AN ABSTRACT OF THE THESIS OF

Nora Egan Demers for the degree of Master of Science in Zoology presented on June 30, 1993

Title: The Acute Effects of Stress on Plasma Proteins of Rainbow trout, *Oncorhynchus mykiss.*

Redacted for Privacy

Abstract approved _____

Christopher J. Bayne

Plasma from resting and stressed rainbow trout were analyzed by crossed immunoelectrophoresis (CIEP) to detect differences in the concentrations of individual plasma proteins. Cortisol was measured as an indicator of the level of stress. Resting fish had plasma cortisol levels of approximately 4 ng/mL. Fish were stressed by being held in a net in the air for 30 seconds and then placed in a shallow bucket of water for 5, 10 or 20 minutes. Blood samples were then collected immediately. Plasma cortisol increased from resting levels to nearly 80 ng/mL within 5 minutes and continued to increase over the course of the experiment. A polyclonal rabbit anti-serum to rainbow trout plasma proteins was used in CIEP. Four of the 12 peaks analyzed by ANOVA had increased in concentration after stress. One of these peaks was identified as the C3 component of the complement cascade. These results provide evidence that components of the innate immune system of rainbow trout may be acutely enhanced as a consequence of stress. Increases in the concentrations of certain plasma proteins may accelerate the speed with which fish inactivate foreign antigen or altered "self" to regain homeostasis after an acute stressor.

THE ACUTE EFFECTS OF STRESS ON PLASMA PROTEINS OF RAINBOW TROUT, *ONCORHYNCHUS MYKISS*.

by

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A

THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the degree of

Master of Science

Completed June 30, 1993

Commencement June, 1994

APPROVED:

Redacted for Privacy

Professor of Zoology in charge of major

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Date thesis is presented June 30, 1993

Typed by Nora Egan Demers for Nora Egan Demers

Acknowledgements

Many people have helped in making this thesis a reality. I would especially like to thank Dr. Christopher Bayne, my major professor, for his support and good advice. Sarah Fryer, Randy Bender, Irja Galvan, Jyun-Hong Chen and Maureen Larson, all members of the Bayne Lab, have been kind and helpful, for which I am grateful.

I owe many thanks to all the support staff especially Susan Gilmont and Janet Webster at the Guin Library, Bill Amberg at the Laboratory for Animal Resources, and Dick Ewing for raising the fish.

I have been fortunate to have help from Dr. Stephen Kaattari and others in his lab, especially Jon Piganell, Leslie Gilkey and David Shapiro.

Dr. Carl Schreck and his lab members Dan Farnsworth and Chris Stevens measured plasma cortisol and glucose levels.

I received great support and guidance from the Fish Disease Wing, or Laboratory for Fish Disease Research, at Hatfield Marine Science Center, especially Marcia House, Margo Whipple, Cathy Lannan, Paul Reno, Prudy Caswell-Reno, Bob Olson, Rich Childers and Bill Kelleher.

Financial support was also provided by the Zoology Department Research Fund (ZoRF), a Lylian Brucefield Reynolds Scholarship, and the Laboratory for Animal Resources.

I would like to thank my husband Roy for his patience and support, and finally my mother; I did it even though she wanted me to!

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Introduction

Stress is a universal feature in the daily lives of fish and other organisms. Although the sources of stress are different, the responses are similar. A number of plasma hormones increase in concentration, and this starts an intricate cascade and amplification process to regain "homeostasis". I suggest that plasma proteins may increase in concentration as a response to the hormones released. The proteins could be pre-synthesized and released within minutes on response to a stress. The release of certain proteins decreases the amount of time it takes for the blood to clot, increasing the chances of survival if injured. Other proteins (opsonins) enhance the uptake and clearance of foreign or damaged material. Activation of white blood cells is enhanced by numerous plasma proteins. Plasma proteins also attract cells to damaged areas. No matter the source of the stress, a quick activation of the body's defense mechanisms should increase chances for survival.

I will briefly review contemporary ideas on host defense, effects of stress on the immune system, and on the release and synthesis of acute phase plasma proteins, and the interaction of systems. Then I will propose an hypothesis and present a set of experiments.

Defense

Host natural defenses serve to limit the spread of potential pathogens and remove damaged tissues (Ellis, 1981a,b; Rijkers, 1982; Lipke and Olson, 1990; Pool et al., 1991). The first line of defense in vertebrates is the tegument. In fish, goblet cells in the skin, gills and mucosa continually replenish the protective mucus, containing lysozyme (Fletcher and White, 1973; Ellis, 1981a,b). Lysozyme digests the peptidoglycan in bacterial cell walls (Ellison and Giehl, 1991). DNA sequence analysis reveales calcium-binding lysozyme *c* in liver preparations of rainbow trout (Dautigny et al., 1991). One variant exhibits substantial anti-bacterial activity against 7 pathogens of rainbow trout (Grinde, 1989). Mock and Peters (1990) reported increases in the lysozyme of trout that were stressed by handling.

If physical barriers are breached, the sites of damage and/or microbial invasion commonly experience inflammatory resonses. During inflammation, vascularized tissues of the body respond to injury by increased flow in blood vessels, increased vascular permeability, and cellular infiltration that allows the delivery of various mediators to the site of inflammation (Larsen and Hensen, 1983). The processes involved are dependent upon an extensive

network of interacting mechanisms, mediators and cells (reviewed by Sundsmo and Fair, 1983; Larsen and Hensen, 1983; Rother and Rother, 1986). Macrophages and neutrophils engulf and destroy foreign materials or damaged cells (MacArthur et al., 1984). Macrophages have receptors on their cell surfaces for hormones, and other mediators of inflammation (reviewed by Bodmer, 1985). 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 such as Complement (C') that mediate inflammation (McPhaden et al., 1985). Trout macrophages generate reactive oxygen species similar to those in mammalian phagocytes, that are capable of damaging endocytosed materials (Secombes et al., 1992). Reactive oxygen species play a role in the killing of *Aeromonas salmonicida* by trout macrophages (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.

These components of innate immunity are distinct from the those of the "classical immune system" characterized by its ability to develop a specific response with the hallmark trait of memory. Specific immune responses require the action of B cells producing antibodies (Ig) interacting with T cells in both humoral and cell mediated immunity. Similar interactions have been documented in teleosts (Finn and Nielson, 1971; MacArthur et al., 1984; Nash et al., 1986).

Stress and the General Adaptation Syndrome

The definition of 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) has been refined by Selye and others. In his description of the General Adaptation Syndrome, Selye (1950, 1973) divided the stress response into 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 performance may be reduced.
3. Exhaustion: with severe stress over a prolonged period the capacity of the organism to adapt is exceeded.

Physiological adaptation in fishes is characterized by changes in blood and tissue chemistry that are similar whether the stress results from handling and transport, pollution or water temperature changes, or behavioral factors (Wedemeyer et al., 1990). The changes are adaptive and are required to achieve acclimation. The stress response can 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.

Stress stimulates the hypothalamus which, by means of corticotropin releasing factor (CRF), stimulates the anterior pituitary to release adrenocorticotropin releasing hormone (ACTH), which causes the release of corticosteroids from the interrenal cells. Cortisol and other corticosteroids are four-ringed structure that pass through the plasma membrane and are thought to act upon cytosolic and nuclear targets, affecting gene transcription (Funder, 1992). A cortisol receptor on the membranes of amphibian neuronal cells activates intracellular enzymes when bound with cortisol (Orchinik et al., 1991, 1992; Moore and Orchinik, 1991). Rainbow trout hepatocyte adrenoreceptors are modulated in part by cortisol (Reid et al., 1992).

