The Effect of Seasonality on the Immune Response of Rainbow Trout (*Oncorhynchus mykiss*)

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for my family

Spring is Nature's way of saying, "Let's party" ~ Robin Williams

Declaration

I, Alison Morgan, declare that this thesis has been composed by myself and that the work presented herein is my own, except where otherwise stated. No parts of this thesis have been accepted in partial or complete fulfilment of any other degree or qualification.

Signed: Date: 30th November 2004

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Abstract

The primary aim of the work carried out in this thesis was to determine if season influences the rainbow trout (*Oncorhynchus mykiss*) immune system. It is already well established that seasonality dominates the life history of fish for example in reproductive activity, food intake, locomotor activity, body weight and body condition.

To accomplish this, a twelve month trial was carried out in which a variety of innate immune and haematological parameters were measured every month (Chapter 2). Several of the parameters studied exhibited seasonal patterns. Further trials were carried out to examine the proximate cues of temperature (Chapter 3) and photoperiod (Chapter 4) and their effect on a variety of innate immune responses. These studies concluded that temperature exerted a significant effect on several of the parameters studied whereas, photoperiod, was found to have little or no effect. Furthermore, winter was generally found to have an immunosuppressive effect on the immune system.

A second twelve month trial was carried out, investigating possible methods of alleviating seasonal immunosuppression. The effect of melatonin on the immune system of *O. mykiss* was examine, firstly to determine if it acts as an immunostimulant and secondly to investigate if it improves vaccination protection. The results of this trial generally corroborated those of the Base Level Trial (Chapter 2) in that generally, parameters were suppressed in winter, with the exception of haematocrits which were suppressed in summer.

To investigate if the immune system of rainbow trout exhibits a circadian rhythm a trial was carried out where a variety of innate immune and haematological parameters were measured every 6 hours over a 24 h period (Chapter 6). None of the innate immune parameters studied were observed to exhibit a circadian rhythm.

It is anticipated that this research investigating the seasonal effects of natural and artificial photoperiods and temperatures on immune function will be of benefit to the aquaculture industry. It will provide information that will allow administration of commercial diets containing functional supplements to be timed effectively and will facilitate our understanding of the epidemiology of specific fish pathogens.

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Glossary of Common and Scientific Names

African Catfish	*Clarias gariepinus* (Burchell, 1822)
Atlantic Salmon	*Salmo salar* (Linnaeus, 1758)
Brown Trout	*Salmo trutta* (Linnaeus, 1758)
Carp	*Cyprinus carpio* (Linnaeus, 1758)
Channel Catfish	*Ictalurus punctatus* (Rafinesque, 1818)
Chicken	*Gallus domesticus* (Linnaeus, 1758)
Chinook Salmon	*Oncorhynchus tshawytscha* (Walbaum, 1792)
Common Vole	*Microtus arvalis* (Pallas, 1779)
Dab	*Limanda limanda* (Linnaeus, 1758)
Deer Mouse	*Peromyscus maniculatus* (Wagner, 1845)
European Whitefish	*Coregonus lavaretus* (Linnaeus, 1758)
Golden Hamster	*Mesocricetus auratus* (Waterhouse, 1839)
Grass Carp	*Ctenopharyngodon idellus* (Valenciennes, 1844)
Gulf Killifish	*Fundulus grandis* (Baird and Girard, 1853)
Halibut	*Hippoglossus hippoglossus* (Linnaeus, 1758)
Jundiá Fish	*Rhamdia quelen* (Quoy and Gairnard, 1824)
Japanese Quail	*Coturnix coturnix* (Linnaeus, 1758)
Lumpsucker	*Cyclopterus lumpus* (Linnaeus, 1758)
Nile Tilapia	*Oreochromis niloticus* (Linnaeus, 1758)
Norway Rat	*Rattus norvegicus* (Berkenhout, 1769)
Pike	*Esox lucius* (Linnaeus, 1758)
Plaice (North Sea)	*Pleuronectes platessa*(Linnaeus, 1758)
Rainbow Trout	*Oncorhynchus mykiss* (Walbaum, 1792) (formerly *Salmo gairdneri* (Richardson, 1836))
Rat	*Rattus rattus* (Linnaeus, 1758)
Sea Bream	*Sparus aurata* (Linnaeus, 1758)
Snapper	*Pagrus auratus* (Bloch and Schneider, 1801)
Sockeye Salmon	*Oncorhynchus nerka* (Walbaum, 1792)
Syrian Hamster	*Mesocricetus auratus* (Waterhouse, 1839)
Tench	*Tinca tinca* (Linnaeus, 1758)

Abbreviations

Ø	Diameter
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumen
°C	Degrees Celsius
CFRV	The killed cell-cultured vaccine against Fish Reovirus
CFU	Colony Forming Unit
DMEM	Dulbecco's Modified Eagle's Media
DMSO	Dimethylsulphoxide
ELISA	Enzyme Linked Immunosorbent Assay
EUS	Epizootic Ulcerative Syndrome
g	Gram
h	Hour
HSW	High Salt Wash
IFN-gamma	Interferon Gamma
Ig	Immunoglobulin
IL-1	Interleukin-1
IL-12	Interleukin-12
Kg	Kilogram
L-15	Leibowitz-15 Media
LD	Light Dark
LPS	Lipopolysaccharide
LSW	Low Salt Wash
M	Molar
MAF	Macrophage Activating Factor
mg	Milligram
MHC	Major Histocompatibility Complex
min	Minute
ml	Millilitre
mM	Millimolar
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAT	*N*-Acetyl-Transferase

NK	Natural Killer Cells
NBT	Nitrobluetetrazolium
OD	Optical Density
PBS	Phosphate Buffered Saline
PI	Phagocytic Index
PKD	Proliferative Kidney Disease
PMA	Phorbol 12-Myristate 13-Acetate
PR	Phagocytic Ratio
ProPo	Prophenoxidase System
RBC	Red Blood Cell
RIA	Radioimmunoassay
SCN	Suprachiasmatic Nuclei
SD	Standard Deviation
SE	Standard Error
SVC	Spring Viraemia of Carp
Th	T Helper Cells
TNF-alpha	Tumor Necrosis Factor-alpha
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
U	Unit
WBC	White Blood Cell
μl	Microlitre
μm	Micrometer

Chapter 1 Introduction

1.1 General Introduction

Seasonality dominates the life history of fish. It co-ordinates reproductive activity, affects body weight and condition, affects food intake and locomotor activity and it co-ordinates the immune system. All of these events are synchronised with seasonal changes in climate, daylength and food supplies (Bromage *et al.,* 2001). This response of eurythermal fish to the cyclic seasonal changes of the environment is a complex mechanism that demands the sensing of physical parameters (e.g. temperature and photoperiod) and the corresponding transduction into molecular signals (Molina *et al.,* 2002). It is known that fish, together with most other known seasonally responsive animals, rely on cues from the environment e.g. photoperiod and temperature. These cues are described as 'proximate' and are the environmental conditions that give an animal the greatest chance of survival (Bromage *et al.,* 2001).

Many organisms display a seasonal response be it physiological, behavioural or both. Studies have shown dramatic seasonal changes in the behaviour of salmonids, for example, in summer, they have been observed to forage during the day, but in winter they seek refuge and are found buried in the gravel bed of their home river or hiding amongst vegetation. Night-time observations in winter have shown, however, that these fish emerge from their daytime sheltering places. It has therefore been suggested that they switch from being predominantly diurnal in the summer to being nocturnal in winter (Valdimarsson *et al.,* 1997). Fish display a strong association to season in their breeding strategies, with young fish being produced under the most favourable environmental conditions. Juveniles of high latitudes are produced in spring, a time of increasing photoperiod and temperature coinciding with an increase in food availability.

Whereas, fish of tropical and sub-tropical regions are produced following seasonal rainfall or changes in oceans currents, again coinciding with a seasonal increase in food availability (Bromage *et al.,* 2001). Immunity is another function in an organism's life history that is thought to be influenced by season. In recent years this area has received increasing interest particularly in mammals (Nelson, 2004).

1.2 The Fish Immune System

During recent years considerable progress has been made in describing and understanding the immune system of fish. Gnathostomes, but not agnatha, contain thymus, spleen and gut-associated lymphoid aggregates, while the bone marrow appears for the first time in urodeles. There is functional and phenotypic evidence of lymphocyte heterogeneity in all vertebrates (Zapata *et al.,* 1996). Fish above the level of the agnatha display typical vertebrate immune responses characterised by immunoglobulins, T-cell receptors, cytokines and major histocompatibility complex molecules. However, the immune system of fish is quite different in its efficiency and complexity from that of higher vertebrates, and is much simpler than that of mammals (Warr, 1997). Recent investigations of the teleost immune system have used similarities with the more characterised defence systems of higher vertebrates to identify genes and gene products. However, while genetic differences may be small and some molecular and cellular agents similar, the morphology, i.e. the structure and form of the immune system, is quite different between fish and mammals (Press and Evenson, 1999). Unfortunately, our knowledge is sufficiently limited that we do not yet have a full understanding of the reasons for this difference (Warr, 1997).

Immunity is generally considered to be mediated by two major systems: the innate (non-specific) system and the adaptive (specific) immune system. The innate system is thought to be of ancient origin, while the adaptive immune system is characteristic of vertebrates above the level of agnathan fish (Warr, 1997). The innate immune response is often regarded as the primary line of defence in the fish immune system. If the pathogen successfully penetrates that first line of defence, it is then dealt with by the various components of the adaptive immune system. In both branches of the immune response, humoral and cellular components are present. This division of the immune system into innate and adaptive systems is simply to create a more concise understanding of immunoregulatory mechanisms. However, in reality they both react with each other and are interdependent (Köllner *et al.*, 2002).

In fish, the first line of defence against infectious microorganisms is based on the innate system, which without prior specific activation can act in forming a more static barrier. This natural resistance is normally effective enough to protect fish from infectious diseases until adaptive immune responses are induced. It has been hypothesised that healthy fish exhibit both innate and adaptive immune responses depending directly on environmental temperature (Köllner *et al.*, 2002).

1.2.1 The Innate Immune Response

Innate immunity is often thought to be more important in fish than adaptive immunity (Ellis, 2001), because it has been proposed that it replaces adaptive immunity at low temperatures (le Morvan *et al.,* 1996). The innate immune system consists of multiple components. Typically it includes inflammation, phagocytosis, release of cytokines, natural killer cell activity, non-classical complement activation, release of acute phase proteins and the production of broad spectrum antimicrobial substances, frequently mediated through peripheral blood leucocytes (Smith *et al.,* 2000). Fish are in intimate contact with their environment, which can contain very high concentrations of bacteria and viruses. Many are saprophytic, some are pathogenic and both bacteria and viruses are capable of digesting and degrading the fish's tissues. However, under normal conditions the fish maintains a healthy state by defending itself against these potential invaders though the innate immune system. These mechanisms are both constitutive and responsive and provide protection by preventing the attachment, invasion or multiplication of microbes in or on the tissues. The protection is innate and does not depend upon recognition of the distinctive molecular structure of the invading species. Secondly, there is little or no time lag for them to act. However, even as recently as 2001, the innate system was considered to be unaffected by water temperature (Ellis, 2001). For ectothermic vertebrates, these characteristics are very important because the adaptive immune system defences take considerable time to respond (Ellis, 2001). It is well established that the adaptive system is very temperature dependent. Therefore the innate responses may be described as being more important in fish than in endothermic vertebrates. Indeed some of them such as lysozyme activity and complement appear to be more potent in fish than in mammals (Ellis, 2001).

Physical barriers include the skin or scales of fish that vary from the thin layers of certain tropical species to the leather-like hides of sharks. Unless damaged, the skin provides ample protection against pathogenic invaders (Weber, 1999). Mucus also acts as a method of defence (Alexander and Ingram, 1992). Lysozymes are synthesised in both the liver and extra-hepatic sites, however the kinetics of synthesis and secretion are not yet clear (Bayne, 2001). Cell lysis is also accomplished by complement, following activation by immunoglobulins or other materials. Complement interacts with lysozyme activity and other components of fish mucus, such as C-reactive protein and it is possible that many significant facets of the 'complement cascade' are initiated in the mucus (Shephard, 1994). In addition, fish tissues and body fluids contain naturally occurring proteins or glycoproteins of a non-immunoglobulin (Ig) nature that react with a diverse array of environmental antigens and may confer an undefined degree of natural immunity to fish (Alexander and Ingram, 1992).

1.2.2 The Adaptive Immune System

The defining characteristics of the adaptive immune system are "exquisite specificity and memory" (Warr, 1997). The essential cells of adaptive immunity are the lymphocytes. One type of lymphocyte is thymic-derived (the T-cells) which recognises fragments of antigen bound to molecules encoded in the major histocompatibility complex (MHC). The MHC molecules that present processed antigens to T cells occur on most cell types, however antigen-presenting cells tend to be specialised, namely; macrophages, dendritic cells or B-lymphocytes. B-lymphocytes produce and secrete antibodies (known collectively as immunoglobulins Igs). T cells, in contrast, have multiple functions (mediated by different T-cell subsets) the two most important of which are direct killing of target cells and co-operating with B cells to help their

production of antibodies. The interactions between T cells, B cells, and other cells involved in immune reactions, are mediated by at least two other classes of molecule: (1) cell surface molecules (other than MHC, TCR or Ig) that are involved in cell-cell adhesion and signal transduction; and (2) cytokines (including the interleukins) which are polypeptide hormones that mediate cell growth division and differentiation in the immune system (Warr, 1997).

1.3 Seasonality of Disease Prevalence

In addition to the well-documented seasonal cycles of mating and birth, there are also significant seasonal cycles of illness and death among many animal populations. Challenging winter conditions (i.e., low ambient temperature and decreased food availability) can directly induce death via hypothermia, starvation or shock. Many environmental challenges are recurrent and thus predictable. Animals could enhance their survival, and presumably increase their fitness, if they could anticipate immunologically challenging conditions in order to cope with these seasonal threats to health (Nelson *et al.,* 1995). As the seasons change animals face alterations in environmental stressors. In particular, the prevalence and intensity of pathogenic infection are often seasonal.

Seasonal fluctuations of illness and death among humans and non-human animals have been recognised for centuries (Table 1.1). Hippocrates observed that "in autumn, diseases are most acute, and most mortal on the whole". The spring is the most healthy, and least mortal (Nelson *et al.,* 2002). Fish also exhibit these seasonal fluctuations in disease prevalence. In a study of fish farms and rivers of northeast Spain, seasonal patterns of bacterial and viral diseases were revealed (Ortega *et al.,* 1995).

Bacterial diseases were found to be more prevalent during periods of high temperature, whereas viral infections were highest during seasons with low temperatures. Importantly the periods of greatest risk for any diseases were times of substantial temperature change i.e. spring and autumn (Ortega *et al.*, 1995).

Table 1.1 Seasonal variation in peak prevalence of human illness and disease (adapted from Nelson, 2004)

Disease	Peak Prevalence
Malaria	Winter-early spring
Influenza	Winter-early spring
Human reovirus	Winter
Respiratory syncytial virus	Winter-early spring
Coronaviruses	Winter-summer
Enteroviral infection	Summer
Tuberculosis	Winter
Legionnaires disease	Summer
Brucellosis	Spring-early summer
Pneumonia	Winter-spring
Coronary heart disease	Winter
Stroke	
Cerebral infarction	Spring-summer
Ischemic attacks	Winter-spring
MS	Spring-summer
IDDM	Autumn-winter
Rheumatoid arthritis	Autumn-winter
Breast cancer	
No. of cases diagnosed	Winter
Initial detection	Spring-summer
Risk of death	Summer
Season of removal	Winter
Season of birth	Summer
Lung cancer	Summer-Autumn
Melanoma	Spring-summer
Urinary bladder carcinoma	Autumn-winter

Vibrio salmonicida is an example of a bacterial disease that has a seasonal pattern. Also known as cold water vibriosis, it is generally considered to be non-pathogenic at water temperatures above 10°C. It is a haemorrhagic septicaemia that has remained an important disease in Norwegian marine salmonid aquaculture since its first recorded occurrence in 1977 (Colquhoun and Sørum, 2001). Cold water disease, as the name suggests, is commonly observed when the fish are held in cold water typically at temperatures between 4 and 10°C and it is caused by the Gram negative Myxobacterium *Flavobacterium psychrophilum* (Nematollahi *et al.,* 2003).

Fish parasites can also exhibit seasonal patterns of infection (Gbankoto *et al.,* 2001; Clifton-Hadley *et al.,* 1986). For example, the myxosporean parasite *Myxobolus sp.* demonstrated a clear seasonal pattern in the gills of two tilapia species from Lake Nokoué (Bénin, West Africa). In this case it was found that these patterns could not be directly explained by temperature variations. Instead, it was found to be due to fluctuations in salinity, temperature, and pH between dry and wet seasons probably affecting the parasite cycle by causing modifications in host behaviour (Gbankoto *et al.,* 2001). Proliferative Kidney Disease (PKD) is a temperature dependent disease of freshwater salmonid fish. Studies have shown that naturally infected fish subsequently held under laboratory conditions had clinical PKD at 12-18°C but not at 9°C (Clifton-Hadley *et al.,* 1986). Water temperature regimes have been used to prevent the occurrence of PKD. Rainbow trout were exposed to the parasite for 4 weeks in May at an average temperature of 15°C and then kept at 12°C for a year under laboratory conditions. No mortalities were observed in the fish and furthermore, when re-exposed to enzootic water at a permissive temperature the fish did not develop PKD (de Kinkelin and Loriot, 2001).

Fungal infections have also been shown to demonstrate a seasonal pattern. Epizootic ulcerative syndrome (EUS) outbreaks across Asia-Pacific usually occur during the colder seasons of the year when the temperature is below 25°C. It is not clear whether this results from a reduced immunity in fish at low temperatures making them more prone to infection or from reduced infectivity of the pathogen at higher temperatures or a combination of both (Miles *et al.*, 2001).

Some viral infections are also documented to have seasonal patterns of occurrence. Spring viraemia of carp (SVC) for example, shows a seasonal pattern of prevalence. SVC is a disease of several species of cyprinid fishes caused by *Rhabdovirus carpio*. It typically occurs when water temperatures are below 18°C and is most common in the spring. At 20-22°C infection occurs but clinical disease does not develop (Jeney and Jeney, 1995).

A suggested hypothesis adapted from Nelson *et al.* (2002), would be that the complex interactions between ambient temperature and altered immunocompetence throughout the seasons will likely lead to seasonal changes in disease and death among fishes.

1.4 Seasonal Fluctuations in Immune Function

Studies have produced evidence that infection is cyclical with the seasons and that this phenomenon is mirrored in cycles of immune function (Nelson *et al.,* 2002). Changes most often reported include; histological changes in the lymphoid system, changes in the number of circulating lymphocytes and their response to mitogens, and changes in resting antibody titre and response to antigenic challenge. In general, all parameters are suppressed in winter and are highest in summer. This pattern, and the very nature of ectothermic metabolism, has led many researchers to investigate the role of temperature in the seasonal variation of immune response (Slater and Schreck, 1998).

The habitat of most organisms is subject to pronounced seasonal fluctuations. Literally, all physical environmental factors important to an organism, such as; temperature, daylength, and rainfall, vary with season. Animals have, therefore, to adapt themselves to these more, or less, large fluctuations of the environment, depending upon latitude (Csernus *et al.,* 1998).

It is well documented that environmental parameters like temperature and dissolved oxygen affect the immune system of crustaceans. An increase in temperature has been reported to increase total haemocyte counts in several crustaceans, and high temperature is known to activate the prophenoxidase (ProPo) system (Johansson and Söderhäll, 1989). A relationship between salinity and infectious hypodermal and haematopoetic necrosis has been documented recently (Cheng and Chen, 2000). Consequently, there is increasing interest in the possibility that seasonal neuroendocrine rhythms govern the immune system of ectothermic vertebrates. In addition, the underlying mechanisms may also be of relevance to environmental modulation, such as

the immunomodulatory effects of low temperatures, stress, and pollution seen in fish. Immune responses of ectothermic animals are known to vary seasonally. Changes most often reported include histological changes in the lymphoid system (Nakanishi, 1986).

Most studies on the effect of temperature on the immune system of ectothermic vertebrates do not take the seasonal period into account. Even at a constant temperature, seasonal variations affect the structure and function of the ectotherm immune system, possibly because of the influence of neuroendocrine rhythms on immunity (Collazos *et al.,* 1994a). Seasonal variation, affecting the structure and function of the ectotherm immune system, is an excellent 'natural' model of the influence of neuroendocrine rhythms on immunity (Zapata *et al.,* 1992).

1.4.1 Leucocyte Responses

Immune responses are produced primarily by white blood cells. In a study carried out by Collazos *et al.* 1998, it was found that leukocyte counts for both male and female tench were significantly lower in winter and spring when compared with summer and autumn. Shortened daylength may be priming the immune system for winter, since the highest white cell count was observed in the autumn (Weber, 1999).

It has been revealed that there are statistically significant changes throughout the year in the number of lymphocytes in the lymphoid organs (Álvarez *et al.,* 1998). The spleen and pronephros have similar annual patterns of lymphocyte distribution with high numbers in spring and autumn and two periods of lymphoid involution in summer and winter (Álvarez *et al.,* 1998). In wild brown trout the highest numbers of thymocytes have been shown to occur in trout caught in May and August, and the lowest in winter (Álvarez *et al.,* 1998). In addition to normal lymphocytes, degenerated

lymphoid cells that show pale cytoplasm devoid of cell organelles also occurred in all the lymphoid organs. A negative correlation exists between the numbers of normal lymphocytes and that of degenerated lymphoid cells (Álvarez *et al.,* 1998).

1.4.2 Lysozyme Activity

Lysozyme is an antibacterial enzyme that catalyses the hydrolysis of (glycosaminoglycan) walls of bacteria, leading to rupture and death of the remaining protoplast (Abercrombie *et al.,* 1992). The enzyme predominantly attacks Gram positive bacteria, and to some extent, also Gram-negative bacteria. Lysozyme is localised in the lysosomes of neutrophils and macrophages and is released into the blood from these cells. Although the kidney appears to be the major site of lysozyme activity in teleosts, the blood is recommended as a more practical, less variable tissue for monitoring lysozyme activity in fish (Hutchinson and Manning, 1996). Seasonal changes in lysozyme activity have been reported in lumpsuckers, Fletcher *et al.* (1977), observed that between February and June there was a statistically significant decrease in lysozyme activity in the sera of males, but an increase in the females. This seasonal change was hypothesised to be related to the stage of the breeding cycle. In dab it has been shown that there is a generally consistent seasonal trend in serum lysozyme activity, with low values being associated with reduced sea temperatures, time of spawning and poor condition factor (Hutchinson and Manning, 1996). These results are in general agreement with the seasonal trends of serum lysozyme activity measured in North Sea plaice (Fletcher and White, 1976).

1.4.3 Mortality Trends

Cold weather and the associated short photoperiod of winter, environmentally 'programs' fish for reduced activity and food intake, and they do not respond to stressors with increased feeding. If the elevated metabolic demands persist, stored body lipid necessary for overwintering is depleted, body condition drops and the fish may die (Lemly, 1996). As the energetically challenging conditions can directly induce death via hypothermia, starvation or shock; surviving these demanding conditions likely puts individuals under great physiological stress (Nelson and Demas, 1997).

1.4.4 Phagocytosis

Phagocytosis is considered to be a prominent mechanism in antimicrobial natural defences (Michel and Hollebecq, 1999). Phagocytic cells include tissue macrophages, circulatory monocytes and neutrophils (Chen *et al.,* 1996). Phagocytosis is the process whereby the plasma membrane of a cell encloses a particle in the external environment and traps it within a food vacuole. This is normally converted to a heterophagosome as lysosomes fuse with it and enable digestion of the contents (Abercrombie *et al.,* 1992).

Few data are available with regard to the importance of phagocytosis in the defence mechanisms at low temperatures. In channel catfish the primary impact of temperature on phagocyte function was concluded to be due to the assay temperature, although phagocytes appeared to be more resistant to low temperature than lymphocytes (Scott *et al.,* 1985; Ainsworth *et al.*, 1991). However, adaptation to low temperature did lead to an improvement in the respiratory burst activity, which in turn would imply a greater bacterial killing ability. This greater effectiveness of phagocytosis at low

environmental temperature has also been reported in tench (Collazos *et al*, 1994a). In addition, macrophages isolated from rainbow trout, cultured at low temperatures were responsive to macrophage activating factor and showed a higher relative increase in respiratory burst activity compared to counterparts cultured at higher temperatures (Hardie *et al.*, 1994). This information emphasises the potential importance of innate immune functions with regard to the susceptibility of fish to disease at low temperatures (le Morvan *et al.*, 1997).

1.4.5 Specific Antibody Titre

The general view is that higher temperatures enhance the adaptive immune response whereas lower temperatures inhibit it (Ellis, 2001). Hoare *et al.* (2002), observed that anti-*Vibrio* lipopolysaccharide (LPS) antibody levels of halibut increased with temperature. This observation is consistent with findings for other teleost species including carp, (Rijkers *et al.*, 1980; le Morvan *et al.*, 1996) and dab (Secombes *et al.*, 1991). This is corroborated by Avtalion *et al.* (1970), who demonstrated that carp immunized against bovine serum albumin had a suppressed primary antibody response at low temperatures. However, the secondary response can be elicited if immunological memory is established at high temperatures (Avtalion *et al.*, 1970).

1.4.6 Thymus Size

The teleost thymus is considered to be analogous to the mammalian thymus, and is therefore thought to constitute an antigen-free thymic micro-environment in which thymocytes mature (Castillo *et al.*, 1998). The size of the thymus in wild brown trout, as well as that of the subcapsular, inner and outer thymic zones, undergoes very significant changes over the year (Álvarez *et al.*, 1998). The thymic area has been shown to have

three periods of maximum development: spring, September and mid-late autumn (November and December), while in March has the smallest rate of development for the year. The development observed in the subcapsular zone in December-January, and between June-October was interrupted by two periods of minimal development from February to April and in November. The inner zone (which always shows the greatest level of development) reaches its maximum size in April and November and the minimum in January and August. The outer zone has its maximal values in winter, between December-March, with low values over the rest of the year (Álvarez *et al.*, 1998).

1.5 The Effect of Temperature on the Immune System of Fish

Fish are poikilothermic animals, and as such are subject to the changes in temperature in which they live, as they are unable to regulate their internal temperature. Therefore, the levels of circulating blood cells and proteins can be affected by seasonal cycles. In particular, it has been observed that during cold periods, poikilothermic animals suffer immunodepression due to the low temperature (Collazos *et al.*, 1998). Although water temperature is known to be an important regulator of the immune response of fish, its effect has been investigated in very few species. It has been suggested that the innate immune system is not affected by temperature, whereas the adaptive system is dependent upon it (Ellis, 2001).

In a study examining the effects of rearing temperature on immune functions in sockeye salmon the results obtained suggested that the immune apparatus of sockeye salmon reared at 8°C relied more heavily on the innate immune response, while the adaptive immune response was used to a greater extent when the fish were reared at

12°C. However, in this particular study a seasonal effect was not detected, although fish maturation affected the results (Alcorn *et al.,* 2002). Temperature has been shown to affect T and B cell function, antibody production and has been implicated in serum lysozyme activity and C-reactive protein fluctuations. Studies have demonstrated seasonal differences in immune response, even when temperature remained constant, suggesting that other factors such as photoperiod may be important. Furthermore, other studies have demonstrated continued seasonal variations in immune response even when both temperature and photoperiod have been held constant. It has been hypothesised that seasonal variations in the immune system may be driven by endogenous endocrine rhythms (Slater and Schreck, 1998).

It is generally accepted that higher temperatures enhance immune responses whereas lower temperatures adversely affect their expression. The fact that some fish like catfish and carp can survive extremely low temperatures implies that there is a means of defence other than adaptive immunity (le Morvan *et al.,* 1997).

Winter is energetically demanding and stressful; thermoregulatory demands increase when food availability usually decreases. The stress of coping with energetically demanding conditions may increase adrenocortical steroid levels that could indirectly cause illness and death by compromising immune function (Nelson and Demas, 1997).

1.6 The Effect of Photoperiod on the Immune System of Fish

The annual cycle of changing photoperiod provides an accurate indicator of time of year and thus allows immunological adjustments prior to the deterioration of conditions (Nelson *et al.,* 1995). It may be considered to be the most reliable proximate cue in terms of predictability. In recent years, researchers have found an association between the duration of environmental light and some immune parameters of birds (Moore and Siopes, 2000).

To date very little research has been carried out on the effect of photoperiod on the immune response of fish. Initial research suggests that a prolonged change in the natural photoperiod adversely affects the immune function of rainbow trout (Leonardi and Klempau, 2003). This was illustrated by the elevated cortisol levels of the experimental trout in this study, suggesting an effect due to stress. This is corroborated by Olsen *et al.* (1993), who hypothesised that cortisol levels during the smolting period of Atlantic salmon are probably controlled more by photoperiod than by endogenous rhythms.

Short day lengths appear to be more effective at mediating immune function in individuals with robust reproductive responses to photoperiod (Nelson *et al.,* 1995). For instance, splenic weights of deer mice were reduced in short days (Demas and Nelson, 1998). Splenic masses, total splenic lymphocyte numbers and macrophage counts were significantly higher in hamsters exposed to short days as compared to animals exposed to long photoperiods (Yellon *et al.,* 1999). Animals maintained on short day lengths (8:16 LD) possessed more white blood cells than animals maintained on long day

lengths (16:8 LD); neutrophil numbers were unaffected by day length in adult female mice (Nelson *et al.*, 1995).

Most of the research in mammals has indicated that on short photoperiods both the cellular and the humoral components of the immune system were enhanced compared to mammals kept on long photoperiods (Mahmoud *et al.*, 1994; Blom *et al.*, 1994; Nelson and Blom, 1994; Demas *et al.*, 1996; Demas and Nelson, 1996). The pineal hormone melatonin codes day length information (Nelson *et al.*, 1995) and may be the mediator of these photoperiodic effects due to its indirect effects on immune function photoperiods (Moore and Siopes, 2000).

1.7 Proximate Mechanisms Underlying Seasonality

In recent years, it has become increasingly apparent that the mammalian immune and neuroendocrine systems are intimately linked and that bi-directional communication between the two is essential for the maintenance of homeostatic function (Table 1.2) (Harris and Bird, 2000).

Many fish species go through distinct life cycle stages that are associated with changes in levels of circulating hormones. The interactions between different hormones are often complex, but in many cases changes in plasma hormone levels correspond with changes in the immune status and the health of the fish. The modulatory effects of hormones on fish immune responses may have important implications for fish health and aquacultural practice and the study of these effects may lead to a better understanding of the interactions between the immune and endocrine systems in other

Table 1.2 Neurotransmitters/hormone with immunomodulatory properties found in humans (taken from Roberts, 1995)

Hormone/Neurotransmitter	Effect
Immune Enhancers	
Prolactin	Macrophage activation, proliferation of NK cells, IL-2 Production.
Growth Hormone	Activates antibody synthesis, IL-2 production.
Somatostatin	Proliferation of T, NK B cells and macrophages.
Vasointestinal Protein	Proliferation of T, NK, and macrophages.
Substance P	Proliferation of T, NK, and macrophages.
α Melanin stimulating hormone	Proliferation of NK cells, downregulates IL-1, TNF-alpha, upregulates IL-10.
Thyroxine	T cell activation
B-Endorphin	Activates T and macrophages, suppresses B cells.
Acetylcholine	Stimulates T and NK cells increases γ-IFN.
Melatonin	Activates T, NK and B cells, upregulates IL-2.
Seratonin	T-cell proliferation.
Dopamine	Stimulates T and NK through acetylcholine stimulation.
Oestrogen	Promotes γ-IFN activates autoimmune response.
Immune Suppression	
Serotonin	Impairs T, NK, macrophages, blocks antibody production, inhibits IL-4; Ig-E.
Epinephrine/norepnephrine	Blocks IL-1; IL-2.
Testosterone	Impairs immune function through enhanced cortisol production.

animals, including mammals. There is increasing interest in the possibility that seasonal neuroendocrine rhythms govern the immune system of ectothermic vertebrates. It has been documented that even at a constant temperature, seasonal variations occur in the fish humoral immune responses (Collazos *et al.*, 1994a).

1.8 Melatonin

The hormone melatonin is synthesised in the pineal gland and retinas of many vertebrate species. In many non-mammalian vertebrates, the hormone producing cells of the pineal organ are the directly light sensitive, pineal photoreceptors. Galen (130-200AD) documented the pineal gland first. It has been a subject of interest and speculation for more than 2000 years, since the Greek anatomists observed that the pineal gland as an unpaired structure in the brain. The French philosopher Descartes proposed an important role for this organ in brain function and regarded it as the "right hand" of the soul (after Zrenner, 1985). In 1958, Lerner and his co-workers were able to isolate melatonin from bovine pineals, initiating the modern era of pineal research (Lerner *et al.*, 1958). The pineal organ is now considered as a component of the circadian system in vertebrates and is thought to be involved in the timing and control of rhythmic functions and behaviours (Hastings *et al.*, 1989). This seems to be related to the ability of the pineal organ to transform environmental stimuli, mainly photoperiodic information, into nervous and hormonal signals. Among the hormonal signals, the indoleamide melatonin is considered to be an internal "zeitgeber" (time-giver) in vertebrates (Armstrong, 1989; Falcón and Colin, 1989; Underwood, 1989; Zachmann *et al.*, 1992).

The pineal gland is a neural structure in vertebrates that transduces environmental information into rhythmic endogenous signals via periodic secretion of its hormone melatonin (Okimoto and Stetson, 1999). The pineal organ and the retina of vertebrates are two components of the circadian system, primarily influenced by the light : dark (L:D) cycle, which are involved in the control of various rhythms (circadian, circannual). Both the pineal and retina produce the neurohormone melatonin in a rhythmic manner. The nocturnal rise in melatonin production results from the increase in the activity of serotonin *N*-acetyl-transferase (NAT), which catalyses the conversion of serotonin to *N*-acetylserotonin. This compound is then methylated by hydroxyindole-*O*-methytransferase to give melatonin (Begay *et al.,* 1994).

Melatonin has been studied extensively in vertebrate endocrinology and reproductive physiology. Initially, the contention that melatonin is a hormone was based on circumstantial evidence (Wurtman *et al.,* 1968) until the demonstration and quantification of melatonin in the chicken serum by bioassay and gas chromatography-mass spectrometry (Pang, 1985).

1.8.1 Environmental Effects on Melatonin

1.8.1.1 Light

Environmental lighting is a major regulatory factor of melatonin synthesis and secretion in the pineal gland (Pang, 1985). It is obvious that melatonin has direct links with seasonality as its production is controlled by photoperiod. Consequently, it may cause an effect on the immune response that can be associated with seasonality.

Melatonin is a hormonal signal for photoperiod and is intricately involved in many aspects of circadian and seasonal physiology (Falcón, 1999). In many vertebrates

the pineal gland converts information relating to the daily light or dark cycle into a daily rhythm of melatonin secretion, with levels low during the day and raised for all or part of the night (Randall *et al.,* 1995). Circadian oscillators located within the pineal are reset daily by the ambient light: dark cycle (Okimoto and Stetson, 1999). It has been suggested that the change in light intensity between light and dark phases is more important as an environmental zeitgeber than absolute intensity or wavelength (Kadri *et al.,* 1997). Melatonin peaks at night, even in nocturnal animals (Reiter, 1986). The duration of the nocturnal increase in pineal and circulating melatonin is generally proportional to the length of the night (Randall *et al.,* 1995). Consequently, daily and seasonal fluctuations of melatonin occur as the amount of light decreases in the autumn and increases in the spring, serving to regulate the circadian clock (Arendt, 1998). In certain photoperiodic vertebrates, it is this feature of the melatonin signal which determines the timing of a variety of seasonal events (Randall *et al.,* 1995). Many, if not most, biochemical, physiological and behavioural events are rhythmic. Rhythms with a periodicity of 24 h (daily) or one year (annual) represent major components in the adaptation of organisms to their environment.

Environmental factors such as light and temperature play an important role in the synchronisation of these rhythms. In vertebrates, synchronisation is mediated through the circadian system, which is composed of sensors and circadian oscillators. These include the lateral eyes, the suprachismatic nuclei of the hypothalamus and the pineal organ (Falcón *et al.,* 1989).

1.8.1.2 Temperature

Apart from illumination, pineal melatonin is, apparently, affected by other diverse environmental conditions. One of the most common environmental changes encountered by animals is the daily or seasonal temperature variability.

In poikilothermic vertebrates, the pineal and plasma rhythms have been shown to be thermosensitive as well as photosensitive. It has been suggested that with regard to the poikilotherm's pineal gland, photoperiod controls the duration of the nocturnal rise in melatonin and temperature regulates the amplitude of the daily rhythm, and in the case of very low temperatures (during the winter), the melatonin rhythm disappears (this has been observed in the frog, *Rana perezi* (Delgado and Vivian-Roels, 1989). This dual regulation of melatonin synthesis would have an adaptive significance considering that very similar photoperiods, but different temperatures are experienced by animals twice during the seasonal cycle (Delgado *et al.*, 1993).

1.8.2 Melatonin Rhythms in Salmonids

Pronounced seasonal changes have been described for pineal *N*-acetyl-transferase activity and pineal/plasma melatonin levels in many mammals (Steinlechner *et al.*, 1991). In some reptiles and amphibians dramatic seasonal variations in the amplitude of the nocturnal increase in pineal and circulating melatonin have been reported (Mayer, *et al.*, 1997). These variations appear to be related to seasonal changes in the environmental temperature, with the abolition of melatonin rhythms occurring in several species at low temperatures. In contrast, a study carried out by Randall *et al.* (1995), found well-defined melatonin rhythms were always present in Atlantic salmon, irrespective of time of year, photoperiod and temperature. The study also suggested that

daylength per se did not influence the amplitude of the nocturnal increase in melatonin concentrations. However, night-time melatonin levels did tend to be higher in samples taken in June and August, when water temperatures ranged from 13 to 19.5°C, than during the other sampling periods when water ranged from 1.75 to 8.25°C.

Studies *in vitro* in the rainbow trout have demonstrated that pineal NAT activity and melatonin production are temperature dependent (Max and Menaker, 1992). The activity of pineal ganglion cells is also temperature dependent in the rainbow trout (Randall *et al.,* 1995). Thus, the pineal of the Atlantic salmon and other salmonids may be able to integrate information on both photoperiod and temperature (Randall *et al.,* 1995).

1.8.3 The Role of Melatonin

Melatonin is a molecule to which an unusually large number of functions and have been ascribed (Gern and Karn, 1983). These functions include pigment aggregation and calcium-activated dopamine release in the retina, (Vernadakis *et al.,* 1998). Experiments carried out to ascertain the different functions of melatonin, have generated somewhat contradictory results as its function can differ from species to species (Hoffmann, 1981a). This has been shown in experiments where melatonin has been either implanted or injected into animals of different species. Some of these results are listed in Table 1.3. Unfortunately, only a few studies have examined melatonin effects in non-mammalian vertebrates (Gern and Karn, 1983).

Table 1.3 Some melatonin effects listed by group (adapted from Gern and Karn, 1983).

Group	Species	Effect of Melatonin
Cyclostoma	*Geotria australis*	Induces pallor
	Lampetra	Increases lightening in amocoetes
Osteichthyes	*Mystus tengara*	Depresses ovarian recrudescence in February and April
	Heteropneutes fossilis	Inhibits vitellogenesis. Causes ovarian regression in spawning catfish
	Gasterosteus aculeatus	Increases female gonadal weights under long photoperiods
Amphibians	*Xenopus leavis*	Induces retinal cone contraction in larvae
	Hyla	Inhibits gonadal enlargement in long photoperiods
	Rana	Decreases *in vitro* ovulation
	Ambystoma	Delays endogenous clock
Reptiles	*Columbia*	Increases growth hormone
	Coturnix	Increases oviductal weights during sexual maturation

1.8.3.1 Melatonin Circadian Rhythms

Since ancient times it has been documented that there are several seasonal and/or environmental variables on which animals can rely to cue changes in their biological systems e.g. reproduction, daylength, temperature, rainfall, food availability. Depending on the biotope however, some are more accurate predictors of the season than others. In temperate and arctic zones, for example, in which drastic climatic conditions impose a precise endocrine cycle, one of the most regularly changing phenomena is the daily change in daylength (photoperiod) (Csernus *et al.,* 1998). Other environmental factors, however, should be considered. In the tropical and equatorial zones of the world, where the fauna is immensely rich in species, more irregularly recurring external stimuli such as rainfall are equally precise and used for timing seasonal functions (Csernus *et al.,* 1998). Moreover, in the field there is always a co-variation of all these potential proximate factors. For example, in temperate zones in autumn the decrease in photoperiod is always associated with a decrease in mean temperature and food availability, and it is evident that interactions exist between the effects of all these factors (Csernus *et al.,* 1998).

The understanding of photoperiodic and circadian systems in fish is important not only from the viewpoint of comparative physiology, but also from applied science because this may help the management of fisheries and aquaculture. As in mammals, melatonin could be a key molecule in the regulation of photoperiodic and circadian systems in fish (Iigo *et al.,* 1997).

Despite having phylogenetically evolved for each class of vertebrate animals, rhythmic secretion of melatonin by the pineal gland has remained a vital component of the biological clock mechanism throughout the vertebrate phylum (Korf, 1994). The

benefits of requiring a single hormone, such as melatonin, for controlling or initiating circadian rhythms in humans, are that potential treatments can be more readily discovered and synthesised for ailments such as jet lag, insomnia disorders, shiftwork or sleep problems associated with blindness (Bubenik *et al.,* 1998).

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.,* 1982a). Careful consideration needs to be given to the time of day at which immuno-reactive substances are administered (Hrushesky, 1984) as well as other conditions such as the lighting regime and the 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).

Immune defences are organised along both 24-hour and yearly time scales. Two circadian systems have been isolated in both experimental animals and man: (Levi *et al.,* 1991) (1) the circulation of T, B or NK lymphocyte subsets in peripheral blood (Kronfol *et al.,* 1997) and (2) the density of epitope molecules at their surface (Mikolajczak *et al.*, 2000), which may relate to cell reactivity to antigen exposure. In a study examining For example, in mice, macrophage spreading and ingestion ability were significantly lower at the beginning and higher at the end of the dark period, while a significant increase in blood T-lymphocytes and helper-inducer T-lymphocyte percentages occurred during the dark period (Cardinali *et al.,* 1997).

1.9 The Effect of Immunostimulants on the Immune System of Fish

Many occasions arise in fish culture in which it is desirable to enhance the immune response. These include strengthening the normal immune response in order to enhance protection and the treatment of immunosuppressive conditions. Several substances are capable of increasing the immune response, and these can be classified into two main categories – adjuvants and immunostimulants (Galeotti *et al.,* 1998). An adjuvant is a substance which, when given with an antigen, enhances the immune response to that antigen. In contrast, immunostimulants need not be administered together with an antigen, they are generally given to induce a non antigen-specific enhancement of the immune system (Galeotti *et al.,* 1998).

Multitudes of substances have proven effects in enhancing one or more aspects of the immune response. These compounds are applied to boost or stimulate the innate immune system of cultured fish Cook *et al.,* 2003). These compounds include; bacteria and bacterial products (Dalmo and Seljelid, 1995), complex carbohydrates (Castro *et al.,* 1999), nutritional factors (Obach *et al.*, 1993; Clerton *et al.*, 2001), animal extracts, cytokines, lectins, plant extracts (reviewed by Galeotti, 1998)and synthetic drugs such as levamisole (Mulero *et al.*, 1998). The use of immunostimulants in fish diets has been considered for many years, and there are a large number of academic publications on the subject (Burrells *et al.*, 2001a). In a study investigating the effects of prolonged administration of a commercial ß-glucan based immunostimulant preparation, EcoActiva™, in the form of a feed supplement, on innate immune parameters and the growth rate of snapper (*Pagrus aurata*) it was found that fish fed on a diet supplemented with EcoActiva™ and held at a winter temperature had a significant enhancement of macrophage superoxide anion production upon stimulation with

phorbol myristate acetate. However, EcoActiva™ failed to increase either classical or alternate complement activity (Cook *et al.*, 2003). Despite the availability of effective vaccines for the most important aquaculture diseases, the potential of immunostimulants to enhance disease resistance to generalised infection remains attractive (Burrells *et al.*, 2001b).Those discussed here will be studied during the course of this project.

1.9.1 Innate Immunostimulants

1.9.1.1 Immunomodulation by the Pineal Gland and Melatonin

Pinealectomy or any other experimental procedure that inhibits melatonin synthesis and secretion induces a state of immunosuppression that is counteracted by melatonin in several species (Maestroni and Conti, 1993). Melatonin *in vivo* displays an immunoenhancing effect, particularly apparent in immunodepressive states on various immune parameters (Cardinali *et al.*, 1997)

Among melatonin's versatile functions, immunomodulation has emerged as a major effect of the hormone in vertebrates (Cardinali *et al.*, 1997). Melatonin is a natural hormone, which has been studied extensively in vertebrate endocrinology and reproductive physiology. Recently, it has been purported as a panacea for various human ailments ranging from jet lag (Oxenkrug and Requintina, 2003) to cancer (Leon-Blanco *et al.*, 2003). It is sold over the counter in the United States as a health food supplement. Research in the last decade has elucidated immunoregulatory properties of this hormone in human and rodent studies (Weber, 1999). However, a literature search failed to produce any studies investigating the interaction of melatonin and immunoregulation in fish. Based on data obtained for seasonal variations of immune function in mammals, birds and fish, coupled with current studies on immune function

in vitro in mammals, neuroendocrine regulation of the fish immune system can also be explored (Weber, 1999).

Melatonin has been reported to enhance a variety of cellular and humoral immune responses, for example, administration has been shown to enhance lymphocyte proliferation (Huang *et al.,* 2003); rejuvenate degenerated thymus and redress peripheral immune functions in aged mice (Tian *et al.,* 2003). In addition, it had been shown that exogenous melatonin enhances the activities of B and T lymphocytes in immature chickens (Brennan *et al.*, 2002). Furthermore, inhibition of melatonin synthesis has been observed to depress cellular and humoral immune responses in mice (Maestroni *et al.,* 1986).

Melatonin affects immune function both indirectly, acting through other hormones, and directly by acting on components of the immune system (Nelson and Drazen, 1999). Melatonin affects tumourigenesis and tumour development. Many of the indirect effects of melatonin on immune function are mediated through glucocorticoids and these in turn maybe part of an integrated series of adaptations to manage energy. Direct effects of melatonin on immune function appear to be mediated by melatonin receptors on lymphatic tissue or on immune cells in circulation. Melatonin may be part of an integrative system to; co-ordinate reproductive, immunologic and other physiological processes, to cope successfully with energetic stresses during winter (Nelson and Drazen, 1999).

The nature of the mechanisms involved in the immunomodulatory activity of melatonin remains unclear. There has been some suggestion of the existence of membrane specific binding sites for melatonin in immune cells, for example, within the

spleen (Yu *et al.*, 1991). Melatonin is also a potent anti-oxidant, acting by itself rather than through specific binding sites (Cardinali *et al.*, 1997)

1.9.2 Specific Immunostimulants

1.9.2.1 Vaccination

Vaccination is classed as a specific immunostimulation because it is educating the immune system against a specific pathogen. Fish farming has grown significantly during the last 20 years. Very often fish, like trout and salmon, are kept at high population densities. This will increase the risk for dramatic disease outbreaks (van Muiswinkel, 1992). Vaccines are inappropriate for juveniles. This is because the secondary immune response in teleosts is comparatively short lived and takes place only if the animals are above a certain size or age and are above their immunological permissible temperature. For salmonids the permissible temperature is often close to or above average winter temperature in the U.K. so it is likely that fish must spend a considerable proportion of their life without the benefit of specific immunoglobulins. At such times the fish are totally dependent upon the innate immune system to help in the maintenance of homeostatic integrity against pathogenic or opportunistic microbial invaders (Smith *et al.*, 2000).

Injection is the most often used route of immunisation in mammals. However, this can be somewhat impractical for fish. As a result various other methods have been developed to allow mass vaccinations to take place. These include immersion, hyperosmotic immersion, bath, spray and oral modes of delivery. Although impractical, intraperitoneal injection has been used and in many instances is the most effective method (Kaattari and Piganelli, 1996).

1.9.3 Limitations of Some Immunostimulants

Although there are numerous positive effects of immunostimulatory compounds on the immune system in fish, in comparison with specific immunisation, the effect of innate immunostimulants (single application) is normally of short duration. The yeast ß-glucan gave an increase in respiratory burst activity 4-7 days after treatment (Jørgensen and Robertsen, 1995). However, the duration of protection offered through a single application of an immunostimulant is usually very brief. For example, Anderson and Siwicki (1994) gave groups of trout the immunostimulants glucan and chitosan. Those groups given the immunostimulants 1-3 days before the challenge were well protected, but 14 days after the immunostimulation the protection in all fish was greatly reduced (Galeotti *et al.*, 1998).

In addition, it is also worthwhile considering that in several species, immunostimulants appear not to confer any objective advantage. For example, long-term oral administration of ß-glucan to turbot does not reduce the level of mortality in fish after a challenge with a virulent *Vibrio anguillarum* (Galeotti *et al.*, 1998).

There is a current trend to administer immunostimulants, especially in the diet, for prolonged periods of time or with doses which exceed the recommended levels, with the aim of obtaining better and faster productivity results. It is best to consider the possible negative effects of some immunostimulants, especially in the case of overdosage. Additionally, the permanent application of immunostimulatory substances may lead to sensitisation, with allergic reactions (over-dosage resulting in anaphylactic reactions) (Galeotti *et al.*, 1998).

1.10 The Use of Rainbow Trout in the Proposed Study

Salmonids (order Salmoniformes, Class Osteichthyes) were originally native to the northern Temperate Zone. However, they are now found in almost all waters of the worlds continents with the exception of Antarctica (Porter, 1996). The proposed study will look at the effect of seasonality on the immune response of rainbow trout which is now a recognised member of the Pacific Salmonidae (Kendall, 1988)

Transmissible diseases are known to have devastating effects on both wild and cultured stocks of fish. As with many animals, epidemics often strike juveniles or larvae, and this can pose serious economic problems for fish farming (Smith *et al.*, 2000). Fish farming has grown significantly during the last thirty years. Trout and salmon are often kept at high stocking densities. This can increase the risk of disease outbreaks. Although antibiotics can be used to treat bacterial diseases, there are some drawbacks with their use. Repeated use of these drugs can induce antibiotic resistance in microorganisms (Anderson and Levin, 1999). Moreover, harmful residues may be present in the fish brought to market. In this context it is not surprising that there is increasing prophylaxis of salmonid fish diseases by vaccination and immunostimulation (Sakai, 1999).

1.11 Aims of Study

- The principle aim of this thesis is to determine if immunity displays seasonal patterns in rainbow trout. This is investigated in Chapter 2.

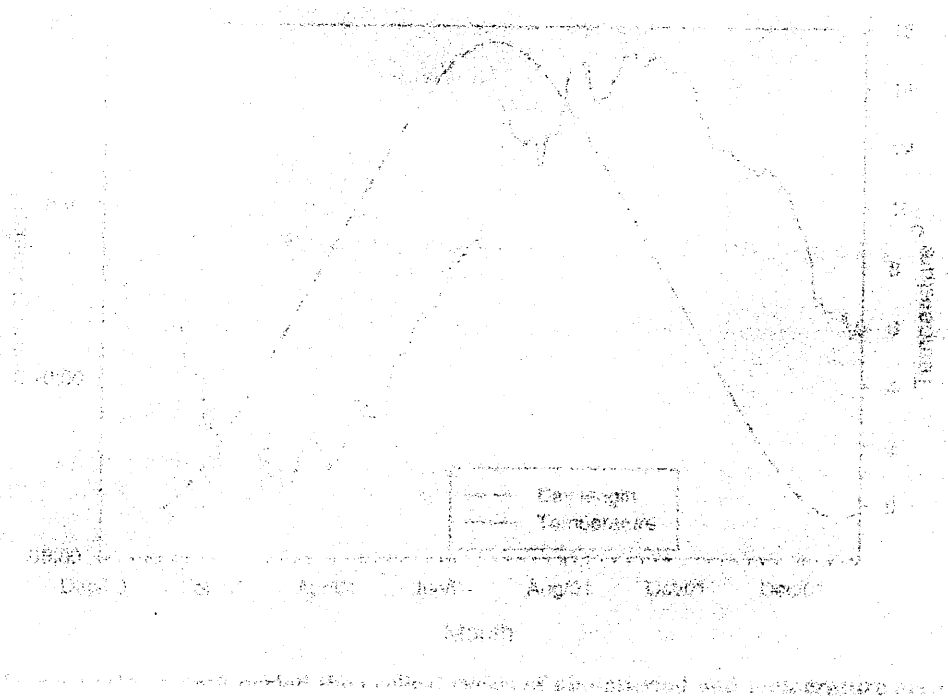
- To determine which environmental cue(s) is used to detect changes in seasonality i.e. temperature (Chapter 3) and/or photoperiod (Chapter 4)

- If seasonal immunosuppression is observed, methods of alleviating it will be examined. This is presented in Chapter 5.

