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This thesis tests the hypothesis that innate immunity may be enhanced immediately following a stressful event. The experiments characterize the acute effects of the fight or flight response on some immunological and endocrine parameters in rainbow trout (*Oncorhynchus mykiss*). Plasma cortisol and catecholamines were elevated within seconds of the initiation of an acute handling stressor consisting of 30 seconds in the air and five, 10 or 20 minutes in a shallow bucket of water. Plasma lysozyme activity increased after stress, however, the increases were not statistically significant unless variation was reduced by serial bleeding of the same individual trout before and after stress. A more "resting" fish was achieved by use of the anesthetic 2-phenoxy-ethanol which was surreptitiously introduced into the tanks before the initial bleed. Individual fish were then revived in freshwater and stressed as before. Enhancement of lysozyme activity was evident although levels of plasma stress hormones in fish that were anesthetized, revived and stressed were less than when fish were similarly stressed without anesthetic. Levels of cortisol and catecholamines increased within seconds of capture and aerial exposure, returned to near pre-stress levels after the fish had been placed in a shallow bucket of water for 30 seconds, then increased again. Evaluation of the influence of acute stress on survival following challenge with the pathogen *Vibrio anguillarum* yielded equivocal data. Results presented here suggest that

enhancement of innate defenses as part of the fight or flight response merits further evaluation.

Immediate Effects of Acute Stress on Innate Immunity in Rainbow Trout (*Oncorhynchus mykiss*).

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Nora Egan Demers, Author

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Chapter I

Introduction, Literature Review and Hypotheses

Survival requires maintenance of the "milieu interior" (Bernard, 1859). Homeostatic mechanisms detect, process and respond to perturbations (Cannon, 1929). Of the many systems interacting to maintain the balance required for existence, the immune system plays a lead role in detecting and responding to "foreign" particles and repairing damage after bodily injury. We propose that innate defenses are enhanced immediately after the fight or flight response.

The study of the immune system has traditionally been divided into two arms. One component of the immune system is capable of specific immune responses; the evolutionarily older arm involves innate defenses. Among living organisms, only vertebrates possess the ability to mount a specific immune response. The lymphocytes, B and T cells, ultimately responsible for the specificity of the response, are present only in mammals, amphibians, reptiles, birds and the jawed fishes. Elements of innate defense systems, however, are present in most living organisms.

The primary innate defense effector cell is the macrophage. It is a circulating and an extra-vascular cell constantly encountering and clearing potentially dangerous particles from the internal environment. Both macrophages and dendritic cells are imperative to the development of a specific

antibody response. They present degraded antigens to lymphocytes, a necessary step in the specific immune response of vertebrates.

Stress has been shown to influence immune responsiveness. One well documented effect of stress is suppression of the lymphoid system. There are indications that the suppression may be mediated by cortisol acting on B and T cell populations. However, although the specific immune response is suppressed, partially due to the actions of cortisol, there are also indications that innate defense responses may be enhanced (see below). Activation of innate immunity as part of the fight or flight response which occurs during a stress event could help an organism identify and clear foreign material or damaged self from the body as the animal regains homeostasis.

Evidence is mounting that the release of catecholamines in stressful situations serves to activate systems that are necessary for the defense of an organism. As early as the 1920's, Cannon described a plethora of instances of bodily changes in response to fear, pain and hunger. Among those changes were increased heart rate, increased circulation to the brain and muscle, decreased blood clotting time and a releases of red blood cells from the spleen. Most of these changes are modulated by catecholamines (Cannon, 1929). Cannon postulated that the changes were the adaptive response of the body to an insult. In the General Adaptation Syndrome, Selye defined stress as "the sum of all physiological responses by which an animal tries to maintain or reestablish a normal metabolism in the face of a physical or chemical force" (Selye, 1950). Only in the past few years has research begun to focus again on the fight or flight response, and attempted to explore the adaptive elements of acute stress. This body of work conveys a distinctly different message from that conveyed by the bulk of recent literature which emphasizes the detriments of chronic stress. The

message here is that enhancement of innate defenses is part of the fight or flight response.

Teleosts have been used as models in biomedical research. Also, in the field of toxicology, fish are being used as indicator species for the potential consequences of pollution (Zelikoff et al, 1991). In the research described here rainbow trout are used as a comparative model for innate defense mechanisms since the innate response mechanisms of trout are similar to those of other vertebrates, including humans. The trout is also important in its own right as a major aquaculture species and a critical component of the fishing industry. Information about trout immunity and innate defenses is now quite extensive (Fletcher, 1982; Rijkers, 1982, Alexander and Ingram, 1992). In the following pages, I will briefly review some aspects of acute stress's effect on immunity in humans, rodents, and teleosts. I will review the roles of several plasma proteins in immunity, then propose a set of hypotheses on the nature of the innate responses of trout to stress, and then propose a set of experiments designed to test them.

STRESS AND THE GENERAL ADAPTATION SYNDROME

Selye (1950, 1973) divided the stress response into the following three stages:

1. Alarm: the pituitary is activated and "stress hormones" such as corticosteroids and catecholamines are released and initiate changes to regain homeostasis.

2. Resistance: the organism has responded and attempts to acclimate, but its performance may be reduced.

3. Exhaustion: with severe stress over a prolonged period, the capacity of the organism to adapt is exceeded, resulting in death.

In fish, physiological adaptation to stressors is characterized by changes in blood and tissue chemistry that are similar whether the stress results from handling and transport, pollution, water temperature changes, or behavioral factors, such as social interactions (Wedemeyer, et al, 1990). The changes are adaptive and are required to achieve acclimation. The stress response can also be considered in terms of primary, secondary and tertiary changes that involve sequentially higher levels of biological organization (Mazeaud, et al, 1977, 1981; Wedemeyer, et al, 1990).

1. Primary changes concern the endocrine system, resulting in the synthesis and release of hormones.

2. Secondary changes, mostly evident in the blood and tissues, include elevated blood sugar and reduced blood clotting time. Other changes include diuresis, electrolyte loss, and depletion of liver glycogen.

3. Tertiary changes include reduced growth and reproduction, reduced resistance to disease, and increased mortality.

Stresses, perceived by sensory components of the peripheral nervous system, stimulate the hypothalamus, which, through the release of corticotropin releasing factor (CRF), stimulates the anterior pituitary to release adrenocorticotropin releasing hormone (ACTH, Axelrod and Reisine, 1984) which elicits the release of corticosteroids from the adrenal cells. Cortisol and other corticosteroids pass through the plasma membranes of target cells and are thought to act upon cytosolic and nuclear targets, affecting gene transcription (Funder, 1992). Glucocorticoids apparently modulate the action of Nuclear-

Factor κB, a transcription activator (Scheinman, et al. 1995). By a separate mechanism of action, a cortisol receptor, first described on the membranes of amphibian neuronal cells (Orchinik, et al, 1991, 1992; Moore and Orchinik, 1991), immediately activates intracellular enzymes. This demonstration that cortisol also modulates behavior *via* a G-protein signal opens a whole new avenue of mechanisms of action for corticosteroids, acting within seconds, in addition to the more clearly defined role of modulating transcription (Moore, et al, 1995; Rose, et al, 1995; Wehling, 1995).

During stress in teleosts, the hypothalamus, by direct neuronal pathways to the head kidney, also stimulates the chromaffin cells to release catecholamines (Mazeaud and Mazeaud, 1981). Catecholamines, such as adrenaline and noradrenaline, are small, charged molecules that interact with cell membrane adrenergic and noradrenergic receptors. The cellular responses to catecholamines require seconds or less to be manifested. Adenlyate cyclase enzymes are triggered to generate cyclic AMP that acts on protein kinases, resulting in activation or suppression of enzymes. Some consequences of receptor binding of catecholamines in teleosts include decreased liver glycogen, upset electrolyte balance, increased blood glucose and lactate levels, increased heart rate, and water content changes (Mazeaud and Mazeaud, 1981; Janssens and Waterman, 1988). For example, the release of glucose from trout hepatocytes is modulated in part by catecholamines acting on beta-adrenergic receptors (Reid, et al, 1992).

STRESS, HORMONES AND IMMUNITY

Humans

Epinephrine alters the response of human mononuclear cells to mitogens (Crary, et al, 1983a). It also alters the spectrum of circulating lymphocytes in the peripheral blood (Crary, et al, 1983b). In a study using cultured endothelium (Benschop, et al, 1993), adrenergic stimulation caused detachment of natural killer (NK) cells, indicating that the release of epinephrine during stress served to recruit cells of the first line of defense (i.e. NK cells). More recently, the same group has demonstrated that propranolol, a β-receptor antagonist, ingested one hour prior to a stressful mental task, abolished the sympathetic response caused by the task. Significant increases in the number and activity of NK cells in the circulation in the stress group were not present in the propranolol group (Benschop, et al, 1994). Additionally these investigators have also recently documented that the change in lymphocyte distribution with stress is related to the change in cardiovascular variables such as blood pressure and heart rate (Benschop, et al, 1995).

Other descriptions of modulation of immune parameters by psychological stress include the following: Lymphocyte subsets and the proliferative response to phytohemagglutinin were changed within 5 minutes of an acute psychological stressor, with those individuals showing the greatest cardiovascular reactivity also showing the greatest immune alterations (Herbert, et al, 1994). Rapid and substantial immune changes to brief psychological stressors (an arithmetic exam) in humans were apparent after only 12 minutes (Naliboff, et al, 1991), including increased numbers of natural killer (NK) cells and of circulating CD8

suppressor/cytotoxic T cells. Confrontational role-playing in male humans led to significant increases in circulating NK cells, large granular lymphocytes, and suppressor T cells (Naliboff, et al, 1995), lending further support to the hypothesis that sympathetic activation is a primary mechanism for increases in NK cell numbers at times of alarm. A study which classified women as high or low sympathetic reactors found that the high reactors experienced the greatest change in number of NK cells and T cells after a stressful event (Matthews, et al, 1995). Individual differences in immune changes appear highly correlated with cardiovascular activity (see also Manuck, et al, 1991). It seems clear that short term stressors can produce transient immunological alterations (Kiecolt-Glaser, et al, 1992).

Exercise has also been used as a model of the stress response, with similar modulation of cell functions reported. Selective administration of epinephrine to obtain plasma concentrations identical with those seen during exercise mimicked the exercise-induced effects on NK cell activity, lymphocyte proliferative response and blood mononuclear cell subsets. That information, as well as cytokine modulation effects after exercise stress, have been reviewed (Pedersen, 1991). Among elite sportswomen, circulating neutrophils showed a higher ingestion capacity for *Candida albicans* than did neutrophils from sedentary women (Ortega, et al, 1993a). The greater phagocytic capacity was correlated with a higher plasma cortisol concentration and lower ACTH concentration. In sedentary men, phagocytic function was similarly stimulated after acute moderate exercise (Ortega et al, 1993b). The changes in humans after exercise have been documented and the value of exercise as a model for acute stress endorsed (Hoffman-Goetz and Pederson, 1994).

The literature on the effects of stressors on immunity is expanding rapidly. A recent review in which the literature was evaluated using meta-analytical

methods supports the observations of lymphocyte redistribution after acute stress (Herbert and Cohen, 1993). Each physiological model of stress has its specific contributions and weaknesses. Individual differences in the adrenergic response appear to be the most influential factor in determining alterations of immunological parameters following acute stress, with gender and race having limited influence (Mills, et al, 1995). The responsiveness among individuals, however, appears to be consistent over time (Marsland, et al, 1995).

Rodents and birds

There is a growing realization that the effects of stressors on immunity are far from simple. Studies dating from the 1960's show that chickens maintained in a stressful social environment were more resistant to bacterial infections, but more susceptible to viral infections (Gross and Siegel, 1965), and that selection for high corticosteroid response was associated with increased susceptibility to viral infection but reduced susceptibility to bacterial infection (Gross and Colmano, 1971). Macrophages from mice stressed 8 or 14 days appeared activated, as evidenced by increased protein production, but were less cytotoxic (Aarstad, et al, 1991), yet more evidence of the complex role of stressors on immune function. At least two other groups (see below) have documented modulation of rat macrophage activity as a result of stressors, or stress hormones.

In macrophages from rat spleen, analysis revealed that Tumor Necrosis Factor (TNF) production was suppressed by norepinephrine following activation of the macrophages with lipopolysaccharide (Hu, et al, 1991). Stimulation of β-adrenergic receptors on macrophages decreases production of macrophage-derived TNF in rats, while production is stimulated by α-adrenergic receptor stimulation (Spengler, 1994). The macrophages apparently produce and respond

to the epinephrine in an autocrine fashion. Additionally, a reduction in the mixed lymphocyte reaction of rat cells after stress is due to changes in macrophages, perhaps due to the release of a suppressive factor (Fleshner, et al, 1995).

In rat hepatocytes, the synthesis and secretion of protein increased in response to physiological levels of catecholamines in a manner that appeared to be receptor mediated (Silove, et al, 1991). Acute-phase reactants in rats were also increased by catecholamines (Kahl and Schade, 1991). Cortisol, however, does not always impair immune function (Klein, et al, 1992).

Steroid hormones play an active role in immune modulation. Endogenous corticosteroids mediate the neutrophilia caused by platelet activating factor (Harris, et al, 1995), and reduction of TNF synthesis in macrophages from stressed mice also follows treatment with corticosteriods (Fantuzzi, et al, 1995). In rats, the redistribution of peripheral blood cells after acute stress is mediated in part by corticosterone (Dhabhar, et al, 1995). These changes include a decrease in white blood cell number, and an increase in neutrophil numbers in the circulation. The authors hypothesize that

> "An important function of endocrine mediators released under conditions of acute stress may be to ensure that appropriate leukocytes are present in the right place at the right time to respond to an immune challenge that might be initiated by the stress-inducing agent. The modulation of immune cell distribution by acute stress may be an adaptive response designed to enhance immune vigilance and increase the capacity of the immune system to respond to challenge in immune compartments which serve as major defense barriers for the body (Dhabhar, et al, 1995)."

Teleosts

It is clear that suppression of the immune system occurs as a result of corticosteroid release during stress (Tripp, et al, 1987; Maule, 1989; Maule, et al, 1989; Espelid, et al, 1996). Under many circumstances, the effects of stress on fish are immunosuppressive (Robertson, et al, 1963; Pickering and Pottinger, 1983, 1987; Tripp, et al, 1987; Maule, et al, 1989). Ellsaesser and Clem (1986) concluded that immunosuppression was caused primarily by a direct disturbance of the T and B lymphocytes.

Maule (1989) noticed that acute stress can have a transient **positive** effect on immune function and disease resistance in salmonids. He suggested that apparent stress-related enhancement of resistance was the result of increased function of nonspecific immune mechanisms. Using handling and social conflict as stressors, Peters and Schwarzer (1985) studied how these stressors changed the populations of cells in the spleen and head kidney of rainbow trout. They found that blast cells became rare, immature polymorphonucleocytes and small lymphocytes decreased in numbers, and macrophage-like cells increased in number. The high number of macrophage-like cells may have indicated that the stressors initially stimulated the innate arm of the defense system in an adaptive manner. Increased abundance of neutrophilic granulocytes and reduced numbers of lymphocytes were typical. A subsequent report (Peters, et al, 1991) upheld these results, showing that, under social stress, phagocytes became activated.

The cortisol stress response is fairly well developed in rainbow trout within two weeks of hatching (Barry, et al, 1995). By 3 weeks post-hatching, trout fry experience an "adult-like" stress response. Alevins (5-9 weeks post hatching) mimicked the stress response seen in adults, albeit on a smaller scale (Krumschnabel and Lackner, 1993).

Although trout respond at an early age, there is notable variation in the stress response among populations and strains. Changes in plasma cortisol, glucose and chloride in response to confinement stress were more extreme in wild trout than in hatchery-reared trout (Woodward and Strange, 1987). A genetic basis for high and low responsiveness has been documented in rainbow trout and Atlantic salmon (Pottinger, et al, 1992). Different strains of rainbow trout appear to have quantitative differences in the functioning of the HPA axis (Pottinger and Moran, 1993). Incidentally, plasma cortisone was significantly higher within 5 minutes of the handling stressor, perhaps indicating that the initial secretion of cortisol is greater than indicated by measurements of plasma cortisol (since the source of plasma cortisone is believed to be plasma cortisol, Pottinger and Moran, 1993).

Among rainbow trout populations selected for high or low cortisol responses to stress, challenge by three bacterial pathogens led to overhall higher mortality and poorer performance in the high-stress response group (Fevolden, et al, 1991, 1992). Similar observations were made in Atlantic salmon (Fevolden, et al, 1993). More specifically, in the rainbow trout, high stress responders had higher survival after *Vibrio anguillarum* exposure but decreased survival after *Aeromonas salmonicida* exposure, while the opposite was true for low responders! Hemolytic activity was higher in low stress response fish while lysozyme activity was higher in high stress fish. Genetic heritability has been documented for plasma lysozyme activity, but not yet for plasma hemolytic activity, levels of IgM or specific antibody in salmonids (Lund, et al, 1995). Corticosteroid responses among individuals appear to remain stable over time (Pottinger, et al, 1992). At the cellular level, cortisol enhances the metabolic potential of fish red blood cells by up regulating β- adrenoreceptors (Perry and Reid, 1992). Trout hepatocytes are also upregulated by cortisol (Vijayan, et al, 1994).

Other factors affected by stress include apoptosis and neutrophil function. An acute confinement stress resulted in decreased apoptosis in channel catfish peripheral leukocytes, although the mechanism and significance remains unknown (Alford and Tomasso, 1994). Channel catfish stressed by handling and transport had changes in blood lymphocyte percentages and neutrophil function (Ainsworth, et al, 1991), similar to changes documented in humans. Kidney and spleen phagocytic cells of the flatfish *Limanda limanda* are similarly enhanced with acute stress (Pulsford, et al, 1994).

Catecholaminergic modulation of immunological processes has also been reported in teleosts. Flory (1988) found that selective reduction of catecholamine levels in the sympathetic nervous system of coho salmon was accompanied by dramatically reduced levels of catecholamines in the spleen, and reduced capacity to produce antibody. The implied involvement of catecholamines in this immune modulation was supported when catecholamine agonists and antagonists were administered to responding immunocyte cultures *in vitro* (Flory, 1990). Catecholamines also modulate the respiratory burst, an observation which has been further examined (Bayne and Levy, 1991a, 1991b).

Liver membrane receptors for catecholamines can increase intracellular levels of cyclic AMP in catfish (Fabbri, et al, 1992). This suggests that the mechanisms exist in teleosts for modulation of acute-phase reactants similar to those seen in rats (Kahl and Schade, 1991). The β-adrenergic binding sites in catfish liver modulate adenylate cyclase, as in humans, but the receptors are not functionally identical between humans and trout (Fabbri, et al, 1992). Modulation of cell calcium by epinephrine has also been documented in fish hepatocytes (Zhang, et al, 1992).

The spleen:somatic index is a good indicator of the state of activation of the sympathetic nervous system, and by extension the degree of stressor experienced

by the organism. In yellowtail, *Seriola quinqueradiata*, fitted with a window in the body wall near the spleen, contraction of the spleen was observed within two minutes following exercise stress or hypoxia (Yamamoto, et al, 1985). In trout, the spleen released 21% of total body hemoglobin content after an air stressor or chase. The greatest release occurred within three minutes of the onset of the stressor (Pearson and Stevens, 1991). Furthermore, it appears the splenic contractions may be modulated *via* an α-adrenergic mechanism since the α-antagonist phentolamine blocked the spleen contraction in acutely stressed rainbow trout (Pearson, et al, 1992).

The effects of an acute stress are obviously quite different from those of long-term or chronic, inescapable stress. Adrenaline receptors on hepatocytes and macrophages may help elicit the immediate release of proteins, well before activation of an acute phase response, and other mediators that help an organism identify and clear foreign or damaged material that may enter as a result of an injury sustained after being stressed.

DEFENSE

Bodily damage is often an outcome of a stressful situation. If physical barriers are breached, the sites of damage and/or microbial invasion commonly experience inflammatory responses. During inflammation, vascularized tissues respond by increased flow in blood vessels, increased vascular permeability, and cellular infiltration that allows the delivery of various mediators to the site of inflammation (Finn and Nielson, 1971; Larsen and Hensen, 1983). The processes involved are dependent upon an extensive network of interacting mechanisms,

mediators and cells (reviewed in mammals by Sundsmo and Fair, 1983; Larsen and Hensen, 1983; Rother and Rother, 1986).

Among the cells infiltrating areas of inflammation are macrophages and neutrophils, which engulf and destroy foreign materials and damaged cells (MacArthur, et al, 1984). Mammalian macrophages have receptors on their surfaces for hormones and other mediators of inflammation (reviewed by Bodmer, 1985). As previously mentioned, the processing and presentation of phagocytosed materials by these antigen presenting cells (APC) and their release of cytokines may cause activation and proliferation in both the natural and specific arms of the immune system (Berzofsky and Berkower, 1989; Unanue, 1989). Phagocytes are known to produce components of the complement cascade that mediate inflammation (McPhaden, et al, 1985). In trout, macrophages generate reactive oxygen species, capable of damaging endocytosed materials, similar to those generated by mammalian phagocytes (Secombes, et al, 1992). The reactive oxygen species play a role in the killing of potential pathogens such as *Aeromonas salmonicida*, causative agent of furunculosis (Sharp and Secombes, 1993).

The recognition of viruses, bacteria, or damaged host tissue for destruction by phagocytes is enhanced by a variety of plasma proteins such as complement components, fibronectin, and C-reactive protein. These are components of innate immunity and are distinct from those involved in specific lymphoid immune responses. The specific immune responses require interactions among B cells, T cells, and macrophages in both humoral and cell mediated immunity. Although the same cell types and interactions have been documented in teleosts (MacArthur, et al, 1984; Nash, et al, 1986; Clem, et al, 1991; Vallejo, et al, 1992; Kaattari, 1992), the focus of this review is on innate factors, including plasma proteins that may affect survival.

PLASMA PROTEINS OF INTEREST

Plasma proteins differ from another well studied group of stress proteins, the heat shock proteins, in that the heat shock proteins function intracellularly while the plasma proteins are distributed throughout the body in plasma and lymph. They mediate effects in cells other than those producing them. In all vertebrates, plasma proteins are diverse. Over one hundred proteins have been characterized in the plasma (Putnam, 1975; 1984). The plasma proteins include several that are termed acute-phase reactants. Concentrations of these proteins change after bodily injury. Schreiber (1987) divided the acute phase proteins into two categories: positive, which increase in concentration, and negative, which decrease. Of the positive acute phase proteins in mammals, some are opsonins, enhancing phagocytosis. Included among the opsonins in the plasma are complement components, C-Reactive protein, antibody, and fibronectin. There is a remarkable degree of inter-dependence and cooperation among these molecules.

The complement cascade comprises at least 14 proteins, and others are necessary to modulate its activity. Functions of complement include cytolysis and opsonization. Complement and coagulation systems interact, releasing and activating vasoactive amines, kinins and prostaglandins, all of which play a part in regulating vascular permeability and chemotaxis (for a review of effects in trout see: Ellis, 1981a). Other roles include increasing smooth muscle contraction (Rother and Rother, 1986). The bulk of human complement proteins are made in hepatic parenchymal cells. Some are made by monocytes and macrophages for use in the immediate area (McPhaden, et al, 1985). There is general agreement

that the more activated a macrophage, the greater its secretion of complement proteins (McPhaden, et al, 1985).

The complement cascade has been traditionally divided into two pathways of activation. The alternate pathway begins with the cleavage of component C3. C3a, a vasoactive chemoattractant fragment, and C3b are produced. C3b is covalently bound to membranes of a bacterium, virus, fungus or damaged host cell. Then other complement components are activated in an amplification cascade that leads to the deposition of the membrane-attack-complex, MAC, C5-C9 (reviewed by Rother and Rother, 1986). Among the complement components, C3 is the major phagocytosis-promoting factor (Matasuyama, et al, 1992). C3 is in the highest concentration of all complement proteins in mammals and is also an acute phase reactant (Schreiber, 1987). It is cleaved by the serine proteases trypsin, plasmin, thrombin, and elastase as well as by other complement components.

In the classical pathway, component C1q binds to immunoglobulin (Ig). This complex, with additional components, cleaves C4. Other components are split, and these eventually degrade C3 and initiate the MAC as in the alternate pathway. The classical pathway is not activated solely by Ig. C1q also interacts with C-Reactive Protein, DNA, heparin, collagen, fibronectin, fibrinogen and fibrin, thus identifying cells, cell products, bacteria, or virus for clearance (Jiang, et al, 1991; Sim and Reid, 1991). The receptor for complement component C3b, CR3, is also the receptor for β-glucan (Thornton, et al, 1996).

Interactions of complement components with lectins also augment phagocytosis (Kurashima, et al, 1991). For example, rat serum mannan binding protein (MBP) activates the classical pathway (Ikeda, et al, 1987). The complement cascade interacts with the clotting, kinin-forming (studied by Lipke

and Olsen, 1990, in rainbow trout), and fibrinolytic pathways in inflammation (reviewed by Kaplan, et al, 1982; Frank and Fries, 1991).

Complement components have been isolated and purified from trout (Nonaka, et al, 1984) and from carp (Nakao, et al, 1988; Yano, et al, 1988; Nakao et al, 1989; Uemura et al, 1992). The mechanism of activation of rainbow trout complement is similar to that involved in activation of mammalian complement (Sakai, 1984a, 1984b). There is evidence for both complement receptors and Ig receptors on fish macrophages (Sakai, 1984a, 1992). C3 receptors on carp neutrophils enhance phagocytosis when C3 is bound to particles (Matsuyama, et al, 1992). The bactericidal effects of catfish alternative pathway complement have been documented by electron microscopy (Jenkins, et al, 1991). Interaction among systems is equally evident in teleost homeostatic mechanisms. Both complement and antibody augment macrophage activity in the clearance of *Vibrio anguillarum* from rainbow trout (Honda, et al, 1986). It was recently demonstrated that multiple C3d fragments linked to an antigen markedly reduce the amount of antigen required to effect a specific antibody response (Dempsey, et al, 1996). Clearly, changes in concentrations of complement proteins could have a major impact on the host's ability to respond to infection or tissue damage.