During stress, the hypothalamus also stimulates the chromaffin cells of the head kidney to release catecholamines (Mazeaud and Mazeaud, 1981). Catecholamines, such as adrenaline and noradrenaline, are small, charged molecules that interact with cell membrane proteins. The actions of 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. Secondary effects of catecholamines include decreased liver glycogen, upset electrolyte

balance, increased blood glucose and lactate levels, increased heart rate, and water content changes (Mazeaud and Mazeaud, 1981). Catecholamines were also shown to modulate rat acute phase reactants (Kahl and Schade, 1991).

Stress and Immunity

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). However, Maule (1989) also noticed that acute stress can have a transient positive effect on immune function and disease resistance. 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 lymphoid hemopoietic organs, specifically 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 stressors initially stimulated the innate arm of the defense mechanism in an adaptive manner. Increased abundance of neutrophilic granulocytes and reduced numbers of lymphocytes were typical. A more recent report (Peters et al., 1991) upheld these results and showed that, under social stress, phagocytes became activated. Ellsaesser and

Clem (1986) concluded that immunosuppression was primarily caused by a direct disturbance of the T and B lymphocytes. They considered that adaptive elements of the initial alarm phase (proliferation and activation of immunocytes) were overwhelmed in the exhaustion phase by cellular degeneration and accelerated autophagocytosis. Chavin (1973) noted that teleost interrenal and chromaffin cells had a markedly different appearance after saline was introduced into the fish tanks.

In evaluating the levels of cortisol as indicators of stress, Maule and Schreck (1991) noted significant increases in plasma cortisol concentrations in fish that were acutely stressed (by handling), chronically stressed (by handling), or fed cortisol-treated food. These measurements were made three to four hours after the stressor. Barton et al., (1986) noted significant increases in cortisol concentrations in juvenile chinook salmon after multiple acute handling stresses, however, samples were taken no earlier than 1 hour post treatment. Responses to confinement stress have been compared in wild and hatchery-reared rainbow trout (Woodward and Strange, 1987). Cortisol concentrations increased in both stocks, with changes being more extreme in the wild fish. The earliest time point measured in this study was 30 minutes after the stress, at which time there was a significant elevation in plasma cortisol.

Few have measured cortisol levels within minutes of a stressor. Singley and Chavin (1971, 1972, 1975) reported increases in both hormones after only 15 seconds of saline stress in goldfish. However, Singley noted that the time to bleed the first fish was greater than 1 minute (Spieler, 1974). Schreck (1976) and Strange et al. (1977),

reported that corticosteroids increase "almost instantaneously" with the onset of stress. Plasma cortisol levels rose within 10 minutes in atlantic salmon after an injection of ACTH (Nichols and Weisbart, 1984). Sumpter et al., (1986), using handling and confinement to study the effects of stress on coho and rainbow trout, found plasma ACTH levels were significantly elevated after 2 minutes of stress, and were peaking within 5 minutes. Plasma cortisol levels in both species, however, were unchanged after 2 minutes and began to rise only after 8 minutes. Plasma cortisol values of red drum were not significantly higher until 30 minutes after transport in low salinity sea water (Roberston et al., 1988). Flounder and atlantic salmon were fitted with catheters to draw blood samples at various time points without disturbing the fish (Waring, et al., 1992). Significant increases in plasma cortisol concentrations were noted in both species within 9 minutes of handling and confinement stressors. An increase of 200% was noted in the salmon, and an increase of nearly 1000% in the flounder at the 9 minute sample. Although many have studied the effects of stress on cortisol levels (reviewed by Barton and Iwama, 1991), few have looked at the kinetics of the short-term response. Those that have are not in agreement. More data are needed to improve our understanding of the stress response.

While it is clear that suppression of the immune system occurs as a result of corticosteroid release during stress (Tripp et al., 1987; Maule, 1989; Pickering et al., 1989), less is known about the effects of catecholamines on disease resistance. The synthesis and secretion of protein by rat hepatocytes 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 rat were increased by catecholamines, but whether the response is receptor-mediated or non-specific remains to be clarified (Kahl and Schade, 1991). Flory (1988) found that sympathectomy of coho salmon was accompanied by dramatically reduced levels of catecholamines in the spleen, and altered 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 also found that catecholamines can modulate the respiratory burst, an observation which has been further examined (Bayne and Levy, 1991a and 1991b). Characterization of catfish liver membrane receptors for catecholamines that increase intracellular levels of cyclic AMP (Fabbri et al., 1992) suggests that teleosts may experience a modulation of acute-phase reactants similar to that seen in rats (Kahl and Schade, 1991).

The levels of plasma catecholamines reported in resting rainbow trout vary widely between studies (Woodward, 1982) probably due to the difficulty of obtaining blood from truly unstressed fish. The release of catecholamines from the chromaffin cells upon stimulation is very fast. Mazeaud et al. (1977) found significant increases in adrenaline within 5 minutes of struggling in salmonids. Adrenaline concentrations in the plasma and head kidney of rainbow trout rose within 2 minutes of a handling disturbance (Nakano and Tomilson, 1967).

Another indicator of stress is the time it takes for the blood to clot. Blood of trout that had been stressed coagulated faster than

blood from minimally stressed fish, and phagocytes from stressed fish were more phagocytic than those from control fish (Ruis, personal communication). Blood clotting time and changes in leucocyte counts are among the most sensitive indicators of acute stress (Casillas and Smith, 1977; Wedemeyer et al., 1990).

Adrenaline receptors on hepatocytes and macrophages may help elicit the immediate release of proteins and other mediators. Their release could continue the activation process, improving an organism's ability to respond to stressful situations and their potential consequences.

Plasma Proteins of Interest

The plasma proteins include several that are termed acute-phase plasma proteins. Concentrations of these proteins change after the body has been stressed. Schreiber (1987) divides the acute phase proteins into two categories: positive acute phase proteins, which increase in concentration, and negative acute phase proteins, which decrease. Of those proteins termed positive acute phase reactants in mammals, Complement (C'), C-Reactive Protein, antibody, and fibronectin enhance phagocytosis, i.e. they are opsonins. Plasma proteins also increase vascular permeability and have other roles in enhancing immunity. There is a remarkable degree of interdependence and cooperation among these molecules.

The Complement cascade comprises at least 14 proteins, and others proteins are necessary to modulate its activity. Three vital functions of C' are cell activation, cytolysis and opsonization. C' and coagulation systems interact, releasing and activating vasoactive amines, kinins and prostaglandins, all of which play a part in vasodilation, vascular permeability and chemotaxis (Ellis, 1981). Other roles include increasing vascular permeability and smooth muscle contraction (Rother and Rother, 1986). The bulk of human C' 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 C' cascade has been traditionally divided into two pathways. The "alternate" pathway begins with the cleavage of component C3. C3a, a vasoactive chemoattractant fragment, and C3b are produced. C3b is deposited on membranes of a bacterium, virus or damaged host cell. Other C' 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 C' components, C3 is the major phagocytosis-promoting factor (Matasuyama et al., 1992). C3, which is in highest concentration of all C' proteins in mammals and is an acute phase reactant (Schreiber, 1987), is cleaved by the serine proteases trypsin, plasmin, thrombin, and elastase as well as C' factors.

"Classical pathway" component, C1q, binds to Ig. This complex activates C1r and results in cleavage of C4. Other components are cleaved that eventually cleave C3 and initiate the MAC as in the alternate pathway. Classical pathway activation is not regulated 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).