- The pineal hormone melatonin will be investigated to determine if it plays a role in relaying seasonal information to the fish immune system. This is investigated in Chapter 5.

- To determine if immunity displays circadian patterns of immunity. This is explored in Chapter 6.

Chapter 2 Variations in Innate Immune Activity over a 12-Month Period



2.1 General Introduction

2.1.1 Background

During a continuous twelve month period the temperate aquatic environment will be affected by the two primary components of season i.e. temperature and photoperiod. These components are interlinked and follow very similar cycles as shown in Fig. 2.1. Typically during the spring quarter, temperatures and photoperiod increase and the reverse occurs in autumn. This seasonality affects the life history of fish with the timing of developmental and maturational events dominated by and synchronized with seasonal changes in climate, daylength and food supplies (Bromage *et al.*, 2001).

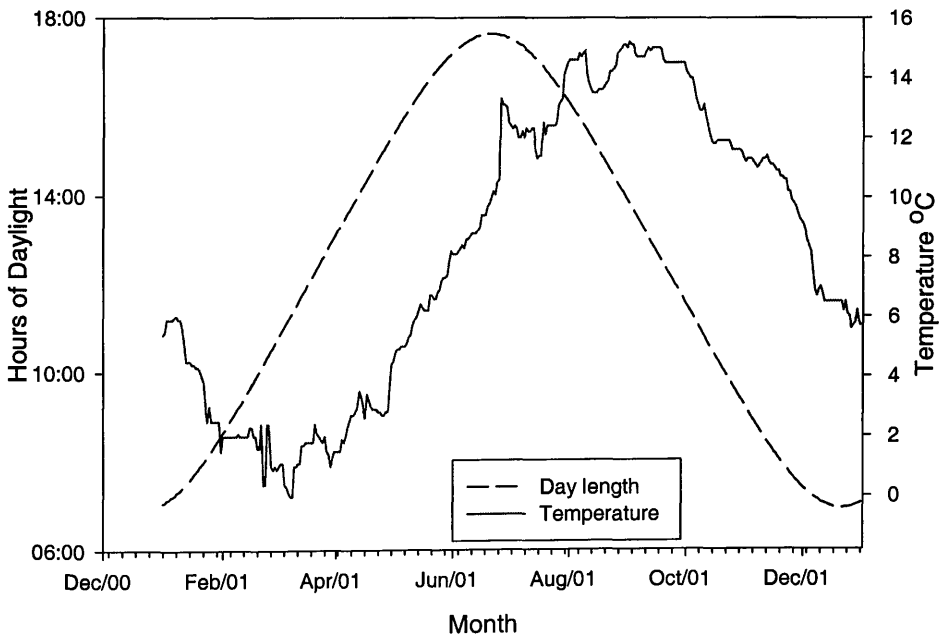


Fig. 2.1 Over a twelve month period the cyclical cycles of photoperiod and temperature are closely correlated in the temperate environment. Consequently, any effect of season observed could in response to either or both of these parameters. Data presented is for the Niall Bromage Freshwater Research Facility (NBFRF).

It is well documented that seasonality also affects the immune response of vertebrates (Zapata, 1992). It is already well established that during a twelve month period adaptive immunity exhibits a seasonal cycle in fish, in particular, changes in resting antibody titre and response to antigenic challenge (Nakanishi, 1986). Other studies state seasonal changes, in particular in the lymphoid system (Wojtowicz and Plytycz, 1997; Álvarez *et al.*, 1998) and in the numbers of circulating lymphocytes (Slater and Schreck, 1998). It has generally been assumed that the innate immune response does not exhibit this seasonal cycle but remains constant throughout the year (Ellis, 2001), always providing a defence to invading pathogens.

Many environmental challenges are recurrent and thus predictable. Animals could enhance their survival and presumably increase their fitness, if they could anticipate immunologically challenging conditions in order to cope with these seasonal threats to health (Nelson *et al.*, 1995). A potential mechanism to anticipate changes in season may involve the pineal hormone melatonin. It exhibits a strong circadian rhythm as the majority of the hormone is produced during the dark phase of the day. Consequently, its production is affected by the seasonality of photoperiod. During the winter months, when the dark phase is at its longest, melatonin is produced for a greater length of time compared to the shorter dark phase of summer days (Randall *et al.*, 1995). In addition, melatonin production is affected by temperature with higher levels reported in Atlantic salmon maintained at 12°C compared to fish maintained at 4°C (Porter *et al.*, 2001).

2.1.2 Aims

The primary aim of the work presented in this chapter was to establish a base line of data. The reasons for this are two fold. 1) To determine if the innate immune system of rainbow trout is affected by seasonality and what these effects are, and 2) Work carried out later in this thesis to examine the effect of different seasonal components, i.e. temperature and photoperiod could be compared to this baseline data set.

2.2 Materials and Methods

2.2.1 Trial Set-Up and Fish Maintenance

This 'Base Level' trial was set up at the Niall Bromage Freshwater Research Facility (NBFRF) (Institute of Aquaculture, University of Stirling). Fish used at the NBFRF were all supplied from the farm itself. The tanks were set up in a flow through system supplied by a reservoir situated 1 km from the facility. Water was supplied to the tanks at a rate of approximately 2 L per second at ambient seasonal temperature. Light was supplied by 60-watt pearl, tungsten filament light bulbs housed within waterproof lamps providing an intensity of 17-19 lux at the water surface (Skye Instruments, Powys, UK). The photoperiods were controlled by 24 h digital electronic time switches and were set to simulate the natural photoperiod by a light sensor located on the outside of the building. Fish were fed a standard commercial pelleted trout feed (EWOS), and were fed according to the manufacturers tables. Fish showing any signs of disease or that were outside the required weight range were humanely euthanised using phenoxyethanol (Sigma Chemical Company Ltd.). Fish were held in 3 tanks (2 m Ø x 1 m) with 100 fish per tank at the start of the experiment. All fish used were female. This was to prevent precocious males with a potentially compromised immune system from skewing the results.

Five fish were sampled per month. All fish were weighed and measured. A variety of immunological and haematological techniques were carried out, the methods for which are described in Section 2.2.3.

2.2.2 General Experimental Procedures

2.2.2.1 Chemicals and Reagents

The chemicals, reagents and antibodies used in experimental procedures were purchased from Sigma Aldrich Chemicals, England, BDH, England and Diagnostics Scotland unless otherwise stated. Buffers and solutions were prepared using high quality or tissue culture grade components. All recipes are provided in Appendix A.

2.2.2.2 Anaesthesia

Anaesthesia was used whenever the fish were handled to reduce stress and damage to the fish. A 1:20,000 concentration of 2-phenoxyethanol (Sigma Aldrich Chemical Company Ltd) was used. Once anaesthetised, fish were measured, weighed and bled accordingly, then humanely sacrificed using a Schedule 1 method, in accordance with the Animals (Scientific Procedures) Act 1986, by severing the anterior spine. This was to ensure that the same fish was not sampled more than once.

2.2.2.3 Blood Sampling

Anaesthetised fish were bled by caudal venepuncture using 1 ml or 2 ml syringes and a 25 G or 23 G needle (Terumo Europe N.V. Belgium). Prior to sampling, syringes were rinsed with heparin (4 mg ml^{-1}, Sigma Chemical Company) to allow collection of plasma and avoid coagulation. A complete venesection was performed. The blood was dispensed into dried, individual Eppendorfs and stored on ice for transporting to the laboratory. Plasma haematocrit and blood counts were carried out prior to centrifugation at 7000 rpm for 10 min at 4°C. The plasma from each fish was individually aliquoted and stored at -70°C until required.

On several occasions blood sampling was carried out during the dark period for analysis of plasma melatonin levels. In total darkness fish were removed from the tank and placed in an anaesthetic bath for 2 min. The fish was then removed from the anaesthetic and the blood sample taken under a dim red light (λ= 670-800 nm, 0.2 lux at 0.5 m) (Porter, 1996). The samples were then transported back to the lab on ice and plasma collected as before.

2.2.3 Measurement of Innate Immune Parameters

2.2.3.1 Total White Blood Cell Counts

Ten μl of blood was added to 990 μl PBS (Appendix A) (a 1:100 dilution). An aliquot of diluted blood was placed on a Neubauer haemocytometer (Hawksley & son, England). White blood cells were discriminated from red blood cells since the former are rounded and refractile (Garbi, 1998). The average numbers of white blood cells per large square of the haemocytometer were counted under a phase contrast microscope at 100 x magnification (Fig. 2.2). The counts were expressed as white blood cells (WBC) ml^{-1} (Equ. 2.1)

Equ. 2.1 White Blood Cell Count (ml^{-1})

$$WBC\ ml^{-1} = WBC \times \text{dilution factor} \times 10^4$$

2.2.3.2 Total Red Blood Cell Counts

One hundred μl of the solution of blood prepared in Section 2.2.3.1 was added to 900 μl of PBS (1:1000 dilution). The red blood cells were then counted as described for Section 2.2.3.1 and the red blood cells ml^{-1} calculated accordingly.

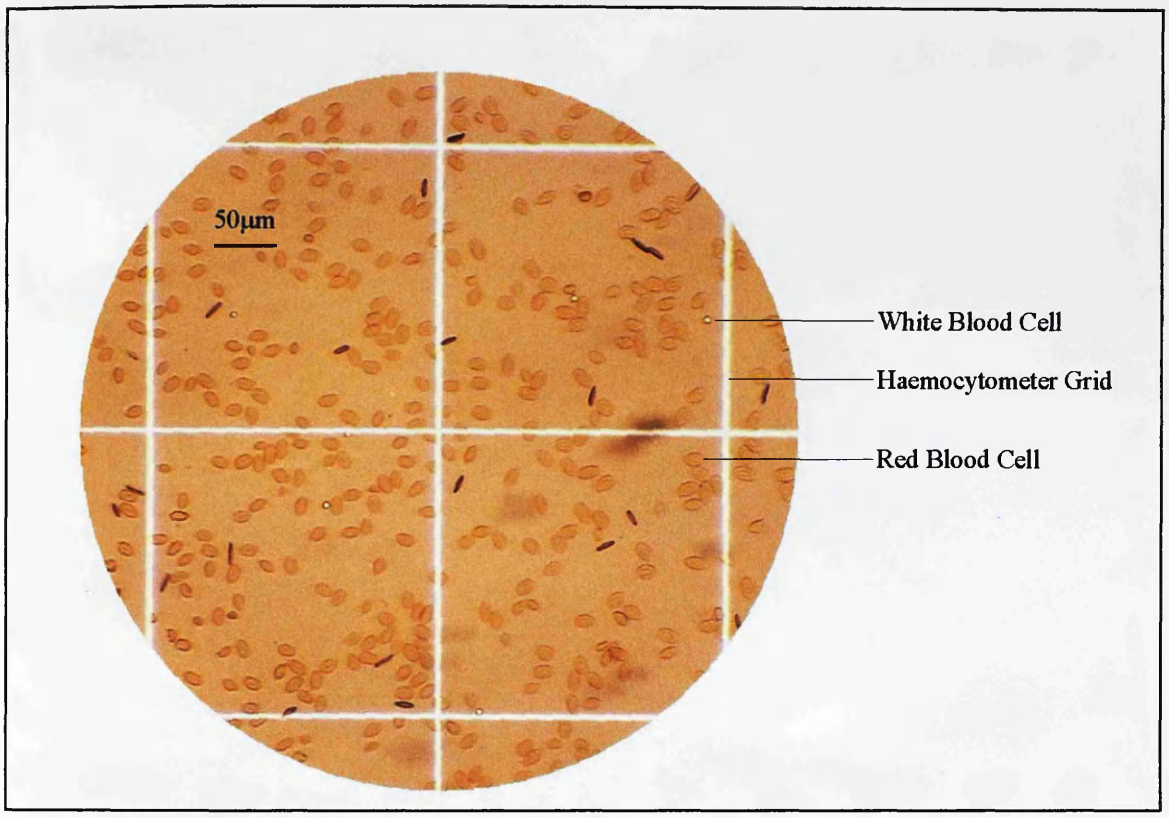


Fig. 2.2 Measuring total white blood cell count.

2.2.3.3 Lysozyme Activity

The method used to detect lysozyme activity in trout plasma was based on the ability to lyse the Gram-positive bacterium *Micrococcus lysodiekticus* (Parry *et al.*, 1965; Peddie and Secombes, 2003). Lysozyme activity in plasma was measured turbidimetrically. A 0.04 M Sodium Phosphate Buffer (pH 5.8) was prepared. Lyophilised *M. lysodiekticus* was added at a concentration of 0.2 mg bacteria ml^{-1} and this was then incubated at 25°C for 20 min. Twenty mls of bacteria suspension was enough for one 96 well microplate.

Ten µl of plasma was added to 5 replicate wells of a non-coated 96 multiwell plate (NUNC, BDH). Two columns of the plate containing buffer only was used as a negative control. One hundred and ninety µl of the bacterial suspension was added to all but the control wells. This suspension was added quickly using a multi-channel pipette.

Care was taken not to introduce bubbles into the samples. Using a Dynex MRX II plate reader absorbance was read at 540nm one min after buffer was added to all wells and then again after 5 min. Lysozyme activity was expressed as the amount of sample causing a decrease in absorbance of 0.001 min^{-1}. Units used were units min^{-1} ml^{-1}.

2.2.3.4 Isolation of Rainbow Trout Pronephros Macrophages

Aseptic techniques were used at all times using a laminar flow hood. The removal of the head kidney (pronephros) from rainbow trout renders a supply of macrophages available for immunological assays (Braun Nesje *et al.,* 1981). As much blood as possible was removed from the sacrificed fish prior to macrophage collection via total venesection. In a laminar flow cabinet 5 ml of Leibovitz-15 (L-15) medium containing 10 μl heparin, was added to a 30 mm Ø Petri dish. Using blunt sterile forceps the dish was covered with a square of pre-cut, sterile 100 μm mesh. Using a sterile scalpel an incision was made at the gill arches and sliced backward towards the anus just below the lateral line. This clearly exposed the swim bladder. The swim bladder was pierced and pulled away from above the kidney. The edges of the head kidney were cut and then scraped together using a sterile spatula. The head kidney was removed using sterile forceps and placed on the mesh overlaying a Petri dish.

The kidney was teased through the mesh using the sterile plunger from a syringe. The mesh was rinsed with 1 ml of L-15 and the mesh dragged backwards off the petri dish to ensure the maximum of macrophages were collected. Using a sterile plastic Pasteur pipette the macrophage solution was gently drawn up and dispensed into a sterile bijou and stored on ice. The collected macrophages were stored temporarily on ice ready for immediate use in respiratory burst and phagocytosis assays.

2.2.3.5 Respiratory Burst Activity of Head Kidney Macrophages.

Method adapted from Rook *et al.* (1985). One hundred μl of the macrophage suspension was aliquoted into 8 replicate wells of a sterile 96-well microtitre plate (NUNC) and left for 1 h at 21°C to allow the cells to adhere to the plate. Plates were then washed 3 times with L-15 medium to wash off non-adherent cells. To the first set of 3 replicate wells, 100 μl of 1 mg ml^{-1} nitrobluetetrazolium (NBT) in L-15 was added. To the next 3 replicate wells 100 μl of 1 mg ml^{-1} NBT solution containing 1 μl ml^{-1} of phorbol myristate acetate (PMA) was added. To the remaining 2 wells, 100 μl of lysis buffer (Appendix A) was added. Plates were incubated at 21°C for one hour. The solutions from the first 6 replicate wells were removed and the plates were then washed 3 times with L-15 medium. To the first six replicate wells 100μl of neat methanol was added for 5 min to stop the reaction. They were then washed 3 times with 70% methanol and the wells left to air-dry (a minimum of 30 min).

One hundred and twenty μl of dimethyl sulfoxide (DMSO) and 140 μl of 2M Potassium Hydroxide (KOH) were added to each of the washed wells. The absorbance of each well was read at 610 nm using an ELISA plate reader.

The average numbers of adherent cells in the wells containing lysis buffer was determined using a Neubauer haemocytometer. Numbers were adjusted to 1×10^5 cells.

The average weight of fish was 20.5 g (SD = 2.84; SE = 0.73) at the first month of sampling (July 2002). Fish were maintained at an ambient temperature and a simulated natural photoperiod, throughout the 12 month period.

2.2.4 Measurement of Melatonin

Blood samples were taken using heparinised syringes, twice daily, every three months at midday and two hours after darkness had fallen and transported back to the laboratory on ice. Collected plasma samples were collected were stored at –70°C as soon as possible until analysed using a melatonin radio immunoassay (RIA) method adapted from Randall (1992). The sensitivity of the assay is 3.9 pg ml^{-1} (Randall, 1992). Recipes for tricine buffer, charcoal solution, antibody, radiolabel and standards are provided in Appendix A.

A standard curve was produced for all assays carried out (Fig. 2.3). Standards were prepared in a range of dilutions from 0-500 pg/250μl in polystyrene tubes (LP3; Luckhams Ltd). Two hundred μl of tricine buffer was added to each of the sample tubes. A further two tubes had 450 μl buffer added to them, and these were used to measure non-specific binding (NSB). An additional 250 μl of buffer was added to the NSB and standard tubes. Two hundred and fifty μl of sample plasma was added to the sample tubes. To all but the NSB tubes 200 μl of antibody (sheep anti-melatonin) was added. All tubes were incubated for 30 min at 21°C. Tritiated melatonin (200 μl) was added to all tubes, which were then vortexed. Tubes were incubated for 18 h at 4°C.

Charcoal solution was made immediately prior to use and stirred on ice for 30 min. Five hundred μl of this solution was added to all tubes which were then vortexed. They were incubated for 15 min at 4°C, then centrifuged at 4°C, 2000 rpm for 15 min. One ml of the supernatant was collected from each tube and aliquoted into6 ml polyethylene scintillation vials (Canberra Packard Ltd), to which was added 4 ml.

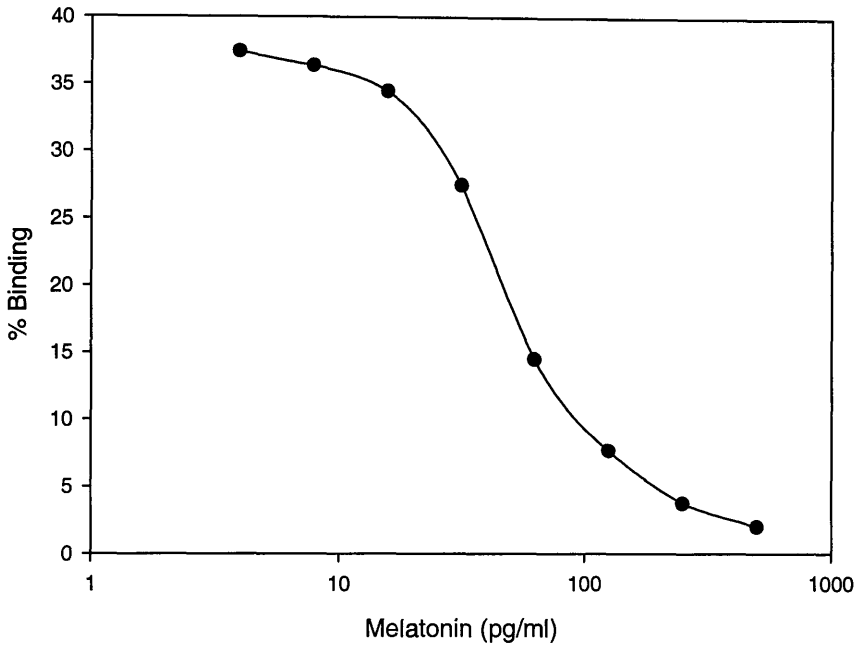


Fig. 2.3 Typical melatonin radioassay standard curve. The concentration of melatonin within a sample is obtained from the intersect of the percentage binding in the sample.

of scintillation fluid (Ultima Gold; Canberra Packard Ltd). A further three tubes were prepared, each with 4 ml of scintillation fluid. One hundred μl of triatiated melatonin was added to two of these vials, this was to enable calculation of total radioactivity. The remaining tube was left blank, for the calculation of background radioactivity. All tubes were vortexed and radioactivity measured for 4 min in a scintillation counter (1900TR LSA; Canberra Packard Ltd).

2.2.5 Statistical analysis

Before analysis data was found to be normally distributed and homogenous without transformation. This test was performed using the appropriate function on the Minitab statistical Package (V. 10). Data was analysed ($P<0.05$) using an ANOVA General Linear Model, Tukey Pairwise comparison tests (Post-hoc) and Pearson's correlation coefficient all using Minitab (V. 10).

2.3 Results

2.3.1 Weight

At the time of first sampling (July 2002) fish the average weight of the fish was 20.53 g and at the end of the 12-month trial (June 2003) average weight was 248.55 g (Fig. 2.4).

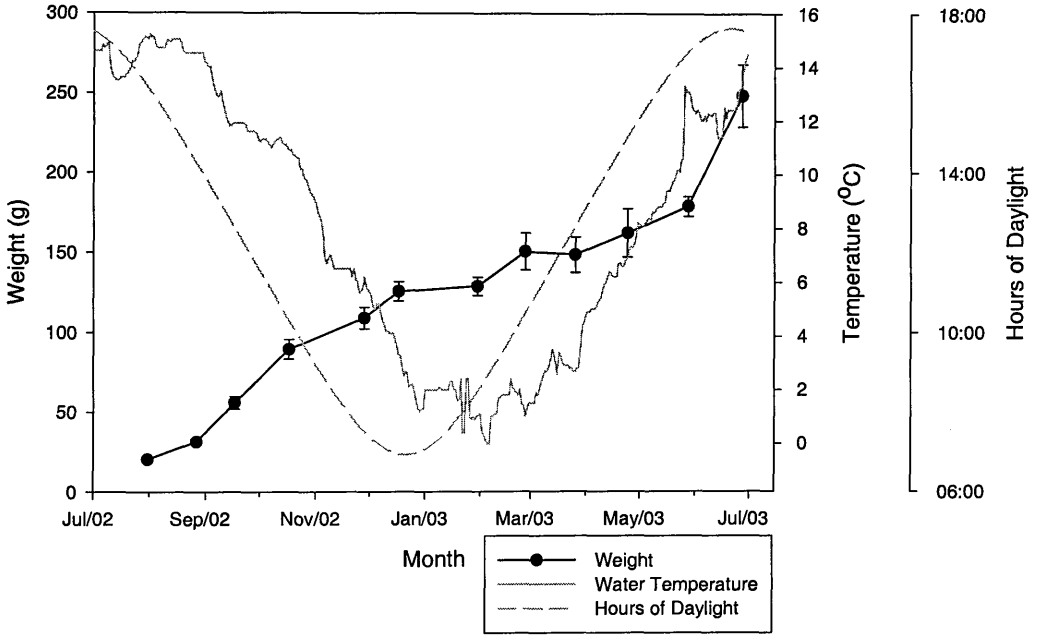


Fig. 2.4 Weight of fish during the 12-month trial period (n=15; mean ± SE).

2.3.2 Condition Factor

Condition factor had improved from 1.49 in July (2002) to 1.19 in June (2003) (Fig. 2.5). This represents a general decrease in condition factor over the year towards a more desirable condition factor of 1.

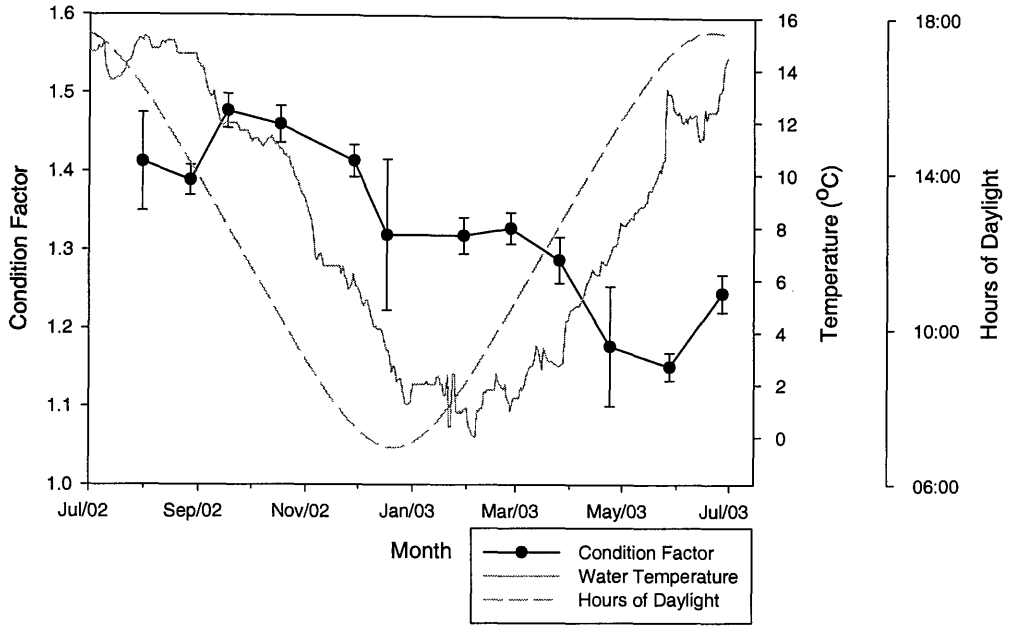


Fig. 2.5 Variations in condition factor over a twelve month period (n=15; mean ± SE).

2.3.3 Variations in Innate Immunity and Haematological Parameters Over a 12-Month Period

2.3.3.1 White Blood Cell Counts

Total white blood cell counts were measured every month over the twelve month period (Fig. 2.6). There was a general increase in white blood cell counts during the spring months peaking in July (2002)/June (2003). Total white blood cell numbers decreased during the autumn and winter months to their lowest in March (2003). Using an ANOVA (GLM) a significant effect by month was found on white blood cell counts (P=0.000). Using a Tukey post-hoc test pair wise comparisons test (Table 2.1) no significant differences were observed between the summer months of May (2003), June (2003) and July (2002) (P>0.05). There was also no significant difference in white blood cell numbers between the winter months of November (2002), December (2002)

and January (2003) (P>0.05). However, total white blood cell counts in the winter months were significantly lower than that of the summer (P=0.000).

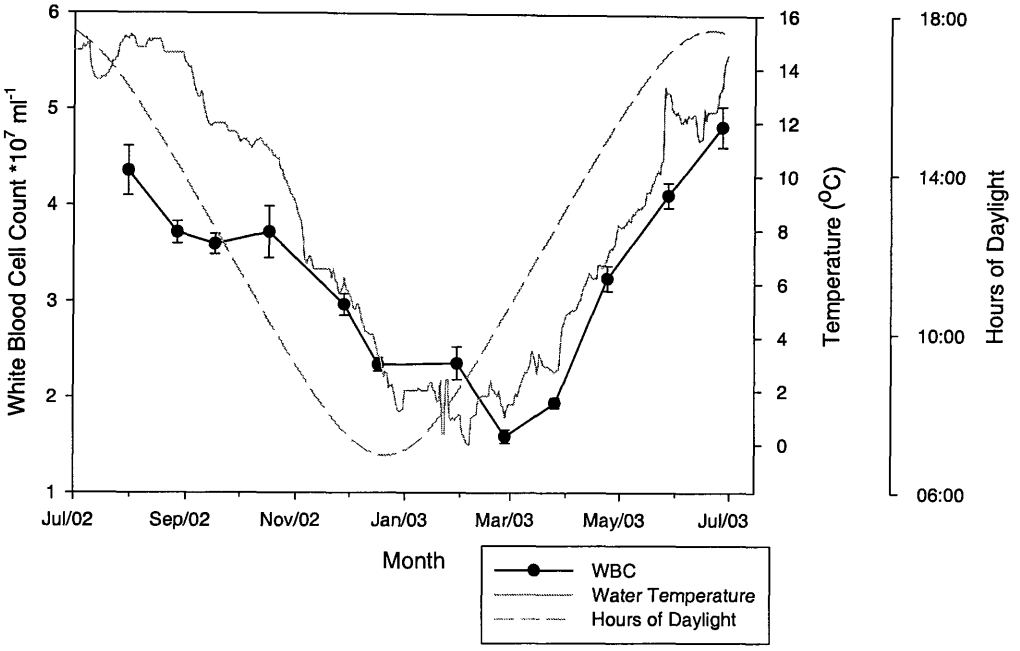


Fig. 2.6 Variations in white blood cell counts over a twelve month period (n=15; mean ± SE).

Table 2.1 Tukey Pairwise Comparison Tests investigating changes in total white blood cell counts with season (n=15)

	Jul '02	Aug'02	Sep'02	Oct'02	Nov'02	Dec'02	Jan'03	Feb'03	Mar'03	Apr'03	May'03	Jun'03
Jul'02	X											
Aug'02	P>0.05	X										
Sep'02	**P=0.039**	P>0.05	X									
Oct'02	P>0.05	P>0.05	P>0.05	X								
Nov'02	**P=0.000**	**P=0.047**	P>0.05	**P=0.043**	X							
Dec'02	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	X						
Jan'03	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	P>0.05	X					
Feb'03	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.043**	**P=0.036**	X				
Mar'03	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	P>0.05	P>0.05	X			
Apr'03	**P=0.000**	P>0.05	P>0.05	P>0.05	P>0.05	**P=0.004**	**P=0.005**	**P=0.000**	**P=0.000**	X		
May'03	P>0.05	P>0.05	P>0.05	P>0.05	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.0082**	X	
Jun'03	P>0.05	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	X

2.3.3.2 Red Blood Cell Counts

Red blood cell counts peaked during July (2002) and June (2003) (Fig. 2.7), although very little difference was observed between the other sampling months. Statistical analysis revealed a significant effect with month on red blood cell counts (P=0.000). This was investigated further Using a Tukey post-hoc pairwise comparisons test (Table 2.2), and it was observed that the red blood cell counts obtained in July (2002) were not significantly different from those taken in October (2002) or April (2003) (P>0.05), but significantly lower than those taken in June (2002) (P=0.000) and significantly higher than those taken during the remaining sampling months (P>0.05).

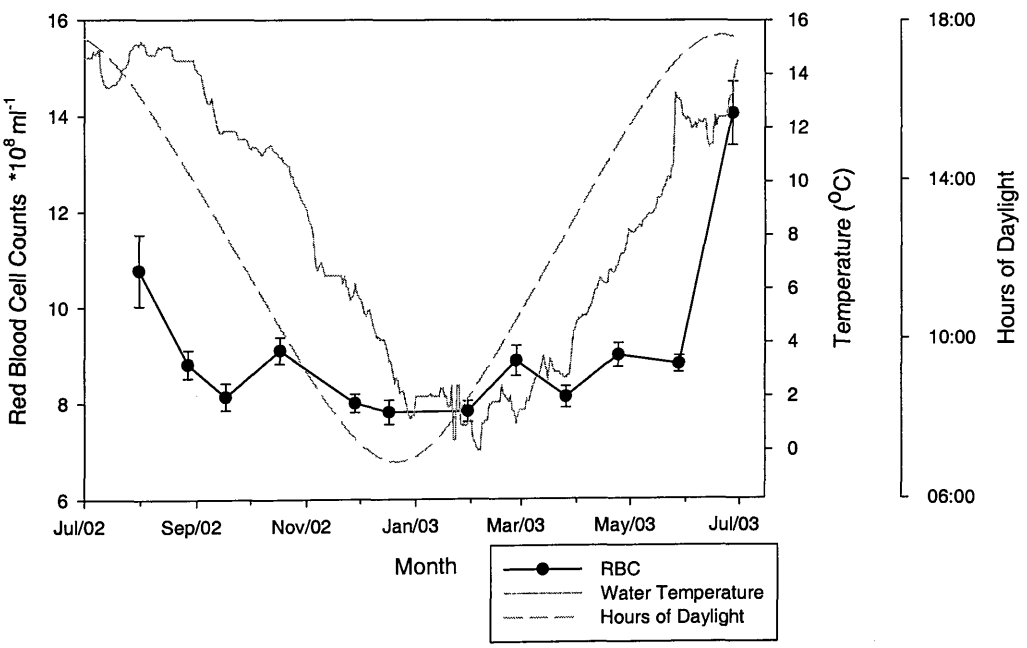


Fig. 2.7 Variations in red blood cell counts over a twelve month period (n=15; mean ± SE).

Table 2.2 Results of Tukey Pairwise Comparison Tests investigating red blood cell counts with mouth (n=15)

	Jul'02	Aug'02	Sep'02	Oct'02	Nov'02	Dec'02	Jan'03	Feb'03	Mar'03	Apr'03	May'03	Jun'03
Jul'02	X											
Aug'02	P=0.016	X										
Sep'02	P=0.000	P<0.05	X									
Oct'02	P<0.05	P<0.05	P<0.05	X								
Nov'02	P=0.000	P<0.05	P<0.05	P<0.05	X							
Dec'02	P=0.000	P<0.05	P<0.05	P<0.05	P<0.05	X						
Jan'03	P=0.000	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	X					
Feb'03	P=0.025	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	X				
Mar'03	P=0.000	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	X			
Apr'03	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	X		
May'03	P=0.015	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	X	
Jun'03	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	P=0.000	X

2.3.3.3 Lysozyme Activity

Plasma lysozyme activity was also measured every month for twelve months throughout the trial period (Fig. 2.8). A clear seasonal pattern of activity was observed as plasma lysozyme activity decreased from October (2002) to April (2003) and increased from May (2003) to July (2003). The lowest levels of activity were recorded in April (2003). A significant effect on plasma lysozyme activity by month was found (P=0.000). Using a Tukey post-hoc test, it was revealed from pair wise comparisons (Table 2.3) that plasma lysozyme activity in September (2002) was not significantly different from that of August (2002), October (2002), or June (2003) (P>0.05), but it was significantly greater than all other months. Lysozyme activity measured in March (2003) and April (2003) were significantly lower than those measured in; August (2002), September (2002) and June (2003) (P<0.05).

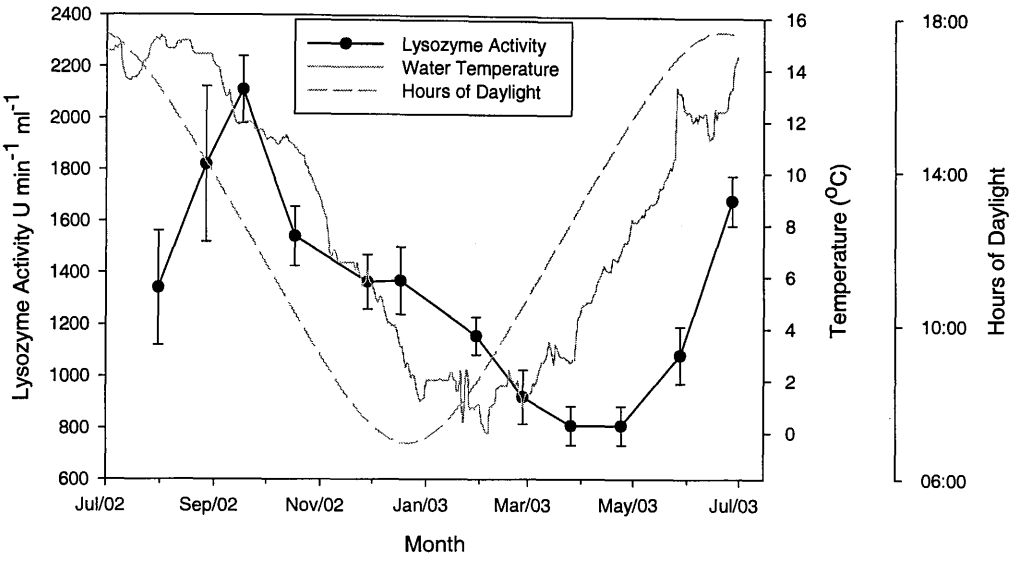


Fig. 2.8 Seasonal lysozyme activity over a twelve month period (n=15; mean ± SE).

Table 2.3 Results of Tukey Pairwise Comparison Tests investigating the effect of month on lysozyme activity (n=15).

	Jul'02	Aug'02	Sep'02	Oct'02	Nov'02	Dec'02	Jan'03	Feb'03	Mar'03	Apr'03	May'03	Jun'03
Jul'02	X											
Aug'02	P>0.05	X										
Sep'02	**P=0.01**	P>0.05	X									
Oct'02	P>0.05	P>0.05	P>0.05	X								
Nov'02	P>0.05	P>0.05	**P=0.015**	P>0.05	X							
Dec'02	P>0.05	P>0.05	**P=0.017**	P>0.05	P>0.05	X						
Jan'03	P>0.05	P>0.05	**P=0.000**	P>0.05	P>0.05	P>0.05	X					
Feb'03	P>0.05	**P=0.005**	**P=0.000**	P>0.05	P>0.05	P>0.05	P>0.05	X				
Mar'03	P>0.05	**P=0.004**	**P=0.000**	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	X			
Apr'03	P>0.05	**P=0.000**	**P=0.000**	**P=0.019**	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	X		
May'03	P>0.05	**P=0.018**	**P=0.000**	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	X	
Jun'03	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	**P=0.045**	**P=0.033**	**P=0.0015**	P>0.05	X

2.3.3.4 Respiratory Burst Activity

Respiratory burst activity (Fig. 2.9) was not observed to exhibit an obvious seasonal pattern. Activity was observed to be greatest during the winter month of February (2003) and lowest during the winter month of January (2003). This is corroborated by the GLM ANOVA which revealed significant differences of respiratory burst activity by month (P=0.000). However, using a Tukey post-hoc test, pairwise comparisons revealed that no significant difference in activity was observed between August and February (P=1.000) (Table 2.4). The pairwise comparisons further revealed that respiratory burst activity levels recorded in February were significantly greater than all other months (P=0.000) except July and August (P>0.05). Respiratory burst activity in August was significantly greater than all months except October, February, and March (P<0.05). There was no significant difference between March, April, May and June (P>0.05).

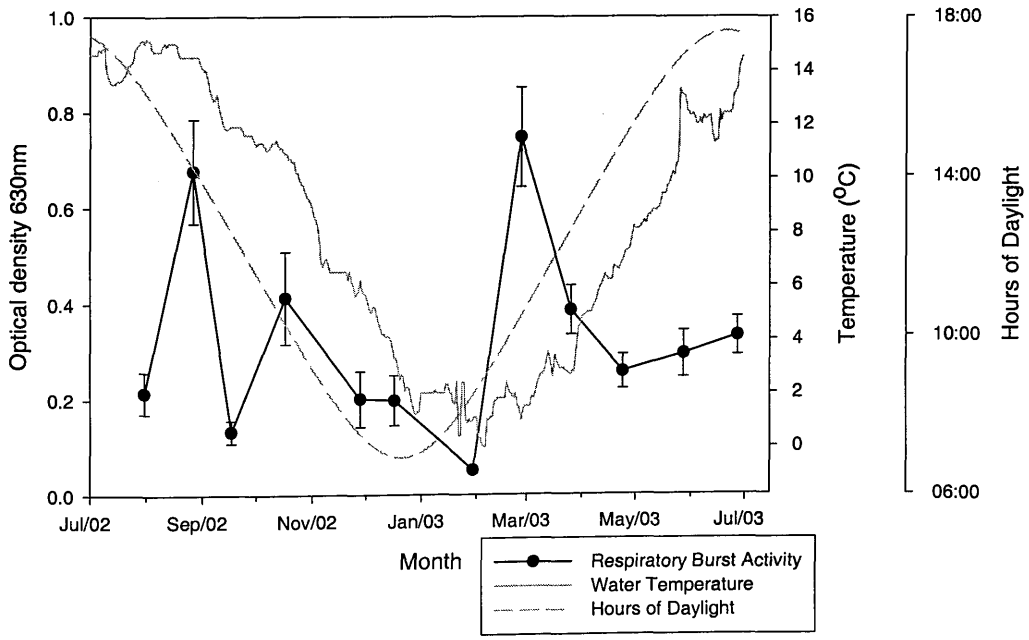


Fig. 2.9 Variations in respiratory burst activity over a twelve month period (n=15; mean ± SE).

Table 2.4 Tukey Pairwise Comparisons examining the effect of month on respiratory burst activity (n=15)

	Jul'02	Aug'02	Sep'02	Oct'02	Nov'02	Dec'02	Jan'03	Feb'03	Mar'03	Apr'03	May'03	Jun'03
Jul'02	X											
Aug'02	P=0.000	X										
Sep'02	P>0.05	P=0.000	X									
Oct'02	P>0.05	P>0.05	P>0.05	X								
Nov'02	P>0.05	P=0.000	P>0.05	P>0.05	X							
Dec'02	P>0.05	P=0.000	P>0.05	P>0.05	P>0.05	X						
Jan'03	P>0.05	P=0.000	P>0.05	P=0.005	P>0.05	P>0.05	X					
Feb'03	P>0.05	P>0.05	P=0.000	P=0.012	P=0.000	P=0.000	P=0.000	X				
Mar'03	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P=0.013	P=0.004	X			
Apr'03	P>0.05	P=0.000	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P=0.000	P>0.05	X		
May'03	P=0.000	P=0.002	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P=0.000	P>0.05	P>0.05	X	
Jun'03	P>0.05	P=0.000	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P=0.000	P>0.05	P>0.05	P>0.05	X

2.3.3.5 Correlation of Seasonal Cues with Innate Immune Parameters

Seasonal patterns in the innate immune parameters were further investigated using Pearson's Correlation Coefficient (Table 2.5). The profiles of photoperiod and temperature are very similar, and without specific investigation into each, it is impossible to determine which the true environmental cue is. However, together these cues are used to represent the typical seasonal pattern.

As would be expected there was a strong positive correlation between hours of daylight and temperature (P=0.000). Positive correlations (P=0.000) were also observed between both seasonal cues and total white blood cell counts and red blood cell counts. Lysozyme activity was positively correlated with temperature (P=0.000) but not photoperiod (P=0.069). Respiratory burst activity was not positively correlated with any

of the seasonal cues, or any of the other innate immune parameters investigated (P>0.05).

Positive correlations are observed between all the immune and haematological parameters investigated with the exception of respiratory burst activity (P<0.05). However, although significant, the correlation between red blood cell counts and lysozyme activity was the weakest of all the significant correlations.

Table 2.5 Pearson Correlation Coefficients for immune parameters VS seasonal cues

	Hours of Daylight	Temperature	White Blood Cell Counts	Red Blood Cell Counts	Lysozyme	Respiratory Burst
Hours of Daylight	X					
Temperature	0.691 **P=0.000**	X				
White Blood Cell Counts	0.644 **P=0.000**	0.747 **P=0.000**	X			
Red Blood Cell Counts	0.531 **P=0.000**	0.378 **P=0.000**	0.455 **P=0.000**	X		
Lysozyme	0.069 P>0.05	0.386 **P=0.000**	0.232 **P=0.002**	0.149 **P=0.047**	X	
Respiratory Burst	0.078 P>0.05	0.050 P>0.05	-0.091 P>0.05	0.056 P>0.05	0.047 P>0.05	X

Cell Contents: Pearson correlation
P-Value

2.3.4 Variations in Melatonin Levels over the 12-Month Trial Period

Plasma melatonin was measured quarterly at midday and two hours after darkness over the twelve month trial period (Fig. 2.10). Melatonin levels were highest at night throughout the trial period. Summer plasma melatonin levels were higher than winter levels both during the night and the day. As expected there was a significant difference between night time melatonin levels and day time levels (P=0.000). It was determined from an ANOVA GLM that there was a significant difference between the summer melatonin samples of July (2002) and May (2003) sampling points and the winter months of November (2002) and January (2003) for both the day and night sampling (P=0.000).

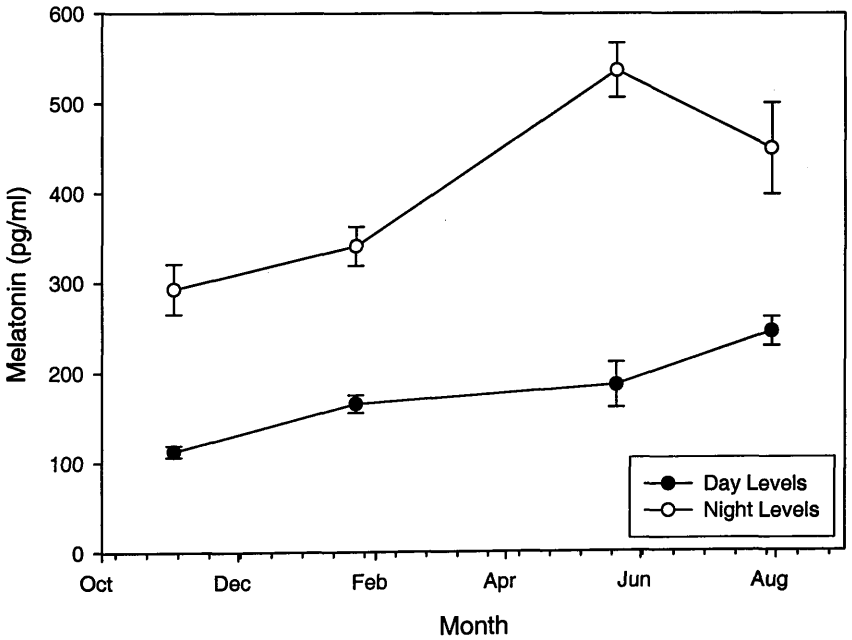


Fig. 2.10 Day and night melatonin levels over the twelve month trial period (n=15; mean ± SE).

2.4 Discussion

An effect of season was observed on several of the innate immune and haematological parameters studied. The immune response of rainbow trout exhibited significant difference between months for all of the innate immune parameters studied. A general seasonal effect could be described for total white blood cell counts, lysozyme activity and red blood cell counts. However, seasonality appeared to have little or no effect on respiratory burst activity. This is corroborated by the results of the Pearsons Correlation Coefficients which revealed a positive correlation between the seasonal cue of temperature and values for all the parameters studied with the exception of respiratory burst activity. A positive correlation between hours of daylight and the values for the parameters studied was repeated with the exception of lysozyme activity (P=0.069). Plasma lysozyme activity was shown to be significantly positively correlated with temperature, but not photoperiod. This suggests from this data that temperature is potentially the more dominant seasonal cue for this parameter.

White blood cell counts increased steadily during the spring months peaking in July (2002)/ June (2003). They then decreased during the autumn and winter months to their lowest level in March (2003). This corroborates the work of previous studies in that white blood cell counts exhibited a seasonal rhythm (Slater and Schreck, 1998). It was observed in this trial that total white blood cell counts generally increase during spring, peaking in the summer and then decreasing in activity in autumn to their lowest levels in winter. This coincides with the seasonal increase in water temperature and day length. However although seasonal, the results of previous studies are somewhat different to those presented here i.e. the pattern of seasonality seen is not the same for

example, Collazos *et al.* (1998), reported that leucocyte counts for both male and female tench were significantly lower in spring and winter when compared with summer and autumn. However, the seasonal variation in white blood cell counts corroborates with the hypothesis presented by Slater and Schreck (1998). "In general, all immune parameters are suppressed in winter and highest in summer".

A slight seasonal effect was observed for red blood cell counts as lowest values were recorded in the winter months of November (2002), December (2002) and January (2003) whereas the highest red blood cell counts were recorded during July (2002) and June (2003). It is well documented that cold water contains a greater quantity of dissolved oxygen compared to warmer water. Consequently, fewer red blood cells are required to carry oxygen around the body of the fish in colder weather as the oxygen is more readily available. However, in the warmer summer months of July (2002) and June (2003) the reduced availability of oxygen means that more red blood cells are required to absorb available oxygen. Furthermore, winter is a period of reduced activity and therefore less energy is expended i.e. a reduced metabolism, thus less oxygen would be required. This is another reason for the observed seasonality of red blood cell counts.

Lysozyme activity exhibited a seasonal pattern during the 12-month period. The pattern is very similar to that of the white blood cell counts in that activity increased in spring peaking in late summer and then decreased over autumn to its lowest level in late winter. This has also been shown in dab, in which a generally consistent seasonal trend in plasma lysozyme activity was observed, with low values being associated with winter (Fletcher and White 1976). Again, this corroborates the hypothesis of Slater and Schreck (1998) "In general, all parameters are suppressed in winter and highest in summer".

Although significant differences were recorded in respiratory burst activity ($P<0.05$) according to month, these cannot really be said to be seasonal. The highest levels of respiratory burst activity occurred in August (2002) and February (2003) and the lowest in January (2003). No known studies have been carried out to examine the effect of season on respiratory burst activity. Rather trials have concentrated on the parameter of temperature although disparate results have been published (Nikoskelainen *et al.*, 2004). In this study the peaks observed in August (2002) and February (2003) were significantly higher than at all other sample times ($P<0.05$). Respiratory burst activity has been reported to increase in kidney leucocytes isolated from dab following a stress event (Pulsford *et al.*, 1994). It may be that the fish in the trial had underwent a stressful event in the 24 h period prior to sampling, e.g. perhaps the tanks had been cleaned. Unfortunately, there is no record of when such cleaning events were carried out.

A general seasonal pattern of the innate immune response and haematological parameters studied with the exception of respiratory burst activity was observed. Generally immune parameters were highest in summer and lowest in winter. The seasonality exhibited may be in response to seasonal increases in the level of potential pathogens in the environment. The seasonal patterns in immunity maybe correlated to seasonal patterns in pathogen load. For example, several diseases of the aquatic environment present a seasonal pattern, including proliferative kidney disease (PKD). One of the most economically important diseases among commercially reared rainbow trout in Europe. The disease is often seasonally dependent, occurring at water temperatures above 15°C in the summer and autumn months of the year (Hedrick *et al.*, 1993). Furunculosis (*Aeromonas salmonicida*) is also generally a seasonal disease, with acute outbreaks occurring when water temperatures are about 20°C and chronic

infections occur when temperatures are 13°C (Hjeltnes *et al.*, 1995). This co-ordinates well with the seasonal patterns exhibited by lysozyme activity and white blood cells as the immune system is being built up in preparation for potential attack by seasonal pathogens. If this is the case, the change in climatic conditions must be being anticipated otherwise any preventative measures to combat the increased pathogen load would occur too late. However, the mechanisms that regulate these seasonal components have yet to be completely identified.

The closer the value of Pearson's correlation coefficient is to 1 or -1 the stronger the correlation. Unsurprisingly, there is an extremely strong positive correlation between photoperiod and temperature, Pearson's value = 0.691. White blood cell counts, red blood cell counts and lysozyme activity all exhibit a positive correlation with both temperature and photoperiod. Respiratory burst activity is the only parameter to exhibit no correlation with either of the seasonal cues. This could be that it is always maintained at a background level and only increases when it is required.

The strongest correlation was observed with white blood cell counts and were very strongly correlated with temperature, Pearson's value = 0.747. Plasma lysozyme activity also showed a stronger correlation with temperature than for photoperiod. Red blood cell counts however, showed a greater correlation with photoperiod. These results suggest that temperature is the principle environmental cue for the seasonality for the immune parameters of white blood cell numbers and lysozyme activity. This will be investigated further in Chapters 3 and 4.

Melatonin is known to mediate the effects of daylength on both daily and seasonal behavioural and physiological events in certain vertebrates (Randall, *et al.*, 1995). It is well documented that there is a cyclical daily rhythm of melatonin

production, with the majority of the hormone produced during the dark phase (Hazlerigg *et al.*, 2001). This is corroborated by the results of this trial, as there is a significantly higher level of melatonin (P=0.000) in the night time samples compared to day time samples. In addition, the results indicate that melatonin levels change with season, with levels being significantly higher during the summer months compared to winter in both the day and night time samples. This may be an effect of temperature. Porter *et al.* (2001), reported that plasma melatonin levels in Atlantic salmon were significantly higher in fish maintained at 12°C compared to those at 4°C. This corroborates the results of the trial presented in this chapter, as temperature in the summer months was obviously greater in summer than winter. Consequently, it is not unexpected that melatonin levels would be higher during the warmer, summer months.

The increasing age of the fish may have affected melatonin levels. The majority of studies examining the effect of melatonin and age have been carried out in mammals. A study in rats concluded that pineal and plasma melatonin concentrations decline with age (Djeridane and Touitou, 2001). This pattern was not observed in the trial presented here. A strong seasonal pattern was observed with the lowest values being observed at the first sampling point which took place in winter and the highest in the early summer month of May.