C-Reactive protein (CRP) was identified over 60 years ago by its ability to bind the C-polysaccharide of *Streptococcus pneumoniae* (Tillet and Francis, 1930). CRP binds to phosphocholine-containing molecules, commonly found in the cell walls or surface structures of invading microorganisms (White, et al, 1981). Functions of CRP include recognition and targeting for defensive mechanisms (opsonization) and modification of effector cells such as polymorphonuclear cells (PMN), lymphocytes, monocytes and platelets (Rijkers, 1982; Gotschlich, 1989).

Human CRP binds to receptors on mononuclear phagocytes and lymphocytes, stimulates phagocytosis, and inhibits the aggregation of platelets (Schreiber, 1987). CRP-like molecules have been isolated from trout (Murai, et al, 1990) and channel catfish (Szalai, et al, 1992).

After a physical injury, CRP levels in the teleost *Tilapia mossambica* were increased within 24 hours (Ramos and Smith, 1978). In trout, production of CRP was significantly increased 3 to 10 days after immunization with Freund's complete adjuvant (Kodama, et al, 1989a). CRP concentrations in sera increased dramatically within 24 hours after an intraperitoneal inoculation of *Aeromonas salmonicida* (Kodama, et al, 1989b). Injection of *E. coli* lipopolysaccharide (LPS) and rapid increases in water temperature (both stressors) also caused a dramatic increase in the amount of CRP detected in the serum of trout within the first few days after treatment and the levels remained elevated for up to two weeks. Channel catfish respond similarly (Szalai, et al, 1994). Purified CRP increased the consumption of rainbow trout complement in the presence of *Streptococcus pneumoniae* C-polysaccharide, indicating the activation of complement by CRP (Nakanishi, et al, 1991). CRP may be important to the innate defenses of fish by enhancing phagocytosis and activating complement.

It is believed that natural defenses are especially important in fish since these animals exhibit relatively slow lymphoid immune responses against bacterial and viral infections (Sakai, 1976; Murai, et al, 1990). The interaction of components from distinct systems is demonstrated again with the evidence that CRP increases translation and secretion of the inflammatory cytokines, interleukins, by macrophages (Ballou and Lozanski, 1992; Rochemonteix, et al, 1993). Furthermore, deposition of complement components, resulting in damage to membranes, exposes C-Reactive protein binding sites on those cell membranes (Li, et al, 1994).

Fibronectin, a high molecular weight glycoprotein, occurs in the blood, lymph, tissue fluid, and extracellular matrix. Soluble and membrane bound forms of fibronectin play roles in cell-cell interactions such as wound healing, embryonic development, oncogenesis and metastasis (reviewed by Saba, 1989; Hynes, 1990). Fibronectins are also important in the adherence of microorganisms to white blood cells. They play roles in the formation of immune complexes, phagocytosis, and blood coagulation (Saba and Jaffe 1980; Saba, 1989). A fragment of human fibronectin is opsonic and augments monocyte phagocytosis (Czop, et al, 1981, 1985). Fibronectin may also influence the outcome of parasitic diseases by mediating adherence of parasites to the host extracellular matrix (Wyler, 1987; Klotz and Smith, 1991). Fibronectin appears to modulate macrophage-mediated immunoregulation, affecting the eventual production of antibodies (Rybski, et al, 1989).

The presence of fibronectin has been documented in trout plasma through its cross-reactivity with antiserum to *Xenopus* fibronectin (Lee and Bols, 1991). It has also been purified from carp (Uchida, et al, 1992) and from rainbow trout (Takahashi, et al, 1992). Both polyclonal and monoclonal antibodies specific for trout fibronectin have been generated (Sadaghiani, et al, 1994). Fibronectin metabolism in rainbow trout cell culture is also influenced by cortisol (Lee and Bols, 1991). The role of fibronectin in repairing injuries and in fighting infections warrants its inclusion in this list of plasma proteins that are relevant to innate immune mechanisms, and that may increase following acute stress.

Alpha2-macroglobulin (α_2-mac) is an acute phase protein, that, in mammals, is produced by the liver and macrophages. Synthesis is increased in response to endogenous mediators released from neutrophils and macrophages

(Putnam, 1975). The rat α_2-mac gene contains both IL-6 and glucocorticoid response elements, which influence its synthesis in myoepithelial cells (Warburton, et al, 1993).

Mammalian α_2-mac is a tetramer that complexes with a wide spectrum of proteases including serine, thiol, carboxyl and metalloproteases (Putnam, 1984). Upon encountering a protease, α_2-mac undergoes conformational changes and "entraps" it. The complex of protease and α_2-mac is then rapidly cleared from the circulation (Sottrup-Jensen, 1987). Proteases and other components bound by α_2-mac often retain their activity. Such macromolecular complexes may transport biological mediators into cells (James, 1990; Borth, 1992). Following endocytosis of the complex, changes in the pH within endocytic vesicles may release bound cytokines with effects on cellular metabolism (Borth, 1992). Transforming growth factor-β (TGF-β) influences CRP production. In human hepatoma cell lines, the induction of CRP by TGFβ was neutralized by α_2-mac (Taylor and Mortensen, 1991). Interleukin-2 appears to be bound by human α_2-mac (Legras, et al, 1995), apparently retaining its biologic activity. At least nine other cytokines are also bound by α_2-mac (Liebl and Koo, 1993), perhaps influencing, among other things, nerve growth. Clearance of plasminogen activators and inhibitors by α_2-mac has also been documented (Andreasen, et al, 1994), with the observation that the receptor mediated endocytosis utilized is not the typical clathrin coated pit pathway. Another growth factor, PDGF-BB is also inhibited by its clearance, utilizing the α_2-mac receptor/low density lipoprotein receptor-related (LDR) mechanism (Bonner, 1993; Bonner, et al, 1995). The LDR receptor also mediates the cellular internalization and degradation of thrombospondin (Mikhailenko, et al, 1995). Also noteworthy is the observation that removal of lactoferrin from the plasma is mediated by the LDR receptor, and removal is

highly effective in an acidic environment. Inflamed tissues are often acidic (Melinger, et al, 1995).

Alpha$_2$-mac is regulated by cytokines including insulin (Brown and Brown, 1994). Alpha$_2$-mac has also been reported to regulate cell-line macrophage nitric oxide synthesis and "profoundly affect cellular function" without gaining entry into the cell and without binding specific plasma membrane receptors (Lysiak, et al, 1995).

The multifunctionality of this plasma anti-protease is further revealed with the discovery of a second receptor for α_2-mac (Misra, et al, 1993, 1994a, 1994b). This receptor appears to be involved in the modulation of intracellular levels of inositol phosphates, calcium and cyclic AMP (Misra, et al, 1993). Also of interest to immunologists in particular, the presentation of antigens is enhanced when they are bound to α_2-mac (Chu and Pizzo, 1993), as is the production of antibody (Chu and Pizzo; 1993, Chu, et al, 1994; Mitsuda, et al, 1995). Clearly this plasma protein has roles in a variety of homeostatic mechanisms including immunity (Borth, 1994).

In other vertebrates, including rats, similar functions have been documented for this highly utilized proteinase inhibitor (Webb, et al, 1995). Influenza A Virus is inhibited by the α_2-mac in the serum of horse, rabbit, and pig (Ryan-Poiner and Kawaoka, 1993), demonstrating another potential role in defense. It is also the major defense component in acute pseudomonal septic shock as recently described in the guinea-pig model (Khan, et al, 1994). Clearance of elastase produced by bacteria appears imperative to survive shock. Alpha$_2$-mac is of primary importance in this function (Khan, et al, 1995). It has been proposed that a modification makes α_2-mac a macrophage activating factor. The acquisition of activating property is the result of exposure of mannose

residues by enzymes (Murai, et al, 1995a), which enhances immunoglobulin receptor mediated phagocytosis (Murai, et al, 1995b).

In fish, α_2-mac may suppress inflammatory and immunologic responses (Ellis, 1981b). Proteases released by the bacterium *Aeromonas salmonicida* and which contribute to its pathogenicity can be inhibited by the α_2-mac of rainbow trout serum (Ellis, 1987). Freedman (1991) found a qualitative difference in the inhibitory activities of α_2-mac from brook and rainbow trout, corresponding to the greater susceptibility of brook trout to furunculosis. Processing of α_2-mac by trout liver cells has recently been documented (Gjøen and Berg, 1994). Clearly, α_2-mac may play a vital role in regaining homeostasis after any acute stress in which proteinases - endogenous or exogenous - are released into the blood.

Host natural defenses serve to limit the spread of potential pathogens and remove damaged tissues. Such defenses have been reviewed in teleosts (Ellis, (1981a, 1981b; Rijkers, 1982; Lipke and Olson, 1990; Pool, et al, 1991; Alexander and Ingram, 1992). The first line of defense in vertebrates is the tegument and its secretions. In fish, goblet cells in the skin, gills and mucosa continually replenish the protective mucus, containing **lysozyme** (Fletcher and White, 1973; Ellis, 1981a, 1981b). Lysozyme is also present in the plasma. It digests the peptidoglycan in bacterial cell walls (Ellison and Giehl, 1991). Specifically, it hydrolyzes the β(1-4) linkages between N-acetylglucosamine and N-acetylmuramic acid, which are exposed on gram-positive bacteria but covered by an outer layer on gram-negative bacteria (Phillips, 1966). Most bacterial pathogens of fish are gram-negative. One variant of rainbow trout lysozyme exhibited substantial anti-bacterial activity against seven bacterial pathogens, five of which were gram-negative (Grinde, 1989). Lysozyme has been used as a genetic marker in breeding programs (Røed, 1993a, 1993b). Lines of trout selected for low stress response, as indicated by a low cortisol response,

displayed significantly lower lysozyme activity than the high responders (Fevolden, et al, 1991, 1992).

In addition to the genetic influence on lysozyme activity in trout, Mock and Peters (1990) reported increases in the plasma lysozyme activity as a result of handling. Røed (1993b) found that lysozyme activity was influenced by the amount of time the trout were confined prior to sampling. In Atlantic salmon, lysozyme activity was correlated to bleed time following confinement stress (Fevolden, et al, 1994). Those authors propose that lysozyme increased with stress, however, "evidence that elevation of lysozyme level is immuno-suppressive remains to be documented". That statement was perhaps an attempt to fit the observation into the prevailing paradigm of immunosuppression with stress. As illustrated, lysozyme is an important component of innate defenses attacking bacteria cell walls.

Bacterial breakdown products, such as β-glucans, are also potent immunostimulators (Jørgensen, et al, 1993; Rørstad, et al, 1993). In addition, immunization with a bacterial pathogen (Rainger and Rowley, 1993) or glucan administration (Engstad, et al, 1992) increased lysozyme activity in salmonids. Non-specific defense mechanisms of rainbow trout were enhanced after either injection of or bath exposure to glucans (Jeney and Anderson, 1993). The enhancement of non-specific mechanisms includes neutrophil and macrophage activation, including enhanced phagocytic capacity and enhanced lysozyme activity (Brattgjerd, et al, 1994). Glucans have also been shown to activate salmon pronephros macrophages *in vitro* (Sveinbjørnsson and Seljelid, 1994). Moreover, protection against *Aeromonas salmonicida* is enhanced in brook trout immunostimulated with glucans (Anderson and Siwicki, 1994).

It is important to consider the potential for short term regulation of plasma protein secretion. The constitutive secretory pathway is commonly used for

plasma proteins produced by the mammalian liver. Historically it was assumed that the same cell type could not have both constitutive and regulatory secretory pathways. However, the secretion of the acute phase reactant CRP, posttranslationally controlled in rabbit hepatocytes, is more efficient during the acute phase response (Macintyre, et al, 1985). The half-time for secretion of newly synthesized CRP was found to decrease from as much as 18 hours in rabbit hepatocytes from unstimulated animals to as little as 75 minutes in cells from acute phase animals. The regulated export of CRP from the endoplasmic reticulum (ER) is modulated by a binding site within the ER (Macintyre, 1992). The enhanced secretion of CRP during the acute phase response appears to be due to modifications to glycoproteins in the ER which retain CRP (Macintyre, et al, 1994).

Other forms of posttranscriptional modification which could affect short term secretion of plasma proteins have been documented. For example, secretion of human serum amyloid A, another acute phase reactant, is modulated by mRNA accumulation and the length of the poly A tail on mRNA (Steel, et al, 1993). In astrocytes, cytokines cause a 30% decrease in angiotensinogen mRNA, but the rate of angiotensinogen secretion is increased (Brown and Brown, 1994). β-adrenergic receptors are responsible for the secretion of cholecystokinin (Scott, et al, 1996).

In summary, cell types in addition to hepatocytes are involved in production of plasma proteins. Concentrations of plasma proteins are subject to short term control by both posttranscriptional and posttranslational modifications. Other cells, such as macrophages, endothelial cells and thrombocytes could also play a role in the immediate secretion of plasma proteins when activated by the sympathetic nervous system. In accordance with the body of information reviewed here, and our interest in the possible influence

of acute physiological stressors on innate defenses, the research reported in this thesis was done to test the following hypotheses.

HYPOTHESES

1. ***As components of innate immunity, plasma proteins change within minutes of an acute handling stressor***. Individual fish were sampled for analysis of plasma hormone levels and plasma lysozyme and hemolytic activity. This work is documented in Chapter II.

2. ***Serial bleeding may enhance our ability to detect and evaluate changes in components of innate immunity.*** These experiments are described in Chapter III.

3. ***The kinetics of plasma hormonal increase are more rapid than previously reported***. The short term kinetics of trout plasma hormonal levels are described in Chapter IV.

4. ***Increases in innate immunity after a short term stress enhance an organisms' ability to survive pathogens.*** These experiments are described in Chapter V.

Chapter II

Immediate Effects of Acute Stress on Plasma Lysozyme Activity, Hemolytic Capacity, Cortisol and Catecholamine Levels in Rainbow Trout (*Oncorhynchus mykiss*).

ABSTRACT

The immediate effects of an acute handling stress on several physiological parameters were characterized in rainbow trout. Rainbow trout were bled immediately after the fish had been netted and concussed or they were bled after being subjected to a brief handling stressor consisting of thirty seconds in the air and ten minutes in a shallow bucket of water at ambient temperature. Plasma samples were analyzed for cortisol and catecholamine levels, and the lysozyme activity and hemolytic capacity were evaluated. Crossed immunoelectrophoresis (CIEP) was used in an attempt to quantify changes in the plasma protein profile. Cortisol, noradrenaline and adrenaline levels were significantly increased over initial levels after 5 minutes stress. The rate of accumulation of plasma cortisol was 17.15 ng/mL/min within the first five minutes of the handling stressor. The rate slowed within 20 minutes to 2.82 ng/mL/min. The rates of accumulation of plasma adrenaline and noradrenaline were 298.12 pmol/mL/min and 89.96 pmol/mL/min respectively, in the first five minutes. Mean plasma lysozyme activity increased over the initial level at ten minutes, although the differences were not statistically significant. No significant change in hemolytic capacity of the trout plasma was detected in stressed samples. CIEP

revealed no consistent changes in the concentration of individual plasma proteins, and was not reproducible.

INTRODUCTION

As the fight or flight response appears to have been selected to help an organism respond to potentially dangerous situations, we predicted that it should include enhancements that help the organism to deal with and to survive the potential invasion of pathogens and damage to tissue that may follow acute stress (Demers, 1993; Demers and Bayne, 1994). Such enhancement should be evident as increases in the concentration of particular plasma proteins. The rainbow trout (*Oncorhynchus mykiss*) is an evolutionarily old lineage of vertebrates and seems to rely more heavily than more recently evolved taxa on innate defenses to withstand insult (Murai, et al, 1990; Sakai, 1992). Knowledge of the physiology of the species is quite extensive. For these reasons, and because it is readily available, it was chosen as the subject for our studies.

Previous work (Demers, 1993; Demers and Bayne, 1994) demonstrated that four of twelve rainbow trout plasma proteins, measured by crossed immunoelectrophoresis, increased in concentration after confinement of the fish in a shallow bucket of water following a brief aerial exposure. One of the proteins that increased was identified as complement component C3. In that study, plasma samples from five individual trout were pooled from each of four groups sampled before, or five, ten or twenty minutes after initiation of

the handling stressor. CIEP gels of the pooled plasmas were evaluated by measuring the areas under the peaks formed when the antigens in the trout plasma were precipitated by antibodies in a polyclonal antiserum. The pooling was done because preliminary gels revealed extensive variation among "identical" samples. It was possible that a single fish in any one of the pooled samples could have been the source of the detected changes in plasma protein concentrations. Therefore, in this current study, the goal was to evaluate more individual fish and do CIEP on individual samples. The analysis also included further characterization of trout plasma samples from resting or stressed trout including plasma catecholamine levels and plasma lysozyme activity and hemolytic capacity. This experiment was designed to test the hypothesis that innate defenses are enhanced after a brief handling stress in individual fish, and to expand that characterization among individual trout.

MATERIALS AND METHODS

Fish

Shasta strain rainbow trout (*Oncorhynchus mykiss*) were raised at the Food Science and Technology laboratory in Corvallis, Oregon. Two cohorts of fish were used. The offspring of adults that were spawned December, 1992 (69 fish), and fish raised from the June, 1993, spawn (18 fish). This strain of rainbow trout has been reared in the lab since 1964 with the occasional introduction of wild caught trout to increase the genetic diversity of the broodstock. The trout were maintained on a 12 hour light/dark cycle, in 400

liter flow-through tanks supplied with approximately 12 liters well water per minute (12°C ± 2°C) and fed daily on Oregon Test Diet (prepared at the laboratory). The fish were sampled between 07:30 and 09:30, before the daily feeding, during July of 1994. Fish weighed 65-450 g and were 12-18 months of age.

Stress regime and sampling

Ten fish were stocked in each of six 120 liter tanks. Sampling commenced after the fish had undergone a two week acclimation period. Individual tanks were used to allow three initial samples to be collected on each day. (Netting fish disturbs the fish remaining in a tank which can result in high plasma cortisol levels in subsequently sampled fish.) Three tanks were sampled on each Wednesday (A, C, and E) and the other three tanks (B, D, and F, refer to Table II.1) were sampled on Fridays. The first fish netted and killed by a quick blow to the head (cerebral concussion) from each tank was called the initial sample. The time taken to capture, concuss and bleed each fish was recorded. Then, three fish from each tank were netted and held in the air together for 30 seconds before being placed in a shallow bucket of water. After 5, 10 or 20 minutes, individual stressed fish were captured from the shallow bucket, killed by cerebral concussion, and bled from the caudal vessel into a heparinized syringe. Blood samples were kept on ice until centrifuged for 10 minutes (at 4°C) at 400g to separate the plasma from the cells. Aliquots of plasma were analyzed within an hour after collection for lysozyme activity and hemolytic capacity, or immediately frozen at -80°C in siliconized microfuge tubes for later CIEP analyses and quantitative determinations of catecholamine and cortisol levels.

Lysozyme activity

A turbidmetric assay was used to determine lysozyme activity (Ellis, 1990). Dilutions of hen egg white lysozyme (HEWL, Sigma, St. Louis, MO) were prepared fresh daily from a frozen aliquot of a standard solution (1 mg/mL) using a 0.1M phosphate/citrate buffer pH 5.8. Dilutions of the standard or test plasma (25µL) were placed into each well of a 96 well plate in triplicate. One hundred seventy-five microliters of a 0.075% (wt:v) *Micrococcus lysodeikticus* (Sigma, St. Louis, MO) suspension prepared in a 0.1M phosphate/citrate buffer pH 5.8 was then added to each well. The change in turbidity was measured every 30 seconds for 5 minutes at 450 nm. Softmax ™ computer application provided equivalent units of activity of test sera as compared to the HEWL. A sample of lysozyme data and analysis is in Appendix I.

Hemolytic activity

A 0.5% (v:v) suspension of rabbit red blood cells (RaRBC's, obtained from Laboratory Animal Resources, Oregon State University) in Hanks Balanced Salt Solution (HBSS, Sigma, St. Louis, MO) was prepared using RaRBC's that had been previously washed and re-suspended in phosphate buffered saline (PBS) pH 7.4. A serial two-fold dilution of each test serum (in triplicate), with a final volume of 100µL for each dilution, was prepared using HBSS. Then 100 µL of the RaRBC suspension was placed in each well of the 96 well plate. The plate was shaken for 10 seconds and incubated at room temperature, approximately 19°C. After 1 hour, the plate was centrifuged at 400 g for 10 minutes at 4°C to sediment erythrocytes. One hundred microliters of the supernatant from each well were transferred to a new

microtiter plate and the optical density was determined at 540 nm for each well. Wells containing red blood cells and distilled water were used to obtain values for 100% lysis, while wells containing RaRBC's in HBSS buffer were used as 0% lysis controls. The reciprocal value of the percent plasma that lysed 50% of the RaRBC's was calculated and recorded as Lytic 50. A sample analysis is provided in Appendix II.

Cortisol

Cortisol levels were analyzed by radio immunoassay (Redding et al, 1984) by the laboratory of Dr. Carl Schreck, Department of Fisheries and Wildlife, Oregon State University.

Catecholamines

Catecholamine levels were determined using high pressure liquid chromatography (HPLC) with electrochemical detection (Woodward, 1982). One milliliter of 0.1M aqueous perchloric acid (PCA, Sigma, St. Louis, MO) was added to each thawed plasma sample (100µL). Dihydroxybenzylamine (DHBA) was added as an internal standard (50 µL of a 4.5 nmol/mL solution was added to each sample). After a brief vortex, the samples were centrifuged at 8000 g at 4°C. The supernatant was transferred to a clean microfuge tube containing 10 mg acid washed alumina (Sigma), 25 µL 5 mM sodium metabisulfite and 25 µL 10% (wt: v) EDTA (Sigma, St. Louis, MO). Six hundred microliters of Tris buffer (2.0 M) pH 8.0 was added and the samples were vigorously vortexed. The catecholamines were absorbed onto the alumina for 30 minutes, during which time the tubes were incubated at room temperature on a rocking platform. The samples were then washed 3 times

with 1 mL chilled nanopure water with 2 minute centrifugation at 2000 g between each wash. After the final wash, the catecholamines were eluted from the alumina with 200 µL 0.1M PCA. Ten microliters of this suspension were injected into the HPLC apparatus. The levels of adrenaline and noradrenaline were calculated by the relative areas of their peaks compared to the peak area for the internal standard (DHBA). Sample analysis is provided in Appendix III.

Crossed Immunoelectrophoresis

Two dimensional immunoelectrophoresis (Weeke, 1973) was performed in 1% medium EEO agarose (FMC) prepared using a 0.2M Tris-Barbital buffer pH 8.6 using an LKB (Multiphor - 2117) apparatus. The same buffer was used as a conducting solution. Plasma was removed from -70°C storage and thawed immediately prior to analysis. Ten microliters of each sample were electrophoresed. The first dimension electrophoresis was run for two hours at approximately 10 V/cm. A 1.5 cm X 8 cm gel strip containing the separated antigens was cut from the first dimension gel and transferred to an 8 cm X 8 cm glass plate. Nine milliliters of 1% agarose containing 10% rabbit anti-serum to trout plasma proteins (Demers, 1993) were cast on the second dimension plate and allowed to solidify. The second dimension was run for 22 hours at approximately 2V/cm. All steps were carried out in a cool room (10°C) using a re-circulating cooling plate (5°C). Samples were chosen randomly for each run. A total of 180 CIEP gels were run on 60 separate days to visualize in triplicate all the plasma samples. Samples used for CIEP were a subset that excluded non-sibling fish (18 fish) and mature males, since the amount of anti-serum available was limiting.

The gels were rinsed, dried, and stained with Coomassie Brilliant Blue. The area under each peak was measured using the JAVA image analysis system.

Statistics

An initial model fit which included sex and size was run which verified that there were no confounding variables. An analysis of variance (ANOVA) was performed using SAS software after natural log transformation of some data was used to achieve homogeneity of variance. Values were considered significant if $p<0.05$.

RESULTS

A total of 96 fish were sampled from July 1 through July 27, 1994. The weight of the fish ranged from 65 grams to 450 grams. Insufficient blood was collected from some of the smaller fish to perform all the assays. In the limited samples, priority was given to analysis of lysozyme and hemolytic activity. Two fish were sexually mature males. The remaining fish, of both sexes, were immature but it was possible to distinguish the sex by observing the gonads. Twenty-one fish were removed from the data analysis because they were unduly stressed, in the initial samples as indicated by cortisol levels, or were unhealthy.

All components varied among individual fish. The variation was dramatic in the case of lysozyme and hemolytic activity. There was a trend of increasing lysozyme activity after ten minutes stress, and a decrease in

lysozyme activity to near initial values by 20 minutes after initiation of the stressor. The differences were not significant. Plasma cortisol was significantly increased in stressed fish plasma ($p=0.001$). The levels were similar to those obtained when another cohort of fish was similarly stressed (Demers, 1993; Demers and Bayne, 1994). Plasma catecholamine levels were similarly elevated within five minutes of the acute handling stressor. CIEP analysis revealed no consistant increases. Details of each analysis are described below.

