentration of 50pg ml^{-1} was tested on lymphocytes exposed to cortisol concentrations of 0, 10 and 300 ng ml^{-1}. A control group of wells containing the same range of cortisol but without MCH were incubated in parallel.

Cortisol and aMSH

Alpha-MSH ([Nle^{4}, D-Phe^{7}]αMSH; Sigma) dissolved in 0.1M HCl to give a stock concentration of 0.1mg ml^{-1} was serially diluted with L-15 medium, to give a final concentration of 495 pg ml^{-1} in the culture wells. This concentration was chosen to reflect the typical concentration of aMSH found in the blood of trout (Baker *et al.,* 1986). A similar protocol to that used for MCH was adopted in this experiment for cortisol and aMSH. The cells were incubated for two days before the addition of ^{3}H-thymidine, and then for a further five days before being harvested.

Reproductive Steroids

Five teleost reproductive steroid hormones obtained from Sigma (*defines tissue culture grade) were individually tested on spleen and kidney lymphocyte cell cultures *in vitro* namely:

- 11β ketotestosterone
- 17α hydroxy, 20β dihydroxyprogesterone *
- 17α hydroxyprogesterone
- androstenedione
- 17β-oestradiol.*

Each hormone was dissolved in 100% ethanol and a calculated amount of this solution was dried down and resuspended in tissue culture medium to give final concentrations in the culture wells of 10, 30, 100 and 300ng ml^{-1} of each steroid. The cells were incubated for two days before the addition of ^{3}H-thymidine, and then for a further five days before being harvested.

5. Results: *In Vivo*

5.1. Experiment 1: Effects of adaptation to black and white backgrounds on plasma cortisol and immune cell numbers.

To assess whether background colour can influence the immune system, fingerling Rainbow trout were adapted to black and white tanks for periods of 10 and 24 days to observe the effects on plasma cortisol titres and the numbers of lymphocytes in the blood, spleen and kidney.

Blood collected from claudal vessels was used for measurement of plasma cortisol titres, circulating blood cell numbers and for the preparation of blood smears for histological assessment of blood cell types. Spleen and kidney smears were prepared for the estimation of lymphocyte numbers in these tissues. Additional samples were collected from fish killed at the fish farm immediately after capture. The results are shown in Table 5.1.

5.1.1. Cortisol

Plasma cortisol titres were consistently higher in black- compared with corresponding white-adapted fish, although the differences were not significant. Fish killed at the farm had significantly lower plasma cortisol than most groups of adapted fish. These results suggest that in the aquarium, the fish were under greater stress than at the fish farm. Factors such as the additional noise and disturbanc, lower water volume and poorer water quality are the most likely cause.

TABLE 5.1. Experiment 1. The effects of background adaptation on plasma cortisol, red and white blood cell counts and blood, spleen and kidney lymphocyte numbers. Values are means ± 1 standard error, n=6. Bracketed values are significantly different where *=p <0.05, **=p <0.01 and ***=p <0.001.

	Adaptation conditions				
	Farm	Black 10d	White 10d	Black 24d	White 24d
Body weight (g)	42.6±5.0	33.2±6.3	34.0±5.22	29.9±4.4	38.8±7.3
Plasma cortisol (ng/ml)	8.3±1.1	13.4±2.6	10.0±1.7	20.4±7.2	15.0±3.2
Erythrocytes ($x10^6$/μl blood)	2.8±0.9	2.5±0.5	1.5±0.2	15.0±2.0	14.8±2.2
Leucocytes ($x10^3$/μl blood)	31.9±10.8	51.9±12.0	60.4±15.7	231±28	248±43
Lymphocytes Blood (/1000 cells)	30.4±12.2	12.0±1.3	38.0±2.5	15.0±2.9	47.6±4.2
Spleen ($x10^6$/g body wt)	349±23	310±51	232±29	257±56	211±36
Kidney ($x10^6$/g body wt)	184±43	191±38	143±9	207±16	163±34

5.1.2. Blood Cell Counts

Erythrocyte counts were increased significantly in both black- and white-adapted fish after 24d compared to farm values. A even more marked effect was observed for leucocyte numbers which were significantly raised at 10d and dramatically increased by 24d. When lymphocyte numbers were expressed per 1000 cells counted from blood smears, significantly higher numbers of cells were seen in white- compared to black-adapted fish.

5.1.3. Spleen and Kidney Cell Counts.

The highest numbers of spleen lymphocytes were found in fish killed at the farm, but adaptation to different backgrounds did not reveal any obvious trends. No clear pattern was evident for kidney lymphocytes although white-adapted fish had the lowest numbers of cells per gram in this tissue.

5.1.4. Summary

The fish used in this experiment had raised cortisol titres and blood lymphocyte numbers compared to farm controls, these parameters increased with the adaption period .

It is worth noting that, while the classical immunological response seen in this experiment was less marked in black- than white-adapted fish, the stress response, as assessed from cortisol values, was greater in black- than white-adapted fish.

5.2. Experiment 2:
Effects of injection stress and immunological challenge on plasma cortisol and immune cell numbers in black- and white-adapted trout.

The aim of this experiment was three fold. Firstly, it examined the responses that occur when a moderately severe stress is repeated over several days. Secondly, it established an immunisation technique that would be necessary for further investigations of immune function. Thirdly, it revealed differences in the responses to repeated stress and immunisation in fish adapted to black or white backgrounds.

Trout were adapted to black or white backgrounds for two weeks. Three experimental groups were used: uninjected controls, saline-injected and SRBC-injected. Full details of the immunisation regime are given in Section 4.2.1. The results are shown in Table 5.2.

5.2.1. Cortisol

Plasma cortisol levels were lowest in uninjected fish and highest in both injected groups. The differences were not significant however, with the exception of the black saline-injected group whose value was unusually high (15.5ng ml^{-1}). The stressful effects of injection were not obvious in this experiment nor were there any clear differences between saline- and SRBC-injected fish.

TABLE 5.2. Experiment 2. The effects of repeated injections of saline or sheep red blood cells on plasma cortisol and immune cell numbers in black- and white-adapted trout. Values are means ± 1 standard error, n=6. Bracketed values are significantly different where *=p <0.05, **=p <0.01 and ***=p <0.001.

	Adaptation Conditions					
	Uninjected		Saline injected		SRBC injected	
	Black	White	Black	White	Black	White
Body weight (g)	17.8±1.7	14.7±2.0	18.9±2.1	20.3±2.1	19.5±2.4	20.6±2.9
Plasma cortisol (ng/ml)	4.5±0.5	3.1±0.4	15.5±4.5	5.6±0.9	6.9±2.3	5.0±1.3
Erythrocytes ($x10^6$/μl blood)	0.5±0.1	0.4±0.1	2.0±0.5	1.9±0.3	1.6±0.2	2.4±0.1
Leucocytes ($x10^3$/μl blood)	17.3±2.6	31.8±6.3	29.5±7.7	41.1±8.1	45.3±4.6	87.4±6.9
Lymphocytes						
Blood (/1000 cells)	15.0±2.5	41.0±2.8	12.0±2.2	50.0±9.4	26.5±2.4	73.7±5.8
Spleen ($x10^6$/g body wt)	——	——	481±50	1125±208	847±225	1147±236
Kidney ($x10^6$/g body wt)	——	——	479±49.	2007±327	871±231	1698±321

5.2.2. Blood Cell Counts

The number of erythrocytes was 3.2-6.0 times greater in injected compared to uninjected fish, but these increases were not significant. By contrast, white blood cell counts were higher in saline-injected fish than in the uninjected controls and increased further when fish were immunised with SRBCs. Comparisons between black- and white-adapted trout showed that significantly higher numbers of leucocytes were always present in white-adapted groups, especially in immunised fish. Very similar trends were seen when lymphocyte numbers were estimated from blood smears. Thus, lymphocyte counts were much higher in the SRBC-injected trout and there was a significantly greater number of cells in the blood of all white-adapted groups.

5.2.3. Spleen and Kidney Cell Counts

Spleen and kidney lymphocyte numbers were significantly higher in white- than in black-adapted fish for both injected groups. Values for uninjected fish were not obtained.

5.2.4. Summary

This treatment succeeded in raising the number of lymphocytes in the blood and major lymphoid organs following immunising injections of SRBCs. Cortisol values for all categories were relatively low at the time the fish were killed, but would presumably have been much higher shortly after injection. Particularly evident were the much higher lymphocyte numbers observed in white-adapted fish in all three experimental groups.

5.3. Experiments 3 & 4: Effects of injection stress and immunisation on plasma cortisol and immunological response in black- and white-adapted trout.

To confirm the results obtained in Experiment 2 and in order to measure antibody production in response to immunisation, two similar experiments were carried out using black- and/or white-adapted trout. Following immunisation, fish were killed 14 to 16 days after the last antigen injection. The Jerne Passive Haemolytic Plaque Assay was performed on spleen and kidney lymphocytes to assess antibody production, and the Passive Haemagglutination Test used to assay circulating antibody titres. The protocol for these techniques is given in Section 4.2 and the results are shown in Tables 5.3 and 5.4.

5.3.1. Cortisol

The uninjected fish in Experiment 3 had significantly lower cortisol values than either injected group. In Experiment 4 however, cortisol titres were similar in all groups.

5.3.2. Blood Cell Counts

No clear pattern of change was evident in the numbers of circulating erythrocytes in these experiments. Leucocyte counts however, showed the same significant increases as before and were always highest in immunised fish (both experiments). Leucocyte numbers were also higher in white-adapted injected fish than in the corresponding groups of black-adapted injected fish (Experiment 4).

TABLE 5.3. Experiment 3. Immune response to saline injection and immunisation with sheep red blood cells in white-adapted trout. Values are means ± 1 standard error, n=6. Bracketed values are significantly different where *=p <0.05, **=p <0.01 and ***=p <0.001

	Adaptation Conditions		
	Uninjected White	Saline-injected White	SRBC-injected White
Body weight (g)	22.9 ± 1.5	24.6 ± 1.8	22.7 ± 2.7
Spleen weight (mg)	50.0 ± 6.0	37.0 ± 7.0	31.0 ± 5.0
Spleen wt/body wt (x10^3)	1.9 ± 0.2 *	1.2 ± 0.1 *	1.4 ± 0.1
Plasma cortisol (ng/ml)	3.3 ± 1.5 *	16.5 ± 3.3	15.8 ± 3.8 *
Erythrocytes (x10^6/ml blood)	1.5 ± 0.1	1.4 ± 0.1	1.6 ± 0.1
Leucocytes (x10^3/ml blood)	50.6 ± 3.3 ***	52.1 ± 3.7 ***	92.8 ± 5.6

continued.....

Table 5.3. continued

	Uninjected White	Saline-injected White	SRBC-injected White
Lymphocytes			
Blood (/1000 cells)	22.5 ± 2.4	21.4 ± 2.5	49.7 ± 4.2
Spleen ($x10^6$/mg spleen)	245 ± 24	269 ± 79	625 ± 133
Kidney ($x10^6$/g body wt)	532 ± 38	597 ± 63	617 ± 117
Haemagglutination score	1.9 ± 0.6	2.5 ± 0.2	4.3 ± 0.2
Haemolytic plaques (/10^5lymphocytes)			
Spleen (large, >3mm diam.)	0.0	1.7 ± 0.3	2.3 ± 0.5
Spleen (small, <3mm diam.)	0.0	9.0 ± 2.9	56.8 ± 15.8
Kidney (large, >3mm diam.)	0.7 ± 0.7	1.0 ± 0.1	2.3 ± 0.5
Kidney (small, <3mm diam.)	0.0	11.3 ± 3.5	27.5 ± 5.1

TABLE 5.4. Experiment 4. Immune response to saline injection and immunisation with sheep red blood cells in black and white-adapted trout. Values are means ± 1 standard error, n=6. Bracketed values are significantly different where *=p <0.05, **=p <0.01 and ***=p <0.001.

	Adaptation conditions			
	Saline-injected		SRBC-injected	
	Black	White	Black	White
Body weight (g)	53.4 ± 7.7	46.4 ± 2.4	52.2 ± 11.8	41.4 ± 4.6
Spleen weight (mg)	78.0 ± 2.0	52.0 ± 7.0	75.0 ± 2.2	81.0 ± 1.7
Spleen wt/body wt ($x10^3$)	1.5 ± 0.2	1.6 ± 0.2	1.4 ± 0.1	2.1 ± 0.5
Plasma cortisol (ng/ml)	29.5 ± 3.4	31.1 ± 2.0	30.8 ± 6.4	21.9 ± 6.9
Erythrocytes ($x10^6$/ml blood)	2.3 ± 0.2	2.6 ± 0.3	3.4 ± 0.5	2.9 ± 0.5
	*		***	
Leucocytes ($x10^3$/ml blood)	26.9 ± 4.05	38.7 ± 5.8	28.2 ± 3.1	67.3 ± 4.3
		***		

continued.....

Table 5.4. continued

	Saline-injected		SRBC-injected	
	Black	White	Black	White
Lymphocytes				
Blood (/1000 cells)	11.0 ± 1.3	28.3 ± 1.4	17.7 ± 2.2	60.3 ± 5.3
Spleen ($x10^6$/mg spleen)	373 ± 63	625 ± 105	211 ± 33	615 ± 108
Kidney ($x10^6$/g body wt)	354 ± 69	504 ± 93	581 ± 46	632 ± 60
Haemagglutination score	3.0 ± 0.4	2.7 ± 0.5	4.5 ± 0.3	5.0 ± 0.1
Haemolytic plaques (/10^5 lymphocytes)				
Spleen (large, >3mm diam.)	0.2 ± 0.1	0.3 ± 0.2	0.6 ± 0.3	1.2 ± 0.8
Spleen (small, <3mm diam.)	2.8 ± 1.0	2.3 ± 0.3	3.7 ± 1.2	8.3 ± 1.8
Kidney (large, >3mm diam.)	0.3 ± 0.2	0.6 ± 0.3	0.7 ± 0.3	1.8 ± 0.5
Kidney (small, <3mm diam.)	2.5 ± 0.5	5.7 ± 1.4	8.3 ± 3.3	8.4 ± 1.4

5.3.3. Spleen and Kidney Cell Counts

In Experiments 3, spleen lymphocyte numbers increased significantly when fish were injected with SRBCs. In Experiment 4, spleen lymphocyte numbers were significantly higher in white compared to black-adapted fish. Kidney lymphocyte numbers also increased in response to either saline (Experiment 3) or SRBCs (Experiments 3 and 4) but not significantly.

5.3.4.Passive Haemagglutination Test

The scores recorded in Experiments 3 and 4 show that the amount of specific antibody present in the blood increased significantly when trout were injected with SRBCs. Saline injection also caused a rise in the haemagglutination score (Experiment 3). There were no significant differences in the haemagglutination scores between white- and black-adapted fish for either saline or SRBC injected groups (Experiment 4).

5.3.5. Jerne Plaque Assay

Although the number of small plaques (< 3mm diameter) recorded in both experiments was always approximately 10 fold greater than the number of large plaques (> 3mm diameter), the trends seen in the number of both types correspond well in the majority of cases. Thus, in Experiment 3, the highest number of plaques were seen, for both spleen and kidney lymphocytes, in immunised fish. In Experiment 4, except in one case, the number of plaques was always greater in white- than in black-adapted fish and this increase was statistically significant for the counts of small plaques produced by kidney lymphocytes.

5.3.6. Summary

The most important observation from the results of these two similar experiments is that white-adapted fish are more responsive to immunisation with SRBCs than black-adapted fish and produce higher titres of circulating antibodies and a larger number of plaque-forming lymphocytes.

5.4. Experiment 5: Effects of injection and immunisation on plasma cortisol and immunological responses in trout reared on black or white backgrounds.

In view of the difference in the stress and immune responses observed in fish adapted for several weeks to black or white backgrounds, the conditions used in Experiment 4 were repeated using Rainbow trout that had been reared from eggs on black or white backgrounds at Bath University. The aim of this experiment was to establish how permanent exposure to different backgrounds affects stress and immune responses. The immunisation procedure was identical to that employed in Experiment 4. The data obtained for plasma cortisol titres, cell counts and immunological assays are given in Table 5.5.

5.4.1. Spleen and Body Weight

The mean body weight for all the trout used in this experiment was 176.1 ± 7.5g with a range of 124 to 298g, and the mean spleen weight/body weight ratio lay between 1.1 for black-reared saline-injected and 1.5 for black-reared SRBC injected fish. Earlier experiments had used much smaller trout (approximately 20 to 69g body weight) and the spleen weight /body weight ratios for these smaller fish were generally slightly higher (1.2 to 1.9).

5.4.2. Cortisol

In contrast to previous observations on adapted fish, cortisol titres were higher in white-reared than in corresponding black-reared groups. In black- and white-reared fish immunised with SRBCs, the difference was significant.

5.4.3. Blood Cell Counts

No significant differences were found between circulating erythrocytes for any group. Total leucocyte counts and lymphocyte numbers increased, significantly in most cases, in response to immunisation but the highest counts were seen in immunised black-reared fish rather than in immunised white-reared groups. This is in marked contrast to previous results using adapted fish, in which the highest counts were always seen in white-adapted animals.

TABLE 5.5. Experiment 5. Immune response to saline injection and immunisation with sheep red blood cells in trout reared on black or white backgrounds. Values are means ± 1 standard error, n=6. Bracketed values are significantly different where *=p <0.05, **=p <0.01 and ***=p <0.001.

	Adaptation conditions			
	Saline-injected		SRBC-injected	
	Black	White	Black	White
Body weight (g)	181.0 ± 9.7	199.8 ± 0.0	176.3 ± 12.5	146.5 ± 8.6
Spleen weight (mg)	20.5 ± 2.0	23.0 ± 2.0	23.3 ± 5.0	17.7 ± 2.0
Spleen wt/body wt ($x10^3$)	1.1 ± 0.1	1.1 ± 0.1	1.5 ± 0.2	1.2 ± 0.1
			*	
Plasma cortisol (ng/ml^{-1})	15.1 ± 5.7	21.7 ± 2.9	17.7 ± 4.2	29.1 ± 6.2
Erythrocytes ($x10^6$/ml blood)	4.9 ± 0.4	6.7 ± 0.6	5.3 ± 0.6	5.7 ± 0.4
			**	
Leucocytes ($x10^3$/ml blood)	17.9 ± 4.1	22.6 ± 4.8	51.3 ± 3.7	30.7 ± 4.3
	***			

continued.....

Table 5.5. continued

	Saline-injected		SRBC-injected	
	Black	White	Black	White
Lymphocytes				
Blood (/1000 cells)	10.0 ± 0.9	10.7 ± 0.8	18.4 ± 1.7	17.3 ± 1.1
Spleen ($x10^6$/mg spleen)	8.9 ± 1.5	10.1 ± 2.9	31.2 ± 6.6	32.6 ± 5.2
Kidney ($x10^6$/kg body wt)	20.3 ± 2.1	30.8 ± 4.1	27.8 ± 10.1	44.7 ± 6.4
Haemagglutination score	2.5 ± 0.3	1.7 ± 0.3	4.8 ± 0.2	4.3 ± 0.3
Haemolytic plaques (/10^5 lymphocytes)				
Spleen (small, <3mm diam.)	18.7 ± 13.2	15.6 ± 3.7	36.0 ± 9.5	23.8 ± 7.4
Kidney (small, <3mm diam.)	3.5 ± 0.9	4.3 ± 1.9	24.0 ± 0.9	15.2 ± 5.7

5.4.4. Spleen and Kidney Cell Counts

A significant increase in spleen lymphocyte numbers was observed in response to immunisation. A higher number of kidney lymphocytes were present in white- than in black-reared fish for both saline- and SRBC- injected groups, but no obvious response to immunisation was apparent for either group.

5.4.5. Passive Haemagglutination Test

Immunisation resulted in a significant increase in the haemagglutination score for both black- and white-reared fish, but no clear differences were apparent between fish reared on different backgrounds.

5.4.6. Jerne Plaque Assay

Although immunisation increased the number of plaque-forming cells in both spleen and kidney derived lymphocytes, the increases were not statistically significant. Lymphocytes taken from the kidney of immunised black-reared fish however, produced a significantly higher number of plaques than those from immunised white-reared fish. This is in contrast to the results of Experiment 4, in which white-adapted fish gave higher numbers of plaques than black-adapted fish.

5.4.7. Summary

Fish reared on black and white backgrounds show stress and immune responses that are opposite to those seen in fish merely adapted to

similar conditions for a few weeks. In this experiment, it is the animals kept all their lives on black backgrounds that have the lowest plasma cortisol titres but who show the largest immunological responses to immunisation as measured by the haemagglutination test and Jerne plaque assay. The variable responses shown by individual fish and the resulting large standard errors however, make statistical validity difficult to establish although the trend reversal is quite clear.

6. Results *In Vitro*

6.1. Preliminary T cell experiments

In a series of preliminary experiments on black-adapted fish, the conditions for *in vitro* cell growth and the incorporation and recovery of radioisotopes were optimised.

6.1.1. Concanavalin A Concentration

Spleen cells were labelled with ^{3}H-thymidine to measure DNA synthesis and pronephric kidney cells with ^{14}C-leucine to estimate the rate of protein synthesis. The T-cell mitogen, Con A, was added to the cell cultures on day one to give final concentrations in the wells of between 0 and 100mg ml^{-1}. The radiolabels were added to the cell cultures on day three and the cells were harvested and counted for radioactivity on day seven. An assumption was made that the spleen and kidney cells would respond in similar ways to varying concentrations of the mitogen and therefore only one isotope was tested on each cell type. The results presented in Figure 6.1 show that protein synthesis by kidney cells peaked with a dose of 25mg ml^{-1} Con A, while maximum incorporation of ^{3}H-thymidine by spleen lymphocytes occurred with 10mg ml^{-1} Con A. These concentrations were used for all subsequent experiments.

