STUDIES ON THE INTERRELATIONSHIP OF TEMPERATURE, STRESS AND IMMUNITY IN CARP (CYPRINUS CARPIO L.)

by

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THESIS SUMMARY

The University of Aston in Birmingham

STUDIES ON THE INTERRELATIONSHIP OF TEMPERATURE, STRESS AND IMMUNITY IN CARP (CYPRINUS CARPIO L.)

Roderick C. Haynes, Master of Philosophy
January 1989

The thesis examines the effect of temperature and handling stress upon in vitro and in vivo immune functions in the common carp (Cyprinus carpio L.). Fish were subjected to handling stress or to handling stress and temperature change, before monitoring the changes in plasma cortisol and glucose levels.

Plasma cortisol was found to be significantly elevated in both groups after three hours, returning to basal levels after 3 - 6 days. There was no significant difference between the two groups, indicating that temperature change played a subordinate role to the handling stress. The plasma glucose levels were also found to be elevated in both groups, with the fish subjected to handling stress alone exhibiting a greater increase. A return to basal levels occurred over a six day period.

The effects of temperature on immunity were also examined. The haemagglutinin reaction to peritoneally injected sheep erythrocytes was monitored, over a period of five weeks, at 10°C and 20°C. Peak titres were reached on day 17 post - injection at the higher temperature and on day 27 post - injection at 10°C. The levels of the peak titres were not affected by temperature.

The rate of in vitro phagocytic particle clearance was also examined at the two temperatures. Candida albicans was cleared from suspension in 48 hours at an in vitro incubation temperature of 10°C, and in 16 hours at a temperature of 20°C. The temperature at which fish were acclimated (either 10°C or 20°C) had no effect upon the rate of particle clearance.

'Arming' the fish leucocytes by administering a priming dose of Candida albicans 15 days prior to the collection of leucocytes, resulted in a faster in vitro clearance of Candida particles at both temperatures. 'Opsonisation' of Candida albicans, using the immune sera from the fish that received the priming injection, did not appear to affect the clearance rate of the Candida in vitro.

Key Words: Cyprinus carpio ; Temperature ; Stress ; Phagocytosis ; Immunity.

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The work presented in this thesis involving injection of antigens was carried out in conjuction with Dr. J.J. Rimmer.

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1. INTRODUCTION

Over the last fifteen to twenty years, interest in the field of fish diseases and immunology has increased considerably. This is attributable to a number of factors, not least of which is the increase in aquaculture facilities and the need to develop vaccines and prophylactic treatments for fish diseases. Other advantages of studying the fish immune system include the ease with which ontogenetic studies, involving free living eggs and larvae, can be carried out, and the fact, that since fish are poikilothermic, their immune system may be readily manipulated by alteration of the environmental temperature. Many aspects of fish immunity have been reviewed extensively elsewhere, see for instance, Corbel 1975; Ellis 1977a; Elliott 1981; Hart et al 1988; Ingram 1980; Rijkers 1980; Walczak 1985.

Fish exhibit many of the properties of the mammalian immune respose, although not to the same degree of complexity. Both specific and non - specific humoral and cellular mechanisms exist to deal with foreign materials, although it is generally considered that fish depend upon non - specific defences to a greater extent than do mammals.

The primary lymphoid organs in fish are the thymus, pronephros and spleen; they lack the bone marrow and secondary lymph nodes found in the higher vertebrates. Much work has been directed at studying the ontogeny of the lymphoid organs - a function of the ease with which the larval stages can be examined - and the consequent onset of immunocompetence. Lymphopoietic tissue is found in the thymus of young fish shortly after hatching; for example, 5 days post hatch in the carp at 22°C (Botham & Manning 1981). The same study revealed that the spleen was first evident in larval fish on day 5 post hatch, but it remained predominantly erythroid for several months,whereas the pronephros contained lymphocytes on day 6 post hatch.

The presence of lymphocytes, or the development of lymphopoietic tissue, in these organs does not necessarily herald immunological maturity. The study of the onset of immunological competence has also been of prime importance, not only from a comparative point of view, but also to determine the earliest time at which it is possible to vaccinate young fry. For example, van Loon et al (1981) found that 4 week old carp not only failed to respond to sheep erythrocytes, but developed tolerance to the antigen, and could not respond following re - challenge at age 4 months.

There is considerable variability between fish species with regard to the time of onset of immunological maturity, and different aspects of the immune response develop at different times. For example Botham et al (1980) has shown that allograft responsiveness is present in carp 16 days post hatch, and in trout at 14 days post hatch.

Prior to the onset of specific immunological competence, juvenile fish are thought to rely upon non - specific immune mechanisms for defence against pathogens. The first such defensive barrier encountered is the skin and it's overlying mucus layer. The epidermis itself acts as a physical barrier to invading organisms. The mucus layer however, acts not only as a physical barrier, but also contains numerous antibiotic molecules. These are known to include lysozyme (Fletcher & Grant 1968, Fletcher & White 1973), non - specific agglutinins (Di - Conza 1970) and C - reactive proteins (CRP) (Ramos & Smith 1978). The mucus layer itself is constantly being sloughed off and replaced by mucus secreting goblet cells within the epidermis - a process that makes attachment difficult for a waterborne pathogen.

A range of non - specific antibiotic molecules are also found in the serum. The factors contained in the mucus are present ; lysozyme, natural agglutinins and CRP, as well as interferon, transferrin, complement and α– and β– precipitins. Comprehensive reviews of the non - specific components of serum and mucus may be found elsewhere (Alexander 1985, Ingram 1980, El - Feki 1987).

The specific immune response in fish is broadly similar to that found in mammals, with both a cell mediated and a humoral arm.

The humoral side of the response is mounted by the B - lymphocyte, producing an immunoglobulin most similar to the IgM molecule found in mammals. Only this type of antibody is found in fish; the other classes normally found in mammals are absent. Diversity does exist between fish immunoglobulins, however, and this is related to differences in the length of the heavy chain or the degree of polymerisation of the basic four chain IgM unit. In elasmobranchs and chondrosteans, 7S and 19S immunoglobulins are found; in holosteans a 19S form is predominant, and in teleosts both 7S and 19S types occur. In teleosts, the IgM is found to be tetrameric, whereas in the chondrichthyes the molecule is pentameric. (Ambrosius et al 1982 , Jurd 1985).

The exact processes involved in antibody production in fish have yet to be clarified. It is known that, following antigen administration, antigen trapping occurs in the spleen, and, to a lesser extent, in the pronephros (Secombes & Manning 1980). Clusters of protein synthesising pyroninophilic cells are found in these organs and the mesonephros, after administration of antigen (Secombes et al 1982), and it has been hypothesised that

these may be analagous to the germinal centres found in mammals (Pollara et al 1964, Secombes et al 1982), although they are not thought to be the sole site of antibody production. The melano macrophage centres found in the lymphopoietic organs of fish have also been proposed as acting as primitive germinal centres (Ellis 1980, Ferguson 1976).

Antibody production in fish is a temperature sensitive process. In a series of experiments involving the transfer of fish from one temperature to another, Avtalion et al (1976) showed that a temperature sensitive stage occurs early in the immune response; probably in the cell - cell interaction during antigen recognition, which occurs prior to division. The ambient temperature for many fish is often below that at which a rapid antibody response can be mounted, and consequently their non - specific defense mechanisms are thought to play an important role in protection from pathogens.

The cell mediated immune response in fish is manifested in a number of ways, including the ability to reject tissue transplants from genetically dissimilar donors. The advanced bony fish all exhibit acute rejection of first set allografts and accelerated rejection of second set grafts, with an accompanying vigorous cellular immune response. This, and the fact that grafts from related individuals are rejected in chronic fashion or accepted (Nakanishi 1987), is suggestive of a major histocompatability system analagous to that described in mammals. This, however, has yet to be confirmed.

The process of graft rejection in fish - almost entirely limited to scale or skin transplants - is well documented (eg Botham et al 1980, Rijkers & van Muiswinkel 1977), and is known to be temperature dependent.

In vitro indicators of cell mediated immunity in mammals have also been demonstrated in fish. These include the migration inhibition response of macrophages, the mixed lymphocyte reaction and lymphocyte blastogenesis in response to T cell mitogens.

The migration inhibition test depends upon the production of migration inhibition factor (MIF) ; a T cell derived lymphokine that prevents the migration of macrophages in culture. This has been shown to exist in fish by a number of authors, eg McKinney et al (1976), Jayaraman et al (1979). Mixed lymphocyte reactions (MLR) have been interpreted as an in vitro analogue of the allograft response, and occur when leucocytes from two allogeneic individuals are cultured together. MLRs have been demonstrated in a number of fish species, including carp (Ellis 1977b, Grimm 1985, Caspi & Avtalion 1985).

The use of blastogenesis as an indicator of T cell reactivity relies upon the fact that certain mitogens only stimulate T cell populations. In mammals, phytohaemagglutinin (PHA) and concanavalin A (con A) are known T cell mitogens. In fish, these mitogens have been shown to cause blastogenesis in a number of species, including trout and carp (Blaxhall 1985, Liewes & van Dam 1982).

The phagocytic system in fish has been well studied both in vivo and in vitro. Various types of cells have been described as being phagocytic, including blood macrophages / monocytes and neutrophils. However, considerable discrepancies exist within the literature. In the plaice, Pleuronectes platessa, Ellis et al (1976) observed the phagocytosis of carbon particles by blood monocytes, but found that neutrophils did not take up

carbon. MacArthur et al (1984) found that neutrophils in plaice kidney were phagocytic for carbon, glycogen and Vibrio sp.

In carp, both the monocyte / macrophage series and neutrophilic granulocytes have been reported to be phagocytic. Temmink & Bayne (1987) found that these cell types engulfed Bacillus megaterium organisms by endocytosis. They also observed basophilic and eosinophilic granulocytes "wrapping around" bacteria cells using extendable pseudopodia in the manner described by McKinney et al (1977). The phagocytic ability of the neutrophils has been confirmed by Siwicki & Studnicka (1987), using Aeromonas punctata and Pseudomonas alcaligenes.

Temperature is known to have an effect upon phagocytosis. O'Neill (1985) examined the effect of temperature upon the uptake of Candida guilliermondii by brown trout leucocytes and found that their ability to phagocytose the yeast cells was impaired at temperatures below 10°C. The thermal tolerance of trout however does extend down to this level, with the fish only becoming stressed below 4°C (Elliott 1981).

Stress, and it's effects upon fish health has aroused a considerable amount of interest in the past ten years or so, due, in part, to the fact that aquaculturists are rearing fish intensively, and possibly subjecting them to overcrowding, confinement and other stressors. One of the most widely used indicators of stress is the level of cortisol in the blood. Other physiological parameters used to measure stress in fish include blood glucose level, blood cell counts, plasma cholesterol levels and liver glycogen levels (Wedermeyer & McLeay 1981).