Lysozyme activity

The level of lysozyme avtivity in fresh trout plasma ranged from 2.40 to 8.76 equivalent units (μg/mL) of hen egg white lysozyme activity (Table II.1). The mean lysozyme level in the initial sample was 4.70 ± 0.40 μg/mL (mean $\pm$ SE). In the group bled after 30 seconds in the air and five minutes confinement in a shallow bucket, the lysozyme level was 4.67 ± 0.38 μg/mL. In the group bled after 10 minutes confined in the shallow bucket, the mean level of lysozyme activity in the plasma was 5.08 ± 0.31 μg/mL. After 20 minutes in the shallow bucket, the mean level was 4.38 ± 0.27 μg/mL. There was variation within groups and no significant differences were found at any of the times (Figure II.1).

Table II.1 Data from individual rainbow trout plasma on the immediate effects of stress on innate immunity.

sample date	fish code	weight (g)	sex	Lysozyme (μg/mL)	Lytic 50 §	[Cortisol] (ng/mL)	[NA] (pmol/mL)	[A] (pmol/mL)	bleed time §§ (seconds)
Initial samples *									
7/1/94	AR:1	224.30	M	4.75	1.81	5.40	354.11	1751.45	140
7/1/94	CR:1	281.40	M	6.21	1.30	5.70	56.91	214.72	155
7/1/94	ER:1	145.40	F	5.83	1.54	1.60	318.02	970.09	95
7/6/94	BR:1	432.70	M	3.05	2.30	6.30	111.68	458.10	108
7/6/94	FR:1	72.30	F	2.40	1.16				
7/8/94	CR:2	299.20	F	2.90	2.46	3.20	130.66	311.81	71
7/8/94	ER:2	178.80	M	8.53	2.31	7.30	343.13	721.97	60
7/13/94	BR:2	209.20	M	3.94	1.98	7.10			90
7/13/94	DR:2	280.00	F	8.76	4.37	4.00	<	<	48
7/15/94	CR:3	151.90	M	4.76	2.15	8.00	94.20	598.56	48
7/15/94	ER:3	67.50	F	2.82	1.94	1.90	667.88	2445.48	68
7/29/94	AR:4	246.20	M	4.48	2.16	2.20	148.52	582.48	54
7/29/94	CR:4	144.70	M	4.36	2.33	4.20	357.40	1274.78	52
7/29/94	ER:4	233.60	M	3.04	1.95	1.60	164.02	384.48	54
7/27/94	BR:4	258.60	F	4.85	2.51	5.70	348.22	1804.26	61
7/27/94	FR:4	159.20	F	4.60	2.20	1.00	193.79	425.55	44
average				*4.70*	*1.99*	*4.35*	*234.89*	*853.12*	
5 minute samples **									
7/1/94	AS5:1	274.30	F	6.47	2.52	122.20	652.96	4017.46	110
7/1/94	CS5:1	119.80	M	5.37	1.95	55.30	750.81	5288.94	93
7/1/94	ES5:1	213.00	F	5.40	1.73	174.70	519.93	2161.29	90
7/6/94	BS5:1	257.10	F	3.54	2.15	139.20	168.05	1689.66	
7/6/94	DS5:1	161.00	M	6.77	1.42	106.00	5135.80	8272.67	185
7/8/94	AS5:2	222.90	M	3.41	3.11	17.90	804.91	3891.02	82
7/8/94	CS5:2	185.20	F	2.68	2.83	49.30	150.10	1030.97	83
7/13/94	BS5:2	370.90	M	3.97	0.90	30.70	<	348.58	50
7/13/94	DS5:2	183.60	F	7.72	2.50	49.10	<	382.43	50
7/13/94	FS5:2	126.80	F	3.37		160.50	76.97	756.99	62
7/15/94	AS5:3	321.30	F	5.28	3.37		103.62	599.72	53
7/15/94	CS5:3	110.50	F	3.98	2.15	134.90	140.71	651.41	74
7/15/94	ES5:3	115.90	M	2.96	1.76	165.60			63
7/20/94	BS5:3	145.90	M	3.64	2.59		208.90	879.70	87
7/29/94	AS5:4	126.50	F	4.38	2.26	90.50	178.19	1830.80	46
7/29/94	ES5:4	125.20	F	5.92	2.84	22.40	496.60	1931.65	67
7/27/94	BS5:4	75.00	M	3.44	2.05	32.90	406.81	2302.32	82
average				*4.67*	*1.94*	*90.08*	*684.67*	*2343.73*	

* Initial samples were from fish netted, concussed and then bled immediately.

** Samples were from fish netted, held in the air for 30 seconds, confined in a shallow bucket of water for five minutes and then captured, concussed and bled.

§ Lytic 50 is the reciprocal of the percent plasma required to lyse 4.5 X 10^5 rabbit red blood cells.

§§ Indicates the time taken to capture, concuss and bleed each fish.

< indicates catecholamine levels less than the HPLC could detect.

Table II.1 Data from individual rainbow trout plasma
on the immediate effects of stress on innate immunity (continued).

sample date	fish code	weight (g)	sex	Lysozyme (µg/mL)	Lytic 50 §§	[Cortisol] (ng/mL)	[NA] (pmol/mL)	[A] (pmol/mL)	bleed time § (seconds)
10 minute samples ***									
7/1/94	AS10:1	196.10	F	5.49	2.15	52.60	605.99	2211.85	125
7/1/94	ES10:1	165.40	F	7.47	1.17	186.60	2732.35	14867.10	120
7/6/94	BS10:1	246.40	M	4.12	1.92	190.30	333.61	1108.47	136
7/6/94	DS10:1	271.80	M	4.12	1.88	92.60	217.93	1413.75	
7/6/94	FS10:1	80.80	M	4.95	2.28	137.50			
7/8/94	AS10:2	264.30	F	3.96	2.21	151.00			79
7/8/94	CS10:2	230.20	M	4.19	3.55	110.60			87
7/13/94	BS10:2	222.60	F	3.52	1.97	151.70	301.66	3329.93	72
7/13/94	DS10:2	193.90	F	7.61	2.17	114.60	183.05	1644.09	44
7/13/94	FS10:2	178.80	F	5.19	2.22	65.90	516.65	7693.85	102
7/15/94	AS10:3	267.40	F	4.99	2.43	145.70	232.12	872.59	84
7/15/94	CS10:3	124.60	M	3.24	2.06	82.80	358.63	1486.79	57
7/15/94	ES10:3	123.30	F	3.86	1.49	136.80	456.70	1258.70	66
7/20/94	BS10:3	113.20	F	3.99	2.25		171.97	653.43	62
7/20/94	FS10:3	62.00	F	3.34	2.08		499.22	2077.04	68
7/29/94	AS10:4	217.40	M	7.50	2.09	134.90	469.38	835.55	36
7/29/94	ES10:4	125.00	F	4.99	3.75	154.70	295.46	1711.67	51
7/27/94	BS10:4	143.40	F	5.75	1.74	121.80	93.25	158.70	40
7/27/94	DS10:4	43.50	M	5.38	0.89	31.20			46
7/27/94	FS10:4	91.00	F	5.09	2.58	67.70	395.57	1283.28	54
average				*5.08*	*2.09*	*118.27*	*513.74*	*2848.31*	
20 minute samples ****									
7/1/94	AS20:1	312.30	F	6.47	1.96	146.50	1128.40	8537.59	221
7/1/94	CS20:1	302.80	F	5.46	1.20	185.60	844.65	3133.34	202
7/1/94	ES20:1	140.80	F	6.14	1.04	198.00	761.81	2629.02	194
7/6/94	BS20:1	348.90	M	3.83	2.52	28.10	206.32	2476.48	
7/6/94	DS20:1	146.60	F	5.25	0.52	105.30	315.98	439.01	
7/6/94	FS20:1	91.90	M	3.05	1.94		1060.37	2887.03	145
7/8/94	AS20:2	196.20	M	3.06	2.11	124.70			116
7/8/94	CS20:2	192.90	M	3.17	2.46	175.60	427.77	914.58	91
7/8/94	ES20:2	104.80	F	2.65	1.65	140.50			75
7/13/94	BS20:2	329.70	F	5.22	1.71	126.50	276.91	1575.23	57
7/13/94	FS20:2	88.60	M	4.36	2.07	189.30	2639.16	7446.91	75
7/15/94	CS20:3	181.80	M	3.27	1.52	169.50	3072.29	8411.58	63
7/15/94	ES20:3	87.50	F	3.63	1.61	181.50	2040.03	3509.84	53
7/20/94	BS20:3	26.50	M	4.02					77
7/29/94	CS20:4	80.60	F	4.42	1.00		968.90	871.69	50
7/29/94	ES20:4	137.20	M	3.88	1.98	128.80	815.22	2863.73	96
7/27/94	BS20:4	80.80	M	4.97	1.88	140.70	2289.31	6621.70	111
7/27/94	DS20:4	98.00	M	4.24	2.15	180.00	3987.81	6879.80	114
7/27/94	FS20:4	98.40	F	5.73	4.02	202.70	2899.64	2969.99	41
average				*4.38*	*1.83*	*151.46*	*1483.41*	*3885.47*	

*** Samples were from fish netted, held in the air for 30 seconds, confined in a shallow bucket of water for ten minutes and then captured, concussed and bled.

**** Samples were from fish netted, held in the air for 30 seconds, confined in a shallow bucket of water for 20 minutes and then captured, concussed and bled.

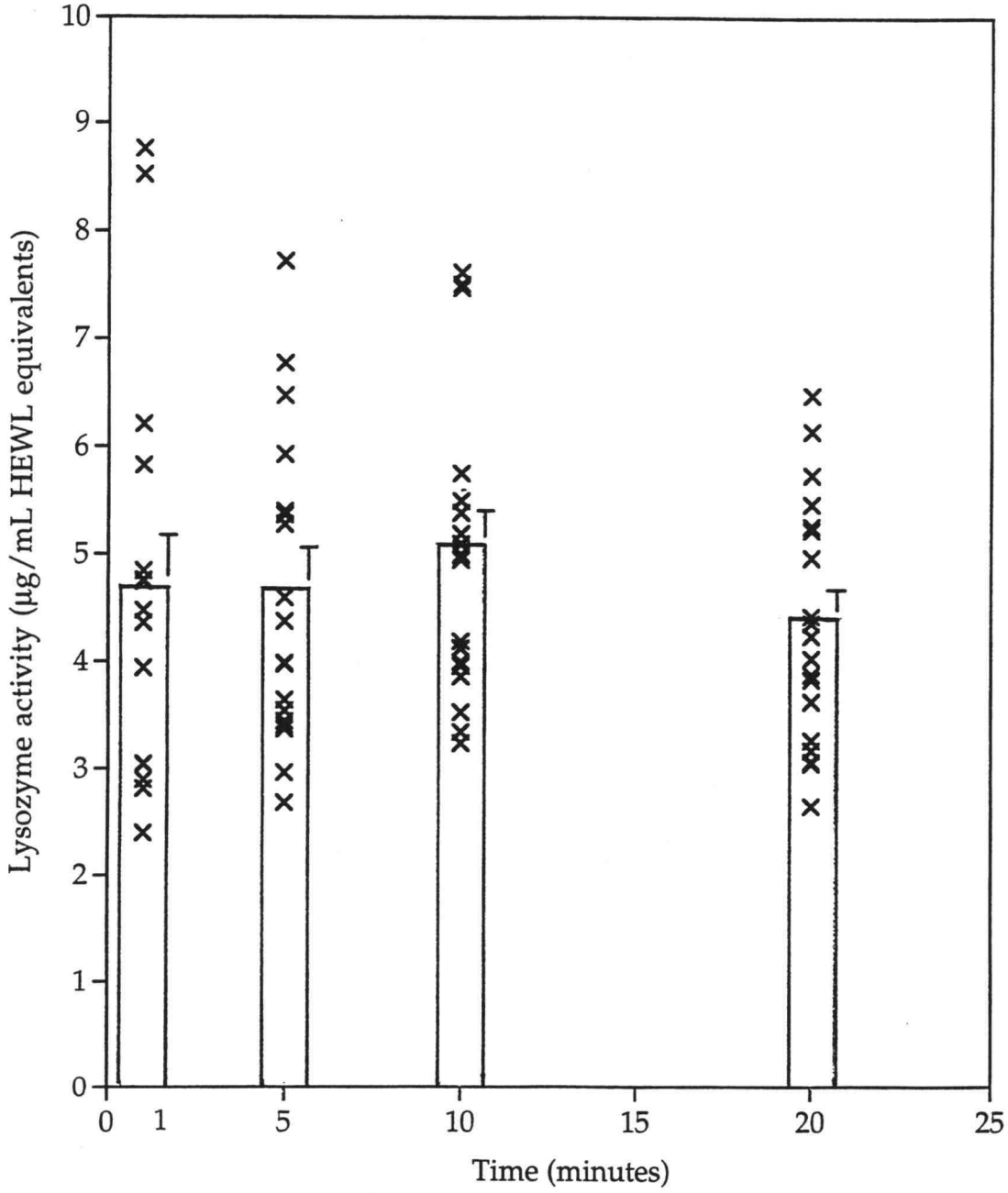

Figure II.1 Plasma lysozyme activity of trout bled quickly without an overt stress or bled after a handling stresssor. Fish were bled immediately after being netted and concussed (1, n=16), or they were bled after being netted, held in the air for 30 seconds, and then placed in a shallow bucket of water for 5 (n=17), 10 (n=20) or 20 (n=19) minutes, then recaptured and concussed. Bars represent mean ± SE.

Hemolytic activity

Median Lytic 50 values for resting, 5, 10 and 20 minute stressed groups were 1.99 ± 0.15 (mean + SE), 1.94 ± 0.18, 2.09 ± 0.14, and 1.83 ± 0.16 respectively (Figure II.2). Trout plasma lysed rabbit red blood cells with Lytic 50 values from 0.52 to 4.37. A natural log transformation was required to normalize the distribution of the data. Although there is an increased activity at 10 minutes and a decrease at 20 minutes, the variation among individuals was large and no significant differences were detected between experimental groups. One fish from the initial group, fish DR:2 had an unusually high hemolytic capacity. Several other fish in the stressed group had high hemolytic capacity; AS5:2, AS5:3, CS10:2, ES10:4 and FS20:4.

Cortisol

Cortisol levels ranged from 1 ng/mL in initial plasma samples to 202.7 ng/mL in fish stressed 20 minutes (Figure II.3). The mean value for resting fish plasma was 4.35 ± 0.60 ng/mL (mean ± SE). The mean plasma cortisol concentration of the five minute group was 90.08 ± 14.58 ng/mL. The mean cortisol level rose to 118.27 ± 10.59 ng/mL by ten minutes after initiation of the handling stressor, and further increased to 151.46 ± 11.08 ng/mL after twenty minutes. Significant increases from resting levels were evident in all stressed fish after log transformation of the data ($p = .0001$).

The rate of cortisol accumulation calculated from the mean cortisol levels at various times after the stressor were as follows: From the initial to the five minute sample, the rate of cortisol accumulation was 17.15 ng/mL/min. Using the difference of the five minute mean and the 10 minute mean, or the difference of the ten minute mean and the 20 minute

mean, rates of increase of plasma cortisol of 5.63 ng/mL/min and 2.82 ng/mL/min were calculated for the intervals mentioned.

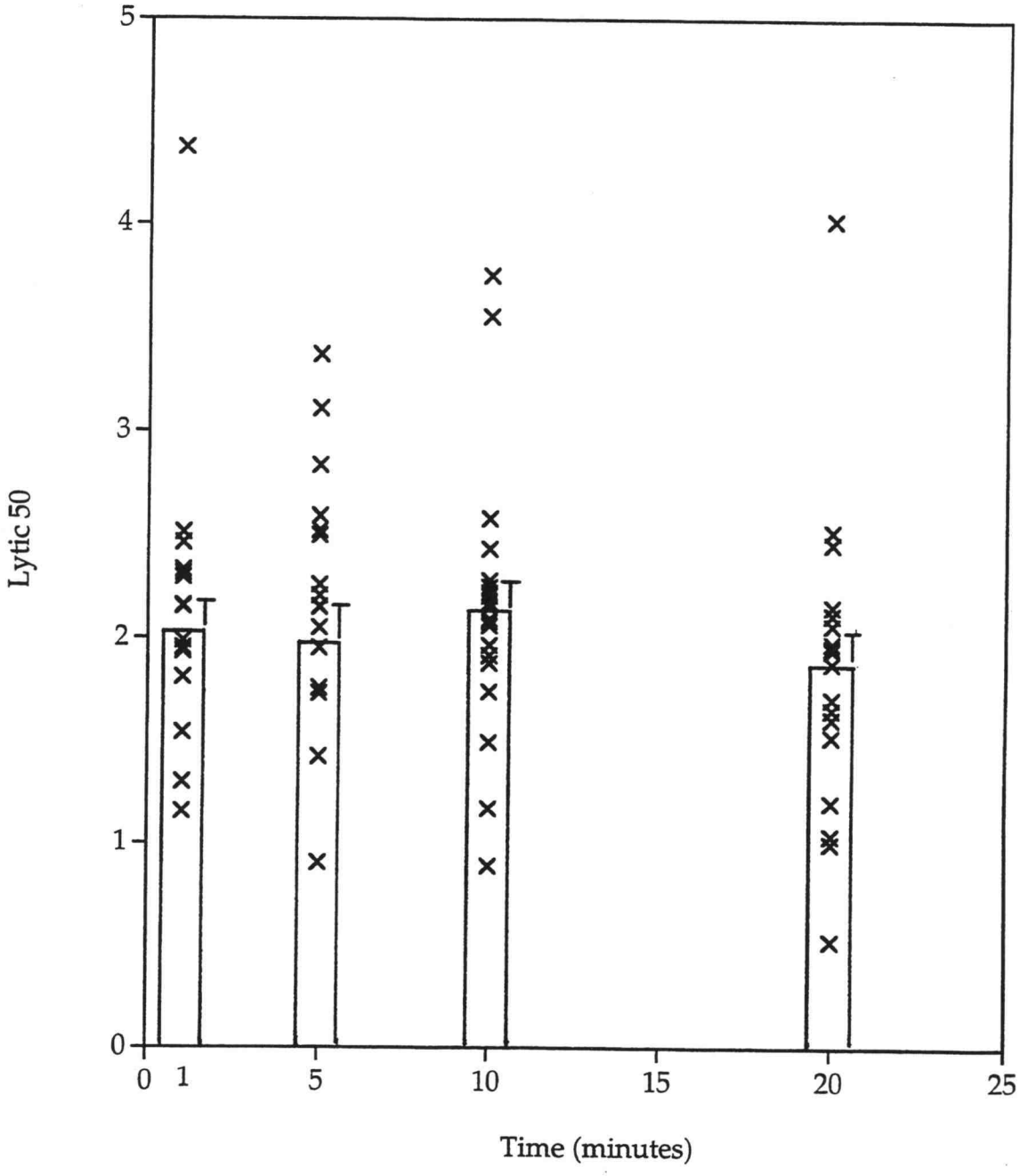

Figure II.2 Hemolysis of rabbit red blood cells (Lytic 50) by trout plasma samples obtained without an overt stress or bled after a handling stressor. Fish were bled immediately after being netted and concussed (1, n=16), or they were bled after being netted, held in the air for 30 seconds, then placed in a shallow bucket of water for 5 (n=16), 10 (n=20) or 20 (n=18) minutes, then recaptured and concussed. Bars represent mean ± SE.

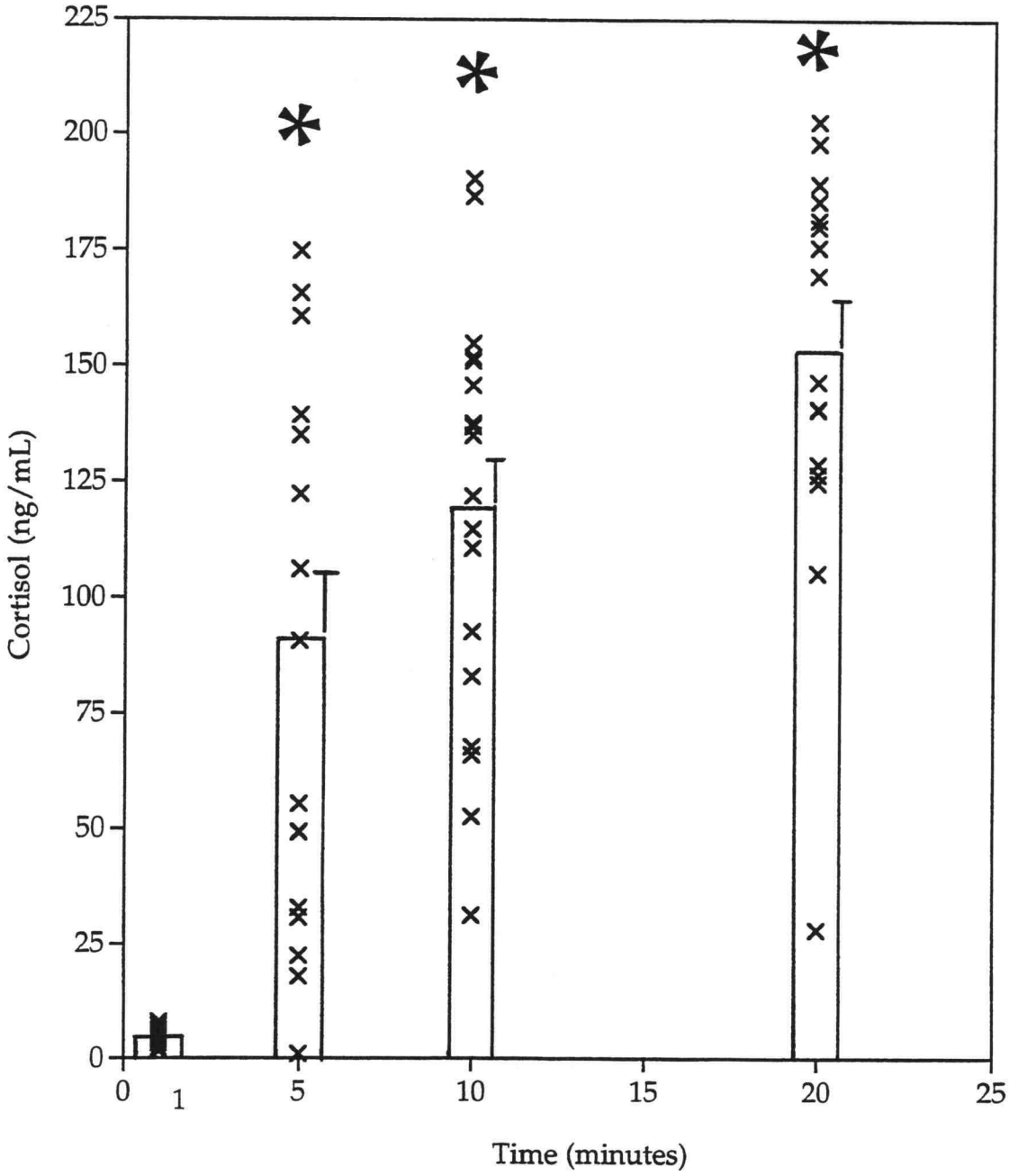

Figure II.3 Plasma cortisol concentrations of trout bled quickly without an overt stress or bled after a handling stressor. Fish were bled immediately after being netted and concussed (1, n=15), or they were bled after being netted, held in the air for 30 seconds, and then placed in a shallow bucket of water for 5 (n=15), 10 (n=18), or 20 (n=16) minutes, then recaptured and concussed. Bars represent mean ± SE. * indicates significant difference from initial levels.

Noradrenaline

Noradrenaline (NA) levels varied from undetectable to 5.14 μmol/mL (DS5:1, Figure II.4A). They were significantly higher in fish sampled after 5 minutes than in resting fish, increasing from 234.89 ± 46.59 pmol/mL in the initial samples to 684.67 ± 349.67 pmol/mL in the five minutes group ($p=0.0016$). There was a significant increase over the five minute value at the 20 minute time period, when noradrenaline was 1483.41 ± 293.01 pmol/mL. Unusually high NA levels were noted in fish DS5:1, ES10:1, and FS20:2.

Adrenaline

Levels of adrenaline in resting plasma samples ranged from undetectable (DR:2) to 2445.5 pmol/mL (ER:3). The mean level of adrenaline in the initial group was 853.12 ± 190.15 pmol/mL. Levels rose to 2343.73 ± 568.59 pmol/mL at the five minute sample and rose to 2848.31 ± 1044.12 pmol/mL by the 10 minute sample. Twenty minutes after the aerial exposure and confinement in the bucket, the plasma adrenaline level was 3885.47 ± 687.81 pmol/mL. Several fish in the five and ten minute groups, and 5 fish in the 20 minute group had high adrenaline levels. Significant differences ($p=.0008$) between resting and stressed levels (Figure II.4B) were evident after the data had been transformed to normalize their distribution. Adrenaline levels increased over resting values at 5 minutes, and there were no further statistically significant increases at the later time points.

Using methods similar to those used to calculate the rate of accumulation of cortisol, noradrenaline accumulated at a rate of 89.96 pmol/mL/min over the first five minutes.