6.1.2. Culture Period

The optimum number of days cells should be cultured to produce a high level of incorporation of radioisotopes was investigated using spleen lymphocytes. It was important for future experiments to ensure that cells were growing exponentially at the time they were harvested, so that any differences in growth response would be maximised and not complicated by any decrease in division rate that occurs as cultured cells become confluent. Splenic lymphocytes were cultured for up to seven days and sample wells were harvested daily from day four. The radioactivity incorporated for each culture period is recorded in Figure 6.2. This shows that radioactivity increased dramatically by day seven. A seven day culture period was used in subsequent experiments since it offered a convenient time scale and gave good levels of incorporation.

6.1.3 Washing Procedure

Separation of the cells from the culture medium involved repeated cycles of centrifugation and removal of the supernatent containing unincorporated radioisotopes. For ^{3}H-thymidine, the radioactivity count stabilised after two washing cycles and for ^{14}C-leucine, after three (Figure 6.3). Further washes did not significantly lower the radioactivity present in the cellular pellet for either isotope and therefore two washes were adopted in the final standard procedure.

FIGURE 6.1. Incorporation of ^{3}H-thymidine by spleen (•) and ^{14}C-leucine by kidney (○) lymphocytes cultured with different concentrations of the T-cell mitogen, concanavalin A. Concanavalin A was added to the cultures on day 1, radiolabels on day 3 and the cells were harvested on day 7. Values are means ± 1 standard error, n=5. * : significantly different (p<0.001) from spleen cells cultured with 25 or 50μg ml^{-1} . ‡ : significantly different (p <0.001) from all other kidney cell cultures**

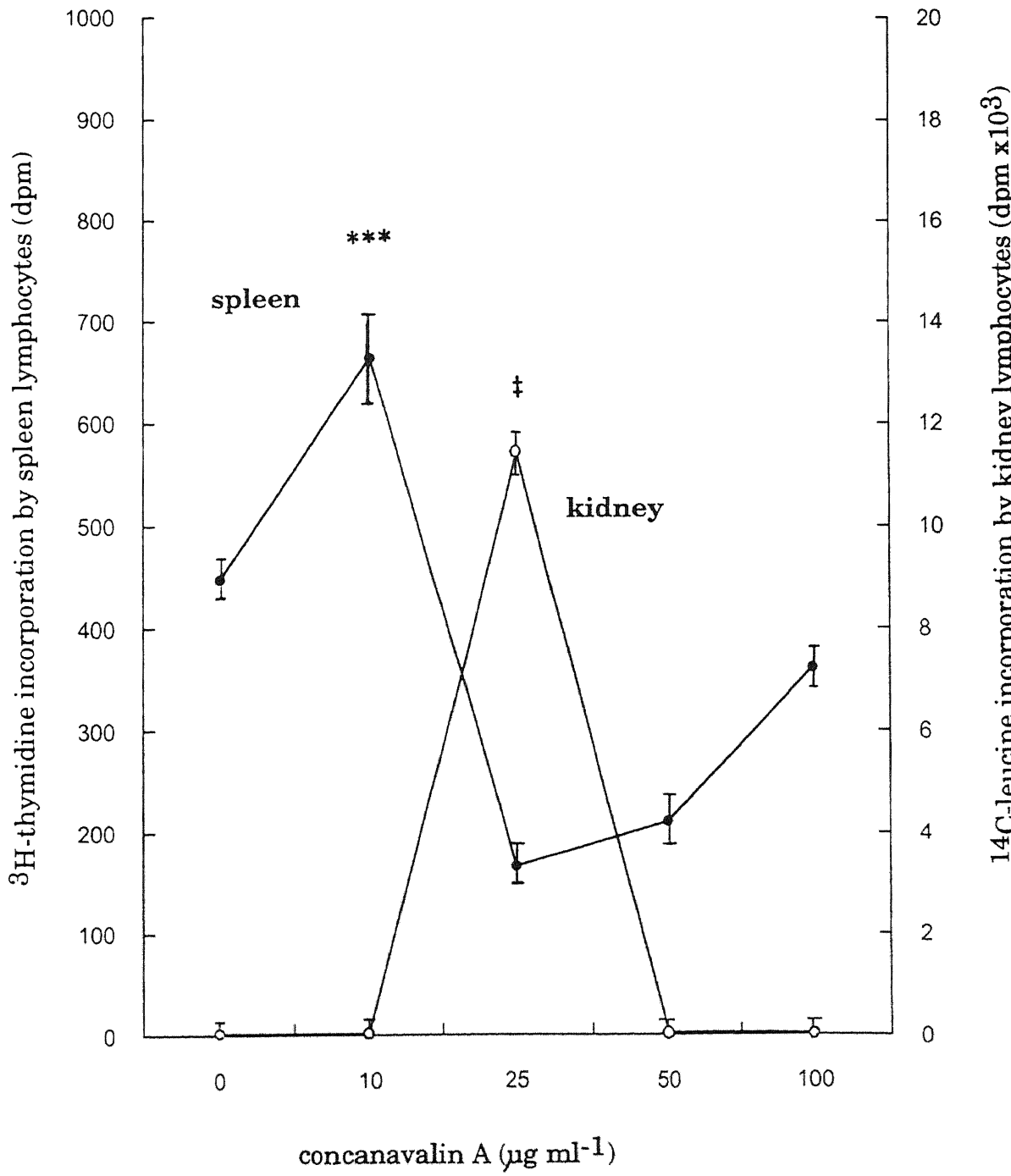


FIGURE 6.2. Incorporation of ^{3}H-thymidine and ^{14}C-leucine by spleen lymphocytes cultured for up to 7 days. 10μg ml^{-1} concanavalin A was added on day 1, and the radiolabels on day 3. Values are means ± 1 standard error, n=5. Significantly greater incorporation compared with shorter periods of culture are shown by the asterisks where *= p <0.05 and ***= p <0.001.

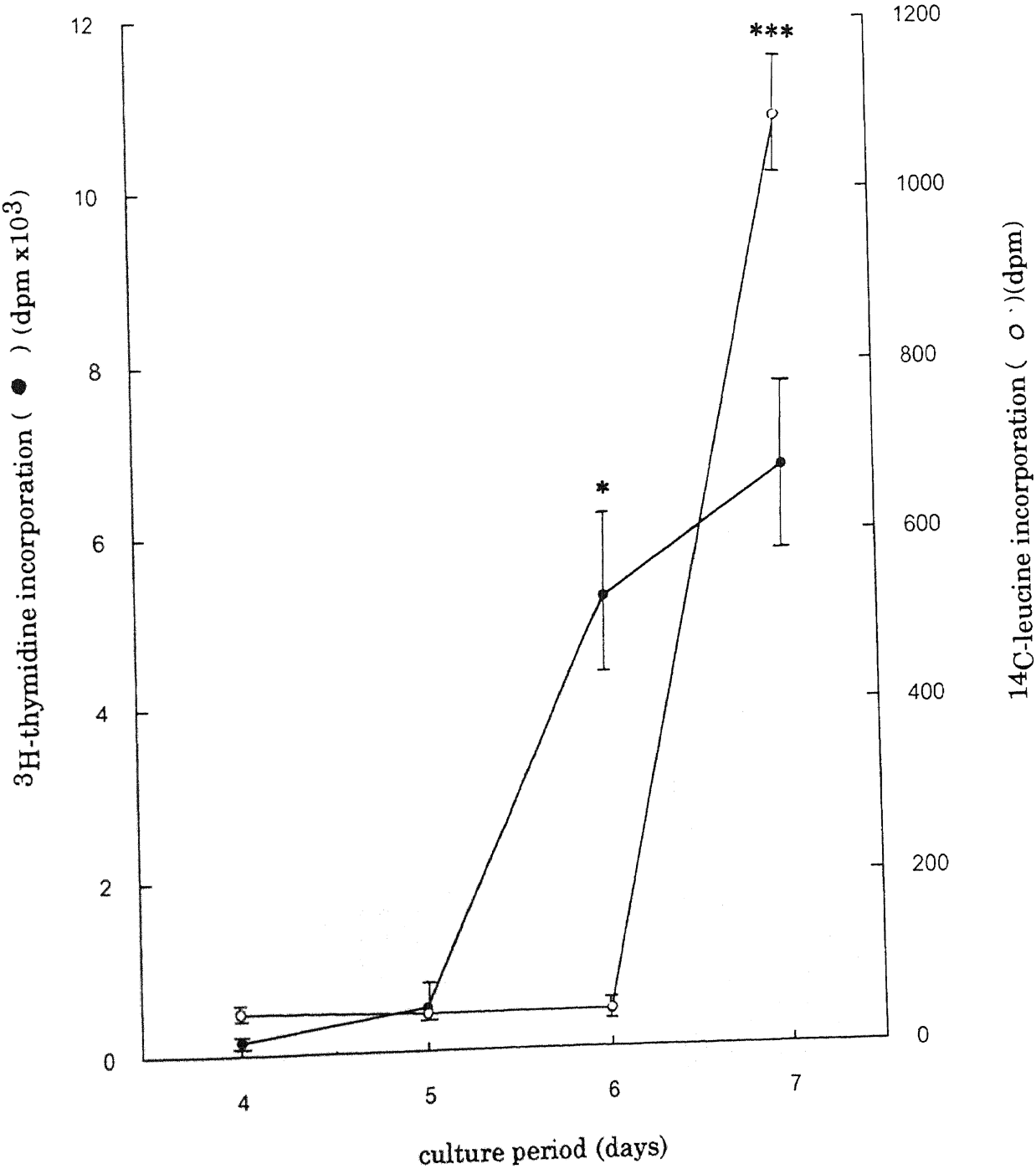
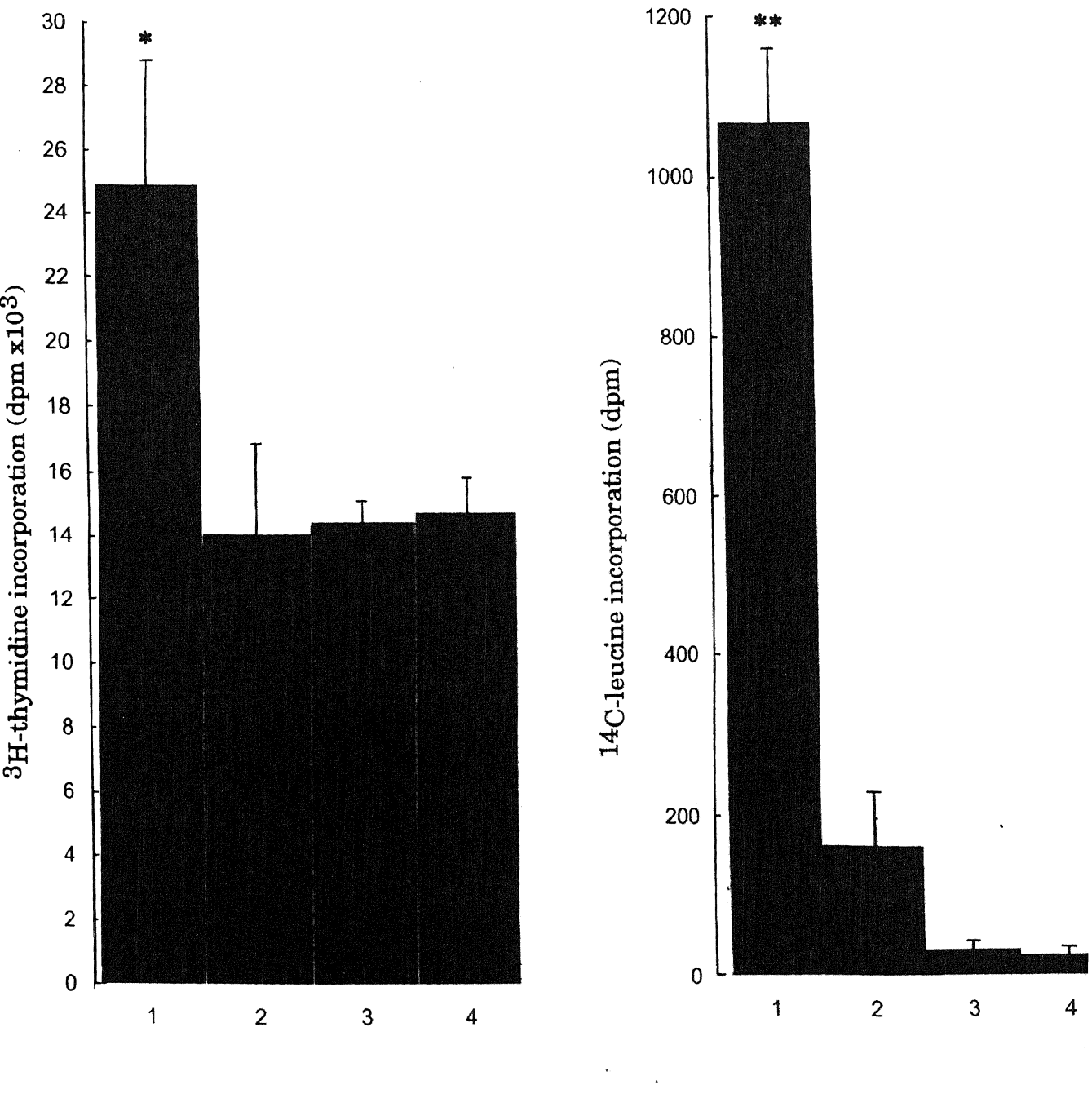


FIGURE 6.3. Effect of repeated washes on radioactivity counts of kidney lymphocytes cultured for seven days with ^{3}H-thymidine or ^{14}C-leucine. Values are means ± 1 standard error, n=5. Asterisks indicate values significantly different from second or subsequent washes where *=P<0.05 and **=p<0.001.



6.2. Background to in vitro experiments 6 to 8.

In the following experiments, lymphocytes taken from trout adapted or reared under different conditions were cultured with Con A and radiosotopes to establish whether their growth *in vitro* was affected by the conditions the fish had been kept under beforehand. As well as measuring the incorporation of radiolabelled thymidine and leucine, other cytological data was collected at the time the fish were killed to allow comparisons with the results of the earlier *in vivo* experiments.

6.3. Experiment 6: The lymphocyte population in fish adapted to black and white backgrounds and their growth responses in culture.

Spleen and kidney lymphocytes were isolated, counted and cultured from fingerling trout adapted for two months to black and white backgrounds.

6.3.1. Spleen and Kidney Lymphocyte Counts

The number of spleen and kidney lymphocytes, expressed per gram body weight, tended to be higher in black- than white-adapted fish but the values were not significantly different (Table 6.1).

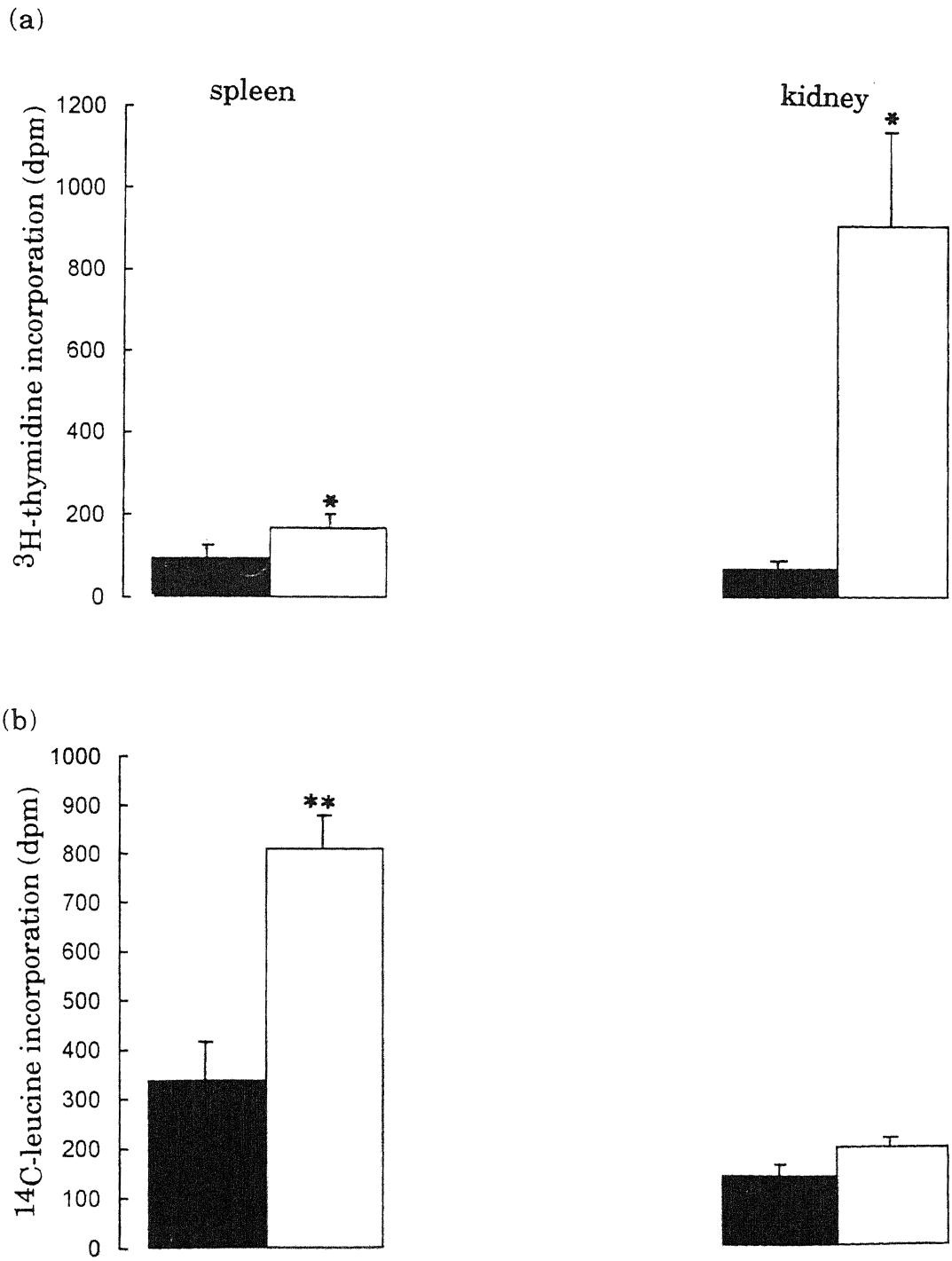
6.3.2. Radioisotope Incorporation

Cell division of Con A stimulated spleen and kidney lymphocytes, as measured by ^{3}H-thymidine uptake, was significantly higher in white-adapted fish. The incorporation of ^{14}C-leucine was also significantly higher for spleen lymphocytes from white-adapted fish but not significant for kidney lymphocytes (Figure 6.4).

TABLE 6.1. Experiment 6. The effects of background adaptation on spleen and kidney lymphocyte numbers. Values are means ± 1 standard error, n=6.

	Black-adapted	White-adapted
Body weight (g)	10.5 ± 1.4	10.3 ± 1.5
Lymphocytes		
Spleen ($x10^6$/g body wt.)	190 ± 80	170 ± 30
Kidney ($x10^6$/g body wt.)	210 ± 40	160 ± 50

FIGURE 6.4. Experiment 6. The incorporation of (a) ^{3}H-thymidine and (b) ^{14}C-leucine by lymphocytes cultured from the spleen and kidney of fish adapted to black (■) or white (□) backgrounds for 2 months. Values are means ± 1 standard error, n=6. Asterisks indicate values are significantly different from black-adapted fish where * = p <0.05 and ** = p <0.01.



6.4. Experiments 7 & 8: Effects of rearing fish on black or white backgrounds under different conditions of stress on blood cell numbers and on the growth responses of cultured lymphocytes.

In two related experiments, adult Rainbow trout that had been reared for 18 months on black or white backgrounds were used. In Experiment 7, the fish were taken directly from the aquarium at Bath University where they had been reared from eggs under as stress-free conditions as possible. Water quality was maintained to a high standard, and the noise, temperature fluctuations, density per tank and general disturbance kept to a minimum. For Experiment 8, a group of Bath-reared fish were transported to tanks at Luton University and held for 12 days in a relatively noisy aquarium. They were also subjected to additional stress by daily netting. The plasma cortisol, red and white blood cell counts, and spleen and kidney lymphocyte numbers obtained in each experiment are shown in Table 6.2. Estimates of the numbers of different blood cells in the circulation were calculated from blood smears taken at the time the fish were killed and are shown in Table 6.3. Con A stimulated lymphocytes isolated from the spleens and kidneys of these fish were cultured with radioisotopes as before and the level of incorporation observed is presented in Figures 6.5. and 6.6.

6.4.1. Body and Spleen Weights

The body weights of white-reared fish tended to be larger (range 503-717g) than those of black-reared fish (range 363-697g) and in the

TABLE 6.2. Experiments 7 & 8. Effects of low and moderate stress on spleen size, plasma cortisol, red and white blood cell counts, and spleen and kidney lymphocyte numbers in fish reared on black or white backgrounds. Values are means ± 1 standard error, n=7. Bracketed values are significantly different were *=p <0.05, **=p <0.01 and ***=p <0.001.