Cortisol, and other corticosteroids, are produced by the interrenal gland in fish, which is functionally homologous to the mammalian adrenal cortex. The interrenal gland is found in teleosts in the anterior kidney and consists, in juvenile fish, of groups of small, densely packed cells with spherical, chromatin - rich nuclei. In adult fish, these cells become hypertrophied, with enlarged nuclei and many small vacuoles (Donaldson 1981).

The secretion of cortisol from the interrenal gland is under the control of the hypothalamic - pituitary - interrenal axis (Figure 1), and, directly, from the pituitary gland mediated by adrenocorticotropin (ACTH). The pituitary, in turn, is under hypothalamic control, mediated by corticotropin releasing hormone (CRH). Cortisol and its' associated corticosteroids are responsible for a number of physiological changes acting upon osmotic regulation, carbohydrate metabolism, behaviour and the immune response.

The immune response is influenced in a number of ways by cortisol. In vitro experiments using mitogens to induce blastogenesis in plaice leucocytes showed that the addition of cortisol to the culture suppressed the mitogenic response (Grimm 1985). Thomas & Lewis (1987) studied haemagglutinin and precipitin production in the red drum (Sciaenops ocellatus) and found that fish fed cortisol in their diet gave reduced responses. Maule et al (1987) found a decrease in the numbers of splenic plaque forming cells, splenic lymphocytes and circulating lymphocytes in coho salmon during the transformation from parr to smolt.

The mechanisms behind the effects of cortisol have yet to be fully evaluated. Kaattari & Tripp (1987) have examined the principle behind the reduction in antibody

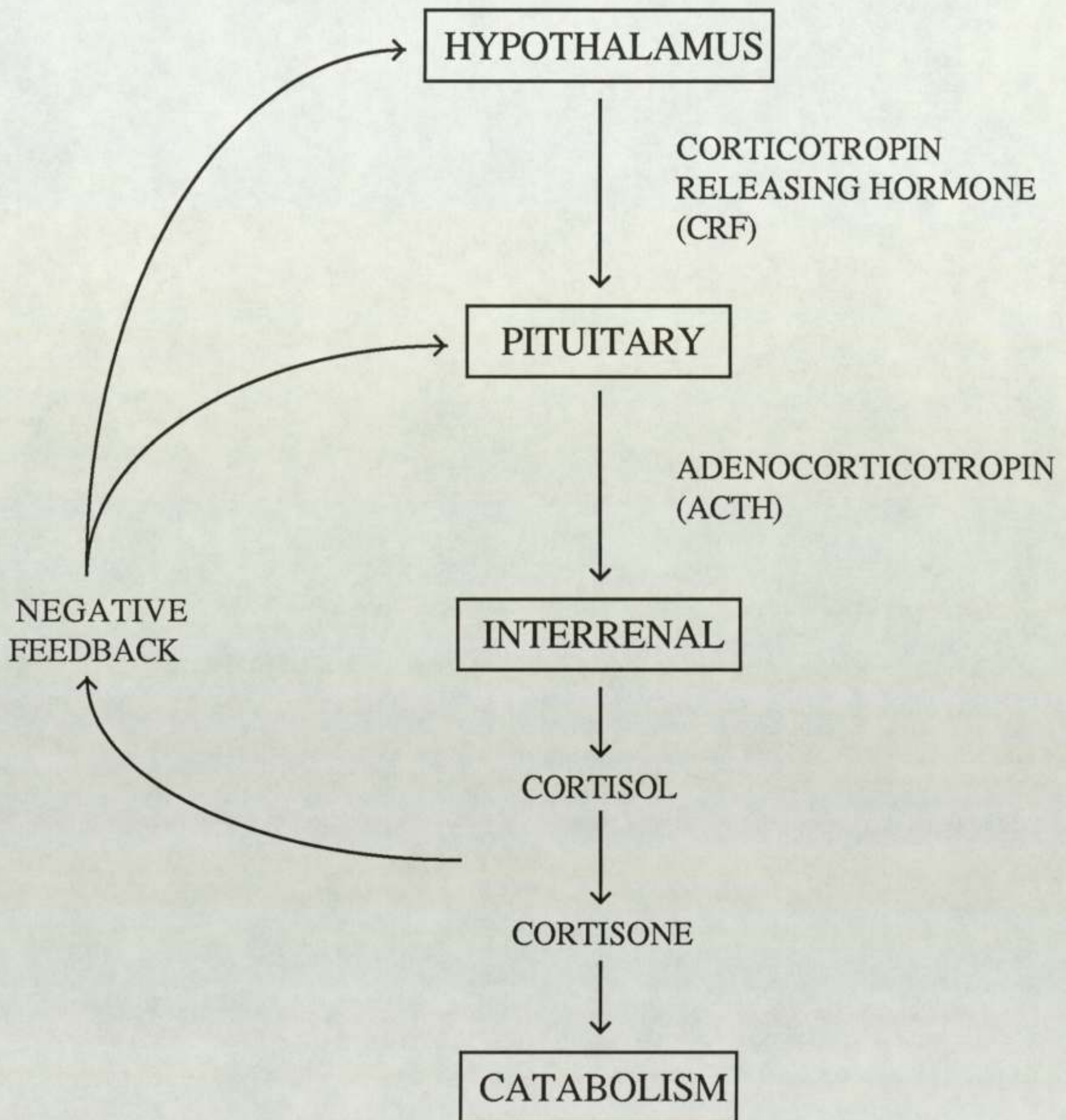


Fig. 1. Diagram illustrating the hypothalamic-pituitary-interrenal (HPI) axis in fish. (After Donaldson, 1981.)

producing cells in salmon, using limiting dilution analysis. They concluded that, in vitro, the suppression induced by cortisol may be overcome by the addition of conditioned medium to the culture, causing, by means of macrophage secreted factors, the previously suppressed reactive cells to become unsuppressed. The mechanism behind this reversible suppression remains unclear.

It can be seen, therefore, that temperature and stress both have a profound effect upon the immune response in fish. Studies have shown that fish given cortisol, either in the diet or by implantation, are more susceptible to infection by pathogenic organisms such as Saprolegnia spp. and Aeromonas salmonicida (Pickering & Duston 1983) and Vibrio anguillarum (Maule et al 1987). Low temperature has also been implicated in outbreaks of fish disease. Wedermeyer & McLeay (1981) list hemorrhagic septicemia (Aeromonas and Pseudomonas spp.), Vibriosis and Spring Viraemia of carp as diseases that have reduced temperature as a predisposing factor.

Whilst both temperature and stress can have an effect upon disease susceptibility, and it is known that both of these factors cause a reduction in the effectiveness of the immune response, the precise relationships between these factors has yet to be clarified.

This work attempts to contribute to the limited literature available on this subject by examining the effects of temperature and stress upon cortisol and glucose metabolism, and correlating this with the humoral immune response. The effects of acclimation at two temperatures upon in vitro and in vivo immune function is also examined.

2. MATERIALS AND METHODS

2.1. Fish Maintenance

Common carp (Cyprinus carpio) weighing between 5g and 10g were obtained from Munton and Fisons Ltd. (Colchester). Prior to experimentation they were kept in large (3' x 18" x 18") oxygenated holding tanks, maintained at 15°C and 20°C, and fed daily on salmon fry food, size 03 (B.P.). Both tanks were maintained on a through flow system, with a constant input of fresh water.

Following acclimatisation in the holding tanks, groups of five control and experimental fish were transferred to smaller experimental tanks, measuring 18" x 8" x 8". These were also oxygenated and maintained on a through flow system at 20° ± 1°C or at 10°± 1°C.

2.2 Anaesthesia

Ethyl-p-aminobenzoate (Sigma) was used to anaesthetise and kill all fish. The standard strength solution was 0.25g ethyl-p-aminobenzoate dissolved in 5ml of acetone and added to 5 litres of water. To kill fish more rapidly (as required for the cortisol assay), 0.4g of anaesthetic was used in the same volume of water.

2.3. The Effect of Temperature Change and Handling Upon Serum Cortisol and Glucose.

Fish were allowed to acclimatise at 20°C and 15°C for two weeks in holding tanks as previously described. Groups of five fish were transferred to experimental tanks in the following manner.

Fish from the 20°C holding tank were placed in a large plastic bag containing water at 20°C and immersed in a 20°C experimental tank for 45 minutes before release. This

procedure was carried out in order to ensure that the 20°C fish also underwent a similar handling "stress" to the fish transferred from 15 to 10°C.

The same procedure was adopted for transferring fish from the 15°C holding tank to experimental tanks at 10°C but the change in water temperature inside the bag was also monitored.

Each group of five fish was kept separate from the other groups. Groups of fish maintained at each of the two temperatures were then killed at the following times after transfer: 3 hours, 1 day, 3 days, 6 days, 10 days and 14 days. In order to minimise diurnal variations, all of the fish were killed at the same time of day (11am). Handling was kept to a minimum, and all fish were killed within two minutes of netting the first fish by placing them in a strong anaesthetic solution (See section 2.2).

After severing the tail, blood was collected from the caudal vein into heparinised haematocrit tubes (Hawkesley and Sons Ltd.). The blood samples were then placed in a microfuge tube and centrifuged for 2 minutes in a Beckmann's microfuge (12,000 rpm). The plasma was removed and stored at -20°C prior to use.

2.3.1 Cortisol Assay.

This work was carried out in conjunction with the Endocrine Department of the "Birmingham and Midland Hospital for Women", who developed the assay. Cortisol was measured using a competitive radioimmunoassay, full details of which are provided in Appendix I.

2.3.2 Glucose Assay

Serum glucose levels were measured using a Beckmann's Glucose Analyser 2 and the glucose oxidase method (Beckman Glucose Analyzer 2 Operating Manual).

2.4. The Effect of Antigen Dose on the Antibody Response to SRBC.

In order to assess the optimal immunising dose of SRBC, five groups of five fish were transferred from the 20°C holding tank to experimental tanks, also maintained at 20°C. Groups of five fish were then injected with either 0.1 ml PBS or 0.1 ml PBS containing $1x10^5$, $1x10^7$, $1x10^8$, or $1x10^9$ SRBC (see section 2.5) via the intraperitoneal route. The fish were killed on day 15 post infection, the blood collected and a haemagglutination test performed, as described in Section 2.5.2.

2.5. Kinetics of Antibody Response to Sheep Erythrocytes at Different Temperatures.

Sheep red blood cells (SRBC) in Alsever's solution (Flow) were washed three times by centrifugation in phosphate buffered saline (PBS) (See Appendix 2) at 2,000 rpm.

Two groups of 35 fish were removed from the holding tanks at 10° and 20°C, allowing the water time to cool (45 minutes) before release. The fish were then allowed two weeks to acclimatise at the new temperatures before injection with SRBC or PBS.

2.5.1 Immunisation

a) 20 fish from each tank (10° and 20°C) were injected with 0.2 ml of a suspension of 5×10^8 SRBC / ml PBS via the intraperitoneal route. They were then returned to an experimental tank at the same temperature.

b) The remaining fish in each group were similarly injected with 0.2 ml PBS and returned to their respective experimental tanks as controls.