Interactions of complement components with bacterial lectins also augment phagocytosis (Kurashima et al., 1991). The C' 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). The mechanism of innate activation of rainbow trout C' is similar to the alternative pathway of activation of mammalian C' (Sakai, 1984 a,b). There is

evidence for both C' and Ig receptors on fish macrophages (Sakai, 1984a). C3 receptors on carp neutrophils enhance phagocytosis when C3 is bound to particles (Matsuyama et al., 1992). 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 bactericidal effects of catfish alternative pathway C' have been documented by electron microscopy (Jenkins et al., 1991). Both C' and antibody augment macrophage activity in the clearance of *Vibrio anguillarum* from rainbow trout (Honda et al., 1986). Clearly, changes in concentrations of C' proteins would 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). Three proposed functions of CRP are 1. recognition and targeting for defensive mechanisms, 2. modification of effector cells such as polymorphonuclear cells (PMN), lymphocytes, monocytes and platelets, and 3. opsonization (Rijkers, 1982; Gotschlich, 1989). Human C-reactive protein binds to receptors on mononucluear phagocytes and lymphocytes, stimulates phagocytosis, and inhibits the aggregation of platelets (Schreiber, 1987).

After a physical injury, CRP levels in *Tilapia mossambica* were increased within 24 hours (Ramos and Smith, 1978). Production of

trout CRP was significantly increased 3 to 10 days after immunization with Freund's complete adjuvant (Kodama et al., 1989a). Rainbow trout 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 these trout within the first days of treatment. Purified CRP increased the consumption of rainbow trout C' in the presence of *Streptococcus pneumoniae* C-polysaccharide, indicating the activation of C' by CRP (Nakanishi et al., 1991). CRP-like molecule have been isolated from trout (Murai et al., 1990) and channel catfish (Szalai et al., 1992). CRP may be an important component of the natural defenses of fish by enhancing phagocytosis and activating C'. Natural defenses are especially important in fish since they exhibit relatively slow specific immune responses against bacterial and viral infections (Murai et al., 1990).

Fibronectin is a high molecular weight glycoprotein found in the blood, lymph, tissue fluid, and extracellular matrix. Soluble and membrane bound forms of fibronectin play roles in cell-cell interactions such as oncogenesis, metastasis, wound healing, and embryonic development (reviewed by Saba, 1989; Hynes, 1990). Fibronectins are 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 play a role in parasitic diseases by mediating adherence of parasites to the host extracellular matrix (Wyler, 1987; Klotz and Smith, 1991). Fibronectin appears to modulate both antibody synthesis by B cells and macrophage-mediated immunoregulation (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), and its metabolism in rainbow trout cell culture was influenced by cortisol (Lee and Bols, 1991). Fibronectin has been purified from carp (Uchida et al., 1992) and from rainbow trout (Takahashi et al., 1992). The potential role of fibronectin in injury and infection warrants its inclusion in this list of plasma proteins of interest.

Alpha 2-macroglobulin (alpha 2-mac) is an acute phase protein produced by the liver and macrophages in response to endogenous mediators released from neutrophils and macrophages (Putnam, 1975). It is an anti-protease and may suppress inflammatory and immunologic responses (Ellis, 1981). Alpha 2-mac is a tetramer that complexes with a wide spectrum of proteinases including serine, thiol, carboxyl and metaloproteases (Putnam, 1984). Many of the clotting proteins are serine proteinases. Upon encountering a proteinase, alpha 2-mac undergoes conformational changes and "entraps" it. The complex is then rapidly cleared from the circulation (Sottrup-Jensen, 1987). Proteinases and other components bound by alpha 2-mac 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 of endocytic vessicles may release bound cytokines with affects on cellular metabolism.

Ellis (1987) found that the proteases released by the bacterium *Aeromonas salmonicida* which contribute to its pathogenicity can be inhibited by the alpha 2-mac of rainbow trout serum. Freedman (1991) found a qualitative difference in the inhibitory activity of alpha 2-mac between brook and rainbow trout which corresponded with the greater susceptibility of brook trout to furunculosis. Clearly, alpha 2-mac may play a vital role in regaining homeostasis after any acute stress in which proteinases are released into the blood, whether they be endogenous or exogenous.

Hypothesis and Experimental Approach

There is convincing evidence that chronic stress causes suppression of the immune response, probably through the action of corticosteroids (Robertson et al., 1963; Pickering and Pottinger, 1983, 1987; Tripp et al, 1987; Maule et al., 1989). There is some evidence, however, that acute stress may enhance immunity, at least initially (Peters and Schwarzer, 1985; Peters, 1985; Maule, 1989; Mock and Peters, 1990; Ruis, personal communication, 1990; Peters et al., 1991). Such enhancement could be due to increases in the concentrations of plasma proteins that stimulate phagocytosis. Also, clotting, vasodilation for entry of cells into damaged tissues, and cell activation could be enhanced by increased amounts of appropriate plasma components.

Although various studies have noted increases in concentrations of cortisol and plasma proteins within hours or days of a stress, very little is known about changes in cortisol or plasma protein concentrations within minutes of an acute stress. I propose that the release of plasma proteins is a very fast process, happening within minutes of a stressor. I propose that at least some of the proteins that increase in concentration are components of natural immunity.

In this study, healthy rainbow trout were bled to obtain resting and stressed plasma. Cortisol values were used as indicators of the stress levels experienced by the fish. A pool of rabbit antiserum to rainbow trout plasma antigens was used to analyze the pooled

plasma of resting or stressed rainbow trout by crossed immunoelectrophoresis (CIEP). Areas under the peaks in samples from stressed and resting fish were analyzed for measurable changes in the concentrations of plasma proteins within minutes of an acute handling stress.

Materials and Methods

Fish

Rainbow trout of the Cape Cod stock from Roaring River Hatchery (Shasta strain) were raised from eggs in 1990 at the Oregon Department of Fish and Wildlife Corvallis Fish Research Lab. The fish, weighing150-450 g at the time of sampling, were raised in a 1.5 m diameter fiberglass tank with flow-through well water (10-12°C), and fed a daily ration of Oregon Moist pellet.

Plasma from coho salmon raised at the Hatfied Marine Science Center was also collected and used in analysis of C3 levels.

Stress regime

Thirty seven fish were sampled from May to December of 1991 between 9:30 am and 12:30 pm. To obtain blood from a resting fish, the fish was netted, sacrificed by a blow to the head, and bled from the caudal vessel within 1 minute of approach to the tank. No further samples from the tank were considered resting until at least 48 hours had elapsed. There were 15 samples collected from resting fish.

Stressed fish were collected immediately after the resting fish were sampled, and within minutes of the first approach to the tank. Each stressed fish was netted, held in the air for 30 seconds and placed in a shallow bucket at ambient temperature for 5 (n=10), 10 (n=9) or 20 (n=4) minutes. The trout were then sacrificed with a blow to the head, and bled from the caudal vessel.

The blood samples were put into a tube containing 10 U/mL heparin and stored on ice, usually less than 1 hour. Samples were centrifuged at 4°C at 400g for 10 minutes to separate the plasma from the cells. Plasma samples were stored in aliquots at -70°C. Five samples of plasma from each group were pooled for analysis by CIEP. The 20 minute stress pool contained plasma from 4 fish. Plasma from individual fish was also analyzed by CIEP, analyzed for cortisol and glucose concentrations, and used for anti-serum production.

Cortisol

Cortisol values were measured by a radioimmunoassay procedure described by Redding et al. (1984).

Glucose

Glucose concentrations were determined by the o-toluidine method (Wedemeyer and Yasutake, 1977).

Rabbit Antiserum to Rainbow Trout plasma antigens

Six female New Zealand white rabbits were used to produce the anti-rainbow trout serum. After initial pre-bleeds, each animal was injected in six subcutaneous and two intramuscular locations with an emulsion of approximately 0.8 mL Freund's Complete adjuvant and rainbow trout plasma from resting or stressed trout. Three rabbits were used for resting and 3 rabbits for stressed antiserum production) Three weeks later, each rabbit was injected with 0.8 mL of Freund's incomplete adjuvant (FICA) emulsified with resting or

stressed rainbow trout plasma. The rabbits were bled by cardiac puncture 7-10 days later. The serum was collected and screened for antigen recognizing capacity using CIEP. Booster injections of rainbow trout plasma emulsified in FICA were given when antiserum titres decreased. Approximately 40 milliliters of blood were collected every two weeks for four months. Sera from rabbits that had more than twelve CIEP peaks were pooled. A pool of approximately 300 mL antiserum against stressed plasma was used in all the gels. The pooled sera were placed in 1 mL. aliquots and stored at -70°C.