	Expt. 7: Low Stress		Expt. 8: Moderate Stress	
	Black-reared	White-reared	Black-reared	White-reared
Body weight (g)	557.9 ± 32.1	611.9 ± 23.3	512.4 ± 42.7	640.1 ± 21.8
Spleen weight (mg)	736 ± 74	863 ±112	1200 ± 27	950 ± 80
Spleen wt/body wt(x10^3)	1.3 ± 0.1	1.5 ± 0.2	3.4 ± 0.8	1.5 ± 0.1
Plasma cortisol (ng ml^{-1})	<1.2	<1.0	30.6 ± 4.4	26.7 ± 7.6
Erythrocytes (x10^6 ml^{-1} blood)	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
Leucocytes (x10^3 ml^{-1} blood)	32.0 ± 4.6	24.0 ± 1.9	28.9 ± 3.3	29.8 ± 1.4
Lymphocytes				
Spleen (x10^6/mg spleen wt)	35.7 ± 3.7	14.3 ± 2.5	42.2 ± 8.0	48.3 ± 10.8
Kidney(x10^6/ g body wt)	53.6 ± 12.6	33.8 ± 5.2	66.9 ± 10.8	66.5 ± 10.3

TABLE 6.3. Experiments 7& 8. The numbers of immature, mature and degenerating erythrocytes and of the most abundant types of leucocyte counted from blood smears taken from low- and moderately-stressed trout reared on black or white backgrounds. Values are means ± 1 standard error, n=7. Bracketed values are significantly different where *=p <0.05, **=p <0.01 and ***=p <0.001.

	Expt. 7: Low Stress		Expt. 8: Moderate Stress	
	Black-reared	White-reared	Black-reared	White-reared
Erythrocytes (x10^3/ml blood)				
immature	12 ± 10	13 ± 5	14 ± 20	37 ± 30
mature	616± 90	730± 30	911± 80	922± 80
degenerate	31 ± 50	44 ± 20	46 ± 80	59 ± 40
Leucocytes (x10^3/ml blood)				
lymphocytes	26.7± 0.4	20.2± 1.0	22.1± 0.6	21.7± 1.2
neutrophils	2.8± 0.4	1.4± 0.6	2.1± 0.5	3.2± 0.8
thrombocytes	2.8± 0.2	2.0± 0.5	4.6± 0.8	4.8± 0.6

** * * ***

moderately-stressed groups this difference was significant. The spleen weight/body weight ratios of moderately-stressed fish were significantly higher in the black-reared group. The moderately stressed black-reared ratio was also higher than the value obtained for low-stressed black-reared fish.

6.4.2. Cortisol

Plasma cortisol titres of low-stressed fish were all less than 1.2ng ml^{-1} and moderate stress increased values more than 25 times (Table 6.2). In contrast to the trends seen in earlier experiments using adapted fish, (see Table 5.5), no differences were apparent between fish reared on different backgrounds.

6.4.3. Spleen and Kidney Cell Counts

A clear trend was apparent from the results of lymphocyte numbers in spleen and kidney tissue (Table 6.2). In low-stressed fish, the black-reared group had significantly higher numbers of lymphocytes in both tissues than the white-reared group, an observation that agrees with the high numbers of these cells observed in the blood (see 6.4.4.).

6.4.4. Blood Cell Counts

When the number of different types of blood cell were counted from blood smears (Table 6.3), it was apparent that the number of lymphocytes in low-stressed fish reared on black backgrounds was significantly higher than counts from the corresponding white-reared

FIGURE 6.5. Experiment 7. The incorporation of ^{3}H-thymidine and ^{14}C-leucine by lymphocytes cultured from the spleen and kidney of fish reared on black (■) or white (□) backgrounds under conditions of low stress. Values are means ± 1 standard error, n=7. Asterisk indicates value is significantly different from corresponding white-adapted group (* = p<0.0 5).

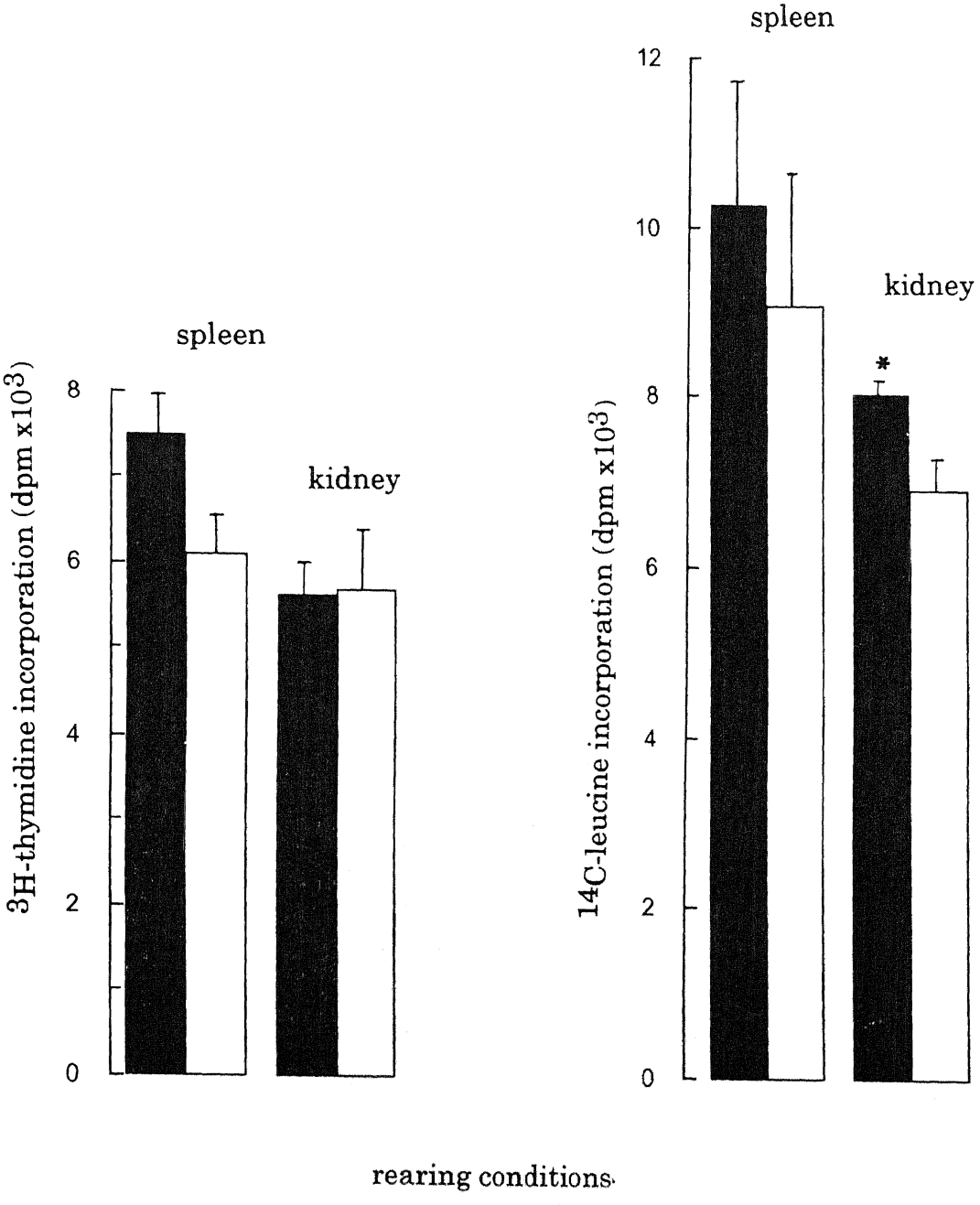
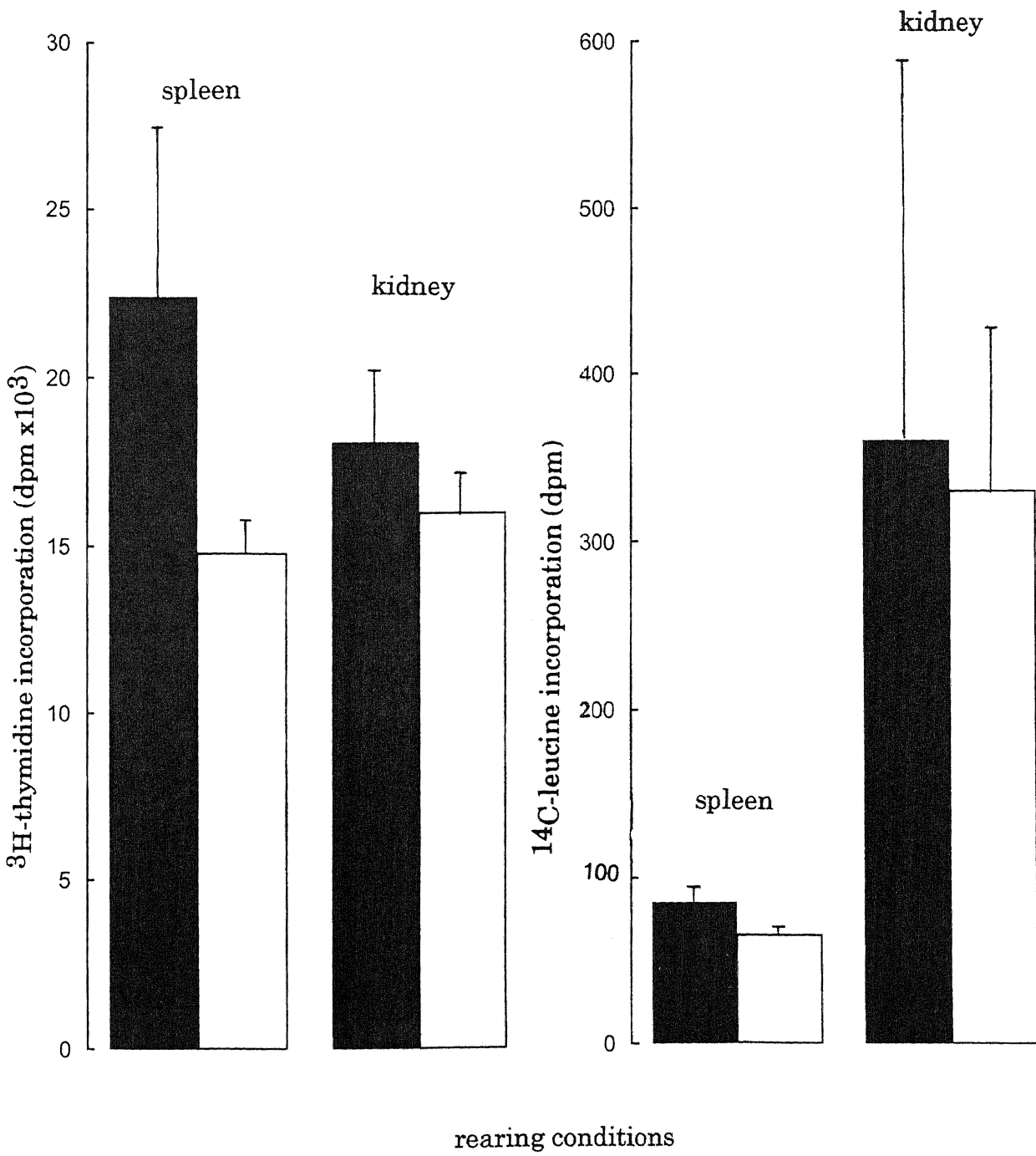


FIGURE 6.6. Experiment 8. The incorporation of ^{3}H-thymidine and ^{14}C-leucine by lymphocytes cultured from the spleen and kidney of fish reared on black (■) or white backgrounds (□) under conditions of moderate stress for 12 days. Values are means ± 1 standard error, n=7.



group and from moderately-stressed black-reared fish. The number of thrombocytes was increased significantly with the level of stress, but black and white differences were not apparent.

6.4.5. Radioisotope Incorporation

The incorporation of both radiolabelled leucine and thymidine by black-reared and corresponding groups of white-reared fish were usually similar, irrespective of the tissue source of the lymphocytes or the level of stress to which the fish had been subjected (Figure 6.5 & 6.6). However, in black-reared low-stressed fish the incorporation of leucine into the kidney lymphocytes was significantly higher compared to their white-reared counterparts (Figure 6.5). This was in marked contrast to the difference between the levels of uptake seen when fish were adapted to different backgrounds, where black-adapted fish consistently showed lower levels of isotope incorporation (see Figure 6.4).

6.4.6. Summary

In these experiments, the reason for using reared, rather than adapted fish, was to enhance the possible influence that MCH might have on stress and immune responses. While some significant differences were observed, in many cases these were opposite to those seen when adapted fish were used. For other data the results were inconclusive. Thus, black-reared fish had increased numbers of lymphocytes in the blood and spleen compared to white-reared fish but the uptake of radioisotopes in Con A stimulated culture was similar and did not show the differences seen in adapted fish.

6.5. Background to *in vitro* experiments 9 to 20.

In this Section, the effects of adding various hormones to spleen and kidney lymphocytes cultured *in vitro* in the presence of Con A or LPS are presented. Full details of the hormones investigated and the culture conditions employed are described in Section 4.3. The rate of cell division was measured using ^{3}H-thymidine using the method previously described.

In the results that follow, the effects of adding various concentrations of hormones to lymphocyte cultures are expressed as the percentage change in ^{3}H-thymidine uptake compared to that measured in control cell cultures. In this way positive values indicate a stimulatory effect, and negative values an inhibitory effect.

In a few experiments, where both black- and white-adapted fish have been used, two sets of control cultures were necessary, one for each type of background. In such cases, the actual amount of ^{3}H-thymidine taken up has been given in one figure and the percentage uptake, compared to the corresponding control cultures, given separately in another. This was necessary to allow comparisons to be made between black- and white-adapted fish for each treatment.

The fish used for this series of experiments were adapted to black or white backgrounds for 14-21 days before use. The pooled body weights, spleen weights and spleen to body weight ratios are shown in Table 6.4. Although black-adapted fish tended to be larger and have bigger spleens than white-adapted, the differences were not significant. The spleen weight to body weight ratios were also similar.

TABLE 6.4. Experiments 9 to 20. Pooled total body weight, spleen weight and body to spleen weight ratios for trout adapted to black or white backgrounds for 14-21 days. Values are means ± 1 standard error.

	Black-adapted (n=48)	White-adapted (n=17)
Body weight (g)	120.8± 10.2	96.4 ±12.8
Spleen weight (g)	0.22 ± 0.03	0.15± 0.02
Spleen/body weight ratio ($x10^3$)	1.62 ± 0.15	1.49± 0.07

6.6. Experiment 9:
Effect of cortisol on lymphocyte proliferation *in vitro*.

The tritiated thymidine incorporated by Con a stimulated splenic lymphocytes taken from black- compared to white-adapted fish for any given dose of cortisol was generally very similar (Figure 6.7a). In Con A stimulated kidney cultures however, white-adapted cells consistently took up more radioisotope than black-adapted cells exposed to the same dose of cortisol, although this difference was not significant (Figure 6.7.b).

Cortisol depressed ^{3}H-thymidine uptake in both types of lymphocyte culture in a dose dependent manner. In general, when cortisol concentrations were 30ng ml^{-1} or above, uptake was significantly lower than control cultures for both black- and white-adapted fish (Figure 6.7.). When expressed as the percentage difference from control cultures however, the percentage inhibition of cell growth was only significant in white-adapted fish (Figure 6.8). The effect was most marked in cultures of white-adapted kidney lymphocytes where a low dose of cortisol (10ng ml^{-1}) depressed uptake by 19.4% and the highest concentration (300ng ml^{-1}) by 37.6%. The corresponding values for spleen lymphocytes were 15.2 and 43.8%.

FIGURE 6.7. Experiment 9. Effect of cortisol on ^{3}H–thymidine uptake by (a) spleen and (b) kidney lymphocytes isolated from black- (■) or white-adapted (□) trout and cultured *in vitro*. Values are mean dpm ± 1 standard error, n=6. Asterisks indicate values are significantly different from corresponding control cultures where * = $p<0.05$ and ** = $p<0.01$.

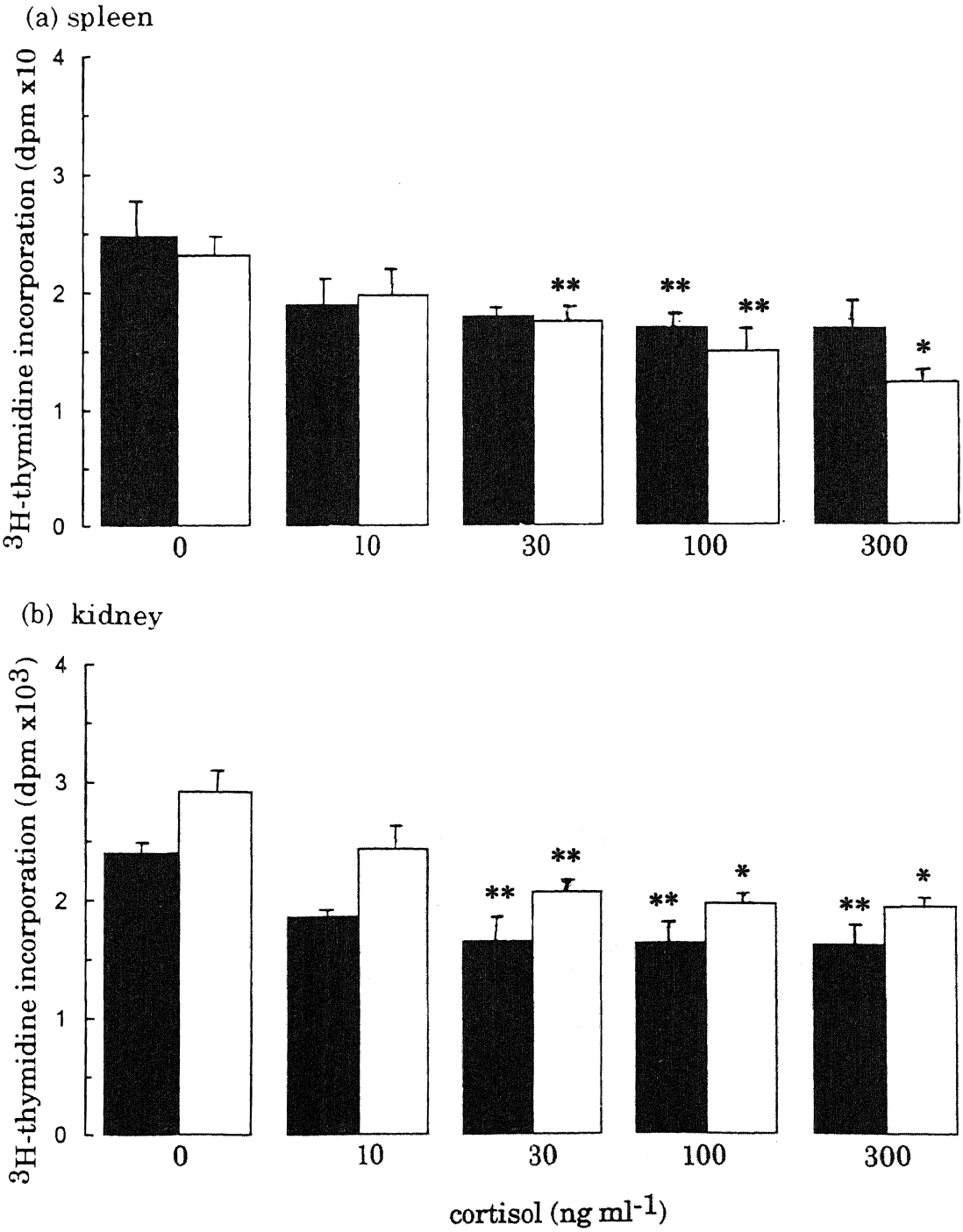
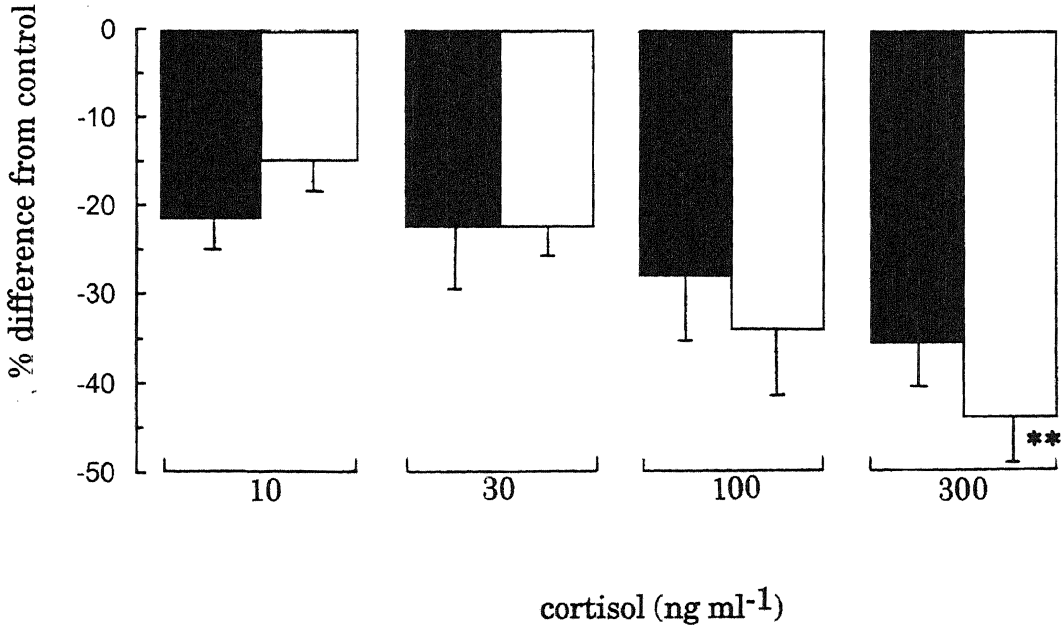
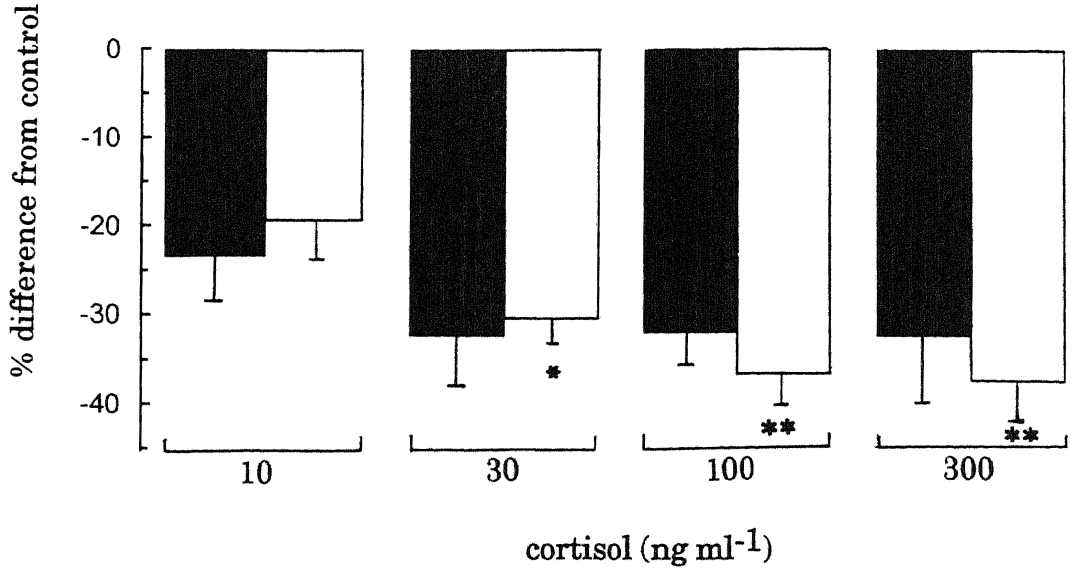


FIGURE 6.8. Experiment 9. Effect of cortisol on ^{3}H-thymidine uptake by (a) spleen and (b) kidney lymphocytes isolated from black- (■) or white-adapted ( ) trout and cultured *in vitro*. Values are expressed as mean % difference from control ± 1 standard error, n=6. Asterisks indicate values are significantly different from corresponding cultures exposed to 10ng ml^{-1} cortisol where * = $p<0.05$ and ** = $p<0.01$.