Groups of five fish from the 20°C tanks (both experimental and control) were killed on days 7, 11, 14, 17 and 27 after injection, and blood was collected as outlined in Section 3. Similar numbers of experimental and control fish kept at 10°C were killed on days 11, 14, 17, 27 and 35. The serum was separated from each blood sample and used immediately to assess the haemagglutinin titre.

2.5.2 Haemagglutination Assay

50 µl of PBS was placed into each well of a 96 well microtitre plate with V-shaped wells (Flow). Into the first well of each row, 50 µl of a serum sample was added, and a twofold serial dilution was made across the twelve wells of the row. 50 µl of a 2% SRBC suspension (packed cell volume) was added to each well, and the plate was sealed and kept at 4°C overnight before examination.

2.6. Effect of Temperature Upon Phagocytosis

2.6.1 Leucocyte Isolation and Preparation

Fish were killed as described in Section 2. Using sterile techniques throughout, the spleen and pronephros were removed and transferred to 2 mls of Liebovitz L-15 tissue culture medium (Sigma). The organs were then teased apart using fine forceps to form a single cell suspension. This suspension was transferred to sterile centrifuge tubes and washed three times by spinning at 2,000 rpm for ten minutes. Cells were resuspended in fresh L-15 medium after each wash. Cell viability was assessed by trypan blue dye exclusion and the final cell concentration was then adjusted to 2.5×10^6 leucocytes ml^{-1}.

2.6.2 Antigen Preparation

Candida albicans was the antigen selected for use in the phagocytosis assay, being readily available, easy to handle and easily distinguishable from the leucocytes. C.albicans was cultured on agar plates at 4°C, subculturing every two months (See Appendix 2).

Colonies of the yeast were removed in sterile fashion by scraping the plate surface, and killed by immersion in formal buffered saline, overnight. The dead yeast cells were then washed three times in L-15 medium by centrifugation in a Beckmann's microfuge. The final concentration of cells was then adjusted to 5×10^6 ml^{-1}.

2.6.3 Test Procedure

Preliminary studies showed that a high concentration of C.albicans produced clumping of the yeast cells. This occurred at concentrations of 10^8 cells ml^{-1}. A final concentration of 2.25 x 10^6 yeast cells ml^{-1} was therefore used, with a leucocyte concentration of 1.125 x 10^6 cells ml.

0.45 mls of yeast cell suspension (5 x 10^6 cells ml^{-1}) were placed in a 1.5 ml microcentrifuge tube, together with 0.45 mls leucocyte suspension (2.5 x 10^6 cells ml^{-1}) and 0.1 ml heat inactivated foetal calf serum (Flow). The mixture was then incubated at 10°C or 20°C whilst constantly rotating (~20 rpm).

At intervals, samples were taken from the microfuge tube and the number of non-ingested yeast cells remaining in suspension was determined by counting under a haemocytometer. An uningested yeast cell was defined as a single yeast particle or a string of particles containing two or more cells. This eliminated the difficult process of determination of the numbers of yeast cells in a string. C.albicans attached to the surface of leucocytes were counted as ingested particles, since distinguishing between intracellular and adherent extracellular particles was visually difficult.

A number of experiments were performed to investigate various aspects of phagocytosis.

2.6.4 i The Effects of In Vivo Environmental Temperature and In Vitro Incubation Temperature

Leucocytes were taken from fish kept at 10°C and at 20°C. Cells taken from both groups of fish were incubated at both 10°C and 20°C in vitro in the presence of Candida albicans. The number of uncleared Candida particles remaining in the culture medium was monitored over a 48 hr period.

2.6.4 ii The Effect of In Vivo Leucocyte 'Arming'

A group of fish acclimatised at 20°C received 0.1 ml of dead C.albicans (5×10^8 C. albicans ml^{-1}) via the intraperitoneal route. They were then maintained at 20°C for 15 days, before being killed and tested for the phagocytic ability of their leucocytes.

Leucocyte suspensions from immunised fish were incubated at both 10°C and 20°C with Candida particles to discover if prior in vivo contact with the antigen altered their phagocytic ability in vitro.

2.6.4 iii. The Effect of 'Opsonisation' of C. Albicans

The serum from the fish used in Section 2.6.4ii was collected, pooled and incubated with C.albicans particles for 30 mins at 20°C. The 'opsonised' Candida cells were washed by centrifugation and resuspension in L-15.

Leucocytes taken from fish acclimatised at 20°C were incubated with the opsonised C. albicans at 20°C. In addition, 'armed' leucocytes (as in 6.4b) were incubated with opsonised Candida at 20°C.

2.7 Statistical Analysis

The results from each of the tests were analysed using the Student's t-test.

3. RESULTS

3.1 Effect of Temperature Change and Handling upon Blood Cortisol

Changes in serum cortisol levels of two groups of fish are shown in histogram form in Fig. 3. The black bars represent cortisol levels in fish subjected to handling stress only. Here, fish were removed from their holding tank (at 20°C), placed in polythene bags in experimental tanks at 20°C and then released after 45 minutes. Since they experienced no change in environmental temperature, any changes in cortisol levels are attributable to the handling procedure.

The second group of fish was transferred from 15°C to 10°C following the same handling procedures. Figure 2 shows the rate of cooling of the water inside the polythene bags. It may be seen that the lower water temperature was reached 20 - 25 minutes post - transfer.

A comparison of the changes in cortisol levels in both groups of fish reveals a transient, but significant ($p < 0.05$) rise in blood cortisol levels some three hours after handling. This change may be ascribed to "handling stress" alone, since the second group of fish (open shaded columns) showed the same increase in cortisol levels despite having also experienced a sudden drop in temperature.

Examination of cortisol levels over a period of 14 days revealed that, in the group of fish maintained at 20°C, there was considerable variation between individual fish, but by 10 to 14 days post handling cortisol levels had returned to control values. There was less individual variability in the fish transferred to 10°C, and their cortisol levels returned to normal by day 3. The increase in blood cortisol seen on day 14 was not statistically significant.

Fig. 2. Cooling Kinetics of Water in Polythene Bags

Transferred from 15°C to 10°C.

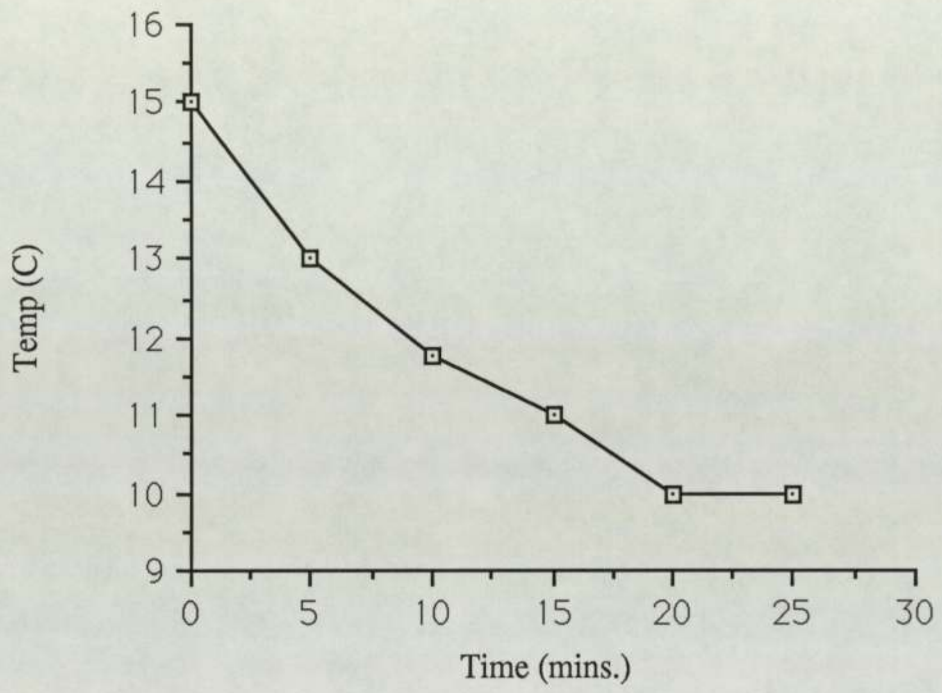
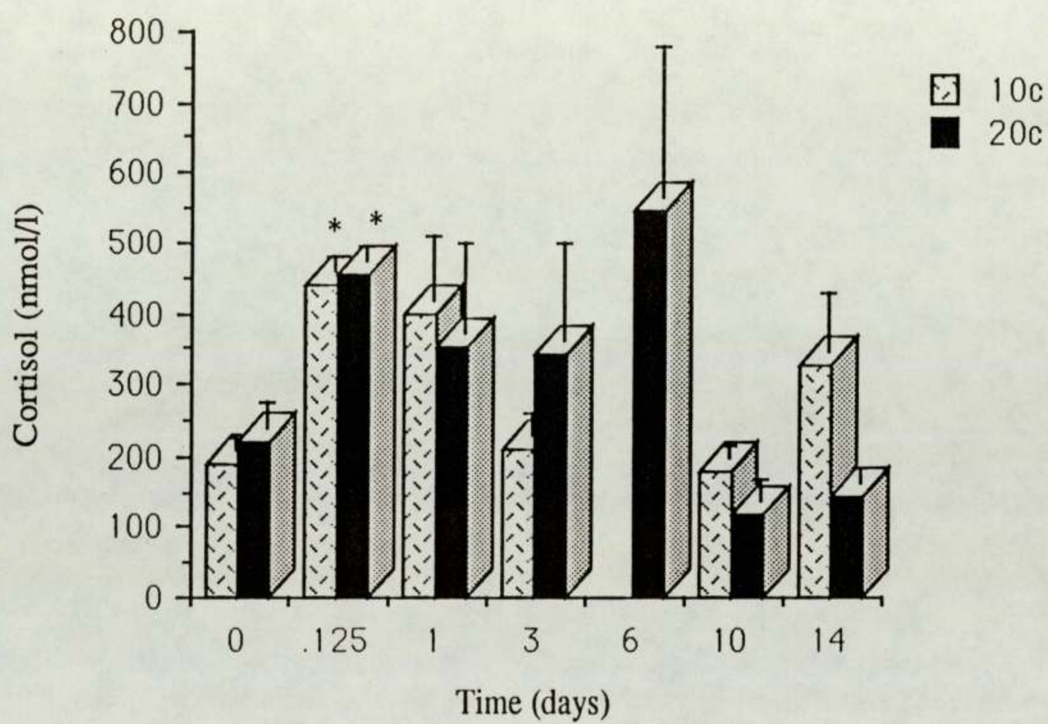

16
15
14
13
12
11
10
9
Temp (C)
0
5
10
15
20
25
30
Time (mins.)

Fig. 3. Effect of Temperature and Handling upon Plasma Cortisol.

Each column represents the mean plasma cortisol level ($n = 5$) + / - SEM (vertical bars). * denotes significance ($p < 0.05$).