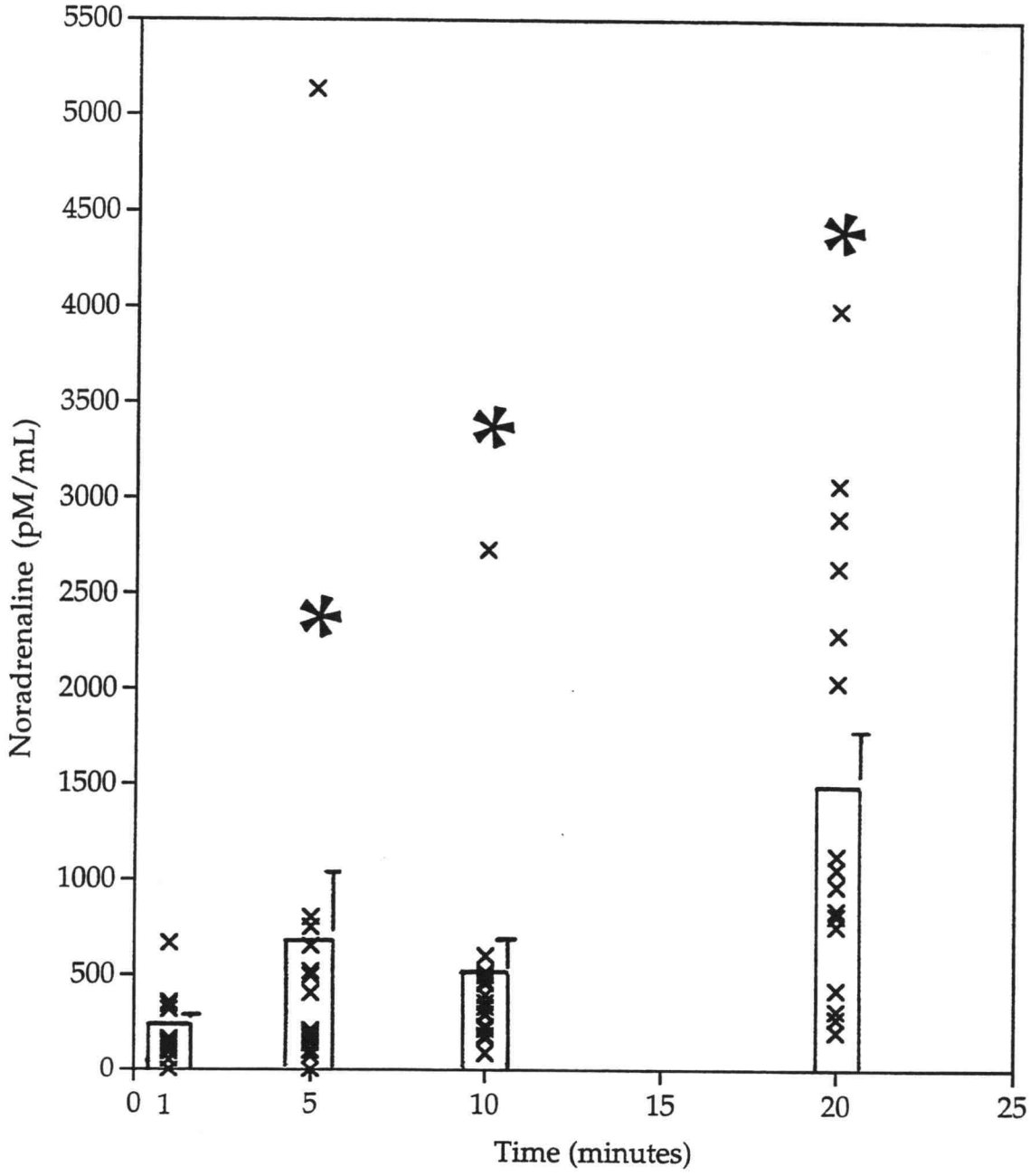

Figure II.4A Plasma noradrenaline levels in trout bled quickly without an overt stress or bled after a handling stressor. Fish were bled immediately after being netted and concussed (1, n=16), or they were bled after being netted, held in the air for 30 seconds, and then placed in a shallow bucket of water for 5 (n=16), 10 (n=13) or 20 (n=14) minutes, then recaptured and concussed. Bars respresent mean $\pm$ SE. * indicates significant difference from initial level.

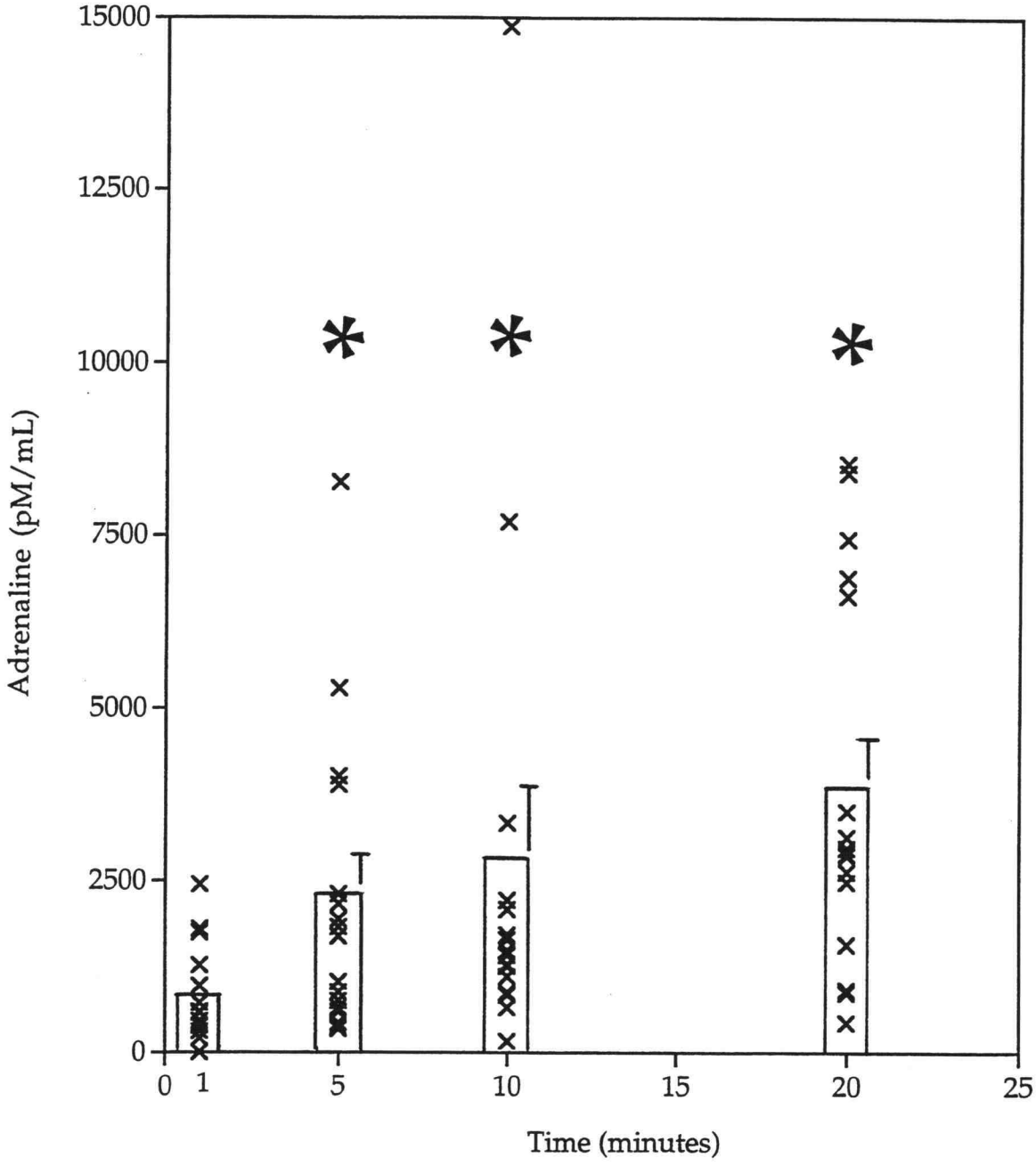

Figure II.4B Plasma adrenaline levels of trout bled quickly without an overt stress or after a handling stressor. Fish were bled immediately after being netted and concussed (1, n=16), or they were bled after being netted, held in the air for 30 seconds, and then placed in a shallow bucket of water for 5 (n=16), 10 (n=13) or 20 (n=16) minutes, then recaptured and concussed. Bars represent mean ± SE.
* indicates a significant difference from initial levels.

Adrenaline levels increased at the rate of 298.12 pmol/mL/min during the same interval. From five to ten minutes, the rate was -34.18 pmol noradrenaline/mL/min and 100.92 pmol adrenaline/mL/min.

A closer analysis of catecholamine levels when compared with the amount of time taken to capture and bleed each fish revealed that increases in catecholamines were correlated with the time taken to bleed the fish in the five and ten minute stressed fish (Figure II.4C). There was a linear relationship between the time take to capture, concuss and bleed the fish and catecholamine levels. This correlation was evident at five and ten minutes for both noradrenaline and adrenaline, but was not in the initial group of fish nor in the 20 minute group.

The rate of change within the described group (Figure II.4C) was calculated to be 1950.9 pmol noradrenaline/mL/min when calculated using the five minutes group data but only 557.52 pmol noradrenaline/mL/min when calculated using the ten minutes group data. The rate of adrenaline accumulation was 3350.6 pmol/mL/min when calculated using the five minutes group data and 3567.0 pmol/mL/min when calculated using the ten minutes group data.

Crossed Immunoelectrophoresis

Replicate gels of the same plasma sample sometimes failed to give identical patterns. Statistical analysis of the data revealed no significant differences in peak height by stress time.

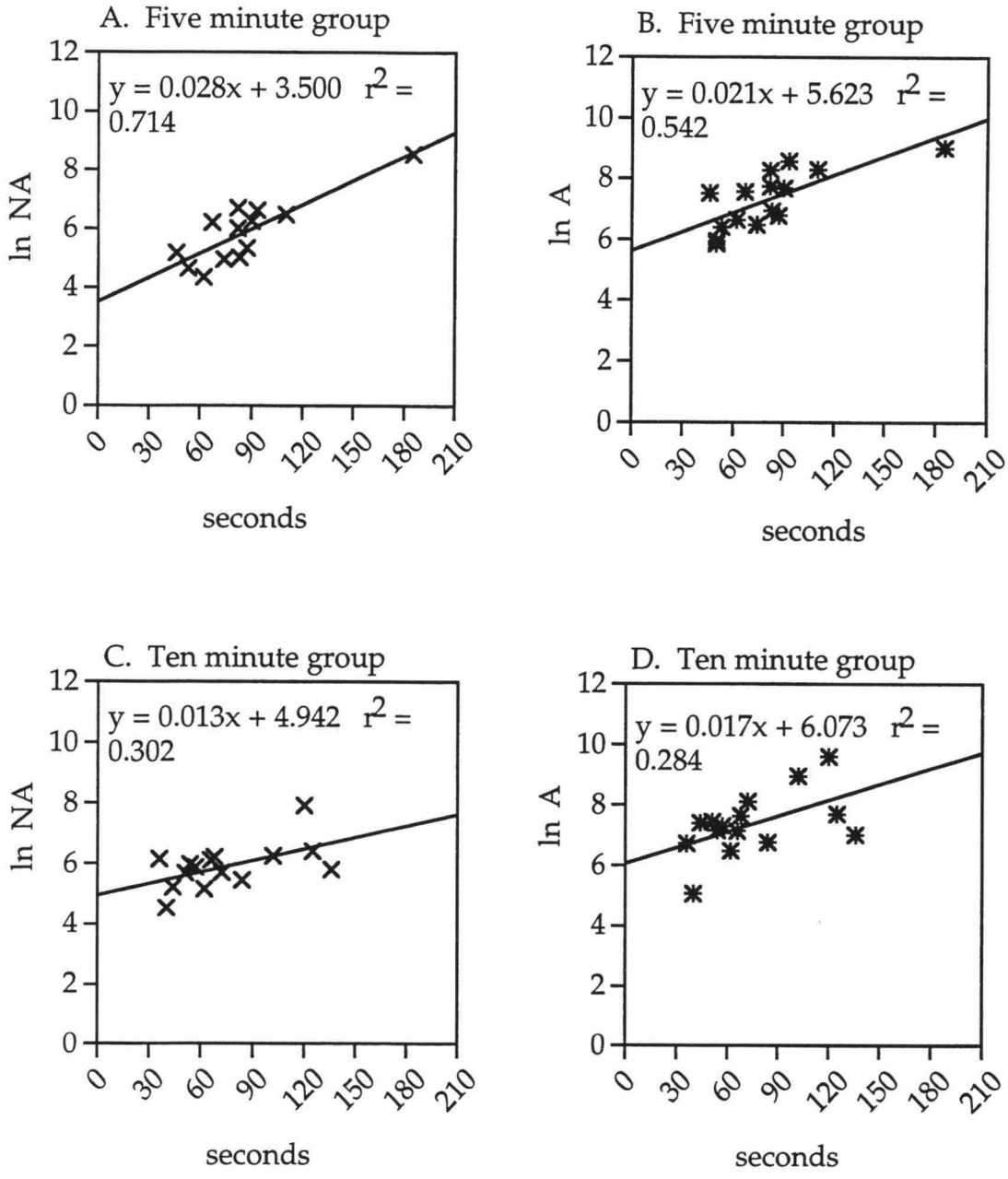

Figure 11.4C Catecholamine levels in trout plasma compared to the time taken to bleed the fish. These figures (A-D) illustrate the time it took to capture, concuss and bleed the animals of the 5 and 10 minute groups versus the natural log of noradrenaline (ln NA) and adrenaline (ln A).

DISCUSSION

Cortisol and catecholamines were elevated within minutes of the handling stressor (see below). Although the differences in the plasma lysozyme activity between the initial and stressed fish samples were not significant, the mean value of lysozyme activity was highest at ten minutes after initiation of the stressor. Two fish in the initial group, ER:2 and DR:2, had unusually high lysozyme levels, but there were no noteworthy comments recorded at the time of bleeding that might explain this. An increase until 10 minutes and then decreased activity by the twenty minute time point is the same pattern that was evident in three of the four plasma proteins previously found to increase after the same handling stressor (Demers, 1993; Demers and Bayne, 1994). Variation within groups was such that changes due to the stressor were not significant. If plasma lysozyme activity does change as a result of an acute handling stressor, a larger sample size or other experimental modifications may be required to detect significant differences. The fish population sampled was genetically diverse and had been raised in the laboratory for only 25 generations, with intentional outbreeding. A more inbred population would probably provide lesser amounts of variation.

Hemolytic activity was highly variable among individuals regardless of the stressor imposed. However, the mean level was highest at ten minutes and dropped to resting levels by twenty minutes after the initiation of the stressor. The stressed fish Lytic 50 levels were not significantly elevated over resting levels, perhaps due to the variation. One factor that may have contributed to variance in the data is that the suspension of rabbit red blood cells used on separate days did not contain exactly the same number of cells.

Rabbit red blood cell counts ranged from 4.2×10^7 cells/mL to 6.1×10^7 cells/mL. When each day's samples were analyzed independently, there were still no significant differences among experimental groups.

Fish plasma cortisol levels rose significantly within five minutes of an acute handling stressor. Levels of plasma cortisol were similar to those previously recorded (Barton and Iwama, 1991; Demers and Bayne, 1994). There is one published report which calculated a rate of plasma cortisol increase of about 5 ng/mL/min in juvenile chinook salmon (*Oncorhynchus tshawytscha*) similarly handled (Strange and Schreck, 1977). The data reported here are more similar to values reported for juvenile walleye, a coolwater teleost (*Stizostedion vitreum,* Barton and Zitzow, 1995). In that study, the rate of increase of plasma cortisol was calculated as being at least 18 ng/mL/min. That rate was calculated using data from a sampling 15 minutes after the initiation of the stressor. It appears that the rate of increase of plasma cortisol among some teleosts, including rainbow trout, may be at least 15 ng/mL/min. According to the data reported here, the rate of **accumulation** of plasma cortisol is fastest, 17.15 ng/mL/min, within the first five minutes of handling. The rate of accumulation slowly decreased to 2.82 ng/mL/min in the ten to twenty minutes interval after stress.

The fact that these rates are calculated from levels in different individuals an each time point should not be overlooked. These calculations are from levels at discrete times and do not account for metabolism or removal from the bloodstream. Furthermore, it may be that there is an immediate release of a prestored pool of hormones and then further synthesis and release following the event.

Catecholamine levels also increased over resting levels after the acute handling and confinement stressor. Adrenaline was the major plasma

catecholamine in trout plasma, as previously described (Nakano and Tomlinson, 1967; Barton and Iwama, 1991). In this experiment, catecholamines were elevated over published resting levels in the initial group of fish sampled after netting and concussion. This is not surprising. "Resting" levels of catecholamines in trout have been attained only by sampling *via* an indwelling catheter (Perry, et al, 1989; Barton and Iwama, 1991; Randall and Perry, 1992; Gamperl, et al, 1994). Cannulation is a stressor we did not want to impose on these fish since it would likely have influenced the plasma protein profile by inducing an acute phase response. Most published values for stressed trout plasma catecholamine levels are at least an order of magnitude lower than those recorded in this experiment (Perry, et al, 1989; Barton and Iwama, 1991; Randall and Perry, 1992; Gamperl, et al, 1994a, 1994b). However there is one report using Coho and Sockeye salmon where the levels of catecholamines in the plasma were elevated following a 10 minutes aerial exposure to levels as high as the levels reported here (Mazeaud and Mazeaud, 1977).

There are at least two possible reasons for the high catecholamines measured here. First, the stressors these fish underwent - air exposure and cerebral concussion - were more severe than the stressors of hypoxia and hypercapnia that are used in the bulk of published research on catecholamine levels in trout plasma (Perry, et al, 1989; Barton and Iwama, 1991; Randall and Perry, 1992; Gamperl, et al, 1994a, 1994b). Second, it is possible that the sampling schedule used in this experiment yielded blood in which catecholamines more closely reflected their maximum levels. Catecholamines not only increase rapidly in the blood, but also fall rapidly, due to clearance and metabolism. In this study, samples were taken within seconds of the stressor. Winder and Yang (1987) showed that plasma

catecholamines were decreased in rat plasma within two minutes of the end of the stressor. This fact makes it imperative that studies designed to measure acute effects sample immediately following the stressor. In this experiment the short-lived catecholamines were probably sampled before significant degradation and removal from the bloodstream. Of those fish with unusually high catecholamine levels, some could be ascribed to experimental conditions. Fish DS5:1, had muscle spasms while being bled. Another fish with high levels, ES10:1, however, had no comments recorded that might explain the high level.

The rate of accumulation of the plasma catecholamines follows a trend similar to that of cortisol. Concerns about calculating rates using these data are even more valid when considering the catecholamines due to their transient existence in the plasma. Valuable information is yielded, however, regarding the levels at these immediate timepoints. The initial rate of accumulation of noradrenaline and adrenaline, 89.96 pmol/mL/min and 298.12 pmol/mL/min respectively, were higher than rates calculated using data collected from later time points. Although reports of plasma catecholamine levels among trout are extensive, none report a rate of increase for plasma catecholamines within minutes of a handling stressor. The modulation of hormone levels in the plasma is effected on a time scale of seconds. Studies characterizing the response, therefore, need to utilize similar intervals for sampling.

The speed with which catecholamine levels change in the plasma when an animal is stressed is emphasized by the correlation found between catecholamine levels and the time taken to bleed the fish. The correlation was evident in only the five and ten minute groups, however. The correlation was probably due to the stress perceived by the fish when it was

being captured from the shallow bucket causing another adrenergic surge. Why the rate of increase was not correlated with time to bleed in initial and 20 minute samples is unclear. What is clear is that plasma catecholamine concentrations are a very sensitive and responsive indicator of stress.

Clearly the rates of accumulation of cortisol and catecholamines are not simply linear. Also, the stressors used in these experiments were several. The initial capture and aerial exposure caused a hormone surge. There could have been some clearance of these hormones during the confinement and another hormonal surge brought on by the stressors imposed when capturing the fish from the bucket after 5, 10 or 20 minutes. A better characterization of the rate of increase of these plasma hormones requires a more detailed study with single stressors as opposed to the complex stressors used here.

CIEP gels failed to reveal any significant differences in peak area between stressed groups. This is contrary to the results of earlier research (Demers, 1993). There are several possible explanations. First, the antiserum used here, produced by pooling the serum from 6 rabbits each of which showed 6 or more peaks in a test run, was weaker than the previous batch which was pooled from sera which showed greater than 12 peaks in the test run. That is, the antiserum used here did not recognize as many rainbow trout plasma proteins. It is possible that there were insufficient antibodies to the plasma proteins that had been found to change previously.

Secondly, in the initial study (Demers, 1993), plasma from 5 fish for each stressor group were pooled, and aliquots were run up to 12 times. In the present study, however, triplicate samples of individual fish plasma were run by CIEP. The antiserum pool was greater than 200 mL, a large quantity of antiserum, but only enough to run CIEP 60 times (three gels per run). It was decided that triplicate gels of each plasma sample would have to suffice for

the evaluation. Unfortunately, replicates of each sample did not always resemble one another.

Another possible explanation for the variation observed between "identical" samples is the plasma samples themselves. Three 15 µL aliquots were taken from each plasma sample, after they were separated by centrifugation from the cells, and placed in individual 0.65 µL siliconized microfuge tubes. It may have been better to remove a larger aliquot from the plasma originally and then put subsets of **that** aliquot into separate tubes. That is, the plasma proteins in each aliquot may have been different due to micro-topographic differences in tubes following centrifugation of the blood. Lastly, some plasma samples were so clotted that a 10 µL aliquot could not be obtained to run CIEP. The clotting may have occurred in other tubes to a lesser extent such that the sample could be obtained, but the profile may have been altered by the clotting. Clotting occurred in spite of the inclusion of heparin to prevent it. Heparin was used since the blood was also being used for hemolytic analysis, and chelators are known to interfere with the complement cascade.

CONCLUSIONS

Plasma cortisol and catecholamine levels were significantly increased within 5 minutes of an acute handling stressor in rainbow trout. Catecholamine levels in these acutely stressed fish were higher than in most previously published reports, perhaps due to the severity of the stressor and the acute time point measured. Mean levels of plasma lysozyme were

increased ten minutes after initiation of stress, then reduced at 20 minutes, as were hemolytic capacities, although not significantly. This pattern is reminiscent of three of the plasma proteins in previously described experiments.

Chapter III

The Modulation of Innate Defenses Following Acute Stress as Detected by Sequential Bleeding of Individual Rainbow Trout.

ABSTRACT

Previous research has shown that variation in plasma lysozyme and hemolytic capacity is considerable among individual rainbow trout (Chapter II). In order to determine if these plasma components, as indicators of innate defenses, were enhanced as a result of stress, experimental fish were anesthetized, bled, revived, and then stressed by being held in the air in a net for 30 seconds and placed in a shallow bucket of water for ten minutes. After the stressor, experimental fish were captured from the shallow bucket, concussed and bled again. Control fish were also bled twice, but were kept anesthetized during the 10 minutes in which the experimental fish were stressed. Plasma lysozyme activity was measured in each fish at both time points. Following 10 minutes of stress, plasma lysozyme activity was significantly increased over control fish ($p=0.016$). Catecholamine levels remained low in anesthetized fish but increased significantly in fish that were revived and then stressed. The experiment was repeated six months later with similar results. Plasma lysozyme activity was again enhanced as a result of the brief handling stressor. Hemolytic capacity was not measurably different among experimental groups. Anesthetization allowed differences in individual levels to be analyzed, but lessened hormonal responses, even in revived fish.

INTRODUCTION

The results of earlier experiments revealed such great variation between individual fish regardless of the stressor that it was necessary to modify the methodology used to measure stress-induced changes in innate defenses which we postulate should be enhanced after the fight or flight response. The previously used method to obtain "resting" fish (i.e. netting and concussion) resulted in unacceptably high plasma catecholamine levels. A new protocol was developed in an attempt to minimize variation and to reduce circulating catecholamine levels in the initial sample of fish.

In the initial experimental planning stages, literature searches revealed that difficulties have often been encountered when attempts were made to obtain repeated measures on the same fish (e.g: Hoffman and Lommel, 1984; Railo, et al, 1985; Garcia, et al, 1992; Gamperl, et al, 1994b). Salmonids and cyprinid fish that were repeatedly sampled by cardiac puncture at weekly intervals experienced mild anemia (Hoffman and Lommel, 1984). These investigators determined that their method of sampling, that is bleeding by cardiac puncture under anesthesia, produced a less severe change in the blood profile than using cannulated fish, bleeding from the ductus Cuvieri or caudal aorta. Railo et al (1985) compared values obtained from resting fish sampled *via* an indwelling cannula to those from fish sampled by cardiac puncture after being stunned by a blow to the head. They found that hematocrit was increased in stunned fish, and blood pH was decreased. Furthermore, the handling of the blood after removal from the animal also significantly affected blood ions. Hematocrit was also found to increase significantly depending on the amount of blood removed, and the size of the fish sampled (Garcia, et al, 1994).

Cannulation provides a means to serially sample the same fish without the stress of capture during each sample. However, cortisol levels in cannulated fish are elevated for at least a week (Gamperl, et al, 1994b). Previous work (Iwama, et al, 1988) showed that catecholamines were increased in stage III anesthetized fish. That is, if anesthetized until opercular movements ceased, all fish had higher catecholamine levels whether the anesthesia was MS-222, benzocaine, 2-phenoxy-ethanol (2-PE), metomidate or carbon dioxide gas. However, catecholamine levels were lowest during all stages of anesthesia and recovery when 2-PE was used as anesthetic.

The population of trout used in these studies has exhibited considerable variation in the parameters of interest (Chapter II, this dissertation). Serial bleeding of individual rainbow trout (*Oncorhynchus mykiss*) appeared to be a method which would reduce the variance within experimental groups enough to test the hypothesis of enhanced innate defenses as a component of the flight or fight response. By using remote infusion of 2-PE into aquaria to anesthetize fish to light anesthesia, a significant enhancement of plasma lysozyme activity as a result of an acute handling stressor was documented.