Crossed Immunoelectrophoresis (CIEP)

In the first step of CIEP, antigens are separated on the basis of electrophoretic mobility. In the second step, the separated antigens are electrophoresed at right angles to the first dimension into gel containing antiserum. Antigens precipitated by specific antibodies in the gel appear as peaks that are detected by Coomassie Blue. The area enclosed by a precipitation line and the line at which the two gels meet is directly proportional to the total amount of antigen (Weeke, 1973; Grubb, 1983).

Agarose (FMC Bioproducts, ME) was cast at 1% in Tris-Barbital buffer, pH 8.6 (see Appendix for details about CIEP reagents) onto a glass plate for CIEP (Figure 1). Electrophoresis was carried out at 10 V/cm for 2 hr. 20 min. on an LKB (Sweden) apparatus cooled to 10-12°C in an 18°C room. Agarose strips containing the plasma components of a single fish, a pool of plasma, or purified antigen

were then cut and transferred for the second dimension overnight run (Figure 2). After completion of this run, gels were rinsed twice in phosphate buffered saline (PBS) pH 7.4 for 15 minutes, transferred to Gelbond (FMC bioproducts), placed on a warming tray, and covered with a piece of dry Whatman filter paper, wet sponges, several centimeters of absorbant paper towels and a weight for 15 minutes. They were then rinsed in PBS for 15 minutes and pressed again. The gels were then rinsed in distilled water for15 minutes and pressed to dry on a warming tray. They were stained with Coomassie Brilliant Blue (CBB) for 10 minutes and destained 1-2 minutes to remove unbound stain (see Appendix for details). The least amount of agarose required to cover the glass plate yielded the most distinct precipitation pattern.

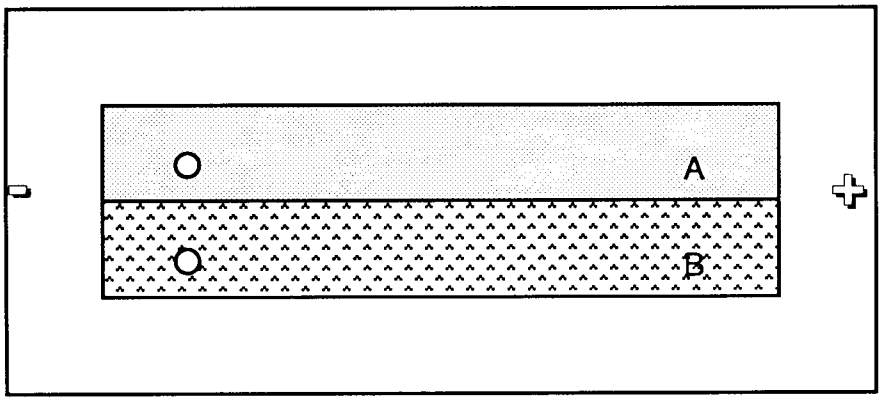


Figure 1. First dimension CIEP. 17.2 mL 1% agarose was cast on a 14.7 cm by 7.9 cm glass plate. Ten µl of sample was placed into each 4 mm well on the cathode side. Constant voltage was applied at approximately 10V/cm for 2 hours 20 minutes. Shaded areas (A & B) indicate portion of gel containing separated antigens that was tranferred to the second dimension plate. Electrophoresis was carried out in an 18°C room. The cooling plate temperature was 10-12°C. + = cathode; - = anode.

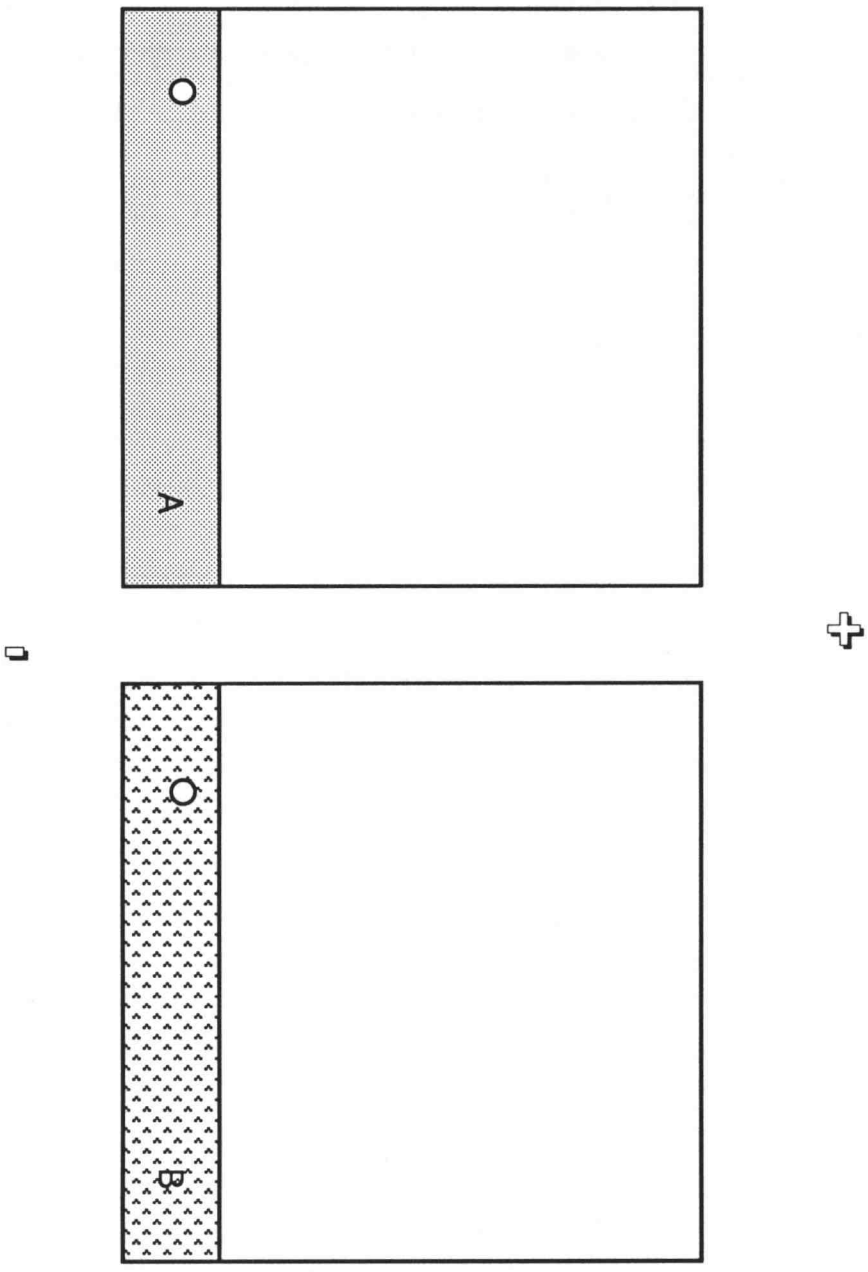


Figure 2. Second dimension CIEP. 18 mL 1% agarose containing 11.1% rabbit anti-rainbow trout serum was cast onto each of two 12.5 cm X 12.5 cm glass plates containing transferred slabs of agarose from the first dimension run (A & B). Gels were electrophoresed at constant voltage (2V/cm) for 22 hours in a humid chamber in an 18°C cold room.