(a) spleen



(b) kidney



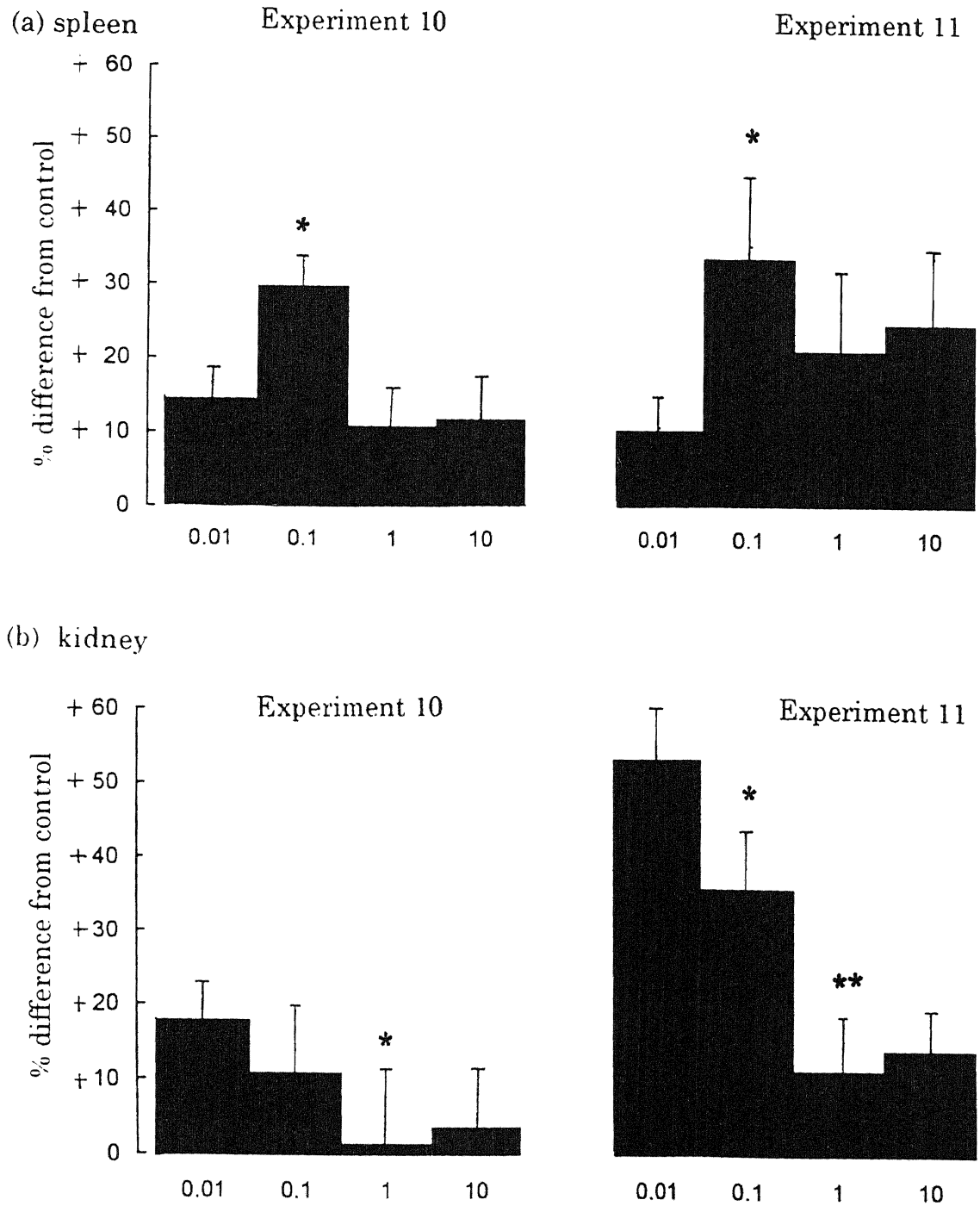
6.7. Experiments 10 & 11: Effects of MCH on lymphocyte proliferation *in vitro.*

In two similar experiments, melanin-concentrating hormone was found to enhance Con A stimulated lymphocyte division at all doses investigated (Figure 6.9). To minimise interference by any endogenous MCH, the lymphocytes were taken from black-adapted fish in which circulating titres of the hormone would be low.

In cultures of spleen cells, a 29.8 and 33.7% stimulation of ^{3}H-thymidine incorporation was observed in Experiment 10 and Experiment 11 respectively with a dose of 0.1ng ml^{-1}. This concentration produced significantly greater stimulation than all other doses tested (Figure 6.9a).

Kidney cell cultures showed maximum enhancement with 0.01ng ml^{-1} MCH in both experiments, and at this dose there was significantly greater uptake than at all other concentrations. With higher doses the positive effects of MCH decreased from 18.3 to 1.7% in Experiment 10 and from 53.6 to 11.7% in Experiment 11 (Figure 6.9b).

FIGURE 6.9. Experiments 10 & 11. Effect of melanin-concentrating hormone on the ^{3}H-thymidine uptake by (a) spleen and (b) kidney lymphocytes isolated from black-adapted trout and cultured *in vitro*. Values are expressed as mean % difference from control ± 1 standard error, n=6. Asterisks indicate values are significantly different from corresponding cultures exposed to 0.01ng ml^{-1} MCH where $*=p<0.05$ and $**=p<0.01$.



6.8. Experiment 12:
Effects of melanin-concentrating hormone on cortisol-suppressed lymphocyte proliferation *in vitro*.

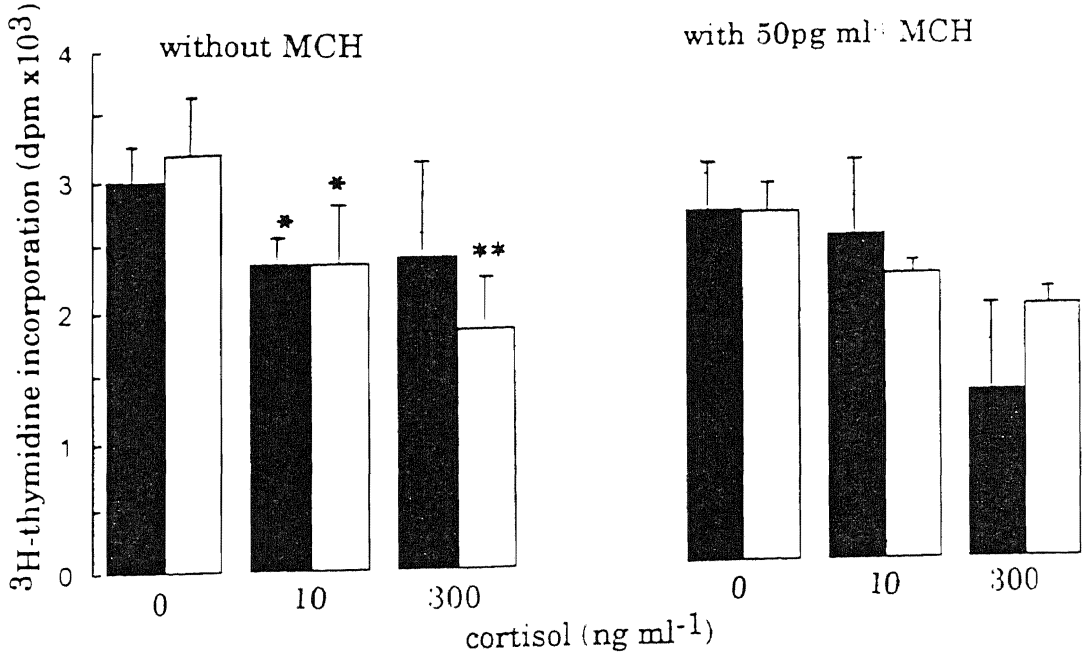
In view of the response seen in experiments 10 and 11 by spleen and kidney cell cultures and the opposing effects of cortisol and MCH on cell division already observed, MCH was added to cortisol-suppressed lymphocyte cultures in order to establish the combined effects of these hormones when both were present at the same time. Based on the results of Experiment 9 (see Figure 6.7), cells were cultured with 0, 10 or 300ng ml^{-1} cortisol with or without the addition of 0.05ng ml^{-1} MCH. In complete agreement with the results from Experiment 9, cortisol inhibited ^{3}H-thymidine uptake by Con A stimulated spleen and kidney lymphocytes whether or not MCH was also present (Figure 6.10). In the absence of additional MCH, cortisol suppression was similar in black and white-adapted trout but became more marked in white-adapted fish at high doses of cortisol (300ng ml^{-1}). The addition of MCH did not change the pattern of response, but in kidney lymphocyte cultures, black-adapted cells now showed significantly greater cortisol inhibition than the equivalent white-adapted cells (Figure 6.10a & b).

The same data are expressed in terms of the percentage difference from their respective controls in Figure 6.11 and these may be compared with the earlier results for Experiment 9 shown in Figure 6.8. In the absence of additional MCH, cortisol suppression increased significantly with the dose and was always greater in white-adapted fish. In kidney cell cultures for example, suppression increased from 26.3% with 10ng ml^{-1} cortisol to 51.8% with 300ng ml^{-1}. These data can be compared with the black-adapted values which, for kidney cells, ranged from

17.2% with 10ng ml^{-1} cortisol to 37.6% with 300ng ml^{-1}. The addition of MCH to the cultures tended to reduce the percentage inhibition especially with the highest concentration of cortisol and in cells from white-adapted fish. When cultured with MCH, spleen cells showed a reduction from 42.3 to 26.2 % inhibition and kidney cells a reduction from 51.8 to 29.3% inhibition. In other words, the addition of MCH flattened the cortisol dose response curve and reduced the inhibition caused by cortisol. This difference between cultures with and without MCH, was statistically significant in white-adapted lymphocytes at most doses (Figure 6.11 a & b).

hormone on ^{3}H-thymidine uptake response to cortisol by (a) spleen and (b) kidney lymphocytes isolated from black- (■) or white-adapted (□) trout and cultured *in vitro*. Values are expressed as mean dpm ± 1 standard error, n=6. Asterisks indicate values are significantly different from corresponding cultures not exposed to cortisol where *=p<0.05 and **=p<0.01. ‡ : significantly different (p<0.05) from black-adapted cells exposed to the same conditions

(a) spleen



(b) kidney

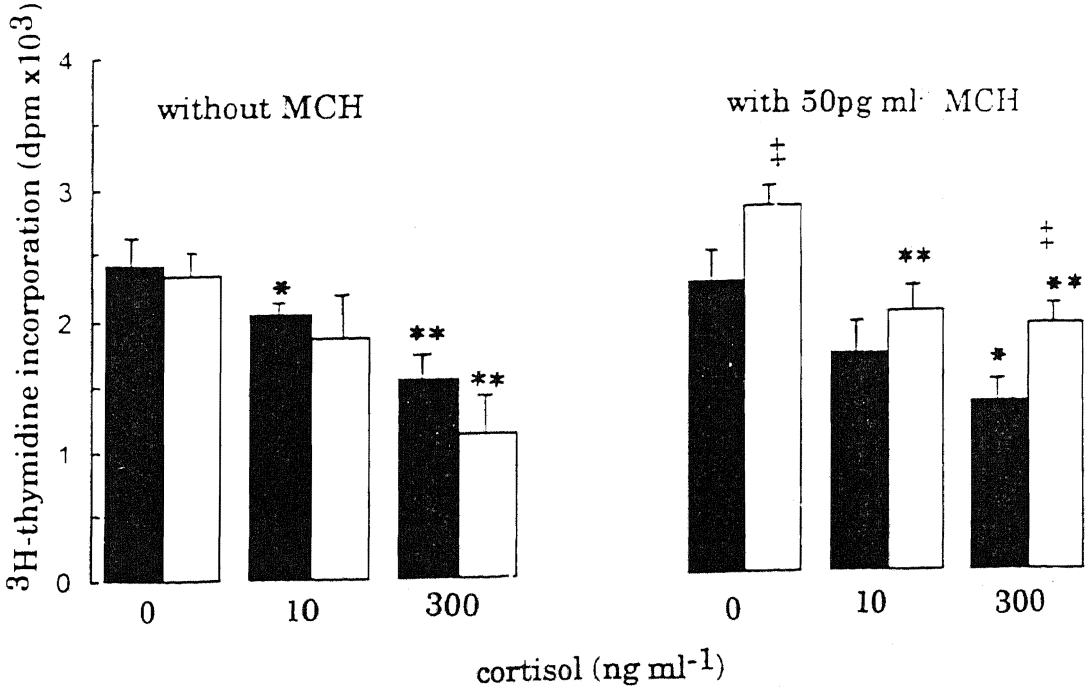
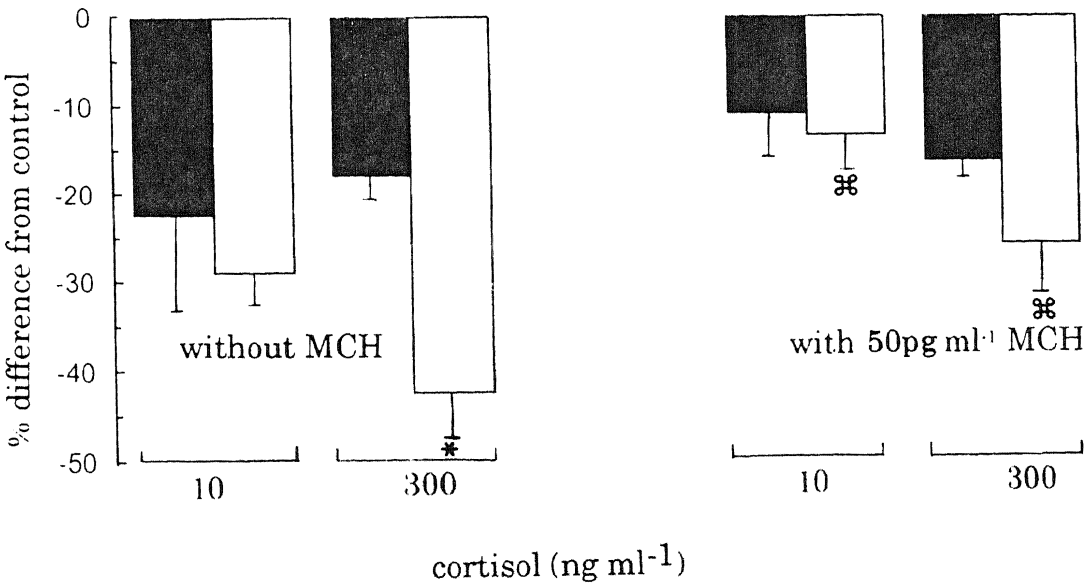
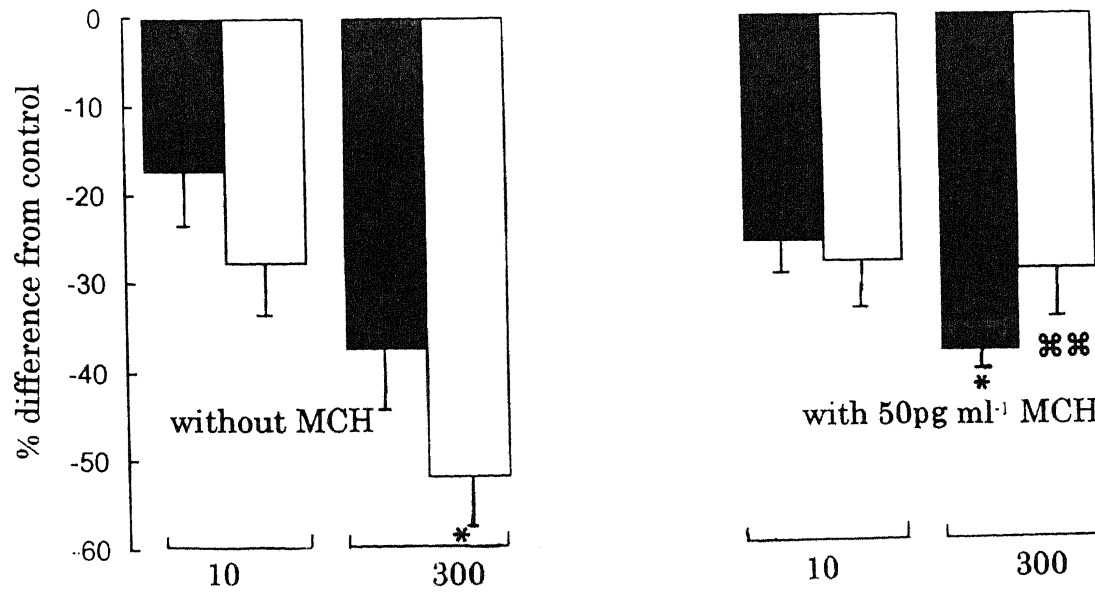


FIGURE 6.11. Experiment 12. Effect of melanin-concentrating hormone on the response to cortisol by (a) spleen and (b) kidney lymphocytes isolated from black- (■) or white-adapted (□) trout and cultured *in vitro*. Values are expressed as mean % difference from control ± 1 standard error, n=6. Asterisks indicate values are significantly different from corresponding cultures exposed to 10ng ml^{-1} cortisol where *=$p<0.05$ and **=$p<0.01$. ⌘ : symbols indicate values are significantly different from corresponding cultures not exposed to MCH where ⌘ =$p<0.05$ and ⌘⌘=$p<0.01$.

(a) spleen



(b) kidney



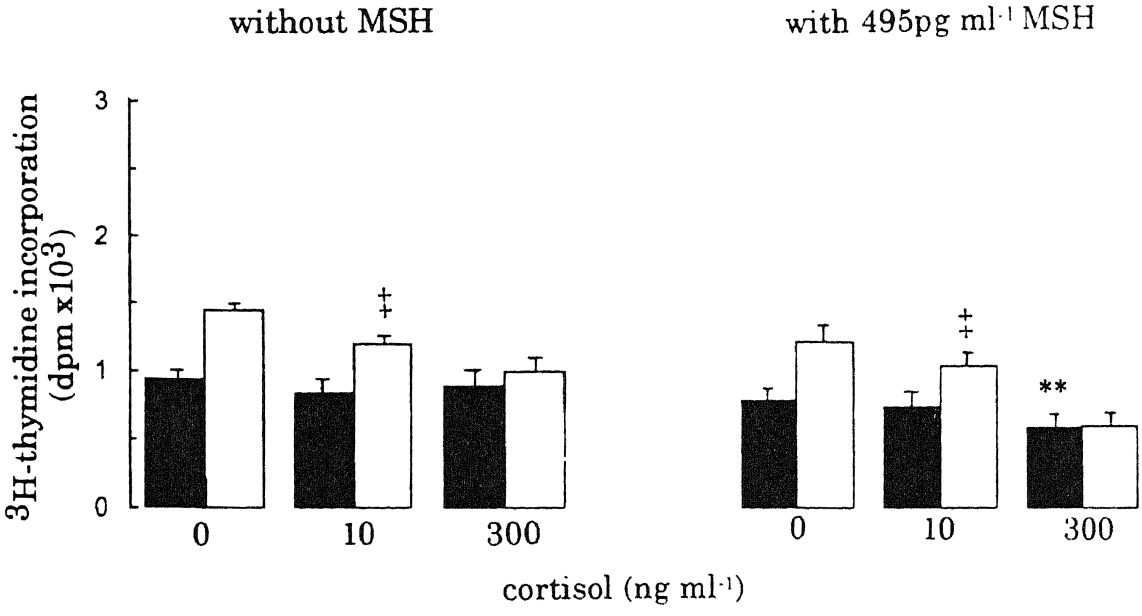
6.9. Experiment 13 : Effects of melanocyte stimulating hormone on cortisol-suppressed lymphocyte proliferation *in vitro*.