Alpha$_2$-mac is a known acute phase reactant in some vertebrates (Putnam, 1985, 1984). It has a variety of functions related to defense and immunity including clearance of proteases and cytokines and enhanced antigen presentation (James, 1990; Borth, 1992; Liebl and Koo, 1993; Bonner, et al, 1993, 1995; Chu and Pizzo, 1993, 1994). Here, in addition to the measurements of lysozyme, hemolytic capacity, cortisol and catecholamines, an antiprotease assay was used to quantify α_2-mac in trout plasma before and after stress.

MATERIALS AND METHODS

Fish

Shasta strain rainbow trout (*Oncorhynchus mykiss*), spawned in June of 1994, were raised at the Food Science and Technology laboratory in Corvallis, OR. The fish were reared on a 12 hour light/dark cycle, maintained in flow through (approximately 12 liters/min) well water (12°C ± 2°C) in 250 liter tanks. They were fed daily on *Biobrood* (Warrenton, OR). Fish were not fed on the days they were sampled. At the time of the experiment, fish weighed 100-450 grams and were approximately 16 months old.

Stress regime and sampling

Experiment III.1

Ten fish were stocked in each of five 400 liter tanks. Sampling commenced after two weeks acclimation. Anesthetic (100 mL of 2-phenoxy-ethanol) was infused into the tank (a final concentration of approximately 0.25 mL 2-PE per liter) *via* a tube in the water inflow without raising the tank lid or otherwise disturbing the fish. After 2 minutes, individual anesthetized fish were removed from the tank and bled from the caudal vessel into a heparinized syringe. Experimental fish were first revived in freshwater (except the first day), then stressed by being held in the air for 30 seconds and then placed in a shallow bucket of water for ten minutes. After the stressor, the fish were captured, killed by cerebral concussion, and bled again. Control fish were bled after anesthesia, returned to the tank containing anesthetic and bled again ten minutes later, after the stressed fish were sampled. Individual fish were tracked using color-coded

nets and buckets. Blood samples were kept on ice until centrifuged for 10 minutes at 4°C, 400 g to separate the plasma from the cells.

Lysozyme activity was assayed within an hour of the end of the experiment using fresh aliquots of plasma. Alpha$_2$ macroglobulin activity was assessed in some trout plasma samples. Catecholamines were assayed from frozen aliquots. Experiment III.1 was performed during October, 1995. Fish were sampled between 8 AM and 10 AM to reduce diel variation in hormone levels.

Experiment III.2

This experiment was basically a repeat of Experiment III.1. Ten fish were sampled on each of three days during February, 1996. Plasma hemolytic activity was also evaluated. Control fish were concussed before the second bleed. Samples from day one of the experiment were evaluated for plasma cortisol levels.

Lysozyme activity

A turbidmetric assay was used to determine lysozyme activity (Ellis, 1990) as described in the previous chapter.

Alpha$_2$-macroglobulin activity

Proteolytic inhibition of thermolysin by trout plasma (a measure of α_2-mac activity) was measured using the substrate hide powder azure (substrate) in a protocol adapted from Bender, R.C. et al (1992). Specifically, trout plasma was step diluted to 1:300 in a hide powder assay buffer (125 mM Tris, 15 mM $CaCl_2$, 0.025% Brij 35, 0.025% NaN_3, pH 7.5). A volume of 100 µL of this diluted plasma was added to a 2 mL siliconized tube containing 0.15 µg thermolysin in 50 µL of assay buffer (250 mM Tris, 30 mM $CaCl_2$, 0.05% Brij 35, 0.05% NaN_3, pH 7.5) and

0.5 mL substrate (20% sucrose, 2.0% hide powder, 0.05% Brij 35, 0.05% NaN_3). The tubes were placed on a rocking platform and incubated for 20 minutes at 37°C. The substrate was removed by centrifugation for 5 minutes at 8000 *g*, and the A_{590} of the supernatant was determined. Proteinase activity in the absence of plasma was determined using standards with 0.025 µg, 0.10 µg, and 0.15 µg of thermolysin. The standard curve allows the decrease of A_{590} in samples containing plasma to be expressed. The decrease is due to the inhibition of thermolysin activity acting on the substrate. The inhibition of this activity is presumed to be effected by the α_2-mac present in the trout plasma. Data are expressed as percent inhibition of thermolysin activity. An inhibition of 100% means the plasma effectively inhibited all of the 0.15 µg thermolysin.

Hemolytic activity

The hemolytic assay was done as previously described (Chapter II). Plasma from Experiment III.2 was evaluated for hemolytic activity.

Cortisol

Cortisol levels in fish #51 through #60 of Experiment III.2 were analyzed by radioimmunoassay (Redding et al, 1984) by the laboratory of Dr. Carl Schreck, Department of Fisheries and Wildlife, Oregon State University.

Catecholamines

Catecholamine levels were determined using high pressure liquid chromatography (HPLC) with electrochemical detection (Woodward, 1982) as described in the previous chapter (Chapter II). Plasma samples from Experiment III.1 were analyzed for catecholamines.

Statistics

An ANOVA was performed on the difference in the change in experimental levels in trout plasma compared to the change in values in control fish. Differences were considered significant when $p<0.05$.

RESULTS

Experiment III.1

The change in plasma lysozyme activity in individual fish after an acute handling stress was significantly greater than that in control fish ($p=0.016$, Table III.1a and Figure III.1). Lysozyme activity increased in 19 of 25 stressed fish, while only 9 of 25 control fish had increases in lysozyme levels. Lysozyme levels varied among individual trout from a low of 2.67 µg/mL to a high of 7.69 µg/mL. There was a mean increase of $+0.274 \pm 0.148$ µg/mL in experimental fish, and a mean decrease of -0.031 ± 0.051 µg/mL in control fish. Two stressed fish had increases of nearly 2 µg/mL, and two other stressed fish had decreases of about 1.5 µg/mL. All other fish (control or experimental) had changes of less than 1 µg/mL in this experiment.

Adrenaline levels ranged from nearly undetectable (< in Table III.1a), to 64.39 pmol/mL among initial samples. Adrenaline rose in all fish that were revived before being stressed (Table III.1a). Adrenaline levels of stressed fish were highly variable, with levels ranging from 243.8 to 2256.3 pmol/mL. Fish #1 through #5, which were not revived between bleeds, had very low adrenaline levels in samples from the second, post-stress, bleed.

Table III.1a Data from individual rainbow trout bled before and after stress.

sample date	no.	wt. (g)	Lysozyme activity (µg/mL) initial*	second**	change §	Adrenaline (pmol/mL) initial*	second**	change §
Stressed trout								
8/10/95	1	287.00	4.58	5.08	0.50	<	31.24	
8/10/95	2	337.00	4.15	4.64	0.49	64.39	65.14	0.75
8/10/95	3	218.00	5.12	5.40	0.28	28.22	86.37	58.15
8/10/95	4	285.00	3.70	3.59	-0.11	9.04	24.25	15.21
8/10/95	5	259.00	n.d.	4.25		nd	nd	
8/17/95	21	202.00	5.60	6.29	0.70	<	392.47	
8/17/95	22	348.00	3.12	3.40	0.28	12.90	165.44	152.54
8/17/95	23	200.00	3.35	5.11	1.76	<	1169.10	
8/17/95	24	231.00	4.53	3.20	-1.33	12.93	405.21	392.28
8/17/95	25	295.00	3.08	3.43	0.35	<	1509.50	
9/6/95	11	367.00	4.91	5.66	0.75	18.57	1679.80	1661.23
9/6/95	12	259.00	4.15	4.48	0.32	21.01	1260.40	1239.39
9/6/95	13	212.00	3.78	4.10	0.31	7.99	1298.60	1290.61
9/6/95	14	373.00	5.77	6.18	0.41	<	2862.80	
9/6/95	15	301.00	6.64	6.09	-0.54	<	nd	
9/7/95	31	224.00	5.72	4.42	-1.30	nd	1542.90	
9/7/95	32	182.00	4.14	4.34	0.21	6.53	461.54	455.01
9/7/95	33	211.00	4.21	6.28	2.08	<	849.30	
9/7/95	34	442.00	6.00	6.29	0.29	<	888.20	
9/7/95	35	374.00	5.31	5.19	-0.12	<	243.84	
9/13/95	41	315.00	5.44	5.75	0.31	<	1503.10	
9/13/95	42	263.00	6.22	6.76	0.54	17.13	660.70	643.57
9/13/95	43	392.00	7.69	7.83	0.14	13.58	1738.40	1724.82
9/13/95	44	228.00	4.33	4.52	0.19	nd	nd	
9/13/95	45	345.00	5.04	5.14	0.10	49.46	2256.30	2206.84

* the initial sample was from fish which were remotely anesthetized with 2-PE before being bled.
** The second sample was from fish which were revived, then stresed by 30 seconds in the air and 10 minutes in a shallow bucket of water. Control fish were bled twice while anesthetized.
§ The change is the difference between the second and the initial levels.
no. refers to fish code.
nd means no data.
<indicates HPLC difficulty.

Table III.1a Data from individual rainbow trout bled before and after stress (continued).

sample date	no.	wt. (g)	Lysozyme activity (µg/mL) initial*	second**	change §	Adrenaline (pmol/mL) initial*	second**	change §
Control trout								
8/10/95	6	361.00	5.50	5.53	0.03	23.35	9.60	-13.75
8/10/95	7	251.00	5.05	4.78	-0.27	10.03	9.58	-0.45
8/10/95	8	215.00	4.74	4.54	-0.20	12.56	<	
8/10/95	9	254.00	5.30	5.16	-0.14	10.49	<	
8/10/95	10	280.00	4.76			nd	nd	
8/17/95	26		4.78	4.72	-0.06	nd	nd	
8/17/95	27	219.00	3.22	2.67	-0.55	nd	nd	
8/17/95	28	135.00	3.13	3.24	0.11	nd	nd	
8/17/95	29	216.00	5.97	5.56	-0.42	nd	nd	
8/17/95	30	236.00	3.59	3.53	-0.06	nd	nd	
9/6/95	16	417.00	5.33	5.31	-0.01	10.84	37.40	26.56
9/6/95	17	257.00	4.64	4.55	-0.09	nd	31.50	
9/6/95	18	330.00	5.65	5.46	-0.20	16.58	28.34	11.76
9/6/95	19	112.00	4.42	5.20	0.78	nd	168.75	
9/6/95	20	413.00	6.55	6.34	-0.21	22.93	nd	
9/7/95	36	416.00	4.01	3.90	-0.11	<	<	
9/7/95	37	298.00	4.97	4.88	-0.08	<	49.96	
9/7/95	38		4.99	5.10	0.11	<	<	
9/7/95	39	306.00	4.14	4.28	0.14	<	22.26	
9/7/95	40	240.00	4.24	4.15	-0.09	12.79	21.08	8.29
9/13/95	46	328.00	5.78	6.00	0.23	14.93	248.80	233.87
9/13/95	47	352.00	4.64	4.60	-0.04	36.91	nd	
9/13/95	48	201.00	5.67	5.76	0.09	25.46	66.96	41.50
9/13/95	49	450.00	4.86	5.04	0.19	12.26	139.95	127.69
9/13/95	50	402.00	6.25	6.38	0.13	13.94	30.64	16.70

* the initial sample was from fish which were remotely anesthetized with 2-PE before being bled.
** The second sample was from fish which were revived, then stresed by 30 seconds in the air and 10 minutes in a shallow bucket of water. Control fish were bled twice while anesthetized.
§ The change is the difference between the second and the initial levels.
no. refers to fish code.
nd means no data.
<indicates HPLC difficulty.

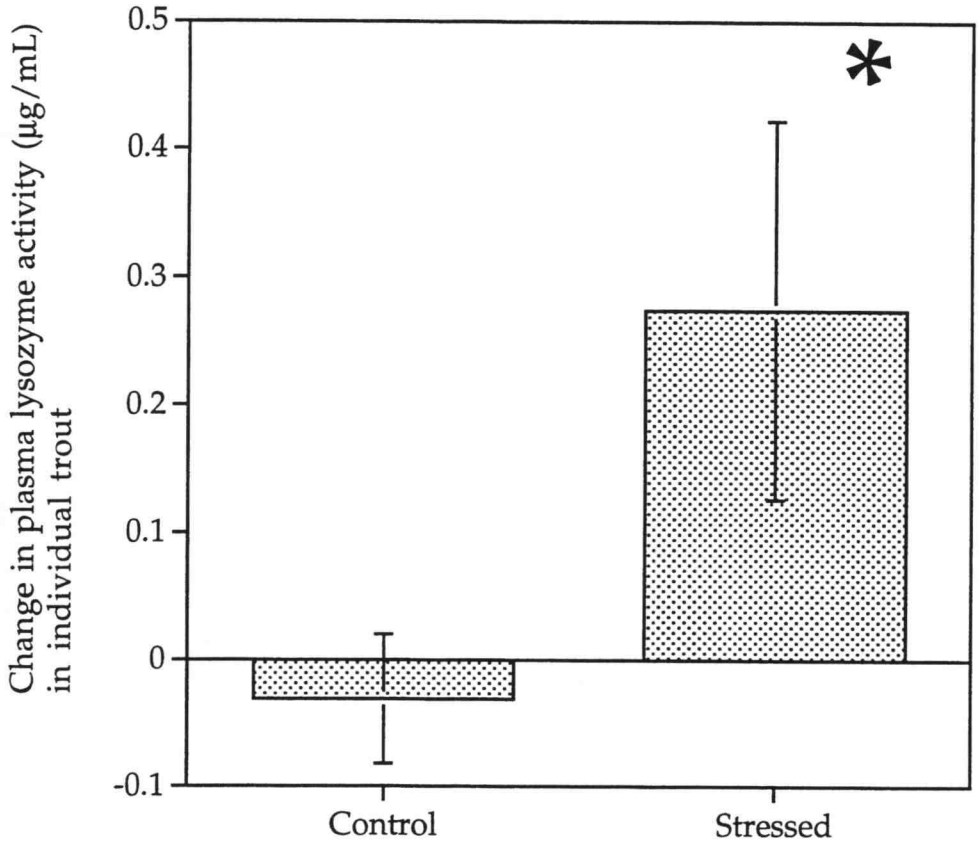

Figure III.1 Change in lysozyme activity of individual trout at two timepoints. Control fish (n=24) were bled twice while anesthetized. Stressed fish (n=24) were anesthetized, then revived and stressed by handling. The stress was 30 seconds in the air and 10 minutes in a shallow bucket of water at ambient temperature. * indicates a significant difference from control level.

The adrenaline level in the plasma of each of those fish was less than 87.0 pmol/mL. In the remaining experimental fish, those that were revived before being stressed, the plasma adrenaline levels were significantly higher ($p=0.001$). The average adrenaline level in the plasma of stressed fish was 782.31 pmol/mL, although variation among animals was marked. Adrenaline levels remained low in most control fish, with a mean level of 66.53 pmol/mL, although there were increases of over 100 pmol/mL in two fish. Catecholamine levels were not obtained for several samples.

Alpha2-mac activity was highly variable among fish and no significant changes were detected. Mean values decreased in both control and stressed fish (Table III.1b).

Experiment III.2

Most fish that were stressed (12 of 15) had increased plasma lysozyme activity levels, while levels in only 6 of 15 control fish were increased (Table III.2a and Figure III.2). Lysozyme activity ranged from 2.82 µg/mL to 6.76 µg/mL, except in one fish, #73, which had a lysozyme activity of 15.96 µg/mL in the initial sample that rose to 17.38 µg/mL after stress! The mean change in plasma lysozyme activity of stressed fish was $+0.184 \pm 0.11$ µg/mL. In the control group, the change in lysozyme activity was -0.058 ± 0.105 µg/mL. The difference was statistically significant ($p=0.0081$).

Hemolytic activity was highly variable. There were no significant differences between resting and stressed fish (Table III.2a). The fish which had high lysozyme activity, #73, also had a hemolytic capacity higher than any other fish assayed.

Plasma cortisol levels in Fish #51 to fish #60 were low (mean 5.4 ng/mL) at the initial sampling (Table III.2b), and increased significantly in experimental

animals ($p=0.0001$). The mean plasma cortisol rose to 79.5 ng/mL in stressed fish. In control animals, the mean plasma cortisol was elevated to 27.0 ng/mL after ten minutes.

Table III.1b Activity of α_2-macroglobulin in trout plasma before and after stress.

sample date	no.	wt.	α_2-mac (percent inhibition of thermolysin) initial	second	change
Stressed fish					
9/6/95	11	367.00	54.13	51.78	-2.35
9/6/95	12	259.00	69.09	61.36	-7.73
9/6/95	13	212.00	44.39	46.91	2.52
9/6/95	14	373.00	64.21	55.81	-8.4
9/6/95	15	301.00	25.31	10.43	-14.88
Control fish					
9/6/95	16	417.00	35.38	26.51	-8.87
9/6/95	17	257.00	34.17	31.92	-2.25
9/6/95	18	330.00	51.76	41.38	-10.38
9/6/95	19	112.00	52.81	39.59	-13.22
9/6/95	20	413.00	57.92	48.76	-9.16

Table III.2a Experiment III.2: Data from individual trout plasma before and after stress.

				Lysozyme activity (µg/mL)			Hemolytic activity (reciprocal of % plasma lysing 50% cells)		
sample date	no.	wt (g)	sex	initial*	second**	change §	initial*	second**	change §
Stressed trout									
2/9/96	51	593	M	6.729	6.645	-0.084	0.815	0.8366	0.0216
2/9/96	52	363	M	6.708	6.155	-0.553	1.042	1.063	0.021
2/9/96	53	713	M	6.155	6.684	0.529	0.986	1.142	0.156
2/9/96	54	545	F	6.483	6.756	0.273	1.017	1.079	0.062
2/9/96	55	572	F	5.82	6.247	0.427	0.845	0.9068	0.0618
2/23/96	61	508	F	3.749	3.842	0.093	1.121	1.285	0.164
2/23/96	62	847	F	3.794	3.568	-0.226	1.121	1.335	0.214
2/23/96	63	734	F	4.61	4.847	0.237	1.535	3.7499	2.2149
2/23/96	64	670	F	3.911	3.914	0.003	3.043	2.087	-0.956
2/23/96	65	594	M	2.832	2.975	0.143	2.979	2.0867	-0.8923
3/1/96	71	756	F	2.817	2.856	0.039	1.4712	1.4481	-0.0231
3/1/96	72	302	M	4.369	4.546	0.177	0.7045	0.825	0.1205
3/1/96	73	532	F	15.96	17.38	1.42	3.06	3.223	0.163
3/1/96	74	527	F	3.529	3.557	0.028	1.6089	1.636	0.0271
3/1/96	75	718	F	2.701	2.958	0.257	1.1125	1.1736	0.0611
Control fish									
2/9/96	56	1010	M	4.399	5.076	0.677	0.5393	0.7796	0.2403
2/9/96	57	717	M	5.525	4.374	-1.151	0.9164	0.4942	-0.4222
2/9/96	58	585	M	6.051	6.149	0.098	0.984	1.098	0.114
2/9/96	59	701	F	6.374	6.346	-0.028	1.121	1.069	-0.052
2/9/96	60	478	F	5.878	5.79	-0.088	1.113	1.077	-0.036
2/23/96	66	728	F	3.724	3.161	-0.563	1.142	1.46	0.318
2/23/96	67	339	F	4.511	4.503	-0.008	1.0896	1.4238	0.3342
2/23/96	68	292	?	4.035	3.752	-0.283	1.4079	1.2336	-0.1743
2/23/96	69	734	F	3.84	3.812	-0.028	1.732	3.229	1.497
2/23/96	70	278	F	3.567	3.58	0.013	1.732	3.229	1.497
3/1/96	76	216	M	2.933	2.768	-0.165	0.852	1.142	0.29
3/1/96	77	727	M	4.004	4.22	0.216	1.5076	1.6158	0.1082
3/1/96	78	801	F	3.825	4.003	0.178	1.6264	1.6511	0.0247
3/1/96	79	1005	M	2.406	2.694	0.288	0.8929	0.8923	-0.0006
3/1/96	80	439	F	3.994	3.968	-0.026	1.211	0.958	-0.253

* The initial sample was from fish which were remotely anesthetized with 2-PE before being bled.
** The second sample was from fish which were revived, then stresed by 30 seconds in the air and 10 minutes in a shallow bucket of water.
Control fish were bled twice while anesthetized.
§ The change is difference between the second and the initial level.
no. refers to fish code.

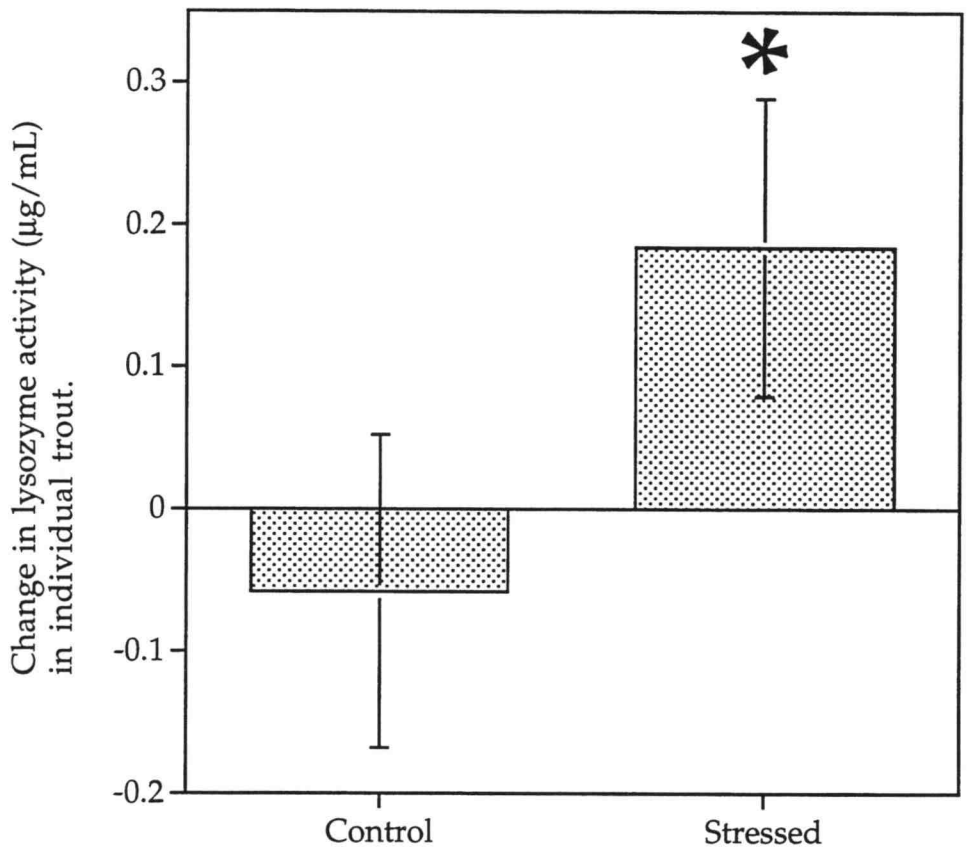

Figure III.2 Change in lysozyme of individual trout from Experiment III.2. Control fish (n=15) were bled twice while anesthetized. Stressed fish (n=15) were subjected to 30 seconds in the air and ten minutes in a shallow bucket of water after being anesthetized, bled and then revived. * indicates significant difference from control levels (p=0.0081).

Table III.2b Cortisol levels in individual trout plasma before and after acute stress.

no.	wt (g)	sex	Cortisol (ng/mL) initial*	second**	change §
Stressed fish					
51	593.00	M	3.20	65.80	62.60
52	363.00	M	0.60	49.30	48.70
53	713.00	M	0.00	55.40	55.40
54	545.00	F	6.20	111.80	105.60
55	572.00	F	3.70	115.30	111.60
Control fish					
56	1010.00	M	1.25	23.35	22.10
57	717.00	M	1.30	22.20	20.90
58	585.00	M	15.30	34.35	19.05
59	701.00	F	3.95	25.10	21.15
60	478.00	F	17.60	30.00	12.40

* the initial samples were from fish which were remotely anesthetized.
** The second sample was from fish which were revived then stressed by 30 seconds in the air and 10 minutes in a shallow bucket of water.
Control fish were bled twice while anesthetized.
§ The change is the difference between the second and the initial levels.
no. refers to fish code.

DISCUSSION

Serial bleeding of trout was successful as a means to follow plasma components in individual fish by reducing variation. It was reduced to the point where significant increases in plasma lysozyme activity as a result of acute stress could be determined. In one fish, the plasma lysozyme activity was nearly three times higher than the average. Two other trout (in Experiment III.1) had increases in plasma lysozyme activity greater than 1.5 μg/mL. However, the average increase in plasma lysozyme activity after stress was 0.2-0.3 μg/mL. In Experiment III.1, the average increase was 0.274 μg/mL, compared to an average decrease of -0.031 μg/mL in control animals. In Experiment III.2, there was an average increase in plasma lysozyme activity of 0.184 μg/mL in experimental animals, while the average change in activity of control animals was -0.058 μg/mL. These data support the hypothesis that acute stress enhances this component of innate immunity. As seen in the data, anesthetization and bleeding in the absence of an intentional stressor did not increase plasma lysozyme activity in control fish.

Some experimental animals did not respond with increased levels of plasma lysozyme activity. In Experiment III.1, six of 25 stressed fish, nearly 25%, had decreased plasma lysozyme activity. In Experiment III.2, three of 15 stressed fish, 20%, had decreased plasma lysozyme activity. Notes kept during the experiment reveal handling difficulties with some fish. For example, fish #31, which had a dramatic decrease in activity after stress was noted as having jumped during the first bleed, and was difficult to bleed on the second sampling. Whether these events have a cause and effect relationship is unclear since there are no obvious trends. Fish #24 also had a dramatic decrease, but experimental difficulties were not noted.