2.5 Conclusion

The results of the trial have achieved the original aims of this study. Primarily, a base line of data for a variety of innate immune responses was compiled for a 12-month trial period. In addition, a seasonal pattern was observed for several of the parameters measured i.e. white and red blood cell counts and lysozyme activity has been recorded. However, the seasonal response observed for these parameters cannot be attributed to a specific seasonal parameter such as photoperiod or temperature because the annual profiles of photoperiod and temperature are closely correlated, consequently any association with season could be due to either photoperiod and/or temperature (Fig. 2.1). Consequently, studies examining the seasonal parameters of photoperiod and temperature were carried out independently and are presented in the following chapters.

The pineal hormone, melatonin, has been shown to exhibit both a diurnal and a seasonal pattern. Production is known to be affected by both photoperiod and temperature. This trial has demonstrated the seasonality of the hormone melatonin. A trial carried out examining the effect of melatonin on the immune system is discussed in Chapter 5.

During the 12 month trial the seasonal peaks of some of the immune parameters studied may be occurring at times of the year when the rainbow trout are at the greatest risk disease. If this is the case, it would be advantageous to the fish to anticipate these risks and be prepared for them in advance. The mechanism that anticipates these seasonal changes is yet to be completely identified.

Chapter 3 Temperature and Innate Immunity

3.1 Introduction

Most fish species cannot regulate their internal temperature. Consequently, the effects of temperature on the immune response of ectotherms such as teleosts are of particular interest (Baras, 1995). In general, immune parameters are suppressed during winter and are highest in summer (Slater and Schreck, 1998).

Substantial literature is available on the immunological rhythms cell numbers and activity *in vitro* as well as *in vivo,* in both laboratory animals and man (Shifrine *et al.,* 1982a and b). It is only within the last decade that there has been increased interest in the effect of temperature on the immune responses of fish. The susceptibility of fish to disease is partly dependent on their environment. This is a result of the close relationship that exists between teleost fish and their surroundings (Le Morvan *et al.,* 1998). Since fish are poikilothermic, environmental temperature will influence all aspects of their physiology, including the immune response (Collazos *et al.,* 1994a). There is currently a great deal of interest in determining how ectotherms, such as teleost fishes, are able to achieve homeostasis, considering that most fish cannot regulate their internal temperature. Moreover, the nature of the adaptive immune response is dependent on the time scale of the environmental change in relation to the generation time of the organism (Baras, 1995).

It has been observed that during cold periods, poikilothermic animals suffer immunodepression due to the low temperatures, especially in warm water fish such as the tench (Collazos *et al.,* 1998) and sea bream (Tort *et al.,* 2004). Due to the potential impact of this phenomenon on the aquaculture industry, the mechanism of low

temperature-induced immunosuppression in fish has been the focus of considerable research in recent years (Bly and Clem, 1992).

3.1.1 The Effect of Temperature on the Occurrence of Fish Disease

The onset of infectious diseases within the aquaculture industry has often been related to changes in environmental temperature and as a result, a cause and effect relationship between low temperatures and immune competence *in vivo* is frequently inferred. For example, in southern USA, the passage of a severe cold weather front can decrease commercial catfish pond water temperatures as much as 9°C in 12 hours. Moreover, the designs of the catfish ponds are such that they are both shallow and have a large surface area and consequently yearly pond water temperatures can vary between 4°C and 38°C. Winter temperatures are known to show the greatest fluctuations, having a normal range of 6-23°C. The fish within these ponds have to endure these rapidly changing and sometimes physiologically low temperatures. Under normal circumstances their primary behaviour to such hostile conditions would be to swim away from the adverse temperature to a more favourable one. Obviously this response cannot be carried out because the fish are enclosed within a farm pond (Bly and Clem, 1992). This inauspicious environment is a direct cause of immunosuppression in the fish, primarily as a result of stress, leaving the fish more susceptible to disease through the creation of 'immunological disasters' (Bly and Clem, 1992).

Disease organisms themselves are affected by temperature. Many diseases have specific temperatures at which they are either optimised or inhibited. There are several published reports indicating significant temporal differences in the occurrence of infectious disease outbreaks in wild populations of fish (Table 3.1). For example, in the North Sea, populations of dab have been documented as having maximum prevalence's

of epidermal papilloma and lymphocystis lesions during the spawning period (March-May), with minimal prevalence's during June to October (Hutchinson and Manning, 1996).

Table 3.1 Optimal temperature for a selection of bacterial diseases of fish (Adapted from Inglis *et. al.*, 1993)

Bacterial Disease	Causative Agent	Optimal Temperature of Occurrence
Bacterial Coldwater Disease	*Flavobacterium psychrophilum*	15°C
Columnaris Disease	*Flavobacterium columnare*	20.5-22.2°C
Edwardsiella septicaemia	*Edwardsiella tarda*	30°C in catfish
		10-18°C Japanese eels
Enteric septicaemia of catfish	*Edwardsiella ictaluri*	25°C
Vibriosis	*Vibrio anguillarum*	15°C
	Vibrio salmonicida	15°C
Furunculosis	*Aeromonas salmonicida*	22-28°C
Pasteurellosis	*Photobacterium damselae* subspecies *pisicida*	25°C
Bacterial Kidney Disease	*Renibacterium salmoninarum*	15-18°C
Streptococcal septicaemia	*Streptococcus sp.*	20+°C
Mycobacteriosis	*Mycobacterium marinium*	25°C

3.1.2 The Effect of Temperature on the Adaptive Immune Response

It is generally accepted that higher temperatures enhance adaptive immune responses (Bly and Clem, 1992) i.e. the production of antibody is faster and of a higher magnitude, whereas lower temperatures adversely affect antibody expression (Le Morvan *et al.,* 1997). For example, the immune responses of channel catfish have been altered by both *in vivo* and *in vitro* temperature fluctuations. Lower temperatures tended to have an inhibitory effect on the production of specific antibody and warmer temperatures resulted in higher antibody responses (Clem *et al.*, 1984; Bly & Clem, 1992).

It has been suggested that all fish species have a thermal limit, below which there is no adaptive immune response. This 'non-permissive' temperature is dependent upon the species (including age and sex), the type of response being measured, the length of the acclimation period and the interaction with other environmental parameters (Carlson *et al.,* 1995).

Several studies have been carried out looking at the effect of temperature on the adaptive immune response, in one such study it was observed that, in carp, the ability to produce antibodies is clearly suppressed at 10°C (Kurata *et al.,* 1997). A functional decline in helper T-lymphocytes at lower temperatures may cause this suppression in antibody production (Kurata *et al.,* 1997). In a study carried out to investigate temperature dependent-activation of leucocyte populations in rainbow trout after intraperitoneal immunisation with *A. salmonicida* (Köllner and Kotterba, 2002), it was found that the amount of antibodies were higher in sera of trout kept at 10-12°C at day 22 and day 28 post-injection, compared to that of trout kept at 15-17°C. In this case,

immunity was better at a lower temperature, this suggests that there may be an optimum temperature for antibody production.

In a study investigating the immunological assessment of hybrid striped bass at three culture temperatures, it was found that the length of time required to develop a protective antibody response was affected by small temperature variations (3°C). A decrease in water temperature as slight as 3°C can result in suppression of the primary adaptive response for several days, even if this reduced water temperature is within the preferred physiological range of 24-30°C (Carlson *et al.*, 1995). This is corroborated by Cecchini and Saroglia (2002) who reported that sea bass immunized against human-gamma globulins emulsified in Freund's complete adjuvant exhibited a higher antibody response was observed in fish reared at 24 and 30°C, than at 12 and 18°C.

In a study carried out by Xianle and Wengong (1997), grass carp were immunized with the killed cell-cultured vaccine against Fish Reovirus (Vaccine CFRV), The experimental results showed that the water temperature of 10°C is the critical point at which to immunize grass carp with Vaccine CFRV and that the immune response was inhibited below 10°C and enhanced with increasing water temperature. However, immunity decreased above 32°C (the temperature suitable for the growth of grass carp). This study concluded that the water temperature during the inductive phase is one of the key factors that determined the occurrence and strength of the immune response.

The majority of studies report immune suppression at low temperature (Bly and Clem, 1991; Bly *et al.*, 1997). Some studies observed no change at all; for example, seasonal levels of plasma immunoglobulin (presumably representing antibodies) have been assayed in channel catfish and appear to be maintained at ~40 mg l^{-1} whether fish were assayed during summer or winter (Bly and Clem, 1992).

3.1.3 The Effect of Temperature on Innate Immune Response

The effects of seasonal temperatures *in vitro* on the fish's immune response are well established, and there is little doubt that low temperatures can suppress adaptive immune responses (i.e. T and B cell mediated). However, there is still controversy over the effects of temperature on innate immune responses (Bly *et al.*, 1997). This is primarily because there is little data available concerning the thermosensitivity of innate immune responses (Le Morvan *et al.*, 1997).

3.1.3.1 Phagocytosis

It has been documented that in channel catfish, the primary impact on phagocytic function was due to assay temperature, although phagocytes appear to be more resistant to low temperature than lymphocytes which implies the importance of phagocytosis in the defence mechanisms of channel catfish at low temperatures (Ainsworth *et al.*, 1991; Le Morvan *et al.*, 1997). Moreover, in tench it has been found that phagocytic functions were elevated during the colder winter temperatures (Bly *et al.*, 1997).

Tench have been documented to show an increase in phagocytosis activity from autumn to winter, which was due to both a higher number of granulocytes with phagocytic activity and a greater efficiency of phagocytosis (Collazos *et al.*, 1995). In a later study, tench maintained at two different temperatures 12°C (the actual temperature of the natural habitat of these fish during winter) and 22°C (a commonly used temperature within the physiological range) exhibited an increased capacity to ingest inert particles at 12°C (Collazos *et al.*, 1994b).

3.1.3.2 Macrophage Respiratory Burst Activity

Little work has been carried out on the effect of temperature on respiratory burst activity of head kidney macrophages. However, it has been documented that in channel catfish this activity was improved at low temperature (12°C) which would imply improved bacterial killing ability (Dexiang and Ainsworth, 1991; Le Morvan *et al.*, 1997), whereas in colder climates, it has been reported that respiratory burst activity in rainbow trout is stimulated by increased temperature (Nikoskelainen *et al.*, 2004).

3.1.3.3 Plasma Lysozyme Activity

It has been documented that with dab, there is a generally consistent seasonal trend in lysozyme activity with low values being associated with reduced sea temperatures, time of spawning and poor condition factor. This corroborates an investigation carried out on the seasonal trends of lysozyme activity in plaice (Fletcher and White, 1976).

3.1.3.4 Cellular Responses in Trout Skin

External epithelial tissues such as skin form the primary barrier between the internal tissues and the external environment. Consequently, they are the first to experience environmental changes (Iger *et al.*, 1994).

Following a three-hour period of moderate elevation in water temperature, pronounced and prolonged effects on the skin of rainbow trout have been documented (Iger *et al.*, 1994). The most prominent effects of temperature elevation on the trout epidermis were the increased secretory activity of the upper layers of the filament cells and the high incidence of apoptosis in the upper and inner layers of filament cells. Both migration and secretion of mucus cells were stimulated by elevated temperature. In

addition, the thermal shock rapidly initiated extravasation of leucocytes and the penetration of these cells into the epidermis (Iger *et al.*, 1994).

3.1.4 The Effect of Temperature on Haematological Parameters

Several studies have been carried out to investigate the effect of seasonal changes on haematological parameters (Collazos *et al.*, 1998; Ainsworth *et al.*, 1991). For example, Collazos *et al.* (1998), carried out one such study. It was designed to determine changes in red and white blood cell levels and plasma proteins in the blood of tench during the four seasons of the year. The haematological parameters of fish are used as an indicator of their physiological state and the use of these measurements has become widespread in the monitoring of pathologies in fish farming (Martínez *et al.*, 1994). Langston *et al.* (2002), reported that temperature exerts a considerable influence on some blood parameters and on some humoral parameters of halibut. For example, a high temperature of 18°C caused a decrease in the number of circulating blood cells and an increase in serum lysozyme levels.

3.1.4.1 Erythrocytes

Red blood cell counts have been found to be significantly higher in spring than in autumn or winter in male tench. This was thought to be an adaptation of the fish to the warmer seasons, reflecting the decline in the dissolved oxygen content of water making it necessary for improved oxygen uptake within the fish i.e. an increase in erythrocytes (Collazos *et al.*, 1998).

3.1.4.2 Leucocytes

Seasonal changes in white blood cell counts have also been observed. In tench the highest values have been recorded in autumn followed by a sharp drop in winter (Collazos *et al.*, 1998). These values then became noticeably higher (although not significant) by the summer. It was suggested that the increase in total white blood cell levels in tench during the summer, pointed towards a possible protective role for the white blood cells during the warmer seasons. Potentially, this could be the cause of the immunosuppressed state of fish in winter (Collazos *et al.*, 1998).

Lymphocyte proliferation has been documented to exhibit seasonal fluctuations as a result of temperature (Bly *et al.*, 1997). In the tench lymphocyte proliferation was lower during the winter months with best responses obtained *in vitro* with an assay temperature about 5°C above ambient (Bly *et al.*, 1997). Lower temperatures can also cause a functional decline in cytotoxic T lymphocytes. In addition, lower temperatures also reduced production of macrophage activating factor (MAF) by T lymphocytes (Kurata *et al.*, 1997). As result, an adaptive immune system, controlled by T lymphocytes, would not be effective at lower water temperatures (Kurata *et al.*, 1997).

Different water-rearing temperatures have been shown to change the cellular composition of head kidney leucocytes in carp, with the percentage of neutrophilic granulocytes being higher in fish kept at 10°C compared to fish kept at 25°C. Re-acclimation of fish kept at 10°C to 25°C resulted in a reduction in the number of neutrophilic granulocytes (Kurata *et al.*, 1997).

More recently it has been reported that sudden drops in temperature (e.g. over a 3 hour period of 9°C) which can cause a significant stress response which can be measured as a rise in cortisol (Tanck *et al.*, 2000; Engelsma *et al.*, 2003). This in turn

has been shown to affect the dynamics of leucocyte populations, for example relative number of circulating B-lymphocytes in the total leucocyte population decreased significantly within 4 h after the onset of single or multiple cold shocks (Engelsma *et al.*, 2003).

3.1.4.3 Haematocrit Values

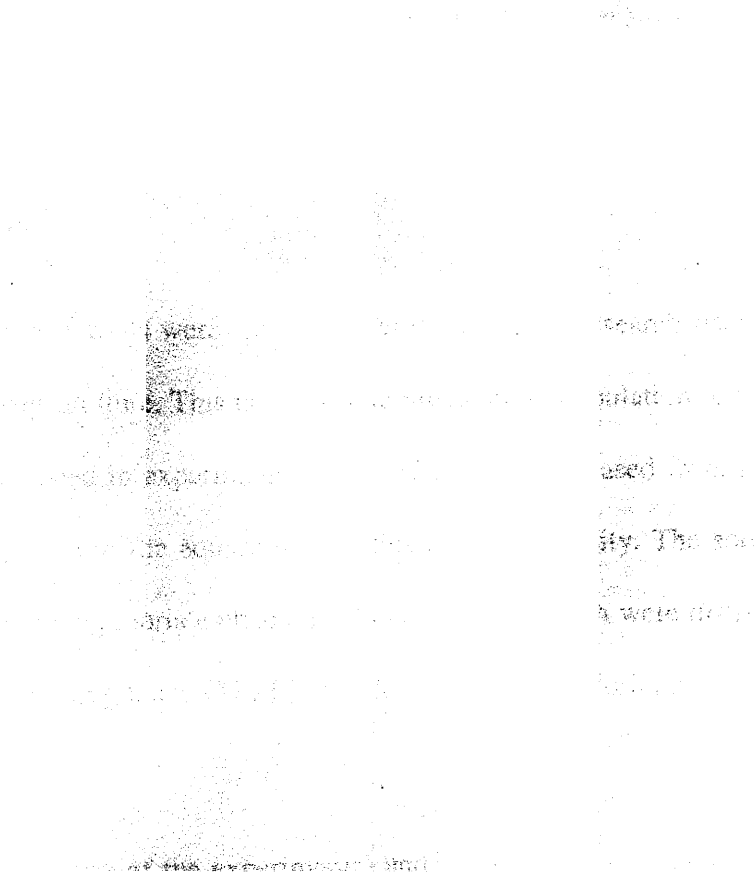
Haematocrit values have been reported to be influenced by season, and these are reported to be lower in winter than spring (North, 2004). This was initially attributed to changes in water temperature. However, when a trial was carried out over a period of twelve months at a constant temperature, haematocrit was still observed to undergo seasonal changes (Denton and Yousef, 1975). Consequently, it was suggested that these seasonal changes were probably related to the degree of physical activity and to metabolic adaptations (Denton and Yousef, 1975). However, correlations have been found between haematocrit and temperature, but only at very high water temperatures (Martínez *et al.*, 1994)

3.1.5 Aims

This chapter is composed of two trials.

- The effect of three different water temperature treatments (5, 10 and 18°C) on a variety of innate immune and haematological parameters, were examined in the first trial (Trial A). The trial was carried out in winter and repeated in summer. The aims of this trial were to examine the effect of temperature and season on innate immunity.

- The effect of acclimating fish held at the three different water temperature treatments (5, 10 and 18°C) to 15°C, was examined in the second trial (Trial B). This temperature was chosen because 15°C is the water temperature most effective for artificially challenging with *V. anguillarum*, which was carried out as part of Trial A. The aim of this study was to determine if acclimating the fish to 15°C affected their innate immunity or haematological parameters. That is, the results observed from the artificial challenge in Trial A, may have been the result of the action of acclimating the fish to 15°C, rather than the effect of the original water treatment temperatures the fish were held at.



3.2 Materials and Methods

Trial A - Effect of Three Different Temperatures on Immunity

In this initial investigation into the effect of temperature on the innate immune response of rainbow trout, three different temperatures were used (5, 10 and 18°C (±2)). The trial was carried out in winter and then repeated in summer. All female fish were used and the same stock of fish was used in each trial. Difficulties were experienced with the chiller and heating equipment in that the system could not be relied upon for longer periods of time, consequently it was decided that sampling would occur at one sampling point during winter and summer. Twenty fish were analysed for each water temperature.

3.2.1 Trial Set-Up

The temperature trials were carried out at the Aquatic Research Facility (ARF) at the Institute of Aquaculture. This is because temperature manipulation is not possible at the NBFRF. Fish used in experiments at the ARF were purchased from local farms and transported by road to the aquarium facilities at the university. The source of the fish is indicated in the appropriate chapters. Upon arrival, the fish were distributed into disinfected aerated holding tanks (370 L) in a flow-through dechlorinated water system at ambient temperature.

The water capacity of the experimental and challenge tanks was 100 L, with a flow through of 1.5 L ml^{-1}. During an acclimation period of three or more weeks, the fish were fed once daily with commercial pelleted trout feed (EWOS, Edinburgh)

according to the manufacturers feed tables. Following acclimation the fish were graded according to their length and distributed into the experimental tanks, which again had been disinfected and were supplied with flow-through dechlorinated water. Fish were fed once daily according to the manufacturers feed tables. Throughout both the acclimation and experimental periods mortalities, feeding behaviour, water temperature and experimental procedures were recorded daily.

Fish were held in 6 circular tanks with 30 fish per tank at the start of the experiment. There were two tanks per temperature treatment. Fish were maintained under a 12:12 LD photoperiod throughout the trial (this is fixed for all research carried out at the ARF). Fish were acclimated to their respective temperatures for 4 weeks prior to sampling.

3.2.2 Measurement of Innate Immunity and Haematological Parameters

Sampling took place after 4 weeks of acclimation with ten fish sampled per tank allowing for a total of twenty fish to be sampled per temperature treatment. Fish lengths and weight were taken along with blood samples. Both fish and blood were transported back to the laboratory on ice.

In the laboratory a variety of innate parameters were measured: macrophage respiratory burst activity, white and red blood cell counts, haematocrit and lysozyme activity. Methods are described in Sections 2.2.2, 2.2.3, and 3.2.3 (haematocrit). In addition, fish were challenged with *V. anguillarum* at the Aquatic Research Facility (Section 3.2.4) at the Institute of Aquaculture to determine if temperature affects survival (20 fish per temperature treatment).

3.2.3 Haematocrit

Heparinised capillary tubes were filled with blood, sealed with Critoseal™ (Hawksley & Son, England) and centrifuged in a Hawksley haematocrit centrifuge (Hawksley & Son, England) for 3 min. The percentage of packed cell volume (haematocrit) was calculated using a Hawksley reader (Hawksley & Son, England).

3.2.4 Bacterial Challenge with *V. anguillarum*

Bacterial challenge was used to measure the adaptive immune response in fish that had been previously vaccinated against *V. anguillarum,* by looking at the level of protection elicited by the vaccine in the different treatment groups. However, it was also used to examine the innate immune response in non-vaccinated fish not previously exposed to the bacterium.

3.2.4.1 Preparation of *V. anguillarum* Broth Culture for Injection Challenge.

Sixty ml of TSB + NaCl was prepared and inoculated with colonies taken from a stock culture plate of *V. anguillarum* (Serotype 1). The broth culture was incubated overnight at 22°C.

On Day 2 the optical density of the bacteria was measured at 610nm using a Cecil CE 2041 2000 Series spectrophotometer and the bacterial concentration of the suspension was calculated using the appropriate regression equation from a standard curve (Appendix B), and then diluted to 7×10^7 CFU ml^{-1} using sterile PBS.

3.2.4.2 Bacterial Challenge by Injection

The challenge was carried out in the challenge suite of the ARF. Tanks were set up with an ambient water supply and a 12:12 LD photoperiod. Fish from replicate tanks were mixed together and then divided into six separate tanks to reduce possible tank effects.

The fish were anaesthetised prior to injection with bacteria, and they received a dose, decided from pre-challenge results (the dose used in each challenge is described during the relevant trial). Bacteria were delivered by intraperitoneal injection. Fish were then allowed to recover in aerated baths before being returning them to their respective tanks.

Fish were monitored twice daily, and any mortalities recorded. Fish were analysed according to Section 3.2.4.4 to confirm that they had died as a result of infection with *V. anguillarum*.

3.2.4.3 Calculation of the Exact Dose of Bacteria Administered

Calculating the dose administered using optical density based on the regression equation alone is inaccurate because the suspension can contain both live and dead bacteria. The exact dose of bacteria was calculated using colony-forming unit (CFU) counts. One ml of the 7 x 10^7 CFU ml^{-1} sample was aliquoted into 9 mls sterile PBS. This results in a tenfold dilution of the original dose that was injected into the fish (10^{-1} dilution). This serial dilution was repeated down to a 10^{-8} dilution. Tryptone Soya Agar (TSA + 2% NaCl) (Appendix A) plates were labelled with the dilution factor and divided equally into 6 segments. A 20 μl aliquot of each prepared dilution was placed into the appropriate segment of the agar plate.

The plates were incubated at 22°C overnight until small colonies that had not overlapped could be observed easily. The average number of colonies on the plate was calculated and multiplied by 50 to give CFU ml^{-1}.

3.2.4.4 Confirmation of Specific Mortalities

Kidney swabs from fish that had died following artificial challenge with *V. anguillarum* were taken using a sterile loop. TSA 2% NaCl plates were streaked 4 times. This allows colonies to be observed. The plates are then sealed with Nescofilm and incubated at 22°C overnight.

Gram Staining

A single bacterial colony was selected from the agar plate streaked with the kidney swab and emulsified in a single drop of sterile PBS on a clean microscope slide. This was spread around the slide using a sterile loop to obtain a thin bacterial film that was allowed to air dry. The slide was then gently heat fixed by passing the slide twice through a hot bunsen flame. The slide was stained for 1 min with crystal violet solution, fixed for 1min with iodine solution and then destained with acetone for 5 sec. The slide was immersed in cold water for 5 sec and then counter stained in safarin for 2 min. Once dry, bacteria were observed under oil immersion x 1000 magnification and their colour and shape was recorded. Gram positive organisms stained blue/purple and Gram negative organisms stained red/pink. *Vibrio. anguillarum* is a Gram negative, rod shaped bacteria.

Sensitivity Discs

Two colonies of bacteria were suspended in 2 ml of sterile PBS, and 5-10 drops were placed onto the appropriate agar plate and then spread around the plate using a sterile glass spreader to allow a bacterial lawn to develop. Sensitivity discs specific to *Vibrio* 0129 (0 μg and 150 μg) were placed on top of the lawn. Plates were incubated at 22°C overnight. Clear zones were evident around the discs if the bacteria were of the family *Vibrio.*

3.2.5 Measurement of Melatonin

Blood samples were taken during the final month of each trial at midday and two hours after darkness had fallen using heparinised syringes and transported on ice to the laboratory. Plasma was collected, samples were frozen at –70°C as soon as possible and later analysed using a melatonin radio immunoassay (RIA) as described in Section 2.2.4.

3.2.6 Statistical Analysis

Before analysis, data was tested for normality and homogeneity of variance using fits and residuals. All data was found to be normal and homogenous without transformation. Data was analysed using an ANOVA General Linear Model (Minitab) and post hoc Tukey tests measuring Pairwise comparisons.

Statistical analysis of the survival to challenge data was performed using the survival test Kaplan Meier (SPSS).

Trial B -Acclimation Trial

During the original investigation into the effect of temperature (Trial A) general resistance to disease was assessed by artificial challenge with *V. anguillarum*. In order to successfully challenge with the bacterium, the water temperature was adjusted to 15°C. The action of changing the temperature from the original three treatment temperatures (5, 10 and 18°C) to 15°C on total red and white blood cell counts and lysozyme activity was investigated.

3.2.7 Trial Set-Up

The investigation was carried out at the ARF. Five fish from each tank i.e. ten fish per treatment (5, 10 and 18°C) were slowly acclimated to 15°C over one week. The trial was carried out using duplicate tanks. Fish were maintained under the original 12:12LD photoperiod. This experiment was carried out during the summer. Measurement of immune parameters and statistical analysis were carried out as per Sections 2.2.2, 2.2.3 and 3.2.6.

3.3 Results

Trial A - Effect of Three Different Temperatures on Immunity

The same stock of fish was used for both the winter and summer trials. Consequently the fish used in the summer trial were significantly larger than those of the winter trial. Average fish weight during the winter trial was 16.25 g (SD = 3.45; SE = 0.77) and 80.04 g (SD = 21.09; SE = 4.71) during the summer trial.

3.3.1 The Effect of Temperature on Immune and Haematological Parameters

3.3.1.1 White Blood Cell Counts

No significant difference was recorded in relation to the total white blood cell counts obtained with season (P=0.817), however, white blood cell counts were significantly affected by temperature (P=0.000) (Fig. 3.1). To determine where this significance occurred, Tukey tests were carried out. Fish held at 5°C exhibited a significantly lower white blood cell count compared to fish held at either 10 or 18°C (P=0.000), regardless of the season. No significant difference was recorded between the winter and summer 5°C groups of fish (P=1.000). Fish held at 10°C exhibited a significantly lower white blood cell count compared to fish held at 18°C (P=0.000), regardless of season. No significant difference was recorded between the winter and the summer with groups of fish held at 10°C (P=1.000). Fish held at 18°C exhibited a significantly higher white blood cell count compared to fish held at either 5 or 10°C (P=0.000), regardless of season. No significant difference was recorded in the white blood cell counts of fish held at 18°C between winter and summer groups (P=1.000).

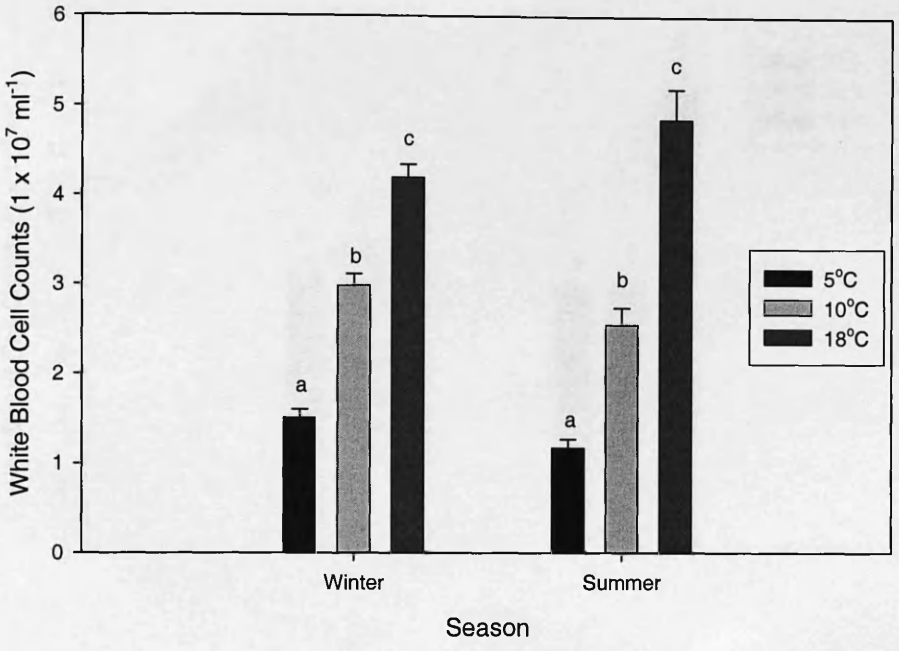


Fig. 3.1 Effect of season and temperature on total white blood cell counts for fish held at three different water temperatures (5, 10 and 18°C) (n=20/treatment/season; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b' and 'c'; 'b' is significantly lower than 'c' (P<0.05).

3.3.1.2 Red Blood Cell Counts

Red blood cell counts (Fig. 3.2) were found to be significantly different according to both the season (P=0.045) and the water temperature (P=0.000). Further examination of the data using Tukey Pairwise comparisons determined that the red blood cell counts of fish held at 5 and 10°C were significantly lower than those of fish held at 18°C (P=0.000). This was revealed to be a season/temperature interaction, since the red blood cell counts of fish held at 18°C were only significant greater than fish held at 5 and 10°C during summer (Table 3.2).

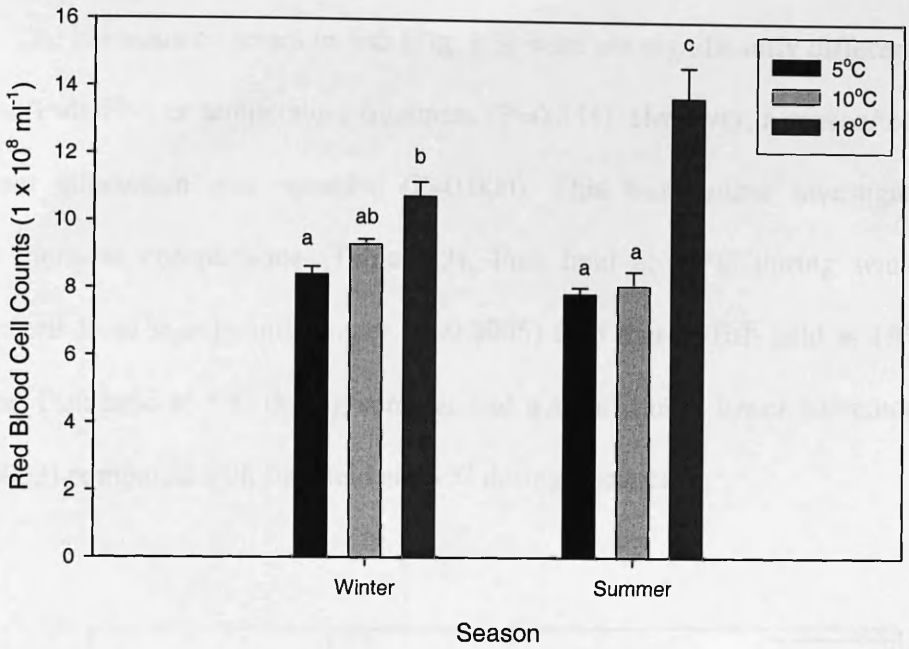


Fig. 3.2 Effect of season and temperature on red blood cell counts for fish held at three different water temperatures (5, 10 and 18°C) (n=20/treatment/season; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b'; 'c' is significantly greater than 'a' and 'b'; 'ab' is significantly lower than 'c' but not significantly different from either 'a' or 'b'(P<0.05).

Table 3.2 Tukey Pairwise Comparisons for season / temperature (n=20)

	Winter / 5°C	**Winter / 10°C**	**Winter / 18°C**	**Summer / 5°C**	**Summer / 10°C**	**Summer / 18°C**
Winter / 5°C	X					
Winter / 10°C	P>0.05	X				
Winter / 18°C	**P=0.005**	P>0.05	X			
Summer / 5°C	P>0.05	P>0.05	**P=0.000**	X		
Summer / 10°C	P>0.05	P>0.05	**P=0.000**	P>0.05	X	
Summer / 18°C	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	X

3.3.1.3 Haematocrit Value

The haematocrit levels in fish (Fig. 3.3) were not significantly different between seasons (P=0.079) or temperature treatment (P=0.355). However, a season/temperature treatment interaction was recorded (P=0.000). This was further investigated using Tukey Pairwise comparisons (Table 3.3). Fish held at 18°C during winter had a haematocrit level significantly lower (P=0.0006) than that of fish held at 18°C during summer. Fish held at 5°C during summer had a significantly lower haematocrit value (P=0.0033) compared with fish held at 18°C during summer.

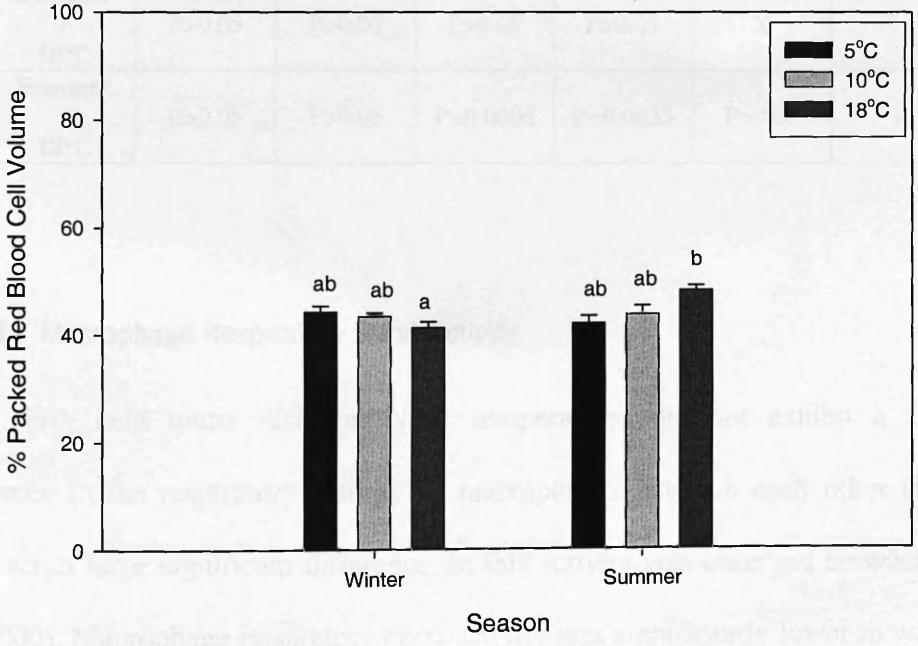


Fig. 3.3 Effect of season and temperature on haematocrit values for fish held at three different water temperatures (5, 10 and 18°C) (n=20/treatment/season; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b'; 'ab' is not significantly different from either 'a' or 'b' (P<0.05).

Table 3.3 Tukey Pairwise comparisons investigating the interaction between season / temperature was observed (n=20/treatment/season)

	Winter / 5°C	Winter / 10°C	Winter / 18°C	Summer / 5°C	Summer / 10°C	Summer / 18°C
Winter / 5°C	X					
Winter / 10°C	P>0.05	X				
Winter / 18°C	P>0.05	P>0.05	X			
Summer / 5°C	P>0.05	P>0.05	P>0.05	X		
Summer / 10°C	P>0.05	P>0.05	P>0.05	P>0.05	X	
Summer / 18°C	P>0.05	P>0.05	**P=0.0006**	**P=0.0033**	P>0.05	X

3.3.1.4 Macrophage Respiratory Burst Activity

Fish held under different water temperatures did not exhibit a significant difference in the respiratory activity of macrophages between each other (P=0.783). However, a large significant difference, in this activity was observed between seasons (P=0.000). Macrophage respiratory burst activity was significantly lower in winter than in summer (Fig. 3.4).

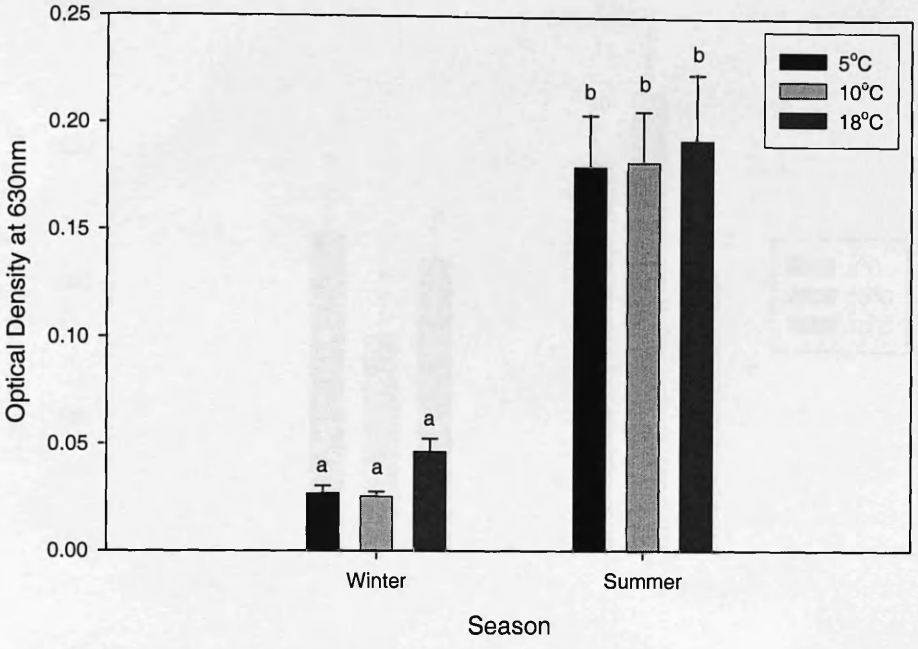


Fig. 3.4 The effect of season and temperature on macrophage respiratory burst activity for fish held at three different water temperatures (5, 10 and 18°C) (n=20/treatment/season; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b' (P<0.05).

3.3.1.5 Plasma Lysozyme Activity

Plasma lysozyme activity of fish held under three different temperature treatments (Fig. 3.5) was found to be significantly affected by temperature (P=0.000) and the seasons of winter and summer (P=0.04). In winter, fish held at 10°C exhibited the lowest lysozyme activity however, during the summer the opposite was true. An interaction between temperature and season was revealed from Tukey Pairwise Comparisons (Table 3.4). Fish held at 10°C in summer exhibited a significantly higher plasma lysozyme activity compared to fish held at 5, 10 and 18°C in winter, and this was significantly higher than fish held at 18°C in summer.

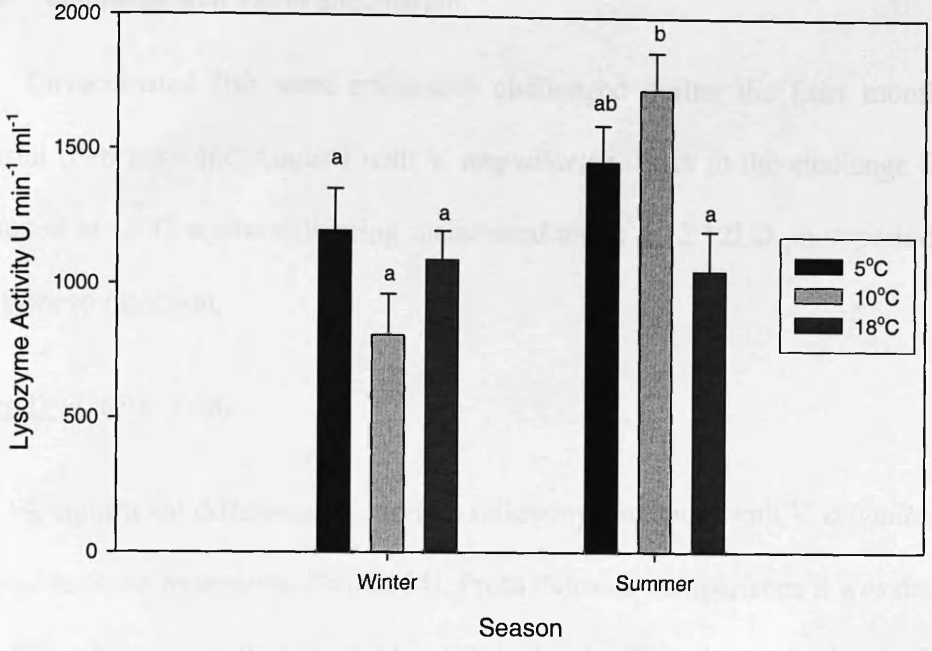


Fig. 3.5 Effect of season and temperature on lysozyme activity for fish held at three different water temperatures (5, 10 and 18°C) (n=20/treatment/season; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b'; 'ab' is not significantly different from either 'a' or 'b' (P<0.05).

Table 3.4 Results of Tukey Pairwise Comparisons investigating a season / temperature interaction (n=20).

	Winter / 5°C	**Winter / 10°C**	**Winter / 18°C**	**Summer / 5°C**	**Summer / 10°C**	**Summer / 18°C**
Winter / 5°C	X					
Winter / 10°C	P>0.05	X				
Winter / 18°C	P>0.05	P>0.05	X			
Summer / 5°C	P>0.05	**P=0.0004**	P>0.05	X		
Summer / 10°C	**P=0.0069**	**P=0.000**	**P=0.0005**	P>0.05	X	
Summer / 18°C	P>0.05	P>0.05	P>0.05	P>0.05	**P=0002**	X

3.3.1.6 Challenge with *Vibrio anguillarum*

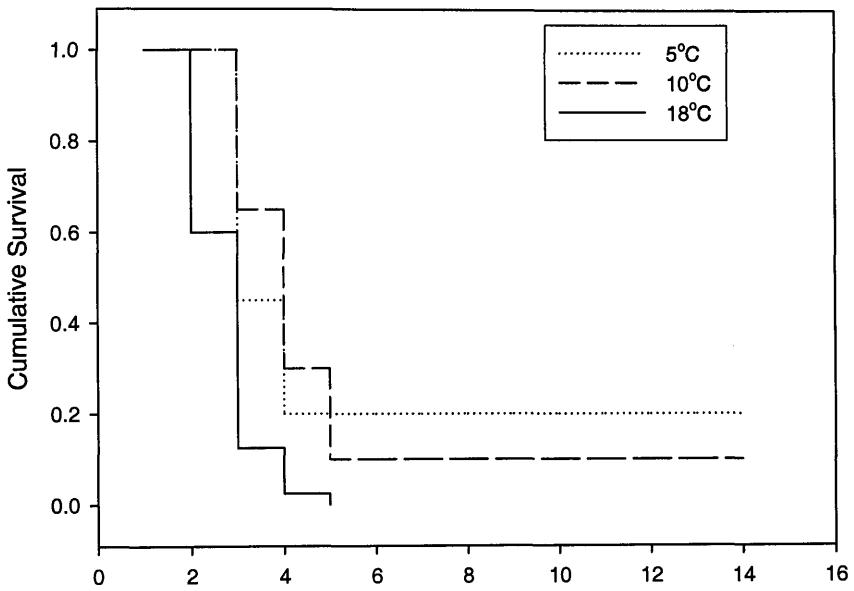
Unvaccinated fish were artificially challenged during the final month during each trial (February and August) with *V. anguillarum*. Prior to the challenge fish were acclimated to 15°C whilst still being maintained under a 12:12LD photoperiod for one week prior to injection.

Winter Trial. (Fig. 3.6a)

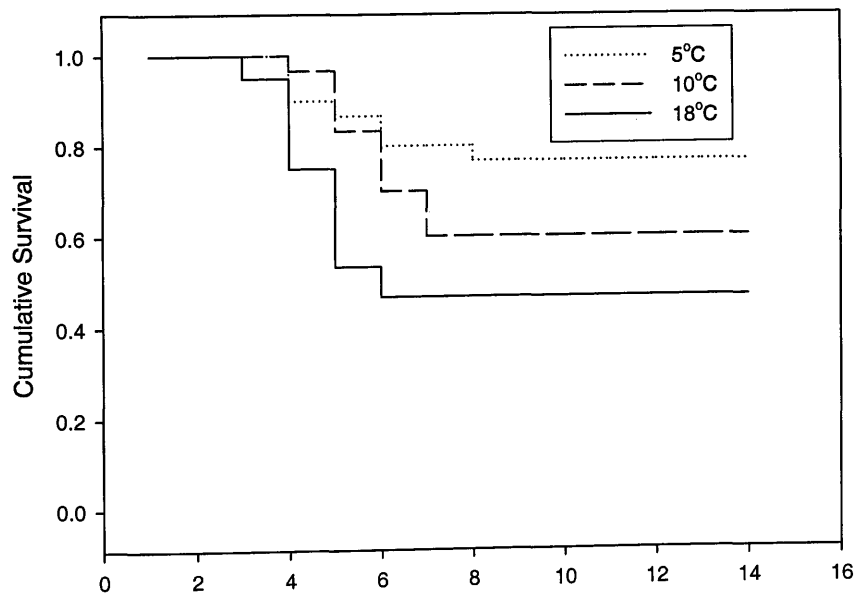
A significant difference in survival following challenge with *V. anguillarum* was observed between treatments (P=0.0184). From Pairwise comparisons it was determined that fish that had originally been held at 5°C had a significantly greater level of survival compared to fish held at 18°C (P=0.0298); and that fish held at 10°C had a significantly greater level of survival compared to fish held at 18°C (P=0.0037). Median survival was day 3 for fish from both the 5 and 18°C treatments; and day 4 for fish from the 10°C treatment.

Summer Trial (Fig. 3.6b)

A significant difference in survival following challenge was observed between treatments (P=0.0094). It was determined using Pairwise comparisons that fish from the 5°C treatment had a significantly higher level of survival compared to fish from the 18°C treatment (P=0.0056); and that fish held at 10°C had a significantly greater level of survival compared to fish held at 18°C (P=0.0507). Median survival was day 5 for fish from the 10°C treatment; and day 7 for fish from the 18°C treatment. No median survival day could be calculated for the 5°C group because there were too few mortalities.



a)



b)

Fig. 3.6 Mortality following artificial challenge with *V. anguillarum* for fish from the winter (a) and summer (b) challenges. Fish were challenged under a 12:12 LD photoperiod following acclimation to 15°C for a week. Fish were originally held at three different temperature treatments (5, 10 and 15°C) for three months. Increased bacterial dose for the summer challenge is because of the increased size of the fish.

3.3.2 The Effect of Temperature on Melatonin

The fish used in the winter trial were too small to collect sufficient plasma to determine the level of plasma melatonin in the individual fish. Consequently, the plasma sample from the fish was pooled. Although five fish were sampled per treatment there was only enough plasma to carry out one melatonin assay per treatment on fish in winter experiment. This explains the lack of error bars on the winter treatment bars (Fig. 3.7). This is also the reason why statistical analysis has only been carried out on the summer results

For the summer experiment, light (am) or dark phase (pm) photoperiod did not show any significant effect on plasma melatonin levels ($P=0.086$). However, water temperature did exert a significant effect on melatonin levels ($P=0.032$). Post-hoc analysis using Tukey Pairwise comparison tests on the data from the summer trial (Table 3.5) revealed that plasma melatonin levels of fish from the 10°C group were not significantly different from either the 5 or 18°C treatment fish. Fish maintained at 18°C had a significantly higher plasma melatonin level regardless of am or pm sampling compared to fish held at 5°C. No significant interactions of am, pm or treatment were observed ($P>0.05$).

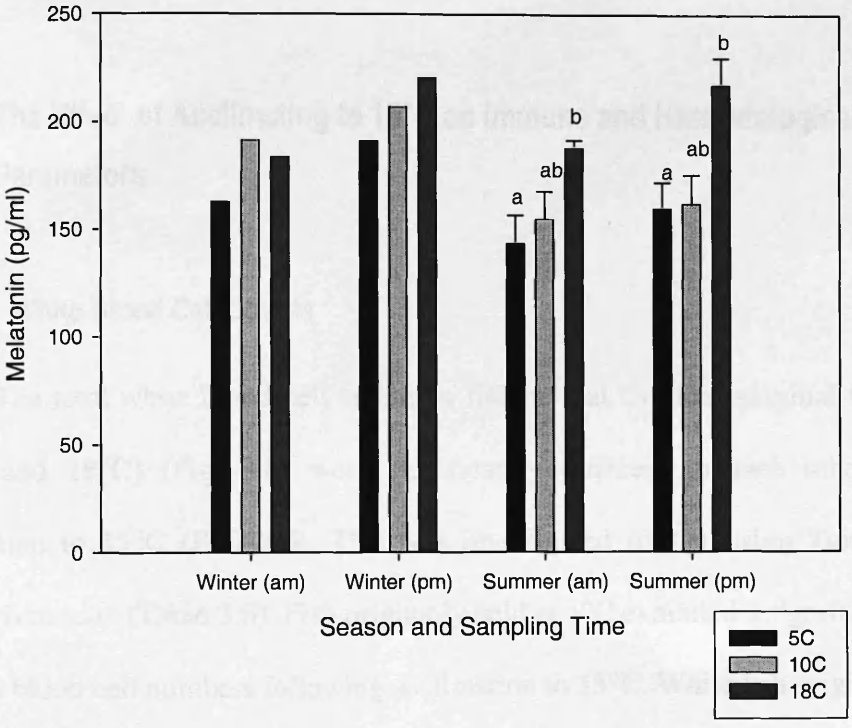


Fig. 3.7 Effect of season and temperature on plasma melatonin levels for fish held at three different water temperatures (5, 10 and 18°C) (n=5; mean ± SE,). Subscripts denote statistical significance, 'a' is significantly lower than 'b'; 'ab' is not significantly different from either 'a' or 'b' (P<0.05). Statistical analysis not carried out for the Winter trial as plasma was pooled from five fish to obtain one result per treatment.

Table 3.5 Tukey Pairwise comparison results for the effect of temperature on melatonin levels during the summer trial (n=5)

	5°C	10°C	18°C
5°C	X		
10°C	P>0.05	X	
18°C	**P=0.0261**	P>0.05	X

3.3.3 The Effect of Acclimating to 15°C on Immune and Haematological Parameters

3.3.3.1 White Blood Cell Counts

The total white blood cell counts of fish held at the three original temperatures (5, 10 and 18°C) (Fig. 3.8) were significantly different to each other following acclimation to 15°C (P=0.000). This was investigated further using Tukey Pairwise Comparison tests (Table 3.6). Fish originally held at 5°C exhibited a significant increase in white blood cell numbers following acclimation to 15°C. While fish originally held at 18°C exhibited a significant decrease in white blood cell numbers following acclimation to 15°C. However, fish originally held at 10°C did not exhibit a significant change in white blood cell counts following acclimation.

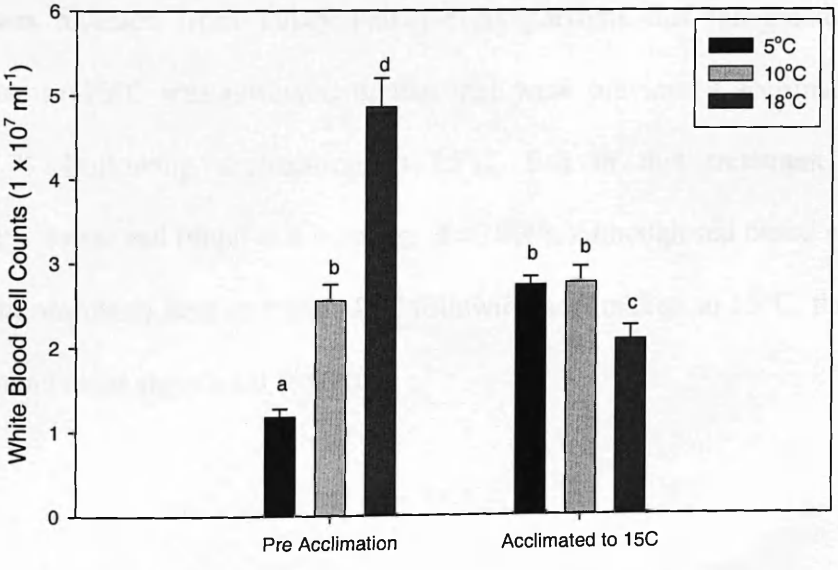


Fig. 3.8 The effect of acclimating the three treatment groups (5, 10 and 18°C) to 15°C on white blood cell numbers (n=20 pre-acclimation and n=10 acclimated; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b'; 'a', 'b' and 'c' are significantly lower than 'd' (P<0.05).