800
700
600
500
400
300
200
100
0
Cortisol (nmol/l)
*
*
10c
20c
0
.125
1
3
6
10
14
Time (days)

3.2 Effect of Temperature Change and Handling on Blood Glucose.

The blood collected from the same fish as used in section 3.1, was analysed for its glucose content, and the results are presented in Figure 4. After 3 hours, both the fish kept at 20°C and those transferred to 10°C showed a significant ($p < 0.05$) increase in the plasma glucose levels, although the fish kept at the lower temperature exhibited a less pronounced increase than those at 20°C. Variation between individual fish within a group did occur, but to a lesser degree than that found in the cortisol measurements (section 3.1). The blood glucose levels returned to that of the control fish over a period of 6 days.

3.3 The Effect of Antigen Dose Upon Antibody Titres

Figure 5 shows the haemagglutinin titres observed in fish injected with different doses of sheep erythrocytes and tested for agglutinating antibody, on day 15 post-injection. The levels of antibody produced show an increase with increasing dose of erythrocytes, and are significantly higher in the fish injected with the highest dose (1×10^9 erythrocytes) when compared to those receiving the lowest dose (1×10^5 erythrocytes). All doses produced a significantly higher ($p < 0.05$) agglutinin titre than that found in the controls.

Fig. 4. The Effect of Temperature and Handling on Blood Glucose.

Columns represent mean plasma glucose levels ($n = 5$) +/- SEM (vertical bars). * indicates significantly ($p < 0.05$) different to control

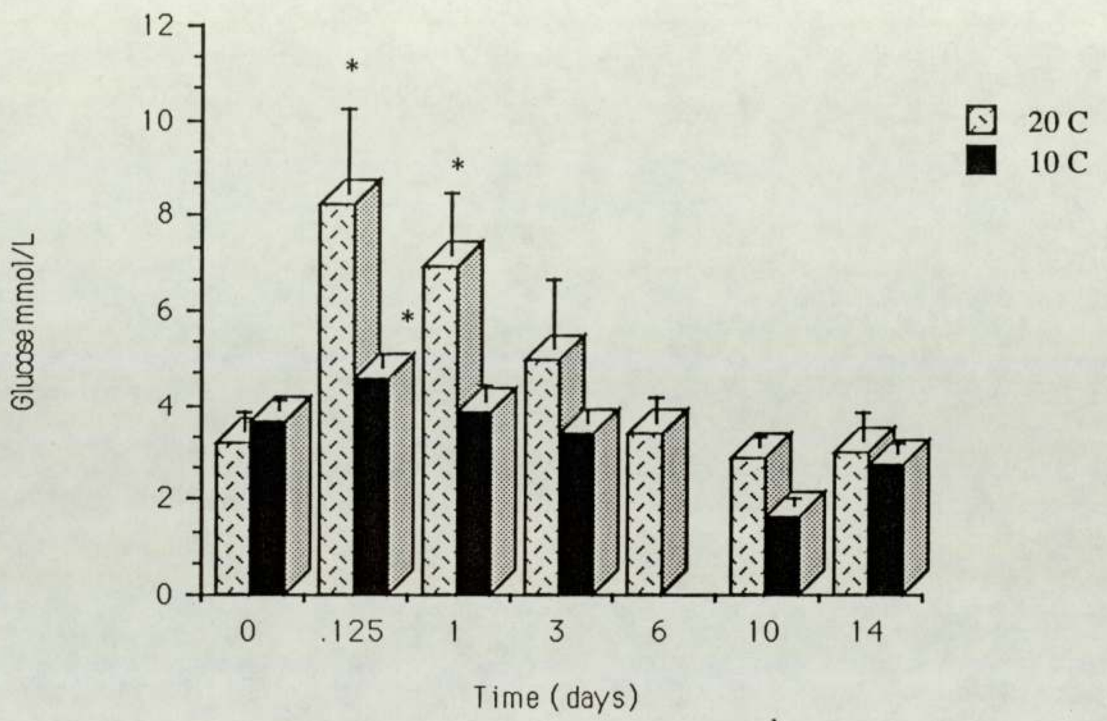
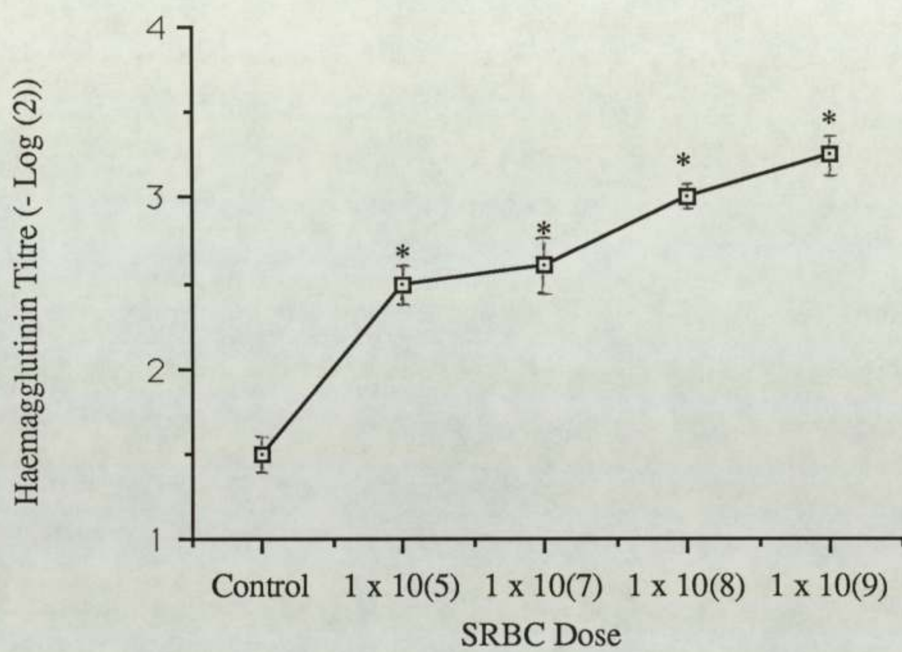

12
10
8
6
4
2
0
Glucose mmol/L
20 C
10 C
*
*
*
0
.125
1
3
6
10
14
Time (days)

Fig. 5. Haemagglutinin Production in Response to Various Doses of Sheep Erythrocytes.

Each point represents the mean agglutination titre ($n = 4$ or 5). Fish maintained at 20°C. * represents titres significantly above control level ($p < 0.05$).



Haemagglutinin Titre (- Log (2))
4
3
2
1
Control
1 x 10(5)
1 x 10(7)
1 x 10(8)
1 x 10(9)
SRBC Dose

3.4 Kinetics of Antibody Response to Sheep Erythrocytes

The agglutinating antibody response to sheep red blood cells, as measured by a haemagglutination test, is shown in Figure 6. Background levels of agglutinins were present in all fish samples; in some cases up to serum dilutions of 1 in 8. At 20°C, the fish exposed to SRBC produced peak titres on days 14 and 17 post injection, whereas at 10°C the antibody response did not reach a maximum until day 27. The peak levels of agglutinating antibody did not differ significantly between the two temperatures.

Lytic activity also appeared to be affected by the injections. Lysis of sheep erythrocytes only appeared on day 14 post injection at 20°C and on day 17 at 10°C. Lytic activity was also seen in the PBS injected fish serum. Levels remained low in all cases.

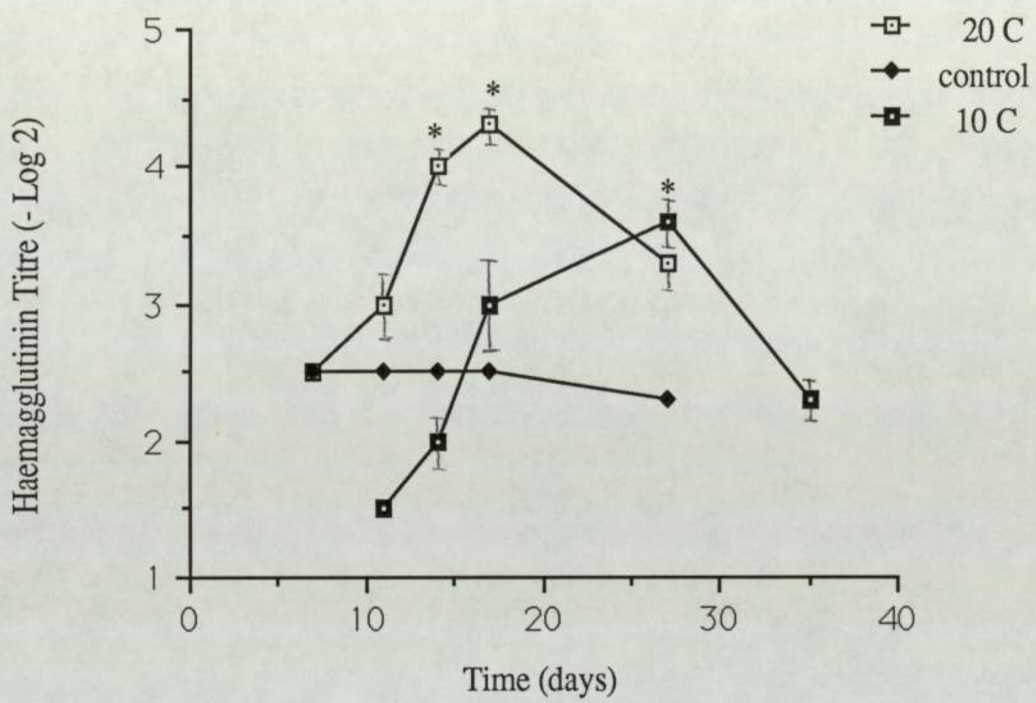
3.5 The Effect of Temperature upon Particle Clearance

3.5.i Environmental Temperature

Figures 7a and 7b illustrate that the temperature at which the fish are maintained in vivo has no effect upon the rate of particle clearance in vitro. Figure 7a shows that cells taken from fish maintained at 10°C and 20°C take the same length of time to clear particles from suspension when both are cultured at 10°C. After 48 hours, some Candida cells were still present in the medium. At 20°C, however (Figure 7b), cells taken from fish maintained at either 10°C or 20°C have removed all of the yeast cells after 16 hours in vitro.

Fig.6. Kinetics of the Haemagglutinin Response to Sheep Erythrocytes at 10°C and 20°C.

Each point represents the mean agglutinin titre ($n = 4$ or 5). * denotes significantly ($p < 0.05$) higher than control.