Java Image Analysis

To facilitate analysis, each gel was photographed, and the print was used for identification and measurement of the peaks. Identification of the same peaks on different gels was a subjective procedure. A video monitor was used to project the original gel image onto the Java Image Analysis System. With photograph and marker in hand, each peak was labelled and measured. Peaks were identified according to how far from the origin (placement of sample) they had travelled, their general shape, sharpness and resolution, and their location compared to other peaks on the gel (Figure 3). The areas under those peaks that could be confidently identified in a majority of the gels were measured. If the entire peak could not be visualized on any given gel, that peak was not measured.

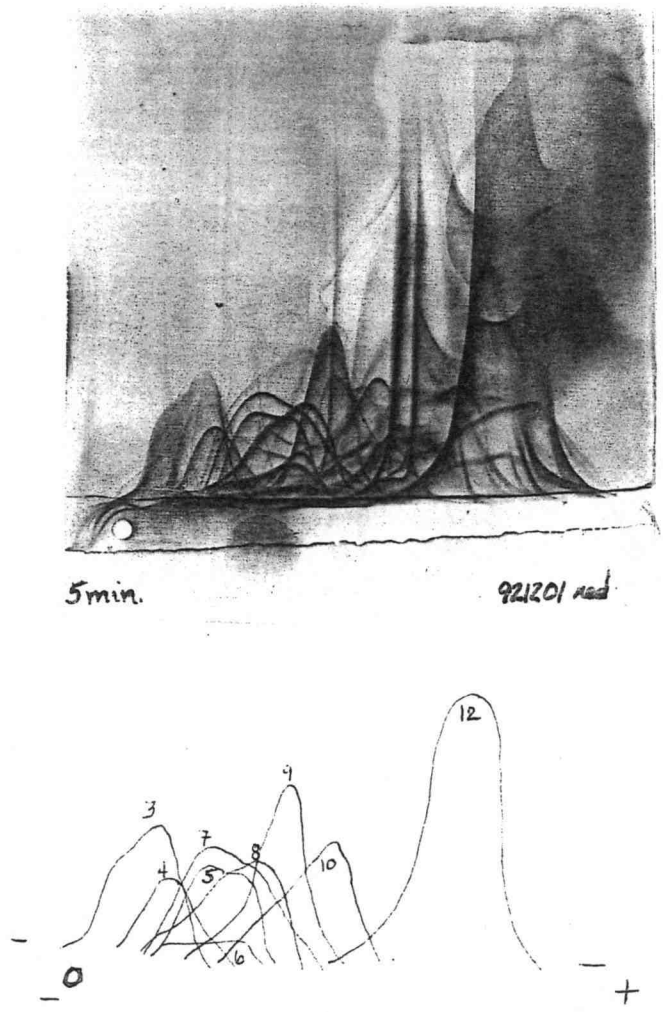


Figure 3. Photograph of one representative gel that was included in the analysis. Peaks that were identified in most gels were numbered 1-12 and correspond to the peaks identified in the lower tracing. Peaks 1 and 2 were not measured on this gel. As can be seen, several peaks were not analyzed.

Antigen detection on Nitrocellulose

Purified C3 and rainbow trout plasma were electrophoresed in agarose gel under the first dimension conditions. The proteins were passively transferred, by mechanical blotting, to a 0.2 μm nitrocellulose membrane (see Appendix for reagents and details). The membrane was blocked for 45 minutes at room temperature with 3% gelatin-TBS. Protein A purified mouse monoclonal anti-rainbow trout C3 (HYB-433, 0.328 mg/mL in 0.5 M NaCl, provided by Lisbeth Goering-Jensen, State Serum Institute, Copenhagen) was used as the probe in an overnight incubation. The membrane was washed in distilled, deionized water, rinsed twice for 10 minutes in TBS then probed with 1:3000 horse-radish peroxidase labelled goat anti-mouse-Ig for 1 hour. After a final wash, Bio-Rad Color Development Reagent was applied and allowed to develop for about 30 minutes.

C3 in the samples was also quantified by placement of five, one μl each, serial, five-fold dilutions of purified C3, and five, one μl each, 1:5 dilutions of rainbow trout plasma onto a nitrocellulose membrane, followed by probing as above.

Statistics

One way analysis of variance was used to analyze cortisol, glucose and peak area information for separate group means. A natural log transformation was required of the peak area data to acheive homogeneity of variance.

Results

Cortisol data

Mean cortisol concentrations increased from less than 5 ng/mL to almost 80 ng/mL within 5 minutes of an acute handling stress ($p<.0001$). They continued to rise during the following 20 minutes (Figure 4 and Table 1).

Glucose

Mean plasma glucose concentrations were significantly higher 10 minutes after stress when compared to resting levels (Figure 5). After 20 minutes, blood glucose was significantly increased ($p<.0001$) over the 10 minute value.

CIEP

Due to the limited supply of anti-serum, and variation in patterns obtained with identical samples, pools of rainbow trout plasma were prepared for electrophoresis (Table 1). Fifteen microliter aliquots of plasma from each pool were stored separately at -70°C until analysis. CIEP runs were carried out with pools and plasma from individual trout (Table 2). For the analysis of resting plasma, 10 replicates of the pool were used. For the 5 minute pool, 8 replicates were analyzed. Seven and 8 replicates of pools for 10 and 20 minute stress groups respectively were analyzed (Table 2). Four of the 12 peaks that were measured (Figure 6) showed significant increases in area from resting (0 minutes) values (Figure 7). None of the

measured peaks significantly decreased in area over the time-course of this study.

Peak 3 was among those peaks whose area changed within 5 minutes of stress. The electrophoretic mobility of Peak 3 is similar to that of purified C3 (Figures 8). This peak was identified as C3 by adding trout C3 (purified by T. Yano in the laboratory of Dr. Steve Kaattari, and solubilized at 1.32 mg/mL) to a plasma sample and running that gel under the same conditions as used in other gels (Figure 9).

Since the area under the peak is directly proportional to the concentration of protein in a sample, it is possible to quantify concentrations of proteins by CIEP (Weeke, 1983). A sample of steelhead trout C3 was run in two-fold dilution by CIEP (Figure 8). Several gels containing dilutions of C3 were run to obtain a best fitting line in order to determine the amount of C3 in resting and stressed fish plasma. By this analysis, resting fish plasma contained approximately 0.65 mg of C3 per milliliter. After 10 minutes, C3 concentrations increased to 1.195 mg/mL. By 20 minutes the concentrations of C3 were near the level of fish stressed for 10 minutes, approximately 0.95 mg/mL (Figure 10).

Attempts were made to identify other plasma components, namely fibronectin, trout Ig and CRP. Purified bovine fibronectin was run under the same conditions as other gels, as was a plasma sample spiked with bovine fibronectin. The fibronectin peak visualized was small and indistinguishable in the spiked plasma

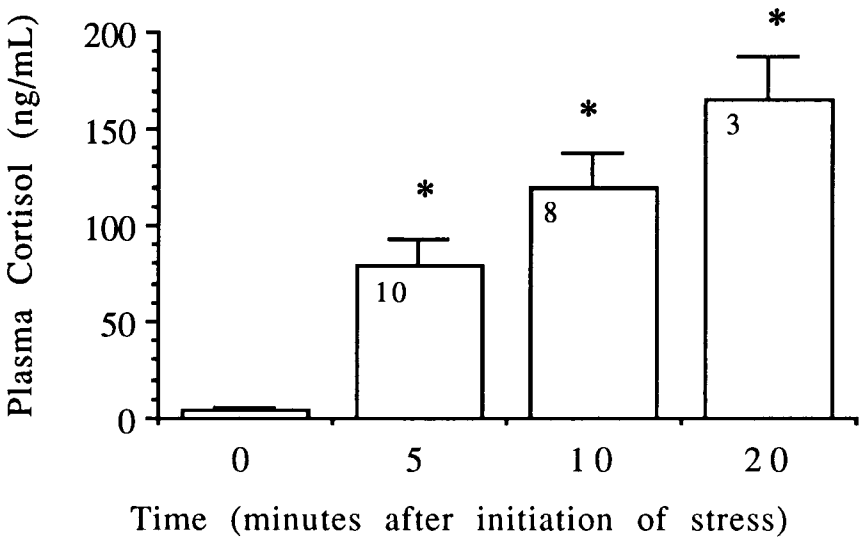


Figure 4. Mean concentration of cortisol in the plasma of resting (0) and stressed rainbow trout. Sample sizes are given inside each bar except group 0; n=16. Internal standard errors are indicated by a bar over each column. * indicates a significant difference from group 0 (resting). $p<.05$.