Table 3.6 Investigation into the interaction of acclimation and original temperature on white blood cell counts using Tukey Pairwise Comparisons.

	5°C	10°C	18°C	Acclimated from 5°C	Acclimated from 10°C	Acclimated from 18°C
5°C	X					
10°C	**P=0.000**	X				
18°C	**P=0.000**	**P=0.000**	X			
Acclimated from 5°C	**P=0.000**	P>0.05	**P=0.000**	X		
Acclimated from 10°C	**P=0.000**	P>0.05	**P=0.000**	P>0.05	X	
Acclimated from 18°C	P>0.05	P>0.05	**P=0.000**	P>0.05	P>0.05	X

3.3.3.2 Red Blood Cell Counts

The effect of acclimating fish from the three different water temperatures (5, 10 and 18°C) to 15°C, significantly affected their red blood cell numbers (P=0.000) (Fig. 3.9). It was revealed from Tukey Pairwise comparisons that the greatest effect of acclimation to 15°C was observed in fish that were previously acclimated to 18°C (Table 3.7). Following acclimation to 15°C, fish in this treatment group had significantly lower red blood cell numbers (P=0.000). Although red blood cell numbers rose in fish originally held at 5 and 10°C following acclimation to 15°C, the difference was not found to be significant (P>0.05).

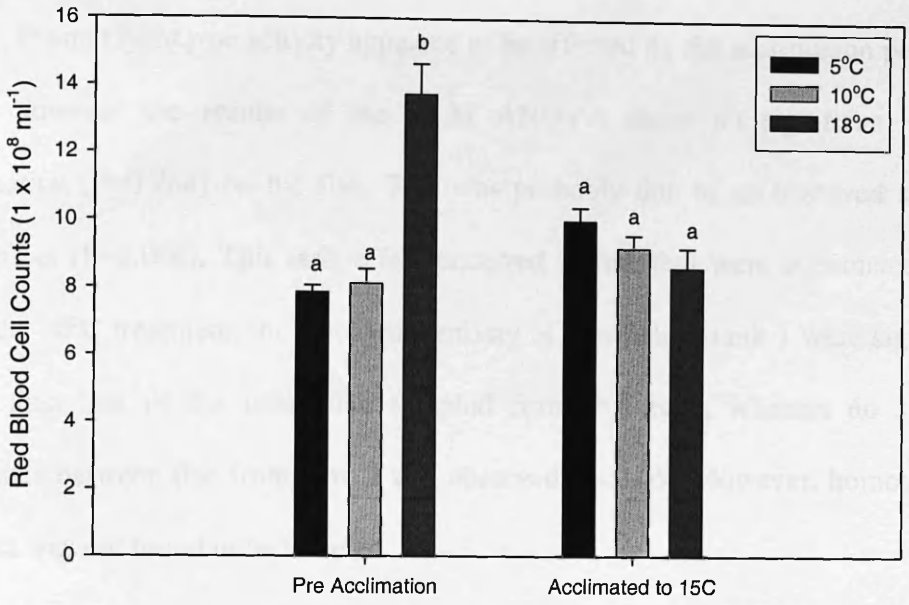


Fig. 3.9 The effect of acclimating the three treatment groups (5, 10 and 18°C) to 15°C on red blood cell numbers (n=20 pre-acclimation and n=10 acclimated; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b' (P<0.05).

Table 3.7 Investigation into the interaction of acclimation and original temperature on red blood cell counts using Tukey Pairwise Comparisons

	5°C	10°C	18°C	Acclimated from 5°C	Acclimated from 10°C	Acclimated from 18°C
5°C	X					
10°C	P>0.05	X				
18°C	**P=0.000**	**P=0.000**	X			
Acclimated from 5°C	P>0.05	P>0.05	**P=0.000**	X		
Acclimated from 10°C	P>0.05	P>0.05	**P=0.000**	P>0.05	X	
Acclimated from 18°C	P>0.05	P>0.05	**P=0.000**	P>0.05	P>0.05	X

3.3.3.3 Plasma Lysozyme Activity

Plasma lysozyme activity appeared to be affected by the acclimation period (Fig. 3.10), however the results of the GLM ANOVA show no significant effect of acclimation (P=0.764) on the fish. This was probably due to an observed significant tank effect (P=0.006). This tank effect occurred in fish that were acclimated to 15°C from the 10°C treatment, the lysozyme activity of two fish in tank 1 were significantly lower than that of the other fish sampled from that tank, whereas no significant difference between fish from tank 2 was observed (P<0.05). However, homogeneity of the data was not found to be affected.

Although not significant, fish originally held at 5 and 10°C exhibited an increase in plasma lysozyme activity following acclimation to 15°C. Fish originally held at 10°C exhibit a lower plasma lysozyme activity following acclimation. It was shown from Tukey Pairwise comparison tests that this difference was not significant (Table 3.8).

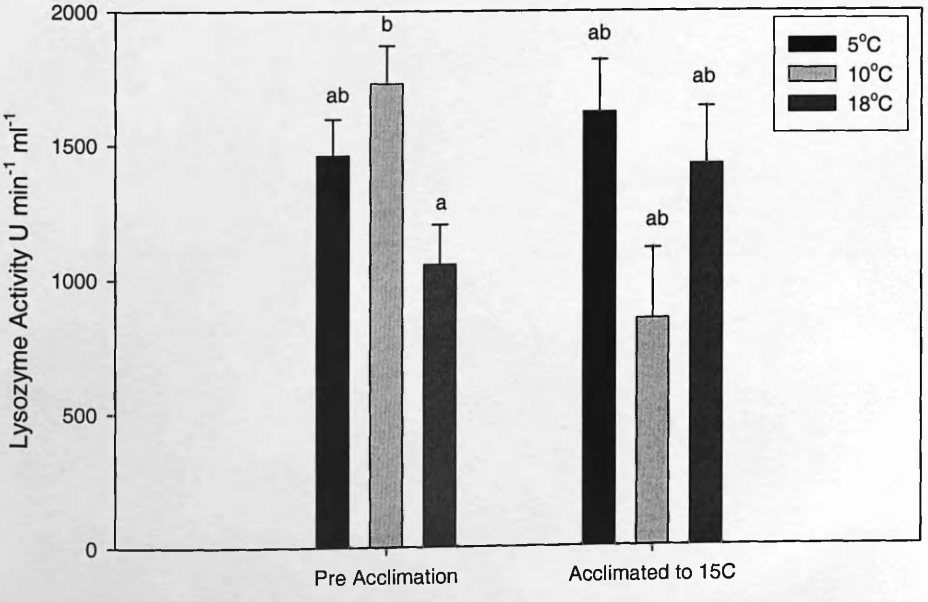


Fig. 3.10 The effect of acclimating the three treatment groups (5, 10 and 18°C) to 15°C on plasma lysozyme activity (n=20 pre -acclimation and n=10 acclimated; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b'; 'ab' is not significantly different from either 'a' or 'b' (P<0.05).

Table 3.8 Results of Tukey Pairwise Comparisons investigating the effect of acclimating fish from three treatment temperatures to 15°C on plasma lysozyme activity.

	5°C	10°C	18°C	Acclimated from 5°C	Acclimated from 10°C	Acclimated from 18°C
5°C	X					
10°C	P>0.05	X				
18°C	P>0.05	**P=0.000**	X			
Acclimated from 5°C	P>0.05	P>0.05	P>0.05	X		
Acclimated from 10°C	P>0.05	P>0.05	P>0.05	P>0.05	X	
Acclimated from 18°C	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	X

3.4 Discussion

According to the results shown in this chapter, there is obviously a direct effect of temperature on the kinetics of the innate immunological responsiveness of rainbow trout, corroborating the work of Carlson *et al.*, (1995). It is often reported that regardless of the fish species examined, elevated water temperatures within the physiological range of the fish (i.e. ~5-10°C above ambient temperature) enhance immune functions (Bly *et al.*, 1997). This is generally corroborated by the data presented here. White blood cell counts regardless of the trial season exhibited a positive correlation with temperature with the lowest counts observed in fish maintained at 5°C and the highest in fish maintained at 18°C. This is further corroborated by the results of the acclimation trial. Fish maintained at 5 and 10°C, and then acclimatised to the higher temperature of 15°C, exhibited an increased level of white blood cell counts, whereas fish originally maintained at 18°C exhibited a drop in white blood cell counts following acclimation to the lower temperature of 15°C. This was also found for red blood cell counts.

Macrophage respiratory burst activity was significantly affected by the season in which the measurements were taken. Since the fish were maintained under the same photoperiod and temperature for each trial, it appears that neither photoperiod or temperature are the principle cue in controlling respiratory burst activity. This does not corroborate Nikoskelainen *et al.*, (2004) who reported that respiratory burst activity is stimulated by increased temperature. Macrophage respiratory burst activity may be affected by the size of the fish i.e. the larger the fish the greater the level of macrophage respiratory burst activity. However, in the Base Level Trial (Chapter 2) macrophage

respiratory burst activity was measured over a twelve month period and there was no correlation between fish size and macrophage respiratory burst activity. It is therefore suggested that the observed response was due to an as yet unknown endogenous rhythm. Further studies are required to determine what this rhythm may be i.e. investigation into the effect of seasonal hormones such as cortisol and melatonin on respiratory burst activity. However, because there was only one sampling point per trial the possibility of human error must not be disregarded.

Plasma lysozyme activity was significantly affected by water temperature, however, unlike the haematological parameters it was not correlated with increasing temperature. Lysozyme activity was greatest at the temperature most relevant to the particular season in which sampling took place. For example, during the winter trial, lysozyme activity was highest in fish maintained at 5°C. Lysozyme activity was shown to exhibit a seasonal effect in the Base Level Trial (Chapter 2). During the trial, lysozyme activity was observed to be at its greatest in summer and at its lowest in winter. Consequently, an endogenous rhythm is suspected to be involved in maintaining its seasonal pattern. Changes in lysozyme activity were observed following acclimation to 15°C, however they were not significant. It maybe that the seasonal cues of photoperiod and temperature have to be taken together to elicit a true seasonal response for this immune parameter. Bowden *et al.,* (2004) corroborates this idea as they reported that serum lysozyme measured in halibut was not significantly affected by either temperature or photoperiod however a seasonal influence was observed. It was suggested that taken individually photoperiod and temperature are not capable of mimicking the seasonal pattern of lysozyme activity and that other unknown factors may be involved.

Following artificial challenge with *V. anguillarum*, a significant difference in survival was observed between fish from the original treatment temperatures (5, 10 and 18°C). The pattern of survival was the same for the winter and summer challenges. It would seem that the higher the original water temperature treatment, the higher the level of mortality, regardless of season. Fish held at 18°C exhibited the highest level of mortality following challenge with *V. anguillarum*, whereas fish held at 5°C exhibited the lowest levels of mortality. To successfully challenge the fish with *V. anguillarum*, fish from the three treatment temperatures (5, 10 and 18°C) were acclimatized to 15°C. It is suspected that the action of acclimating to this new temperature stimulated the immune response and this maybe mimicking the "Spring Effect" i.e. the movement from the cold water temperatures of winter to the rapidly increasing temperatures of spring. This is corroborated by the seasonal effect observed in the Base Level Trial (Chapter 2).

Temperature obviously plays an important role in the seasonal effect seen on the innate immune response and haematology of rainbow trout. Healthy fish exhibit innate immune responses depending directly on environmental temperature (Köllner et al., 2002). Particularly with the haematological parameters, there appears to be a strong correlation between increasing temperature and increasing blood cell counts. To examine the effect of gradually increasing temperature on immune response a third trial was set up. Fish were to be held at 5°C and every week the water temperature was increased by 2°C. At the time of setting up the experiment, only very small tanks were available such that it was only possible to sample one fish per tank per week. Consequently it was necessary to set up 15 tanks. Unfortunately, the combination of the size of the tanks, the fact that they were not opaque and the size of the available fish led to fighting within the tanks, and high levels of mortalities occurred. Consequently, the

trial was terminated in the sixth week. This made the data unusable as it would not have been accurate to state that any observed change in immune response was due to the increase in tank temperature and not the effect of stress. It is hoped that this trial will be repeated under more suitable conditions.

The results of the temperature trials carried out in Trials A and B, suggest that generally the immune response of rainbow trout is lower under cold water temperatures. This corroborates work by Tort *et al.* (2004), who concluded that for sea bream the lower the temperature, the lower the immune response.

Previous studies suggest that in non-mammalian species, temperature is an important modulator of pineal function. This is corroborated by the data of Trial A and the earlier work of Porter, *et al.* (2001), where the higher the water temperature the greater the level of measured plasma melatonin in fish. It is surprising that although melatonin production was affected by photoperiod this was not significant. Typically melatonin production is significantly greater during the dark phase (Porter *et al.*, 2001). A possible explanation for the lower than expected plasma melatonin levels maybe due to the fact that it was not possible to fit a lamp to the underside of the tanks and consequently tanks were illuminated through the observation hatch using overhead lighting. As a result this limited the light from illuminating the whole tank area, and it was restricted to the area directly below the observation hatch. Therefore, although a difference was observed between light and dark plasma melatonin, it is not significant as would have normally be expected. This effect on plasma melatonin was not known until after the experiments were completed. Unfortunately, there was not sufficient fish available to measure plasma melatonin levels during the acclimation experiment.

Plasma melatonin production also varied with season, this influence was almost significant (P=0.062). A study carried out by Porter *et al.* (2001), found that plasma melatonin increased with increasing temperature. However, in Trial A, a seasonal influence on plasma melatonin was observed, even though photoperiod and temperature were controlled. This may be a result of an as yet unknown endogenous rhythm involved in melatonin production.

3.5 Conclusion

The results of these studies investigating the effect of water temperature, suggest that temperature is the principle cue governing the seasonal effect seen with some of the immune parameters measured in rainbow trout. The exceptions are macrophage respiratory burst activity which may be governed by an as yet unknown endogenous rhythm and lysozyme activity which may require the presence of both photoperiod and temperature to induce a seasonal response.

Chapter 4 Photoperiod and Innate Immunity

4.1 Introduction

In Chapter 2, it was observed that over a twelve month period the innate immune system of rainbow trout exhibited a seasonal pattern in its response. Generally, the greatest activity was observed during the summer and it was suppressed in winter. Seasonality consists of two major principle cues, photoperiod and temperature. In this chapter the effect of photoperiod on the innate immune response is examined. Few studies have been carried out examining the effect of photoperiod on the immune response of fish, consequently there is little work available to compare with the results of the trials carried out in this chapter. The effect of photoperiod on the immune response of other vertebrates is described here as an introduction to the topic.

Photoperiodic information is used to initiate or terminate specific seasonal physiological processes, including reproduction, to maintain a positive energy balance. The annual cycle of changing photoperiod is a very accurate temporal cue for determining the time of year (Nelson and Demas, 1997). Indeed, photoperiod has been defined as being the light fraction of the 24 h day and its seasonal changes (Hoffmann, 1981a and b). Photoperiodism was first proposed by the botanists Garner and Allard in 1920 to describe the response of plants to the length of day and night. Today, photoperiod is defined as the ability to determine day length in both plants and animals. Photoperiodism has evolved in virtually all taxa of plants and animals that experience seasonal changes in their habitats (Nelson *et al.*, 2002)

It has already been well established that photoperiod influences growth, feeding, smoltification and reproduction in salmonids (Taylor *et al.*, 2002; Berrill *et al.*, 2003). It is therefore feasible that photoperiod also influences immune function in fish.

Annual cycles are seen to occur for a host of different functions in poikilothermic vertebrates. Though these have been studied much less intensively compared with birds or mammals, photoperiodic effects have been reported in a number of species of fish, Reptilia and some Amphibia (Licht, 1969; Delgado *et al.,* 1987). It has been stated that it is very difficult to make generalisations on the photoperiodic mechanism of poikilotherms (Hoffmann, 1981a). This is because temperature also has a major influence on annual cycles, replacing or often superseding the effect of photoperiod. The same photoperiod may have drastically different effects, depending not only on the phase of the annual cycle but also on ambient temperature (Hoffmann, 1981a).

At a given latitude, the annual alteration in daylength is extremely predictable. If the internal neural event tracking daylength is equally predictable, and bears a constant relation to the duration of daylength (or night-time) then the animal will have a logical and reliable reference system representing daylength (Herbert, 1989). Using such a system, events such as growth, feeding, parr-smolt transformation, reproduction and immune function would all be cued in response to changes in seasonality.

4.1.1 The Effect of Photoperiod on Immune Response

4.1.1.1 Mammalian Immunity

One of the earlier studies carried out investigating the effect of photoperiod on immune response was performed in dogs (Shifrine *et al.*, 1982b). The study concluded that dogs maintained under a 12:12LD light regime had a significantly lowered immunity relative to dogs with a natural photoperiod (Shifrine *et al.*, 1982b). More recent photoperiod studies have also reported slight photoperiodic induced changes in

the splenic masses of rats, deer mice and syrian hamsters (Blom *et al.*, 1994). Furthermore total lymphocyte and macrophage counts were significantly elevated in hamsters exposed to short day lengths compared with their long day counterparts (Blom *et al.*, 1994).

The majority of studies looking at the effect of photoperiod on the immune response have used mice. One such study was carried out by Hayashi and Kikuchi (1982), in which the effects of photoperiod on humoral immune responses against sheep red blood cells (SRBC), a thymus dependent antigen, were investigated. It was observed that the maximal immune response against SRBC is seen in the early light part of the day.

Examples of work examining the effect of photoperiod on immune response in mammals are presented in Table 4.1.

Table 4.1 Laboratory based studies investigating the effect of photoperiod on various immune parameters (adapted from (Nelson *et al.*, 1995).

Immunological Parameters Studied	Species	Enhanced Under Short Day Photoperiod
Splenic Mass	Norway rats	Yes
	Deer mice	Yes
	Golden hamsters	Yes
Thymic Mass	Norway rats	Yes
Lymphocyte Count	Deer mice	Yes
Neutrophil Count	Deer mice	Yes
White Blood Cell Count	Deer mice	Yes
	Common Voles	Yes
Antibody Levels	Deer mice	Yes
Wound Healing Rates	Deer mice	Yes

4.1.1.2 Avian Immunity

The majority of literature relating to the effects of photoperiodic effects on avian immunity is indirect, reflected by changes in growth, performance and reproductive changes (Moore and Siopes, 2000). However, it has been reported that immature cockerels reared under constant light compared to 12:12LD daily cycles, exhibit a suppressed cellular immunity and secondary antibody response (Moore and Siopes, 2000; Kirby and Froman, 1991).

The practice of exposing broiler chickens to continuous light has been used by the poultry industry for many years. A study was set up to determine if a light:dark cycle would improve immune function in these birds. It was subsequently reported that birds reared under controlled light:dark cycles exhibited milder reactions to live respiratory vaccines, higher ELISA titres from vaccines, fewer secondary bacterial infections, lower mortality rate and lower airsacculitis condemnations (Davis and Siopes, 1996).

4.1.1.3 Teleost Immunity

Photoperiod and the teleost immune response have received very little attention. Moreover, it would appear that there are only two papers specifically dealing with this topic, both published in the past two years (Melingen *et al.*, 2002; and Leonardi and Klempau, 2003). Furthermore, the papers focused primarily on leucocyte counts. Leonardi and Klempau (2003) reported that constant light adversely affects the T-cell immune responses of rainbow trout. Melingen *et al.* (2002), reported lower B-cell populations following the onset of a winter photoperiod. Both papers provided evidence that photoperiod affects the immune system of teleost fish. Olsen *et al.* (1993), whilst examining gill Na+, K, –ATPase activity, plasma cortisol level, and non-specific

immune responses in Atlantic salmon during parr-smolt transformation, hypothesised that cortisol levels during the smolting period of Atlantic salmon are probably controlled more by photoperiod than by endogenous rhythms.

4.1.2 The Effect of Photoperiod on Fish Life History

Although little had been published on the effect of photoperiod on the immune response of fish, much work has been carried out investigating the effect of photoperiod on several aspects of fish life history. It has even been hypothesised that virtually all biochemical processes, physiological functions and behaviours are rhythmic in nature and are synchronised by the 24 h light/dark cycle (Boeuf and Falcón, 2001).

4.1.2.1 Growth and Survival

Photoperiod has been documented to have a significant effect on both growth and survival of teleost fish. Available data indicates that fish growth follows a seasonal pattern which changes as a function of variations in daylength (Boeuf and Falcón, 2001). For example, Atlantic halibut exhibit improved growth when exposed to continuous light, but only when the natural daylength is less than 18 h (Simensen *et al.*, 2000).

Giri *et al.*, (2002) reported reduced survival in larval *Wallago attu* when reared in permanent darkness. The same study reported that for highest survival and maximum biomass production, 24 h red light exposure was most effective. In 2002, Hemre *et al.* reported that juvenile Atlantic salmon had reduced growth when maintained under a typical winter photoperiod regime compared to fish maintained under constant light. It has been proposed that reduced activity and anabolic effects of photoperiod contribute

in explaining the increased growth and growth efficiency in fish subjected to continuous light (Jonassen *et al.*, 2000).

The majority of studies report that photoperiod manipulation can be used to enhance growth and survival. However there are some exceptions. No significant growth of larval *W. attu* was observed when reared under different light and photoperiodic regimes when fed a live diet (Giri *et al.*, 2002).

4.1.2.2 Parr-Smolt Transformation

Photoperiod can be used to delay smoltification when Atlantic salmon are exposed to continuous light in autumn and winter (Björnsson *et al.*, 1995). This is in accordance with the hypothesis that the endogenous circannual cycle may 'free-run' under periods of continuous light, causing a progressively longer phase delay (Saunders *et al.*, 1989; Björnsson *et al.*, 1995).

4.1.2.3 Sexual Maturation

It is well known that photoperiod affects sexual maturation. Photoperiod alterations are frequently made to manipulate reproductive cycles in the aquaculture industry. For example, hatcheries control photoperiod to manipulate maturation and spawning time to produce all-year-round supplies of eggs and fry. A second example are 'grow-out' farms where programs of environmental control are set up that can prevent or delay maturation until after fish harvest, thus avoiding the deterioration in flesh quality which often accompanies maturation. Consequently farms can produce the year-round continuity of supply of product of consistent size and quality, which is demanded by the retail markets (Bromage *et al.*, 2001).

4.1.2.4 Fish Behaviour

Photoperiod has been documented to influence a selection of fish behaviours including feeding activity, aggression and locomotor activity. The African catfish when maintained under constant light had an increase of 27.1% in bite marks on the body when compared to fish on a 12:12LD photoperiod (Almazan-Rueda *et al.*, 2001).

Under a long day photoperiod, rainbow trout have been observed to have a daily demand-feeding profile that is always confined to the light phase, and is chiefly composed of two main episodes, directly after lights on (light-elicited) and in anticipation of lights off (endogenous) (Sánchez-Vázquez and Tabata, 1998).

In contrast to this documented feeding regime, the diel locomotor activity profile of rainbow trout has been observed to vary remarkably. A diurnal activity was observed at the tank bottom while a clearly nocturnal pattern was recorded at the water surface (Sánchez-Vázquez and Tabata, 1998).

4.1.3 Aims

The primary aim of this study was to determine if different photoperiod regimes affect the innate immune system of rainbow trout. Secondly, to determine if there was any seasonal effect in this response, the trial was carried out in winter (December-February) and then repeated in summer (June-August).

4.2 Materials and Methods

Three different photoperiodic regimes were used in the trial presented in this chapter, namely constant light (24:00 LD), long day (16:8LD) and short day (8:16LD). In addition to measuring the effect of photoperiod, the effect of seasonality was also examined in the same study. To accomplish this, a three month trial was carried out in winter (December 2001-February 2002), and this was then repeated in summer (June-August 2002). The average temperature during the winter trial was 2.5°C and 13.8°C during the summer trial. Fish from the same Niall Bromage Freshwater Research Facility (NBFRF) stock were used in both trials.

4.2.1 Trial Set-Up

The trials were carried out at the NBFRF and fish maintenance was as described in Section 2.21. Fish were held in 12 tanks (1 m Ø x 1 m) with 30 fish randomly allocated per tank with three tanks per photoperiodic treatment. Although the fish used in the trial were mixed sex, only immature fish were sampled. This was to prevent precocious males with a compromised immune system from potentially skewing the results. Fish were maintained under ambient temperature during each three month trial. Fish were acclimated to their respective photoperiods for one month prior to commencing the trial. The lengths and weights of all fish sampled were measured. Fish were sampled at random between 10 am and 12 pm each month. All tanks were in light phase at that time.

4.2.2 Measurement of Innate Immunity and Haematological Parameters

Sampling of fish took place every month with three sample points per season (winter/summer). Four fish were sampled, per tank at each time point with a total of twelve fish sampled per treatment. As per the description in Section 2.2.2 fish lengths and weights were measured when blood was taken. Both fish and blood were transported back to the laboratory on ice.

A variety of innate parameters were measured, using the methods described in Sections 2.2.3 and 3.2.3, i.e. respiratory burst activity of head kidney macrophages, total white and red blood cell counts, haematocrit and plasma lysozyme activity.

In addition, fish were challenged at the Aquatic Research Facility at the Institute of Aquaculture (Section 3.2.4) with *Vibrio anguillarum* to determine if photoperiod affects survival to an experimental challenge with the bacterium (30 fish per treatment). The challenge took place in February of the winter trial and August of the summer trial. The fish challenged in winter were significantly smaller than those of summer, consequently bacterial dose was larger for the summer fish (winter $5*10^6$ CFU ml^{-1}; summer $7*10^6$ CFU ml^{-1}). Pre-challenges were used to determine the appropriate dose..

4.2.3 Measurement of Melatonin

Plasma melatonin levels of the experiment fish were measured during the February of the winter trial and August of the summer trial. This sampling took place on a different day to the normal sampling. Samples were taken at midday and two hours after darkness using heparinised syringes. Blood samples were transported to the laboratory on ice, from which plasma samples were collected and frozen at –70°C as

soon as possible until analysed using a melatonin radio immunoassay (RIA) as described in Section 2.2.4.

4.2.4 Statistical Analysis

Before analysis, the data was tested for normality and homogeneity of variance using fits and residuals. All data was found to be normal and homogenous without transformation. Data was analysed using an ANOVA General Linear Model (Minitab). Where significant differences ($P<0.05$) were observed Post-hoc Tukey tests were carried out.

Statistical analysis of the survival to challenge data was performed using the Kaplan Meier survival test (SPSS).

4.3 Results

Fish used in both the summer and winter phases of this trial were from the same original NBFRF stock. Fish weight and length were significantly different between the two sampling periods of winter and summer. Fish from the winter trial (at trial start average of 84.09 g; SD = 4.37; SE = 1.26) were significantly smaller and lighter (P=0.000) compared to fish from the summer trial (at trial start average of 158.54 g; SD = 28.9; SE = 8.344).

4.3.1 The Effect of Photoperiod on Immune and Haematological Parameters

4.3.1.1 Total White Blood Cell Counts

Photoperiod does not appear to affect fish white blood cell counts (P=0.199) (Fig. 4.1). The measured white blood cell counts of fish were significantly greater in summer than in winter, irrespective of photoperiodic treatment (P=0.000) There was no significant difference in the white blood cell counts between the three winter months (P=1.000) or between the three summer months (P=1.000). Although the photoperiodic regime did not significantly affect white blood cell counts (P=0.199), there was a significant effect due to season and photoperiod (P=0.023). Tukey Pairwise comparison tests were used to analyse this further using a series of pair wise comparisons (Table 4.2). The results of these pairwise comparisons corroborate the results of the earlier statistical analysis as photoperiodic treatments within a particular season do not have any significant effect on white blood cell counts. However, between the seasons there is a difference. Significant differences in white blood cell count levels are a direct result of season and not photoperiod.

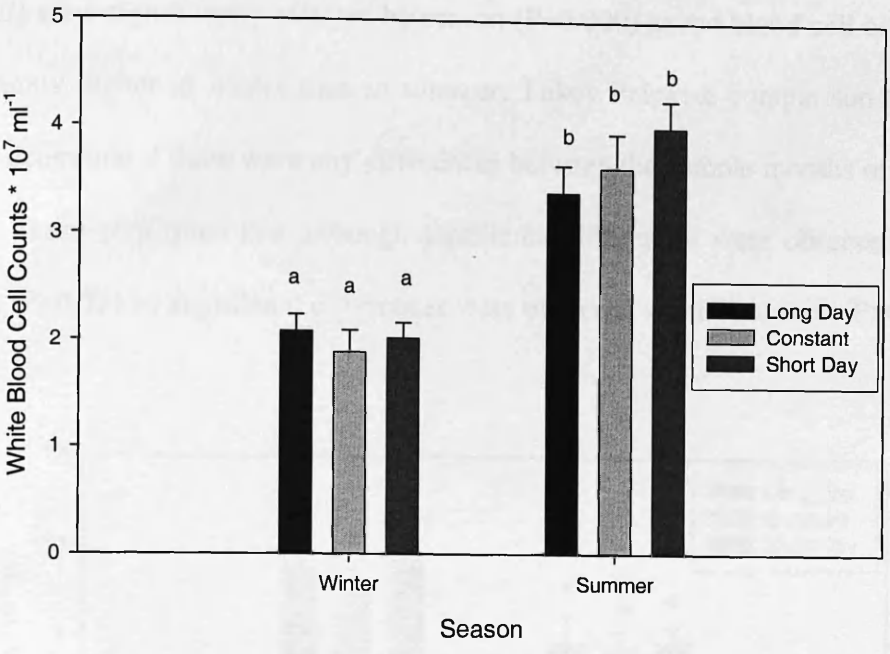


Fig. 4.1 The effect of three different regimes (18:6 LD, 6:18 LD and 24:00 LD) and season on total white blood cell counts (n=36/treatment; mean ± SE), Subscripts denote statistical significance, 'a' is significantly lower than 'b' (P<0.05).

Table 4.2 Results of Tukey tests examining pairwise comparisons between the different photoperiod regimes within and between season for total white blood cell counts (n=36/treatment)

	Winter / Long Day	**Winter / Constant**	**Winter / Short Day**	**Summer / Long Day**	**Summer / Constant**	**Summer / Short Day**
Winter / Long Day	X					
Winter / Constant	P>0.05	X				
Winter / Short Day	P>0.05	P>0.05	X			
Summer / Long Day	**P=0.000**	**P=0.000**	**P=0.000**	X		
Summer / Constant	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	X	
Summer / Short Day	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	P>0.05	X

4.3.1.2 Total Red Blood Cell Counts

As with white blood cell counts no significant effect of photoperiod was observed on measured total red blood cell counts (P=0.435). Total red blood cell counts

(Fig. 4.2) were significantly affected by season (P=0.000) as red blood cell counts were significantly higher in winter than in summer. Tukey Pairwise comparison tests were used to determine if there were any differences between the sample months of each trial season. These confirmed that although significant differences were observed between seasons (P<0.05) no significant differences were observed within a season (P>0.05).

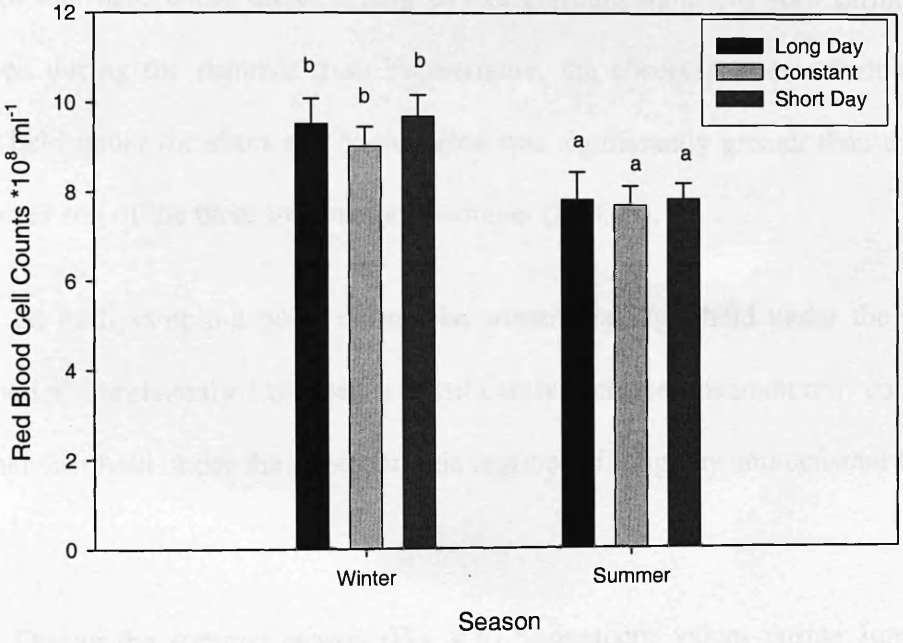


Fig. 4.2 The effect of three different photoperiodic regimes (18:6 LD, 6:18 LD and 24:00 LD) and season on total red blood cell counts (n=36/treatment; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b' (P<0.05).

4.3.1.3 Haematocrit Values

Haematocrit values (Fig. 4.3) were significantly affected by photoperiod ($P=0.001$). To determine where this significant difference occurred Tukey Pairwise comparisons were carried out. It was found that haematocrit values taken from fish held under a short day photoperiod during the winter were significantly greater ($P<0.05$) than those of fish held under either a long day or constant light. No such difference was observed during the summer trial. Furthermore, the observed haematocrit of fish in winter held under the short day photoperiod was significantly greater than that of fish held under any of the three treatments in summer ($P>0.05$).

At each sampling point during the winter trial, fish held under the short day photoperiod consistently exhibited a significantly increased haematocrit, compared to the other fish held under the photoperiodic regimes of long day and constant light (Fig. 4.4).

During the summer season (Fig. 4.5) haematocrit values during June for fish held under the short day photoperiod were significantly lower than that of July and August, as confirmed using a Tukey test. The haematocrit of fish held under the short day photoperiod in June was found to be significantly lower than that of all other photoperiodic treatments irrespective of sampling point or photoperiodic treatment during the summer trial. However, fish held under a short day photoperiod did not exhibit the lowest haematocrit levels during the July or August sampling points (Fig. 4.5).

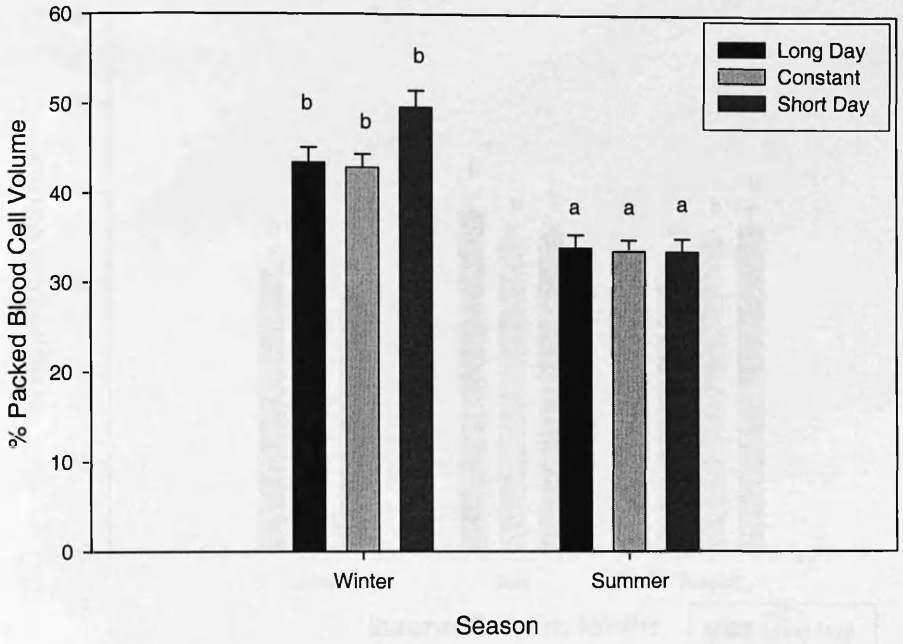


Fig. 4.3 The effect of photoperiod (18:6 LD, 6:18 LD and 24:00 LD) and season on haematocrit (n=36/treatment; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b' (P<0.05).

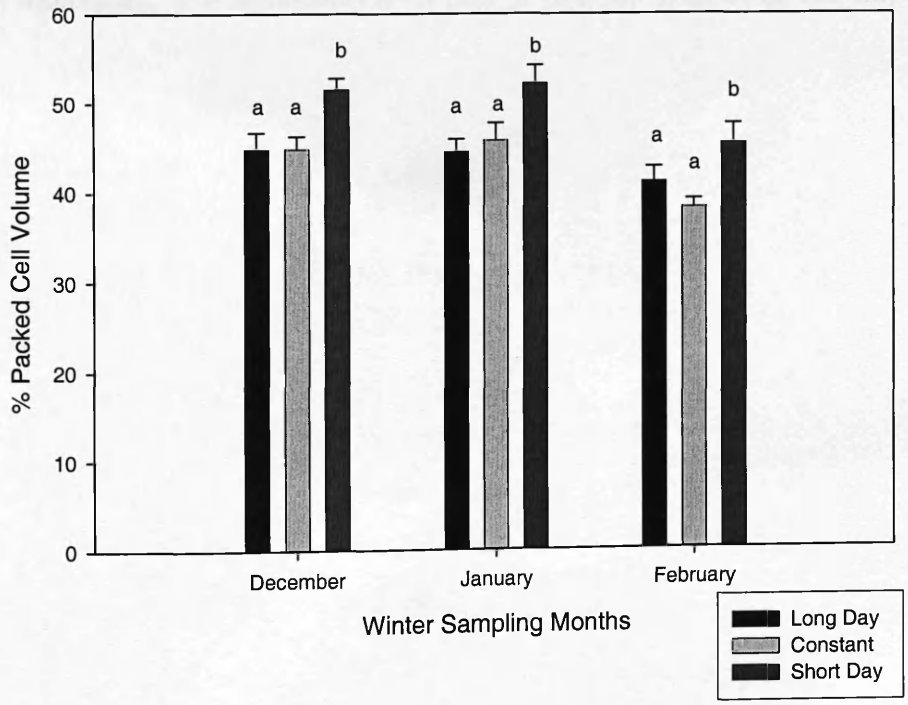


Fig. 4.4 Haematocrit values for each of the winter sample months for the three photoperiodic treatments (18:6 LD, 6:18 LD and 24:00 LD) (n=12/treatment; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b' (P<0.05). Treatments; 18:6 LD, 6:18 LD, 24:00 LD.

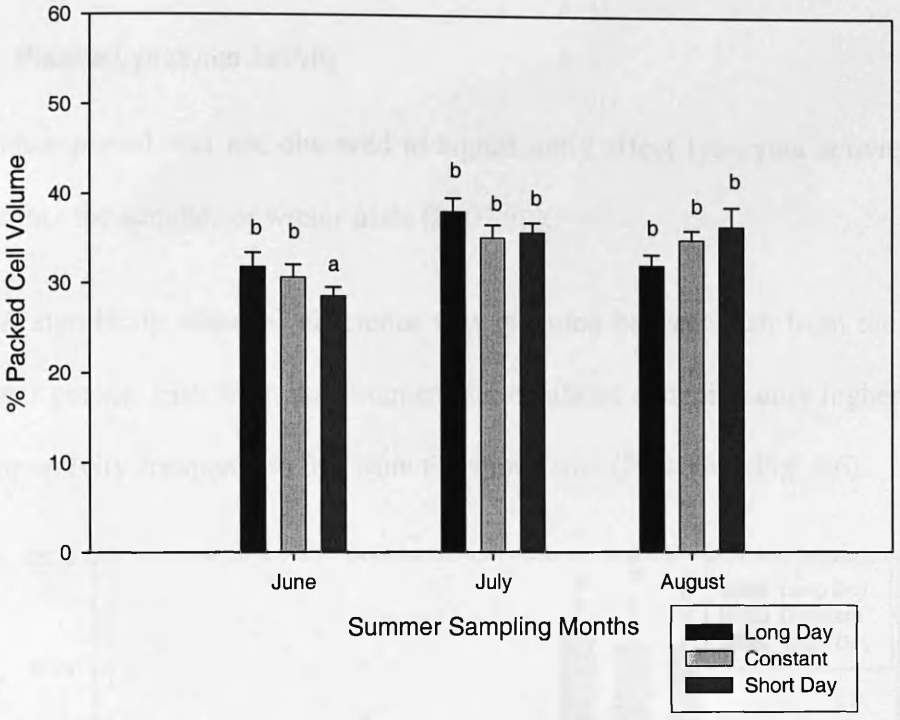


Fig. 4.5 Haematocrit values for each of the summer sample months for the three photoperiodic treatments (18:6 LD, 6:18 LD and 24:00 LD) (n=12/treatment; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b' (P<0.05). Treatments; 18:6 LD, 6:18 LD, 24:00 LD.

4.3.1.4 Plasma Lysozyme Activity

Photoperiod was not observed to significantly affect lysozyme activity of fish during either the summer or winter trials (P=0.770).

A significant seasonal difference was recorded between fish from the summer and winter phases. Fish from the summer trial exhibited a significantly higher level of lysozyme activity compared to fish from the winter trial (P=0.000) (Fig. 4.6).

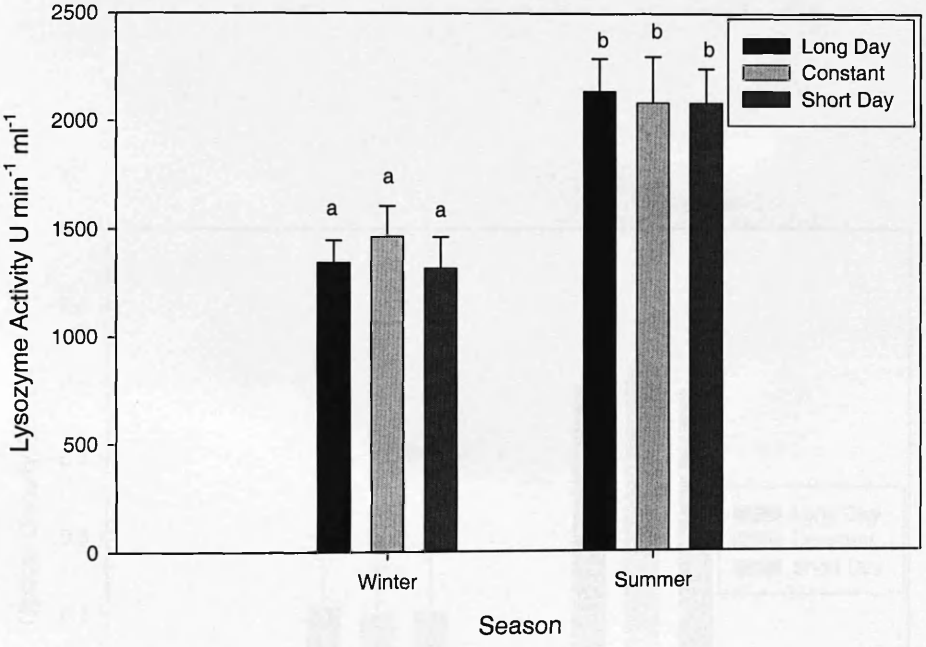


Fig. 4.6 Comparison between seasons for lysozyme activity of fish held under the three different photoperiod treatments (18:6 LD, 6:18 LD and 24:00 LD) (n=36/treatment; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b' (P<0.05).

4.3.1.5 Respiratory Burst Activity

Photoperiod did not significantly affect the respiratory burst activity of head kidney macrophages (Fig. 4.7) (P=0.870). However, respiratory burst activity was significantly affected by season (P=0.000). Respiratory burst activity was significantly greater in summer compared to winter. There is no significant differences between the months sampled within each season (P>0.05).

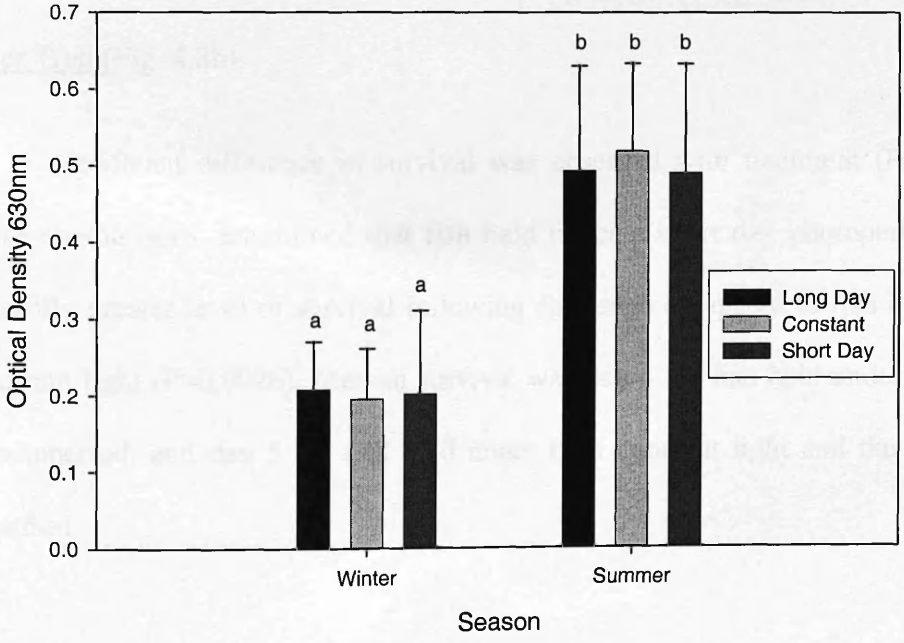


Fig. 4.7 Comparison between seasons for respiratory burst activity of fish held under the three different photoperiod treatments (18:6 LD, 6:18 LD and 24:00 LD) (n=36/treatment; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b' (P<0.05).

4.3.1.6 Challenge with *Vibrio anguiliarum*

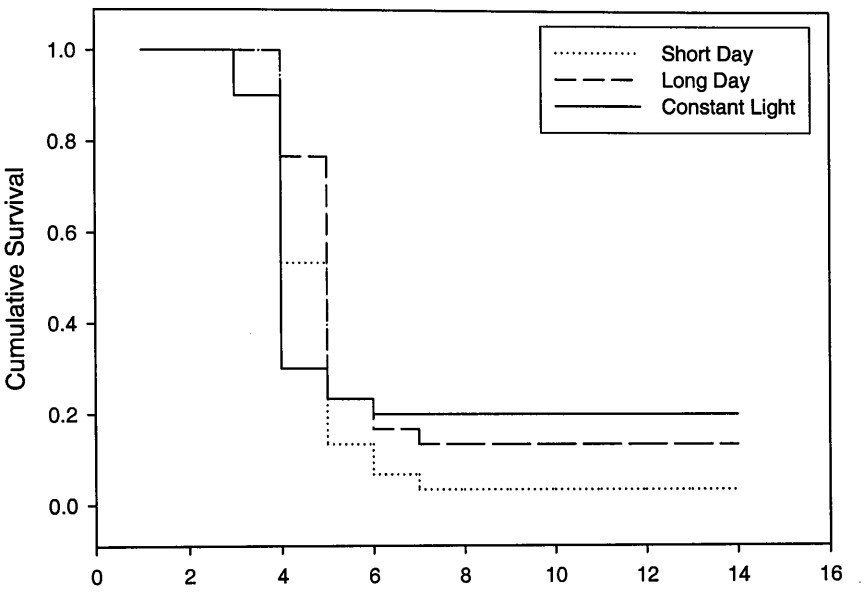
Fish were artificially challenged at the end of each season with *V. anguillarum.* Prior to challenge fish were acclimated to 15°C and a 12:12LD photoperiod for one week prior to injection.

Winter Trial (Fig. 4.8a)

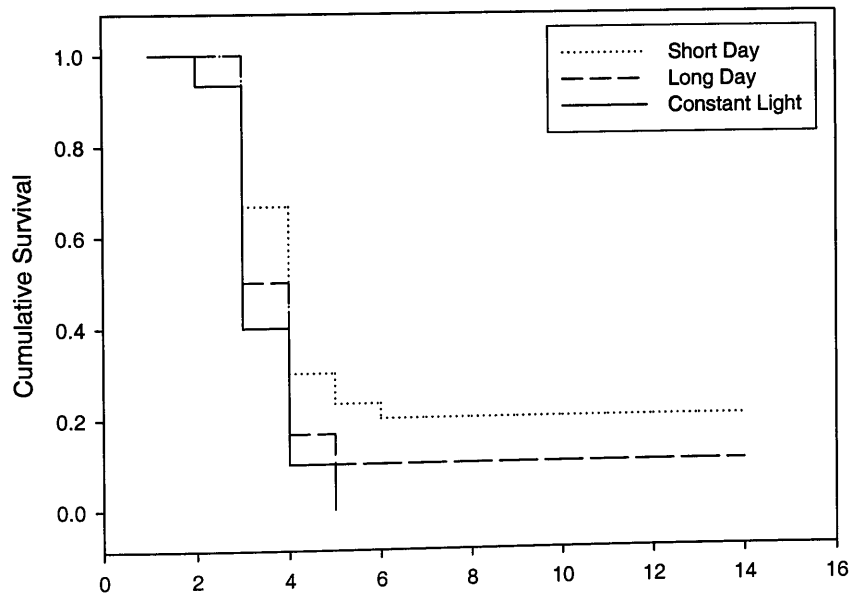
A significant difference in survival was observed with treatment (P=0.0068). Pairwise comparisons determined that fish held under constant light had a significantly greater level of survival following challenge compared to fish held under the short day photoperiod (P=0.0021). Median survival was day 5 for all treatments.

Summer Trial (Fig. 4.8b).

A significant difference in survival was observed with treatment (P=0.0114). Pairwise comparisons determined that fish held under a short day photoperiod had a significantly greater level of survival following challenge compared to fish held under the constant light (P=0.0026). Median survival was day 4 for fish held under the short day photoperiod, and day 5 for fish held under both constant light and the long day photoperiod.



a)



b)

Fig. 4.8 Survival following artificial challenge with *V. anguillarum* for fish from the winter (a) and summer (b) challenges. Fish were challenged under a 12:12 LD photoperiod. Fish were originally held under three different photoperiods (long day, short day and constant light) for three months, they were then acclimated to the standard 12:12 LD photoperiod of the ARF aquarium over a period of one week prior to challenge.

4.3.1.7 The Effect of Photoperiod on Melatonin

Plasma melatonin levels were significantly affected by photoperiod treatment (P=0.000) and by night and day (P=0.000) (Fig. 4.9). Fish held under the constant light regime irrespective of season had significantly lower plasma melatonin levels compared to fish from the short and long day photoperiods night time sampling (P=0.000). The typical diurnal rhythm of melatonin (i.e. the majority of melatonin is normally produced during the night phase, is overridden by the constant light treatment). Consequently fish from the constant light treatment exhibited little difference between day and night time melatonin production. The fish held on the short day and long day photoperiods exhibited a typical diurnal rhythm, with the majority of melatonin production occurring during the dark phase. Season was not observed to significantly affect plasma melatonin production (P>0.05).

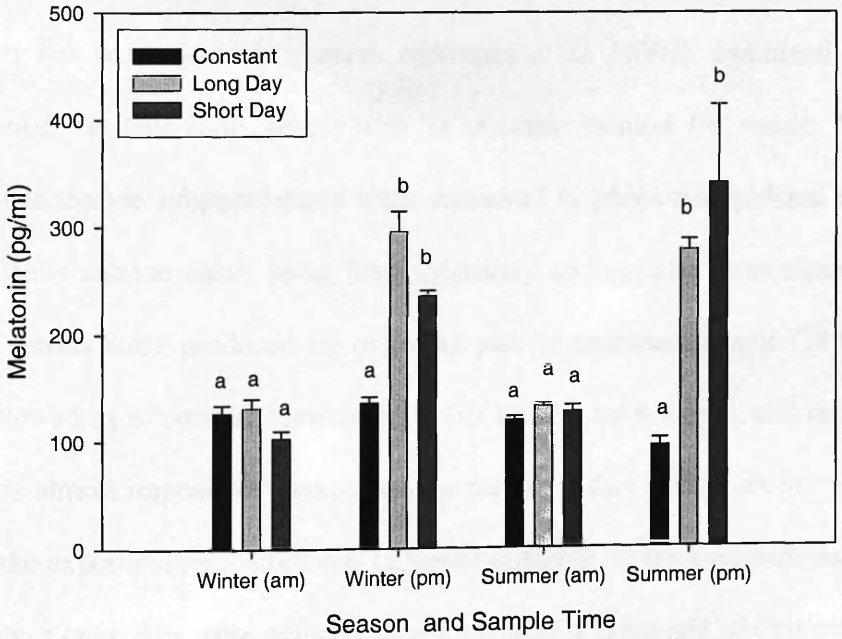


Fig. 4.9 The effect of photoperiod (18:6 LD, 6:18 LD and 24:00 LD) and time of sampling on plasma melatonin levels for fish from the summer and winter trials (n=5; mean ± SE). Subscripts denote statistical significance, 'a' is significantly lower than 'b' (P<0.05).