Genetic variation among fish may explain some differences. Some laboratories have attempted to breed trout that have high (or low) plasma lysozyme activity levels (e.g. Fevolden, et al, 1992; Røed, 1993). A single fish had a level over three times higher than any other fish in this experiment. A few fish in the experiments described in Chapter II had unusually high lysozyme activity also. These are indications that there are individuals with high basal plasma lysozyme levels in this population as well. Regardless, it appears that plasma lysozyme activity is increased after a brief aerial exposure and 10 minutes in a shallow bucket of water.

As previously mentioned, increases in specific plasma protein concentrations as detected by crossed immunoelectrophoresis were greatest after ten minutes (Demers, 1993; Demers and Bayne, 1994). If one of the proteins that significantly increased at ten minutes was lysozyme, then the kinetics of increase of plasma lysozyme activity in trout may be maximal near 10 minutes. This conclusion is possible since three of the four protein concentrations were decreased at 20 minutes in that experiment. These results support the previous observations of enhanced plasma lysozyme activity immediately following stress (Mock and Peters, 1990; Røed, 1993; Fevolden, et al, 1994). Further experiments are needed to more fully characterize the kinetics of change of lysozyme activity as a result of the fight or flight response.

To provide another means of comparing these data to data obtained in other laboratories, fresh plasma from three humans (two male, one female) was analyzed for plasma lysozyme activity. The lysozyme activity of human plasma evaluated in the same manner as trout plasma was 8.23, 8.8, and 7.67 μg/mL. These levels are comparable to the activity measured in human serum using a lysoplate technique (Hankiewicz and Swierczek, 1974). The activity level was

higher in the human plasma tested than in most of the trout plasma samples run under the same conditions.

Comparison of these data to previously published data for teleosts reveals that these levels are lower than those reported by Mock and Peters (1990). Those experimenters also used a lysoplate technique. Since they used HEWL as a standard, however, comparison between experiments is possible. Other investigators used HEWL as a standard (Rainger and Rowley, 1993), but did not record the data in terms of units of HEWL activity. Other investigators report lysozyme activity in comparison to plasma from an individual trout in their experiment (Røed, et al , 1993a, 1993b; Fevolden, et al, 1994) or as a change in Optical density (Grinde, 1989) and the data are not directly comparable to those reported here. Another report that compared lysozyme activity among fish species did not use HEWL standards, and therefore cannot be compared directly (Lie, et al, 1989).

Adrenaline levels in **initial** samples in this experiment suggest that the fish were far less stressed than were those fish in the previously described experiment (Chapter II). Also, the levels of adrenaline in **stressed** fish were not as high as those in stressed fish in the earlier experiment (Chapter II). Surreptitiously introducing the anesthetic into the tank before handling, as done in the experiments reported in the present chapter, dramatically reduced adrenaline levels relative to those obtained after netting and concussing without anesthetic, as were done in the experiments reported in Chapter II, but prevented plasma hormones from rising as high as if fish were stressed without prior anesthetization.

In fish that were not revived before being stressed (Experiment III.1, #1-#5) adrenaline levels barely increased as a result of the handling stressor. In revived fish, however, levels increased, but not to levels as high as those determined

when using the same stressor as that used in the earlier work (Chapter II). This is probably because the anesthetic reduced the effect of the stressor by blocking the response. Although the fish were revived before being stressed in this experiment, they may not have been fully recovered from the anesthetic.

Caution is required when comparing catecholamine levels measured in different experiments. The samples were analyzed at different times. The data in Chapter II were obtained by HPLC in August of 1994. Data for the fish reported in the present experiment were obtained in January of 1996. In 1994, 10 μL of the sample was injected into the HPLC apparatus for the analysis. In 1996, however, nearly 50 μL was injected into the HPLC apparatus for some samples. All samples had DHBA as an internal standard. All samples were extracted following the same procedure. Albeit at different times, all samples were extracted in a timely manner using plasma that had been stored at -80°C. One reason for the need to inject a larger volume was that the adrenaline levels from the initial sample of fish in this experiment were lower due to the anesthetic reducing the fight or flight response. Even with the increased volume of sample injected, difficulties with equipment prevented accurate levels from being measured for some samples. Some additional samples were not extracted; others were extracted, but not run. For those data that are available, however, adrenaline levels increased dramatically in asssociation with stress ($p=0.0001$).

The cortisol data on a subset of the fish in Experiment III.2 also indicated that stressed fish may not have been fully revived. The average cortisol concentration in the plasma of the stressed fish measured from Experiment III.2 was 79.5 ng/mL. In the previous experiment (Chapter II), the average plasma cortisol level from fish which had been similarly treated was 122.0 ng/mL. Although some fish (6) from that initial experiment (Chapter II) had plasma cortisol level less than 100 ng/mL, most (15) had levels greater than 100 ng/mL.

Six of the 15 fish had plasma cortisol levels greater than 150 ng/mL. A previous study (Demers and Bayne, 1994) reported an average plasma cortisol levels of 119.1 ng/mL in trout stressed in the same manner. Therefore, anesthetic may have prevented some of the increase in plasma cortisol concentration in trout, even after they had been revived in freshwater. Furthermore, the control animals had increases in plasma cortisol concentration after ten minutes in the anesthetic bath when compared to the initial samples. This result differs from those described by Iwama, et al. (1989), which showed decreases in plasma cortisol following anesthetization.

An evaluation of hemolytic activity revealed high variability among fish. The data obtained on the first day of Experiment III.2 showed hemolytic capacity increasing in all five experimental fish and decreasing in three of five control fish. On the following day, the results were entirely different. Four of five control fish showed higher hemolytic activity. The increase of hemolytic activity of two control fish plasma samples was dramatically higher. Variability in the red blood cell targets may explain some of this variability. On the first (and third) days of sampling, the rabbit red blood cells were approximately eight days old. They had been stored at 4°C and showed no sign of autolysis. On the second day of sampling, however, the rabbit red blood cells were only two days old. The fresher cells were not as easily lysed as older cells as revealed by the higher values on the second day. To provide a means of comparing these data to data obtained in other laboratories, fresh plasma from three humans (two male, one female) was analyzed for plasma hemolytic activity. Only the plasma from the female subject showed any hemolytic capacity against the rabbit red blood cells used in this experiment (data not shown). This may be due to differences in blood type. The plasma that lysed the rabbit red blood cells was A+. One plasma that did not lyse was O-; the blood type of the other sample that did not

lyse the rabbit red blood cells is unknown. It is possible that differences of this nature are also present in trout blood. That is, some individuals are better capable of lysing rabbit red blood cells than others. The difference may be related to recognition of sugar moieties on the red blood cell target surfaces. The variation in rabbit red blood cell age helped in preventing any definitive conclusions from being drawn from the hemolysis study.

The quantification of specific plasma protein concentrations or activity usually depends on the use of antibodies or a characterization of an activity ascribed to the protein of interest. As the number of monoclonal antibodies against specific plasma proteins and cytokines in human and mammalian research continues to expand, tests for characterization of differences among individuals in health and sickness are being developed. The same is true in teleost research. In the future, more specific tests will be available to determine the plasma concentrations of specific plasma proteins in trout as well. For instance, both monoclonal antibodies and polyclonal antisera to trout fibronectin are now available (Sadaghiani, et al, 1994). Trout CRP levels are being characterized using antibodies (Kodama, personal communication). A better evaluation of changes in plasma protein concentrations or activites may require the development of specific tests designed to determine the concentrations of the particular proteins of interest.

In summary, serial bleeding of individual fish before and after an acute handling stressor revealed that plasma lysozyme activity, adrenaline and cortisol levels were significantly elevated in stressed fish. Plasma hemolytic and α_2-mac activities were not measurably changed compared to control fish.

Chapter IV

Immediate Acute Kinetics of Plasma Catecholamine and Cortisol Increases in Rainbow Trout .

INTRODUCTION

The kinetics of change of cortisol and catecholamine levels in teleosts are fairly well characterized on a diel and hourly scale (for reviews see Perry, et al, 1989; Barton and Iwama, 1991). Plasma catecholamine levels in trout (*Oncorhynchus mykiss*) are known to increase within two minutes when fish are disturbed by repeatedly grasping them by the tail and forcing movement within the tank (Nakano and Tomlinson, 1967). Cortisol, likewise, is increased in the plasma of trout but only after two minutes (reviewed by Barton and Iwama, 1991). In one publication (Strange, et al, 1977), a rate of increase of 5 ng/mL/min was given for juvenile chinook salmon (*Oncorhynchus tshawytscha*). Walleye (*Stizostedion vitreum*), a cool water teleost important for sport fishing and aquaculture, were stressed by a 30 second aerial exposure and then confinement in a bucket. In those fish, the rate of increase in plasma cortisol levels was calculated to be 18 ng/mL/min. This was calculated from the first timepoint measured, 15 minutes. The authors proposed that walleye may have an interrenal response that is faster than those of salmonids (Barton and Zitzow, 1995). Other studies document plasma hormonal increases in trout after handling stressors, however, they seldom examine the very acute changes (Perry, et al, 1989; Barton and Iwama, 1991). In the earlier research on immediate changes in trout plasma (Chapter II), a few fish from the initial group, which had

not been exposed to overt/intentional stress, had cortisol levels higher than that which is normally considered resting. The stress of capturing the fish for bleeding, more difficult with some fish, could have caused the cortisol levels to increase. The documented increases in cortisol require at least two minutes to be evident (Barton and Iwama, 1991). This study attempted to better characterize the acute physiological changes which occur within seconds of the handling stressor up to two minutes later. The fish were netted and concussed for the initial sample. Stressed fish were netted, held in the air and then placed in a shallow bucket of water for 30, 60 or 90 seconds.

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss*) were raised and maintained at the Food Toxicology Lab, Corvallis, Oregon. Animals were fed thrice weekly with *Biobrood* (Warrenton, OR). They were not fed on the days they were sampled. The experiment was conducted between 07:30 and 09:30 on two separate days in late July, 1995. Fish weighed between 137-298 grams and were approximately 13 months of age.

Experimental regime

Ten trout were stocked in each of three 400 liter circular tanks. They were acclimated to the tanks for one week before sampling. On each day, one fish from each tank was netted, killed by cerebral concussion, and bled from the caudal arch into a syringe containing heparin. That fish was the initial sample.

Then four fish were netted and held in the air for 30 seconds. One fish was bled as before, and the rest were placed in a shallow bucket of water (at ambient temperature). After 30, 60, or 90 seconds, a fish was removed from the shallow bucket, concussed and bled. This was repeated in each tank on two separate days. There was a sample size of 6 for each group. Blood was kept on ice until centrifuged. Plasma was collected and stored at -80°C until assayed.

Catecholamine determination

Catecholamine levels were determined using high pressure liquid chromatography with electrochemical detection as previously described (Chapter II).

Cortisol

Cortisol levels were determined by radioimmunoassay (Redding et al, 1984) by the laboratory of Dr. Carl Schreck, Oregon State University, Department of Fish & Wildlife.

Statistical analysis

A randomized block design was used with checks for confounding variables, then ANOVA was performed testing the null hypothesis that levels were the same as initial levels. Levels were considered significant if $p<0.05$.

RESULTS

Catecholamines

The mean noradrenaline (NA) level was 131.9 ± 26.12 (mean ± SE) pmol/mL in trout in the initial group (Table IV.1 and Figure IV.1A). After 30 seconds in the air, the NA level increased to 160.46 ± 47.45 pmol/mL. This decreased to 70.83 ± 12.3 pmol/mL after 30 seconds in the bucket and increased again to 98.37 ± 12.75 pmol/mL and 106.97 ± 23.0 pmol/mL at the final two time points. There were no statistically significant changes in noradrenaline level in this experiment.

Table IV.1 Data from individual trout plasma samples collected to determine the kinetics of hormone increase within seconds of a handling stressor.

code	sex	Time (seconds) (since air stress)	Cortisol (ng/mL)	Adrenaline (pmol/mL)	Noradrenaline (pmol/mL)
ARA*	F	-	0.60	132.52	98.46
BRA*	M	-	10.80	178.20	135.62
CRA*	M	-	0.40	233.15	104.35
ARB*	F	-	1.00	267.83	245.72
BRB*	F	-	0.60	225.38	149.00
CRB*	F	-	0.80	140.19	58.87
average			*2.36*	*196.21*	*131.90*
AS30A**	F	0	1.10	680.90	381.81
BS30A**	F	0	6.40	509.92	167.87
CS30A**	F	0	0.30	644.21	190.29
BS30B**	F	0	32.90	214.10	68.12
CS30B**	F	0	0.10	275.92	123.04
average			*16.84*	*394.97*	*160.46*
AS1A***	F	30	0.60	305.70	77.92
BS1A***	F	30	0.30	244.45	106.44
CS1A***	M	30	0.70	249.33	83.59
BS1B***	F	30	4.30	127.05	42.35
CS1B***	F	30	1.00	219.16	43.83
average			*1.80*	*229.14*	*70.83*
AS1.5A §	F	60	5.90	498.14	105.83
BS1.5A §	F	60	4.90	681.55	86.72
CS1.5A §	F	60	2.00	796.88	70.89
AS1.5B §	F	60	13.10	n.d.	n.d.
CS1.5B §	F	60	55.60	352.09	130.04
average			*13.58*	*582.16*	*98.37*
AS2A §§	F	90	2.40	322.39	80.80
BS2A §§	F	90	10.50	404.93	65.54
CS2A §§	F	90	23.70	364.13	217.20
AS2B §§	F	90	19.10	259.89	70.91
BS2B §§	F	90	19.10	662.70	63.28
CS2B §§	F	90	7.20	553.79	107.48
average			*22.53*	*444.79*	*106.97*

n.d. indicates no data

* indicates trout that were netted and concussed before being bled.

** indicates trout that were netted and held in the air for 30 seconds before being concussed and bled.

*** indicates trout that were netted, held in the air for 30 seconds, then placed in a shallow bucket of water for 30 seconds before being captured, concussed and bled.

§ indicates trout that were netted, held in the air for 30 seconds, then placed in a shallow bucket of water for 60 seconds before being captured, concussed and bled.

§§ indicates trout that were netted, held in the air for 30 seconds, then placed in a shallow bucket of water for 90 seconds before being captured, concussed and bled.

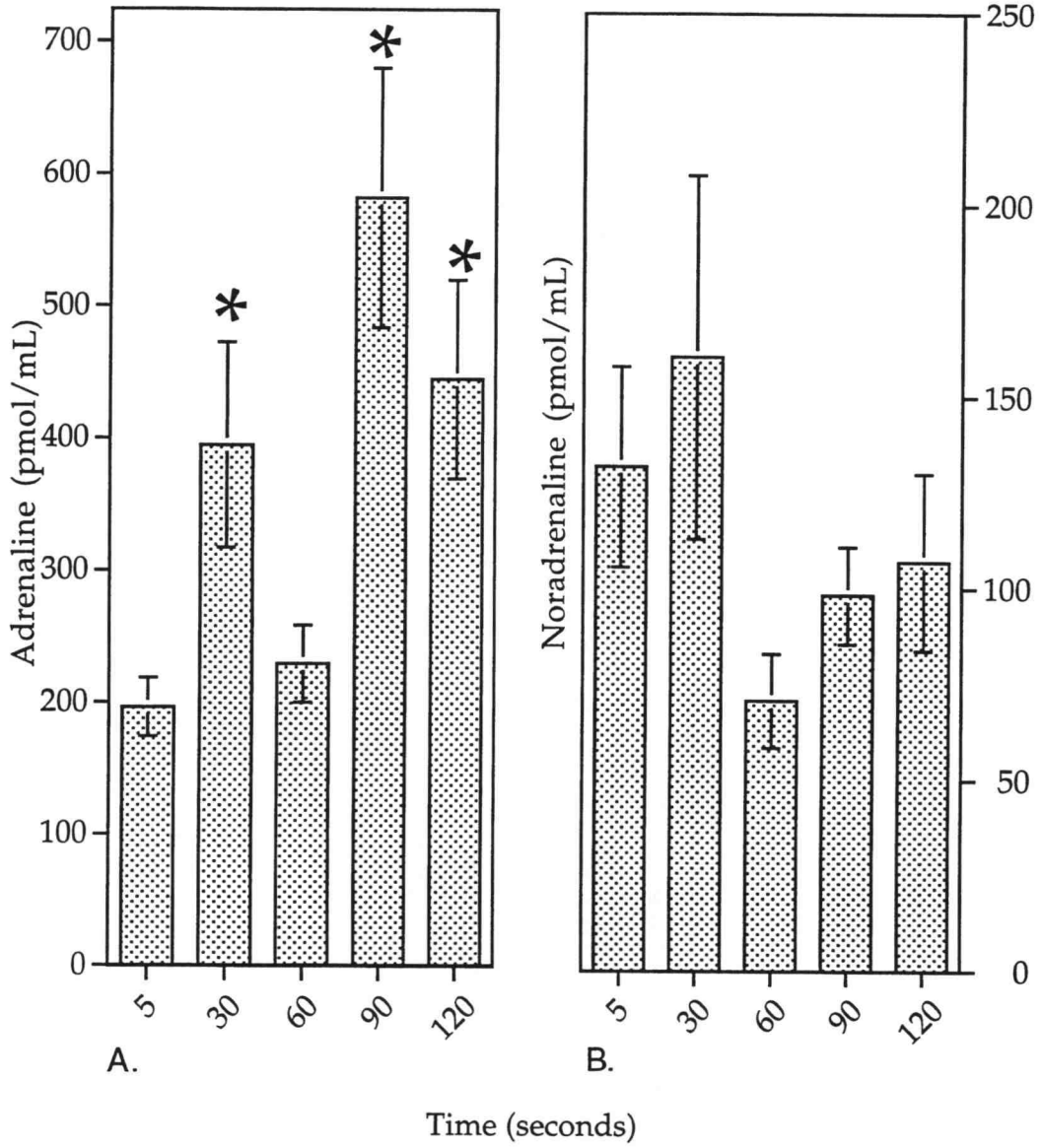

Figure IV.1 Adrenaline (A.) and noradrenaline (B.) levels in trout plasma after stress. Fish were netted and concussed (5) or netted and held in the air for 30 seconds (30), then placed in a shallow bucket of water for 30 (60), 60 (90), or 90 (120) seconds before being bled. Bars indicate means ± SE.
* indicates a significant increase over initial levels.

The mean adrenaline level was 196.21 ± 22.26 pmol/mL in the initial group. After 30 seconds in the air, adrenaline had increased to 394.97 ± 77.68 pmol/mL. The adrenaline level decreased to 229.14 ± 29.18 pmol/mL after 30 seconds in the bucket, and increased again to 582.16 ± 98.3 pmol/mL and 444.79 ± 75.45 pmol/mL at the final two time points (Figure IV.1B). Significant differences were noted when comparing initial samples to the samples from stressed fish ($p=0.0067$). Although the drop at 60 seconds (after 30 seconds in the bucket) was not statistically significant, the level obtained at 90 seconds (after 60 seconds in the bucket) was significantly higher than that obtained at 60 seconds (after 30 seconds in the bucket, $p=0.0063$).

Adrenaline accumulated in the plasma at a rate of 13.25 pmol/mL/min when calculated using the mean levels at 30 seconds and in the initial samples. Noradrenaline accumulated at a rate of 1.90 pmol/mL/min when calculated similarly. As mentioned, there was a drop after 30 seconds in the bucket for all hormones measured. The rate of that decrease was -11.04 pmol/mL/min for adrenaline and -5.98 pmol/mL/min for noradrenaline. The subsequent rate of accumulation after 30 seconds in the bucket was 23.53 pmol/mL/min for adrenaline and only 1.84 pmol/mL/min for noradrenaline. The rate of accumulation for noradrenaline was very similar over both the initial 30 seconds (1.9 pmol/mL/min) and after capture from the bucket (1.84 pmol/mL/min).

Cortisol

The initial plasma cortisol level was 2.36 ± 1.69 ng/mL (mean ± SE, Figure IV.2). After 30 seconds in the air, the plasma cortisol concentration was 16.84 ± 10.08 ng/mL. After 30 seconds in the bucket the mean was only 1.80 ± 0.79 ng/mL.

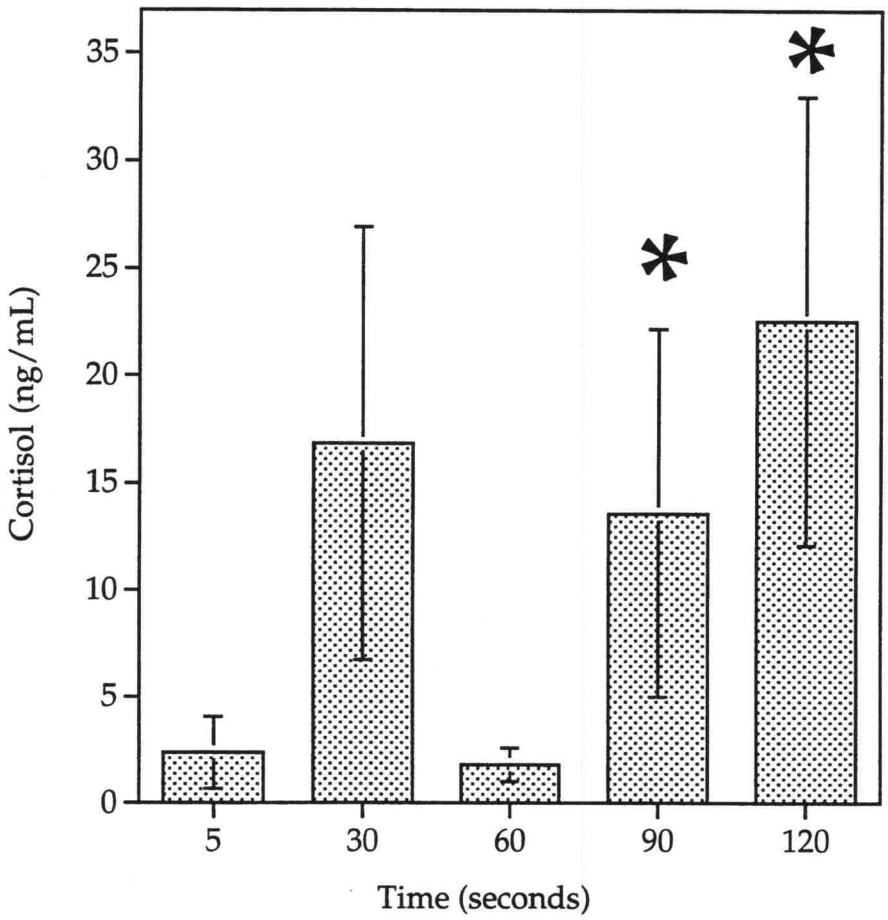

Figure IV.2 Plasma cortisol levels in trout sampled within seconds of stress. Fish were netted and concussed (5) or netted and held in the air for 30 seconds (30), then placed in a shallow bucket of water for 30 (60), 60 (90), or 90 (120) seconds before being bled. Bars indicate means $\pm$ SE. * indicates a significant increase over initial levels.

Plasma cortisol was 13.58 ± 8.0 ng/mL after 60 seconds in the bucket and then increased to 22.53 ± 10.45 ng/mL at the final time point. When comparing initial samples to those from all fish which were stressed and then confined, there was a statistically significant difference ($p= 0.007$). The level of cortisol was significantly increased over the initial value within 90 seconds of the initiation of the stressor ($p=0.0138$).

The kinetics of cortisol accumulation in this experiment was calculated using the difference of the mean values at the final and initial time points. The average overall rate of accumulation within the first two minutes of handling was 10.08 ng/mL/min but the rate of increase was greatest after a full minute in the bucket: 20.73 ng/mL/min.

DISCUSSION

Trout responded very quickly to aerial exposure by rapid increases in plasma stress hormones. More surprising, however, was the speed at which those hormones were apparently depleted from the plasma. This study showed high plasma catecholamine levels after 30 seconds of aerial exposure and a marked reduction (significantly in the case of adrenaline) after 30 seconds in a shallow bucket of water. Levels then increased again in fish held in the bucket for an additional 60 or 90 seconds. This suggests that return to water allowed at least some recovery over aerial exposure, and that the stress of being captured after being held in a shallow bucket may be responsible for the later increased levels by causing another hormone surge. This is further supported by the results previously illustrated in Figure II.4C. In that figure, a linear relationship

between time to bleed and plasma catecholamines was documented. The longer it took to capture and bleed the fish, the higher their plasma catecholamine levels.

The ability of rainbow trout to increase plasma cortisol appeared to be quicker than previously reported (Barton and Iwama, 1991). In this experiment, levels were higher within 30 seconds of handling. Cortisol levels were significantly increased over the initial level within 90 seconds of handling. The higher levels of cortisol seen at 30 seconds may be due to the response to air exposure. It is possible, however, that the two fish with elevated cortisol concentrations were otherwise stressed before the start of this experiment. It is also possible that within the population of trout used for these studies some individuals may have constitutively high resting levels. Studies with larger sample sizes and less complex experimental stressors would clarify the immediate hormonal response to stressors.

The rate of 10.08 ng/mL/min for plasma cortisol increase calculated from the overall data is more than twice the rate previously described for steelhead (Strange, et al, 1977), but less than the rate reported from walleye (Barton and Zitzow, 1995). The value of 20.73 ng/mL/min, calculated from the final timepoint compared to the level after 30 seconds in the bucket, is slightly higher than the rate of 18 ng/mL/min reported for walleye (Barton and Zitzow, 1995). The Chapter II data show plasma cortisol increased at a rate of 17.15 ng/mL/min during first five minutes. The rate of plasma cortisol increase in the 10 to 20 minutes interval was only 2.82 ng/mL/min. If the overall change from the initial to the 20 minute time point were used to calculate the rate, in a manner similar to that used with previous data (Strange and Schreck, 1977; Barton and Zitzow, 1995), the rate of cortisol increase would have been only 6.5 ng/mL/min.