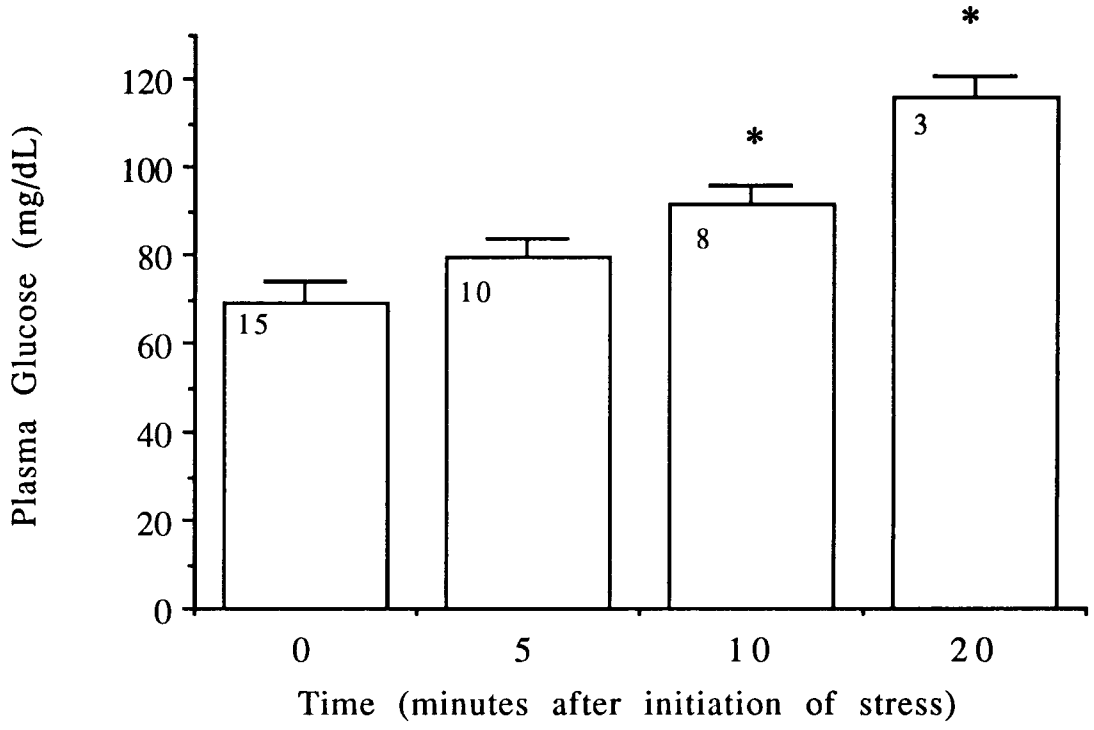


Figure 5. Mean concentration of glucose in trout plasma. Sample sizes are given inside each bar. Resting samples are shown as 0 Time. Internal standard errors are indicated by a bar over each column. * indicates a significant difference from resting values. $p<.0001$.

Table 1. Data on individual rainbow trout sampled. Information is included on each fish's size, sex, date and time of collection, plasma cortisol and glucose concentrations. Stress times indicated as 0 are resting fish. Stress times of 5, 10 or 20 minutes refers to the amount of time the fish were kept in a shallow bucket of water after being netted and held in the air for 30 seconds.

TABLE 1

Date	pool	Sample	Stress	Cortisol	Glucose	wt (g)	L(cm)	sex	time sampled
910506		D1	0	15	73	211	29	m	10:52 AM
910513		E1	0	4.3	66	329	30	f	11:20 AM
910515		F1	0	3	91	256	29	m	10:20 AM
910529		G1	0	3	72	163	26	?	11:00 AM
910603	*	I1	0	3.7	93	290	34	m	10:30 AM
910711		J1	0	1	69	386	37	f	12:30 PM
910724		J1	0	2	64	349	34	f	10:45 AM
910813	*	K1	0	1	79	190	29	f	11:15 AM
910827	*	L1	0	8.2	49	378	33	f	9:30 AM
910919	*	M1	0	1.8	37	251	28	m	not recorded
910930		N1	0	3	79	359	31	f	12:30 PM
911007		O1	0	2.8	70	350	32	m	12:10 PM
911014	*	P1	0	3.6	57	382	34	m	12:00 PM
911028		Q1	0	3	71	244	30	f	11:45 AM
911204		R1	0			348	35	m	10:00 AM
910506		D2	5	92	88	461	38	f	10:52 AM
910711	**	J2a	5	78	91	182	30	m	12:30 PM
910724		J2a	5	18	68	208	28	m	10:45 AM
910724		J2c	5	32	63	263	29.5	f	10:45 AM
910813	**	K2	5	64	76	221	29	f	11:15 AM
910827		L2	5	125.9	68	178	26	f	9:30 AM
910919	**	M2	5	58.1	64	309	34	m	not recorded
910930		N2	5	169.3	101	276	29	f	12:30 PM
911007	**	O2a	5	92.9	90	294	32	m	12:10 PM
911028	**	Q2a	5	59	88	250	29	f	11:45 AM
910513		E2	10	184.9	87	290	32	m	11:20 AM
910515	***	F2	10	138.9	89	305	32	m	10:20 AM
910529	***	G2	10	95	97	342	35	f	11:00 AM
910603		I2a	10	56	110	350	33	f	10:30 AM
910711		J2b	10	50	74	415	37	m	12:30 PM
910724	***	J2b	10	110	99	138	27	f	10:45 AM
911014	***	P2a	10	191.2	80	318	32	f	12:00 PM
911028	***	Q2b	10	126.6	100	264	30	m?	11:45 AM
911204		R2a	10			458	36	m	10:00 AM
910603	****	I2b	20	161	122	417	37	f	10:30 AM
911007	****	O2b	20	205.7	120	324	33	m	12:10 PM
911014	****	P2b	20	130.3	106	423	36	f	12:00 PM
911204	****	R2b	20			354	35	f	10:00 AM

Date example: 910724 is July 24, 1991

* indicates resting plasma pooled for CIEP analysis, ** pooled for 5 minutes, *** plasma pooled for 10 minutes, **** pooled for 20 minutes.

Stress is minutes after 30 seconds in the air.

Cortisol concentration is ng/mL.

Glucose concentration is mg/dL.

Table 2. Peak areas of each CIEP gel. The area under the peak is given under the column heading for that peak and is measured in square mm. Note that some values, especially in gels containing stressed plasma, are missing. Missing values resulted from gels whose peak pattern was incomplete (the top was not visible), and therefore the peak was not measured.