Since melanin-concentrating hormone (MCH) and melanocyte-stimulating hormone (αMSH) have many antagonistic physiological actions, the possibility that αMSH may also be involved in the ability of lymphocytes to proliferate in the presence of cortisol was investigated. The experimental protocol was identical to Experiment 12 except that cultures were grown with or without the addition of 495pg ml^{-1} αMSH.

The results show that, as before, cortisol suppressed Con A stimulated lymphocyte division by cells from both black- and white-adapted fish. There were some significant differences between black and white groups at 10ng ml^{-1} cortisol. The addition of 495pg ml^{-1} αMSH did not have any significant effect on cell growth (Figure 6.12 a & b). When the data are expressed in terms of the percentage difference from control cultures, lymphocytes from white-adapted fish showed maximum inhibition with the highest dose of cortisol and this was significant for spleen cells (Figure 6.13 a & b). Smaller changes in inhibition with increased cortisol were noted for cells cultured from black-adapted fish but the differences were less clear cut. None of the cells cultured with 495pg ml^{-1} αMSH showed any significant changes in inhibition compared with cultures without αMSH.

FIGURE 6.12. Experiment 13. Effect of melanin-stimulating hormone on ^{3}H-thymidine uptake response to cortisol by (a) spleen and (b) kidney lymphocytes isolated from black- (■) or white-adapted (□) trout and cultured *in vitro*. Values are expressed as mean dpm ± 1 standard error, n=6. Asterisks indicate values are significantly different from corresponding cultures not exposed to cortisol where *=p<0.05 and **=p<0.01. ‡ : significantly different (p<0.05) from black-adapted cells exposed to the same conditions

(a) spleen



(b) kidney

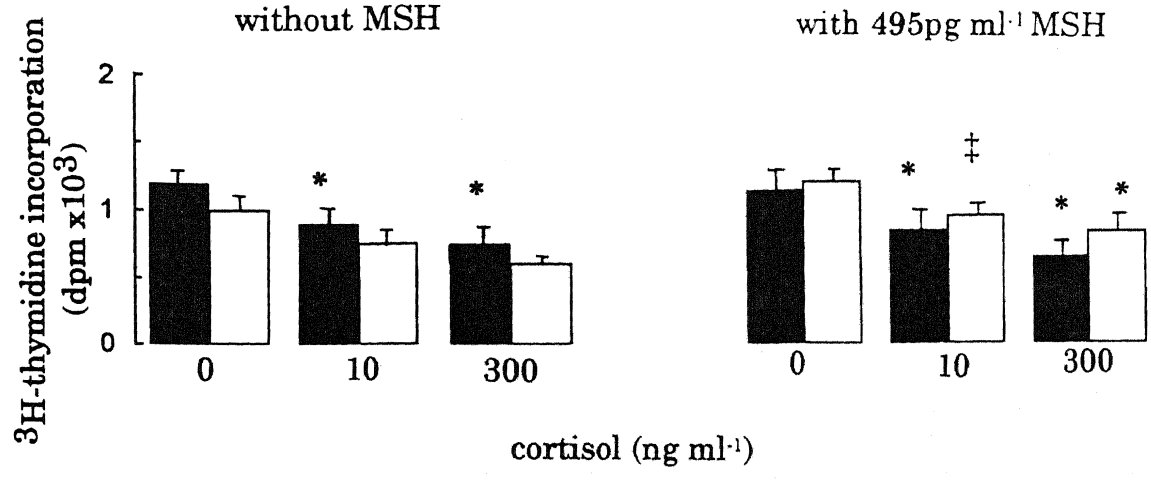
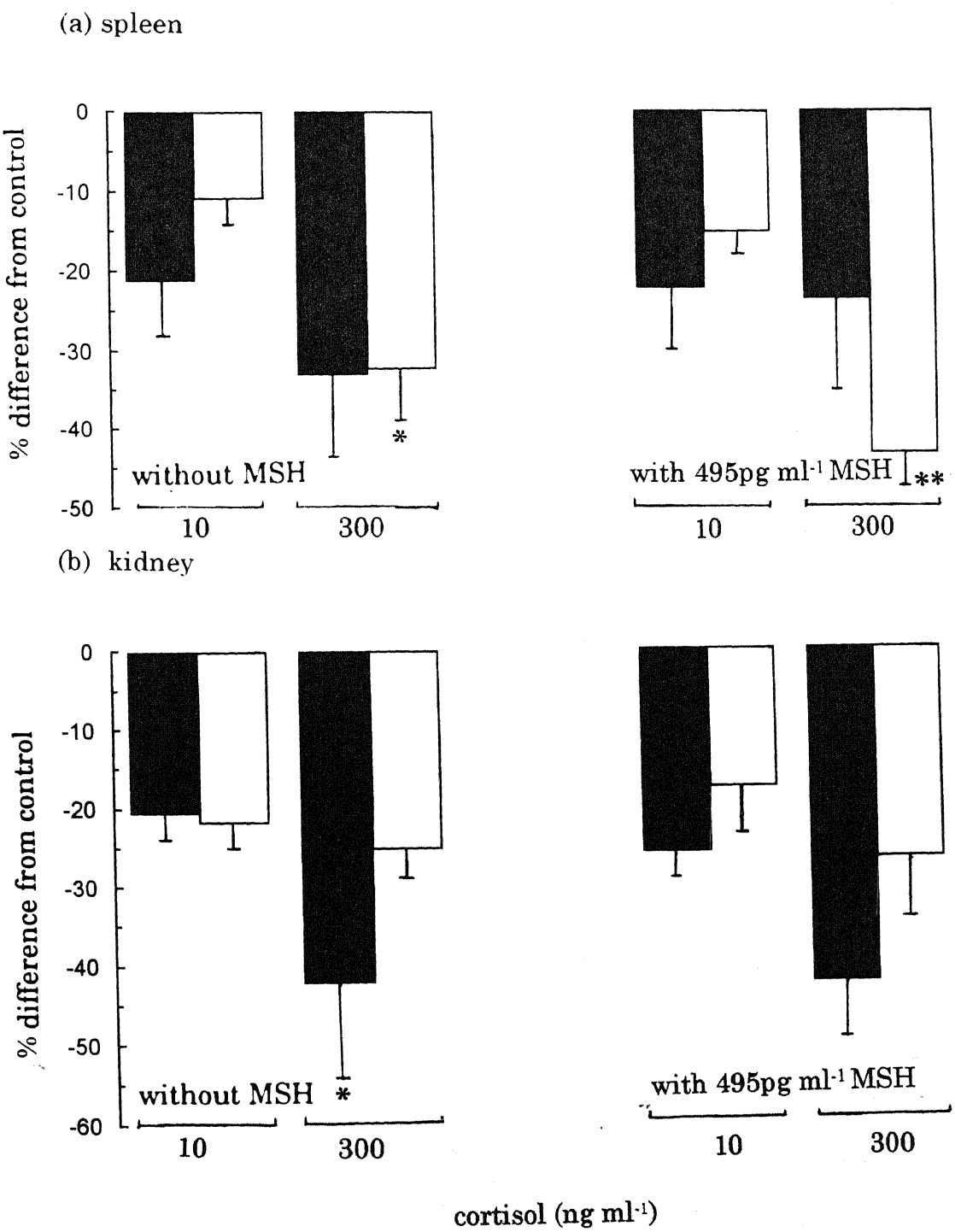


FIGURE 6.13. Experiment 13. Effect of melanin-stimulating hormone on the response to cortisol by (a) spleen and (b) kidney lymphocytes isolated from black- (■) or white-adapted (□) trout and cultured *in vitro*. Values are expressed as mean % difference from control ± 1 standard error, n=6. Asterisks indicate values are significantly different from corresponding cultures exposed to 10ng ml^{-1} cortisol where *=$p<0.05$ and **=$p<0.01$.



6.10. Experiments 14-18: Effects of reproductive steroids on lymphocyte proliferation *in vitro.*

In a series of similar experiments, five different teleost reproductive steroid hormones were added to Con A stimulated spleen and kidney lymphocyte cultures. Their effect on ^{3}H-thymidine uptake was measured at four concentrations of each hormone and the results recorded as the % difference compared to control cultures without steroid (Figure 6.14). While some steroids tended to be either primarily stimulatory or inhibitory, others gave mixed responses depending on the dose and on whether spleen- or kidney-derived lymphocytes were tested.

6.10.1. 11β ketotestosterone

When added to cultures of spleen lymphocytes, this androgen inhibited uptake by -28.5% at a concentration of 10ng ml^{-1}. The inhibitory effect increased to -47.7% at a concentration of 100ngml^{-1}. At 300ng ml^{-1} however, the inhibition was only -23.5%. In kidney lymphocyte cultures 11β ketotestosterone showed a significant dose dependent inhibition of isotope incorporation from -18.3% at 10ng ml^{-1} to -2.6% at 300ng ml^{-1}.

6.10.2. 17a hydroxyprogesterone

In kidney lymphocyte cultures, this steroid showed a dose dependent inhibition of ^{3}H-thymidine uptake reaching -27.4% with 300ng ml^{-1}. The inhibition was less marked in spleen cell cultures at the highest

dose (-13.2%) while at 30ng ml^{-1} or less, the hormone became slightly stimulatory.

6.10.3. *Androstenedione*

In spleen cell cultures, this steroid tended to be more or less inhibitory with a maximum inhibition of -35.7% with 300ng ml^{-1} By contrast, when added to kidney lymphocytes the hormone was a powerful stimulant and lymphocytes showed a clear dose dependant increase in ^{3}H-thymidine uptake from +14.8% at 10ng ml^{-1} to +114% at 300ng ml^{-1}.

6.10.4. *17α hydroxy, 20β dihydroxyprogesterone*

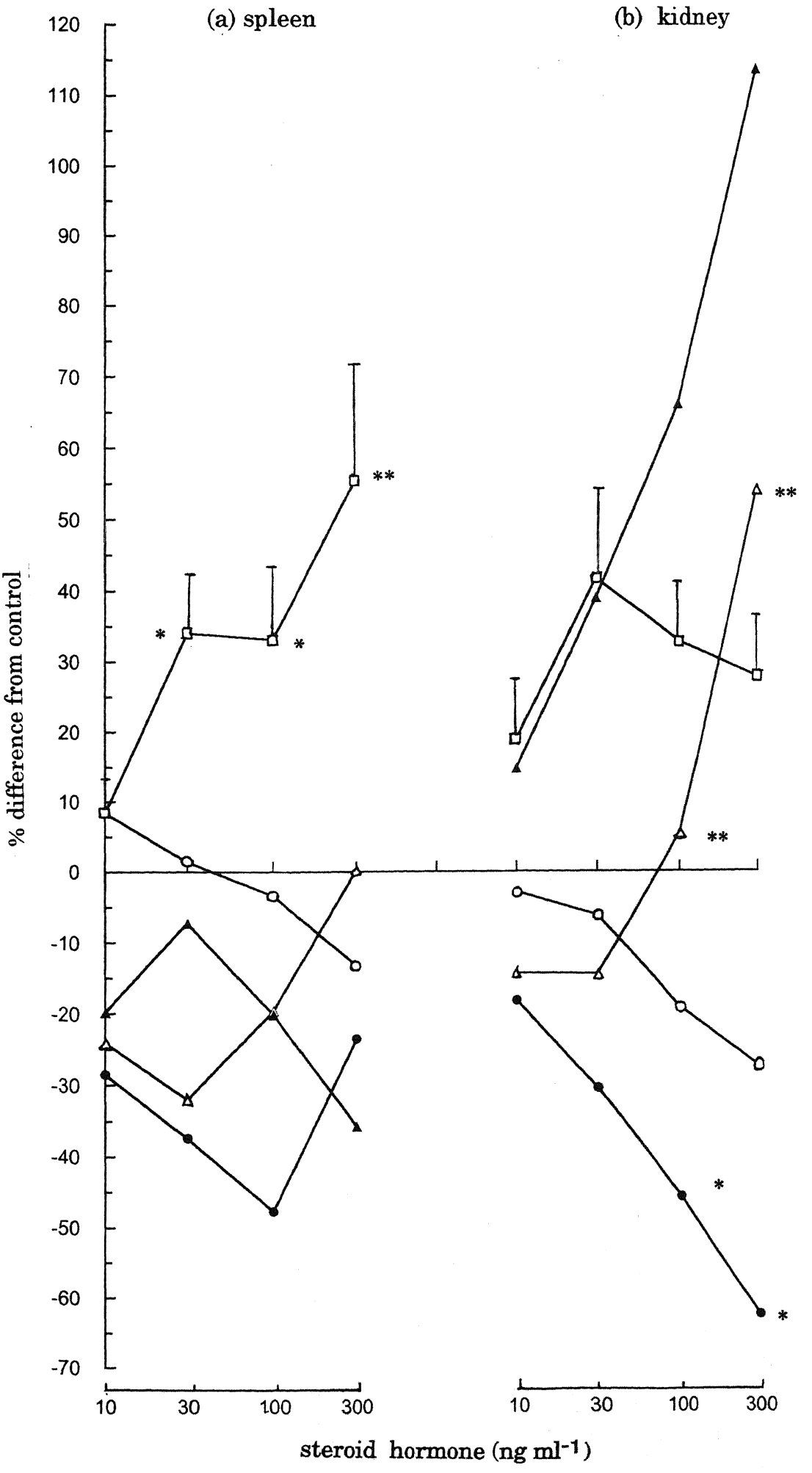
The pattern of response seen in spleen lymphocyte cultures was variable. Whereas concentrations between 10 and 100ng ml^{-1} all inhibited uptake by between -19.6 and -32.1%, the highest concentration had little effect. In kidney lymphocytes the response was more obvious. Low concentrations between 10 and 30ng ml^{-1} inhibited cell division by approximately -14% but higher concentrations stimulated growth by as much as +54.2% at 300ng ml^{-1}

6.10.5. *17 β oestradiol*

Oestradiol consistently stimulated uptake of labelled thymidine by both spleen- and kidney-derived lymphocytes. In spleen cell cultures, the effect was more or less dose dependent, with significant increases in stimulation as the dose increased. Uptake was greatest at 300ng ml^{-1} when incorporation increased by +55.5%. In kidney cell cultures, the

FIGURE 6.14. Experiments 14-18. Effects of reproductive steroids on ^{3}H-thymidine uptake by (a) spleen and (b) kidney lymphocytes isolated from black-adapted trout and cultured in *in vitro* with Con A. Values are expressed as mean % difference from controls. Standard errors are shown for 17b estradiol on spleen lymphocytes and are typical of those for all other means which have been omitted for clarity. Asterisks indicate values significantly different from cells exposed to 10ng ml^{-1} in each case, where *=p <0.05 and **=p <0.01.

- ● 11β ketotestosterone
- ▲ androstenedione
- △ 17α hydroxy, 20β dihydroxyprogesterone
- ○ 17α hydroxyprogesterone
- □ 17β-oestradiol.


(a) spleen
(b) kidney
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
% difference from control
*
*
**
**
**
*
*
10
30
100
300
10
30
100
300
steroid hormone (ng ml-1)

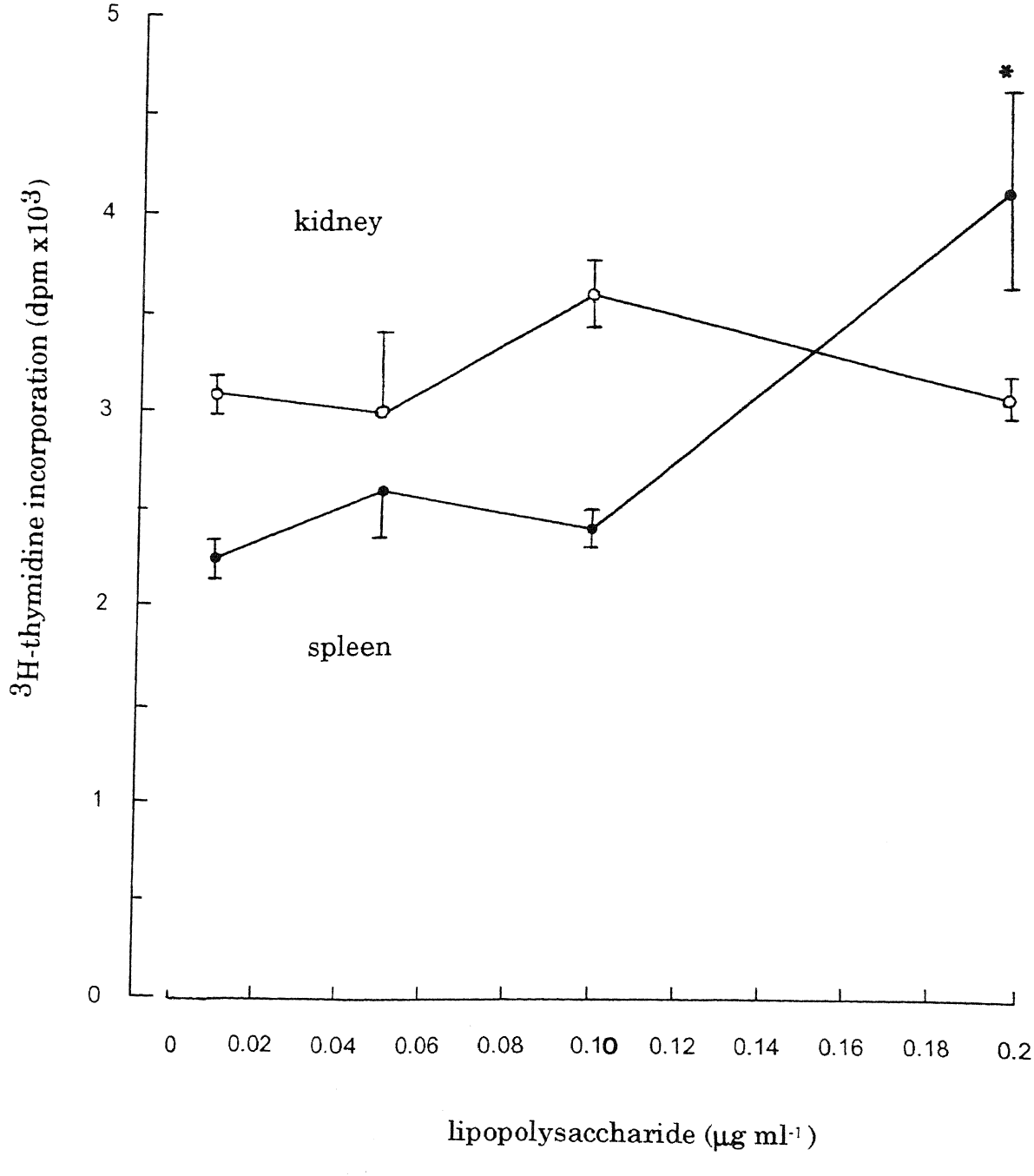
relationship was not linear, with stimulation ranging from +18.9% at 10ng ml^{-1} to +41.8% at 30ng ml^{-1} (Figure 6.14).

6.11. Experiment 19: Preliminary B cell experiments.

In a preliminary experiment, the concentration of lipopolysaccharide required *in vitro* for optimum cell growth and incorporation of radioisotopes was assessed.

Spleen and kidney cells were labelled with ^{3}H-thymidine to measure DNA synthesis. The B cell mitogen lipopolysaccharide (LPS) was added to the cell cultures on day one to give final concentrations in the wells of between 0 and 200mg ml^{-1}. Radiolabelled thymidine was added to the wells on day three and the cells were harvested and counted for radioactivity on day five. The results presented in Figure 6.15, show that ^{3}H-thymidine incorporation by spleen and kidney cells was maximal at 200 and 100 mg ml^{-1} respectively. The culture period and number of washes used in subsequent experiments was the same as adopted for T cell cultures using Con A.

FIGURE 6.15. Experiment 19. Incorporation of ^{3}H-thymidine by spleen and kidney lymphocytes cultured wih different concentrations of the B cell mitogen lipopolysaccharide (LPS). LPS was added on day 3 and the cells were harvested on day 5. Values are expressed as mean dpm ± 1 standard error, n=6. Asterisk indicates value is significantly different ($p<0.05$) from spleen cells cultured with 0.05µg ml^{-1} LPS.