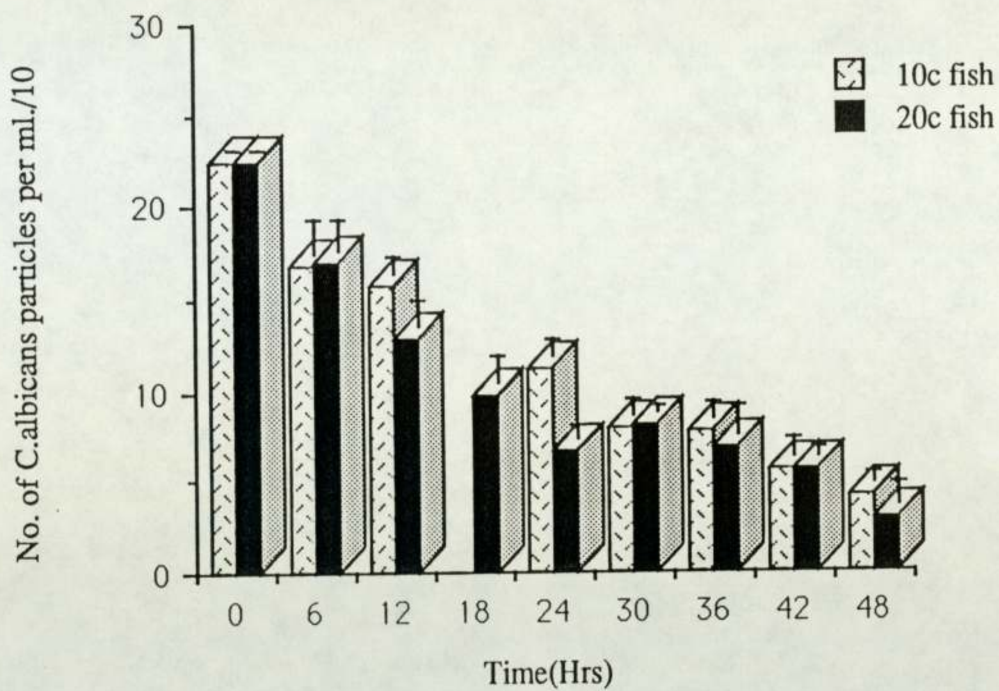
20 C
control
10 C
Haemagglutinin Titre (- Log 2)
Time (days)
5
4
3
2
1
0
10
20
30
40
*
*
*

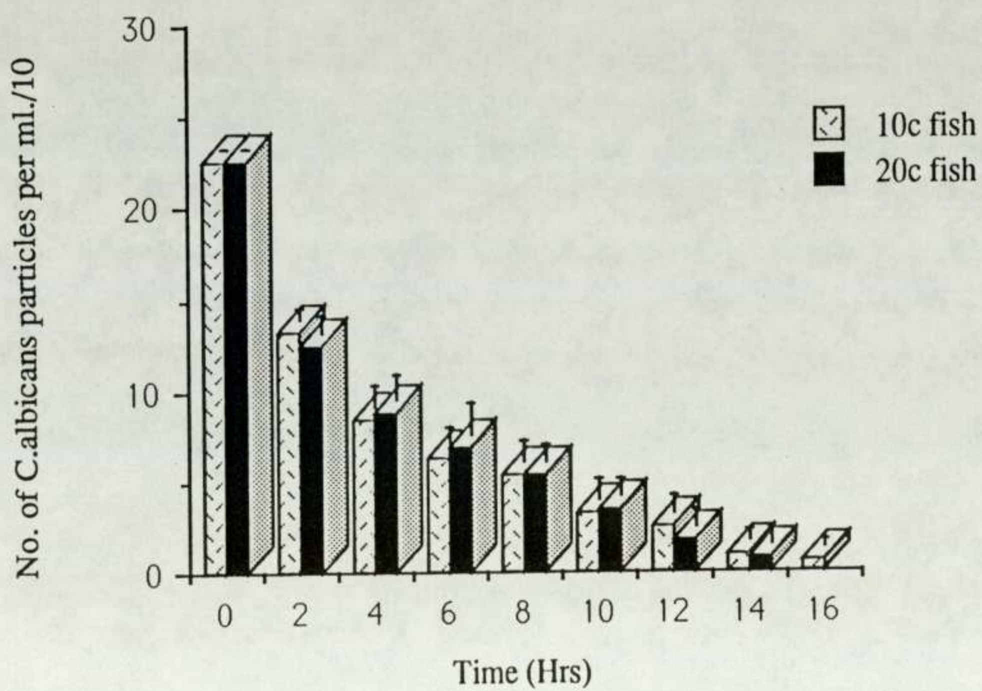
Fig. 7. Effect of Environmental Temperature upon Particle Clearance

by Phagocytes In Vitro.

a) In vitro clearance of Candida at 10°C by cells taken from fish maintained at 10°C and 20°C. Each column represents mean + / - SEM.

b) In vitro clearance of Candida at 20°C by cells taken from fish maintained at 10°C and 20°C. Each column represents mean + / - SEM.


No. of C.albicans particles per ml./10
30
20
10
0
0
6
12
18
24
30
36
42
48
Time(Hrs)
10c fish
20c fish


No. of C.albicans particles per ml./10
30
20
10
0
0
2
4
6
8
10
12
14
16
Time (Hrs)
10c fish
20c fish

3.5.ii Incubation Temperature

The effect of in vitro incubation temperature can be clearly seen in Figure 8 (a and b). The upper graph (8a) shows the difference in clearance rates at the two different incubation temperatures, 10°C and 20°C. The cells in this experiment were taken from fish maintained at 10°C. In cultures maintained at 10°C, Candida particles are still present 32 hours after all the particles have been removed by cells maintained at the higher temperature. Similar results were observed for cells taken from fish kept at 20°C (Figure 8b): at an incubation temperature of 20°C, particle removal is complete after 14 hours, whilst at 10°C, particles are still present after two days of culture.

3.6 The Effect of 'Arming' on Particle Clearance

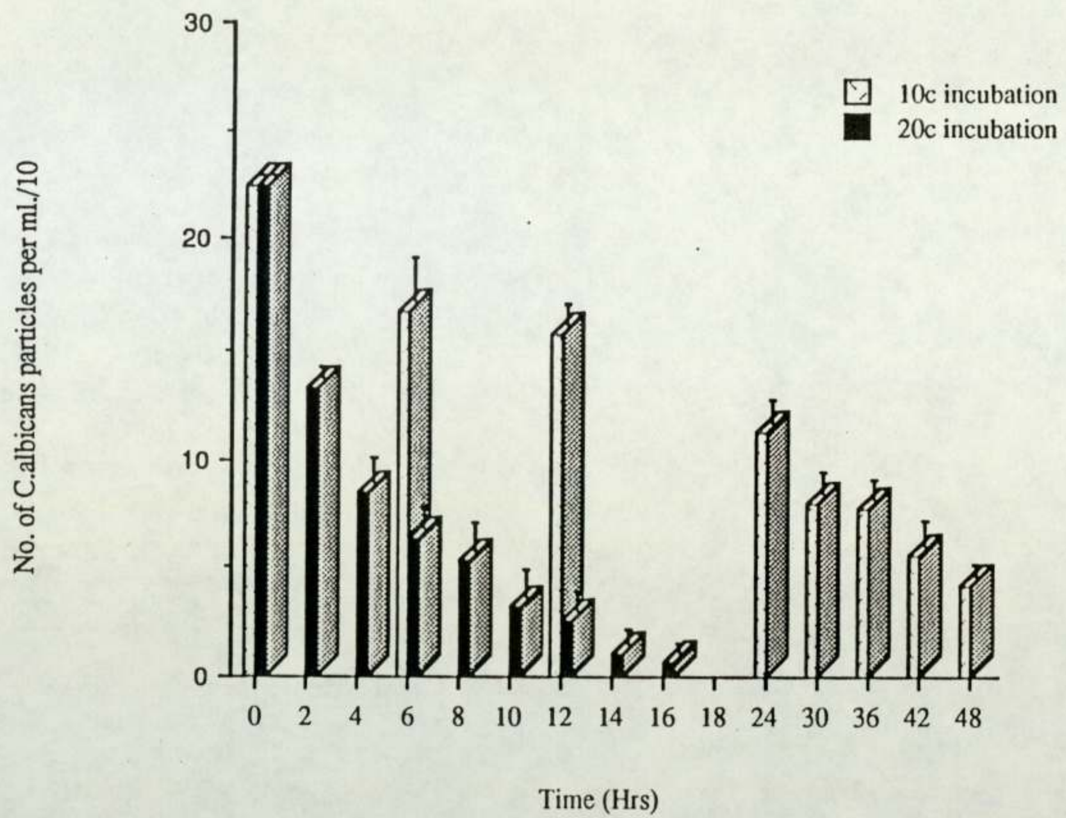
'Armed' leucocytes were obtained from fish maintained at 20°C and injected two weeks prior to sacrifice, with Candida albicans. Their ability to clear Candida particles from suspension was compared with that of normal leucocytes from uninjected fish kept at 20°C. The results are presented in Figure 9. At an incubation temperature of 10°C (Figure 9a), cells from fish that had not been injected with C. albicans; ('unarmed' cells) had not cleared the yeast particles after 24 hours (as would be expected given the results shown in Figure 8). The 'armed' macrophages however had removed all of the Candida cells after 18 hours. A similar effect was seen at an incubation temperature of 20°C; the 'armed' cells had cleared the Candida albicans after 6 hours, whereas 'unarmed' cells took 14 hours.

Figure 10 shows that, although 'arming' increases the clearance rate, temperature still plays an overriding role in the speed at which the Candida are removed. Thus, 'armed'

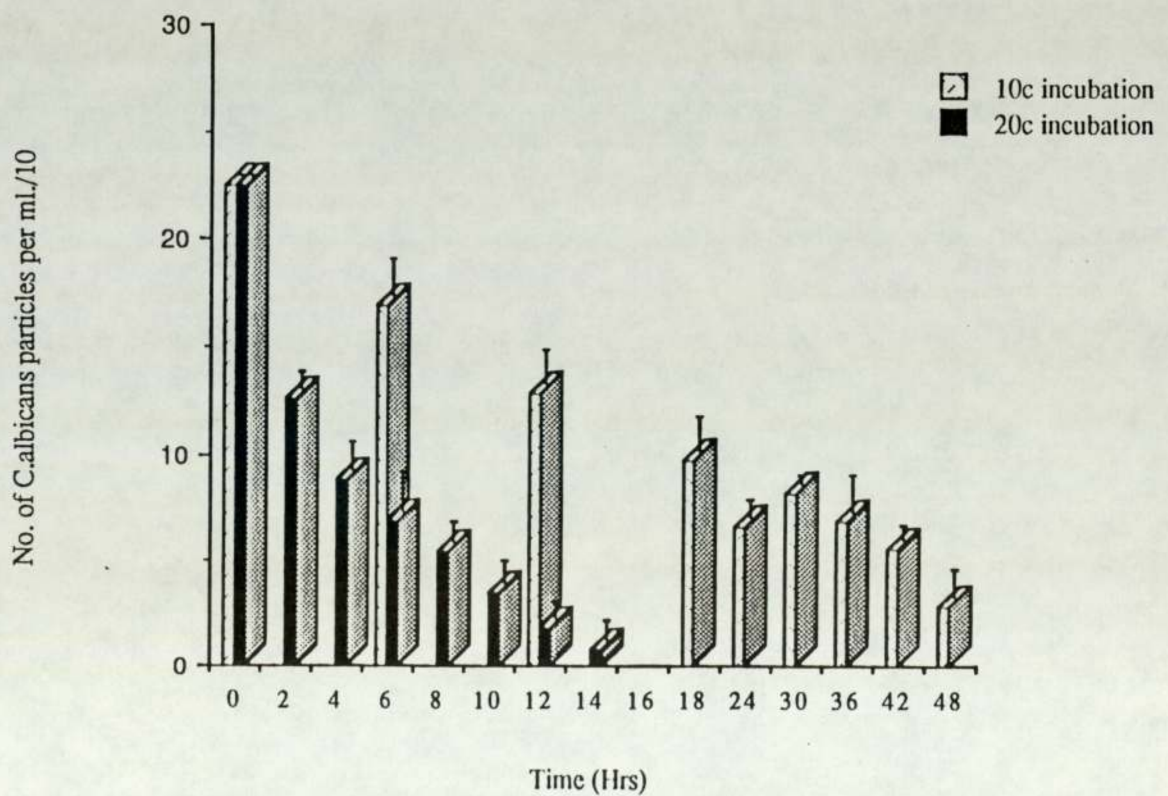
Fig.8. The Effect of In Vitro Temperature on Particle Clearance.

a) In vitro clearance of Candida particles at 10°C and 20°C, by cells obtained from fish maintained at 10°C. Columns represent means + / - SEM (n = 5). The time taken to clear the particles differs significantly between the two in vitro temperatures.

b) In vitro clearance of Candida particles at 10°C and 20°C, by cells obtained from fish maintained at 20°C. Columns represent means + / - SEM (n = 5). The time taken to clear the particles differs significantly between the two in vitro temperatures.