4.4 Discussion

Photoperiod manipulation is extremely popular in finfish culture. The increasing importance of photoperiod manipulation makes it crucial that potential effects on the immune system are investigated. The results of these trials examining the effect of photoperiod on innate immune response suggest that photoperiod has little if any effect on the immune parameters studied. The haematological parameter, haematocrit, was the only parameter studied that was observed to be significantly affected by photoperiod. Fish held under a short day photoperiod exhibited a significantly greater haematocrit compared to the long day and constant photoperiods during the winter trial.

Few papers have been published, specifically examining the effect of photoperiod on immune response. Furthermore, the effect of photoperiod on teleost immunity has received little interest. Melingen *et al.* (2002), examined the effect of photoperiod on leucocyte populations in Atlantic salmon 0+ smolt. Variations in different leucocyte subpopulations were measured in photo-manipulated out-of-season (0+) Atlantic salmon smolt using flow cytometry and specific monoclonal antibodies. The 0+ smolts were produced by exposing parr to continuous light (24:00 LD) until June followed by a ''winter'' photoperiod (12:12 LD) for 6 weeks, and then continuous light. It is almost impossible to compare the results of this work with the data presented here as the experiment structure was radically different. In the experiments described in this chapter once fish were assigned a photoperiod it remained unchanged throughout the trial. However, it is important to note that the effect of changing photoperiod from continuous to short day did result in changes in leucocyte populations, for example neutrophils increased and B-cells decreased (Melingen *et al.,* 2002). This is

corroborated by Leonardi and Klempau, (2003) who examined the effect of photoperiod on the immune system of juvenile rainbow trout in the Southern Hemisphere. Their work corroborates the findings of Melingen *et al.* (2002), in that a significant effect of photoperiod on immune response was observed, in particular, changes in leucocyte populations. It was recorded that constant light adversely affects the T cell immune responses of rainbow trout. If the trials were to be repeated, assays measuring T-cell responses would certainly need to be carried out. Furthermore, it is unfortunate that immune parameters were not measured before acclimating the fish to their respective photoperiods. It is therefore impossible to know if changes in immune response took place as a direct result of changing the photoperiodic regime. If the experiment was to be repeated this would certainly take place.

White blood cell counts have been shown to be affected by photoperiod in an avian study carried out by Moore and Siopes (2000). They observed that in Japanese Quail, constant light suppressed white blood cell counts compared to birds held under a long day photoperiod. This does not corroborate the results of this trial as there was no significant difference found between the photoperiod treatments and total white blood cell counts.

Unfortunately, a literature search found no previous studies examining the effect of photoperiod on respiratory burst or lysozyme activity. In this study photoperiod was not found to significantly affect either respiratory burst or lysozyme activity.

The results of the challenge with *V. anguillarum* are interesting as the results for each season are contrary to each other. During the winter trial, fish held under constant light photoperiod exhibited the greatest level of survival whereas during the summer trial, fish held under short day exhibited the greatest level of survival. The method of

challenge required fish to be moved from their original photoperiodic treatments to a 12:12 LD photoperiod. Challenge was carried out one week after the fish were moved to their new photoperiod regimes. This could therefore be the reason for the observed photoperiod effect, particularly as challenge took place relatively quickly after starting the new photoperiod regimes. Sprague* (pers. comm., 2004), reported that cortisol levels are significantly increased when changing to a constant light photoperiod from a natural situation. This increase in cortisol may have affected the immune system (Slater and Shreck, 1993; Slater *et al.,* 1995; Hassig *et al.,* 1996), and consequently caused the observed significant results in the challenges.

Haematocrit values were also affected by photoperiod, where the correct seasonal photoperiod for each trial resulted in the highest percentage of packed cell volume. For both trials fish held under a constant light photoperiod exhibited the medium level of survival. Unfortunately, again there are no studies available to compare this work to. However, the effect of photoperiod on general survival has been studied. Nile Tilapia fry (but not fingerlings) survival has been shown to be significantly affected by photoperiod. Fish fry subjected to long light periods (24 and 18 h) had significantly better growth and feed utilization efficiency than those exposed to intermediate or short light periods (12 or 6 h) (El-Sayed and Kawanna, 2004). Furthermore, the results this study indicated that the response of Nile tilapia to photoperiod cycles depends on fish developmental stage. Tilapia fry were more sensitive to photoperiod than fingerlings and juveniles (El-Sayed and Kawanna, 2004). The suggestion that photoperiod affects survival only in the earliest developmental phase (El-Sayed and Kawanna, 2004) may also apply to immune response.

* Sprague, M. (2004). Institute of Aquaculture, University of Stirling

Photoperiod was observed to significantly affect plasma melatonin levels. Fish held under constant light had significantly lower levels of melatonin compared to fish during the dark phase periods of both the short and long day photoperiodic regimes. No seasonal difference in plasma melatonin levels was recorded for any of the photoperiodic treatments investigated. The fact that no seasonal difference was observed is surprising as the results of Chapter 3, suggest that temperature does affect plasma melatonin, with increasing levels as temperature increases. However, this also corroborates the work of Porter *et al.* (2001), which concluded that temperature does affect plasma melatonin levels.

Significant seasonal differences were observed in several of the parameters studied. This seasonal difference could be a result of fish age/size as the fish of the summer trial were several months older than those of the winter trial and were significantly larger. Conversely, the seasonality observed could be due to an unknown endogenous rhythm that was not studied during the course of this study. The results of Chapter 3, examining the effect of temperature on the innate immune response and haematological parameters, indicated that temperature does significantly affect the majority of these parameters. It is therefore likely that the significant differences observed between the summer and winter trials are a result of temperature.

The results of this trial corroborate those of the 'Base Level Trial' in Chapter 2 for white blood cell counts, haematocrit and lysozyme activity. Respiratory burst activity exhibited a clear seasonality effect, more so than during the 'Base Level Trial', with activity being significantly greater in summer. However, the red blood cell counts were contrary to what might have been expected, with levels being greatest in the winter compared to the summer. The red blood cell counts of the 'Base Level Trial' showed

little seasonality effect, although the greatest levels were recorded in the summer month of July which had a significantly higher red blood cell counts than all other months. These increased levels of red blood cells may be due to the increased metabolism of these fish at this time.

The annual cycle of changing photoperiod provides an accurate indicator of time of year and if it is utilised as a seasonal cue would allow immunological adjustments to be made prior to the deterioration of conditions experienced in winter (Nelson *et al.*, 1995). Although photoperiod has not been shown to act as a principle seasonal cue for the parameters of this study, other teleost studies have observed photoperiodic effects on leucocyte populations (Leonardi and Klempau, 2003) and cortisol levels (Leonardi and Klempau, 2003; Melingen *et al.*, 2002). Together these results suggest that photoperiod may mediate seasonal changes for some aspects of the teleost immune system (Blom *et al.*, 1994). The enhancement of immune response is an adaptation that has evolved to increase the chances of survival, thereby increasing the chance of reproduction. Winter survival may be dependant on an immunological balance between photoperiod-mediated enhancement and winter environment immune suppression (Blom *et al.*, 1994).

4.5 Conclusion

In conclusion, photoperiod was not found to act as a cue for the innate immune parameters of respiratory burst activity, plasma lysozyme activity and total white and red blood cell counts. The only parameter that was significantly affected by photoperiod was haematocrit and that was only during the winter trial. Photoperiod may act as a cue for seasonality for other parameters not studied in these trials. It has already been shown

to significantly affect the production of cortisol (Olsen *et al.*, 1993). Season was shown to affect several of the parameters studied regardless of treatment e.g. total white blood cell counts were significantly higher in summer compared to winter. The cue for this seasonality effect could be temperature or an as yet unknown endogenous rhythm. Several studies have demonstrated continued seasonal variations in immune response even when both temperature and photoperiod have been held constant (Leceta and Zapata, 1986; Zapata *et al.*, 1983), this suggests that that seasonal variations of the immune system may be driven by endogenous rhythms (Zapata *et al.*, 1992).

Sampling took place after a minimum of four weeks acclimation to the treatment photoperiods (18:6 LD, 6:18 LD and 24:00 LD). Sampling did not take place either prior to, or during this acclimation period, it is possible that a change in photoperiod does affect the parameters studied, but only for a very short period of time before a natural balance is resumed. If this is the case, this short term effect could have been missed as sampling took place only when the fish had been acclimatized to their respective treatments. It is suggested that in future trials samples should be taken both prior to and during the acclimation period.

During this study, the element of photoperiod examined was the effect of day length. However, photoperiod may affect immunity through light intensity and wavelength. These were not investigated during the course of the trial. Although relatively few studies have investigated the effect of these aspects of photoperiod on teleost life history, one study has shown that feeding incidence increases with light intensity (Puvanendran and Brown, 2002).

Chapter 5 Melatonin, Immunity and Seasonality

5.1 Introduction

5.1.1 Hormones and Seasonality

Seasonality of the immune system has been the principle subject of investigation during this thesis. The principle cues of seasonality have been studied in previous chapters i.e. temperature (Chapter 3) and photoperiod (Chapter 4) However, how this seasonal information is transduced to the brain and integrated to co-ordinate the immune system requires further investigation. The mechanisms that synchronise immune activity with the appropriate time of year are not yet understood. Furthermore, it was observed from the earlier work carried out in this thesis that seasonal immunosuppression tends to occur in winter. It may be possible to alleviate this seasonal immunosuppression; for example using dietary immunostimulants.

Of particular importance is the maintenance of the synchronicity of the physical immunological components with the appropriate time of year. There is now considerable evidence that both neural and endocrine factors act to maintain homeostasis of the immune system (Nelson *et al.,* 2002). The hormone melatonin produced mainly by the pineal gland has been implicated in the mechanism that regulates the seasonality of the immune system. "Seasonal variation, affecting the structure and function of the ectotherm immune system, is an excellent 'natural' model of the influence of neuroendocrine rhythms on immunity" (Zapata *et al.,*, 1996) (Fig. 5.1, Fig. 5.3).

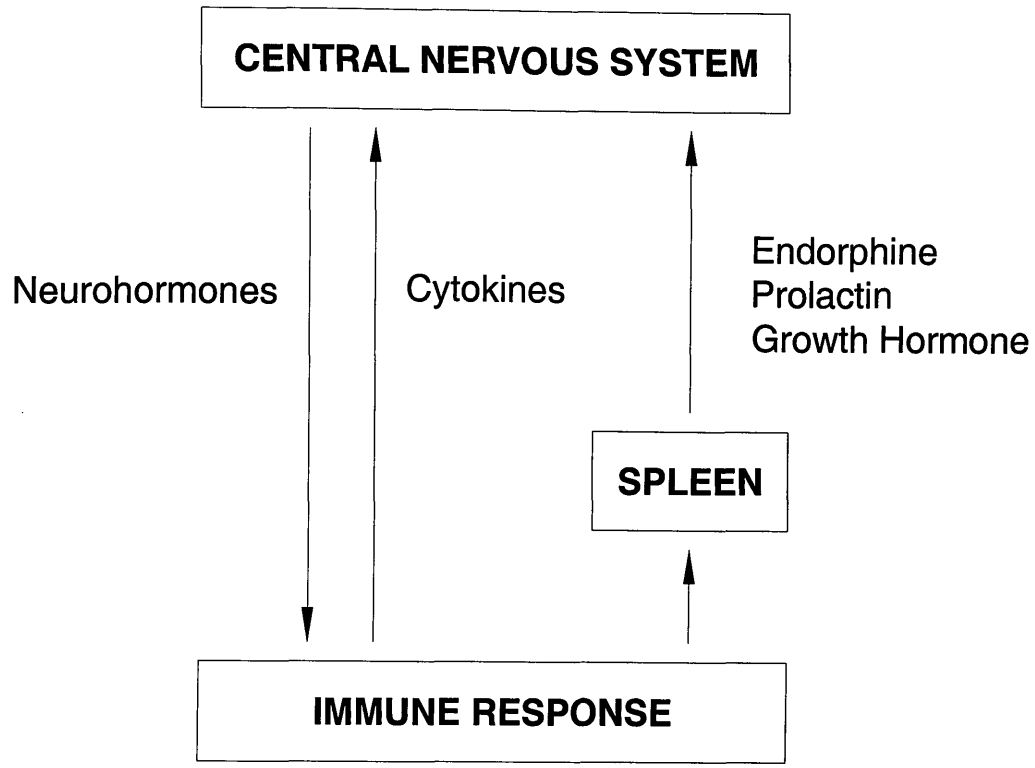


Fig. 5.1 Neuroendocrine immune modulation is bidirectional. In mammals, cytokines released from immune activated cells modify neurohormones. The spleen can produce neurohormones in response to immune stimulation. The central nervous system produces a variety of neurotransmitters and neurohormones that bind to receptors in immune cells and modify their activities (Adapted from Roberts, 1995).

5.1.1.1 Cellular and Molecular Biology of Melatonin Synthesis

Melatonin is a product of tryptophan (TRP) metabolism produced by the pineal gland (Reiter, 1991) (Fig. 5.2). As pineal melatonin production increases at night, there is a parallel rise in blood levels of the hormone. Evidence that the nocturnal surge of blood melatonin is a consequence of pineal secretion comes from the fact that both pinealectomy and the sympathetic denervation of the gland by bilateral superior cervical ganglionectomy prevents the night-time rise in plasma melatonin. Although there are other organs that produce melatonin such as the retina, blood concentrations of the hormone derive primarily from the pineal gland (Reiter, 1991).

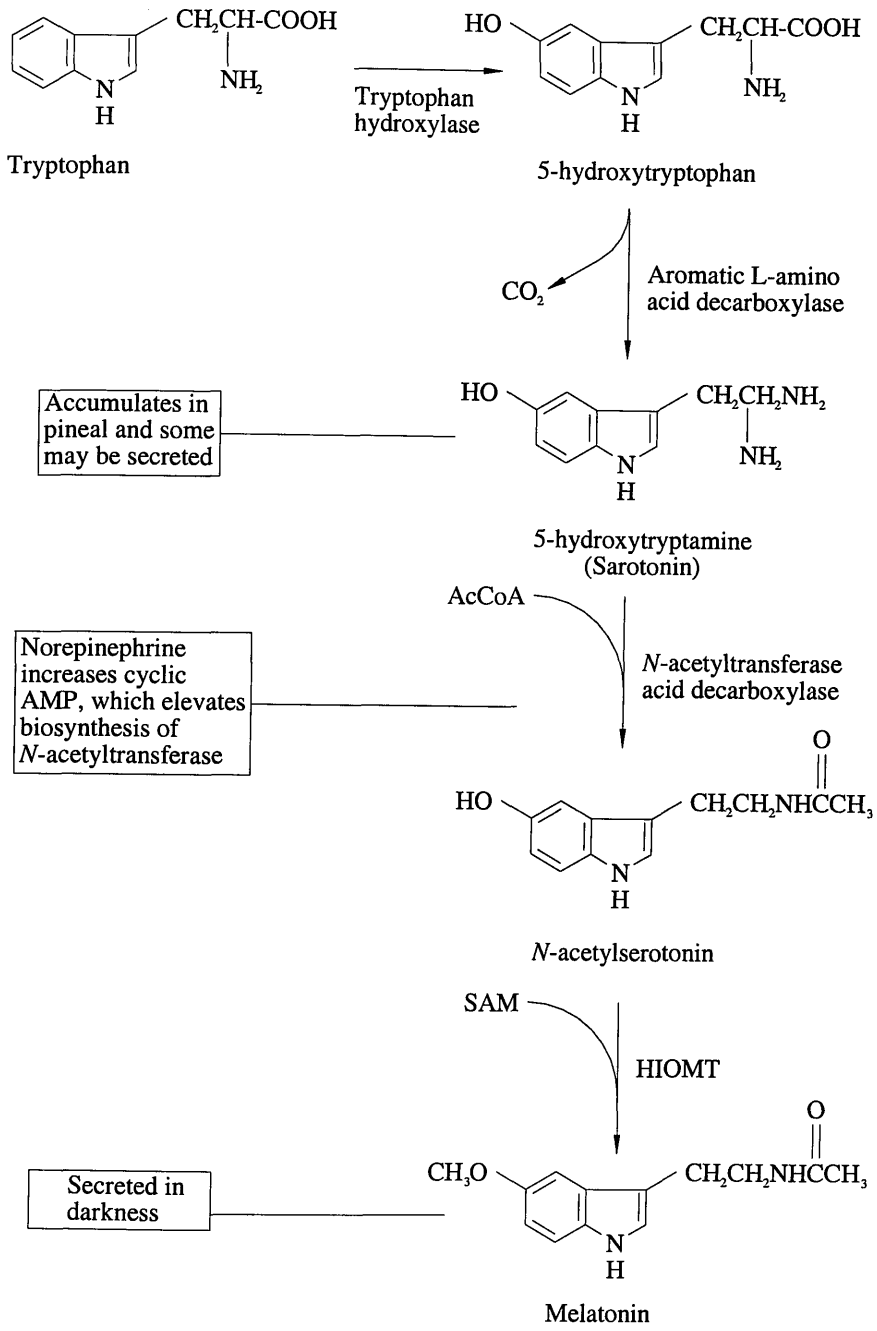


Fig. 5.2 Melatonin synthesis pathway. Serotonin is formed from trytophan. During the light hours of the day, tryptophan is converted in a two-step reaction to serotonin. In darkness (shaded area), increased norepinephrine secretion causes an increase in *N*-acetyltransferase, the first of two enzymes that convert serotonin to melatonin. SAM, *S*-adenosylmethionine; HIOMT, hydroxyindole-*O*-methyltransferase (taken from Nelson *et al.*, 2002)

The characteristic of melatonin that apparently allows for its rapid escape from pinealocytes into the blood vascular system is its lipophilicity, which also probably helps its rapid entrance into other body fluids. Even though the bulk of the melatonin in

the blood (~70%) is bound to albumin, there seems to be no impediment in its entrance into all body fluids and, indeed its presence has been found in every fluid tested for the hormone (Reiter, 1991).

5.1.1.2 Photoperiod as a Modulator of Pineal Function

Melatonin is synthesised and secreted by the pineal gland in a precise, regulated temporal pattern. The best known and probably best-studied biological function of melatonin is its provision of annual day length information. Melatonin encoding day length (photoperiod) information, appears to be the primary hormone orchestrating the seasonal changes in reproductive function observed among many mammals living in mid to high latitude habitats (Nelson and Demas, 1997). Low levels of melatonin synthesis and secretion occur during the day, but this increases at night to result in elevated levels of melatonin in the blood. The levels of melatonin remain high for a duration that is directly related to the length of the dark period. Consequently, winter and summer photoperiods are reflected in long and short duration melatonin signals respectively. Studies have established that the duration of elevated melatonin is the essential characteristic which conveys the photoperiodic message (Morgan and Mercer, 1994). The secretory pattern of melatonin allows individuals to ascertain the time of year and thus anticipate predictable seasonal environmental changes (Nelson and Demas, 1997). Research into this mechanism in fish is limited, and although it has been shown to exist, there is still a lack of understanding into the role of melatonin.

Rainbow trout is one of the few known fish species where an absence of an endogenous component regulating melatonin secretion has been reported (Randall *et al.*, 1991). Unlike many other fish species studied, melatonin production in rainbow trout does not follow a persistent rhythm. Melatonin production in rainbow trout appears to

be a direct response to darkness (Randall *et al.,* 1991). It has been suggested that the rainbow trout pineal does not contain a circadian oscillator capable of regulating melatonin production (Gern and Greenhouse, 1988; Randall *et al.,* 1991). Increased levels of melatonin have been reported in the common carp before lights have been switched off (Kezuka *et al.,* 1988). While cultured pineals of pike do not exhibit an increase in melatonin production when exposed to darkness at midday (Falcón *et al.,* 1989).

5.1.1.3 Temperature as a Modulator of Pineal Function

Unlike mammalian pineal glands which respond primarily to photoperiodic cues perceived by the eyes, there is evidence that the pineal complex of many non-mammalian species extracts both thermal and photoperiod information directly from the environment. As a consequence, the pineal generates a rhythm of melatonin production which reflects the changes in time, duration and amplitude of the complex variations of the daily and seasonal photothermal environment (Moyer *et al.* 1995). There have been very few studies reported in which the effect of temperature on melatonin production in fish have been investigated. However, one such study carried out by Porter *et al.* (2001), found that juvenile Atlantic salmon maintained at 12°C showed significantly higher levels of dark phase plasma melatonin compared with groups of fish maintained at 4°C. Moyer *et al.* concluded from his 1997 study, that both light and temperature are important modulators of pineal function although the combined effects of these on pineal melatonin production is complex and unclear. Obviously, a great deal more study is required in this area.

In addition to responding to changes in environmental temperature melatonin has been shown to affect the body temperature of animals itself. This corroborates the

hypothesis that temperature and melatonin are inextricably linked. For example experiments where Japanese quail were injected with melatonin resulted in reduced locomotor activity in the birds as well as a simultaneous decrease in their body temperature (Nakahara *et al.*, 2003).

5.1.1.4 Neuroendocrine System and Immunity

In recent years evidence has been collected which confirms that the neuroendocrine and immune systems communicate and cooperate extensively to form a single, complex regulatory network that ensures homeostasis (Weyts *et al.*, 1999). Corticosteroids and the sex steroids are thought to play a vital part in this system (Fig. 5.3).

It has been found in humans that physiological levels of estrogen stimulate humoral and cell-mediated immune responses, while the male hormone, testosterone, does the opposite (Weinstein *et al.*, 1984). This is corrobated in fish as testosterone in Chinook salmon has been shown to have a significant immunosuppressive effect *in vitro* (Slater and Shreck, 1997).

Cortisol is biologically the most active corticosteroid in the circulating blood of teleost fish (Esteban *et al.*, 2004). Cortisol has also been found to have an immunosuppressive effect on the fish's immune system (Slater and Shreck, 1993).

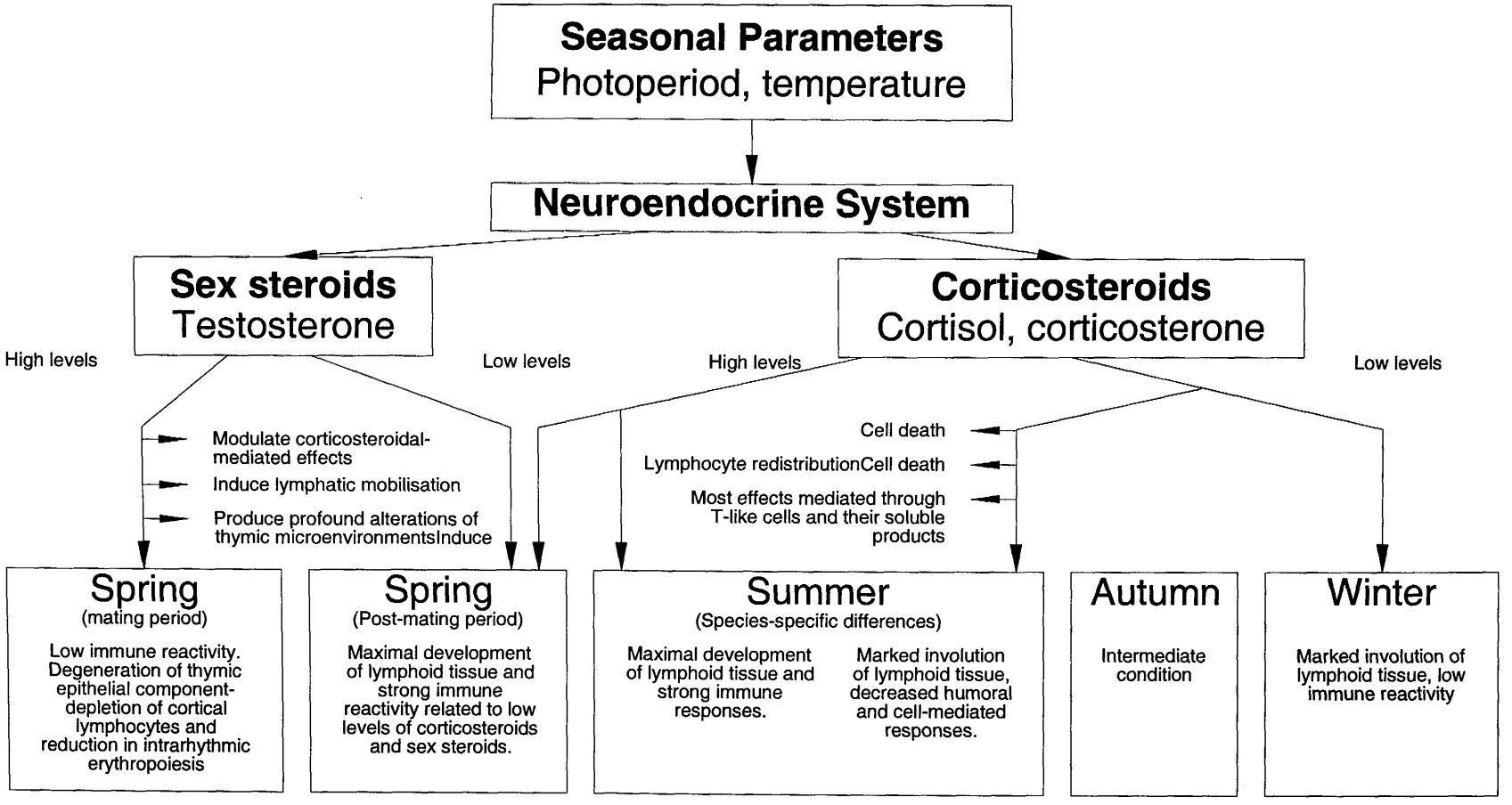


Fig. 5.3 Modulation of seasonal changes in ectotherm immune function mediated by endogenous neuroendocrine rhythms (Figure taken from Zapata, 1992)

5.1.2 Melatonin and Immunity

It has already been well established that the central nervous system plays an important role in modulating the immune response. Cytokines, neurohormones and neurotransmitters are essential factors in this neuro-immuno-communication. The neuroendocrine network which modulates immune response is bidirectional as shown in Fig. 5.2. Several papers have recently demonstrated that the hormone melatonin exerts important immunoregulatory functions in mammals (Pioli *et al*, 1993; Beskonakli *et al*, 2000) and birds (Moore *et al.*, 2002; Moore and Siopes, 2002; Moore and Siopes, 2003). However, little work has been carried out looking at the effect of melatonin on the immune response of fish.

Many vertebrates display profound changes in their physiology and behaviour, coincidental with their seasonal environment (Morgan and Mercer, 1994). Changes in biometeorological conditions signal the endocrine system to respond with an adaptive alteration to its physiological status in anticipation of the upcoming seasonal change (Pévet, 1984). In addition to the well-documented seasonal cycles of mating and birth (Rosa and Bryant, 2003), there are also significant seasonal cycles of illness and death among many animal populations (Kevan, 1979). Challenging winter conditions (i.e., low ambient temperature and decreased food availability) can directly induce death via hypothermia, starvation or shock. Many environmental challenges are recurrent and thus predictable. Animals maybe able to enhance their survival and presumably increase their fitness, if they could anticipate immunologically challenging conditions in order to cope with these seasonal threats to health (Nelson *et al.*, 1995).

The habitat of most organisms is subject to pronounced seasonal fluctuations. Literally all physical environmental factors important to an organism, such as temperature, daylength, and rainfall, vary with season. Animals therefore have to be able to adapt to these environmental fluctuations, depending upon their latitude (Pévet, 1984) as seasonal effects usually intensify with increasing latitude (London, 1987).

The precise mechanisms, by which the central nervous system is able to perceive, differentiate and ultimately integrate this complex of various stimuli has not yet completely been identified. However, melatonin and the pineal gland are thought to be greatly implicated in such a mechanism (Pévet, 1984). Consequently, there is increasing interest in the possibility that seasonal endocrine rhythms are involved in the co-ordination of the immune system of ectothermic vertebrates. However, at present the linkage between the environment and the controlling neuroendocrine cascade remains unclear (Porter *et al.*, 2000).

5.1.3 Alleviation of Seasonal Immunosuppression

One of the most promising areas of development for strengthening the immune defences of fish is the administration of immunostimulants. Knowledge of when to administer these immunostimulants is obviously of great importance. Immune parameters are not maintained at the same levels throughout the year, instead they fluctuate with season. Previous studies indicate that immune parameters tend to be suppressed during winter (Zapata, 1992, Slater and Schreck, 1998). This has been corroborated by the results of previous chapters in this thesis investigating the effect of season (Chapter 2) and temperature (Chapter 3). Therefore it could be beneficial to administer immunostimulants just prior to periods of immunosuppression. Furthermore,

immunosuppression occurs at other times of the fish life history, for example at times of spawning, when more energy is put into reproduction instead of maintaining body condition and health, or moving fish as part of aquaculture production.

Several methods to alleviate immunosuppression are used in the aquaculture industry. It is well known that the immune system in fish can be immunostimulated using dietary supplements including β glucans (Engstad *et al.*, 1992; Jorgensen and Robertsen, 1995; Couso *et al.*, 2003), β glucans plus vitamin C (Verlhac *et al.*, 1996) or levamisole (Siwicki, 1989).

5.1.3.1 Vaccination

Vaccination is used as a method of educating the fish immune system to a specific pathogen. In this study, the vaccination used is against *Vibrio anguillarum.* A commercial vaccine against *V. anguillarum* as used in this study has been available in Europe since the late 1970's (Lillehaug, 1989). In addition to its role in protecting the fish against a specific disease threat, vaccination has also been found to stimulate the innate immune system for example respiratory burst activity is generally reported to increase following vaccination, with peak activity being observed 30 days post-injection (Secombes, 1994).

5.1.3.2 Melatonin Administration

In recent years the role of melatonin as a potential immunostimulant has been investigated in mammals and birds. Melatonin has been shown to positively enhance both humoral and cell mediated immunity (Champney *et al.*, 1997; Akbulut *et al.*, 2001; Brennan *et al.*, 2002; Moore and Siopes, 2002; Moore and Siopes, 2003). For example it has been shown that melatonin activates T helper cells in mice by means of binding

directly to melatonin receptors on both Th1 and Th2 cells (Currier *et al.*, 2000). Administration of melatonin during a pineal inactive periods such as daytime was found to enhance various aspects of immune function (Nelson and Drazen, 2000). In addition, degenerated immune function caused by retrovirus infection can also be restored by melatonin supplementation (Zhang *et al.*, 1999). Age related immunosuppression has been shown to be partially restored following administration of melatonin in rodents. It is therefore thought that administration of melatonin may augment the depressed humoral immune responses seen in elderly patients (Tian *et al.*, 2003; Akbulut *et al.*, 2001). The studies use a variety of methods to administer melatonin including injection, dissolved in drinking water and implantation of slow release capsules.

5.1.4 Aims

In the present chapter a variety of immune (both innate and adaptive) and haematological parameters were measured over a twelve month period to ascertain if seasonality influences their levels and to determine when the immune system is at its weakest and therefore more susceptible to disease. The information collected from this trial will allow administration of immunostimulants to be timed more effectively.

The trial also aimed to determine is melatonin could be used as an immunostimulant. Fish were also vaccinated against *V. anguillarum* firstly to investigate the effect of season on the adaptive immune response, and secondly in conjunction with administration of melatonin to determine if vaccine efficacy is enhanced.

5.2 Materials and Methods

5.2.1 Trial Set-up

A 12-month trial was carried out at the Niall Bromage Freshwater Research Facility (NBFRF) using pre-yearling (hatched June 2001), mixed sex, rainbow trout all originating from the University of Stirling's freshwater research facility. First sampling took place in January 2002. Average weight of fish at the start of the trial was 82.07 g (SD = 12.89; SE = 0.911). At the start of the trial each tank contained 500 fish.

The fish were initially maintained in four replicate, round fibreglass tanks (1 m Ø x 0.70 m water depth,) at the University of Stirling's freshwater research facility at the NBFRF. During May the fish were moved to larger tanks to prevent overcrowding (2 m Ø x 1 m). The tanks were set up in a flow through system supplied by a reservoir situated 1 km from the facility. The water was supplied to the tanks at a rate of approximately 2 L per second at ambient seasonal temperature. Light was supplied by two 60-watt pearl, tungsten filament light bulbs housed within waterproof lamps providing an intensity of 17-19 lux at the water surface. The photoperiods were controlled by 24 h digital electronic time switches, which were set to imitate natural photoperiod.

5.2.1.1 Treatments

The treatments were carried out in duplicate. Each tank contained four treatment groups within it. The fish were fin clipped to allow easy identification of the different groups. A quarter of the fish were vaccinated against *V. anguillarum*, a quarter were implanted intra-muscularly with a slow release melatonin implant, a quarter of the fish were both vaccinated and had a melatonin implant and the remaining quarter of the fish were untreated (Fig. 5.4). Vaccination, melatonin implantation and fin clipping took place on the same day in early December 2001. This took place at the same time, whilst fish were under anaesthetic (1:20,000 concentration of 2-phenoxyethanol). Fish were placed in aerated water to recover and full recovery was usually seen within five minutes. Five mortalities were recorded the day following this procedure, no further mortalities were recorded.

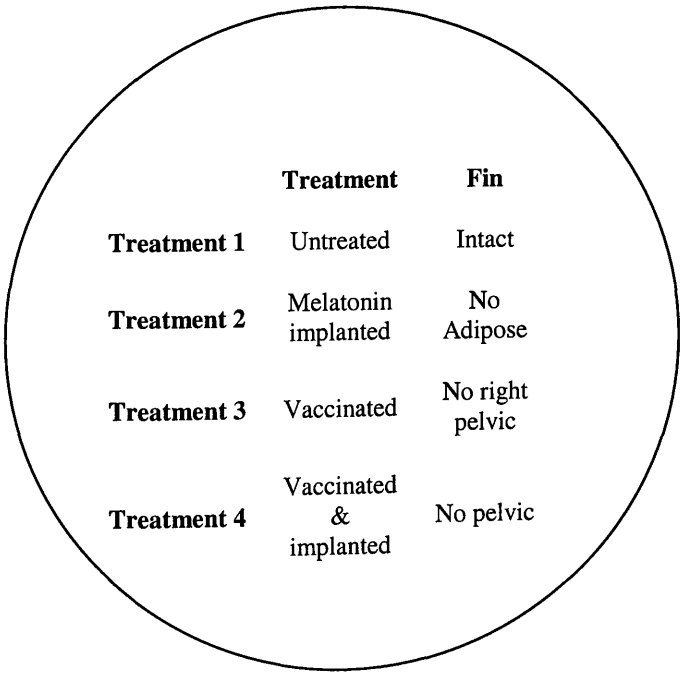


Fig. 5.4 Treatment groups within any one tank

5.2.1.2 Vaccination

Fish were vaccinated (0.1 ml/fish) intraperitoneally against *V. anguillarum,* with a commercial vibriosis vaccine, provided by Schering Plough Aquaculture.

5.2.2 Melatonin Implantation

Melatonin implants (18 mg, Regulin, Schering Pty. Ltd) were used in this trial. The implants permanently elevated plasma melatonin in excess of night-time physiological rhythms with the aim of 'masking' the natural rhythm in circulating melatonin levels (Porter, 1996). Regulin implants contain 18mg of melatonin and were coated in a polymer that allowed a slow and constant release of the hormone.

An implanter (Schering Pty. Ltd) was used to administer the implants intramuscularly 1 cm below the dorsal fin. Previous studies report that the level of melatonin released is dependent on the body weight of the fish (Porter, 1996). In addition, there is no significant difference between day and night melatonin levels of implanted fish, although night time levels are generally slightly higher (Porter, 1996).

5.2.2.1 Sampling

The duration of the trial was twelve months. Once fish were acclimated for four weeks prior to first sampling took place. Fish blood was sampled for antibody titre and lysozyme activity every month during the trial period. Macrophage respiratory burst activity, phagocytic activity, total blood counts and haematocrit were measured every alternate month. Weight and length of fish were measured each month. Methods used to measure these parameters are described in Sections 2.2.2, 2.2.3, 3.2.3, 3.2.4. and 5.3 Forty fish were sampled at each sampling (10/treatment).

5.3 Measurement of Innate Immune Parameters

5.3.1 Phagocytosis Activity of Head Kidney Macrophages

Microscope slides were dipped in 100% ethanol. Two circles were drawn on each slide using a PAP pen (AGAR Scientific) (one slide per fish). One hundred μl of the macrophage suspension (Section 2.2.3.4) was placed within both circles on the slide. The slides are then left for 1 h at 21°C to allow the cells to adhere to the glass. Following incubation, the slides were gently washed with L-15 using a sterile Pasteur pipette. A suspension of bakers' yeast was prepared in L-15 at a concentration of 5 mg ml^{-1}. To one circle of cells was added 100 μl of this suspension was added, while to the second well only L15 medium was added. Phagocytosis was allowed to take place for 1h, after which time the slides were washed with L-15. The slides were dipped in 70% methanol to fix the cells and then stained using a Quick Stain Kit (Raymond Lamb Ltd) and air dried.

The slides were mounted using Pertex-mounting medium. The cells were examined under oil immersion x1000 magnification and the number of yeast engulfed by 100 macrophages determined where possible. Fig. 5.5 illustrates the appearances of macrophages engulfing yeast through phagocytosis. This information was used to calculate Phagocytic Ratio (PR) (Equ. 5.1) and the Phagocytic Index (PI) (Equ. 5.2)

Equ. 5.1 Phagocytic Ratio

$$PR = \frac{\text{No. of macrophages with one or more ingested yeast cells}}{100}$$

Equ. 5.2 Phagocytic Index

$$PI = \frac{\text{No. of ingested yeast cells in 100 macrophages}}{100}$$

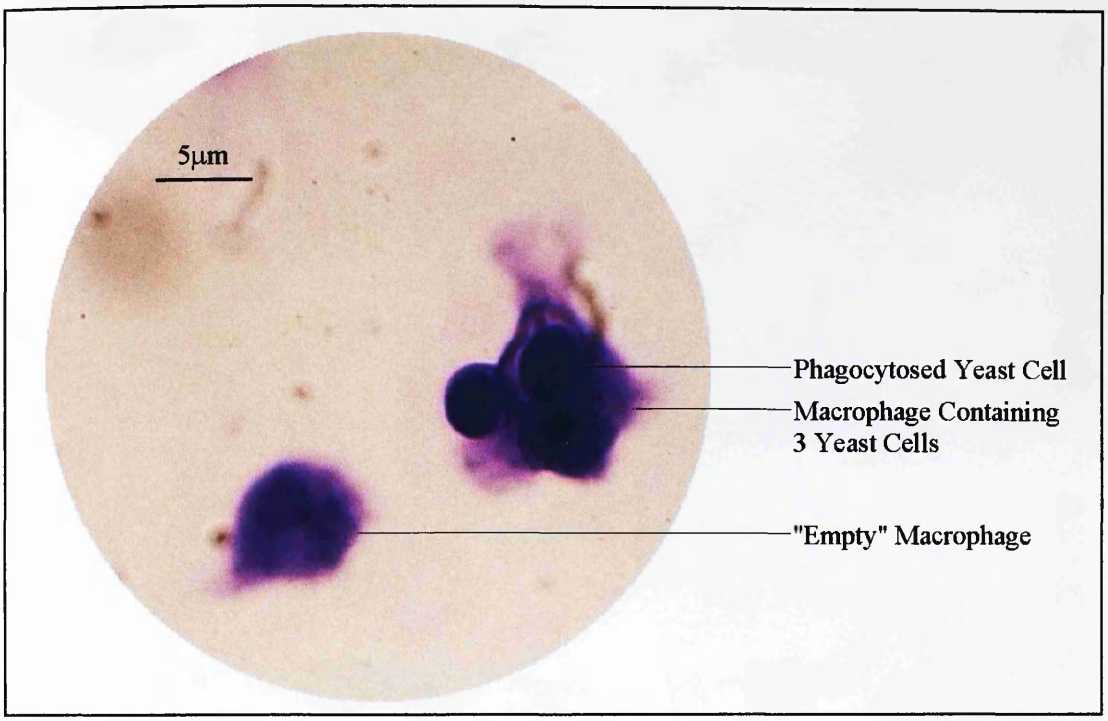


Fig. 5.5 Measuring phagocytic activity of head kidney macrophages using yeast. Field of view under oil immersion.

5.4 Measurement of Adaptive Immune Parameters

The specific antibodies produced in response to vaccination against *V. anguillarum* were measured at various time points after vaccination and the level of protection was determined using an experimental challenge with the bacterium.

5.4.1 Measurement of Specific Antibody Titres Using an Indirect Enzyme Linked Immunosorbent Assay (ELISA)

5.4.1.1 Antigen Preparation

Cultures of *V. anguillarum* (Serotype I) obtained from Schering Plough Aquaculture were grown in tryptone soya broth + 2% NaCl (TSB+NaCl) for 24 h at 22°C prior to use. The following day bacteria were collected by centrifugation at 3000 rpm, for 30 min at 4°C. The broth was then decanted and the remaining bacterial pellet

resuspended in sterile PBS. The bacteria were washed twice with sterile PBS by centrifuging as above. After washing the remaining pellet was resuspended in 2 ml PBS. The optical density of the bacterial suspension at 610 nm was determined, and its concentration adjusted to 1 x 10^8 CFU ml^{-1} in sterile PBS using a standard curve of CFU vs OD 610 (Appendix B). The bacterial suspension was then heat killed by incubating in a water bath at 60°C for 60 min.

5.4.1.2 ELISA Protocol

- **Day one**

A 0.05% (weight/volume) solution of poly-L-lysine in carbonate-bicarbonate buffer (coating buffer) was freshly prepared. Fifty μl $well^{-1}$ of this was aliquoted into the ELISA plates and left for 1 h at 21°C. The plates were washed three times with low salt wash (LSW) buffer (Appendix A). One hundred μl of the heat-killed bacteria were aliquoted into each well. The plates were covered and stored at 4°C overnight.

- **Day two**

Gluteraldehyde in PBS (0.05% v/v) was added to the bacterial suspension at 50 μl $well^{-1}$. The plates were covered and left to incubate for 1 h at 21°C for 20 min. Plates were then washed three times with LSW.

To block non-specific binding sites, plates were post-coated with a 1% bovine serum albumin (BSA) solution. They were covered and left to incubate for 2h at 21°C, and then washed three times with LSW.

Plasma samples were diluted 1/5 in PBS and added to the first well in each row. One sample per row. Two fold dilutions were then made along the row up to and including column 11. Plates were then covered and incubated overnight at 4°C. Positive

control wells consisted of blood plasma taken from fish that survived challenge with *V. anguillarum*. Negative control wells consisted of blood plasma taken from fish that had never been exposed to *V. anguillarum*. PBS was used to measure background levels on the plate.

- **Day three**

Plates were washed five times with high salt wash buffer (HSW) (Appendix A). The fifth addition of HSW was left in the plate for five min before adding 100 μl of neat anti-trout monoclonal antibody (supernatant) (4C10, Appendix B) was aliquoted into all wells. Plates were covered and incubated for 1h at room temperature. Again plates were washed five times with high salt wash buffer (HSW), which was left for 5 min on the last wash.

Anti-mouse IgG horse radish peroxidase (HRP) conjugate (Diagnostics Scotland) was diluted 1/1000 in conjugate buffer (Appendix A). One hundred μl well^{-1} of this solution was aliquoted into all of the wells. Plates were covered and incubated for 1 h at 20°C temperature. Again plates were washed five times with high salt wash buffer (HSW) to remove any unbound conjugate and incubated for 5 min on the last wash.

Substrate buffer (Appendix A) was added to the plates at 100μl well^{-1}. Plates were incubated at 21°C for 10 min. Positive results were indicated by a blue colour. The reaction was stopped by adding 50 μl well^{-1}of 2M H_2SO_4, where upon the colour changed from blue to yellow. The optical density was read at 450 nm using an ELISA plate reader.

5.4.2 Statistical Analysis

Before analysis data was tested for normality and homogeneity of variance using fits and residuals. All data was found to be normal and homogenous without transformation. Data was analysed using an ANOVA General Linear Model (Minitab). The Post-hoc Tukey test was used to carry out Pairwise comparisons (Minitab). Correlation statistics were performed using Pearson's Correlation Coefficient (Minitab). Statistical analysis of the survival to artificial challenge was carried out using the Kaplan Meier survival test statistic (SPSS).

5.5 Results

The profile for temperature at the NBFRF over the sampling period (January 2000-01) has been added to each of the following graphs. This is because the results of the previous two chapters suggest that temperature is the primary environmental cue used by the rainbow trout immune system.

5.5.1 Weight

Fish were weighed every month throughout the trial. At the start of the trial average weight was 82.7g. Treatment had no affect on weight until August, from which point to the end of the trial the vaccinated group were the heaviest of the treatment groups (P=0.030) (Fig. 5.6).

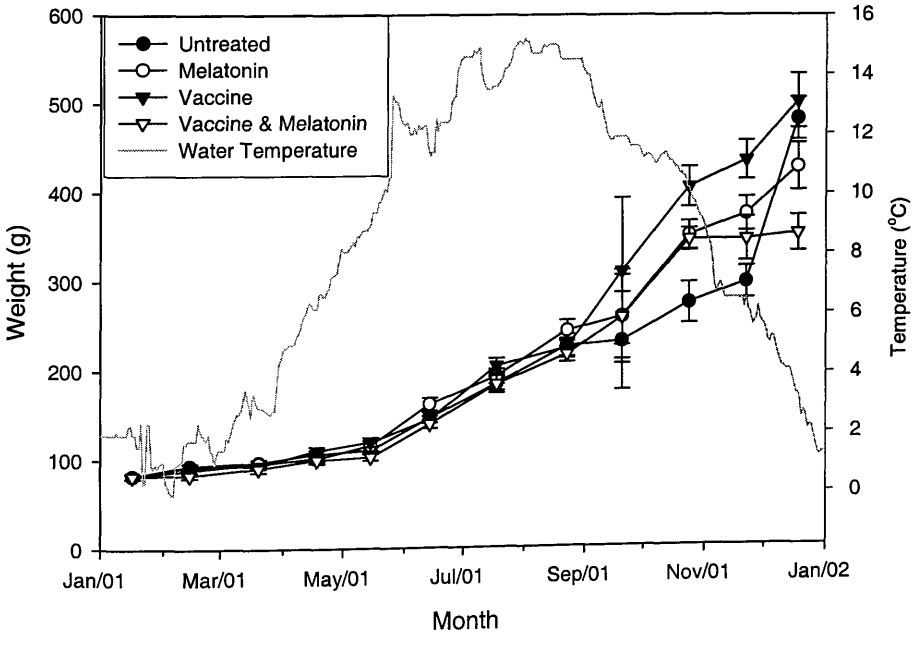


Fig. 5.6 The weight of fish measured every month for the duration of the trial for fish from the four treatment groups. (n=20; mean ± SE)

5.5.2 Changes in Immune and Haematological Parameters

5.5.2.1 White Blood Cell Counts

No significant difference was recorded in white blood cell counts between fish in the four different treatment groups (P=0.306) (Fig. 5.7). An analysis of the GLM ANOVA results revealed a significant difference in white blood cell counts with month (P=0.000). Tukey Pairwise comparison tests were carried out (Table 5.1), analysis of which determined that a seasonal pattern in white blood cell counts was present. The white blood cell counts recorded in the summer months of June and August were significantly greater than all other months, but counts were not significantly different from each other at these two sampling times (P=0.9998). This summer peak is statistically very important because all white blood cell counts measured during the other sampling months are not significantly different from each other (P>0.05). There appears to be a strong relationship between total white blood cell counts and water temperature, particularly in June in August where rapid increase in white blood cells corresponds to a rapid increase in temperature.

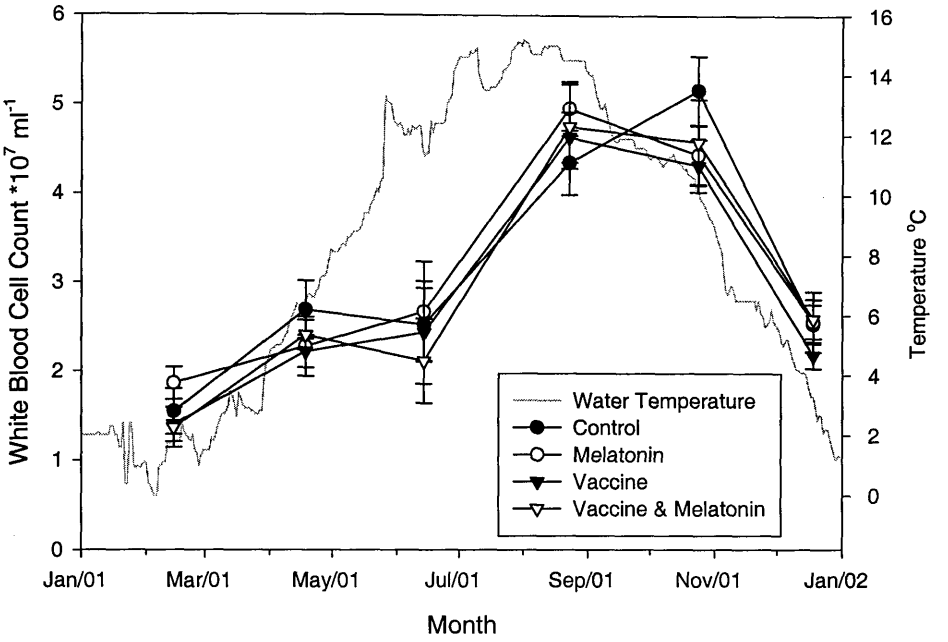


Fig. 5.7 White blood cell counts of fish from the four treatment groups (n=20; mean ± SE), there is no significant difference between the groups (P=0.306).

Table 5.1 Tukey Pairwise Comparisons used to determine the months in which white blood cell counts were significantly different to each other (n=80).

	February	April	June	August	October	December
February	X					
April	P>0.05	X				
June	**P=0.000**	**P=0.000**	X			
August	**P=0.000**	**P=0.000**	P>0.05	X		
October	P>0.05	P>0.05	**P=0.000**	**P=0.000**	X	
December	P>0.05	P>0.05	**P=0.000**	**P=0.000**	P>0.05	X

5.5.2.2 Red Blood Cell Counts

Red blood cell counts of fish from the various treatment groups were found to vary during the twelve month trial period, however there was no obvious affect due to treatment (Fig. 5.8). This was confirmed using a GLM ANOVA. Significant differences were found with month P=0.000, but not with treatment (P=0.141). The significant effect with month was further investigated using Tukey Pairwise Comparison tests (Table 5.2). Red blood counts in February were significantly greater than at any other point during the trial (P=0.000) for all four treatments. Red blood cell counts in June were significantly greater than in August, October and December (P<0.05), while no significant differences were observed in red blood cell counts between October, August or April (P>0.05).

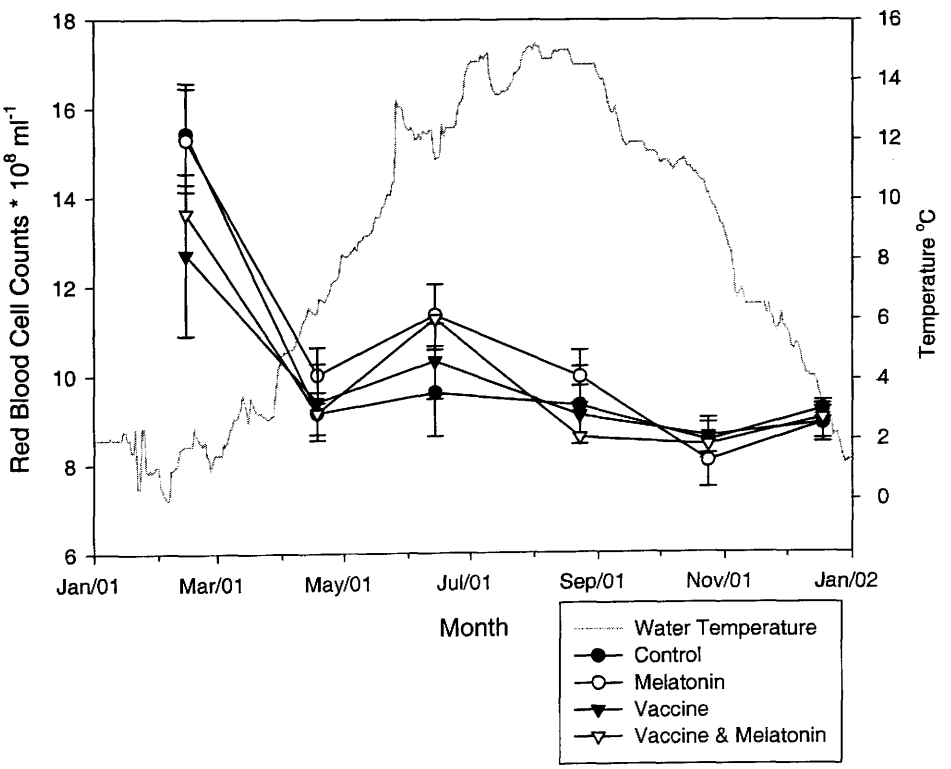


Fig. 5.8 Red blood cell counts of fish from the four different treatment groups over the 12-month trial period (n=20; mean ± SE).