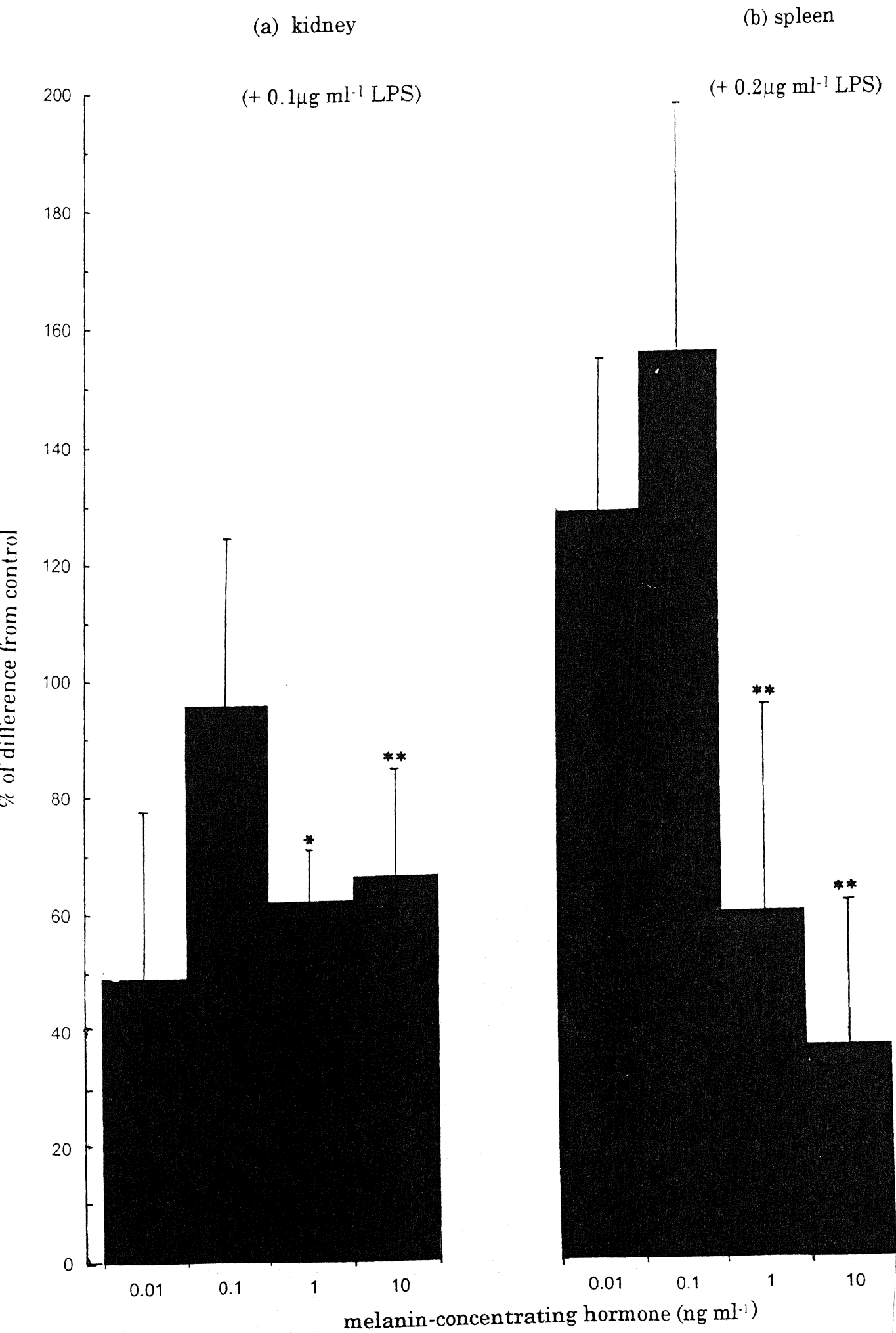
6.12. Experiment 20:

Effects of MCH on B cell lymphocyte proliferation *in vitro.*

To minimise interference by any endogenous MCH, lymphocytes were taken from black-adapted fish in which circulating titres of the hormone would be low. Melanin-concentrating hormone was found to stimulate B cell lymphocyte division at all doses investigated (Figure 6.16).

A maximum stimulation of 95% compared to cultures without MCH was obtained for kidney cells at a dose of 0.1ng ml^{-1} MCH. Higher doses of MCH were significantly less effective. Spleen cell cultures showed an even more marked response to 0.1ng ml^{-1} MCH and thymidine uptake increased by 155%. With higher doses, the stimulatory effect on spleen cells decreased significantly in a dose dependent manner to only 34% of the untreated control cultures with a dose of 10ng ml^{-1} (Figure 6.16).

FIGURE 6.16. Experiment 20. Effect of melanin-concentrating hormone on ^{3}H-thymidine uptake by (a) kidney and (b) spleen lymphocytes isolated from black-adapted trout and cultured *in vitro* with lipopolysaccharide (LPS). Values are expressed as mean % difference from controls ± 1 standard error, n=6. Asterisks indicate values are significantly different from cells exposed to 0.1ng ml^{-1} where * =p <0.0.5 and **=p <0.01.


(a) kidney
(+ 0.1μg ml-1 LPS)
(b) spleen
(+ 0.2μg ml-1 LPS)
% of difference from control
200
180
160
140
120
100
80
60
40
20
0
*
**
**
**
0.01
0.1
1
10
0.01
0.1
1
10
melanin-concentrating hormone (ng ml-1)

7. Discussion

This thesis has described an investigation on the interactions of melanin-concentrating hormone with the hypothalamo-pituitary-interrenal axis of Rainbow trout and the cellular actions of this neuropeptide and thus examined how this neuropeptide may be involved in modifying the immune response. The results have confirmed previous findings that trout adapted to white backgrounds have lower cortisol levels than black-adapted fish. They have further shown that circulating lymphocytes are more numerous and more active in white-adapted fish and when these fish are immunised with SRBCs, the rise in antibody titre is greater than in fish adapted to black tanks. When fish are reared, rather than adapted, to black and white tanks however, the black and white differences are either no longer evident, or are reversed compared to the results obtained with adapted fish.

The main findings of these experiments are summarised in Tables 7.1 and 7.2 and possible mechanisms of how these observations can be explained are discussed in the following pages. Although the inhibitory effect of corticosteroids on the immune system are well known, other steroids have not received the same attention. This study has also obtained preliminary data on the effect of reproductive steroids on lymphocyte responses to T-cell mitogens.

7.1. Stress and cortisol

When fish were held under conditions of mild chronic stress, the plasma cortisol titres were higher in black- than in white-adapted fish, and these differences were generally significant (Table 7.1). Baker and Rance (1981) and Gilham and Baker (1985) have reported similar results and concluded they were due to the ability of MCH to inhibit hypothalamo-pituitary-interrenal axis activity. MCH has been shown to act as a CRF-inhibiting factor, either by depressing the release of CRF from the hypothalamus or by rendering the corticotrophs less sensitive to CRF (Baker *et al.*, 1985a). Thus, when black-adapted fish, in which MCH titres are much lower, are exposed to stress, inhibition of the HPI axis is not so evident.

In fish reared on different backgrounds, no obvious differences were found between plasma cortisol titres of black- and white-reared fish, even after exposure to the chronic stress of regular disturbance. Moreover, white-reared fish had significantly higher plasma cortisol titres than black-reared fish after exposure to the stress of injection (Table 7.2). Thus, rearing fish on black or white backgrounds eliminates or reverses the trend seen in adapted fish. In contrast to these results, Green *et al.* (1991) found that black-reared fish secreted more plasma cortisol than white-reared fish when undisturbed and, also, when subjected to daily disturbance, or the stress of injection. However, they found no difference in the release of ACTH and CRF in black and white-reared fish, even when these fish were stressed. The hypothalamic content of bioactive CRF was greater in white- than in black-reared fish. One possible explanation for the results obtained

here is that, because white-reared fish are exposed throughout their lives to very high levels of MCH, the CRF and ACTH cells are desensitised to the inhibitory influence of MCH and so respond in a similar way to black-reared fish. The MCH receptors on these cells may become less sensitive to MCH, the receptor numbers may decline as the concentration of MCH increases or the receptors may fail to develop. Down regulation of receptor number is well established for peptide hormones (Archer *et al.*, 1994). Significantly higher levels of bioactive CRF are released from the hypothalami of white-reared fish when endogenous MCH is removed by immunoabsorbtion (Green *et al.*, 1991). Since these fish contain more releasable CRF than black-reared fish they may become more responsive to stress once MCH inhibition is removed.

Although plasma MCH titres were not measured in these experiments, it has already been established that fish adapted to white tanks have a five fold greater plasma MCH titre than fish adapted to black-tanks (Kishida *et al.*, 1989). It is perhaps surprising that plasma MCH titres in white- and black-reared fish are very similar to those found in adapted fish (Kishida *et al.*, 1989; Green & Baker, 1991; Green *et al.*, 1991). The hypothalamic content of MCH however, is 10 fold higher in white-reared than in white-adapted fish, and 10 fold lower in black-reared compared with black-adapted fish (Baker, 1991). Similar differences in the MCH content of the pituitary gland of reared and adapted fish is also evident (Baker,1991).

Although any stress that threatens homeostasis will stimulate a rapid secretion of glucocorticoids, the results of experiments that use the rise in plasma cortisol as a measure of stress must be interpreted with

caution. In general, the magnitude and extent of corticosteroid secretion usually reflects the severity and duration of the stressor (Barton *et al.*, 1980; Pickering and Pottinger, 1989; Pickering, 1989). Low plasma cortisol titres however, may not necessarily mean the absence of stress, certain toxicants and parasitic infections fail to elicit an increase in corticosteroid levels (Schreck and Lorz, 1978; Laidley *et al.*, 1988). Low doses of anaesthetic can cause increases in cortisol titres to the level found in acutely stressed fish, but when higher doses are given that result in rapid immobilisation, corticosteroid titres remain low (Strange and Schreck, 1978).

An increase in the number of lymphocytes in the kidney and spleen was noted as the degree of stress increased from low to moderate (Table 7.2). This increase in organ lymphocyte numbers and concomitant fall in circulating lymphocyte titres may be due to the effect of stress. In mammals, stress causes the redistribution of lymphocytes from the blood into the bone marrow and to a lesser degree into the spleen (Fauci and Dale, 1975a; b). In fish the interrenal gland is a homologue of the bone marrow, so the increase in the number of spleen and kidney lymphocytes following stress could represent a comparable redistribution of lymphocytes from the circulation.

In all experiments, plasma cortisol titres were in the range of 1 to 30ng ml^{-1} . These values fall well within the range reported for Rainbow trout in the literature (Barton *et al.*, 1980; Barton and Peter, 1982; Pickering and Pottinger, 1989). The upper end of this range scale is indicative of mild chronic stress (Pickering and Pottinger, 1989).

TABLE 7.1. A summary of the results of the experiments on black- and white-adapted fish highlighting the predominant trends. Bold arrows indicate significant ($p \leq 0.05$) increases or decreases, and small arrows statistically non significant trends in plasma cortisol titres, leucocyte numbers and the results for immunological assays and radioisotope incorporation. A dash indicates experiments where no clear differences were apparent. The relevant experiment numbers are shown as superscripts.

ADAPTED FISH			
	Black compared to White	Result of Stress	Result of SRBC immunisation
Plasma cortisol	↑2 ↑1,2,4	↑3 ↑1	
Leucocytes	↓2,4 ↓1	↓1	↑2,3,4
Lymphocytes			
Blood	↓1,2,4		↑2,3,4
Spleen	↓2,4	↓1	↑3 ↑2 —4
Kidney	↓2 ↓4	—1	↑3,4
Haemolytic plaques			
Spleen	↓4		↑3,4
Kidney	↓4		↑3,4
Haemagglutination assay	—4		↑3,4
Radioisotope incorporation			
Thymidine Spleen	↓6		
Leucine	↓6		
Thymidine	↓6		
Kidney			
Leucine	↓6		

TABLE 7.2. A summary of the results of the experiments on black- and white-reared fish highlighting the predominant trends. Bold arrows indicate significant ($p \leq 0.05$) increases or decreases, and small arrows statistically non significant trends in plasma cortisol titres, leucocyte numbers and the results for immunological assays and radioisotope incorporation. A dash indicates experiments where no clear differences were apparent. The relevant experiment numbers are shown as superscripts.

	REARED FISH		
	Black compared to White	Result of stress	Result of SRBC immunisation
Plasma cortisol	↓5 ↓7,8	↑7,8	↑5
Leucocytes	↑5 ↑7 —8	—7,8	↑5
Lymphocytes			
Blood	↑7 —5,8	↓7,8	↑5
Spleen	↑7 —5,8	↑7,8	↑5
Kidney	↑7 —5,8	↑7,8	—5
Haemolytic plaques			
Spleen	↑5		↑5
Kidney	↑5		↑5
Haemagglutination assay	—5		↑5
Radioisotope Incorporation			
Thymidine Spleen	↑7,8		
Leucine Spleen	↑7,8		
Thymidine Kidney	↑8 —7		
Leucine Kidney	↑7 ↑8		

In Experiment 2, cortisol titres were low for all fish except the black-adapted saline-injected group, these being the last to be sampled. It is likely that the removal of the other fish had disturbed those that remained, sufficiently to raise their cortisol levels. Pickering and co-workers (1982) reported that the sampling order was correlated with increases in plasma cortisol in Brown trout. It is worth noting that when working with fish it is very difficult to control and standardise procedures because even the mildest disturbance can greatly increase cortisol titres. Uncontrollable disturbances such as fire alarms, noisy cooling systems *etc.* can also upset the fish and interfere with any differences between experimental groups.

Another factor to consider is the diurnal variation in plasma cortisol that has been reported for several species. Rance *et al.* (1982) found the highest cortisol titres in Rainbow trout held in outdoor ponds occurred during the hours of darkness, in the summer months, with an additional second peak occurring in aquaria-held fish early in the morning. Several factors are believed to influence this second peak, including the light-dark cycle, the feeding regime and the lunar cycle. During the winter months, titres in aquaria-held fish were low throughout the 24 hour period, even when the fish were exposed to an extended light phase. Similar results have been reported for the Brown trout (Pickering and Pottinger, 1983). By contrast, Barton *et al.* (1980) failed to find any rhythm in fingerling Rainbow trout. In Experiment 5, diurnal variation may have been partly responsible for the raised cortisol titres, although since Experiments 1-3 and 7-8 were carried out during the winter months, and in Experiments 1-4 fingerling trout were used, any circadian or seasonal interference is likely to be minimal.

The position of a fish within a dominance hierarchy is another important consideration, because it can affect plasma cortisol titres as well as interrenal activity (Noakes and Leatherland, 1977), lymphocyte numbers (Peters and Schwarzer, 1985), growth (Li and Brocksen, 1977) and even lead to increased mortality (Laidley and Leatherland, 1988). Pottinger and Pickering (1992) have shown that the dominance hierarchy was most pronounced in fish held in pairs, its importance declining as the number of fish increased. They suggested, that in fish confined in groups of ten, social interaction was not an important consideration. In our experiments, the minimum number of fish per group was seven and therefore only minimal disturbance can be attributed to social dominance in these experiments.

Finally, the stress response of fish is also determined by genetic factors, with differences both within and between species (Gjedrem and Aulstad, 1974; Refstie,1986; Fevolden *et al.*, 1991). Rainbow trout are relatively insensitive to stress when compared to Brown trout (Pickering *et al.*, 1989) and Atlantic salmon (Fevolden *et al.*, 1991).

7.2. Blood cell numbers

The interpretation of the data for blood cell densities is difficult, because these values can be affected by changes in blood cell volume that follow stress (Soivio *et al.*, 1977; Pickering *et al.*, 1982). Wedemeyer (1970) has reported an altered osmotic balance post stress which changes the blood cell volume and causes spleen contraction that releases additional RBCs into the circulation. This may explain the

increase in the number of erythrocytes and leucocytes observed as the adaptation period increased (Experiment 1). Other factors, such as testosterone in mature males (Slicher, 1958; Mirand *et al.*, 1965) and seasonal changes (Lehman *et al.*, 1976) have been claimed to stimulate erythropoesis.

The raised leucocyte counts observed in Experiment 1 may be due to blood volume changes, or neutrophilia resulting from infection and/or stress. Lymphocytopenia and simultaneous neutrophilia following stress have been demonstrated by Johensson-Sjobeck *et al.*, 1978 and Ellsaesser and Clem, 1986. Other workers have failed to demonstrate any effect of acute or chronic administration of cortisol on neutrophils in Brown or Rainbow trout (Pickering *et al.*, 1982; Pickering, 1984). Neutrophilia usually indicates infection, (Watson *et al.*, 1956; Manhjan *et al.*, 1979; Pickering, 1986), although neutropenia has been reported following bacterial infections (Amend and Smith, 1975; Lester and Budd, 1979). In Experiment 1, the ten fold difference in the total leucocyte count seen between fish adapted for 10 and 24 days is not reflected by a similar increase in the number of lymphocytes. This suggests that the differences seen in the leucocyte counts are due to volume changes or changes in another subset of the leucocyte population. The results imply that counting blood smears and assessing the number of each cell type per 1000 cells, gives a more accurate picture than leucocyte counts alone, although the procedure is very time consuming.

7.3. Stress and the response to immunisation

It has been well established for over forty years that stress and cortisol inhibit the immune response (Weinreb, 1958). The leucocyte counts of black-adapted fish were depressed compared with those of white-adapted fish (Table 7.1). The lymphocyte counts per 1000 cells were also significantly depressed in black-adapted fish. Both these parameters vary inversely with the plasma cortisol concentrations and this suggests that MCH probably affects the leucocyte population indirectly, by lowering plasma cortisol concentrations in white-adapted fish, although a direct affect of MCH on the immune system cannot be ruled out (see Section 7.6). These results, and those of others, show that raised plasma cortisol concentrations, due to stress and/or administered glucocorticoids, are associated with an reduction in the number of circulating WBCs (McLeay, 1975; Pickering *et al.*, 1982; Pickering, 1984; Ellsaesser and Clem, 1986; Pickering and Pottinger, 1987a; b; Wiik *et al.*, 1989). Specifically, it is the change in the number of lymphocytes that affects the overall leucocyte count (Weinreb, 1958; Pickering, 1986; Pickering and Pottinger, 1987a). The number of thrombocytes and neutrophils showed no clear correlation to cortisol and this data has not been included in the results. Pickering and coworkers (1982; Pickering, 1984) have also reported that cortisol does not affect the levels of circulating thrombocytes or neutrophils. Fish reared on black backgrounds had greater or equal numbers of leucocytes and lymphocytes than their white-reared counterparts (Table 7.2), a finding that may be correlated with the similar cortisol levels in these reared fish. In Experiments 7 and 8, a marked increase in thrombocyte numbers was recorded in fish exposed to moderate but not

to low stress. This observation may be due to thrombocyte clustering, a phenomenon seen by other workers in stressed and in glucocorticoid-treated fish (Wiik *et al.*, 1989).

Immunisation with SRBCs caused a significant increase in the number of circulating lymphocytes compared to saline and uninjected controls (Table 7.1). The magnitude of this response was related to the original number of lymphocytes, i.e. black-adapted fish showed a smaller increase in lymphocyte numbers than white-adapted fish, even after immunisation, and the maximum immune response always occurred in white-adapted fish. These findings are in keeping with previous work on fish, which has shown that immunosuppression reduces the increase in the number of lymphocytes that follows immunisation (Anderson *et al.*, 1982).

In reared fish, a similar pattern of stimulation was seen, immunisation causing a significant increase in the numbers of circulating lymphocytes. Once again, this increase was proportional to the number of lymphocytes in the controls. It is noteworthy that irrespective of the adaptation or rearing conditions, the response to SRBC immunisation was always stimulatory.

The density of spleen lymphocytes was significantly lower in black compared to white-adapted fish (Table 7.1), presumably because of the lower plasma cortisol titres in the latter group. A similar trend was evident for the kidney, with lower lymphocyte populations in the pronephros of black- compared to white-adapted fish. Chilmonczyk (1982) has previously shown that corticosteroid treatment reduces the lymphocyte populations in the spleen, kidney and thymus.

These differences in lymphocyte numbers in the spleen and kidney of black- and white-adapted fish persist even when fish are immunised. Antigenic challenge induces cell mitosis and increases the number of lymphocytes in both organs (Du Pasquier, 1976; Etlinger *et al.*, 1978). In reared fish, there was a significant increase in the number of spleen lymphocytes, but no obvious difference in the kidney following immunisation. Ingram and Alexander's (1981), data on organ to body weight ratios in Brown trout, revealed an increase in the spleen /body weight ratio after SRBC injection, and a slight but insignificant decrease in the kidney /body weight ratio. The difficulty of extracting the whole interrenal gland in comparison to the relative ease of removing the discreet structure of the spleen may explain the lack of response recorded for the kidney of reared fish. It is also possible that since interrenal tissue is a major site of steroid synthesis, the local levels of corticosteroids in this organ may limit the antigenic response of kidney lymphocytes. The great variation in the number of lymphocytes in the blood and lymphoid organs reported here, has also been described by others (Chilmonczky, 1982; Ingram and Alexander, 1981). The cause of the differences in lymphocyte number between fish are still unclear, but genetic, physiological and immunological parameters must play an important role.

In addition to changes in cell numbers, antibody responses depended on a fish's hormonal balance. Immunisation with SRBCs always produced an increase in the number of plaque forming cells, irrespective of the nature of the background, and this increase was most marked in white-adapted fish.