30
20
10
0
No. of C.albicans particles per ml./10
10c incubation
20c incubation
0 2 4 6 8 10 12 14 16 18 24 30 36 42 48
Time (Hrs)

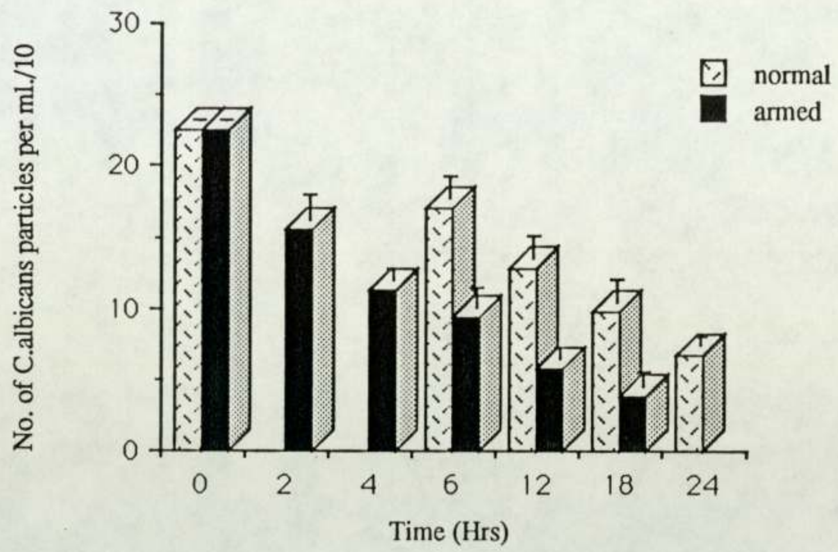


30
20
10
0
No. of C.albicans particles per ml./10
10c incubation
20c incubation
0 2 4 6 8 10 12 14 16 18 24 30 36 42 48
Time (Hrs)

Fig.9. The Effect of "Arming" upon Particle Clearance

a) Clearance rate of Candida by 'armed' and normal phagocytes taken from fish maintained at 20°C. In vitro incubation temp. = 10°C. Each column represents mean + / - SEM (n = 5). Clearance time of by 'armed' cells is significantly ($p < 0.05$) less than that of the normal cells.

b) Clearance rate of Candida by 'armed' and normal phagocytes taken from fish maintained at 20°C. In vitro incubation temp. = 20°C. Each column represents mean + / - SEM (n = 5). Clearance time of by 'armed' cells is significantly ($p < 0.05$) less than that of the normal cells.


No. of C.albicans particles per ml./10
30
20
10
0
normal
armed
0
2
4
6
12
18
24
Time (Hrs)

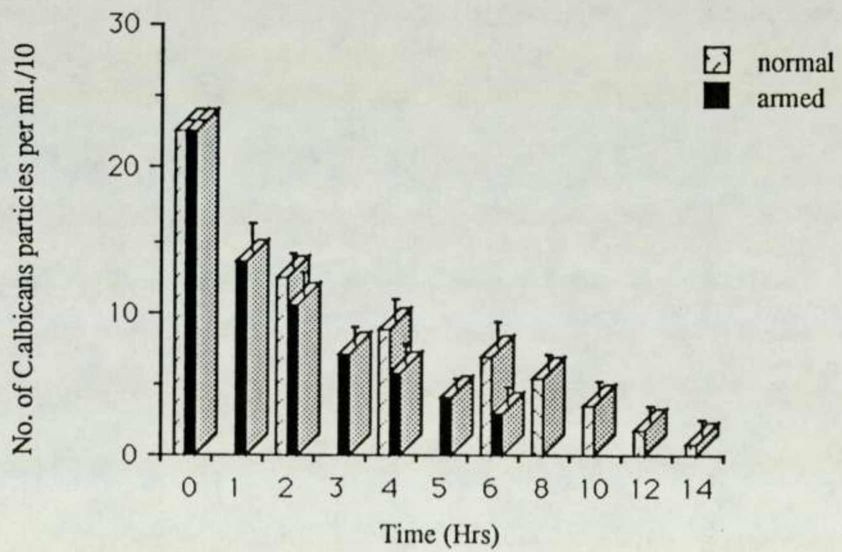
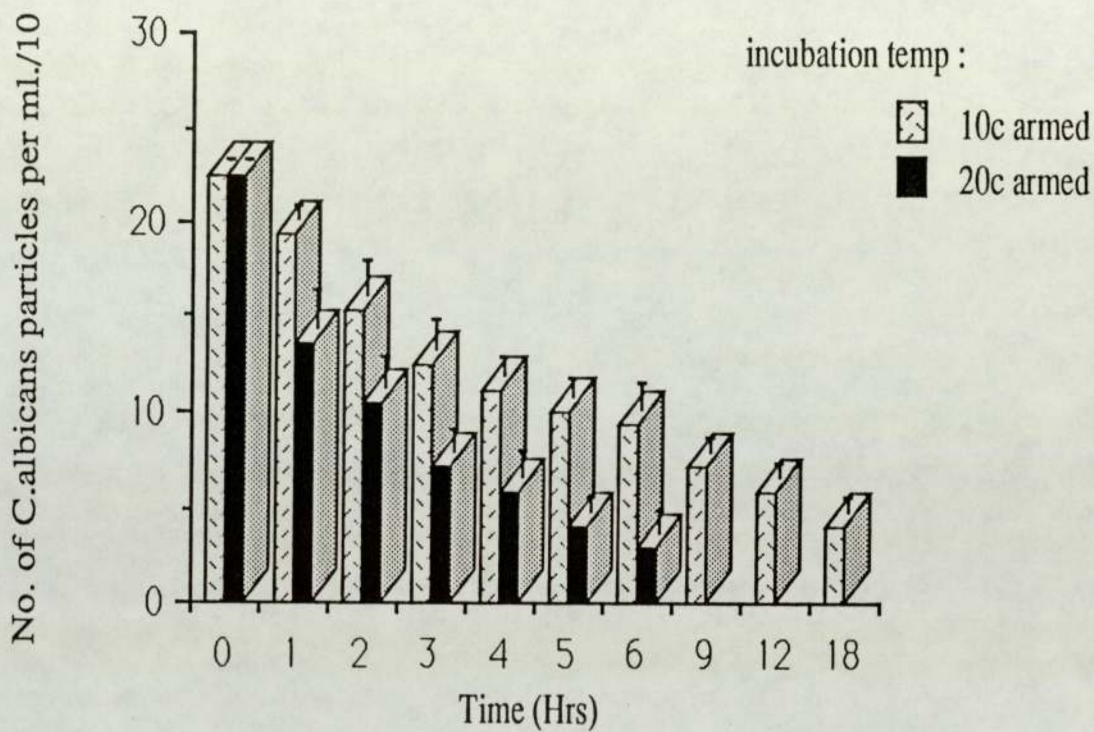

No. of C.albicans particles per ml./10
30
20
10
0
normal
armed
0
1
2
3
4
5
6
8
10
12
14
Time (Hrs)

Fig.10. The Kinetics of Particle Clearance by 'Armed' Leucocytes

at Different In Vitro Temperatures.

'Armed' leucocytes, obtained from fish maintained at 20°C, incubated at 10°C and 20°C. Each column represents mean + / - SEM (n = 5). Differences between clearance rates is significant.


No. of C.albicans particles per ml./10
30
20
10
0
incubation temp :
10c armed
20c armed
0
1
2
3
4
5
6
9
12
18
Time (Hrs)

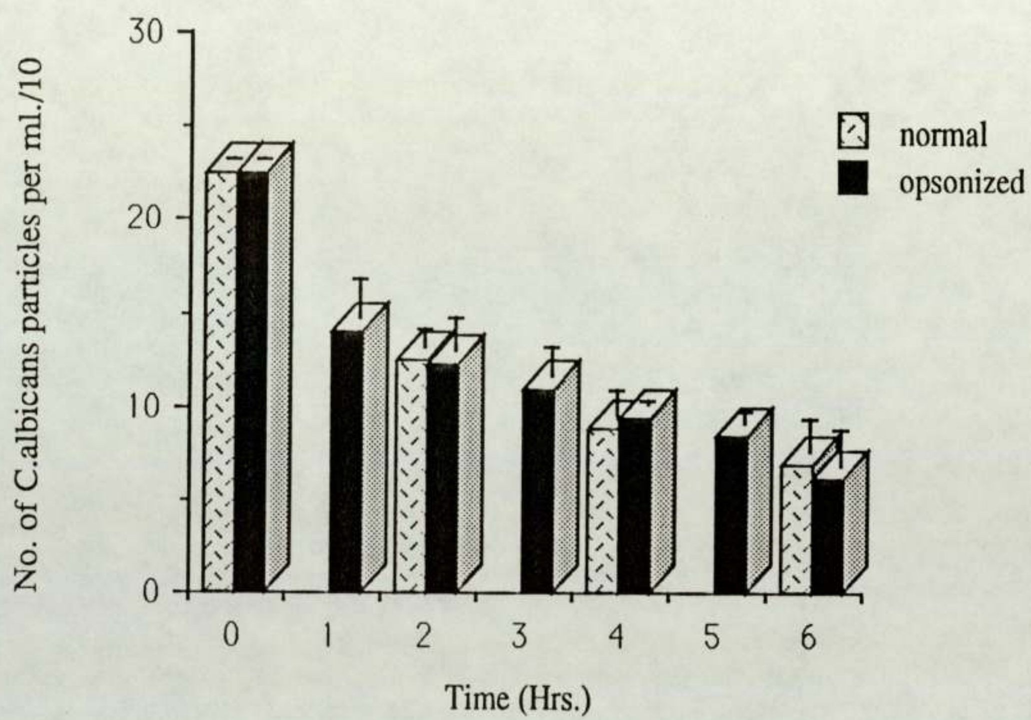
cells cultured at 20°C still remove all particles from suspension in one third of the time taken for similarly 'armed' cells cultured at 10°C.