Table 5.2 Tukey Pairwise comparison tests investigating the effect of month on red blood cell counts (n=80)

	February	April	June	August	October	December
February	X					
April	**P=0.000**	X				
June	**P=0.000**	P>0.05	X			
August	**P=0.000**	P>0.05	**P=0.007**	X		
October	**P=0.000**	P>0.05	**P=0.000**	P>0.05	X	
December	**P=0.000**	P>0.05	**P=0.000**	P>0.05	P>0.05	X

5.5.2.3 Haematocrit

No significant differences were found between the four treatment groups (P=0.147) (Fig. 5.9). Haematocrit values appear to have a negative relationship with season, i.e. values were at their lowest during the summer and greatest during the winter sampling. Significant differences in haematocrit were found in relation to month (P=0.000). This was analysed further using Tukey Pairwise Comparison tests (Table 5.3), from which it was determined that haematocrit values measured in February were significantly higher compared to all other months (P=0.000), for all treatments, haematocrits measured in April were significantly higher than in June, August or October (P=0.0001). Haematocrits measured in October were significantly lower than in December (P=0.000).

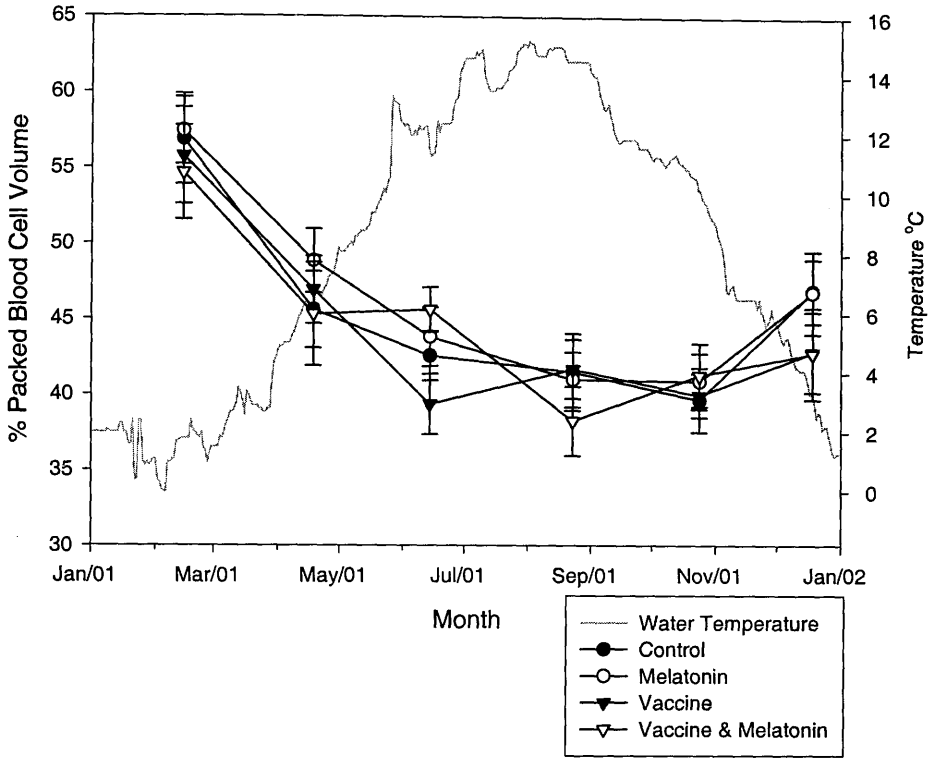


Fig. 5.9 Haematocrit of fish from the four treatment groups over the 12-month period (n=20; mean ± SE). There is no significant difference between the treatment groups (P=0.306).

Table 5.3 Tukey Pairwise Comparisons to determine differences in haematocrit values between the sample months (n=80)

	February	April	June	August	October	December
February	X					
April	**P=0.000**	X				
June	**P=0.000**	**P=0.000**	X			
August	**P=0.000**	**P=0.000**	P>0.05	X		
October	**P=0.000**	**P=0.000**	P>0.05	P>0.05	X	
December	**P=0.000**	P>0.05	P>0.05	**P=0.000**	**P=0.000**	X

5.5.2.4 Macrophage Respiratory Burst

It was established that in June, macrophage respiratory burst activity was significantly greater than at any other point of the trial ($P<0.05$). No significant difference was observed in macrophage respiratory burst activity between the fish under the four treatments ($P=0.660$). However, a significant difference was observed between months ($P=0.000$). This confirms the graphical observations that macrophage respiratory burst activity was at it greatest in June (Fig. 5.10). This activity was significantly higher in fish sampled in June, compared to any other time during the trial ($P=0.000$).

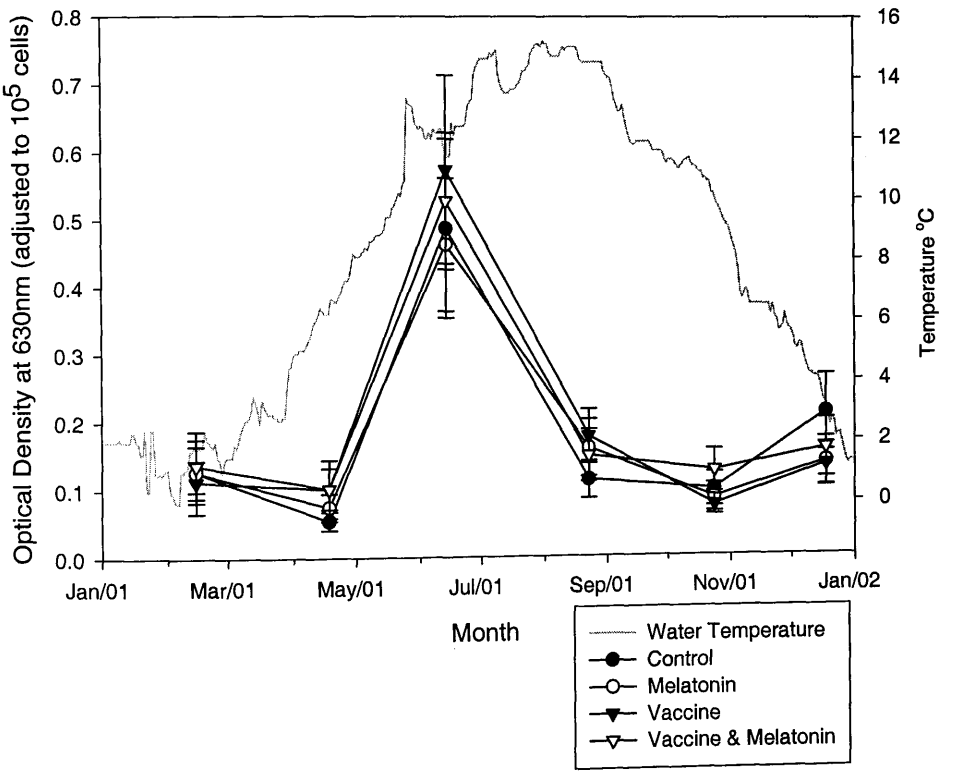


Fig. 5.10 Macrophage respiratory burst activity of fish from the four study treatments. No significant difference was observed in respiratory burst activity (n=20; mean ± SE).

5.5.2.5 Phagocytosis

Phagocytic activity was assessed by determining the number of yeast cells ingested by head kidney macrophages (Phagocytic Index PI) and the number of macrophages that ingested yeast (Phagocytic Ratio PR). PR and PI were significantly affected with month (P=0.000). This was further investigated using the Tukey Pairwise comparison test (Table 5.4, Table 5.6). Treatment had a significant effect on both PR and PI (Fig. 5.11, Fig. 5.13). Analysis of data using Tukey Pairwise comparison tests revealed that fish with melatonin implants had a significantly higher PR and PI (P=0.000) than those fish without an implant (Table 5.5, Table 5.7). Melatonin significantly enhanced phagocytic activity by both increasing the number of macrophages ingesting target yeast cells and increasing the number of target yeast cells ingested by a macrophage cell (Fig. 5.12, Fig. 5.14)

Phagocytic Ratio

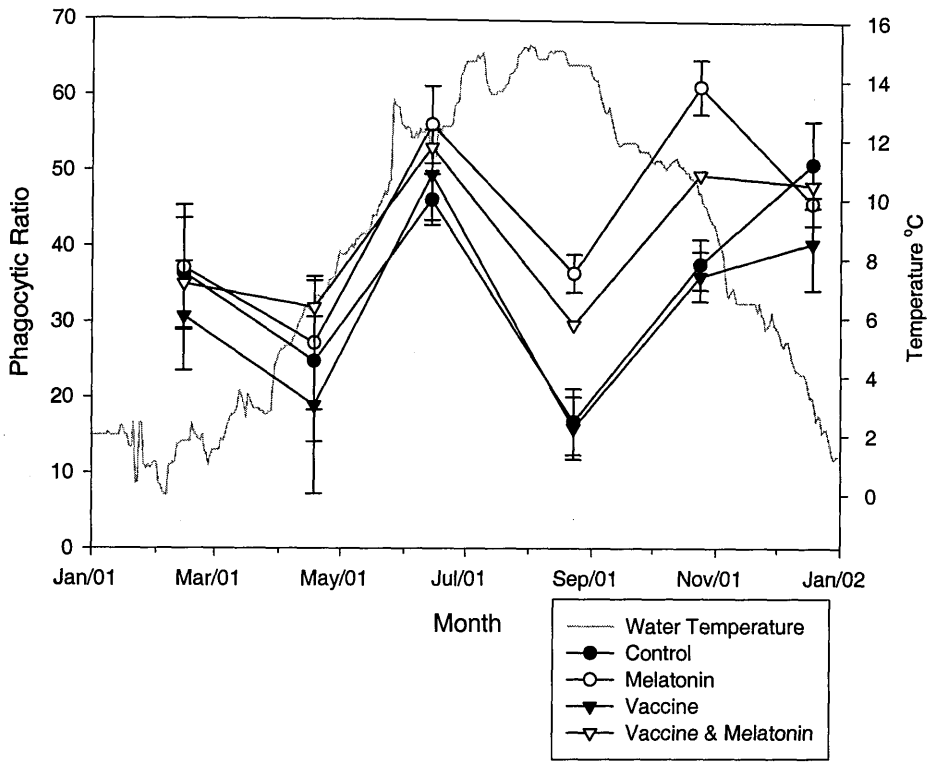


Fig. 5.11 Phagocytic ratio of fish from the four treatment groups over the 12-month trial period (n=20; mean ± SE)

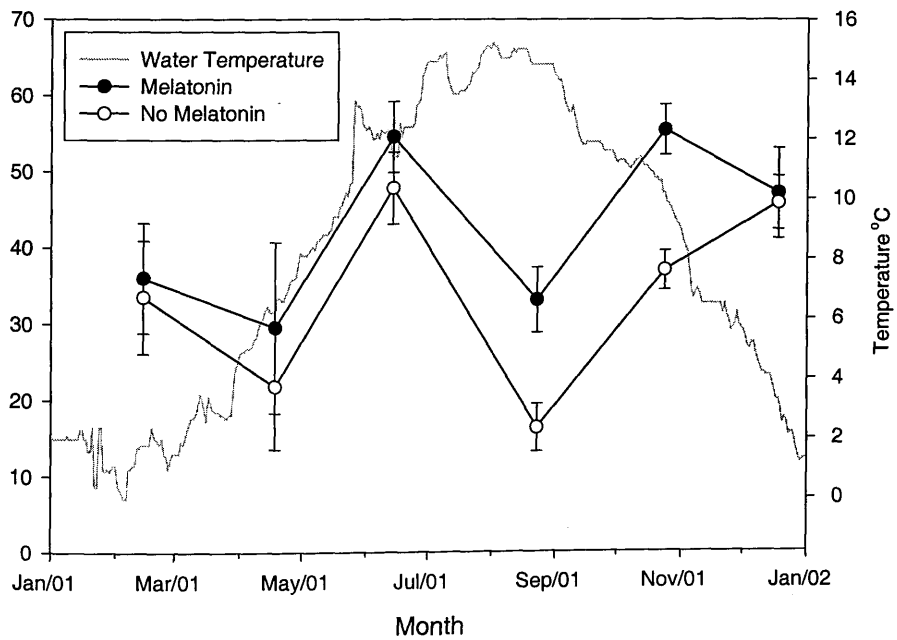


Fig. 5.12 Phagocytic ratio for fish with a melatonin implant and compared to fish without (n=20; mean ± SE).

Table 5.4 Tukey Pairwise comparison test results for phagocytic ratio by month (n=80)

	February	April	June	August	October	December
February	X					
April	P>0.05	X				
June	**P=0.000**	**P=0.005**	X			
August	**P=0.000**	**P=0.000**	**P=0.000**	X		
October	P>0.05	P>0.05	P>0.05	**P=0.000**	X	
December	**P=0.035**	P>0.05	P>0.05	**P=0.000**	P>0.05	X

Table 5.5 Tukey Pairwise comparisons investigating difference in phagocytic ratio by treatment over the 12-month trial period (n=20).

	Untreated	Melatonin	Vaccinated	Vaccinated & Melatonin
Untreated	X			
Melatonin	**P=0.000**	X		
Vaccinated	P>0.05	**P=0.000**	X	
Vaccinated & Melatonin	**P=0.000**	P>0.05	**P=0.000**	X

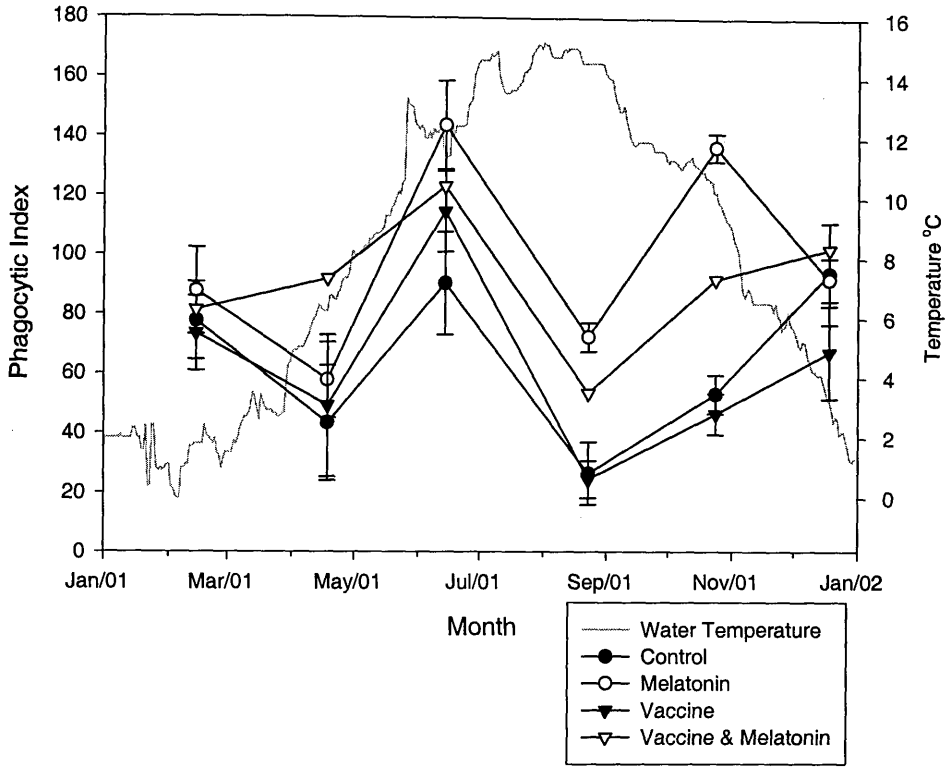


Fig. 5.13 The effect of treatment on phagocytic index over the 12-month trial period (n=20, mean ± SE)

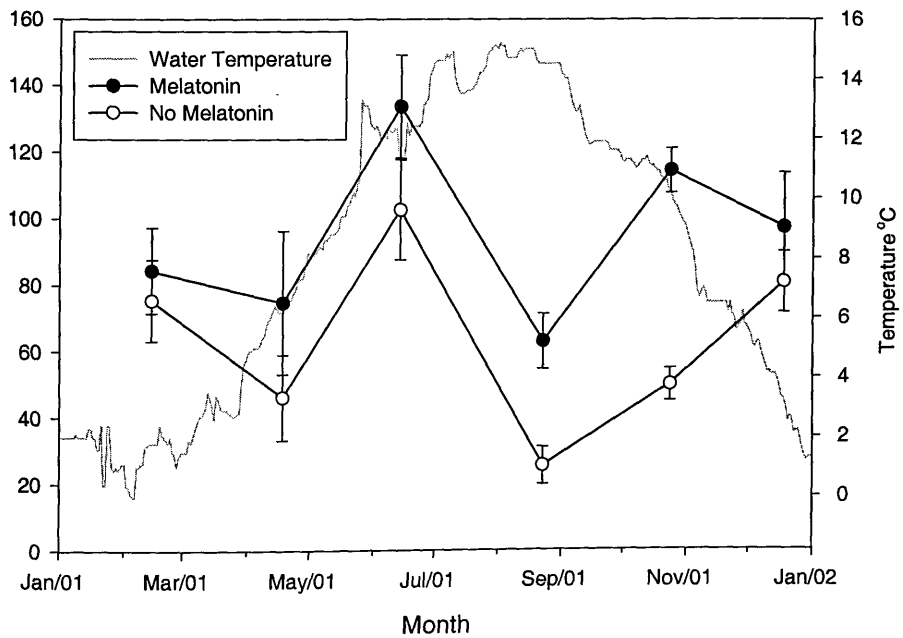


Fig. 5.14 Phagocytic index of fish with a melatonin implant compared to those without. (n=20, mean ± SE).

Table 5.6 Tukey Pairwise comparison test results for phagocytic index by month (n=80)

	February	April	June	August	October	December
February	X					
April	P>0.05	X				
June	**P=0.000**	**P=0.000**	X			
August	**P=0.000**	**P=0.000**	**P=0.000**	X		
October	P>0.05	P>0.05	**P=0.000**	**P=0.000**	X	
December	P>0.05	P>0.05	**P=0.000**	**P=0.000**	P>0.05	X

Table 5.7 Tukey Pairwise comparisons investigating difference in phagocytic index by treatment over the 12-month period (n=20/treatment).

	Untreated	Melatonin	Vaccinated	Vaccinated & Melatonin
Untreated	X			
Melatonin	**P=0.000**	X		
Vaccinated	P>0.05	**P=0.000**	X	
Vaccinated & Melatonin	**P=0.000**	P>0.05	**P=0.000**	X

5.5.2.6 Lysozyme Activity

No significant difference was observed in lysozyme activity between the treatment groups (P=0.058). Lysozyme activity differed significantly with month (P=0.000). Tukey Pairwise Comparisons were used to determine when these differences

occurred (Table 5.8). Fish sampled in January, February, March and April had significantly lower lysozyme activity than months May to December inclusive (P=0.000). Fish sampled in May had a significantly lower lysozyme activity than the months June to November inclusive (P=0.000). Fish sampled in August, October and December had a significantly higher lysozyme activity than November and December (P<0.05) while fish sampled in September had a significantly higher lysozyme activity than December (P=0.000).

Graphically lysozyme activity showed a very good positive relationship with temperature and exhibited a seasonal pattern (Fig. 5.15)

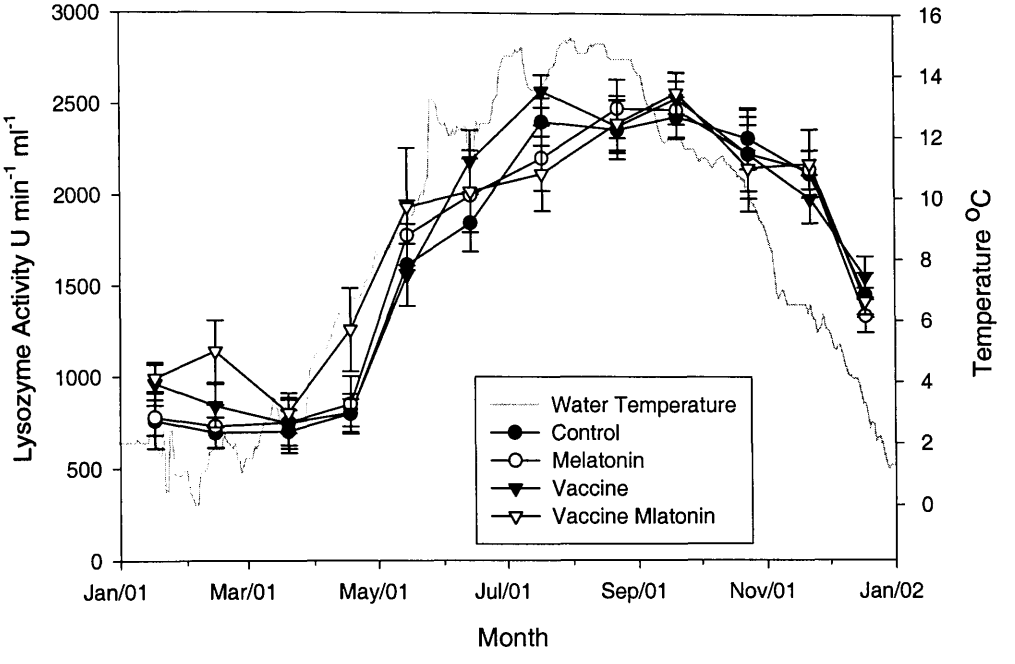


Fig. 5.15 Lysozyme activity of fish held under the four different treatment groups (n=20, mean ± SE). No significant difference was observed between the treatment groups (P=0.058)

Table 5.8 Tukey Pairwise Comparisons to determine when the significant differences in lysozyme activity occur (n=80).

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Jan	X											
Feb	P>0.05	X										
Mar	P>0.05	P>0.05	X									
Apr	P>0.05	P>0.05	P>0.05	X								
May	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	X							
Jun	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	X						
Jul	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	X					
Aug	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	X				
Sep	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	P>0.05	P>0.05	X			
Oct	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	P>0.05	P>0.05	X		
Nov	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	P>0.05	P=0.000	P>0.05	**P=0.000**	X	
Dec	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	X

5.5.2.7 Measurement of Antibody Titre with ELISA

Antibody titre against *V. anguillarum* in the plasma of experimental fish was measured every month throughout the 12-trial. Significant differences in antibody titre were observed between the treatment groups (P=0.000) (Fig. 5.16). Tukey Pairwise Comparison tests were used to determine where these significant differences occurred, and as would be expected, differences were found between the vaccinated and unvaccinated fish (P=0.000) (Fig. 5.17).

Seasonality of antibody titres was observed. This is clearly illustrated in Fig. 5.16 and Fig. 5.17. Temperature is used on the graphical figures to illustrate season and there is an obvious close positive correlation to season, with the highest antibody titres observed during the summer months and the lowest during the winter. This was studied further using Tukey Pairwise Comparisons (Table 5.9). There was no significant difference (P>0.05) in antibody titre between the summer months (May-September),

and no significant difference between the winter months of October-December (P>0.05). The antibody titre of the winter months were significantly lower (P=0.000) than the summer months (P=0.000). These results are true regardless of diet or treatment.

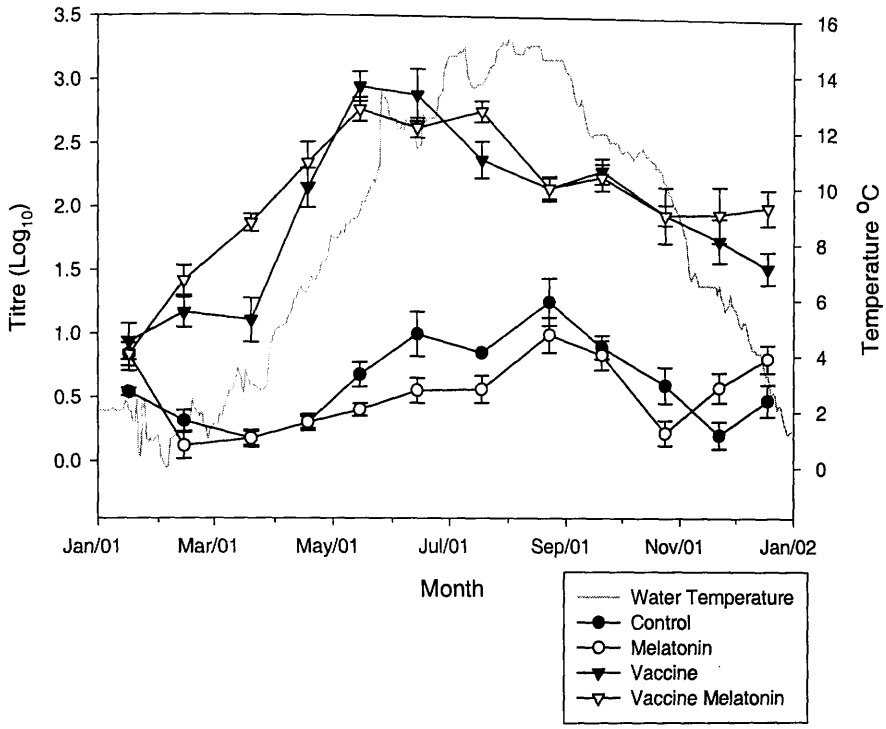


Fig. 5.16 Antibody titre against *V. anguillarum* of fish from the four different treatment groups measured throughout the twelve month period (n=20; mean ± SE)

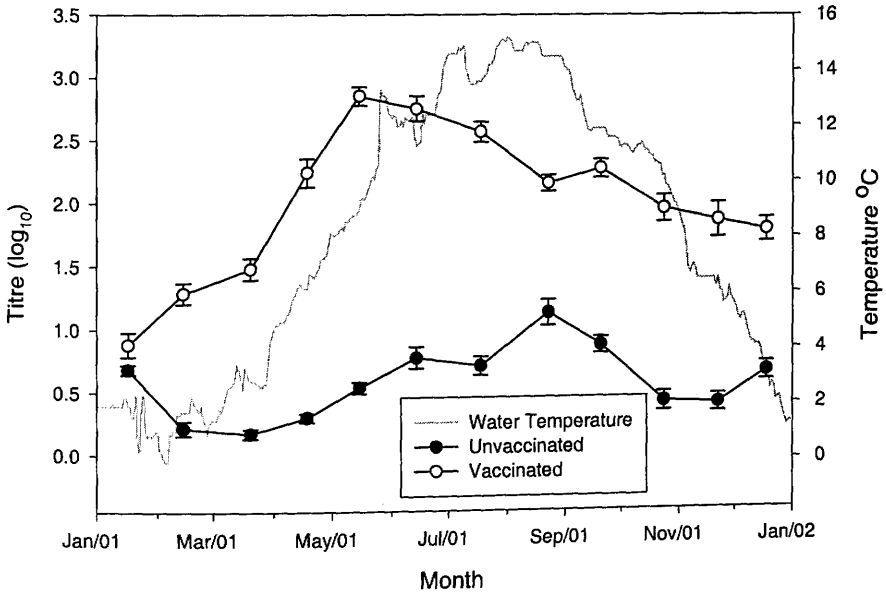


Fig. 5.17 Comparison between the antibody titre of fish vaccinated against *V. anguillarum* and unvaccinated fish (n=40; mean ± SE).

Table 5.9 Tukey Pairwise Comparisons to determine when the significant differences in antibody titre occur (n=80).

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Jan	X											
Feb	P>0.05	X										
Mar	**P=0.000**	P>0.05	X									
Apr	**P=0.000**	P>0.05	P>0.05	X								
May	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	X							
Jun	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	X						
Jul	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	P>0.05	X					
Aug	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	P>0.05	P>0.05	X				
Sep	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	P>0.05	P>0.05	P>0.05	X			
Oct	**P=0.000**	**P=0.000**	P>0.05	P>0.05	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	**P=0.000**	X		
Nov	**P=0.000**	P>0.05	P>0.05	P>0.05	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	X	
Dec	**P=0.000**	**P=0.000**	P>0.05	P>0.05	**P=0.000**	**P=0.000**	**P=0.000**	P>0.05	**P=0.000**	P>0.05	P>0.05	X

5.5.2.8 Challenge with *Vibrio anguillarum*

Fish were challenged in March, June and September. A December challenge was planned but unfortunately the fish did not survive being transported from the fish farm to the challenge suite at the Institute of Aquaculture. Veterinary investigation concluded that this was probably due to the large size of the fish being transported.

March Challenge (Fig. 5.18)

A significant difference in the levels of survival were observed between the treatment groups (P=0.000). Fish that were vaccinated exhibited a significantly greater level of survival compared to fish that were not (P=0.000). Pairwise comparisons revealed a significant difference in survival between the untreated fish and the melatonin implanted fish (P=0.003). No significant difference between the vaccinated fish and the vaccinated fish with a melatonin implant was recorded (P=0.3173). Fish that were vaccinated and had a melatonin implant were the only group to exhibit 100%

survival. The median survival time for both the untreated and the melatonin implanted fish was 2 days. However, the median survival time for both the vaccinated fish and vaccinated with a melatonin implant could not be calculated; this is because so little mortality was recorded.

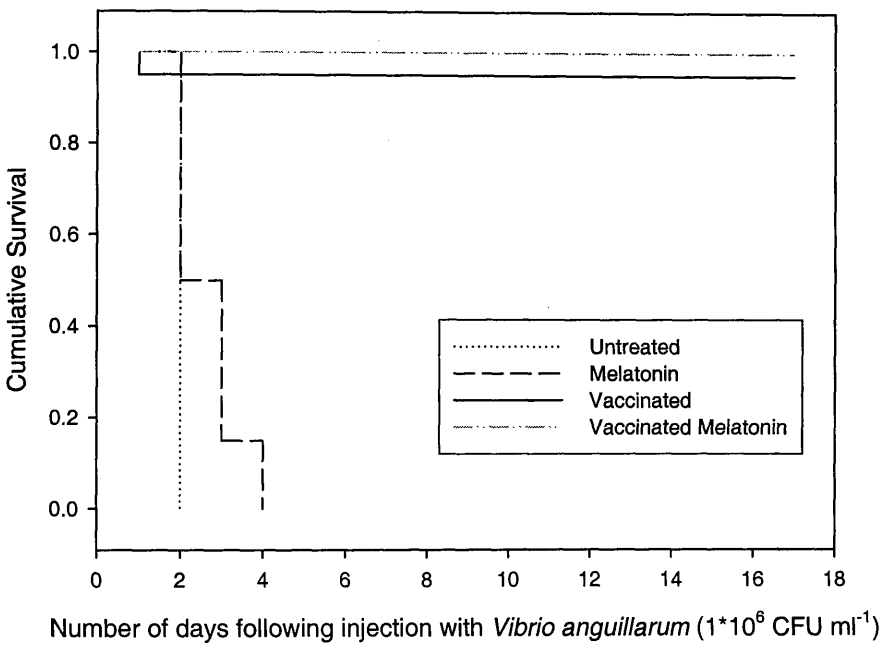


Fig. 5.18 Cumulative survival of fish from the four different treatment groups following artificial challenge with *V. anguillarum* in March (n=20/treatment).

June Challenge (Fig. 5.19)

The June challenge was not very successful as only a total of six fish died as a result of challenge. A significant effect of treatment was recorded (P=0.0477). Pairwise comparisons determined that both vaccinated treatment groups had a significantly higher level of survival compared to untreated fish (P=0.0375). Levels of survival for melatonin implanted fish were not significantly different from untreated fish (P=0.3856); nor from either of the vaccinated treatments (P=0.152). Median survival could not be calculated as too few fish died as a result of challenge.

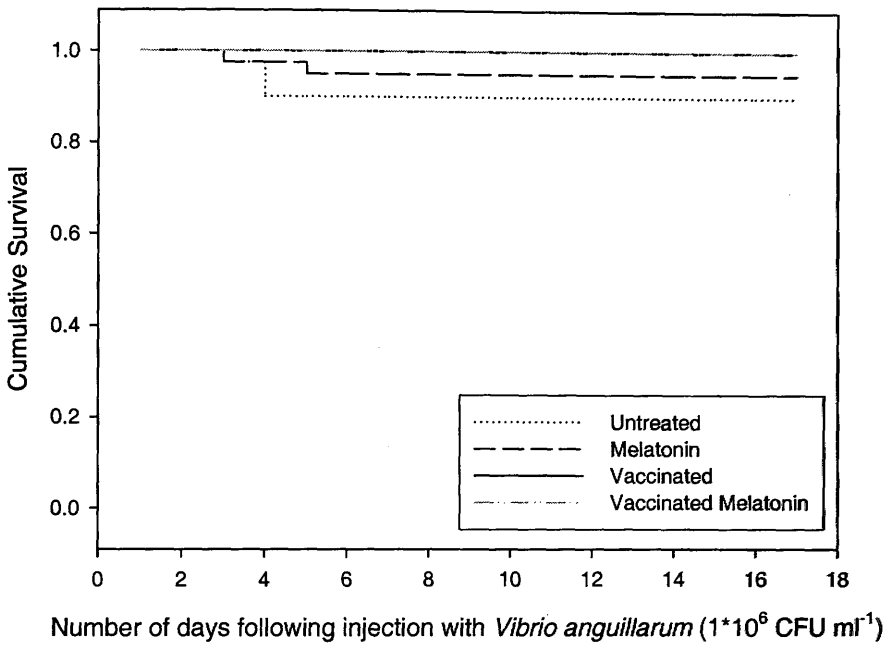


Fig. 5.19 Cumulative survival of fish from the four different treatment groups following artificial challenge with *V. anguillarum* in June (n=20/treatment).

October Challenge (Fig. 5.20)

A significant difference in survival following challenge was recorded between treatments (P=0.000). Pairwise comparisons revealed that both vaccinated and vaccinated/melatonin implanted fish had a significantly higher level of survival following challenge, compared to both untreated fish, and fish with a melatonin implant (P=0.000). Untreated fish had a median survival of day 5; melatonin implanted fish had a median survival of day 6. Fish from both of the vaccinated treatments had no mortalities, consequently no median of survival could be calculated.

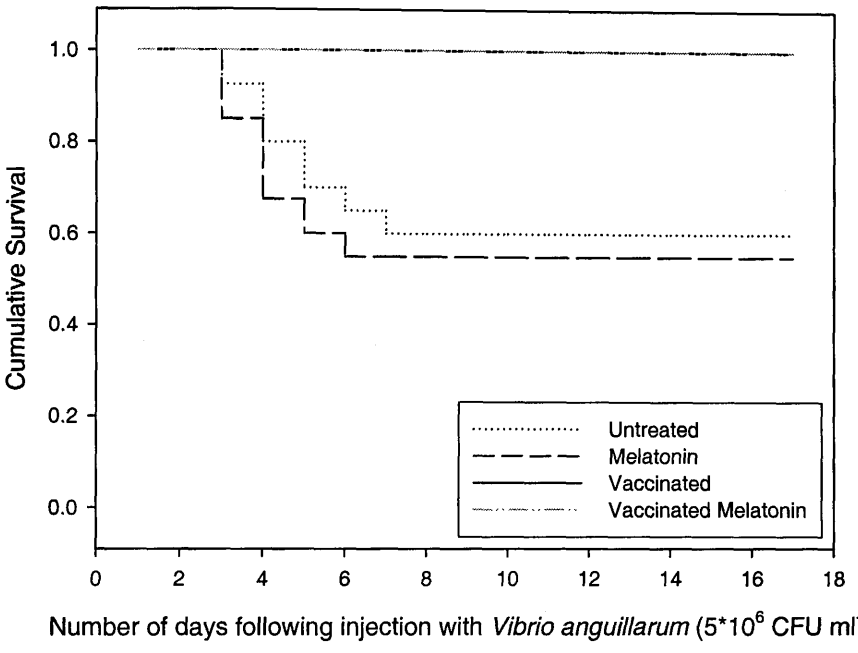


Fig. 5.20 Cumulative survival of fish from the four different treatment groups following artificial challenge with *V. anguillarum* in October (n=20/treatment).

5.5.3 Correlation Between the Immunity and Seasonal Cues

Pearson correlation coefficients were used to determine if the seasonal cues of photoperiod and temperature were correlated with any of the immune parameters studied (Table 5.10). The majority of cues were correlated with temperature rather than photoperiod. The immune parameters which exhibited the strongest positive seasonal correlations were plasma lysozyme activity, antibody titres to *V. anguillarum* (ELISA), and total white blood cell counts. Haematocrit values exhibited a strong negative seasonal correlation.

Table 5.10 Correlation coefficients between seasonal cues and immune parameters studied

	Hours of Daylight	Temperature	WBC	RBC	Lysozyme	Resp. Burst	Haematocrit	Ab Titre	PI	PR
Hours of Daylight	X									
Temperature	0.691 **P=0.000**	X								
WBC	0.492 **P=0.000**	0.583 **P=0.000**	X							
RBC	**-0.023** P=0.619	-0.279 **P=0.000**	0,050 P=0.275	X						
Lysozyme	0.266 **P=0.000**	0.673 **P=0.000**	0.363 **P=0.000**	-0.305 **P=0.000**	X					
Resp. Burst	0.366 **P=0.000**	0.215 **P=0.000**	0.336 **P=0.000**	0.117 **P=0.011**	0.134 **P=0.000**	X				
Haematocrit	-0.176 **P=0.000**	-0.488 **P=0.000**	-0.223 **P=0.000**	0.456 **P=0.000**	-0.441 **P=0.000**	-0.076 **P=0.000**	X			
Ab Titre	0.323 **P=0.000**	0.362 **P=0.000**	0.204 **P=0.000**	-0.069 **P=0.138**	0.252 **P=0.000**	0.191 **P=0.000**	-0.186 **P=0.000**	X		
PI	-0.122 **P=0.024**	-0.270 **P=0.000**	-0.187 **P=0.001**	0.023 P=0.676	-0.145 **P=0.008**	0.130 **P=0.017**	0.045 P=0.410	-0.016 P=0.779	X	
PR	-0.000 P=0.999	-0.195 **P=0.000**	-0.132 **P=0.015**	0.027 P=0.618	-0.122 **P=0.025**	0.161 **P=0.003**	0.072 P=0.187	0,034 P=0.541	0.916 **P=0.000**	X

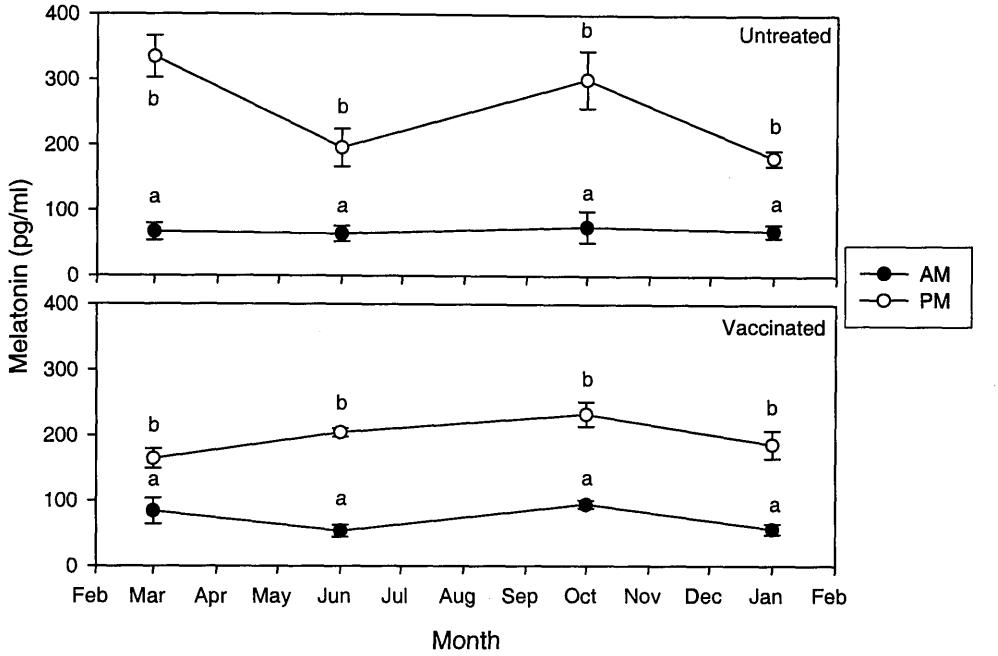
Cell Contents: Pearson correlation
P-Value

Table Abbreviations: WBC = Total white blood cell count; RBC = Red blood cell count; Resp. Burst = Respiratory burst of head kidney macrophages; Ab Titre = antibody titre to *V. anguillarum*; PI = Phagocytic index and PR = Phagocytic ratio

5.5.4 Measured Plasma Melatonin During the Trial Period

A significant difference in plasma melatonin levels was recorded between fish from the different treatment groups (P=0.000). Fish with a melatonin implant had significantly higher levels of plasma melatonin compared to those without (P=0.000). Fish without a melatonin implant exhibited clear differences in plasma melatonin levels by month and time of sampling (am/pm) (P=0.000) (Fig. 5.21a) (Table 5.11). Whereas treatments in which fish had a melatonin implant, no difference was recorded between samples taken during the day or night (P=0.641). Plasma melatonin levels were significantly higher in March than at any other sample point (P=0.000) (Fig. 5.21b).

a)



b)

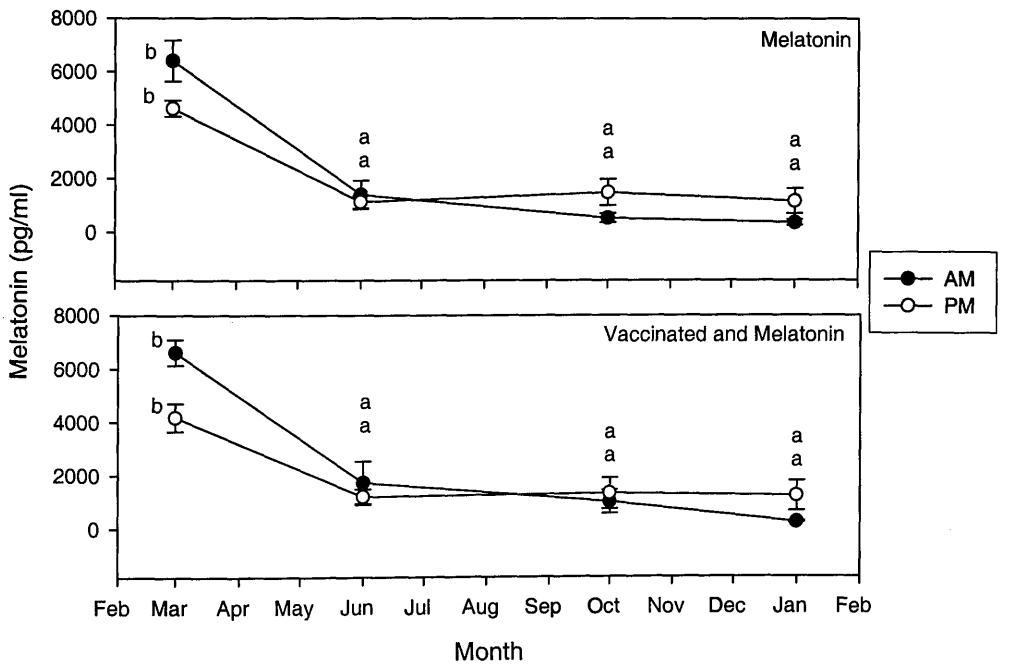


Fig. 5.21 Melatonin levels of treatment groups without melatonin implants (a) and with melatonin implants (b) measured at both AM and PM over the trial period (n=16; mean ± SE). Subscripts denote significance, 'a' is significantly lower than 'b'.

Table 5.11 Tukey pairwise comparisons between treatments (n=8; mean ± SE).

	Untreated	Melatonin	Vaccinated	Vaccinated & Melatonin
Untreated	**X**			
Melatonin	**P=0.000**	**X**		
Vaccinated	P>0.05	**P=0.000**	**X**	
Vaccinated & Melatonin	**P=0.000**	P>0.05	P>0.05	**X**

5.6 Discussion

One of the aims of this trial was to investigate melatonin administration as a method of alleviating the winter immunosuppression observed in the 'Base Level Trial' in Chapter 2. However, phagocytosis was one of only two parameters studied which were found to be significantly enhanced by the melatonin implant. The second parameter was challenge; fish from the June challenge with a melatonin implant survived longer compared to untreated fish. A possible explanation as to why so few parameters were enhanced by the melatonin may have been because the first sampling point did not take place until 6 weeks after administering the melatonin implants. Any resulting immunostimulatory effect by the melatonin implant may have only been for a very short period of time after implantation and as the fish may have quickly adapted, elicited by the implant. Thus an effect may have been missed by Week 6. Weber (1999), in a preliminary study carried out at the Institute of Aquaculture, University of Stirling, found that fish given a melatonin implant exhibited a higher, although not significant, level of survival following challenge with *V. anguillarum*. Weber also found significant differences in lysozyme activity and cytochrome C activity, with implanted fish exhibiting an increased level in these activities. This might be because the trial was shorter than the trial carried out here and sampling also took place four weeks after implantation rather than the six weeks performed here. Another reason why melatonin had no real effect on stimulating the immune parameters measured may have been the quantity of melatonin used. High doses of melatonin (~200mg/Kg) have been reported to suppress immunological responses (Maestroni, 1988). This information relates to mammalian studies, and no data is available for teleosts, but it is likely that melatonin levels above these physiological levels may suppress the immune response of fish. The

implants used in this trial were the only ones commercially available at the time of setting up the experiment and were originally developed to regulate reproduction in sheep. Obviously being designed for sheep, the implants are large and may have been inappropriate for this particular experiment. Since setting up this trial, technology has been developed that would allow the production of melatonin implants in the laboratory to the scientists specifications and these maybe more suitable for use in fish. It is recommended from this work that further trials examining the effect of melatonin on the immune response of fish use less than 18 mg melatonin.

The melatonin implants constantly released melatonin throughout the course of the 12 month trial. However, the amount of melatonin measured in March in implanted fish was significantly greater than at any other time of year. It can therefore be concluded that although the implants released melatonin constantly, it was not released uniformly. This was also observed by Taylor *et al.* (2004). However, this does not corroborate earlier work carried out by Porter (1996) who reported that plasma levels of melatonin in implanted fish did not decrease significantly over a twelve month period after implantation. This may have had a detrimental effect on the fish, and could account for the lack of any noticeable responses throughout the trial.

Melatonin implantation affected the appearance of the fish. An observation made during the trial was that melatonin implanted fish were much lighter in colour by the end of the sampling period, to the extent that it was possible to visually select a melatonin implanted fish just by its colour. A suggested reason for this marked colour change is the role melatonin plays in pigmentation i.e. melatonin is an important determinant of an individual's production of melanin (Constantinescu, 1995). Melatonin is a weak dose-independent lightening agonist in fish skin (Filadelfi and Castrucci,

1994). Furthermore, melatonin is successfully used as a treatment for *Acanthosis nigricans*. This is a disorder of hyperpigmentation (an increase of the dark pigment melanin) in the skin. Injections of melatonin are used to reverse the effects of this hyperpigmentation (Scott *et al.,* 1995). It is therefore not surprising that the implanted fish had become much lighter in colour.

Vaccination significantly increased survival to challenge and antibody titre to *V. anguillarum*. Fish which had a melatonin implant that were also vaccinated did not exhibit any significant enhancement in antibody titre to *V. anguillarum*. Secombes, in 1994, reported that vaccination stimulates respiratory burst activity, with peak activity being recorded 30 days post-injection. Unfortunately, immune activity was not measured until Week 6 of setting up the trial, and any significant effect of the melatonin implants on the innate immune response, may have been missed by this time.

It is interesting to note that in the final five months of the trial, with the exception of the last sample point in December, fish that were vaccinated (without a melatonin implant) were significantly larger than fish from the other treatment groups. This is the opposite of previously published data where it has been reported that vaccination has a negative effect on fish growth performance ($P<0.05$) (Kitlen *et al.,* 1997). However, the trial carried out by Kitlen was not as long as the trial described here. It is possible that if their trial had continued, the vaccinated fish may have exhibited increased growth.

The results of this trial produced further evidence that many of the immune and haematological parameters studied in rainbow trout are influenced by season. For example the strongest seasonal correlation was exhibited by plasma lysozyme activity, while the phagocytic ratio had the weakest seasonal correlation and was only correlated

with temperature not photoperiod. It was impossible to measure the effect of seasonality during the challenge as any seasonal difference that was observed may have been due to the quantities of bacteria injected into the fish at each challenge. The challenge procedure used in this investigation was intraperitoneal injection of the pathogen. This ensured that each fish received the same dose of bacteria. However, this method does not take into account how well a fish takes up a pathogen, as this too may be influenced by season. Therefore to examine this effect it is recommended that an immersion technique be used instead.

Haematocrit was the only parameter examined which exhibited a strong negative correlation with season, i.e. the highest level was observed during the winter months and the lowest during the summer. This data corroborates the work of North (2004), who reported similar results for both lysozyme activity and haematocrit levels.

The results of this trial suggest that over the course of a year some of the parameters examined may not have only one peak a year, but in fact have two or three peaks. This may contradict the statement that 'immune parameters are generally suppressed in winter and greatest in summer' (Nelson, 2004). For example, Phagocytic Index appeared to exhibit a biannual rhythm. The periods of greatest temperature change i.e. autumn and spring were the times when PI was greatest. However as sampling only took place every second month it is possible that the drop in PI observed in August was the result of a stress event or sampling error.

5.7 Conclusion

The results of this study confirmed the presence of seasonal patterns of immunity, as described in the 'Base Level Trial' (Chapter 2).

Melatonin has been shown to significantly enhance phagocytic activity however with all other parameters studied it did not appear to act as a significant immunostimulant. The reason why no other immune parameters were affected maybe because melatonin was administered at a non-physiological dose, and although it was not enough to induce an immunosuppressive effect, it may have counteracted any positive effect on the immune response of rainbow trout. Alternative methods and timing of melatonin administration need to be established, with a better control over the levels of melatonin released. There is certainly evidence to support the immunostimulatory effects of melatonin in previous studies (Akbulut *et al.*, 2001; Brennan *et al.*, 2002; Champney *et al.*, 1997). It is possible that any immunostimulatory effect occurred within the first couple of weeks of administration. As the first sample was not taken until Week 6 of the trial, any immunostimulatory effect of melatonin may have been missed. Melatonin was not found to enhance the effects of vaccination to *V. anguillarum*. Neither was vaccination found to enhance any of the innate immune parameters studied.

Chapter 6 Circadian Rhythms and Innate Immunity

6.1 Introduction

6.1.1 Circadian Rhythms

Circadian rhythms are endogenous or self sustaining rhythms with period lengths of approximately twenty-four hours (Nelson *et al.*, 2002). They are regulated by 'clocks' located in specific structures of the central nervous system, such as the suprachiasmatic nucleus (SCN) in mammals, and by peripheral oscillators present in other various tissues (Cermakian and Sassone-Corsi, 2002). Environmental cues such as light and temperature are thought to control these circadian rhythms and to be able to reset the daily phase of molecular rhythms, thus ensuring that the organisms behaviour remains tied to the environment (Cermakian and Sassone-Corsi, 2002).

6.1.2 Hormonal Entrainment of Circadian Rhythms

Melatonin is produced in a diurnal rhythm, with the majority of melatonin being produced during the dark phase of the day. Melatonin is thought to have an active involvement in the entrainment of circadian rhythms because of its diurnal pattern of production. This is corroborated by Maestroni and Conti (1989) who reported a circadian rhythm in analogue with the effects of melatonin. Melatonin plays an important role in the regulation of physiological functions in fish such as body colouration, seasonal reproduction and circadian locomotor activity. These actions are mediated via melatonin receptors (Eström and Meissl, 1997). Melatonin receptors are also subject to circadian rhythms. In goldfish, the density of melatonin binding sites exhibited day-night changes (Iigo *et al.*, 2003). In rainbow trout the greatest abundance or melatonin receptors occurs in the early afternoon. According to Futter (2003), this

occurred at 14:00 (under natural photoperiod) at the Niall Bromage Freshwater Research Facility (NBFRF). In addition to its role in the entrainment of seasonal rhythms, melatonin has also been demonstrated to act as an immunostimulant (Nelson and Drazen, 1999; Moore and Siopes, 2000). Furthermore, it has been hypothesized that a circadian rhythm of immune response may exist (Davies *et al.*, 2001), the circadian rhythm of melatonin may be part of the mechanism eliciting the potential circadian rhythm of immunity.