In general, fish that were more sensitive to stress, *i.e.* black-adapted or white-reared, produced lower plaque numbers. Others have shown that various types of stress, for example, toxic chemicals such as phenol (Gonchoarov and Mikryakov, 1970), heavy metals such as zinc, copper and mercury (Sarot and Perlmutter, 1976) and cortisol (Tripp *et al.*, 1987) can inhibit the production of antibodies. Miller and Tripp (1982) found that increasing periods of captivity could also inhibit immune responses.

In the present experiments, plaque numbers were always counted after 24 hours incubation. Anderson *et al.*, (1979) found that further plaques develop if counts are carried out a day later, as cells secreting small amounts of antibodies, only produce visible plaques after an extended period .

Although it is surprising that saline-injected fish produced some plaques, this is unlikely to be due to contamination of the syringe with antigenic material. Other authors have reported the production of plaques in saline-injected trout (Ingram and Alexander, 1981), especially by spleen rather than kidney lymphocytes (Chiller *et al.*, 1969b; Anderson *et al.*,1979). No explanation as to the cause of non-specific plaque formation has been proposed although they may be heterophilic antibodies against microbial antigens

In immunised fish, the number of plaques produced was higher for spleen than for kidney lymphocytes, an observation that has reported for other poikilotherms (Chiller *et al.*, 1969b; Ambrosius and Hanstein, 1971). Cortisol production by the interrenal gland *in vivo* may be responsible for the decrease in the antibody production of lymphocytes

in vitro. The fact that glucocorticoids are known to reduce the proliferative response of lymphocytes to antigens supports this hypothesis (Cupps and Fauci, 1982). The quality of plaque forming cells is also known to vary with age and season (Pontius and Ambrosius, 1972).

The occurrence of small and large diameter plaques has been reported for fish (Chiller *et al.*, 1969a; Sailendri and Muthukkaruppan, 1975) and other vertebrates (Jerne and Nordin, 1963). This phenomenon may be due to the presence of two distinct populations of cells with different antibody producing properties (Chiller *et al.*, 1969a), or to the pouring temperature of the overlaying medium (Ingram and Alexander, 1981). In Experiment 5, using reared fish, only small plaques were recorded and the latter explanation for the lack of large plaques in this experiment, seems the more likely explanation.

The haemagglutination assays showed that immunisation with SRBCs increases the antibody titres in the circulation 1.5 to 2.5 fold, but no significant differences emerged for fish adapted or reared on black or white backgrounds (Table 7.1 and 7.2). The haemagglutination score tended to vary inversely with the plasma cortisol level, the higher the cortisol titre the lower the antibody titre. In these experiments a series of doubling dilution's were carried out, with the failure of SRBCs to agglutinate being used as the end point of the assay. A finer series of graded dilutions may have revealed small differences in plasma antibody titres between groups of fish.

It is worth noting that mammalian lymphocytes are capable of secreting immunoreactive ACTH (Smith and Blalock, 1981). Mitogenic

stimulation of human T cells produces raised levels of POMC mRNA, by causing rapid transcription of the POMC gene. Farrar *et al.*,1987 found that lymphocytes activated by PHA lectin released IL-2 that could also modulate POMC expression in mammalian cell lines. Thus, at least in mammals, lymphocytes exert an effect on the HPA axis, but whether this is true for fish is unknown.

7.4. Factors affecting lymphocyte response *in vitro*

Spleen and kidney lymphocytes taken from white-adapted fish always responded better in culture than lymphocytes from black-adapted fish. How could the previous exposure of fish to different backgrounds influence lymphocyte proliferation *in vitro* ? One possible explanation is that *in vivo*, black-adapted fish are exposed to greater levels of plasma cortisol than white-adapted. Manser (1992) reported that lymphocytes cultured from stressed mammals multiply at a lower rate than those from unstressed animals. Ellsaesser and Clem (1986) also found that lymphocytes from fish exposed to transport stress had dramatically reduced responses to Concanavalin A.

In contrast to the results for adapted fish, lymphocytes isolated and cultured from black-reared fish exposed, either to moderate or low stress, generally responded equally well, or better than white-reared. One possible explanation for this reversal may be related to the environment to which the lymphocytes have been previously exposed. Thus in white-reared fish, CRF cells become desensitised to the inhibitory influence of MCH which no longer effectively modulates the release of corticosteroids. In consequence, the lymphocytes from black-

and white-reared fish are exposed to similar levels of circulating corticosteroids and respond in a similar manner when cultured *in vitro*.

In some experiments there was considerable variation in the proliferative response to Con A of individual fish that resulted in large standard errors associated with the mean incorporation values. Other workers have reported significant variation in mitogen-induced stimulation of individual fish (Tillitt *et al.*, 1988; Ellsaesser and Clem, 1986).

The mitogens used here to stimulate lymphocyte growth in culture are those originally selected for mammalian cells and it is worth reviewing the evidence that fish lymphocytes respond to mammalian mitogens in the same way. In the *in vitro* experiments described in Chapter 6, kidney lymphocytes responded well to the T-cell mitogen Con A. This agrees with work on Rainbow trout by Warr and Simon (1983) who found that lymphocytes from the kidney, thymus, spleen and peripheral blood were responsive to both LPS and Con A. Cuchens and Clem (1977) found similar results with bluegill (*Lepomis macrochirus*). On the other hand, Etlinger *et al.* (1976) found Rainbow trout kidney cells unresponsive to Con A and thymus cells unresponsive to LPS, although lymphocytes from the spleen and blood responded to both mitogens. Different responses to the same mitogen can be due to many factors, including the serum source and concentration of the mitogen which are critical factors in tissue culture. It remains unresolved just how specific these mitogens are in stimulating specific fish cell populations. Even in mammalian lymphocyte culture responses are not always straight forward, a report by Raffel and Sell (1981) suggested that in rabbits, Ig-

positive lymphocyte cells (presumptive B cells) can also respond to the T cell mitogen Con A.

7.5. Corticosteroids and cell proliferation *in vitro.*

The results of the *in vitro* experiments demonstrate the powerful inhibitory actions of cortisol on lymphocyte proliferation in culture that overrides any effect that might be due to background colour. Grimm (1985) has also shown that cortisol suppresses division of mitogen-stimulated lymphocytes in plaice *(Pleuonectes platessa L.)*. Similar responses have been observed in mammals. For example, cortisol greatly reduces the response of mammalian splenocytes stimulated with T-cell mitogens (Westly and Kelley, 1984; Okimura *et al.,* 1986a).

The inhibitory effects of corticosteroids on fish lymphocyte growth are not clearly understood. In this context, a brief review of the events occurring in mammalian systems may be helpful. In mammals, glucocorticoids cause a generalised suppression of cellular metabolism, but more specifically act by inhibiting the mitogen-induced production of the T-cell growth factor IL-2 (Munck *et al.,* 1984). In mammals, the stimulatory properties of lectins and antigens are due to their ability to cause lymphocytes to secrete IL-2. Growth *in vitro* is dependent on the continued production of IL-2 and by inhibiting IL-2, glucocorticoids are capable of completely preventing the clonal expansion of activated lymphocytes (Gillis *et al.,* 1979b). The fact that these effects can be reversed with the addition of IL-2 suggests that corticosteroids do not directly interfere with the action of IL-2 (Gillis *et al,* 1979a; b).

The ability of fish to secrete and respond to interleukin-like substances has been widely reported (Caspi and Avtalion, 1984; Grondel and Harmsen, 1984; 1985; Sigel *et al.*, 1986 and Kaattari and Tripp, 1987; Tripp *et al.*, 1987). Caspi and Avtalion, (1984) reported an IL-2 like substance in carp that promoted T-cell like proliferation. There is also evidence to suggest that cortisol may inhibit IL-like factors necessary for the differentiation of B-like lymphocytes in salmonids (Kattaari and Tripp, 1987).

Glucocorticoids have been reported to cause a reduction in glucocorticoid receptor numbers on mammalian lymphocytes (Crabtree *et al.*, 1980; Shipman *et al.*, 1983), taking approximately three weeks to recover to pre-treatment levels (Shipman *et al.*, 1983). Glucocorticoid receptors have been identified on fish lymphocytes from the spleen and head kidney (Maule and Schreck, 1991). The fact that lymphocytes from white-adapted fish show greater suppression by cortisol than black-adapted fish (see Figure 6.8) can be explained by the differences in sensitivity to glucocorticoids. Since black-adapted fish are exposed to higher cortisol titres than white-adapted, down regulation in the receptor affinity in black-adapted lymphocytes would explain their reduced mitogenic response. Maule and Schreck (1991) reported that chronic stress causes an increase in receptor number but a decrease in the affinity of the receptor.

Another factor to consider when examining the results are the relevance of the *in vitro* model to the *in vivo* system. Some cells appear corticosteroid resistant in the whole animal but become sensitive when grown in culture (Cohen, 1989).

7.6. MCH and the immune system.

Perhaps the most exciting result to emerge from this work is that MCH enhances the division of both spleen and kidney lymphocytes when added to the culture medium *in vitro* (see Figures 6.9 & 6.16). Concentrations between 10pg ml^{-1} or 100pg ml^{-1} MCH were the most effective, especially when LPS was used. These two concentrations are very similar to the plasma levels of MCH in black- and white-adapted fish respectively (Kishida *et al.*, 1989) and suggest that MCH may regulate lymphocyte proliferation *in vivo*. An important point overlooked in this thesis, is the possibility that MCH could stimulate T and B cell prolifferation in the absence of other mitogenic agents. It seems likely, but not proven that MCH could have direct stimulatory effect on cultured lymphocytes and have a similar action in intact fish.

Although 10-100pg ml^{-1} MCH is within the normal range for plasma titres, the situation *in vivo* is complicated by the fact that local concentrations of MCH could be considerably higher. Although maximum enhancement occurred at a dose of 10pg ml^{-1} *in vitro* this does not mean a direct correlation can be drawn between *in vivo* and *in vitro* results. In this context, the fact that mRNA for MCH has been detected in the mammalian spleen, may indicate that paracrine release of MCH by spleen cells could influence lymphocyte function (Nahon *et al.*, 1993).

The interaction of other hormones and neurotransmitters which potentiate the effects of MCH on lymphocytes may also be important *in vivo*. For example, noradrenalin is known to potentiate the action of MCH on melanophores (Baker *et al.*, 1986).

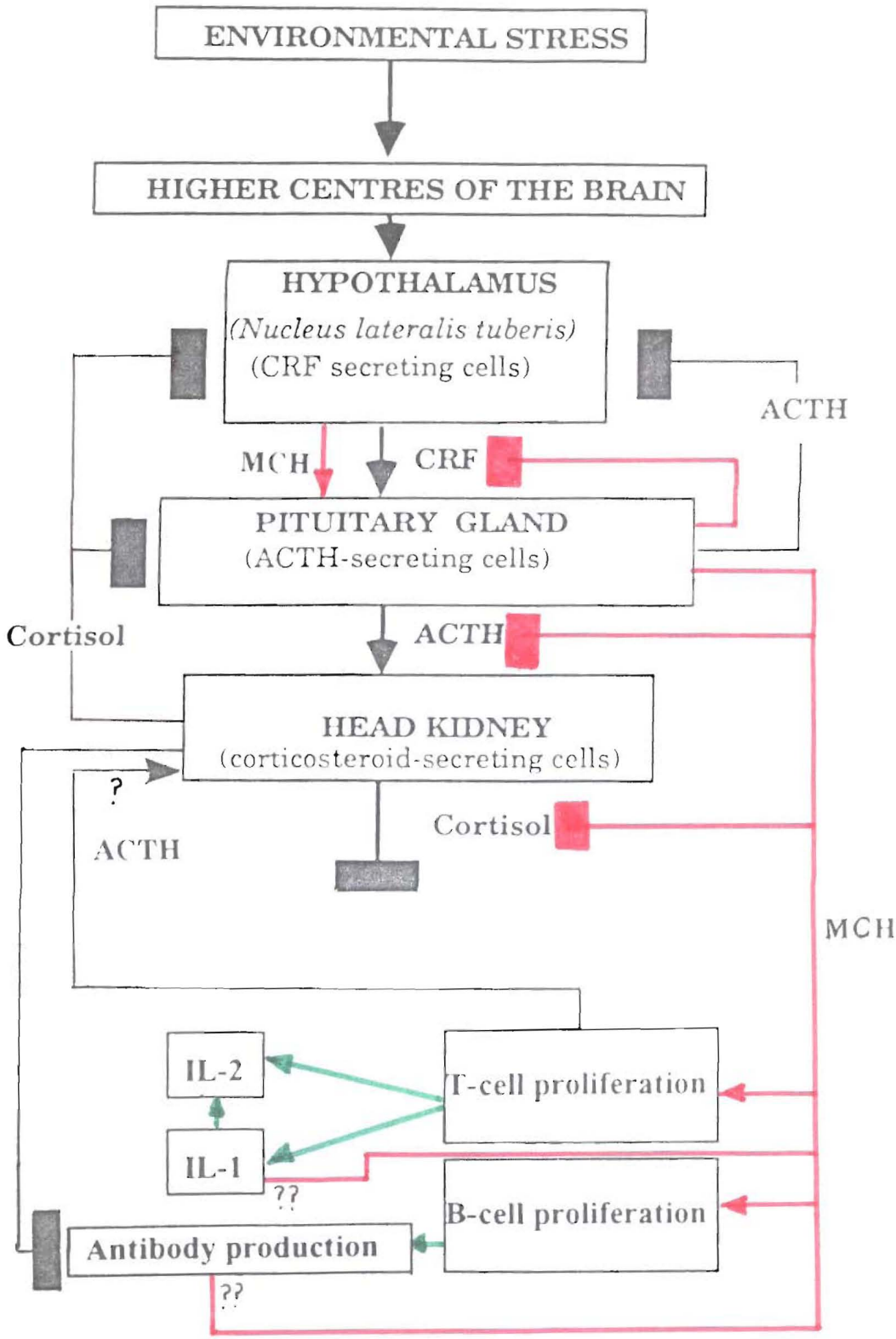
One interaction investigated here was the effect of adding both MCH and cortisol to lymphocyte cultures. The inhibitory effect of cortisol on cell proliferation is still evident, but less marked, in cultures to which MCH has been added. These findings emphasise the fact that, within the normal physiological range of plasma titres of MCH in black- and white-adapted fish, this neuropeptide modulates the primary effects of corticosteroids, but cannot override them.

Thus, it appears that as well as reducing plasma cortisol levels by inhibiting the release of CRF and possibly ACTH, MCH also has a direct action on lymphocyte susceptibility to corticosteroids and may therefore protect the fish from the damaging effects of long term corticosteroid immunosuppression.

The findings *in vitro* that MCH has a positive effect on lymphocyte proliferation can now be considered in relation to the *in vivo* experiments using black- and white-adapted. The plasma MCH levels of black-adapted fish are five fold lower than the levels in white-adapted fish (Kishida *et al.,* 1989), thus part of the improved immune status seen in white-adapted fish may be due to the stimulatory effect of MCH on T and/or B lymphocytes.

Furthermore, in white-adapted fish, MCH is blocking the secretion of CRF and possibly ACTH thus reducing the secretion of cortisol. Overall, these combined actions offer fish with raised titres of plasma MCH a distinct advantage over their counterparts adapted to black tanks. Figure 7.1. summarises the possible effects of MCH on the HPI and immune system in the light of these experiments.

FIGURE 7.1 A development of Figures 1.1 and 2.4 showing additional mechanisms of MCH action on the immune system, based on the results of the present work. Other regulatory pathways for future investigation are also shown (??).


ENVIRONMENTAL STRESS
HIGHER CENTRES OF THE BRAIN
HYPOTHALAMUS
(Nucleus lateralis tuberis)
(CRF secreting cells)
ACTH
MCH
CRF
PITUITARY GLAND
(ACTH-secreting cells)
Cortisol
ACTH
HEAD KIDNEY
(corticosteroid-secreting cells)
?
ACTH
Cortisol
MCH
IL-2
T-cell proliferation
IL-1
??
B-cell proliferation
Antibody production
??

7.7. MSH and the immune system

In many biological systems, the actions of MSH and MCH are antagonistic. As well as their opposing effects on colour change in fish (Baker and Ball, 1975), they have also been shown to have opposing actions in rats, affecting grooming behaviour (Eberle*et al.*, 1989), the response to auditory stimulation (Miller *et al.*, 1993) and passive avoidance (McBride *et al.*, 1994). In view of these antagonistic actions, it was surprising to find that when MSH was added to lymphocyte cultures at concentrations comparable to those levels found circulating in the blood of black-adapted trout, no clear effect on cortisol inhibition of lymphocyte growth was observed. The lack of response to MSH could be due to a lack of MSH receptors on lymphocytes. Cannon and co-workers (1986) also found that MSH had no affect on mitogen or IL-2 stimulated mammalian T cell lines. However they did find that murine T-cell proliferation *in vitro* induced with IL-1 was inhibited, in a dose dependent manner, by the addition of aMSH, and thus implied that aMSH interferes with the binding of IL-1 with its target receptors. By inhibiting the release of IL-1, aMSH could also inhibit the release of IL-2 and thereby prevent T and B cell clonal proliferation *in vivo* . Alpha MSH also prevents the stimulatory effects of IL-1, on corticotrophin-releasing hormone (CRH) in rats (Calogero *et al.*, 1988).

7.8. Reproductive steroids and lymphocyte proliferation *in vitro*

The link between reproductive and immune functions has been known for many years in vertebrates, but most investigations have centred on the overall effects *in vivo*. The possibility that steroid hormones, other than the corticosteroids, could influence the immune system has recently received renewed attention (Slater and Schreck, 1993).

The immune deficiencies known to occur in sexually mature salmonids include lowered bacteriacidal actions of normal serum (Iida *et al.*, 1989), increased bacterial and fungal infections (Pickering and Duston, 1983), and a reduced ability to produce antibodies (Ridgeway, 1962). Sexually mature trout have high levels of many steroid hormones including ketotestosterone, 17β–oestradiol and 17α hydroxy 20β dihydroxyprogesterone. It is well known that cortisol inhibits the immune system, however the effects of the other steroids have not been fully investigated in fish. Preliminary results in Section 6.10 suggest that these reproductive steroids do indeed have marked effects on lymphocyte growth, and these can be either stimulatory or inhibitory.

7.8.1. Androgens

In the plasma of mature male Rainbow trout ketotestosterone is the major androgenic steroid present which reaches a maximum concentration of approximately 250ng ml^{-1} in January-February (Scott *et al.*, 1980b; Matty, 1985). Ketotestosterone is also present in female fish, but at much lower titres (~10ng ml^{-1}). In other teleosts, such as plaice, ketotestosterone is unimportant and testosterone is the major

androgen (Matty, 1985). The effect of ketotestosterone on lymphocyte growth in culture is best interpreted in the light of what is known about the immune status and androgen levels in mature fish (see Figure 7.2).

Sexual maturation is reached in October-November and is marked by a decrease in the number of circulating lymphocytes. This decline coincides with a increase in circulating cortisol and testosterone titres. By February, towards the end of the spawning season, the cortisol concentrations have returned to basal levels, but lymphocyte numbers are still lower than in prespawning fish (Pickering, 1986, Pickering and Pottinger, 1987a), and depression of the lymphocyte population continues until March. Since ketotestosterone reaches its maximum levels in January-February, the fact that ketotestosterone inhibited lymphocyte growth in culture suggests that it may contribute to the continued leucopenia seen *in vivo*. Other factors such as water temperature have also been implicated as a cause of the continued reduction in the lymphocyte population (Pickering, 1986).

Adrenostenedione has been identified in the plasma of salmonids, reaching its maximum concentrations at the time of full maturation during October-November (Matty, 1985). This hormone is believed to play a role in spermatogenesis. Its effect on lymphocyte growth *in vitro* were conflicting, causing stimulation of kidney but inhibition of spleen lymphocyte growth.

The complex action of adrenostenedione was also observed for some other steroids investigated and may indicate that the closely related compounds can interact with more than one type of receptor and therefore exhibit mixed properties.