3.7 The Effect of Opsonisation Upon Particle Clearance

Figure 11 reveals that the attempted opsonisation of the Candida albicans appeared to have no effect upon the clearance rate. Normal cells follow the same clearance kinetics curve whether dealing with normal or opsonised Candida particles. This observation is confirmed by an inspection of Figure 12, which shows that 'armed' leucocytes exhibit identical clearance kinetics of normal or opsonised Candida. This graph combines a study of the effects of 'arming' leucocytes *and* opsonising the Candida. It may be seen that only the 'arming' of the leucocytes has a significant effect upon the rate of clearance.

Fig. 11. Effect of 'Opsonisation' upon Particle Clearance

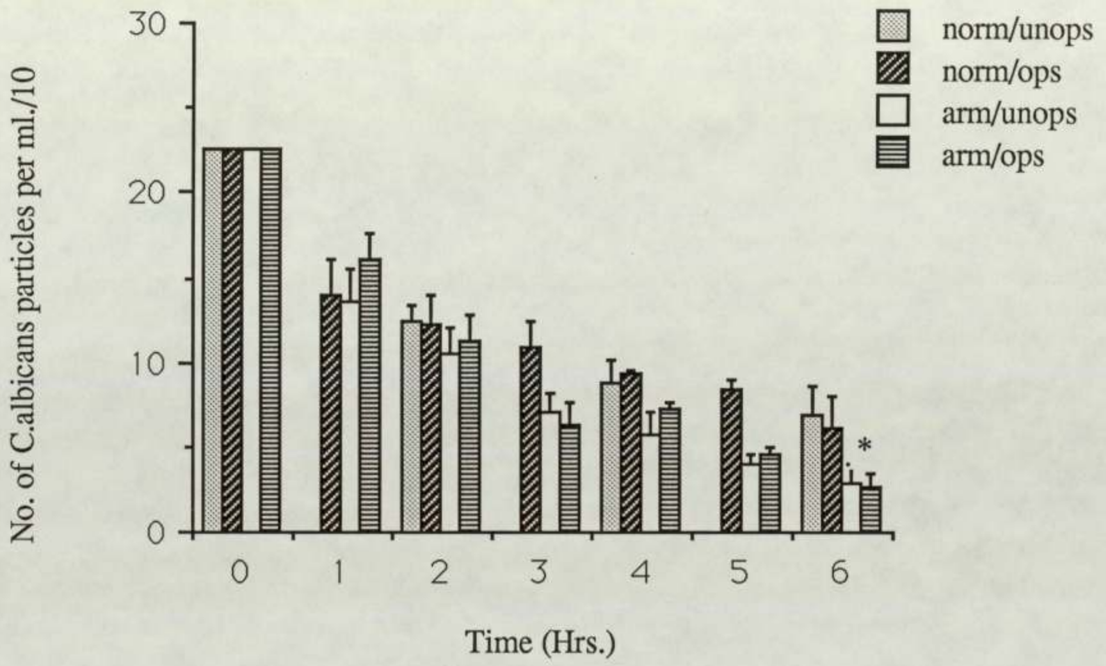
Leucocytes taken from fish maintained at 20°C. In Vitro incubation temperature of 20°C, with 'opsonised' and normal Candida. Columns represent means + / - SEM (n = 5).



30
20
10
0
No. of C.albicans particles per ml./10
normal
opsonized
0
1
2
3
4
5
6
Time (Hrs.)

Fig.11. Effect of 'Opsonisation' and 'Arming' upon Particle Clearance

'Armed' or normal leucocytes incubated in vitro with 'opsonised' or normal Candida. Columns represent mean + / - SEM (n = 5). * indicates significate differences from the control (normal leucocytes, normal Candida).


30
20
10
0
No. of C.albicans particles per ml./10
0
1
2
3
4
5
6
Time (Hrs.)
norm/unops
norm/ops
arm/unops
arm/ops
*

4. DISCUSSION

There are a number of problems involved in the study of stress in fish. Potentially, any manipulation of the environment can be stressful, and consequently it is difficult to study the effects of a single stressor. In this study, temperature was chosen as the stressor for two reasons. Firstly, there is a considerable body of background literature covering the effects of temperature on fish immunity. It was felt that this would form a useful basis upon which to build the present series of experiments. Secondly, temperature is relatively easily controlled in the laboratory, and changes in this environmental parameter can perhaps be more readily quantified than, say, crowding or handling stress.

A number of physiological parameters have been used to measure or indicate stress (Wedermeyer & McLeay, 1981), including the primary responses; for instance cortisol levels (Robertson et al 1987, Barton et al 1985), and the secondary responses, including plasma osmolality (Robertson et al 1987) and plasma glucose levels (Chavin & Young 1970, McLeay & Brown 1975). The tertiary effects (e.g. susceptibility to disease) tend to be too far removed from the stressor to be of direct use in stress measurement.

The levels of serum cortisol found in the carp in these experiments were of a similar magnitude, i.e. 200-600 nmol l^{-1} to those reported by other workers, e.g. Kuhn et al (1986). Cortisol levels are subject to considerable diel changes; Kuhn reported levels in carp rising to over 2,000 nmol l^{-1} during the hours of darkness, with peak levels being reached around 2200 hours. The temperature and photoperiod to which fish are subjected also influences both the levels attained and the diel rhythm pattern, with higher values being found at higher temperatures. Similar photoperiod and temperature induced patterns are found in salmonids, for example Salmo salar (Thorpe et al 1987) and S. trutta (Pickering & Pottinger 1983).

Cortisol has been used as an indicator of stress by many workers. Pickering et al (1982) examined various stress responses of brown trout and found that, after a two minute period of handling stress, an increase in blood cortisol levels had occurred. This increase was transitory, lasting less than one day, although other parameters measured indicated that the fish took up to two weeks to recover. Robertson et al (1987), working with red drum (Sclaenops ocellatus) found a transient increase in cortisol levels, lasting only three hours, after subjecting the fish to acute handling stress. Chronic stress, for example, overcrowding (Robertson et al 1987), or sexual maturation (Pickering & Christie, 1981), leads to a prolonged elevation of plasma cortisol, followed by a gradual return to basal levels.

This study showed a rise in the blood cortisol levels after three hours, in both the fish subjected to handling stress and those subjected to handling and temperature stress. This rise may be attributed to the handling stress alone, as no difference exists between the cortisol levels of the two groups after three hours. After this time however, the variability of the individual fish masks any significant results, although a general trend towards basal levels may be evident.

It might have been expected that, given the fact that two sublethal stresses are being applied to the fish in this study; i.e. handling and temperature, that an initial, transient elevation in plasma cortisol in both the experimental and control fish would have been followed by a rapid reduction in levels in the fish kept at 20°C. The fish maintained at 10°C might be expected to have raised levels of cortisol for an extended period of time, due to the chronic stress. Another possible outcome may have been a greater level of

plasma cortisol in the fish subjected to both handling and temperature stress. Donaldson (1981) suggested that cortisol levels may be particularly useful in the detection of multiple stressors, as the response reflects the combined effect.

The results of the cortisol assay proved very variable, with standard errors within groups of fish of up to 40% of the mean. The reproducibility of the test itself was found to be reliable, with repeat readings of the same serum sample being similar and the standards also being accurate. The reason for such large variation is obscure, but it tends to disguise any trends, unless large numbers of fish are used. There may be a genetic component within the reaction, influencing the susceptibility to chronic stress - hence the large variability in the later stages of the experiment. The experimental procedure was designed to minimise stress within a group, with all the fish being killed within two minutes of the first fish being sacrificed. Preliminary tests showed that the removal of one fish from a tank caused raised serum cortisol levels in the second and third fish removed.

The results obtained for the serum glucose levels showed a much clearer trend, which was also significant. At 20°C, 3 hours after stress, blood glucose levels had more than doubled. The fish transferred to 10°C showed a similar, but less pronounced, increase in glucose levels, although the levels reached were significantly less than those measured at 20°C. Increased serum glucose levels are considered to be one of the secondary stress responses (Wedemeyer & McLeay 1981), and as such have been used as stress indicators (McLeay & Brown 1975, Robertson et al 1987). The role of the hypothalamic-pituitary-interrenal axis (HPI axis) in the regulation of blood glucose has still to be clarified. It is

now thought that increased serum cortisol levels promote the mobilisation of hepatic glycogen (Paxton et al 1984).

The HPI axis is not thought to be the only factor controlling stress related hyperglycaemia. Mazeaud (1965, 1969) has shown that the administration of adrenalin (normally released in vivo under conditions of acute stress) to carp, causes an increased blood glucose level due to the mobilisation of glycogen in muscle and liver.

Temperature has been reported to influence both the HPI axis and the adrenergic response. For example, Ilan and Yaron (1976) showed that the sensitivity of the carp interrenal gland to ACTH is temperature dependent. Paxton et al (1984) found that serum cortisol levels in goldfish were reduced by over 90% in cold (1°C) water when compared to the levels at 20°C. Similarly, Mazeaud (1971) examined catecholamine concentrations in carp, and found blood adrenaline and noradrenaline levels at 25°C greater than those at 10°C.

The onset of hyperglycaemia is generally found to be considerably slower than the reported speed of cortisol production, as might be expected. Chavin & Young (1970) found hyperglycaemia in goldfish 15 minutes after handling or anoxia, rising to a peak at around 1 hour. This appears to correlate with catecholamine production in the related carp, (Mazeaud 1971), where a latent period of 15-30 minutes was found before production of adrenaline and noradrenaline. This latency has been confirmed in other fish, e.g. dogfish (Butler et al 1978) and Chinook salmon (Mazeaud et al 1977). The hyperglycaemia, once developed, is reported to remain for only 6 hours in red drum after

a minor handling stress (Robertson et al 1987) and for 8 hours in brown trout (Pickering et al 1982). The results of experiments presented here suggest that the levels remained higher than basal for up to 3 days in carp. This prolonged elevation of glucose levels may not be entirely due to the single acute stress. Confinement to the smaller experimental tanks may have kept the stress response at a raised level. In addition, after transfer to the experimental tanks, which were made of clear plastic, the fish showed signs of agitation during movement in the laboratory (the 15°C holding tank was opaque). It has been reported that fish will adapt to chronic stress after a period of time. McLeay and Brown (1975) subjected Coho salmon to poor water quality and investigated carbohydrate metabolism as a stress indicator. They found hyperglycaemia after 12 hours, with levels returning to basal after 72 hours.