6.1.3 Endocrine Circadian Rhythms

Circadian rhythms are thought to be regulated by clocks located in specific structures of the central nervous system, such as the suprachiasmatic nucleus and by peripheral oscillators present in various other tissues. Recent discoveries have elucidated the control of central and peripheral clocks by environmental signals. The major synchroniser in animals is light (Cermakian and Sassone-Corsi, 2002). Several endocrine hormones have been investigated in teleost fish and found to exhibit a circadian rhythm.

Daily rhythms of circulating cortisol has been extensively studied in freshwater fish species (Boujard and Leatherland, 1992). Different rhythms in cortisol levels have been reported in different fish species. Peak values have been reported at night in carp and brown trout (Redgate, 1974; Rance *et al.*, 1982; Pickering and Pottinger, 1983). Whereas peak values during the day have been reported in gulf killifish and rainbow trout (Garcia and Meier, 1973; Boujard and Leatherland, 1992). Peak values during both day and night periods have also been reported in the goldfish and carp (Peter *et al.*, 1978; Kühn *et al.*, 1986).

Reproduction in fish is cyclical and timed to guarantee the survival of the offspring. (Bayarri *et al.*, 2004). Studies have investigated both seasonal and circadian rhythms of the endocrine hormones involved in reproduction. A study measuring cyclical patterns in reproductive hormone levels in sea bass observed that daily variations in plasma luteinizing hormone occurred with the highest levels being found in the dark phase, in fish held under natural conditions. This pattern was suppressed under a long photoperiodic regime (Bayarri *et al.*, 2004). Daily variations in pituitary gonadotrophin-releasing hormone of sea bass were also observed (Bayarri *et al.*, 2004). Cyprinid fishes have also been observed to exhibit a reproductive circadian rhythm and that the onset of the pre-ovulatory gonadotrophin hormone surge is determined by a photoperiodic cue (Aida, 1988). The synchrony of the gonadotrophin hormone surge is observed in both sexes and allows ovulation and milt preparation to occur at the same time, thus optimizing the chances of successful fertilization (Aida, 1988). Bayarri *et al.*, (2004), concluded the observed daily rhythms in luteninizing hormone and gonadotrophin releasing hormone, were co-ordinated by the hormone melatonin. Melatonin is thought to be of major importance for the transduction of photoperiodic information and the regulation of reproduction the reproduction in sea bass. They also hypothesized that the pineal might have a role in physiological performance and behavioural regulation to seasonally changing light–dark cycles.

6.1.4 Circadian Rhythms and Immunity

Virtually all immunological variables investigated to date in animals and humans display biological periodicity (Esquifino *et al.*, 2004). Circadian rhythmicity is revealed in circulating cells, lymphocyte metabolism and transformability, circulating hormones, phagocytic activity and other substances of the immune system, cytokines,

receptors, and adhesion molecules (Miyawaki *et al.,* 1984; Melchart *et al.,* 1992; Hriscu *et al.,* 1998; Petrovsky and Harrison, 1998; Esquifino *et al.,* 2004).

Human studies examining the effect of circadian rhythm on immunity have been carried out (Miyawaki *et al.,* 1984; Melchart *et al.,* 1992; Hriscu *et al.,* 1998; Petrovsky and Harrison, 1998; Esquifino *et al.,* 2004). The potential importance of circadian cycles with regard to the incidence of cancer has recently been brought to the publics' attention. Female night shift workers who have been exposed to light during the time of the usual peak in melatonin levels, may have elevated oestrogen production resulting in an increased incidence of breast cancer (Davies *et al.,* 2001). Furthermore, data from recent clinical studies have shown show that release from circadian regulation causes a dramatic acceleration in cancer progression (Sephton and Spiegel, 2003).

In humans there are physiological variations in the levels of leucocytes and lymphocyte subsets, among them the circadian rhythm is very important in terms of magnitude (Elmadjian *et al.*, 1946; Bertouch *et al*, 1983; Suzuki *et al.*, 1997). It has been observed that total white blood cell counts peak in the evening/night (Plytyzc and Seljelid, 1997; Suzuki *et al.*, 1997). All studied leucocytes have been observed to vary in number or proportion with a circadian rhythm and can be classified into two groups. Group one – granulocytes, macrophages, neutrophils, natural killer cells, extrathymic T cells, monocytes, lymphocytes, γδ T cells, and CD8+ subset exhibit an increase in the daytime (Suzuki *et al.*, 1997; Plytyzc and Seljelid, 1997; Smaaland, 1997). The other group T cells, B cells, αβ T cells and the CD4+ subset exhibit an increase at night. (Suzuki *et al.*, 1997; Plytyzc and Seljelid, 1997; Smaaland, 1997). This is further corroborated by Kronfol *et al* (1997), who reported in healthy humans, that the following immune measures exhibited a significant circadian rhythm; the percentages of

neutrophils, CD4+ cells, and CD56+ cells; the absolute number of total lymphocytes, CD3+ cells and CD8+ cells and Natural Killer cells. The study also indicated that there was a strong inverse correlation between the circadian rhythms of cortisol and the different T cell subsets. Furthermore, a strong direct correlation between the rhythms of cortisol and the percentage of CD56+ and NKCA were also reported.

Cytokine production in human whole blood exhibits a diurnal rhythm (Petrovsky and Harrison, 1998). Peak production of the pro-inflammatory cytokines IFN-gamma, TNF-alpha, IL-1 and IL-12 occurs during the night and early morning at a time when plasma cortisol is lowest (Petrovsky and Harrison, 1998). The finding of diurnal cytokine rhythms may be relevant to understanding why immuno-inflammatory disorders such as rheumatoid arthritis or asthma exhibit night-time or early morning exacerbations and to the optimisation of treatment for these disorders (Petrovsky and Harrison, 1998). It has also been reported that the numbers of circulating T cells, in particular CD4+ T cells peak during the early morning hours when plasma cortisol is low (Ritchie *et al.*, 1983; Abo *et al.*, 1981; Lévi *et al.*, 1988). These peaks in circulating lymphocytes and whole blood cytokine production have been found to be synchronous, this raises the possibility that diurnal variation in cytokine production is the consequence of variations in circulating cell numbers (Petrovsky *et al.*, 1998). Diurnal rhythmicity of cytokine production also has implications for the timing of blood samples drawn for diagnostic T-cell assays (Petrovsky and Harrison, 1998).

Diurnal variation of immune function is not restricted to humans but is also present in a wide range of species including mice, rats, birds and fish (Hriscu *et al.*, 1998; Loubaris *et al.*, 1983; Stinson *et al.*, 1980; Nevid and Meier, 1993; Petrovsky *et al.*, 1998). In nocturnal animals like rat and mouse, the number of total white blood

cells, lymphocytes (Th and B cells) peak during the resting period (Deprés-Brummer *et al.*, 1997; Griffin and Whitacre, 1991; Li *et al.*, 1999; McNulty *et al.*, 1990). However, Kurepa *et al.* (1992), reported the reverse of this rhythm as total T and Th cell percentages increased during the activity period rather than during periods of rest. Further examples of circadian rhythms of immunity in these animals include a circadian variation in phagocytosis by mouse blood neutrophils (Hriscu *et al.*, 1998). Total white blood cell counts have been reported to exhibit a predominant semi-circadian period in mice, regardless of gender (Weigl *et al.*, 2004). The migration of T-cells was studied in New Hampshire chickens over a 24 h period. It was reported that the migration of T cells at 2:00 a.m. was markedly lower than at other time periods (Stinson *et al.*, 1980).

In fish, the majority of work has focused on the circadian patterns for demand feeding (Sánchez–Vázquez *et al.*, 2000 and Chen and Tabata, 2002). However, the results of these studies suggest that it is the action of regular periodic feeding acting as the zeitgeber, rather than hormonal entrainment by melatonin. Furthermore, only one paper appears to have been published in reference to circadian rhythms and immunity in teleost fish (Nevid and Meier, 1993). In this paper a day-night rhythm of immune activity during scale allograft rejection in the gulf killifish was studied. Measuring melanophore breakdown as an indicator of immunity, it was reported that breakdown was two to three times greater during the dark than during the light.

6.1.5 Aim

The aim of this study was to determine if any innate immune parameters of rainbow trout exhibit a circadian rhythm under different photoperiod regimes and if the nature of these potential rhythms was endogenous.

6.2 Materials and Methods

6.2.1 Trial Set-up

The trials were carried out at the Aquatic Research Facility and fish maintenance took place as per the description in the Section 3.2.1. Fish were held in 4 tanks (1 m Ø x 1 m) with 25 fish per tank at the start of the experiment. All fish in the trial were female. This was to prevent precocious males with a compromised immune system from potentially skewing the results.

Due to limited tank space it was not possible to have replicate treatments. Consequently, the four different treatments performed were carried out singularly. The four treatments were 12:12 LD, 12:12 DL (reverse of the previous treatment), constant light and 1:23 LD. It was not possible to have a treatment of 24 h darkness because of restrictions in the Home Office Licence at this time. In the first tank held on the 12:12 LD photoperiod the lights came on at midnight and went off at midday. The second tank, held on the 12:12 DL photoperiod, was the reverse of this. In the tank held on the 1:23 LD photoperiod, the lights came on at midday and went off at 13:00.

The Aquatic Research Facility has a programmed photoperiod of 12:12 LD that cannot be overturned. For this reason the tank were lit with individual lamps under a black plastic cover to prevent any outside light shining through when the lamps were turned off (Fig. 6.1). Fish were acclimated to their respective photoperiods for two weeks prior to the commencing of the trial. All sampled fish were weighed at each sampling point, average weight was 106.45 g (SD = 23.89; SE = 11.11). Fish were sampled every six hours starting at 06:30 (Day 1) and finishing at 06:30 (Day 2) the

following morning. They were maintained under an ambient temperature (remained 10°C during the sampling period).

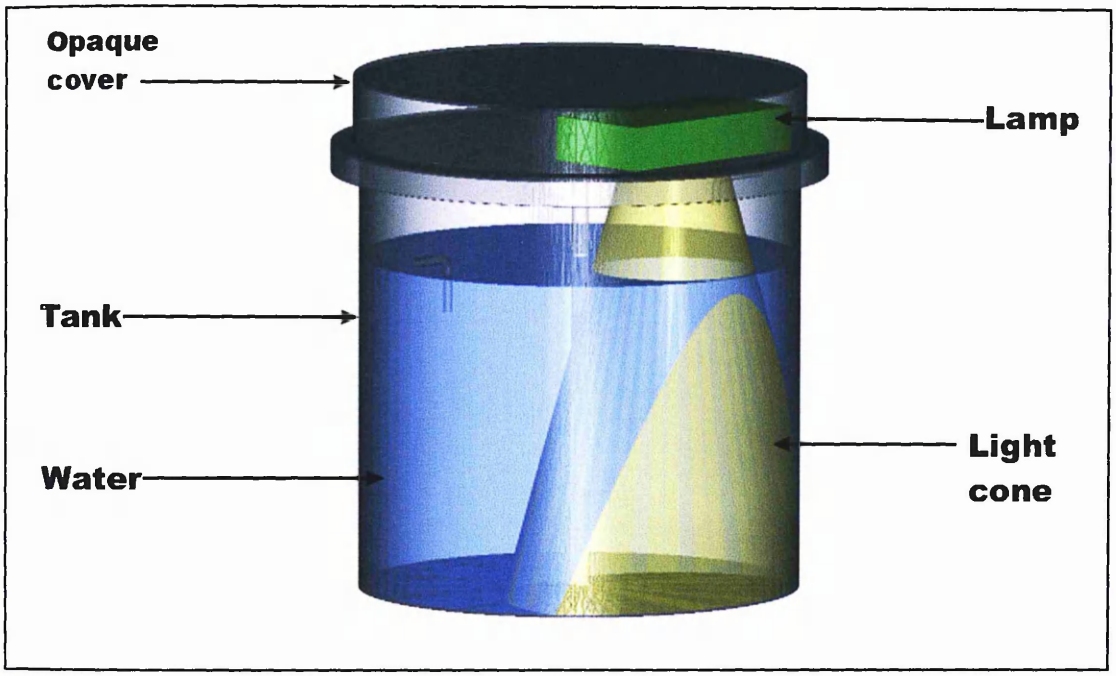


Fig. 6.1 Typical tank set up illustrating the cone of light produced by the lamp shining through the viewing panel.

6.2.2 Measurement of the Immune Response

Sampling took place every 6 h with five fish sampled per tank at each time point. As per the description in Section 2.2.2 fish lengths and weight were taken together along with blood samples. Both fish and blood were transported on ice back to the laboratory. Samples were kept in the fridge until assayed within 6 h of sampling. A variety of innate parameters were measured, methods for which are described in Sections 2.2.3 and 3.2.3. Macrophage respiratory burst activity and total white and red blood cell counts, haematocrit and plasma lysozyme activity were the parameters measured during the trial.

6.2.3 Measurement of Melatonin

Blood samples were taken at every sampling point using heparinised syringes and transported on ice to the laboratory. Plasma samples were collected and frozen at –70°C as soon as possible. Samples were analysed using a melatonin radio immunoassay (RIA) as described in Section 2.2.4.

6.2.4 Statistical Analysis

Before analysis data was tested for normality and homogeneity of variance using fits and residuals. All data was found to be normal and homogenous without transformation. Data was analysed using an ANOVA General Linear Model (Minitab). Tukey Pairwise Comparisons were carried out to further analyse data (Minitab).

6.3 Results

6.3.1 Plasma Melatonin Levels

Plasma melatonin levels were found to be significantly affected by light (Fig. 6.2) (P=0.03). However, fish held under constant light did exhibit significant differences in melatonin levels between the sampling periods, even though there was no dark phase (P=0.001). Using Tukey Pairwise comparisons it was determined that fish held under constant light had significantly higher levels of plasma melatonin at 06:30, on Day 1, than at any other point during the trial. No further significant differences were recorded between the later sampling periods.

Melatonin levels measured in fish held under the 1:23 LD photoperiod also exhibited significant differences over the 24 h period (P=0.000) (Fig. 6.2). Using Tukey Pairwise comparisons it was determined that higher plasma melatonin levels were observed at sampling point 1 (06:30, Day 1), 4 (00:30, Day 2 and 5 (06:30, Day 2) corresponding to dark periods compared to sampling point 2 (12:30, Day 1) performed during the light phase.

Melatonin production by fish held under the 12:12 LD photoperiod also exhibited significant differences over the 24 h period (P=0.019) (Fig. 6.2) with significantly higher plasma melatonin levels during the dark period (sampling point 3, 18:30, Day 1) than at sampling point 4 (00:30, Day 2), taken 30 min after lights were turned on.

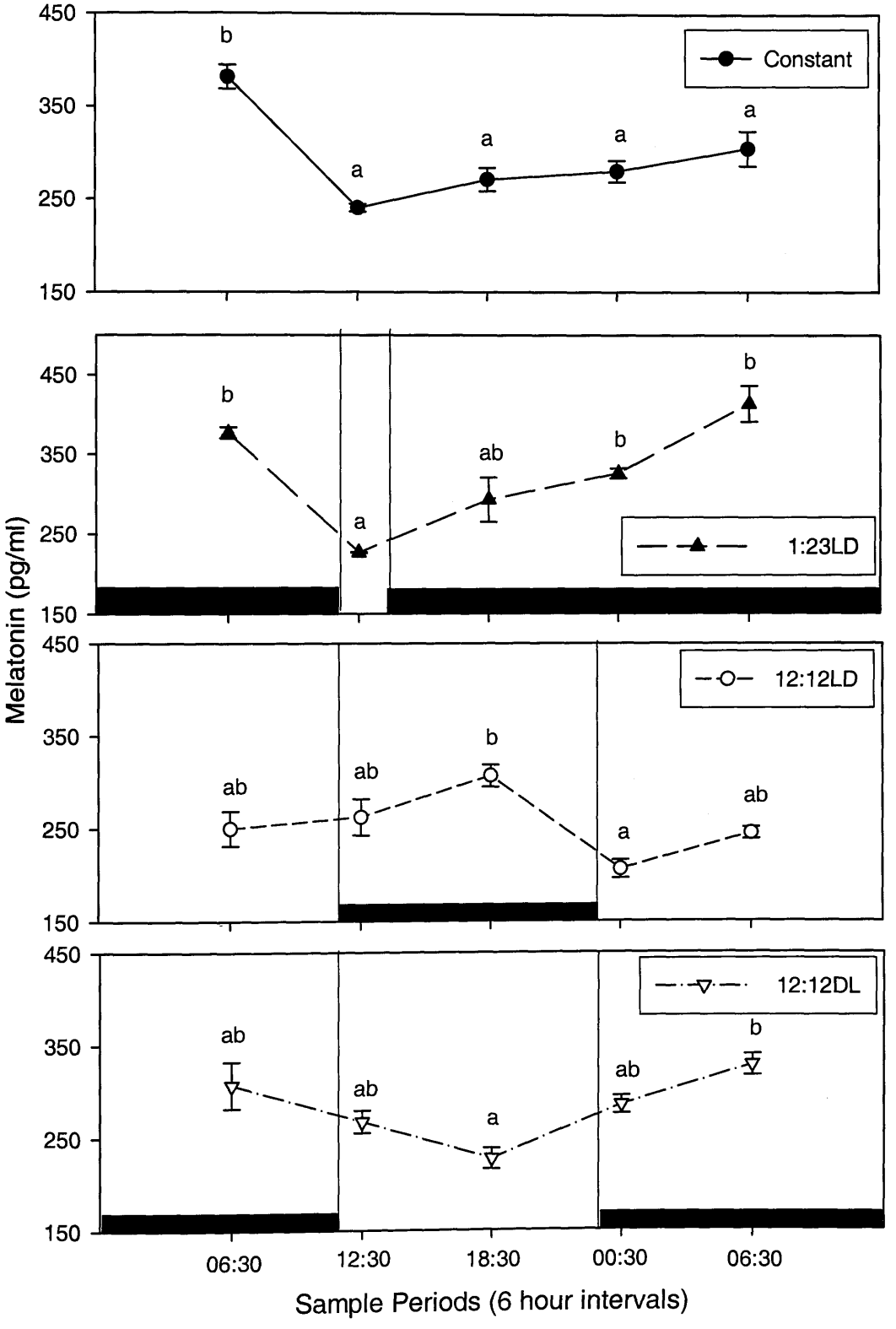


Fig. 6.2 Plasma melatonin levels over the 24 h sampling period for fish held under four different photoperiodic treatments (constant light, 12:12 LD, 12:12 DL and 1:23 LD) (n=5; mean ± SE). Subscripts denotes significance, 'b' is significantly greater than 'a'. 'ab' no significant difference from either 'a' or 'b'.

Melatonin production by fish held under the 12:12 DL photoperiod exhibited significant differences over the 24 h period (P=0.018) (Fig. 6.2). Using Tukey Pairwise comparisons it was determined that melatonin levels measured at 18:30, Day 1, 6.5 h into the light-phase was significantly lower (P=0.0143) than that at 06:30, Day 2, 6.5 h into the dark-phase.

6.3.2 Immune and Haematological Parameters

6.3.2.1 Total White Blood Cell Counts

In this trial the levels of white blood cell counts did not appear to exhibit a circadian rhythm (Fig. 6.3), and there was no significant difference between the four photoperiod treatments (P=0.143).

Fish held under constant light did exhibit a significant difference in white blood cell levels with sampling period (P=0.023). Using Tukey Pairwise comparisons it was determined that the results of the first four sampling periods were not significantly different from each other (P>0.05). However the final white blood cell count (06:30, Day 1) was significantly lower than at the first sampling point (06:30, Day 2) (P=0.0207).

White blood cell counts of fish held under the 1:23 LD photoperiod did significantly differ over the sampling period (P=0.006). However, this was not significantly related to whether the fish were sampled during the dark or light phase (P>0.05). Using Tukey Pairwise comparisons it was determined that the white blood cell counts of fish sampled at 00:30, Day 2 were significantly lower than those of fish sampled at 06:30, Day 1. At both sample points the tank was in darkness.

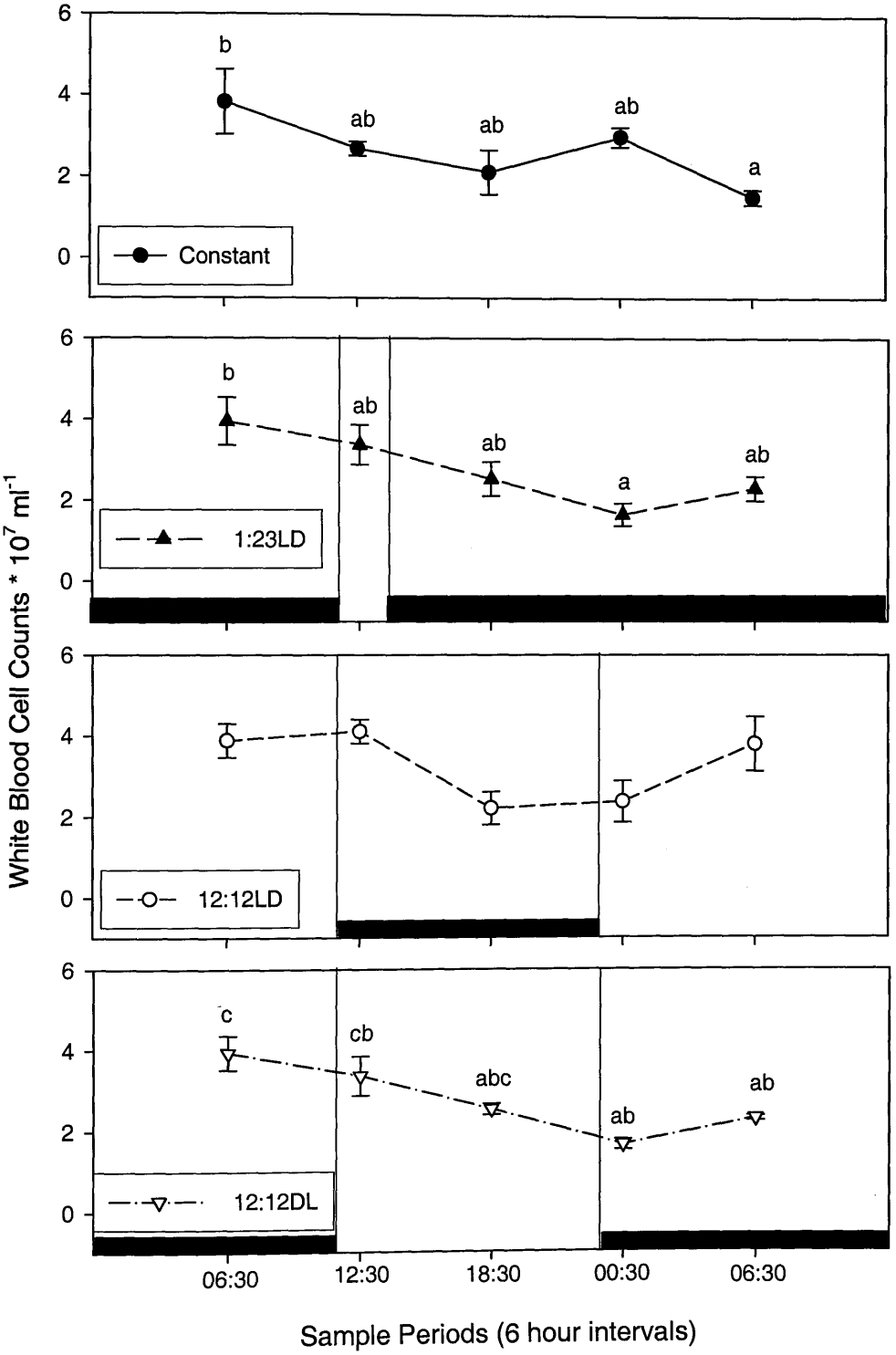


Fig. 6.3 Variations in white blood cell counts over a 24 h period for fish held under four different photoperiodic treatments (constant light, 12:12 LD, 12:12 DL and 1:23 LD) (n=5; mean ± SE). Subscripts denotes significance, 'c' is significantly greater than 'b', 'b' is significantly greater than 'a'. 'ab' no significant difference from either 'a' or 'b'. 'cb' no significant difference from either 'c' or 'b'. 'abc' no significant difference from either 'a', 'b' or 'c'.

White blood cell counts of fish held under the 12:12 LD photoperiod did not exhibit any significant differences between sampling periods (P=0.724). This was verified using Tukey Pairwise Comparisons. However in the reverse 12:12 DL treatment, significant differences were found in white blood cell counts between sampling periods. Tukey Pairwise Comparisons were used to determine when these differences occurred. At the first sampling (06:30, Day 1) when the lights were off the white blood cell counts were significantly higher than at all but the second and third sampling periods (12:30, Day 1; 18:30, Day 1) when the lights were on.

Significant differences were not reported in any of the treatments as a direct result of the lights being either on or off.

6.3.2.2 Total Red Blood Cell Counts

The levels of red blood cell counts in this trial did not appear to exhibit a circadian rhythm (Fig. 6.4) and there was no significant difference between the four treatments (P=0.699).

The red blood cell counts of fish held under the 12:12 LD photoperiod did exhibit a significant difference between sampling periods (P=0.05). However, no differences were observed in the remaining three treatments (Constant light P=0.102; 1:23 LD P=0.358 and 12:12 DL P=0.151). Tukey Pairwise Comparisons were used to determine where the significant difference under the 12:12 LD photoperiod occurred. It was found that the total red blood cell count measured at 00:30, Day 2 was significantly higher than at 06:30, Day 1.

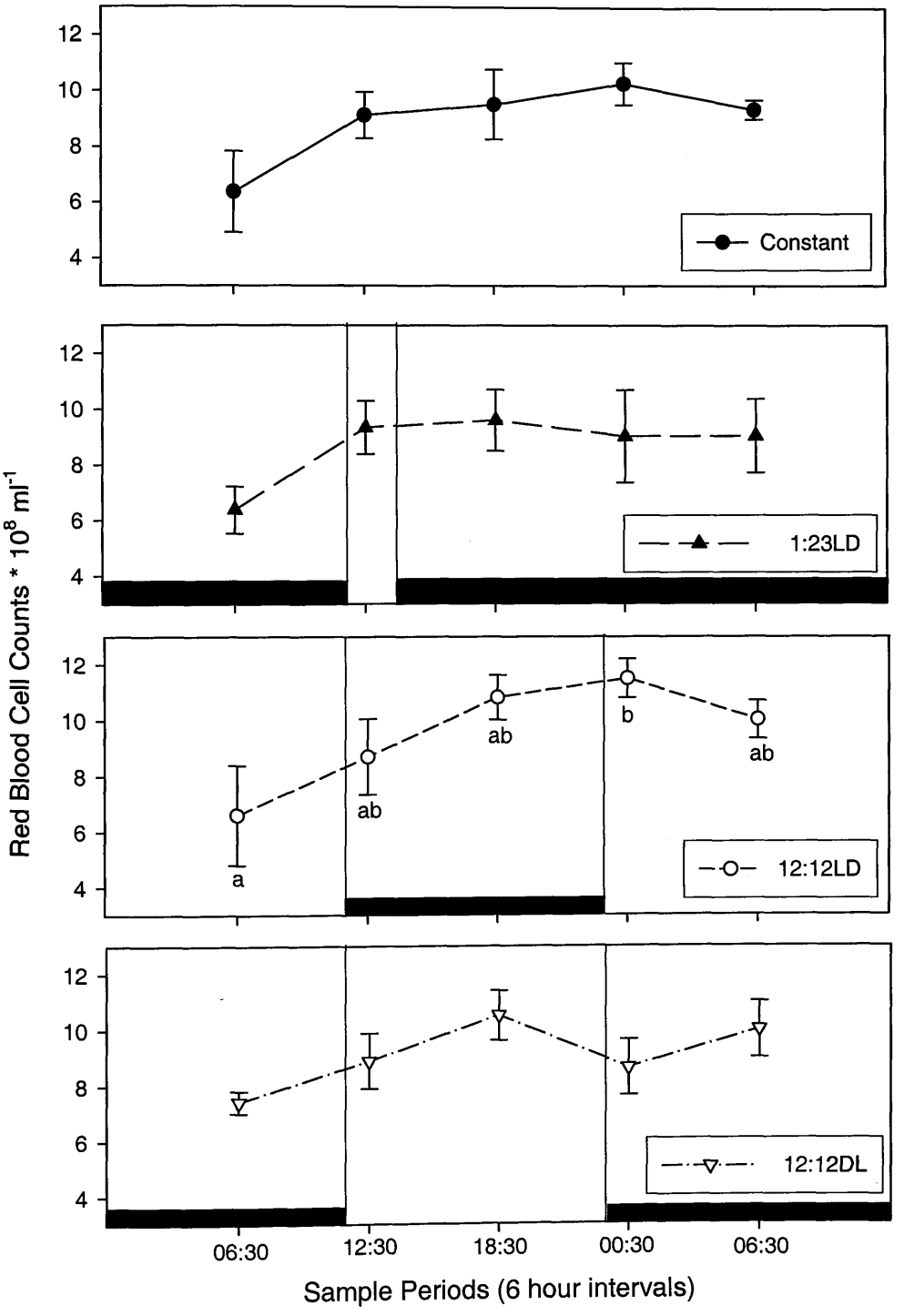


Fig. 6.4 Variations in red blood cell counts over a 24 h period for fish held under four different photoperiodic treatments (constant light, 12:12 LD, 12:12 DL and 1:23 LD) (n=5; mean ± SE). Subscripts denotes significance, 'b' is significantly greater than 'a'. 'ab' no significant difference from either 'a' or 'b'.

Significant differences were not reported in any of the treatments as a direct result of the lights being either on or off (P=0.699).

6.3.2.3 Haematocrit

The levels of haematocrit did not appear to exhibit a circadian rhythm (Fig. 6.5). There was no significant difference between the four treatments (P>0.05).

The haematocrit did not vary significantly throughout the 24 h period for fish held under constant light (P=0.297), 1:23 LD photoperiod (P=0.055), or 12:12 DL (P=0.129). However, significant variations were found in haematocrit levels during the 24 h sample period for fish held under the 12:12 LD photoperiod. Using Tukey Pairwise comparisons to determine where these variations occurred, it was found that haematocrit levels of fish taken at 12:30, Day 1 just after the lights were turned off, were significantly lower than at 18:30, Day 1 (P=0.0233), also during the dark phase, or at 06:30, Day 2 (P=0.03) during the light phase.

Significant differences were not reported in any of the treatments as a direct result of the lights being either on or off.

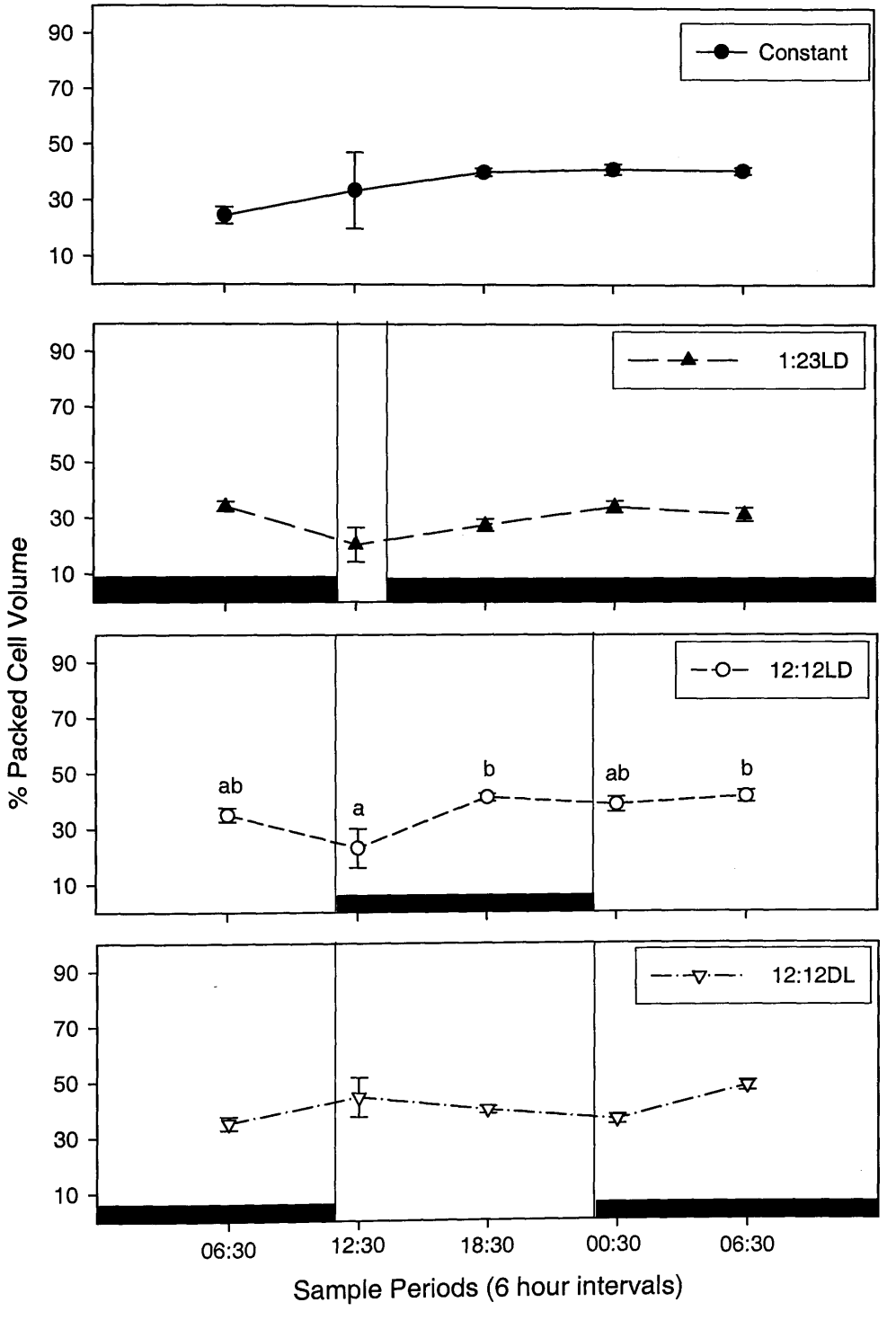


Fig. 6.5 Variations in haematocrit over a 24 h period for fish held under four different photoperiodic treatments (constant light, 12:12 LD, 12:12 DL and 1:23 LD) (n=5; mean ± SE). Subscripts denotes significance, 'b' is significantly greater than 'a'. 'ab' no significant difference from either 'a' or 'b'.

6.3.2.4 Plasma Lysozyme Activity

Plasma lysozyme activity did not appear to exhibit a circadian rhythm (Fig. 6.6), and no significant differences were observed between the four treatments (P=0.735).

Plasma lysozyme activity did not vary significantly throughout the 24 h period for three of the treatments, constant light (P=0.073), 1:23 LD photoperiod (P=0.1) and 12:12 DL (P=0.129). However, in the 12:12 LD treatment plasma lysozyme activity did vary significantly during the 24 h period (P=0.016). To determine where these significant changes took place Tukey Pairwise comparisons were performed. Plasma lysozyme activity measured at 06:30, Day 2 light phase, was significantly lower than that at 12:30, Day 1 (P=0.0365) and 18:30, Day 1 both samplings took place in the dark phase (P=0.0166). Significant differences were not reported in any of the treatments as a direct result of the lights being either on or off

6.3.2.5 Macrophage Respiratory Burst

Respiratory burst activity of head kidney macrophages, did not show a circadian rhythm in any of the photoperiod treatments studied (Fig. 6.7). There was no significant difference observed between treatments (P=0.0296). Neither were significant differences reported between fish sampled during the dark phase and those during the light phase. No significant differences in respiratory burst activity were observed during the 24 h period within any of photoperiodic treatments.

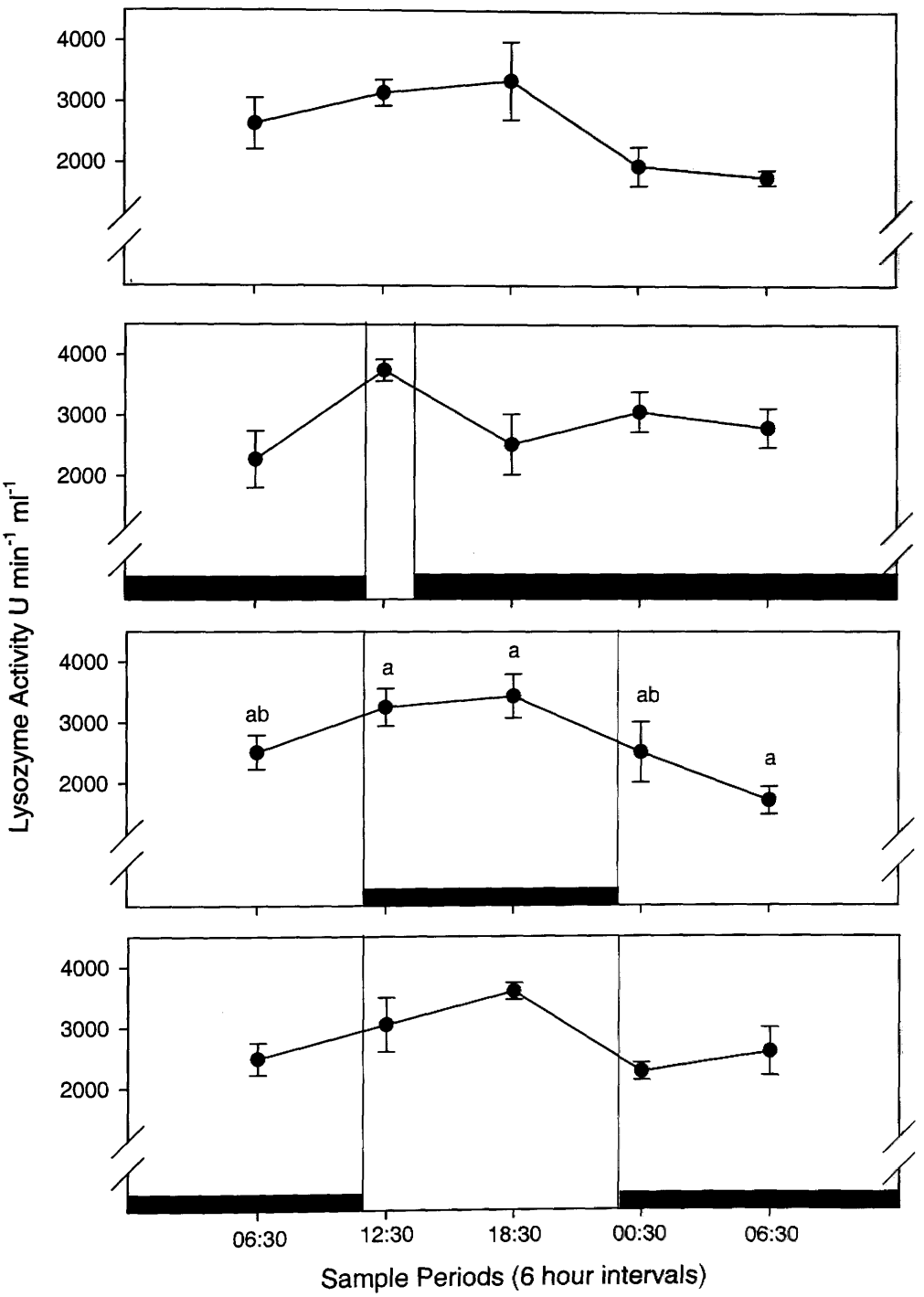


Fig. 6.6 Variations in plasma lysozyme activity over a 24 h period for fish held under four different photoperiodic treatments (constant light, 12:12 LD, 12:12 DL and 1:23 LD) (n=5; mean ± SE). Subscripts denotes significance, 'b' is significantly greater than 'a'. 'ab' no significant difference from either 'a' or 'b'.

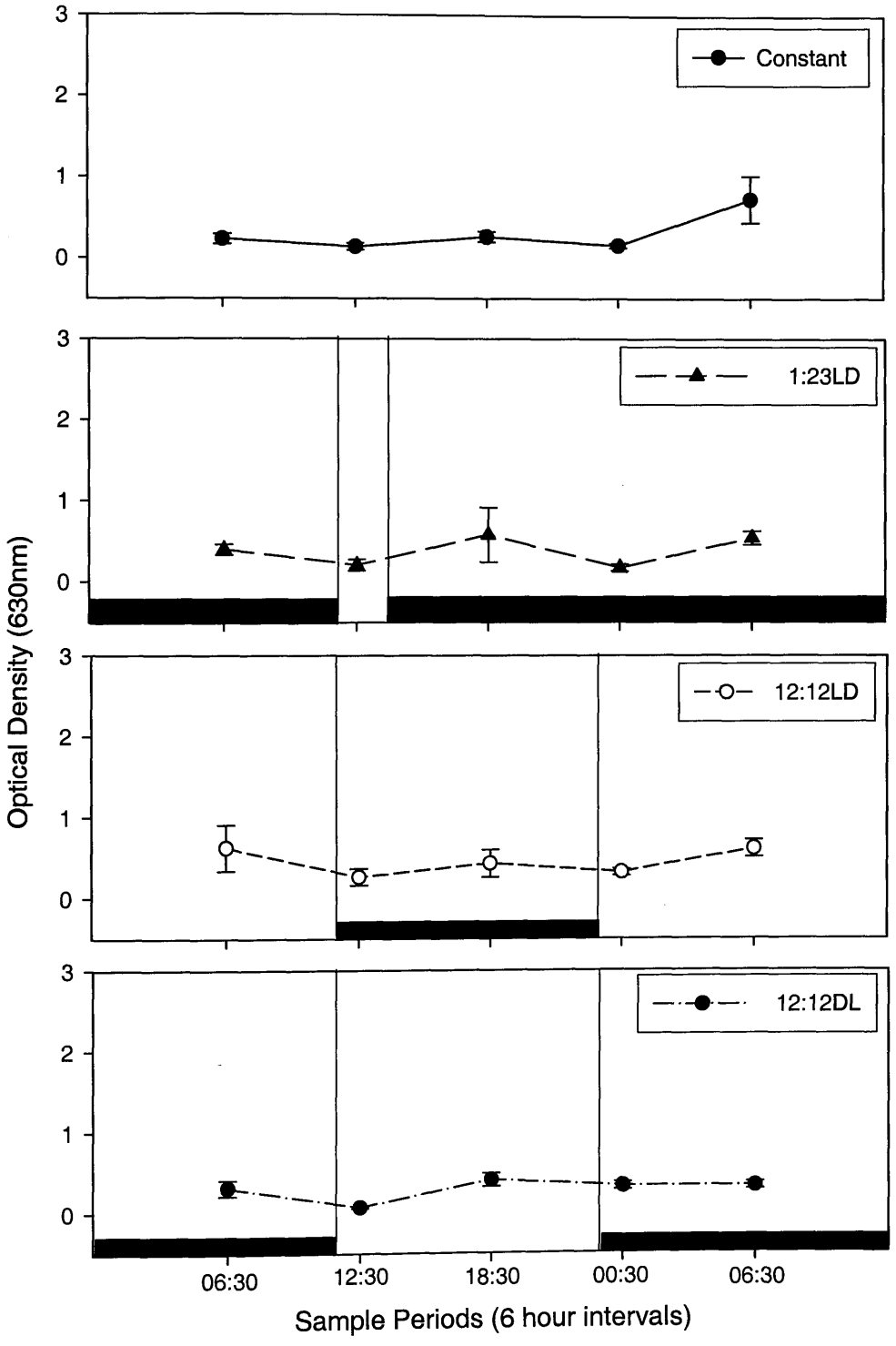


Fig. 6.7 Variations in macrophage respiratory burst over a 24 h period for fish held under four different photoperiodic treatments (constant light, 12:12 LD, 12:12 DL and 1:23 LD) (n=5; mean ± SE)

6.4 Discussion

In most non-mammalian species, the pineal gland serves as a direct photoreceptor, conveying ambient day length information directly to the brain (Nelson *et al.,* 2002). This is corroborated here with melatonin levels being significantly greater during the dark phase compared to the light phase (P=0.03). Previous studies have suggested that circadian rhythms are entrained by the hormone melatonin (Sephton and Spiegel, 2003). The circadian pattern of plasma melatonin was shown in the contrary 12:12 LD and 12:12 DL photoperiod regimes, consequently the patterns of melatonin are the reverse of each other (Fig. 6.2). This was corroborated further by the melatonin levels of fish in the 1:23 LD photoperiod, where melatonin levels were significantly greater during the dark phase. However, melatonin production in the constant light treatment did vary significantly during the 24 h trial even though no dark phase occurred. This was unexpected. Typically fish held under constant light exhibit a low level of plasma melatonin, possibly below the measurable limits of the assay. The lowest level of melatonin that can be measured using the melatonin radioimmunoassay is 3.9 pg ml^{-1} (Randall, 1992). Evidence of this is shown in the Photoperiod Trial of Chapter 4. Melatonin levels measured during the light phase of trial in Chapter 5 were never greater than 136 pg ml^{-1}, however melatonin levels measured during the light phase of any of the treatments in the trial described here never measured less than 205 pg ml^{-1}. This is a sizeable difference, and it is therefore suggested that the pineal gland was being stimulated into producing melatonin, during the light phase. The lighting design of this trial might explain these patterns. The set light regime of the ARF was a 12:12 LD photoperiod, supplied by overhead lighting. To enable the different photoperiods used in this trial, lights were placed above the observation ports of the

tanks. These were then covered with black polythene to prevent any light from the overhead lighting entering the tanks. The light produced by the lamps was restricted by the size and position of the observation port as described in the Materials and Methods (Section 7.2). This resulted in a non-homogenous diffusion of light throughout the water (Fig. 6.1) It is therefore suggested that the fish generally avoided the areas of brightest light, thus resulting in the unnatural melatonin production. This could have resulted in the unexpected melatonin production variation under the constant light regime, and the fact that during the light phase of the other treatments melatonin production was still comparatively high. Light avoidance has been reported in Atlantic salmon where there was a negative correlation between light level and fish density at the surface (Fernö *et al.*, 1995). This behaviour could be attributed to a predator avoidance behaviour i.e. it would be easier for a predator to catch the fish if they were illuminated rather than hidden in the shadows (Fraser and Metcalfe, 1997). However, Migaud *et al.* (2004), have reported that fish are very light sensitive, and even very low light intensities such as 0.5 watts m^{-2} suppress melatonin production.

The trial was designed to examine the possibility of circadian patterns in immune parameter activity. The fact that melatonin was still being produced at an unnaturally high level during the light phase may have affected any circadian rhythms of immune parameter activity, possibly masking them or even over-riding them completely. This may be the reason why the immune parameters studied did not exhibit any obvious circadian rhythms.

Total white blood cell counts were not observed to follow a circadian rhythm. This is converse to previous trials in mice and humans where such rhythms have been reported. It is suggested that the unnaturally high level of melatonin during the light-

phase my have overridden any circadian rhythm present in this parameter (Plytyzc and Seljelid, 1997; Smaaland, 1997; Weigl *et al.*, 2004).

Similar patterns in red blood cell levels were observed for all four treatments. The lowest number of red blood cells was recorded at the first sampling (06:30, Day 1). This was followed by a rise in red blood cell number for the at least the following two sample points. By the fifth and final sampling point (06:30, Day 2), red blood cell counts were lower than those measured at 12: 30.

It is interesting to note that for the haematological parameter, haematocrit the lowest levels were recorded during the dark phase. If melatonin production has been "more natural" i.e. if lower levels of melatonin had been recorded during the light phase, it is possible that the lower levels of haematocrit recorded during the dark phase may have been significant. Likewise, other less obvious circadian rhythms may also have been revealed.

Significant differences in lysozyme activity were only observed in the 12:12 LD photoperiod treatment. However, regardless of treatment similar patterns of lysozyme activity were observed in each of the photoperiodic treatments. In all of the treatments the second sampling (12:30, Day 1) was always higher than at either of the 06:30 samplings.

Respiratory burst activity of head kidney macrophages did not significantly change throughout the 24 h trial period or between treatments. However, within each photoperiodic treatment a similar pattern of peaks and troughs were observed for respiratory burst activity. Furthermore, for each treatment these peaks and troughs occurred at the same time e.g. respiratory burst activity was always lower at the second

(12:30, Day 1) sampling and always higher at the third sampling (18:30, Day 1), for all treatments.

It maybe that these patterns observed for the parameters measured maybe a response to stress, the action of sampling may have stimulated the fish to produce cortisol. Cortisol is a stress hormone and is known to affect immunity in fish (Esteban *et al.,* 2004). For example, it has been reported to play an important role in the down-regulation of phagocytic but not of cytotoxic activity in seabream leucocytes (Esteban *et al.,* 2004). Furthermore, haematological parameters have also been shown to be affected by cortisol. For example, in the jundiá fish, although red and white blood cell and haematocrit levels were not immediately affected by an acute stressor, after 10 days of chronic stimulation, all values changed, with a significant decrease observed in lymphocytes, eosinophils, monocytes and special granulocyte cells, as well in red blood cell and haematocrit levels (Barcellos *et al.,* 2004). It is therefore possible that the cortisol induced by sampling may have over-ridden any circadian rhythm in the parameters studied.

It has been reported that the action of regular periodic feeding, acts as the zeitgeber, rather than hormonal entrainment by melatonin (Sánchez–Vázquez *et al.,* 2000; Chen and Tabata, 2002). This offers a further explanation as to why no circadian rhythms were observed in the parameters studied as during the trial the fish were not fed and had been starved for a day prior to sampling. This was to aid the anaesthesia procedure and it is also easier to collect head kidney macrophages as there is no risk of contamination with uneaten food. However, it is suggested that in future trials periodic feeding be incorporated, so as to investigate if it affects levels of the parameters studied here.

There is a variety of studies examining circadian rhythms and immunity. They provide evidence for rhythms in a variety of immunological parameters including total white blood cell counts, phagocytosis and cytokine production (Weigl *et al.,* 2004; Plytyzc and Seljelid, 1997; Smaaland, 1997; Hriscu *et al.,* 1998; Petrovsky *et al.,* 1998). However, a possible explanation for the lack of circadian rhythms recorded in this trial may be simply due to the fact that the 'wrong' immune parameters were studied. Future studies could investigate parameters such as cytokine production and phagocytosis which have already been shown to exhibit a circadian rhythm in mammals (Hriscu *et al.,* 1998; Petrovsky *et al.,* 1998).

Finally the possible existence of diurnal rhythmicity of immune function suggests that the nature of an immune response, for example in response to vaccination, may be modified by the time of day of antigen administration and raises the possibility that immune responses could be therapeutically manipulated by co-administration of immuno-regulatory hormones such as glucocorticoids (Petrovsky and Harrison, 1998).

6.5 Conclusion

The results of this trial are obviously inconclusive, because, without a typical diurnal rhythm of melatonin production it is unlikely that a circadian rhythm of immune parameters would occur. Consequently, this work must be considered as a preliminary trial and there is obviously a great need for it to be repeated. The first obvious change would be to ensure an equal spread of light across the tank. This could be achieved with either a lamp secured under the lid or illumination from the bottom of the tanks. It may be advantageous to ensure that sampling took place at least an hour after the lights either came on or were switched off. This would give the system producing melatonin to respond accordingly and have the potential to entrain and circadian rhythm of the immune parameters. It would also have been beneficial to run the trial over a 48 h period to examine if any circadian rhythm observed in the first 24 h was repeated, however, this is physically difficult to perform and would therefore have to be carried out on two separate and distinct days.

Chapter 7 General Discussion

The title of this thesis is "The Effect of Seasonality on the Immune Response of Rainbow Trout", which reflects the primary aim of the project. The idea for this follows Nelson's hypothesis (2004) "that some individuals have evolved mechanisms to predict seasonal stressor-induced reductions in immune function, and make appropriate adjustments in anticipation of challenging conditions, as a temporal adaptation to promote survival". The experimental trials in this thesis were aimed firstly at determining if the immune response of rainbow trout is influenced by season (Chapter 2) and if so to determine which environmental cues i.e. temperature (Chapter 3) or photoperiod (Chapter 4) are used to detect changes in season, thus allowing the animal to anticipate the challenging conditions associated with season. Methods of alleviating seasonal immunosuppression in rainbow trout were also investigated (Chapter 5). Finally, the presence of circadian rhythms within the immune response of rainbow trout were examined (Chapter 6).

The results of the initial investigation into the presence of seasonal patterns in immunity corroborated earlier work, primarily carried out in mammals and birds (Nelson *et al.*, 2002). Generally, in mammals immune response is highest in summer and depressed during the winter months (Nelson *et al.*, 2002). It was originally thought that adaptive immunity in fish was seasonal whereas innate immunity remained at a constant background level throughout the year (Ellis, 2001). However, the results of the "Base Level Trial" (Chapter 2) suggest that innate immunity is also influenced by season and exhibits a similar seasonal pattern to that of the adaptive immune response.