FIGURE 7.2. Seasonal changes in (a) plasma lymphocyte numbers and (b) major steroid hormones in mature male Rainbow trout. Shaded region indicates the period of spawning. (After Scott *et al.*, 1980b; Pickering, 1986 and Pickering and Pottinger, 1987a)

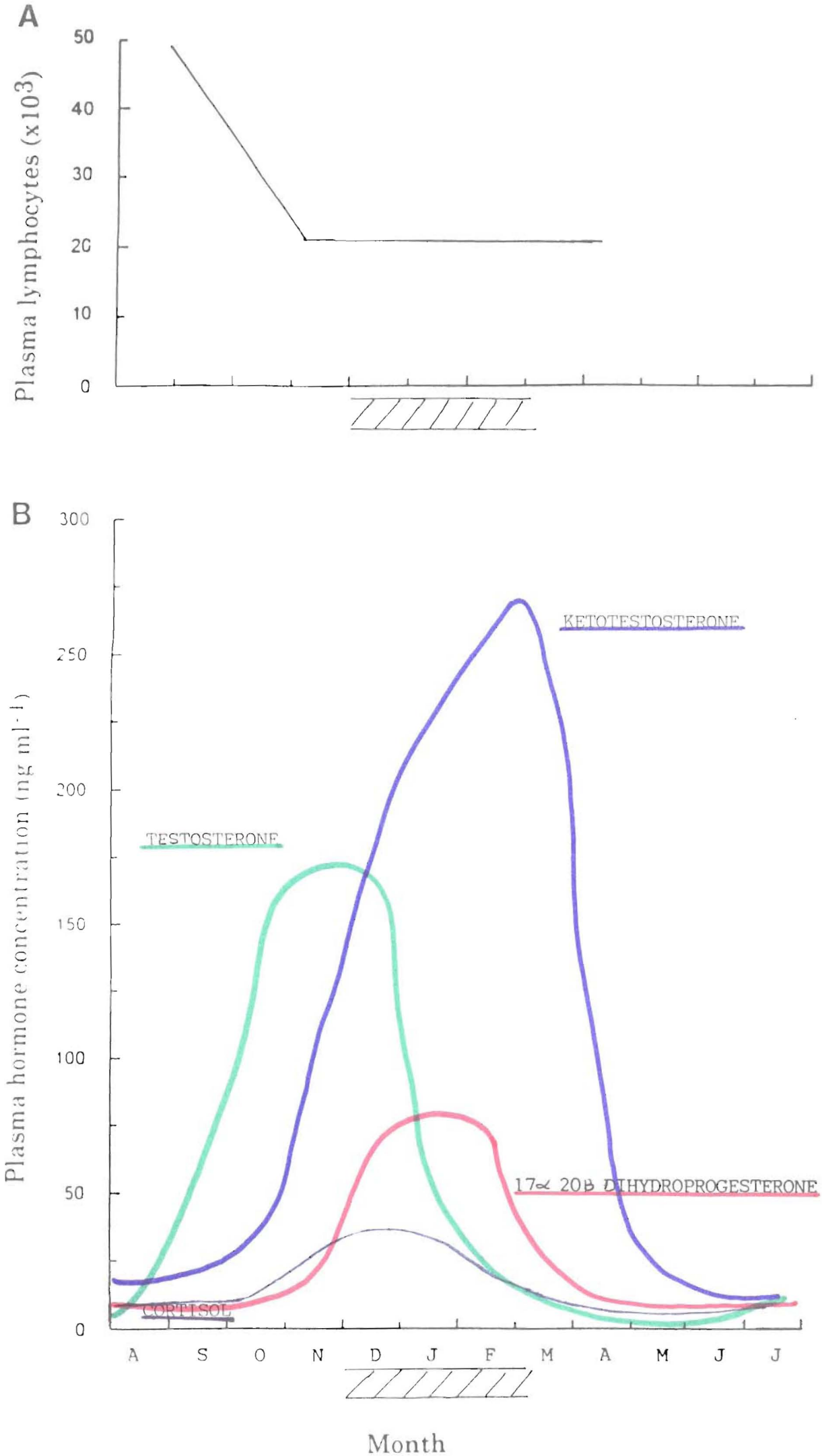
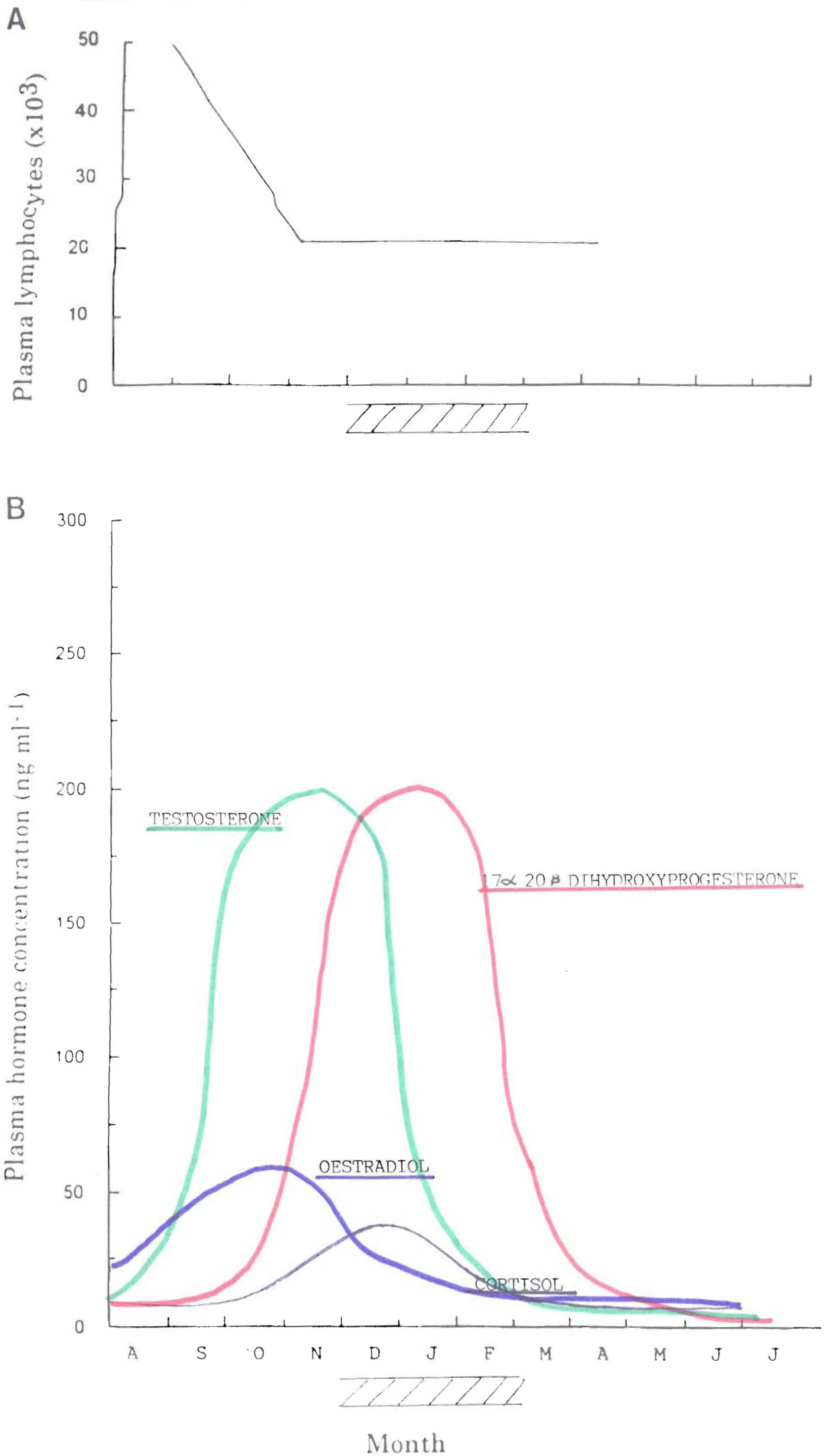

A
Plasma lymphocytes (x10³)
50
40
30
20
10
0
B
Plasma hormone concentration (ng ml⁻¹)
300
250
200
150
100
50
0
KETOTESTOSTERONE
TESTOSTERONE
17α 20β DIHYDROPROGESTERONE
CORTISOL
A
S
O
N
D
J
F
M
A
M
J
J
Month

FIGURE 7.3. Seasonal changes in (a) plasma lymphocyte numbers and (b) major steroid hormones in mature female Rainbow trout. Shaded region indicates the period of spawning. (After Scott *et al.*, 1980a; Pickering, 1980 and Pickering and Pottinger, 1987a)


A
Plasma lymphocytes (x10³)
50
40
30
20
10
0
B
Plasma hormone concentration (ng ml⁻¹)
300
250
200
150
100
50
0
TESTOSTERONE
17α 20β DIHYDROXYPROGESTERONE
OESTRADIOL
CORTISOL
A
S
O
N
D
J
F
M
A
M
J
J
Month

7.8.2. Oestrogens

Oestradiol is the major ovarian steroid present in most teleosts and is normally found in relatively low levels in the circulation rising to 50ng ml^{-1} just before ovulation in December (Scott *et al.*, 1980a; Figure 7.3), and decreasing thereafter (Fostier *et al.*, 1978). This hormone stimulated lymphocyte proliferation *in vitro* (see Figure 6.14). It is possible that the relatively high doses applied in this study have produced pharmacological rather than physiological effects, nevertheless, at doses below 50ng ml^{-1} oestradiol was consistently stimulatory.

In female fish, the oestradiol peak occurs before the peak in cortisol and testosterone (see Figure 7.3). The immune system is affected with leucopenia starting in September and continuing into November, although lymphocyte levels still remain depressed until March. Since cortisol and testosterone titres peak around November, the point of maximum leucocyte inhibition, the raised oestradiol levels in females may provide some protection to the immune system prior to ovulation.

Although oestradiol may stimulate lymphocyte growth it apparently has no significant effect on antibody production in Chinook salmon (Slater and Schreck, 1993). This differs from the situation in mammals, where it has been shown to enhance antibody responses in humans (Trawick and Bahr, 1986) .

7.8.3. Progestagens

In Rainbow trout, 17α hydroxyprogesterone and 17 α hydroxy 20β dihydroxyprogesterone, rather than progesterone, are the active agents of oocyte maturation and reach their highest plasma levels during the spawning period (Campbell *et al.*,1980; Figure 7.3). These two hormones probably have a synergistic effect *in vivo* (Billiard, 1978).

17 α hydroxy 20β dihydroxyprogesterone

17 α hydroxy 20β dihydroxyprogesterone is found in both male and female trout, responsible for spermiation in the former and oocyte maturation in the latter and achieves its highest plasma titres during the spawning season. In this investigation the same pattern of response was shown by cultured spleen and kidney lymphocytes. At low concentrations, comparable to the plasma levels in male trout, (less than 30ng ml^{-1}) the hormone inhibits lymphocyte growth. At high concentrations, (100-300ng ml^{-1}) it stimulates lymphocyte growth especially in kidney cell cultures (Figure 6.14). Since in the male trout levels of 17 α hydroxy 20β dihydroxyprogesterone do not exceed 50ng ml^{-1} it appears this dose would have a mild inhibitory action *in vivo* (Scott and Lilley, 1994). In female trout, where 17 α hydroxy 20β dihydroxyprogesterone titre peaks at approximately 300ng ml^{-1} just prior to ovulation (Scott *et al.*, 1983), the hormone probably acts as a mild lymphocyte stimulant and may counteract the opposing immunosuppressive actions of cortisol and testosterone.

17α Hydroxyprogesterone

The levels of 17α hydroxyprogesterone in the plasma of mature female fish rise slowly and are maintained at about 100ng ml^{-1} during the period of ovulation (Scott *et al.,* 1983). The results shown in Figure 6.14 demonstrate that this steroid is inhibitory to both spleen and kidney lymphocyte proliferation *in vitro*. Thus, during ovulation the titres of this hormone are raised and may partially contribute to the leucopenia observed during the spawning season.

In summary, the results of the work with reproductive steroids have shown that the actions of the major repoductive steroids on lymphocyte growth are quite diverse, some are predominantly stimulatory (oestradiol) or inhibitory (ketotestosterone), others have mixed functions (17 α hydroxy 20β dihydroxyprogesterone).

The interpretation of the results is difficult because the effect of the steroids *in vitro* cannot be directly compared with the complex situation occurring *in vivo*. In the intact animal, steroid hormones do not act in isolation, but are affected by the activity of gonadal, hypothalamo-pituitary and interrenal systems, circadian rhythms and temperature that all influence the immune system. However, these results are of value in helping to elucidate the complex events occurring during maturation and spawning and the role of individual reproductive hormones in the overall picture. It is also clear that the effects of repoductive hormones on the immune system differ between salmonids and other vertebrates (Slater and Schreck, 1993).

7.9. Implications for Aquaculture.

The effects of MCH demonstrated in this work could be economically important to commercial fish production. Although rearing fish on white backgrounds does not reduce the stress response or enhance the immune system, white tanks could be used following an outbreak of disease to provide an immunological boost for short periods of time (2-4 weeks) or to alleviate the harmful effects of raised plasma cortisol levels after stressful events incurred as a result of handling or transport.

MCH has been shown to modulate the HPA axis in all vertebrates and this function probably evolved before its secondary role, adopted only by teleost fish, to enable adaption to pale coloured backgrounds (Baker, 1994). It is interesting to speculate whether these two functions are linked, that is under what conditions fish would naturally benefit from supressed stress responses and enhanced immunological function. Many fish particularly salmonids have been shown to have reduced immunological functions during their reproductive season. Some of the results in this thesis suggest that raised plasma levels of reproductive steroids may be responsible. It is known that many salmonids spawn naturally on gravelly substrates in very shallow water, perhaps the light coloured backgrounds of stony streams and the high level of incident light in shallow water may stimulate increased release of MCH at a time when fish are using these habitats for spawning. Keeping brood fish on white backgrounds during their repoductive season may help protect them from the immunosuppressive actions that arise when plasma levels of reproductive steroids are raised.

8. Conclusions and Future Work

Melanin-concentrating hormone was originally discovered because of its role in the control of colour change in teleost fish. It is now apparent that variants of this neuropeptide are found in all vertebrates, from cyclostomes to mammals, including man, but it is only in teleosts that its primary function is to cause melanin granule contraction within the skin melanophores. The fact that MCH has been highly conserved throughout vertebrate evolution strongly suggests it has other more general functions. One important function to emerge concerns the ability of MCH to modulate the stress response. By affecting the release of CRF from the hypothalamus, and possibly also ACTH from the pituitary gland, MCH can depress corticosteroid levels in the blood. Other actions that are less well established, but are recieving increased attention, include its ability to antagonise the action of MSH, not only in melanophores,but also the behavoiral effects that MSH can have in mammals. Other modulatory actions on growth and osmoregulation are now beginning to be explored.

This study has demonstrated that MCH can also influence the immune system of Rainbow trout in several ways. The *in vivo* experiments have shown that moderately stressed trout show reduced immunological responses. The number of lymphocytes circulating in the blood and the amount of antibody these cells can secrete is reduced by the immuno-suppresive actions of cortisol, but these effects are significantly more marked in black-adaptedthan in white-adapted trout. It is concluded that the modulatory action of MCH on the HPA axis reduces corticosteroid titres in the blood and therefore also limits the degree of immune suppression seen in white-adapted fish.

In addition to this indirect influence of MCH on the immune system, the *in vitro* experiments have demonstrated, for the first time, that MCH can directly stimulate the growth and proliferation of T cell-like and B cell-like lymphocytes grown in culture. This action presumably helps to counteract the inhibition of immune function caused by raised glucocorticoid levels in stressed fish.

Life-long exposure to white backgrounds however, apparently offers no additional protection from the immuno-suppression by glucocorticoids, as might be expected from the enhanced production and release of MCH that occurs in white-reared fish. It seems likely that there is a subtle interplay between the regulatory mechanisms that control the immune and endocrine systems, and that homeostatic control, or specific adaptations during embryonic development, eliminate the differences between black- and white-reared trout that are apparent in adapted fish.

Apart from the effects of cortisol on lymphocyte growth, this study has shown that other steroid hormones can influence T cell-like proliferation. In general terms, androgens were found to stimulate, and oestrogens to inhibit, lymphocyte division. No clear pattern emerged for progesterones. In life, the interaction of the varying steroid hormones would have complex effects that are not easily measured in controlled experiments. Reproduction, even in non-migratory species like Rainbow trout, is one of the most stressful periods in a fish's lif cycle. The present work has demonstrated that cortisol may not be the only steroid responsible for the generalised decline in the immune systemseen

during spawning. Indeed, the stimulatory effect of some steroids may counteract the immuno-suppressive effects of others.

As in any investigation, this series of experiments has raised more questions than it has answered. Further work needs to be carried out to clarify the mechanisms of action of a number of systems:

- The use of radioisotopes to monitor growth and division of lymphocytes is a relatively crude method of assessing the immune system. Other more specific immunological assays would allow more detailed examination of the way in which MCH can affect immune responses. The synthesis of antibodies and the phagocytic activity of granulocytes are two functions that could be measured. Organ culture of lymphoid organs could also provide useful information about the action of MCH in intact tissues.

- Mammalian studies have shown that aMSH inhibits the action of interleukin-1. Since MCH is an antagonist of aMSH, it is possible that experiments designed to look at the effects of MCH on interleukin synthesis, release and action would be a profitable area of investigation.

- Although radioimmunoassays have been developed to measure MCH titres in the blood, antibodies were not available for the work described here. Specific antibodies would allow the tissue and the plasma levels of MCH to be measured under different conditions and at different stages in the life cycle of trout and the levels correlated with the immune status of the fish.

- Many of the effects discussed in this thesis are likely to involve changes in the number and/or affinity of MCH receptors on the surface of lymphocytes and othetr cell types. To date, attempts to assess MCH receptors numbers and distribution have met with little success, but improved techniques may make this possible in the future.

- Although our knowledge of peptides and hormones that are involved in fish reproduction has advanced considerably in recent years, compared with what is known about mammalian systems, the role of steroids in fish immunity is still in its infancy, and deserves further study.

Melanin -concentrating hormone is now well established as an important neuromodulatory peptide in all vertebrates, affecting a wide variety of systems in a subtle but significant ways. It is no longer viewed as a hormone of peripheral interest to fish endocrinologists concerned with skin pigmentation, but has been found to be closely linked to some of the most important systems in all vertebrates, influencing stress and immune functions that are crucial to the survival of individuals, systems that underpin the evolutionary success of the vertebrates.

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Appendix

Dacies Fluid

Formaldehyde

Formalin	10ml
Distilled Water	90ml

Mix the water and fomalin together to make a stock solution of formaldehyde.

Formaldehyde	10ml
Trisodium citrate	31.3g
Brilliant cresyl blue	1.0g
Distilled water	1 litre

Place the stain and trisodium citrate into a beaker mix in the formaldehyde and water. Filter the solution before use.

Phosphate Buffered Saline with Gelatine

Disodium hydrogen orthophosphate	~ 810ml
sodium chloride	9.0g
Thimersal	0.1g
Gelatine	1.0g
Sodium dihydrogen orthophosphate	~ 190ml

Prepare 0.1 molar solutions of disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate at pH 7.4-7.6 at 4°C. Into a beaker add the thimersal, sodium chloride and gelatine, then add appropriate amounts of sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate to maintain the pH at 7.4-7.6. Heat gently, stirring continuously, until gelatine has dissolved. When cooled to 4°C adjust pH to 7.4-7.6 with appropriate buffers.

Leishman-Giemsa Stain

Leishman stain

Leishman	0.15g
Absolute methanol	100ml

Dissolve stain in a small drop of alcohol in a pestle and mortar, pour into a bottle and add fresh alcohol to the pestle and start again. Proceed until all powder is used. Place in incubator at 37°C overnight. Place a few drops of stain on the surface of a slide dilute with water, if a metallic scum forms stain is usable.

Giemsa Stain

Stock solution

Giemsa powder	1g
Glycerin	66ml
Absolute methanol	66ml

Mix together the glycerin and Giemsa powder and then place in an oven at approximately 60°C for 1 hour. After the solution has cooled add methanol.

Phosphate buffer

Sodium dihydrogen orthophosphate	35g
Disodium hydrogen orthophosphate	4.84g
Distilled water	3960ml

Prepare a 1% solution at pH 6.0.

Working Giemsa solution

Stock Giemsa	14ml
Phosphate buffer	200ml

Prepare freshly each day.

Staining method

1 Cover dry unfixed sample with 5-6 drops of absolute methanol. Leave for 5-6 minutes.

2 Stain with Lieshmans stain for 3 minutes.

3 Stain with Giemsa stain for 9 minutes.

4 Flood off stain with phosphate buffer (0.1M; pH 6.0)and differentiate if necessary for 1-2 minutes. Sections should appear just pink.

5 Rinse well in tap water to remove residual stains.

6 Air dry overnight, keeping slides in darkness and mount

Period Acid Schiff Technique

Periodic Acid

Periodic acid	1g
Distilled water	200ml

Schiffs reagent

Basic fuchsin	1g
Distilled water	200ml
Potassium metabisulphite	2g
Concentrated hydrochloric acid	2ml
Activated charcoal	2g

Boil the distilled water remove then dissolve basic fuchsin into the water. Allow the solution to cool to 50°C, and add the potassium metabisulphite, mixing continuously. When cooled to room temperature add the hydrochloric acid and mix before adding the charcoal. Leave overnight in the dark then filter. The solution should be pale yellow, store in a dark container at 4°C

Staining method

1 Place sections in distilled water.

2 Treat with periodic acid for 5 minutes

3 Wash well in distilled water

4 Cover with schiffs reagent and leave for 15 minutes

5 Wash in running tap water for 5-15 minutes

6 Counterstain with Harris's haematoxylin 1-2 minutes

7 Differentiate with 1% acid-alcohol and blue with tap water

8 Wash in water then allow to dry then mount.

Haemotoxylin and Eosin

Harris's Haematoxylin

Haematoxylin	2.5g
Absolute alcohol	25ml
Potassium alum	50g
Distilled water	500ml
Mercuric oxide	1.25g
Glacial acetic acid	20ml

Dissolve haematoxylin in alcohol, add this to alum that has been dissolved in warm distilled water. Boil mixture, add mercuric oxide then cool rapidly. When the solution is cold add acetic acid and stain is usable.

Eosin

Eosin Y	5g
Distilled water	500ml
Thymol	one crystal

Staining method

1 Fix section in methanol

2 Wash section in water

3 Stain in Harris's haematoxylin for 10 minutes

4 Wash well in tap water until sections blue

5 Differentiate in 1% acid-alcohol 5-10 seconds

6 Wash well in water until section blues

7 Stain in 1% eosin for 10 minutes

8 Wash in running water for 10 minutes, then dry and mount.