According to the data reported here, the rate of increase is fastest within the first several minutes of handling, and slowly decreases within minutes. Analysis of data from an earlier report (Demers and Bayne, 1994) yielded similar results. The rate of increase of cortisol in trout plasma calculated from the entire 20 minute interval was 8.1 ng/mL/min. The rate calculated using the initial 5 minute interval, however, was 14.98 ng/mL/min. There was a more rapid increase in plasma cortisol in the first few minutes following an acute handling stressor, and the rate of increase was reduced thereafter. Perhaps these data reflect the release of pre-synthesized hormone stores and their subsequent synthesis.

These results indicate that the handling stressor used in these experiments induced surges of both cortisol and catecholamines within seconds of handling. They also indicate that the handling stressor of aerial exposure and confinement in a bucket is a complex stressor with complex modulation of hormone levels. And they show the need to sample at very short intervals when attempting to characterize hormonal fluctuations.

Modulation of defenses has been shown to occur through the action of epinephrine and corticosteroids in humans (Crary, 1983 a, 1983 b; Benschop, et al, 1993, 1994, 1995; Fantuzzi, 1995; Harris, 1995; Dhabhar, 1995). These changes include redistribution of lymphocytes in the body and modulation of neutrophil and NK activity. Similar modulation of immunity by plasma hormones has been documented in teleosts (Flory, 1988; Bayne and Levy, 1991a and 1991b) including changes in immune response and respiratory burst.

As each hormone acts in concert with the other hormones and cytokines produced by the body, it is impossible to ascribe any immunological changes to the effects of one or the other of the hormones alone. Both must be considered as potentially influencing the physiological responses observed. Published reports

indicate, however, that the catecholamines modulate distribution and activity of lymphocytes (Benschop, et al , 1993; Benschop, et al, 1994), teleost hepatocytes (Kahl and Schade, 1991; Fabbri, et al, 1992) and trout phagocytes (Bayne and Levy, 1991a and 1991b). All of those cells are involved in the fight or flight response.

In conclusion, these data more fully characterize the kinetics of hormonal increases in trout plasma, and revealed modulation of both catecholamines and cortisol within seconds. Levels of cortisol were increased significantly over resting levels within 90 seconds of a handling stressor. Catecholamines were increased within 30 seconds, and decreased after 30 seconds "recovery" time. Thirty or 60 seconds later, both plasma noradrenaline and adrenaline were again elevated. The kinetics and modulation of both of these hormones was faster than previously documented.

Chapter V

Preliminary Studies on the Effects of Acute Stress on the Survival of Rainbow Trout following a Pathogen Challenge.

INTRODUCTION

Previous research has suggested that innate defenses may be enhanced as part of the fight or flight response to alarm or other acute stressors (Røed, 1993; Demers and Bayne, 1994; Fevolden et al, 1994; Chapter II and III, this thesis). The levels of plasma lysozyme are elevated for at least ten minutes following handling (Chapter III), but may decrease within 20 minutes (Chapter II). If the fight or flight response enhances innate defenses, then we may expect that fish challenged with a pathogen after an acute stressor may better be able to defend themselves against invading pathogens. Under some conditions, survival of stressed fish may be better than resting fish. The interactions of host, pathogen and environment are intricate and the outcomes of such interactions are seldom straight forward (Wedemeyer et al, 1990). Although there are many difficulties inherent in combining so many factors into the design of an experiment, several experiments were performed to test the postulate of enhanced survival in acutely stressed fish.

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss*) were raised from eggs at the Food Science and Technology laboratory, Corvallis, Oregon. They were reared on a 12 hour light/dark cycle, maintained in 100 L tanks using flow through well water (12°C ± 2°C), at approximately 6 liters per minute, and fed daily on Oregon Test Diet (OTD). Experiments were performed at the Salmon Disease Laboratory, Oregon State University from July, 1994 through March, 1995. The trout weighed at least one gram, but no more than five grams at the start of each experiment. The fish were raised in an environment without intentional stress and with optimal feed and water quality conditions. The experiments took place at a facility where the tanks and plumbing are uniform. These experiments were set up in the back corner of the laboratory to minimize disturbances made by humans using the facility.

Pathogen

Vibrio anguillarum cultures were provided by Dr. J.S. Rohovec or Dr. Robert Olson. The strains used had been isolated from a natural outbreak of vibriosis in salmonids and stored lyophilized at -80°C. They were grown in sterile tryptic soy broth (TSB, Difco). A 10 mL aliquot of TSB was inoculated with bacteria from a petri-dish culture. After 24 hours growth at 17°C with agitation, this 10 mL was used to inoculate a one liter flask of TSB. The flask was incubated overnight at 17°C with agitation. Aliquots of the culture were diluted with TSB and used in the pathogen challenge that morning. For the first experiment, the culture was spun down and the bacteria resuspended in fresh medium so that the suspension was $O.D._{625}$ =1.0 for the high dose.

Dilutions of this suspension were used for the challenge and plated for overnight growth at 18°C to determine the number of viable bacteria per milliliter. All challenges were done between 09:00 and 11:30. Care was taken to not disturb the fish before and for the first several hours after the experimental challenge.

The choice of which pathogen to use was the subject of extensive evaluation. A pathogen that killed relatively quickly would be preferable to one that was slower acting. This is desirable so that the endpoint evaluated, death, would more faithfully reflect the early events following the experimental stress and challenge. *Vibrio anguillarum* best fulfilled the requirements. This gram negative bacterium causes hemorrhagic septicemia (characteristic of most gram-negative fish pathogens) resulting in discoloration, hemorrhage and a relatively quick death. The bacterium is mainly a marine pathogen, but freshwater epizootics have been documented (Giorgetti, et al, 1981). *V. anguillarum* has been shown to be able to enter the fish and reach the kidney within the time it takes to sample that usually aseptic organ (Nelson, 1982).

Experimental design

Experiment V.1

Approximately 30 juvenile fish (1-5 grams) were placed in each of twelve 20 L tanks at the Salmon Disease Laboratory in Corvallis, Oregon, and allowed to acclimate for seven days before the start of the experiment. The well water (12°C + 2°C) rate of flow was approximately 10 L per minute. Daily feeding continued during acclimation and the experiment. The pathogen was introduced into the tank on Dec. 7, 1994. To obtain data that could be compared between different experiments, the same stressor was used as was

used in the experiment described previously (Chapter II). Previous work had demonstrated that the greatest increases of plasma proteins occurred about 10 minutes after the acute handling stress (Demers, 1993; Demers and Bayne, 1994), and since *V. anguillarum* appears to enter the body within seconds of exposure (Nelson, 1982), it was decided that the fish would be challenged 10 minutes after being netted and held in the air for 30 seconds. After 10 minutes in a shallow bucket of water the fish were returned to their 20 liter tanks and the pathogen was infused into the tank via a syringe in the incoming water supply without changing the water flow. Control fish were exposed to the pathogen in the same way, without ever lifting the lid to their tanks. Duplicate tanks of resting or stressed fish were exposed to high (approximately 3.5×10^{12} CFU in 10 mL media) or medium doses (approximately 3.5×10^{11} CFU in 10 mL media). The low dose (approximately 3.5×10^{10} CFU in 10 mL media) of the pathogen was given to only the stressed fish. Control tanks received media only. Dead fish were removed daily. *V. anguillarum* was re-isolated from kidney streaks of several dead fish. The experiment was continued for 27 days.

Experiment V.2

This experiment closely resembled the previous experiment except that the pathogen was from a different source. *V. anguillarum* was obtained from Dr. Robert Olson at the Hatfield Marine Science Center in Newport, Oregon. The isolate had recently been passaged through salmon and brook trout and found to be highly virulent. The challenge was initiated on March 10, 1995 after the same stress protocol described for Experiment V.1. Dilutions of the pathogen in media were introduced into the tanks *via* a syringe in the water inflow tubing without otherwise disturbing the juvenile fish. The bacteria

were not centrifuged. Control tanks had media infused *via* a syringe in the water inflow tubing without otherwise disturbing the fish. The experiment continued for 24 days.

RESULTS

Experiment V.1

In this experiment, trout were held in a net for 30 seconds and then placed in a shallow bucket of water for ten minutes. Then the fish were returned to their tanks and challenged through the water inflow. The high dose of *V. anguillarum* killed 94% and 97% (replicate tanks) of stressed fish, but only 84% and 86% of resting fish. The medium dose of *V. anguillarum* killed 64% and 69% of stressed fish, while killing 88% and 0% of resting fish. The low dose killed 0% and 76% of stressed fish. More stressed fish died in the tank containing the high dose *V. anguillarum,* yet more stressed fish survived the challenge in the medium dose in one tank. The amount of time it took to kill the fish was different among the doses (Figure V.1). Some replicates yielded quite similar results while others gave quite different results.

Experiment V.2

In this experiment, mortality was restricted to only those tanks given the highest dose of *V. anguillarum.* Due to an error, one tank of stressed fish received a double dose of the most concentrated dilution of pathogen. Mortality in that tank was a little over 50%. The other tank containing

stressed fish which received the high dose had nearly 80% mortality (only half the number of bacteria were introduced into that tank). Resting fish exposed to the high dose of *V. anguillarum* experienced 30% and 45% cumulative mortality. The deaths began about four days after exposure to the pathogen (Figure V.2).

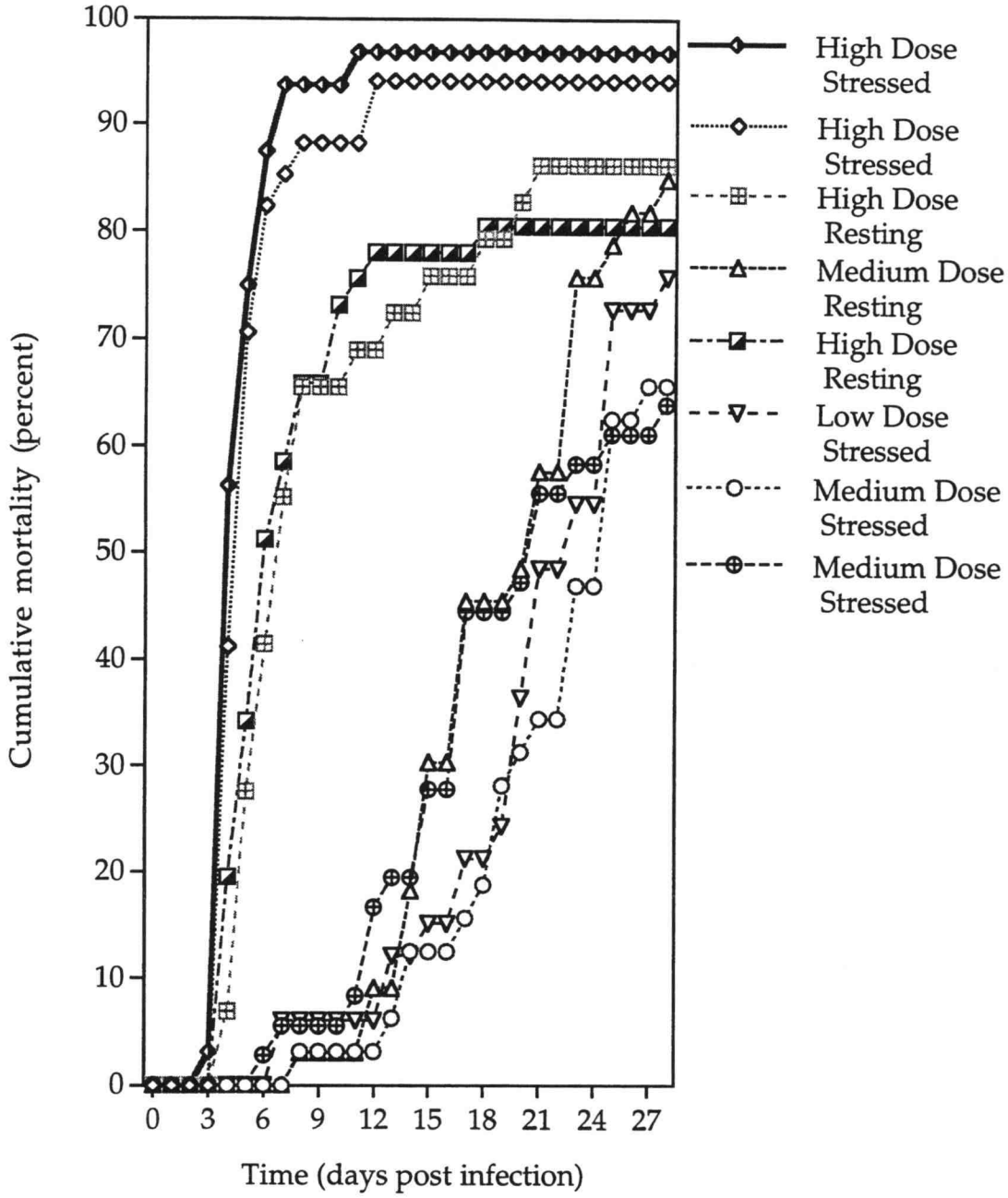

Figure V.1. Experiment V.1. Cumulative percent mortality of juvenile rainbow trout that were bath exposed to *Vibrio anguillarum* at rest or after 30 seconds in the air and 10 minutes in a shallow bucket of water. Fish were challenged *via* the water inflow tubing without disturbing the fish. There was no mortality in tanks without pathogen added.

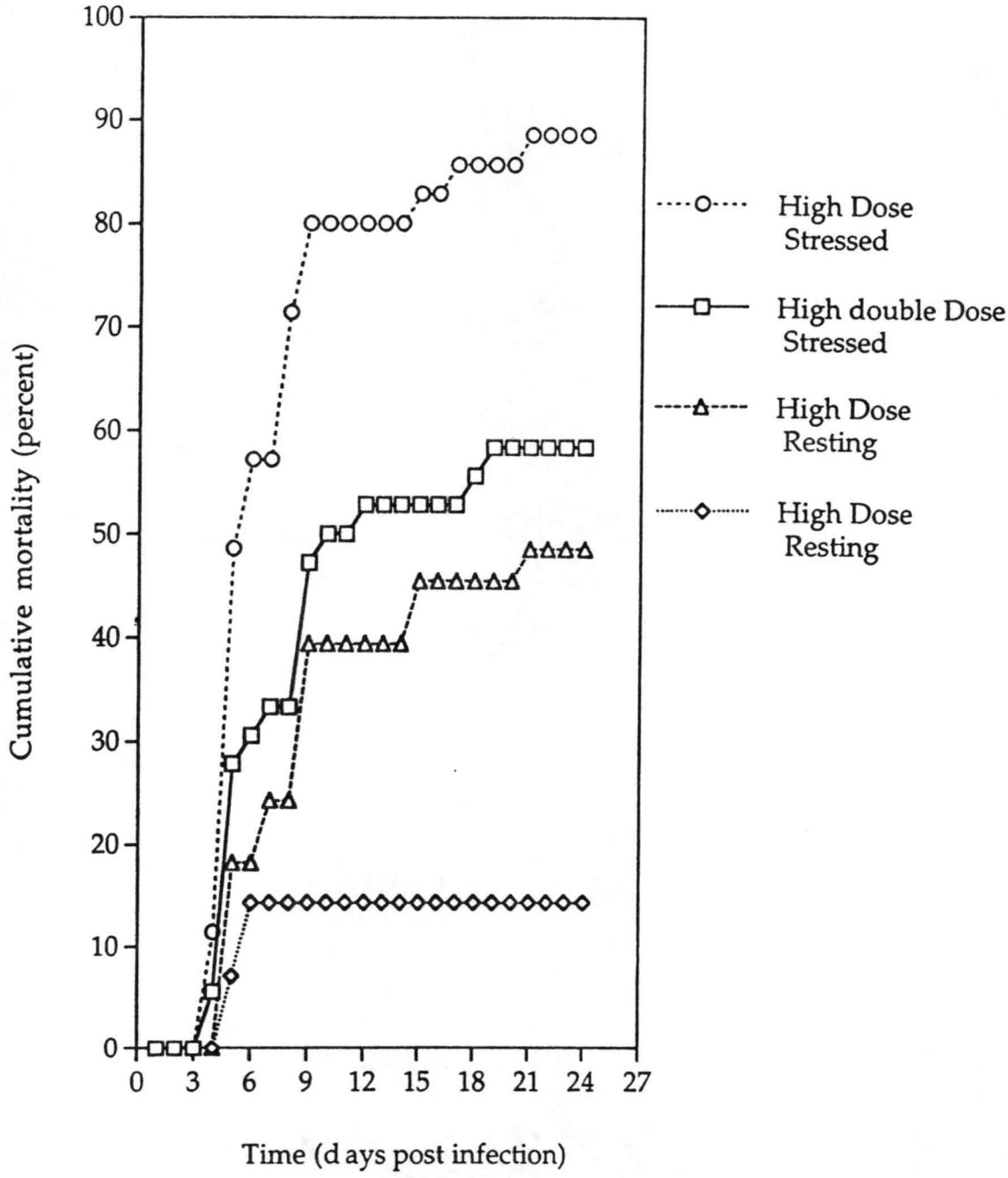

Figure V.2. Experiment V.2 Cumulative percent mortality of juvenile rainbow trout that were bath exposed to *Vibrio anguillarum* at rest or after 30 seconds in the air and 10 minutes in a shallow bucket of water. Fish were exposed *via* a syringe inserted in the incoming water supply.

DISCUSSION

In these experiments, the handling stressor was aerial exposure and confinement followed by bath exposure to a bacterial pathogen. There was a dose dependence to the kinetics of mortality. In fish exposed to the high dose of the pathogen, deaths began about four days after challenge. In those tanks, more stressed than resting fish died (Experiment V.1). Among fish given the medium dose, mortality began several days later than those challenged with the high dose. In one of the medium dose tanks, there was more mortality in the "resting" group than there was in the stressed group. This result is consistent with the hypothesis. However, differences between groups became evident only after three weeks, making any suggestion of enhanced immunity quite tenuous.

Replicate tanks of resting fish challenged with the medium dose, and of stressed fish given the low dose yielded highly variable results. Variability also was more prevalent than any clear indication of a trend in the data from Experiment V.2. There was higher survival among the stressed fish accidentally receiving a high double dose than there was in the tank getting the single high dose (50% and 80% mortality respectively). Resting fish receiving high doses had cumulative mortalities of 20% and 40%. In Experiment V.2 only the highest dose killed, killing began after about four days, and the resting fish experienced better survival than the stressed fish.

In Experiment V.1, stressed fish had slightly higher survival in the medium dose of pathogen challenge. This is consistent with the notion of enhancement of innate defenses as a result on an acute stress. Mortality rates due to *V. anguillarum* were very different between experimental replicates. Use of the same stress protocol as used in previous work, that is, 30 seconds in

a net suspended in the air, may have damaged the first line of defense, the tegument of the fish.

It would be worthwhile to repeat the challenges using a stressor other than the air exposure in a net. By maintaining the integrity of the tegument and mucosal layer, the interaction of the pathogen and host would be more realistic, and more similar between stressed and resting fish. Variation within and among treatments could be lessened with more experience and practice in the techniques of pathogen challenges. As mentioned, experiments with so many interacting systems are inherently difficult to evaluate.

The endpoint of death, occurring more than 20 days after initiation of the experimental variable, may not be optimal. In both experiments, deaths increased in both the low and medium dose tanks after the first several days. One explanation for the later increase is horizontal transmission of the pathogen. Although dead fish were removed at least once a day, the dead fish in the tank could have been a source of pathogen separate from the original source of pathogen at the time of challenge. Although evidence of enhanced survival after an acute handling stress would support the hypothesis presented, experiments *in vitro* may be easier to execute and to evaluate. Some indicator other than death might prove a more sensitive biomarker for the status of the innate immune system.

Chapter VI

Thesis Summary

Increasing the concentrations of plasma proteins which identify and mark foreign cells, viruses and damaged host tissue would ultimately serve to increase the chances for survival in an organism which had recently experienced an alarming or stressful event. Preliminary experiments with rainbow trout (Demers, 1993, Demers and Bayne, 1994) revealed increases in four of twelve plasma proteins within minutes of an acute handling stressor. The work presented in this thesis more fully explored changes in plasma components upon stress and sought possible correlation with stress hormone levels. Whether these changes provide protection was tested using a bacterial pathogen challenge.

Cortisol and catecholamines were present in significantly higher concentrations in the plasma of trout which had been acutely stressed by handling (Chapter II). Plasma lysozyme activity was increased in the plasma of the stressed fish. There was such inter-individual variability, however, that the increases documented were not statistically significant when each sample was taken from a different individual fish. Also, experimental protocols may have precluded detection of differences in hemolytic capacity of rainbow trout plasma.

Several changes in protocol were invaluable in documenting changes in plasma profiles after handling stress in the rainbow trout. The surreptitious introduction of the anesthetic 2-PE was instrumental in reducing plasma levels of stress hormones. By bleeding the same individual before and after the stressor, a component of innate immunity, plasma lysozyme, was found to significantly increase within minutes of stress (Chapter III). This was evident although

plasma catecholamine and cortisol levels in fish that were anesthetized and revived before stressing were lower than those in fish that were not anesthetized before handling. Furthermore, plasma cortisol concentrations were higher in control animals after ten minutes in the anesthetic than were levels in fish that were simply netted and concussed. These results demonstrate that anesthetization, although useful, causes hormonal perturbations that must be considered when evaluating results.

Subsequent experiments (Chapter IV) documented increases of both plasma cortisol and catecholamines within 1.5 minutes of handling and confinement. This is quicker than reported in the literature. The quick degradation of plasma hormones, especially the catecholamines (Winder and Yang, 1987), makes it imperative that studies of kinetics of these hormones be evaluated in increments of seconds instead of minutes or hours. It is possible that a closer evaluation of the hormonal surge caused by stressors in other experimental models such as rats and mice and in humans would reveal a similar modulation. The effects of short-term modulation of plasma hormone levels have been little appreciated by neuroimmunologists simply because experiments have been designed to evaluate differences in hours or days and not immediately after the stressor. In this study, modulation to regain homeostasis after the acute handling stressor appears to have resulted in the return to resting plasma lysozyme activity levels within twenty minutes. Only very short term sampling increments will be effective in determining the validity of these hypotheses in other experimental models.

The final chapter presented experiments designed to determine if survival was enhanced in acutely stressed fish. Variability among replicate tanks made analysis difficult. It may be that evaluation of phagocytic capacity or microbial

killing in acutely stressed animals would provide support for the hypothesis of enhancement of innate immunity following acute stress.

Genetic influences and variability are high in this trout model. A similar situation exists in the human population with which these data can be compared. Trout is a good model for studies of innate defenses if variation among individuals can be overcome. There are presently fish lines being developed which should provide lesser variation among individuals and may make studies like this more rewarding.

Steps taken in these experiments have yielded fish that more closely represent a "resting" state than in previous research. Although catecholamines are not as low as in cannulated fish given a week to recover in a black box (Railo, 1985), the fish used here were not "sensory-deprived" either. Other attempts were made to reduce the plasma hormonal surge by means of injection or oral delivery of drugs (data not shown), however all resulted in compensatory changes including elevations in cortisol. The methods used to obtain a "resting fish" were invaluable in these experiments.

These data demonstrate that physiological perturbations modulate the body's immune system within seconds or minutes. These studies using rainbow trout were successful in demonstrating relevant changes that may occur as a part of the fight or flight response. As more tests are developed for the quantification of specific plasma proteins in trout, such as monoclonal antibodies to trout C3 and trout fibronectin, studies of the effects of stress could address the specific proteins suspected to increase within minutes of a handling stressor. Furthermore, characterization of the proteins which were documented to increase (Demers, 1993) could be undertaken by excising the proteins separated by CIEP and then subjecting the subsequently purified proteins to a N-terminal sequence analysis. Identification of those proteins would likely be fairly

straightforward since the sequences of many plasma proteins of the trout are already described and more are revealed all the time. Homology of trout proteins with proteins from other species should be enough to aid in identification.

These studies have demonstrated enhancement of plasma lysozyme activity and increases in stress hormone levels in trout plasma within minutes of an acute handling stressor. Application of the experimental methods used here to other models should permit further vigorous evaluation of the notion that enhancement of innate immunity is a natural component of the fight or flight response.

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APPENDICES

APPENDIX I

Appendix I. Sample lysozyme data and analysis.

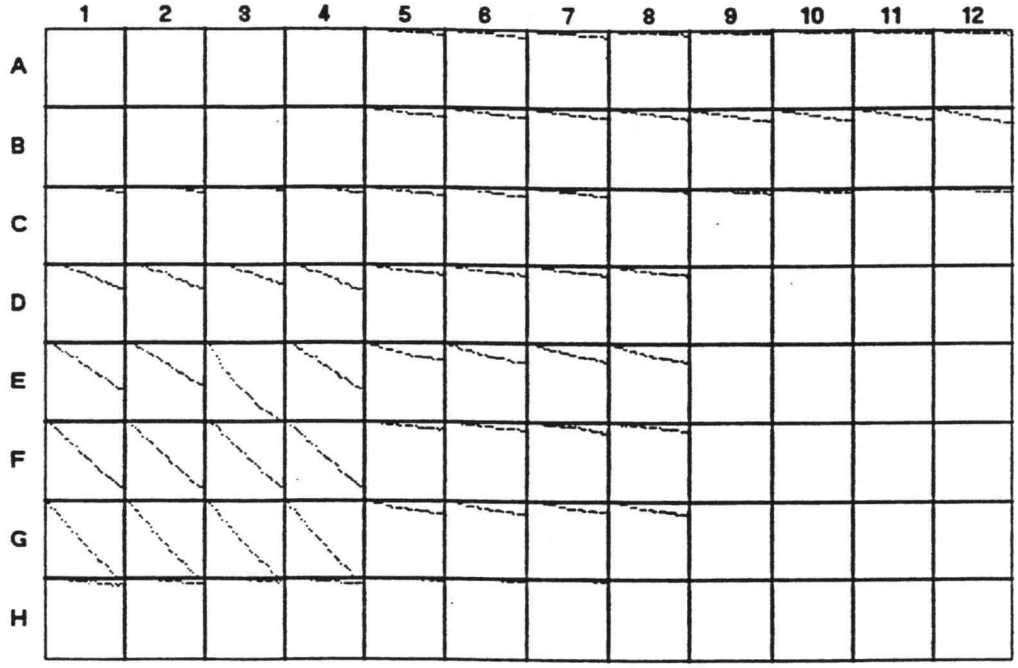
The following are reprints of the Softmax™ computer application printouts. The first figure illustrates the plots of data collected each 30 seconds for 5 minutes. The standards are in the first 4 lanes (in quadruplicate). The next page illustrates a typical standard curve used in the analysis. The following pages show the calculated levels of lysozyme activity in the standards and the plasma samples being tested.