The use of reduced temperature (i.e. 10°C), as a chronic stressor for carp in these experiments did not appear to have a major effect. This is probably due to the acute handling stresses at the beginning of the experiment overshadowing any temperature effects. An external thermostat, enabling the water temperature to be controlled without having to transfer the fish to a different tank, could possibly have overcome this problem. However, the experiment does illustrate the difficulty of working with stress, since any manipulation of the fish, or its environment, may act as a potential stressor. For instance, Pickering and Pottinger (1987) have recently demonstrated that poor water quality can cause a decrease in plasma cortisol levels in brown trout. During a one hour recovery period, the levels returned to 'basal' - i.e. those measured in fish kept in clean water.

The measurement of serum haemagglutinins was initially designed to parallel the stress measurements, in order to investigate whether cortisol levels in the blood - a supposed indicator of stress - affected the antibody response to injected antigen. However, given the facts that only at one point was there a significant cortisol response, and that low temperature did not appear to be a significant stressor, the experiment was of little value as a direct comparison.

The difference between the antibody responses of 10°C and 20°C fish was primarily one of the timing of the response - the 10°C fish had maximum circulating antibody titres on day 27 post injection; 10 days later than the fish held at 20°C, whose levels peaked on days 14-17. The temperature dependence of antibody production in fish is well documented (e.g. Avtalion 1969, Rijkers et al 1980a, Harris 1973). Initially it was thought that fish were not able to produce circulating antibody at low temperatures. Avtalion (1969) reported no circulating antibody in carp kept at 12°C, immunised against soluble antigen (BSA), even after 200+ days. Harris (1973), working on dace at 10°C and 18°C, produced a slight increase in circulating antibody to BSA after multiple injections. However, he also demonstrated the presence of circulating antibody to human erythrocytes at a temperature of 2°C. Similarly, O'Neill (1980) worked on icefish; an Antarctic teleost, and found that antibody to MS2 bacteriophage could be induced at 2°C. Thus, fish seem able to produce antibody to certain antigens over the whole of their normal environmental limits.

The timing of the humoral response is, however, influenced by temperature. Thus, in a variety of fish species, e.g. Coho salmon (Oncorhyncus kisutch) (Paterson & Fryer 1974), carp (Rijkers et al 1980a) and goldfish (Carassius auratus) (Trump & Hildermann

1970), many workers have reported an extended period before antibodies are detected at lower temperatures.

Rijkers et al (1980a) examined the primary response of carp to sheep red blood cells at a number of different temperatures, including 12°C and 20°C; those closest to the temperatures employed in the present study. The method of antibody detection differed from the one used in this experiment, in as much as Rijkers' group used a haemolytic plaque assay to determine the number of antibody producing cells. At 12°C, Rijkers found that maximum plaque forming cell numbers were not present until day 48 post injection, and at 20°C the peak was observed at day 12. The present study disagrees with these findings to an extent, especially at the lower temperature, where a maximum titre was observed on day 27. It is possible that the time course of the present experiment did not extend far enough, and the drop in antibody levels seen after day 27 was anomalous, and that there was a continuing rising titre after this time.

At 20°C, Rijkers found a maximum titre on day 12, whereas in the present study, the peak titre was measured on day 17. On day 14 in this study, however, a titre only slightly below that of the peak was measured. Thus, at 20°C, the findings broadly agree with Rijkers.

The dose and route of administration of antigen also influences the magnitude of the immune response. Rijkers et al (1980b), experimenting with carp, found that a low dose of antigen (SRBC) provoked a poor primary immune response when compared with a higher dose (e.g. 10^9 SRBC). The lower doses however, were more efficient at inducing a better secondary response. This agrees with the results presented here, as far as the primary response is concerned. Rijkers et al (1980b) also found that the route of

administration of antigen influences the magnitude of the response; intravenous injection provoked a higher response than intramuscular injection. Intraperitoneal injection was not investigated in that study.

In general, the findings in this study agree with previous work; low temperature delays the onset of a humoral immune response, but does not affect the magnitude of the response, and, over the range tested here, high doses of antigen were more effective at inducing a response than low doses.

A major part of this project involved the study of the effects of temperature upon the phagocytosis of Candida albicans. A number of techniques were investigated before the one presented in Section (2.7) was decided upon. Enumeration of those cells with ingested C. albicans particles, using light microscopy, proved inadequate, due to the difficulty of distinguishing ingested yeast cells from those adhering to the phagocytic cells' surface. Various staining procedures were investigated to try to clarify this, none of which resulted in any significant improvement. Finally, a modification of the general methodology described by Leijh et al (1986) was used.

C. albicans was selected as a suitable particulate antigen for a number of reasons; it was unlikely that the fish had ever encountered the yeast before, it was readily available and easily cultured and handled. Previous workers have used C. albicans as a target antigen for phagocytosis studies in humans (e.g. Wood & White, 1978), and other Candida have been used in fish (e.g. McKinney et al, 1977, O'Neill 1985).

As shown in Section 3.7 the rate of particle clearance is dependent upon the <u>in-vitro</u> temperature, rather than the temperature at which the fish are maintained. This indicates that, as far as phagocytosis is concerned, there is no acclimatisation of the phagocytes to the lower temperature. O'Neill (1987) suggested that phagocytic ability at lower temperatures, in brown trout, is indicative of an acclimatisation response. However, it seems more likely that the delay in particle clearance is due to the direct effect of temperature upon the biochemical and physical mechanisms of attachment and phagocytosis.

MacArthur and Fletcher (1987), deduced, after reviewing the range of receptors to be found on the surface of fish phagocytes, that the attachment of the antigen to the phagocytic cell is a passive phenomenon. If this is the case, temperature would be unlikely to slow this part of the process in the culture system used in this study.

The process of engulfment, however, is thought to be active, and hence energy-dependent. A number of different methods of ingestion have been observed, all involving the extension of pseudopodia around the attached particle (e.g. McKinney <u>et al</u> 1977, Parish <u>et al</u> 1987). This active process is likely to be temperature dependent; lower temperatures would affect the rate of biochemical reactions and influence the fluidity of the membrane.

This temperature dependent step, however, does not explain why there should be a difference in the rate of particle clearance. <u>C. albicans</u> that were attached to phagocytes were considered to be 'cleared' from the suspension, thus the rates at 10°C and 20°C should be similar. One explanation for this could be concerned with the internalisation

of the receptors with the C. albicans and the subsequent re-expression of receptors for the antigen. If saturation level of the receptors was reached in the system used in this study, the subsequent rate of clearance would be dependent upon the rate of internalisation and re-expression of receptors.

As may be seen from Figure (9), previous in vivo contact with the antigen results in an enhanced rate of clearance of Candida particles during later in vitro tests. Macrophages may be activated directly by soluble substances, such as certain plant lectins and protein components from bacterial cell walls. However, they may also be activated by lymphokines from stimulated T-cells. A number of workers have demonstrated the presence of activated macrophages in fish, e.g. Avtalion & Sharabani (1975), Bartos & Sommer (1981) and Chung & Secombes (1987). Avtalion & Sharabani (1975) examined the mechanism behind this activation, and concluded that the attachment and ingestion process is not affected, but the intracellular killing rate is speeded up. Other studies have noted that other effects of macrophage activation include increased spreading of the cells (Secombes, 1986), increased RNA synthesis and O_2 production (Chung & Secombes 1987) and increased pseudopodial activity (Bartos & Sommer, 1981).

The tests in this study only examined one aspect of macrophage activation; that of particle clearance. The fact that the cells incubated at 10°C, and those at 20°C both showed increased clearance rates indicates that the effect of the previous exposure to antigen was either temperature independent, or took place when all the cells were at the same in vivo temperature; i.e. 15°C. The latter of these two explanations seems the most likely. Which of the two components of phagocytosis was affected by the in vivo priming

is unclear from this study, but there seems to be no reason to disagree with Avtalion & Sharabani's findings that it is the intracellular processes that are speeded up.

Throughout this study, it has been assumed that particle clearance has been achieved solely by the phagocytic cells. The presence and action of cytotoxic T-cells, natural killer (NK) cells has not been considered. This is justified by the light microscopic studies carried out, which showed the yeast particles adhering to, and engulfed by, macrophage like cells; large cells with irregular nuclei, and by neutrophils; smaller cells with lobed nuclei. There may have been some cytotoxic activity mediated by other cell types, but the phagocytic system appeared to be the most important.

Opsonisation of antigens is known to increase the phagocytic ability of phagocytes in mammals. Reports of increased phagocytosis upon opsonisation have also occurred in fish, for example in the lamprey (Fujii 1981), the brown trout (O'Neill 1987) and rainbow trout (Secombes 1986). This study attempted to opsonise C. albicans using serum from fish injected 15 days earlier. A glass slide agglutination test was performed on the serum, but no agglutination was found, even at the highest concentration of serum, possibly indicating a lack of antibody. Incubation of the Candida albicans with the supposedly immune serum did not alter subsequent clearance rates by either normal or 'armed' macrophages.

Thus, much work remains in many fields of fish immunology. Given that current trends continue, research interests will probably parallel the increase in commercial aquaculture, applying the techniques and methods available to the reduction and eradication of disease, with the provision of effective vaccines and prophylactic treatments.

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APPENDICES

APPENDIX 1

Cortisol Assay

Buffer. 0.05M, pH 8.0, Tris with 0.1% BSA and 0.1 % bovine gamma globulin. Store at 4°c.

Radioactive Label. (1 - 2 - 3H) Cortisol. 50μ Ci / ml in ethanol. Store at 4°c, discard after 3 months.

Antiserum, Rabbit. F59311074 raised against G21 - HS - 13SA. Dilute to 1: 80 in buffer and freeze in 200μl aliquots.

Label Antiserum. Evaporate 50 μl of label. Add 200 ml of buffer. Add 1 aliquot of antiserum, mix and allow to stand for 30 mins before use.

Ammonium Sulphate. A saturated solution in distilled water. Mix. Allow to settle and then filter before use.

Standards. 200μl of a stock cortisol in ethanol (5.5 nmol / l) is diluted in 10 mls of buffer to give the top standard of 11pmol / 0.1 ml. Further dilutions are made to yield 0.0069, 0.345, 0.69, 1.38, 2.75 and 5.5 pmol / 0.1ml. Store for up to 2 months at 4°c.

Assay Method

50μl of serum plus quality controls all in duplicate are diluted with 950 mls of distilled water. The diluted samples are then incubated at 60°c for 30 mins. in a water bath. After cooling, 100 μl of the denatured serum is transferred to a 6 ml scintillation minivial. 100 μl of the standards, plus totals in duplicate are also added to minivials. Add 200 μl of antiserum label mixture. Mix on vortex and then incubate for 30 mins. at room temperature. Add 200μl of saturated ammonium sulphate, mix again on the vortex, then add 2 ml of scintillation fluid (Scintran 0). Shake for 1 min., then count after temperature equilibriium in the scintillation counter.

APPENDIX 2

Maintenance Medium for *Candida albicans*

Saboraud Dextrose Agar

Glucose (Dextrose)	40g
Peptone (Mycological)	10g
Agar No. 3	20g
Distilled Water	1 litre

pH to 5.4. Autoclave at 115°c.

Phosphate Buffered Saline

NaCl	8.0g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.2g
Distilled Water	1 litre

pH to 7.2. Autoclave.