TABLE 2 Date	stress time	sample	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
921121	0	pool	02.3654900	00.5184500	02.1396200	01.2958000	02.6973000
921121	10	pool	00.4681600	00.1152100	03.3569600	01.8890000	05.5810900
921123	0	pool	00.7321200	00.0423242	01.1385600	00.8581400	01.9947200
921123	20	pool	00.8025900	00.1284500	02.6829800	01.2192800	03.2085400
921127	0	pool	00.7918300	00.1477600	01.9911600	01.2472300	02.4809900
921127	5	pool	01.6697800	00.3659700	02.6358300	01.0977200	03.1321200
921128	10	pool	01.6178100	00.4366400	02.7754700	01.1575900	03.3892100
921128	20	pool		00.0890771	02.1396000	02.0310600	03.7730600
921201	5	pool			05.3785700	01.8802800	04.1389400
921201	20	pool	05.0111200	00.2722400	05.2484700	02.8398400	07.0618000
921207	5	pool	09.2780000		05.0130000	02.2384200	04.7836000
921207	10	pool	06.9980700	00.2276000	05.2318300	04.3999200	07.7245200
921208	0	M1	02.5530000	00.0858557	01.9020900	01.1021200	02.0555200
921208	5	M2	01.9981300	00.0513688	03.0605800	01.8917100	03.1923100
921210	5	O2a	02.0648600		03.2612300	03.1181600	03.3738500
921210	20	O2B	02.3409100	00.0660815	02.9706100	00.8240100	04.6848900
921214	0	P1			01.7368300	01.3662100	02.8724600
921214	20	P2B			03.2885900	01.9428800	02.5571100
921222	0	pool	02.6181700	00.0508523	02.5006800	01.4028900	02.9370400
921222	10	pool	02.0398700	00.0944225	04.1537300	01.1440200	05.5688300
921223	0	pool			02.9735100	01.1628000	02.3056500
921223	20	pool	02.4869200	00.0910655	01.9310500	01.9858600	04.6277700
921229	0	pool		00.1035400	02.4229700	01.5035900	02.6332800
921229	5	pool	02.8446300	00.0403035	02.9142300	01.5045500	03.1686200
921230	0	pool			02.3925500	01.2706000	02.5222600
921230	20	pool		00.2134000	02.5809200	01.1906600	02.8356000
930102	0	pool	03.1824400	00.0492835	02.4102200	01.2957300	02.5152900
930102	10	pool	02.2410900	00.3940100	03.7729300	01.3711800	04.3625600
930104	0	pool	02.8405500	00.0244029	02.3817200	01.1904900	02.4583600
930104	5	pool	03.7085400	00.0377535	02.3655200	01.1259500	03.8533600
930107	0	pool	03.0711600	00.0311944	02.2029200	01.1204400	02.5297100
930107	20	pool	01.6340300	00.0278309	02.3165700	01.4234900	03.1357900
930111	5	pool	03.6629700	00.0598065	02.3854300	01.5298700	03.0230100
930111	20	pool	02.6121800	00.0587026	03.8763000	01.7575200	03.1092300
930115	5	pool		00.0647323	02.3460200	01.2571700	02.5957100
930115	10	pool	02.5517100	00.1998900	02.8070400	00.7620100	03.2088500
930118	5	pool			02.3328700	01.1795000	02.8409000
930118	10	pool	02.0818500	00.2494500	02.8807100	01.0771100	03.8978200
930121	0	K1			02.1808000	01.8177000	04.4770400
930121	10	K2	06.2159200	00.1058500	02.9411200	01.6939500	04.2747200
930122	0	P1			02.8571300	01.6811200	03.3795800
930122	20	P2b			03.8599800	02.3537300	02.5017900
930123	0	M1	03.9190700	00.1201200	03.5089800	01.9038700	03.0515000
930123	5	M2	04.8993900	00.1093500	03.7635000	02.4170700	04.3948900
930207	0	I1			03.5439800	01.9350400	01.9990100
930207	20	I2b	05.0202900	00.0599200	04.9391400	02.3638900	06.3331700

Peak 6	Peak 7	Peak 8	Peak 9	Peak 10	Peak 11	Peak 12
00.6870700	05.0695600	03.0816100	03.9365500	04.9841900	02.9297400	03.4373200
01.9707100	06.8560800	04.2064500	05.4480700	04.5608700	04.4357100	07.2427900
00.3844300	03.8670400	02.2390700	02.7118600	03.6679200	05.7969400	07.8494400
00.6460600	05.4877200	04.1699600	07.3107500	05.0923300	03.8890600	
00.7863400	04.6793500	02.6878600	03.4377800	04.8365700	06.1097200	08.2100100
00.2742600	04.6177100	03.4264100	04.6123400	03.5716000	04.4065200	07.1821400
00.4784300	03.7321900	02.8031300	06.4919900	03.4429000	04.5367300	09.3005700
01.0532200	06.5417800	04.0975600	08.1229900	04.4012300	07.8608600	
00.5970700	06.1986000	04.8603500	05.2338800	04.8094800		08.3753700
01.3586200	06.1113700	06.6536200	06.6749300	05.1936800	06.9355700	12.2021700
01.5575000	07.5283300	06.6652200	06.8709900	05.7217000	09.0017200	09.6461800
02.0616400	07.3213200	05.3798000	11.2926800	05.4437900	10.3544900	
00.4206900	04.2120300	01.3734900	03.4710100	03.5635400	04.0350500	04.9072300
	05.7159200	03.5702700	06.4044200	05.4775100	06.8395100	08.8425700
00.0946808	04.9670900	02.9792400	08.1491500	08.1306300	05.0357300	
00.3517800	06.1708000		05.7962800	07.1817400	05.2368000	
00.3752800	05.5955600	02.4188300	03.1805400	04.5935100	05.0110700	06.0654500
	06.0653500	03.3157900	06.2337800	04.5545800	06.9935200	10.6883700
	05.7471600	03.6218000	03.9723700	04.7701400	07.4039600	07.5757300
		03.9915700	10.2688000	04.5266200	10.3781300	15.5220900
00.5669800	04.6057400	02.8851400	03.4612900	04.2265100	07.5659300	
00.6817700		05.1203600	07.5016200	06.1556300	11.6754000	
	04.6485800	02.9971500	03.3341700	04.6171900	06.4849100	
00.2118500	04.9365700	03.9533400	04.6794300	05.0959600		08.7865800
00.7603900	04.4976400	03.0754800	03.3559500	04.4598900	08.8842400	07.8513500
00.5635200	04.9655200	02.9106300	04.4157300	03.8547000		
00.7876000	04.2449800	02.8749300	03.1329900	03.9252100	06.6142600	05.6945600
	06.2732700	03.5408400	03.4555200	04.2090500	09.1663800	
	04.2486400	02.7996200	02.9457800	04.0906800	05.8952300	09.2959400
00.6906100	05.0244900	03.6645000	04.4252300	04.2027400		08.8377300
00.4834600	04.3739000	02.5470900	03.1056000	03.6915900	06.5432500	06.0670800
00.3678300	05.2931200	02.7255000	04.1373100	03.8596700	07.6512200	08.2536800
00.2711000	04.6167500	03.2944300	03.9143500	03.7414500	06.1547600	07.7255100
00.4213900	05.8534500	03.0440900	04.7444700	04.7593500	08.6237100	09.9792600
	04.0889300	02.9613800	03.8507500	03.5665800	06.2242700	07.8087100
	03.6681200	01.9467700	02.7113700	03.9916900		10.0199300
	04.5364900	02.2340800	03.7125800	03.7422300	06.6678800	06.9917700
	04.1402900	02.5319200	02.8022200	03.8837300	07.7067200	
00.7314500	05.0638600	04.8342000	05.0686400	06.3706800	08.5133200	07.9514900
00.5327300	04.7253700	04.3634900	04.8871200	"--"	08.2571200	10.9036100
	06.4881900	03.3334600	03.9382500	04.8429800	08.2911200	
	03.5511400	06.6834200	06.1125600	05.9892400		
	06.8451900	03.5292100	03.5291800	05.3694100		
	09.0439200	05.6995900		06.3626700		
	05.7012000	03.2985200	02.8107900			
	07.5159800	05.9936100		04.9548700		