The two principle components of seasonality were investigated to determine which was the primary cue used by rainbow trout to convey seasonal information to the fish's immune system. Photoperiod was found to have little or no effect on innate

immunity (Chapter 4). Photoperiod is used widely in the aquaculture industry to manipulate fish life history (Berrill *et al.,* 2003). For example, constant light has been used to significantly increase the weight of rainbow trout (Taylor, 2004). Photoperiodic regime has also been shown to have a primary effect on altering the timing of maturation in rainbow trout, irrespective of the prevailing water temperature in rainbow trout (Davies and Bromage, 2002). Knowledge of whether the photoperiodic regimes used in the aquaculture industry have a negative or even a positive effect on the fish immune response is of great importance, especially with regard to fish welfare. Challenge with *Vibrio anguillarum*, was found to be significantly affected by photoperiod. During the winter challenge, fish held under constant light exhibited a significantly higher level of survival compared to fish maintained originally under a short day photoperiod. The reverse of this situation was observed during the summer challenge. The method of challenge required fish to be moved from their original photoperiodic treatments to a 12:12 LD photoperiod. Challenge was carried out one week after the fish were moved to their new photoperiod regimes. This could therefore be the reason for the observed photoperiod effect, particularly as challenge took place relatively quickly after starting the new photoperiod regimes. Sprague* (pers. comm., 2004), reported that cortisol levels are significantly increased when changing to a constant light photoperiod from a natural situation. This increase in cortisol may have affected the immune system (Slater and Shreck, 1993; Slater *et al.,* 1995; Hassig *et al.,* 1996), and consequently caused the observed significant results in the challenges.

Temperature as a seasonal cue was investigated in Chapter 3. Most fish species cannot regulate their internal temperature (Baras, 1995). Consequently environmental

* Sprague, M. (2004). Institute of Aquaculture, University of Stirling

temperature has a direct affect on fish life history. The susceptibility of fish to disease is partly dependent on their environment. This is a result of the close relationship that exists between teleost fish and their surroundings (Le Morvan *et al.*, 1998). Since fish are poikilothermic, the environmental temperature influences all aspects of their physiology, including their immune response (Collazos *et al.*, 1994a). The results of the temperature trials in Chapter 3 indicated that temperature affected the majority of the parameters studied. For example, in Trial A the effect of three treatment temperatures (5, 10 and 18°C) on innate immune and haematological parameters was investigated. Total white and blood cell counts were found to increase with increasing temperature. Temperature was also found to affect lysozyme activity. In Trial B, where the affect of acclimating fish from three treatment temperatures to 15°C was investigated, it was found that temperature again affected total red and white blood cell counts although no significant affect on plasma lysozyme activity was recorded. Neither haematocrit nor respiratory burst activity were found to be significantly affected by temperature. The affect of temperature on total white and blood cell counts corroborate the findings of several other studies, in which it was reported that lower temperature elicits a lower immune response in mammals (Nelson, 2004) and fish (Slater and Shreck, 1993). Survival to challenge was also found to be significantly affected by temperature. Prior to challenge, fish were acclimated to a challenge temperature of 15°C from the original treatment temperatures of 5, 10 and 18°C. It was observed that the lower the original temperature, the higher the level of survival following challenge, regardless of season. It is suspected that the action of raising the water temperature from 5 to 15°C, stimulated the immune system, priming it and increasing the fish immune defences, consequently when the challenge took place, these fish were best equipped to survive.

Photoperiod as a seasonal cue for the immune response was investigated in Chapter 4. To date, few studies have been carried out in which the effect of photoperiod on the immune response of fish has been investigated. Olsen *et al.* (1993), hypothesised that cortisol levels during the smolting period of Atlantic salmon are probably controlled more by photoperiod than by endogenous rhythms. This is corroborated by Sprague* (pers. comm., 2004) who reported significantly higher cortisol levels in Atlantic salmon moved from a natural photoperiod to constant light. In Sprague's preliminary study, changing fish from a natural photoperiod to one of constant light resulted in significantly increased plasma cortisol levels. These did not return to the level observed before the change in photoperiod for up to 8 weeks after the photoperiod manipulation. Although, this was only a fairly short term effect, it is possible that the increased cortisol levels may have affected the fish immune system. It is generally accepted that an increase in plasma cortisol is associated with stress (Benfey and Biron, 2000), and increased cortisol levels have been reported to have an immunosuppressive effect in fish (Slater and Shreck, 1993; Slater *et al.,* 1995; Hassig *et al.,* 1996). If the act of changing the photoperiod regime did increase cortisol levels and affect the immune response, the fact that such an affect appears to be short term would mean that in the trials investigating photoperiod (Chapter 4) such an effect could have been missed because sampling did not take place until the fish had been acclimatized to their respective photoperiod treatments (constant light, 18:6 LD, 6:18 LD) for four weeks (with the exception of challenge, where fish were moved to a new photoperiodic regime from the original treatments and challenged after one week).

* Sprague, M. (2004). Institute of Aquaculture, University of Stirling

The results of the investigation discussed in Chapter 4 suggest that the photoperiod is not the principle cue for seasonality for the innate immune and haematological parameters studied i.e. total blood cell counts, respiratory burst, lysozyme and resistance to challenge. However, other parts of the immune system in mammals have been observed to utilise photoperiod as a principle cue e.g. T-Cell populations (Leonardi and Klempau, 2003). It has been hypothesised that these changes in immunity are a direct response to increased cortisol level rather than photoperiod (pers. comm., Ellis*, 2004).

In the natural environment an animal is not only exposed to differing seasonal changes in daylength (as was studied in Chapter 4), but it is also subject to changes in light intensity and wavelength (Fig. 7.1, Fig. 7.2). To date, there are no known studies investigating the effect of wavelength on immunity of fish, but several studies have been carried out investigating the effect of lighting and health of poultry. It has been found that increased light intensity causes stress which results in acts of aggressive behaviour, which in turn leads to the birds being more susceptible to disease from open wounds which result (Sherwin *et al.,* 1999; Pötzsch *et al.,* 2001). Few light intensity studies have been carried out in fish. However, as in the studies on poultry, light intensity has been linked to aggression in African catfish. Fish kept at a high light intensity were observed to suffer 2.46 times as many scars and wounds compared to those kept at low light intensities (Almazán-Rueda *et al.,* 2004). Open wounds are obviously susceptible to infection, increasing the risk of disease. Studies investigating

* Ellis, A.E. (2004). FRS Marine Laboratory, Victoria Road, Aberdeen

the effect of light intensity on feeding, particularly in marine fish larvae have reported that feeding incidence increases with light intensity (Puvanendran and Brown, 2002).

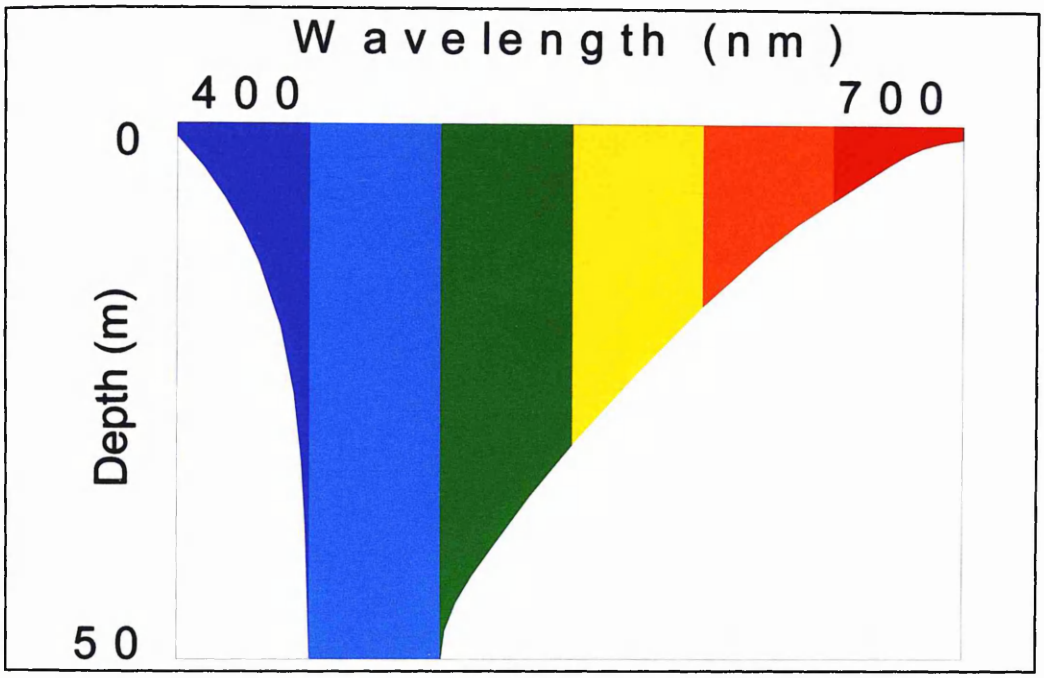


Fig. 7.1 Penetration through water of light from the visible spectrum by depth.

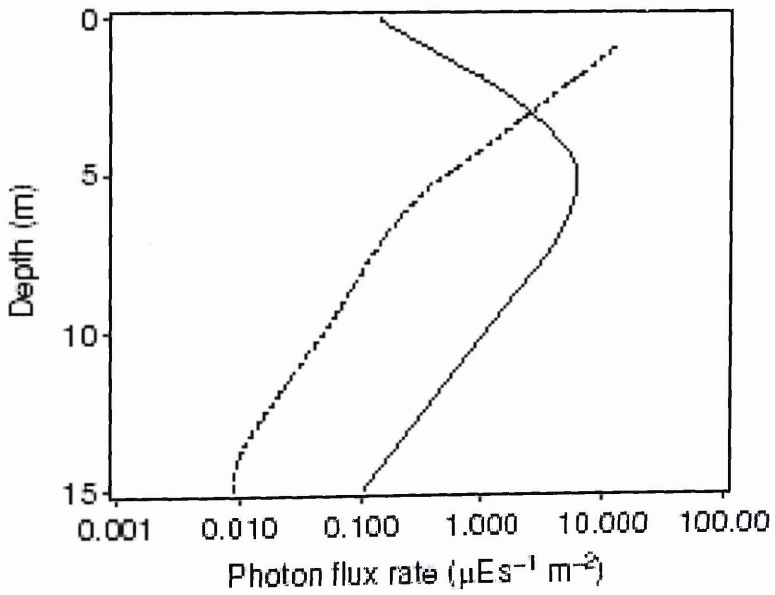


Fig. 7.2 Light intensity decreases with increasing depth. Graph illustrates vertical light intensity gradients with lamps submerged to 3 and 6m depth (solid lines) or lamps mounted above surface (dotted lines) (adapted from Juell *et al.*, 2003).

Seasonal differences were observed in immune activity even when photoperiod and water temperature remained constant (12:12 LD) (Chapter 4). An example of this is the respiratory burst activity of head kidney macrophages. During the first temperature trial (Chapter 3, Trial A), no significant effect of temperature was recorded within either the winter or summer trials i.e. as a result of treatment. However, respiratory burst activity was significantly greater between summer and winter samplings, irrespective of treatment. However, this was not corroborated by the results of the 'Base Level Trial' (Chapter 2) and only partially by the trial in Chapter 5. Lysozyme activity appears to be strongly correlated with season according to the results obtained from the 12-month trials in Chapters 2 and 5 and the photoperiod trial of Chapter 4 (seasonal effect was observed but this was not due to photoperiod). However, when investigated under a controlled temperature and photoperiod (Chapter 3), this influence of season on lysozyme activity is not observed. This is somewhat corroborated by the results of Bowden *et al.* (2004), who reported that serum lysozyme activity in halibut was not significantly affected by either temperature or photoperiod however a seasonal influence was observed. It was suggested that manipulated independently, photoperiod and temperature are not capable of mimicking the influence of season on lysozyme activity and that other unknown factors may be involved. This suggests the presence of an endogenous rhythm (Zapata *et al.*, 1992), which corroborates the results of earlier studies in which, continued seasonal variations in immune response were demonstrated even when both temperature and photoperiod have been held constant (Leceta and Zapata, 1986; Zapata *et al.*, 1983).

The seasonal patterns in immunity measured during this thesis varied between the different trials. For example in the 'Base Level Trial (Chapter 2) the highest total white blood cell counts were recorded in June (2003), however in the trial of Chapter 5,

the highest total white blood cell counts were recorded in October (2002) and August (2003). Although, in both of these trials the lowest total white blood cell counts were recorded in February (2003). Several explanations for these differences are suggested. The first is that temperature profiles are not exactly the same every year and variations do take place. This could have had a direct effect on the immune parameters studied. Secondly although the fish within trials were from the same stock, the fish used at NBFRF had a different origin to those used at the ARF on campus. Thirdly, all trials used female fish with the exception of the trial described in Chapter 5. The differences observed between the seasonal patterns of the parameters measured between trials may also be due to the fact that male fish were present in the Chapter 5 trial whereas in all other trials only female fish were used. Alternatively, the fact that sampling took place every other month could mean that fluctuations were missed compared to the 'Base Level Trial' where sampling took place every month.

The primary aim of this project, to investigate the presence of seasonal patterns in immunity has been achieved. Furthermore, evidence from this project suggests that the proximate environmental cue used by rainbow trout to determine a change in season for some of the parameters studied is temperature rather than photoperiod. Generally, immunity in rainbow trout is at its peak during the summer months and at its lowest during the winter. This leads to the question, why are there seasonal patterns in immunity? If immunity was high throughout the year, wouldn't this be more beneficial in promoting the health of the fish?

Why would it be beneficial to rainbow trout to have a depressed immune system in winter? Immune function is one of several competing life history functions that require substantial metabolic energy (Fig. 7.3). Nelson *et al.* (2002), proposed that when

demands are higher than the immediately available energy, individuals engage in trade-offs among various energy demands to reduce total energy needs. Winter is energetically the most demanding of the seasons. It is a period of reduced activity and foraging (Valdimarsson *et al.*, 1997). This is because the energy required to collect food

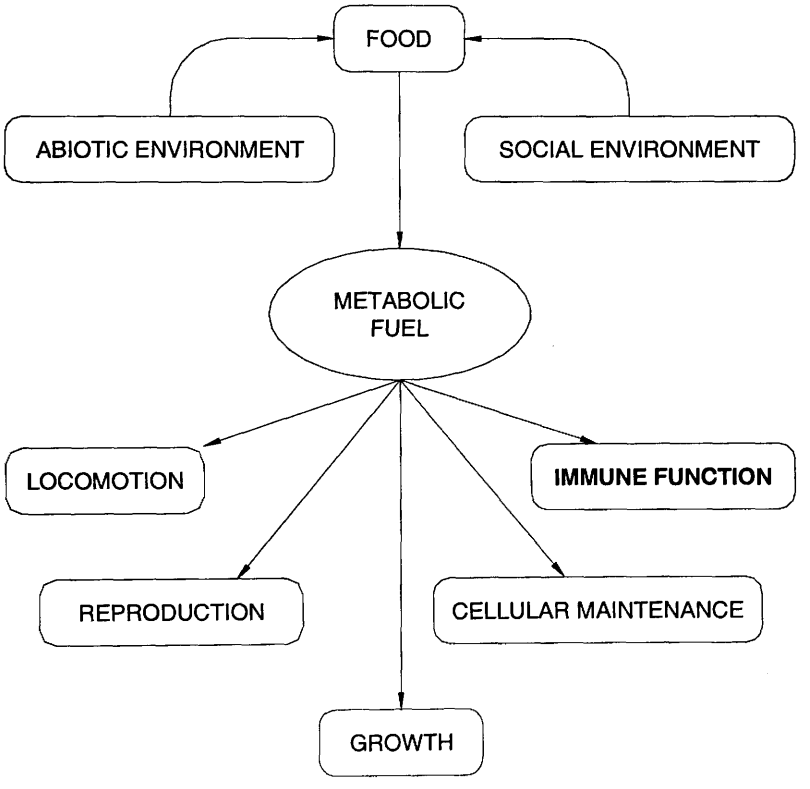


Fig. 7.3 Illustration of competing life history functions that require substantial metabolic energy (adapted from Nelson, 2004).

is greater than the energy gained from the food. The colder water temperatures of winter require fish to expend more energy in activities than they would in the warmer summer months. This is because fish are poikilothermic and the environmental temperature influences all aspects of their physiology (Collazos *et al.*, 1994a). It is therefore likely that the energy required maintaining their immune system in winter is considerably greater than that of summer, as maintaining optimal immune function is energetically

expensive. The production of immune cells and humoral factors all require substantial energy (Demas *et al.*, 1997; Spurlock, 1997; Nelson *et al.*, 2002). Consequently mounting an immune response requires resources that would otherwise be allocated to other functions (Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996; Nelson *et al.*, 2002). In winter it is, therefore, probable that it makes greater energetic sense to maintain body condition rather than maintaining the immune system at an optimal level. This would account for the suppressed immunity of rainbow trout observed in winter.

The converse view of this situation is why would it be beneficial to have an increased immune response during the summer months? Winter is not just energetically demanding for rainbow trout, it affects all organisms of the environment from viruses and bacteria, to plants and animals. The majority of these organisms thrive during the warmer months compared to the winter. Consequently, there is a greater potential threat of disease in the summer than in the winter. It would therefore be extremely beneficial to rainbow trout to have an increased immune system in summer rather than in winter. None of the immune parameters exhibited a seasonal peak during the colder winter months. This could be because there is a lower pathogen load at this time of year. Rather it makes more energetic sense to maintain all immune parameters at a background level. Alternatively, the immune parameters which do increase activity during the winter may not have been studied during this project. Nelson *et al.* (2002), proposed that individuals optimize immune function so that they can tolerate minor infections if the energetic costs of mounting an immune response outweigh the benefits. Evidence of this is shown in precocious male salmonids. In this case, energy is used to promote a reproductive state rather than in maintaining a high immune response (Maule *et al.*, 1987; Maule *et al.*, 1996).

It is easier for an organism to defend itself from a pathogen attack if its immune system is maintained at an optimal level. If pathogen attack is more likely to occur during the summer months the rainbow trout immune system must be primed at this time. In order for it to be successful in doing this, an ability to anticipate a change in season is required i.e. detect that summer is coming. Furthermore, as the season passes into autumn and then winter, rainbow trout must be able to detect that the seasons are changing towards a period where the threat of disease is not as great, therefore maintaining the immune system at an optimal level would be a waste of energy. It is not known what mechanism is involved in mediating this seasonal information, however melatonin was shown to exhibit a seasonal pattern in both the 'Base Level' (Chapter 2) and Melatonin trials (Chapter 5). Melatonin levels were higher during the summer months compared to the winter months. Melatonin production was also shown to be affected by the two principle seasonal cues of temperature (Chapter 3) and photoperiod (Chapter 4). The results corroborated the results of earlier investigations that reported that melatonin is primarily produced during the dark phase (Bayarri *et al.*, 2004) and melatonin production increases with increasing temperature (Porter *et al.*, 2001). It is highly plausible that plasma melatonin is involved in conveying seasonal information to the fish immune system because it is cued by photoperiod and temperature, the principle cues of seasonality.

Enzyme activity involved in the fish immune system, will also be influenced by season. According to the kinetic theory as temperatures increase molecules move faster. In an enzyme-catalysed reaction this increases the rate at which enzyme and substrate molecules meet and hence the rate at which the product is formed. However, eventually as the temperature continues to rise, the hydrogen and ionic bonds that hold the enzyme molecule in shape are broken. When this happens the molecular structure is disrupted

and the enzyme fails to function because the active site no longer accommodates the enzyme. At this point the enzyme is said to be denatured (Fig. 7.4) (Toole and Toole, 1993). Several of the parameters studied involve enzyme activity e.g. the key enzyme in respiratory burst activity is NADPH oxidase, various hydrolytic enzymes are involved in phagocytosis, and the key enzyme for melatonin production is N-acetyltransferase. Lysozyme is a class of enzyme that catalyses the hydrolysis of cell walls of bacteria. In

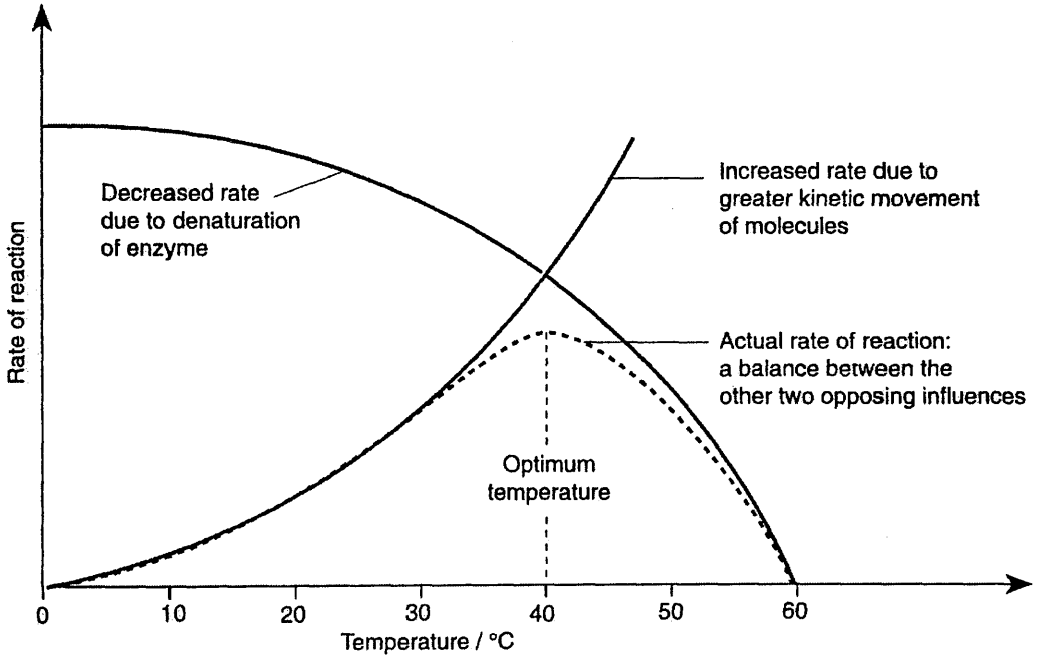


Fig. 7.4 The effect of temperature on an enzyme catalysed reaction (Toole and Toole, 1993).

the 12-month trials (Chapters 2 and 5) plasma lysozyme activity exhibited a strong correlation with temperature. Lysozyme has an optimum temperature of 25°C, so obviously summer water temperatures are closer to this optimum level than in winter, therefore it is not unreasonable to suggest that the organism is more reliant on this immune defence in summer than in winter and consequently produces more of it at that time. There would be little point in producing an immune defence that does not work well, as it would be a waste of energy, more so in winter. Measurement of lysozyme

activity in the laboratory was performed under controlled conditions i.e. all samples were measured at 25°C with different rates of activity measured. This suggests that the quantity of lysozyme in the samples varied with season. Fish are poikilothermic and as such are affected by the temperature of the surrounding water. This will obviously directly affect lysozyme activity and production. However, this idea is perhaps too simplistic. Lysozyme is produced by macrophages which are obviously involved in numerous other aspects of the immune system. The immune system should not be considered as being made up of separate components as they are all integrated and part of a whole. However, temperature obviously affects the parameters studied and probably the enzyme activity associated with them.

Seasonal immunosuppression was observed during the winter months of the 'Base Level Trial' (Chapter 2). It would be beneficial to the aquaculture industry if this seasonal immunosuppression could be alleviated. Fish farming has grown significantly during the last thirty years and very often trout and salmon are kept at high population densities, which are known to increase the risk of disease. This is of particular importance as transmissible diseases are known to have devastating effects on both wild and cultured stocks of fish (Smith *et al.,* 2000). This may increase the risk for dramatic disease outbreaks, particularly when the fish immune system is not at its optimum.

Alleviation of seasonal immunosuppression was investigated as part of the trial described in Chapter 5. Melatonin is known to act as an immunostimulant in mammals and birds (Champney *et al.,* 1997 Moore and Siopes, 2000; Akbulut *et al.*, 2001; Brennan *et al.,* 2002). One group of fish were implanted with an 18 mg implant. Phagocytosis activity and challenge with *V. anguillarum* were the only parameters to be significantly enhanced by melatonin. However, only fish from the June

challenge exhibited a significant difference in length of survival following challenge between untreated and melatonin implanted fish. Phagocytosis was enhanced in fish with a melatonin implant regardless of time of year. A second group of fish were vaccinated against *V. anguillarum* and had a melatonin implant; this was to investigate if melatonin improved the level of protection given by the vaccine. Although vaccinated fish with a melatonin implant did have an increased antibody titre to *V. anguillarum* and an increased level of survival to challenge with the pathogen compared to non-vaccinated fish, the levels were not significantly different from those fish that had been vaccinated alone. Reports have been published providing evidence that vaccination of fish can also enhance the innate immune response of fish. For example, the plasma lysozyme activity of the European whitefish responded strongly following vaccination with two commercial vaccines (Apoject 1800® and Lipogen duo®) (Koskela *et al.*, 2004). However, no such effect was observed during the trial of Chapter 5.

It is possible that any enhancement of the rainbow trout immune system due to the melatonin was short term and as first sampling did not take place until Week 6 after initial administration, this enhancement may have been missed. Furthermore, the melatonin implant was comparatively large (18 mg). The implants used were actually designed to control reproduction in sheep and were the only ones available on the market at the time of setting up the experiment. Maestroni *et al.* (1988), reported that high doses of melatonin (~200 mg kg^{-1}) can actually suppress immunological responses. The high levels of hormones administered in the trial of Chapter 5 may have counteracted any immunostimulatory effects.

Nelson *et al.* (2002), stated that "the most reliable environmental cue for time of year is the annual pattern of changing photoperiod". However, although this may be true

for land animals, this may not necessarily be the case for aquatic life. On land temperature is significantly different in the middle of the night compared to midday. This is not always the case in the aquatic environment. This was demonstrated in the Circadian Trial (Chapter 6), where water temperature was measured every 6 hours over a 24 hour period and remained a constant 10°C. Environmental water temperature changes gradually, exhibiting a very similar pattern to natural photoperiod (Fig. 7.5). A sudden drop in air temperature would not elicit a sudden drop in water temperature

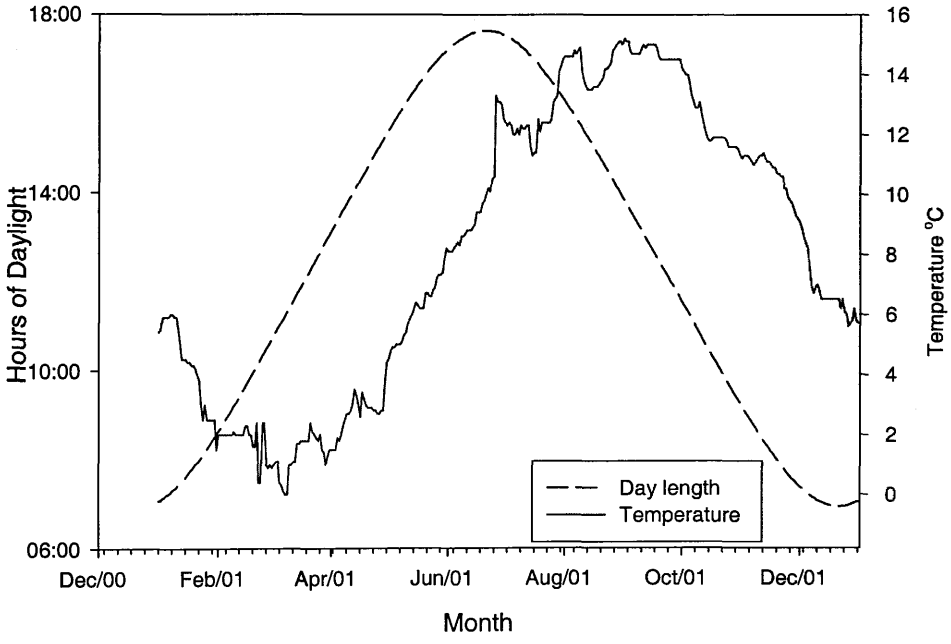


Fig. 7.5 Over a twelve month period the cyclical cycles of photoperiod and temperature are closely correlated in the temperate environment. Consequently any affect of season observed could in response to either or both of these parameters. Data presented is for the NBFRF.

because it takes a significantly longer period of time for a volume of water to change temperature compared to a similar volume of air. Nelson's statement (2004) can therefore be amended, "in the aquatic environment the annual pattern of changing temperature and photoperiod are of similar importance as environmental cues for indicating time of year". However, it would appear that different life history functions

use different environmental cues. For example, photoperiod is the primary cue for reproduction in the Atlantic salmon. However, the results of the trials in Chapter 4 suggest that temperature is the primary cue for lysozyme activity in rainbow trout. Furthermore, although photoperiod was not shown to affect immune response in any of the immune parameters studied in the trials presented here, it has been shown to affect T cell numbers (Leonardi and Klempau, 2003), and it has been shown to affect the stress hormone cortisol (Olsen *et al.,* 1993). Photoperiod and temperature may be of equal importance as seasonal cues to the immune system of rainbow trout. Further study is required to ascertain if this is true.

In conclusion, the results of this study suggest that seasonality does affect both the innate and adaptive immune system of rainbow trout. Furthermore the proximate environmental cue used to determine season by the fish is temperature rather than photoperiod. The hormonal mechanism behind this system may use the hormone melatonin, which itself exhibits seasonality in its production, however, further study is required to determine if this is true.

It is anticipated that research investigating seasonal effects on basic immune function, and the effects of artificial photoperiods and temperature regimes on the immune response, may be of benefit to the aquaculture industry. It could provide information that will allow administration of commercial diets containing immunostimulants to be timed effectively. These methods could be used to alleviate periods of immunosuppression for example, during smoltification. In addition, vaccination strategies and fish movement from one farm to another could be improved by knowing periods of seasonal immunosuppression which could then be avoided.

7.1 Further Study

Although this study has provided evidence for presence of seasonal patterns of immunity further study is still required. The mechanisms that relay the seasonal information of the changing patterns of photoperiod and temperature are not yet known. It is suspected that melatonin plays an important part in this mechanism. However, melatonin is not the only seasonal hormone. The corticosteroids and sex steroids also exhibit patterns of seasonality, and further investigation into immunity and seasonality should also consider these hormones.

Although the majority of parameters studied were unaffected by vaccination or melatonin implantation, the period of first sampling took place in Week 6 of the trial. It is therefore, recommended that in future, sampling should be carried be on a weekly basis rather than monthly. The effect of wavelength and light intensity on the immune response should also be considered in future work. This is of particular interest in the aquaculture industry where deep sea cages are used e.g. cod farming. If challenges are used it is suggested that bath immersion is used to administer the pathogen instead of injecting a calculated dose. How susceptible the fish is to a pathogen may also be seasonal.

Pathogen load of the water is also suspected to be influenced by season. It is possible that the observed seasonal patterns of the immunity observed in rainbow trout maybe in response to this pathogen load. It is therefore, recommended that future studies consider this, and possibly measure how different pathogen populations alter during a twelve month period in the trial environment.

Ultimately, the results of such work, examining the effect of seasonality on the immune response of fish may be used to develop a mathematical model which could be

used to predict periods when fish are most susceptible to disease. By imputing data relating to certain photoperiodic regimes and water temperatures, it may be useful to predict the activity of various aspects of the immune system. This would be an extremely useful tool in the prevention and control of fish disease in aquaculture.

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Appendix A Reagents and Buffers

Unless otherwise stated reagents were bought from Sigma Chemical Company Ltd.

A.1 Bacteriology

A.1.1 Tryptone Soya Agar (TSA) with NaCl

Dissolve the following reagents in 1 L distilled water. Autoclave for 20 min and allow to cool to 50°C before pouring plates. Makes forty plates.

TSA	40 g
NaCl	1.5 g

A.1.2 Tryptone Soyal Broth (TSB) with NaCl

Dissolve the following reagents in 1 L distilled water. Autoclave for 20 min.

TSB	30 g
NaCl	1.5 g

A.1.3 Gram Stain

Dissolve the following reagents in 300 ml distilled water.

Iodine	1 g
Potassium Iodide	2 g

A.2 ELISA

A.2.1 Cell Culture Medium

Prepared under sterile conditions using a laminar flow hood at all times. Dulbecco's Modified Eagles Medium (DMEM) containing additives. To 10 ml DMEM add 0.5mM sodium pyruvate, 20mM L-glutamine and 10000U ml-1 penicillin, 1mg/ml streptomyicin and 20% (v/v) foetal calf serum (heat inactivated for 1 h at 56°C). Before use the cell culture medium was always warmed to 37°C.

A.2.2 Chromagen (Substrate)

Prepare a solution of 1 part acetic acid: 2parts distilled water. For every 3ml of this prepared solution dissolve 0.0394g of 3'3'5'5'-tetramethylbenidine (TMB). The solution is 42mM.

A.2.3 Coating Buffer

Dissolve one carbonate-bicarbonate buffer tablet in 100ml distilled water. Add 1ml Poly-L-lysine . Solution should be pH 9.6.

A.2.4 Conjugate Buffer

1% (w/v) Bovine serum albumin (BSA) in LSW

A.2.5 High Salt Wash Buffer (HSW x10)

Prepared in 1 L of distilled water. Adjust to pH 7.2 and dilute 1/10 to use.

Trisma Base	24.2 g
NaCl	292.2 g
Tween 20	10 ml

A.2.6 Low Salt Wash Buffer (LSW x 10)

Prepared in 1 L of distilled water. Adjust to pH 7.3 and dilute 1/10 to use.

Trisma Base	24.2 g
NaCl	222.2 g
Tween 20	5 ml

A.2.7 Substrate Buffer

Dissolve the following reagents in 1 L of distilled water. Adjust pH to 5.4. Store at 4°C.

Citric Acid	21 g
Sodium Acetate	8.2 g

A.3 General Use Reagents

A.3.1 Heparin

Dissolve the contents of one vial of heparin (containing 25,000 U) to 10 ml of L-15 medium. Sterile filter to 40 μm. Store at 4°C.

A.3.2 Phosphate Buffered Saline (PBS)

Dissolve in 1L of distilled water. Adjust to pH7.2 Autoclave for 20min.

$NaH_2PO_4.2H_2O$	0.876 g
$NaHPO_4.2H_2O$	2.56 g
NaCl	8.77 g

A.4 Macrophage Respiratory Burst Activity

A.4.1 Lysis Buffer

Prepared in distilled water. Store at 4°C.

Citric Acid	0.1 M
Tween 20	1.0 % (v/v)
Crystal Violet	0.05 % (w/v)

A.5 Melatonin RIA

A.5.1 Antibody

Freeze dried sheep anti-melatonin antiserum (Stockgrand Ltd., Surrey, UK) was reconstituted with 2 ml of nanopure water to provide an intermediate solution. This was divided into 100 μl aliquots and stored at -20°C in polystyrene tubes (LP3; Luckhams Ltd). The working solution was prepared by diluting one 100 μl aliquot to 20 ml with assay buffer.

A.5.2 Charcoal Solution

Mix 0.48 g charcoal to 50 ml of tricine buffer. Stir on ice for 30 min.

A.5.3 Melatonin Standards

A stock standard solution of 10 mg melatonin (N-acetyl-5-methoxytrptamine) was dissolved in 10 ml absolute ethanol and stored at -20°C. For each assay fresh standards were prepared from this stock solution. Serial dilutions of 250 μl aliquots were prepared from a working solution of 1 ng ml^{-1} to provide standards from 3.9 - 250 pg $tube^{-1}$. An additional working solution of 2 ng ml^{-1} was used to provide a 500 pg $^{-1}$ standard.

A.5.4 Radiolabel

A stock label of tritiated melatonin ([O-methyl-^{3}H]melatonin) supplied by Aversham International Ltd. In 250 μCi quantities with a specific activity of 70-85 Ci/mol. An intermediate solution was prepared by diluting 20 μl in 2 ml of absolute ethanol (Fisons

Ltd). This solution was stored in 20 ml glass vials (Canberra Packard, Berks., UK) at -20°C. A working solution was freshly prepared for each assay by diluting the intermediate solution with assay buffer to give an activity of approximately 40000 dpm μl^{-100} (~ 20 μl of the intermediate solution in 10 ml of buffer).

A.5.5 Tricine Buffer

The following chemicals were dissolved in 150 ml of nanopure water.

Tricine [N-Tris(hydroxymethyl)methylglycine]	2.688 g
Sodium Chloride	1.35 g
Gelatin	0.15 g

A.6 Serum Lysozyme Activity

A.6.1 Sodium Phosphate Buffer (SPB)

Stock A: Prepare a 0.2 M solution of $NaH_2PO_4.2H_2O$ in distilled water.

Stock B: Prepare a 0.2 M solution of $NaHPO_4.2H_2O$ in distilled water.

Ninety-two ml Stock A was mixed with 8 ml Stock B to create a 0.1M SPB, pH 5.8. One hundred ml distilled water was added. Solution was diluted 2:5 to give a SPB of 0.04 M, pH 5.8.

Appendix B Additional Methods

B.1 *Vibrio anguillarum* Standard Curve

A culture of *Vibrio anguillarum* (Type 1) was prepared overnight. Next day the culture was spun at 3,500 rpm in a centrifuge for 10 min. Supernatant was poured off and the pellet resuspended in sterile phosphate buffered saline (PBS). This was repeated twice and the pellet resuspended in 10 mls sterile PBS. The absorbance of the overnight bacterial suspension was read at 610 nm using a spectrophotometer (Cecil 2041). The value represents the culture at 100%. A ten-fold dilution of this culture was performed from 10^{-1} down to 10^{-7}. For each of the dilutions place six 20 μl drops onto six marked sections of a tryptone soya agar (TSA) plate (separate plate for each dilution). An 80% dilution of the original 100% bacterial solution was made and the serial dilutions repeated as before and the dilutions plated out. Dilutions were repeated at 60, 30 and 20% and serial dilutions for each were performed.

Plates were left on a level surface until the drops dry into the agar. The plates were then inverted and incubated overnight at 22°C. Next day, colonies were counted from each concentration and serial dilution showing 10-50 colonies per drop. Calculate average colonies per drop and calculate for each dilution. Plot optical density against viable bacteria (Fig. B.1).

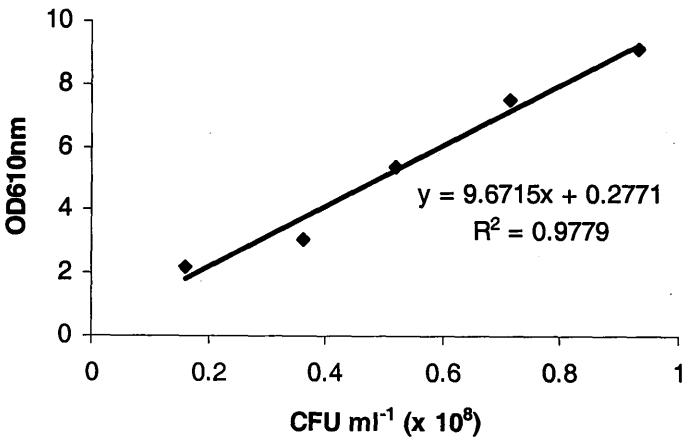

Fig. B.1 Standard curve of the optical density at 610nm of *V. anguillarum* (Type 1), suspension against the number of CFU.

B.2 Cell Culture of 4C10 Hybridoma Cells

Specific antibodies to *V. anguillarum* were detected using the anti-trout monoclonal antibody (Mab 4C10) which was obtained from a 4C10 hybridoma cell line provided by Dr. A. Thuvander and stored in liquid nitrogen (Thuvander *et al.*, 1990). After rapid thawing, hybridoma cells were revived by placing in 10ml cell culture medium (Appendix A) that had been warmed to 37°C. The solution was centrifuged at 1000 xg for 7 min, the resulting supernatant was discarded and the pellet was resuspended in 5 ml cell culture medium. Cells were incubated at 37°C overnight, in an atmosphere containing 5% carbon dioxide using a Galaxy CO_2 incubator (RS Biotech, Finedon, UK). To verify satisfactory growth, cells were observed under an inverted microscope (Carla Zeiss Jena Televal) where a growing stock of hybridoma cells was maintained in 10 ml of cell culture medium in a 25 ml tissue culture flask (Nunc, UK). Stock was kept in the incubator and the media was changed regularly.

Cells were expanded by taking 5 ml of the growing stock of 4C10 hybridoma cells and placing in a 150 ml tissue culture flask, to which was added 15 ml of fresh

culture medium. The flask was maintained in an upright position for 2 days, to encourage cell growth After 2 days, 60 ml cell culture medium was added to the cell solution and the flask was placed flat and kept in an incubator at 37°C for 10 days. The cell solution was then centrifuged at 100 xg for 7 min and the antibody rich supernatant collected and frozen at -20°C until required.

Appendix C Conference Presentations

C.1 Peer Reviewed Oral Presentations

C.1.1 4th International Symposium on Aquatic Animal Health, September 1-5, 2002, New Orleans, Louisiana, USA

The effect of seasonality on the immune responses of rainbow trout (*Oncorhynchus mykiss*)

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Immune responses of ectotherms are known to vary seasonally, although little work has been carried out on the effect of seasonality on immune responses in fish. Environmental factors, mainly temperature and photoperiod, are known to undergo circadian and circumannual rhythms and have been proposed to be direct causative agents of these variations. The effects of variation and/or constant release melatonin implantation on the seasonal variation in the immune response of rainbow trout maintained under a simulated natural photoperiod were examined. Preliminary studies at the Institute have demonstrated that long-term administration (9 weeks) of melatonin via intramuscular implants significantly enhances a number of immune parameters and improves disease resistance. In conjunction with this trial, half the fish were maintained on EWOS "Boost" diet, which is rich in nucleotides, to see if it could alleviate seasonal immunosuppression when compared to a standard commercial diet. Immune parameters of both the specific and the non-specific immune system were assessed over the course of the trial. Seasonality was shown to effect fish haematology and lysozyme activity. An improvement in survival following challenge *Vibrio anguillarum* as a result of using the EWOS "Boost" diet and melatonin implants were observed. It is anticipated that this examination of seasonality on basic immune function will be of benefit to the aquaculture industry. It will provide information that will allow administration of commercial diets containing functional supplements to be times effectively and will facilitate our understanding of the epidemiology of specific fish pathogens.

C.1.2 11th International Conference of the European Association of Fish Pathologists, "Diseases of Fish and Shellfish", 21-26 September 2003, Corinthia San Gorg Conference Center, St Julians, Malta

The Effect of Season on the Immune Response of Rainbow Trout (*Oncorhynchus mykiss*)

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Immune responses of ectotherms are known to vary seasonally, although little work has been carried out on the effect of seasonality on immune response in fish. Four different trials were carried out to determine the principle environmental cue stimulating this seasonal immune response was the ambient photoperiod and/or temperature. Trials were carried out once in winter and once in summer, to determine if time of year affected these potential cues. In trials designed to investigate the effects of temperature the photoperiod was maintained at 12:12 LD and the temperature during photoperiod trials was ambient. Melatonin, a hormone produced in the pineal gland of teleosts, regulates seasonal events and is thought to be involved in the regulation of the immune system was measured at regular intervals. A variety of 'non-specific' immune responses were studied. To date the majority of work in this area has concentrated on the 'specific' immune response as it has been generally assumed that the 'non-specific' immune responses remain constant throughout the year.

Temperature was found to have a significant effect on several of the immune parameters studied including blood counts and respiratory burst. Haematocrit was not affected by temperature. Conversely, photoperiod was found to have no effect on the immune parameters studied with the exception of haematocrit in winter. It is anticipated that the data from this study will lead to the development of a simple model that can be used to predict times of immunosuppression in fish as a result of projected temperature and photoperiod regimes. Knowledge of when immunosuppression occurs in fish allows the farmer to provide adequate immunostimulation to reduce the risk of associated health problems.

C.1.3 6th International Symposium on Fish Immunology, 24-29 May 2004, University of Turku, Turku, Finland

TITLE: THE EFFECT OF SEASON ON THE IMMUNE REPONSE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Seasonality is inextricably linked with the life history of fish. It is becoming increasingly apparent that season affects the immune system of many vertebrates. The data presented here focuses on the effect of season on the non-specific immune response of rainbow trout.

A 12 month seasonality trial was carried out. Fish studied were all female and had an average weight of 20g at the start of the trial. The fish were divided into three tanks and allowed to acclimatize to ambient temperature and photoperiod for one month prior to sampling. Each month fish were sampled and a variety of non-specific immune responses measured including respiratory burst, phagocytosis, red and blood cell counts. A seasonal response was observed however, the environmental cue for the observed response couldn't be identified from this trial. Consequently, trials were set up to determine if photoperiod or temperature (primary seasonal cues) were the environmental trigger for the seasonal changes observed in the immune response of *O. mykiss*. Temperature was found to have a significant effect on several of the immune parameters studied including blood counts and respiratory burst. Haematocrit was not affected by temperature. Conversely, photoperiod was found to have no effect on the parameters studied with the exception of haematocrit in winter. It is anticipated that the data from this study will form the basis of a simple model and together with results from other seasonality trials could be used to predict times of immunosuppression in fish as a result of projected seasonal variation. Knowledge of when immunosuppression occurs in fish allows the farmer to provide adequate immunostimulation to reduce the risk of associated health problems.

C.1.4 Aquaculture Europe 2004, "Biotechnologies for Quality", 20-23 October 2004, Barcelona, Spain

TEMPERATURE AND IMMUNITY IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Introduction

Seasonality dominates the life history of fish. It affects a variety of factors including growth, reproduction and immune response. Environmental factors, mainly temperature and photoperiod, are known to undergo circadian and circumannual rhythms and have been proposed to be direct causative agents of these variations. This work examines the effect of temperature on serum lysozyme activity, blood counts and general disease resistance at three treatment temperatures (5, 10 and 18°C) and then after acclimation to a challenge temperature of 15°C.

Materials and methods

To examine the effect of temperature on the immune response of rainbow trout a trial was carried out consisting of duplicate tanks of fish held at three different temperatures (5, 10 and 18°C). After four weeks at their respective temperatures serum lysozyme and blood cell counts were measured, n = 20/treatment (fish were culled following sampling). The remaining fish were then gradually acclimated to 15°C for one week and serum lysozyme and blood cell counts were remeasured, n = 20/treatment (fish were culled following sampling). The remaining fish were challenged with *Vibrio anguillarum*, n = 30/treatment. Only female fish were used and their starting weight was 65g. Photoperiod was 12:12LD throughout the trial.

Results

White blood cell counts measured in fish maintained at the original three treatment temperatures (5, 10 and 18°C) were significantly affected by temperature (Fig. 1). It was observed that the greater the temperature the higher the number of white blood cells.

Following acclimation to 15°C fish originally held at 5°C exhibited a significantly increased white blood cell count (P=0.000). Whereas fish originally held at 18°C exhibited a significant reduction in their white blood cell counts (P=0.000). This observed pattern of results was repeated for serum lysozyme activity.

Survival to challenge with *V. anguillarum* varied between treatment groups, (Fig. 2). Fish originally held at the lowest treatment temperature of 5°C exhibited the highest level of survival and at 18°C the lowest.

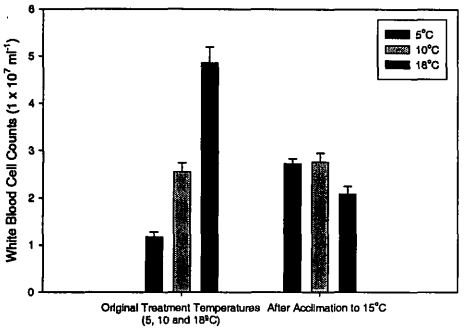

Fig. 1. The effect of acclimating fish originally held at the three original treatment temperatures to 15°C, on white blood cell counts (n=20/treatment, ±SE). Photoperiod 12:12LD.

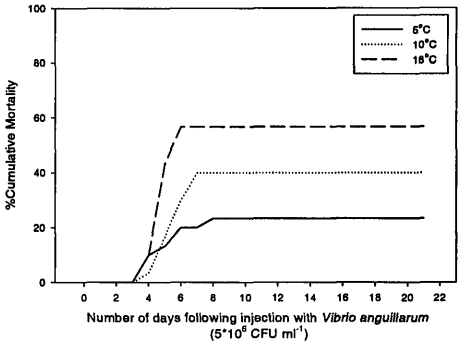

Fig. 2. % Cumulative mortality following challenge with *V. anguillarum*. Fish were gradually acclimated to 15°C for one week prior to challenge (n=30/treatment). Photoperiod 12:12LD

Discussion

This data corroborates the well established theory that temperature significantly affects immunity. A suggested hypothesis for the observed changes in immune activity following acclimation is that the act of raising temperature from 5°C to 15°C maybe mimicking the natural spring immune activity. A previous study carried out at the Institute of Aquaculture observed that the majority of immune responses increase in activity during spring and peak in summer (1). Further work is required, for example a trial where the water temperature is gradual increased and a variety of immune parameters measured at set increments would help to better understand this phenomenon.

References

Morgan, A.L., Thompson, K.D., Migaud, H., Porter., M.J.R., and Bromage, N.D. (2004). The effect of season on the immune response of rainbow trout, *6th International Symposium on Fish Immunology – Turku, Finland*

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C.2 Poster Presentations

C.2.1 10th International Conference of the European Association of Fish Pathologists, 9-14 September 2001, Trinity College, Dublin, Ireland

The Effects of Seasonality on the Immune Response of Rainbow Trout (*Oncorynchus mykiss*)

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Whilst immune responses of ectotherms are known to vary seasonally, little work has been carried out on the seasonal variations in immune function in fish. Many vertebrates display profound changes in their physiology and behaviour coincident with variations in their seasonal environment. These include changes in reproductive activity, immune response, food intake, locomotor activity, body weight and body condition. Due to the long-term nature of these physiological changes, such adaptations cannot be reactive, but instead must be initiated before the anticipated changes in climatic conditions. Therefore, the mechanisms that regulate these seasonal changes demand a temporal component, which ensures that physiological changes remain synchronised with the appropriate time of the year. Photoperiod provides a highly predictable environmental time cue which is used by many vertebrates to co-ordinate their seasonal physiological cycles. In addition, it is well documented that within the animal photoperiodic messages are conveyed by the pineal hormone, melatonin (1).

At present two 12 month trials are being performed. One is examining the effect of temperature on the immune system of *Oncorhynchus mykiss* under a constant 12h light/12h dark photoperiod. The second examines the effects of variation and/or constant release melatonin implantation on the seasonal variation in the immune response of *O. mykiss* maintained under a simulated natural photoperiod. Preliminary studies at the Institute have demonstrated that long-term administration (9 weeks) of melatonin via intramuscular implants significantly enhances a number of immune parameters and improves disease resistance (2). In conjunction with this trial, half of the fish were maintained on EWOS 'Booster' diet, which is rich in nucleotides, to see if it could alleviate seasonal immunosuppression when compared to a standard commercial diet which was fed to the remaining fish. Immune parameters of both the specific and the non-specific immune system are being assessed over the course of the two trials, results of which will be presented here.

It is anticipated that this research investigating the seasonal effects of natural and artificial photoperiods on basic immune function will be of benefit to the aquaculture industry. It will provide information that will allow administration of commercial diets containing functional supplements to be timed effectively and will facilitate our understanding of the epidemiology of specific fish pathogens.

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2. Weber, E.P. (1999). The effects of melatonin on the innate immune system of rainbow trout (*Oncorhynchus mykiss*), *MSc. Thesis*, Institute of Aquaculture, Stirling, Scotland.

C.2.2 3rd International Symposium on Fish Vaccinology, 9-11 April 2003, Bergen, Norway

The Effect of Season on the Specific Immune Response of Rainbow Trout (*Oncorhynchus mykiss*)

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Immune responses of ectotherms are known to vary seasonally, although little work has been carried out on the effect of seasonality on immune responses in fish. Environmental factors, mainly temperature and photoperiod, are known to be subject to circadian and circumannual rhythms and have been proposed to be directly entrained by these variations. Preliminary studies have demonstrated that long-term administration (9 weeks) of melatonin via intramuscular implants significantly enhanced a number of immune parameters and improved disease resistance. The effects of seasonal variation and/or constant release melatonin implants on the specific immune response of rainbow trout were examined when maintained under a simulated natural photoperiod. In conjunction with this trial, half the fish were fed the EWOS 'Boost' diet, which is rich in nucleotides, to assess whether it could alleviate seasonal immunosuppression when compared to a standard commercial diet.

In addition, fish were vaccinated against V. anguillarum. Antibody titres of fish were measured monthly using ELISA. Four months after vaccination, fish were challenged with V. anguillarum; this was repeated three times at 3-month increments. Boost diet and melatonin had no effect on antibody titre. Boost diet improved survival following challenge with V. anguillarum. Season significantly effected antibody titre. Rapidly increasing antibody titres were observed during spring – this correlated with increasing spring temperatures. The implications of season i.e. time and the seasonal fluctuations of temperature and photoperiod will be discussed.