MOLECULAR DEVICES
Raw Data Plots

DATA FILE:	lyse0706		
DESCRIPTION:			
PROTOCOL:	lyse0706.dat		
DESCRIPTION:			
MODE:	Kinetics	AUTOMIX:	Once
WAVELENGTH:	450	RUN TIME:	05:00
CALIBRATION:	On	LAG TIME:	00:00

PRINTED: 7/6/94

OD LIMIT: -0.2000
Vmax Pts: 31

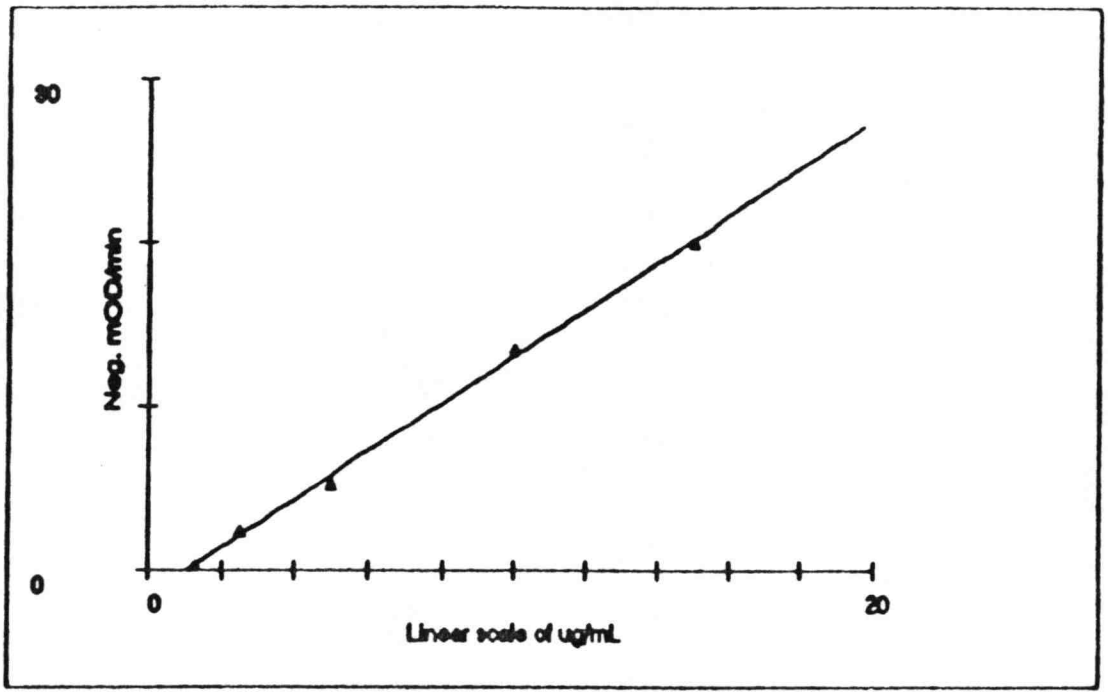


Curve Fit: Linear

Corr. Coeff: 0.999

$y = A + B * x$

A = -1.98 B = 1.44



STANDARD	Std. Value	Well	mOD/min	Mean	Std Dev	CV	Calc. Value
ST2.5	2.5 ug/mL	B1	2.965	2.984	0.273	9.146	2.673
		B2	2.692				2.636
		B3	3.362				2.875
		B4	2.716				2.546
STD05	5 ug/mL	C1	6.727	6.637	0.550	8.280	4.590
		C2	6.797				4.628
		C3	5.863				4.150
		C4	7.160				4.811
STD10	10 ug/mL	D1	15.59	15.22	1.161	7.632	9.105
		D2	15.44				9.028
		D3	13.56				8.074
		D4	16.28				9.457
STD15	15 ug/mL	E1	27.58	31.31	9.105	29.08	15.22
		E2	25.38				14.09
		E3	44.89				24.03
		E4	27.41				15.13
STD20	20 ug/mL	F1	37.06	37.00	0.159	0.429	20.05
		F2	37.16				20.09
		F3	37.01				20.02
		F4	36.78				19.90

MOLECULAR DEVICES

STANDARD	Std. Value	Well	mOD/min	Mean	Std Dev	CV	Calc. Value
STD25	25 ug/mL	G1	44.49	45.26	0.817	1.806	23.83
		G2	45.53				24.36
		G3	44.72				23.95
		G4	46.29				24.75

UNKNOWNS	Mean	Std Dev	CV	Well	Value	mOD/min	Dil.Factor
BS101	4.117	0.064	1.554	B5	4.046	5.660	1.000
				B6	4.096	5.757	
				B7	4.125	5.815	
				B8	4.199	5.960	
BS201	~~3.839~~ 4.138	0.628	16.37	C5	3.966	5.503	1.000
				C6	4.315	6.188	
				C7	4.134	5.832	
				C8	2.916	3.442	
DR1	4.466	0.069	1.534	D5	4.494	6.538	1.000
				D6	4.509	6.567	
				D7	4.496	6.543	
				D8	4.363	6.282	

UNKNOWNS	Mean	Std Dev	CV	Well	Value	mOD/min	Dil.Factor
				F6	3.993	5.556	
				F7	4.537	6.623	
				F8	3.997	5.563	
DS201	5.251	0.207	3.937	G5	5.491	8.496	1.000
				G6	5.340	8.198	
				G7	5.022	7.574	
				G8	5.151	7.828	
FR1	2.400	0.155	6.456	H5	2.361 &	2.353	1.000
				H6	2.588 &	2.798	
				H7	2.435 &	2.498	
				H8	2.216 &	2.067	
FS101	4.950	0.457	9.240	B9	4.668	6.879	1.000
				B10	4.668	6.879	
				B11	4.840	7.218	
				B12	5.626	8.759	
FS201	3.049	0.096	3.157	C9	3.133	3.867	1.000
				C10	3.124	3.850	
				C11	2.936	3.478	
				C12	3.005	3.616	
FS51	2.783	0.150	5.387	A9	2.783	3.180	1.000
				A10	2.583 &	2.788	
				A11	2.821	3.255	
				A12	2.944	3.497	
UBS51	3.543	0.392	11.08	A5	3.505	4.598	1.000
				A6	3.942	5.454	
				A7	3.705	4.990	
				A8	3.018	3.642	
UDS51	6.771	0.244	3.611	E5	6.437	10.35	1.000
				E6	7.023	11.50	
				E7	6.827	11.12	
				E8	6.795	11.06	
UNBR1	3.045	0.273	8.975	H1	3.328	4.250	1.000
				H2	3.142	3.884	
				H3	2.678 &	2.975	
				H4	3.032	3.669	

APPENDIX II

Appendix II. Sample hemolytic data and analysis.

The following pages include the raw data for hemolytic data. The optical density of the supernatant of each well including controls is given in the first figure. The next two pages show the worksheet files used to calculate the Lytic 50 - the reciprocal of the percent plasma required to lyse 50% of the red blood cells present in the well.

MODE: Endpoint AUTOMIX: Off
WAVELENGTH: 540
CALIBRATION: Off

Optical Density

AR4 | CR4 | ER4 | ASS:4

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.152	0.159	0.150	0.150	0.146	0.141	0.150	0.150	0.153	0.153	0.151	0.041
B	0.149	0.150	0.149	0.183	0.166	0.182	0.151	0.149	0.150	0.150	0.149	0.041
C	0.148	0.148	0.148	0.147	0.147	0.148	0.147	0.149	0.154	0.153	0.148	0.042
D	0.146	0.146	0.144	0.146	0.145	0.145	0.140	0.141	0.143	0.145	0.144	0.041
E	0.114	0.118	0.115	0.120	0.120	0.120	0.094	0.101	0.102	0.120	0.119	0.135
F	0.052	0.057	0.055	0.068	0.065	0.064	0.045	0.049	0.048	0.065	0.063	0.132
G	0.039	0.038	0.037	0.038	0.038	0.039	0.038	0.037	0.038	0.038	0.038	0.136
H	0.038	0.038	0.038	0.032	0.037	0.038	0.037	0.037	0.037	0.038	0.037	0.138

comp940729data

% sera	sample	o.d.1	o.d.2	o.d.3	mean O.D.	mean -bkgd	%lysis(Y)	Y/(1-Y)	% sera	sample
10	AR4	0.152	0.159	0.15	0.15367	0.116667	1.19048	-6.25	10	AR4
5	AR4	0.149	0.15	0.149	0.14933	0.112333	1.14626	-7.8372093	5	AR4
2.5	AR4	0.148	0.148	0.148	0.148	0.111	1.13265	-8.5384615	2.5	AR4
1.25	AR4	0.146	0.146	0.144	0.14533	0.108333	1.10544	-10.483871	1.25	AR4
0.625	AR4	0.114	0.118	0.115	0.11567	0.078667	0.80272	4.06896552	0.625	AR4
0.313	AR4	0.052	0.057	0.055	0.05467	0.017667	0.18027	0.21991701	0.313	AR4
0.156	AR4	0.039	0.038	0.037	0.038	0.001	0.0102	0.01030928	0.156	AR4
10	CR4	0.15	0.146	0.141	0.14567	0.108667	1.10884	-10.1875	10	CR4
5	CR4	0.183	0.166	0.182	0.177	0.14	1.42857	-3.3333333	5	CR4
2.5	CR4	0.147	0.147	0.148	0.14733	0.110333	1.12585	-8.9459459	2.5	CR4
1.25	CR4	0.146	0.145	0.145	0.14533	0.108333	1.10544	-10.483871	1.25	CR4
0.625	CR4	0.12	0.12	0.12	0.12	0.083	0.84694	5.53333333	0.625	CR4
0.313	CR4	0.068	0.065	0.064	0.06567	0.028667	0.29252	0.41346154	0.313	CR4
0.156	CR4	0.038	0.038	0.039	0.03833	0.001333	0.01361	0.0137931	0.156	CR4
10	ER4	0.15	0.15	0.153	0.151	0.114	1.16327	-7.125	10	ER4
5	ER4	0.151	0.149	0.15	0.15	0.113	1.15306	-7.5333333	5	ER4
2.5	ER4	0.147	0.149	0.154	0.15	0.113	1.15306	-7.5333333	2.5	ER4
1.25	ER4	0.14	0.141	0.143	0.14133	0.104333	1.06463	-16.473684	1.25	ER4
0.625	ER4	0.094	0.101	0.102	0.099	0.062	0.63265	1.72222222	0.625	ER4
0.313	ER4	0.045	0.049	0.048	0.04733	0.010333	0.10544	0.11787072	0.313	ER4
0.156	ER4	0.038	0.037	0.038	0.03767	0.000667	0.0068	0.00684932	0.156	ER4
10	AS5:4	0.153	0.151		0.152	0.115	1.17347	-6.7647059	10	AS5:4
5	AS5:4	0.15	0.149		0.1495	0.1125	1.14796	-7.7586207	5	AS5:4
2.5	AS5:4	0.153	0.148		0.1505	0.1135	1.15816	-7.3225806	2.5	AS5:4
1.25	AS5:4	0.145	0.144		0.1445	0.1075	1.09694	-11.315789	1.25	AS5:4
0.625	AS5:4	0.12	0.119		0.1195	0.0825	0.84184	5.32258065	0.625	AS5:4
0.313	AS5:4	0.065	0.063		0.064	0.027	0.27551	0.38028169	0.313	AS5:4
0.156	AS5:4	0.038	0.038		0.038	0.001	0.0102	0.01030928	0.156	AS5:4
10	AS10:4	0.141	0.144	0.139	0.14133	0.102333	1.13704	-8.2972973	10	AS10:4
5	AS10:4	0.141	0.137	0.137	0.13833	0.099333	1.1037	-10.642857	5	AS10:4
2.5	AS10:4	0.137	0.136	0.137	0.13667	0.097667	1.08519	-12.73913	2.5	AS10:4
1.25	AS10:4	0.13	0.131	0.139	0.13333	0.094333	1.04815	-21.769231	1.25	AS10:4
0.625	AS10:4	0.103	0.105	0.102	0.10333	0.064333	0.71481	2.50649351	0.625	AS10:4
0.313	AS10:4	0.046	0.048	0.045	0.04633	0.007333	0.08148	0.08870968	0.313	AS10:4
0.156	AS10:4	0.039	0.039	0.038	0.03867	-0.000333	-0.0037	-0.00369	0.156	AS10:4
10	CS10:4	0.139	0.139	0.139	0.139	0.1	1.11111	-10	10	CS10:4
5	CS10:4	0.138	0.138	0.139	0.13833	0.099333	1.1037	-10.642857	5	CS10:4
2.5	CS10:4	0.138	0.136	0.137	0.137	0.098	1.08889	-12.25	2.5	CS10:4
1.25	CS10:4	0.136	0.134	0.138	0.136	0.097	1.07778	-13.857143	1.25	CS10:4
0.625	CS10:4	0.119	0.121	0.121	0.12033	0.081333	0.9037	9.38461538	0.625	CS10:4
0.313	CS10:4	0.077	0.078	0.083	0.07933	0.040333	0.44815	0.81208054	0.313	CS10:4
0.156	CS10:4	0.04	0.039	0.042	0.04033	0.001333	0.01481	0.01503759	0.156	CS10:4
10	ES10:4	0.14	0.14	0.139	0.13967	0.100667	1.11852	-9.4375	10	ES10:4
5	ES10:4	0.141	0.138	0.14	0.13967	0.100667	1.11852	-9.4375	5	ES10:4
2.5	ES10:4	0.144	0.137	0.136	0.139	0.1	1.11111	-10	2.5	ES10:4
1.25	ES10:4	0.141	0.14	0.135	0.13867	0.099667	1.10741	-10.310345	1.25	ES10:4
0.625	ES10:4	0.134	0.131	0.129	0.13133	0.092333	1.02593	-39.571429	0.625	ES10:4
0.313	ES10:4	0.096	0.098	0.096	0.09667	0.057667	0.64074	1.78350515	0.313	ES10:4
0.156	ES10:4	0.041	0.042	0.044	0.04233	0.003333	0.03704	0.03846154	0.156	ES10:4
10	CS5:4	0.137	0.138		0.1375	0.0985	1.09444	-11.588235	10	CS5:4
5	CS5:4	0.141	0.138		0.1395	0.1005	1.11667	-9.5714286	5	CS5:4
2.5	CS5:4	0.139	0.141		0.14	0.101	1.12222	-9.1818182	2.5	CS5:4
1.25	CS5:4	0.136	0.137		0.1365	0.0975	1.08333	-13	1.25	CS5:4
0.625	CS5:4	0.121	0.121		0.121	0.082	0.91111	10.25	0.625	CS5:4
0.313	CS5:4	0.06	0.054		0.057	0.018	0.2	0.25	0.313	CS5:4
0.156	CS5:4	0.04	0.039		0.0395	0.0005	0.00556	0.00558659	0.156	CS5:4
10	CS20:4	0.164	0.154	0.156	0.158	0.119	1.14423	-7.9333333	10	CS20:4
5	CS20:4	0.155	0.152	0.152	0.153	0.114	1.09615	-11.4	5	CS20:4

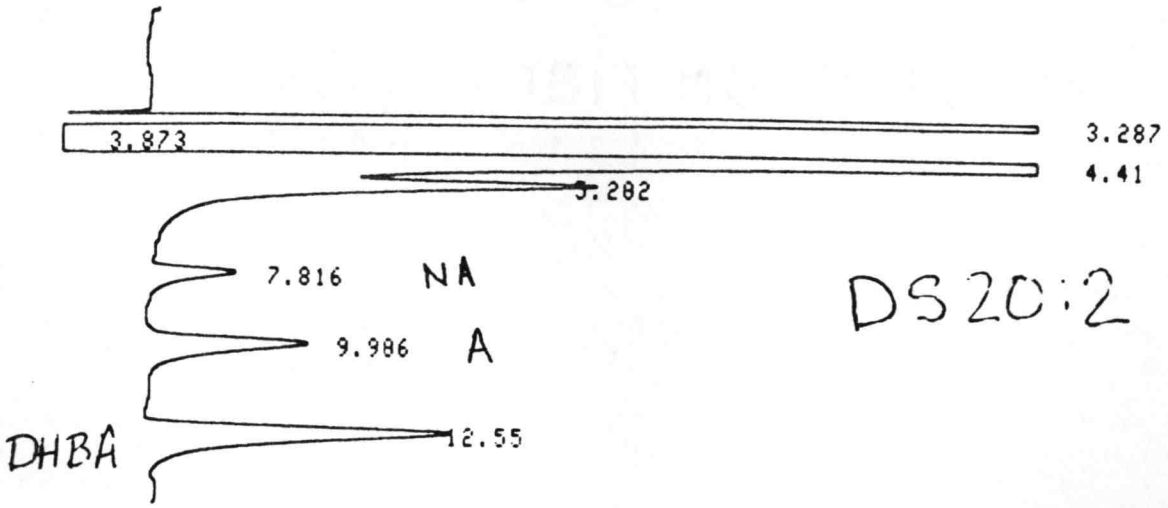
RBT stress. expt. DTA 1994

fish code	PH50	equation (log-log)	comments
AR:1	.65%	zero = .543 log(x) + .0716	.962
AS5:1	~~.475~~	.45 . 45 . 236 log(x) + .476	r² = .952
AS10:1	.538	x10 , 241 log(x) + .538	.995
AS20:1	.572	.204 log x + .572	.941
CR:1	.918	.490 log(x) + .918	.964
CS5:1	.581	.227 " + .581	.995
CS10:1	.969	.436 log x + .969	.919
CS20:1	.965	.436 " + .965	.939
ER:1	.7	.523 log(x) + .807	.963
ES5:1	.640	.205 log x .640	1.0
ES10:1	1.0	.481 log x + 1.0	.901
ES20:1	1.089	.418 log x + 1.089	.937
BR:1	.511	.250 log x + .511	.993
BS5:1	.537	.239 " + .537	.999
BS10:1	.584	.206 " + .584	.971
BS20:1	.475	.259 " + .475	1.0
DR:1	1.009	.424 log x + 1.009	.913
DS5:1	.830	.419 log x + .830	.884
DS10:1	.596	.211 log x + .596	.996
DS20:1	2.169	.836 log x + 2.169	.973
FR:1	1.208	1.137 log x + 1.208	.949
FS5:1	3.764	1.977 log x + 3.764	.978
FS10:1	.511	.240 log x + .511	.995
FS20:1	.584	.230 log(x) + .584	.983
AR:2	.658	.554 log x + .658	.951
AS5:2	.498	.585 " + .498	.928
AS10:2	.527	.244 " + .527	.994
AS20:2	.540	.222 " + .540	.981
CR2	.484	.259 log x + .484	.992
CS5:2	.435	.273 log x + .435	1.0
CS10:2	.368	.287 log x + .368	.993
CS20:2	.482	.250 " + .482	.990
ER2	.507	.248 log(x) + .507	1.0
ES5:2	3.883	2.005 " + 3.883	.991
ES10:2	.845	.552 log x + .845	1.0
ES20:2	.663	.192 log x + .663	.998
BR:2	.567	.209 log x + .567	.959
BS5:2	1.414	1.023 log x + 1.414	.871
BS10:2	.689	.599 " + .689	.996
BS20:2	.745	.528 " + .745	.954
DR2	.430	.668 log x + .430	.950
DS5:2	.579	.594 " + .579	.970
DS10:2	.638	.591 " + .638	.984
DS20:2	.561	.232 " + .561	.997
FR2			

APPENDIX III

Appendix III. Sample catecholamine data and analysis.

The following pages present representative catecholamine data and analysis. The first page provides a copy of the chromatogram and printout obtained by High Pressure Liquid Chromatography. The peak at approximately 8 minutes is noradrenaline, the peak at approximately 10 minutes is adrenaline and the peak at 12.5 minutes is the internal standard, DHBA. The area under the peak is used to calculate the amount of catecholamine present in the sample. A sample printout of the worksheets to caculate the concentration is provided on the following page.



CHROMATOGRAM 1 MEMORIZED

CR501 CHROMATOPAC
CHANNEL NO 1 FILE 0
SAMPLE NO 0 METHOD 2001
REPORT NO 67

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	3.287	142260			15.8227	
		11324hi				
2	3.873	46551	E		5.1776	
		3573hi				
3	4.41	393891			43.81	
		16092hi				
4	5.282	241486	V		26.859	
		5047hi				
5	7.816	9199		NA	1.0231	
		394hi				
6	9.986	21259		A	2.3644	
		731hi				
7	12.55	44444		DHBA	4.9432	
		1400hi				
	TOTAL	899089			100	

CAanalysis940827AJBexpt+

code	area NA	area A	area A toc	area A tota	area DHBA	[NA](pM/mL	[A]total (pM/mL	[A] big peak
CS51	9629	67830		67830	28856	750.80572	5288.93471	5288.935
ES101	54811	298234		298234	45135	2732.3529	14867.0987	14867.1
AS51	16625	101255	1033	102288	57287	652.96228	4017.455967	3976.884
BS101	7749	24744	1003	25747	52262	333.61238	1108.467912	1065.286
DR2				0	47488	0	0	0
FS204	50736	51967		51967	39369	2899.6419	2969.995428	2969.995
DS52		8102		8102	47667	0	382.4343886	382.4344
CR3	1572	9989		9989	37549	94.196916	598.5578844	598.5579
CS53	2419	11199		11199	38682	140.70498	651.4076315	651.4076
FR2	1966	8858	2829	11687	64537	68.542077	407.4523142	308.8228
CS51	6452	51521	477	51998	20432	710.50313	5726.091425	5673.564
ES101	77565	41695	391028	432723	58623	2977.0099	16608.27235	1600.289
DR1	8418	17061		17061	37449	505.76784	1025.054074	1025.054
AR3	9963	42820	734	43554	47997	467.04482	2041.721358	2007.313
CS54	2389	5684	1023	6707	46293	116.11367	325.98341	276.2621
BR3	3957	5494		5494	42257	210.6929	292.5314149	292.5314
ER4	3580	7414	978	8392	49110	164.01955	384.4838119	339.6762
CS51	9629	67830		67830	28856	750.80572	5288.93471	5288.935
BS52		7423		7423	47913	0	348.5849352	348.5849
AR4	4291	16053	776	16829	65007	148.51862	582.4795791	555.6209
ES54	11411	44386		44386	51701	496.60065	1931.655094	1931.655
ES103	11619	30747	1276	32023	57243	456.69776	1258.699754	1208.545
FR4	3577	7855		7855	41531	193.78898	425.5556091	425.5556
AS104	14416	25662		25662	69104	469.37949	835.5449757	835.545
DS202	9199	21259		21259	44444	465.70403	1076.247637	1076.248
FS103	10056	40793	1046	41839	45323	499.21673	2077.041458	2025.114
ES104	5517	31961		31961	42013	295.46212	1711.666627	1711.667
AS104	17361	106854		106854	42857	911.45554	5609.8537	5609.854
AS53	2602	15059		15059	56498	103.62314	599.7159191	599.7159
DS53	30624	43748		43748	50766	1357.2864	1938.955206	1938.955
BS53	11994	50500		50500	129166	208.92882	879.6819596	879.682
AS103	5764	21668		21668	55871	232.124	872.5993807	872.5994
CS103	8017	33236		33236	50297	358.63471	1486.788476	1486.788
CS204	28428	24806	770	25576	66016	968.90148	871.6977702	845.4541
CS53	4691	47220		47220	49247	214.3227	2157.390298	2157.39
CR4	4304	3867	17557	21424	52757	183.55858	913.6986561	164.9212
ES204	21577	75796		75796	59552	815.22451	2863.732536	2863.733
ES104	5355	17571		17571	53719	224.29215	735.9546901	735.9547
AS54	4964	50638	366	51004	62681	178.18797	1830.841882	1817.704
CS104	1579	20096		20096	56874	62.467032	795.0205718	795.0206
FS104	9483	30764		30764	53939	395.57185	1283.282968	1283.283
BS53	3710	18991	699	19690	52540	158.87895	843.2146936	813.2804
CS104	21098	32445		32445	64466	736.36491	1132.399249	1132.399
FS52		33538	622	34160	47025	0	1634.449761	1604.689
FS52	4836	12514	1039	13553	52185	208.50819	584.3489508	539.5516
DS102	5148	45401	836	46237	63277	183.05229	1644.09264	1614.366
BS202	5563	31646		31646	45202	276.907	1575.228972	1575.229
N1A	1646	4776	3106	7882	86090	43.018934	205.9995354	124.8229
N2A	3130	7434		7434	50150	140.42871	333.5294118	333.5294
N2B	2202	3535	791	4326	68232	72.612557	142.6530074	116.5692
N1B	1641	10419		10419	66444	55.569352	352.8196677	352.8197