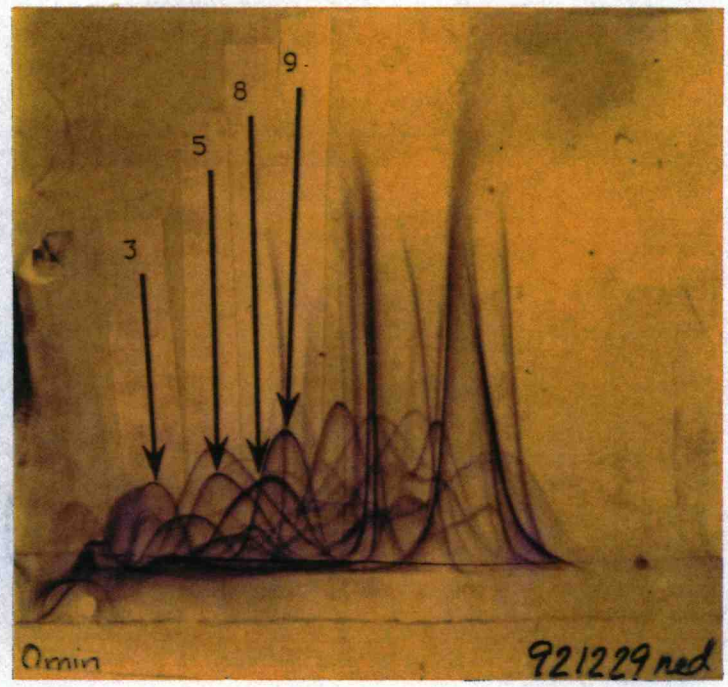
Figure 6. Photographs of crossed immunoelectrophoresis gels. The peaks numbererd 3, 5, 9, and 12 had mean areas that significantly increased from resting values, indicating increased plasma concentrations after 30 seconds in the air and 5, 10 or 20 minutes in a shallow bucket.

A. CIEP gel of resting pool.

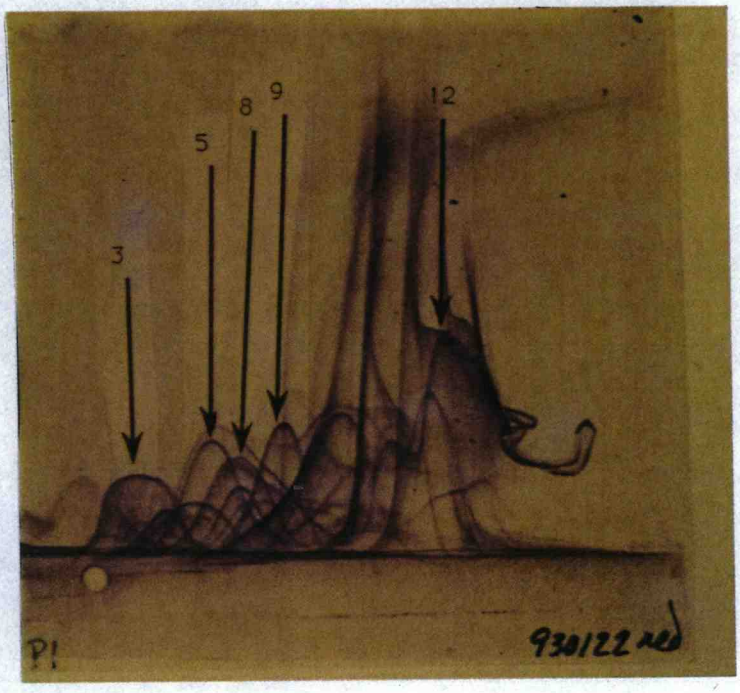
B. CIEP gel of individual resting rainbow trout.

C. CIEP gel of plasma pooled from fish stressed 5 minutes.

D. CIEP gel of plasma pooled from fish stressed 20 minutes.

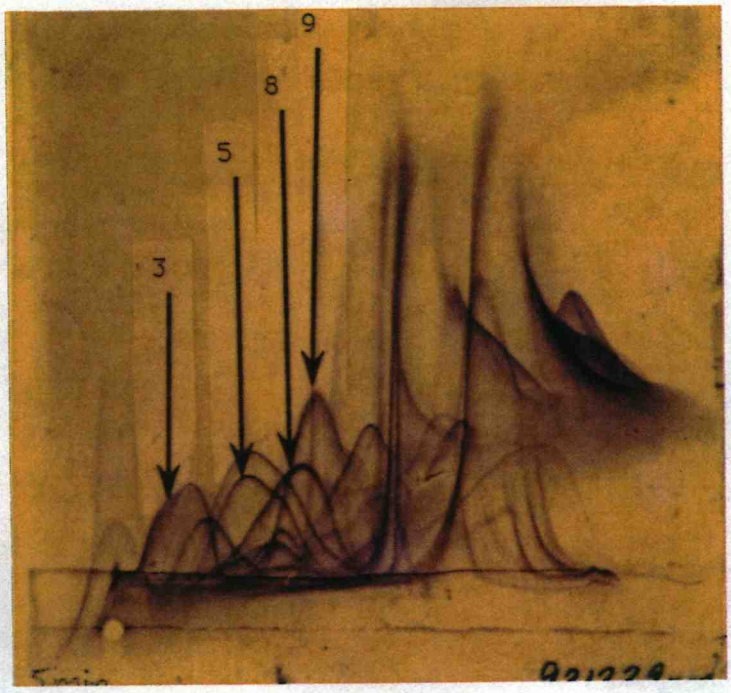


A

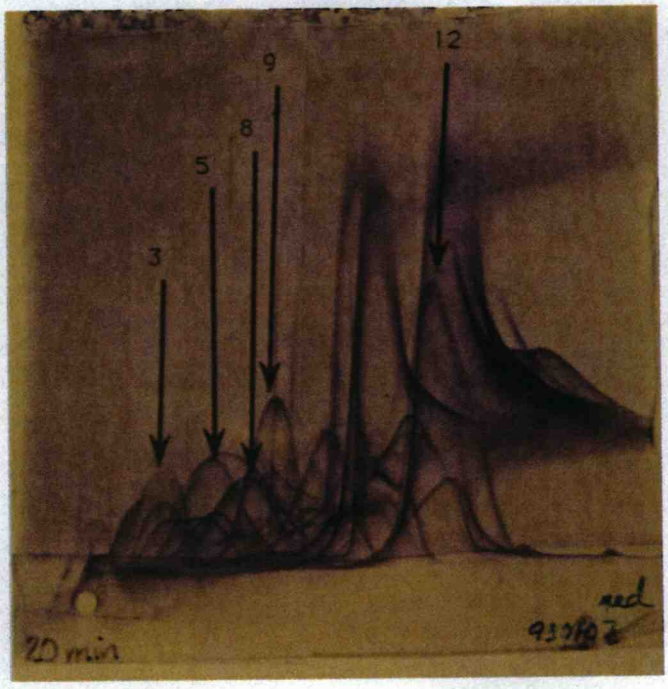


B

Figure 6.



C



D

Figure 6

Figure 7(A and B). Graphical representation of mean peak areas that increased in response to stress. Increases in peak area on CIEP gels from pooled rainbow trout plasma correspond to increases in concentrations of plasma antigens. Mean areas are natural log transformations of the data. Time refers to resting sample pools (0) and rainbow trout kept 5, 10 and 20 minutes in a shallow bucket after 30 seconds in a net in the air. Sample sizes inside bars indicate the number of measured peaks included in the analysis of variance. * indicates a significant difference from resting (0 minutes). ** in Peak 5 graph indicates that10 minute samples were significantly higher than 5 minute samples. A. Peak 3 and Peak 5 display similar trends: concentrations of these proteins appear to increase after stress for 10 minutes, but are in lesser concentrations by 20 minutes after stress. B. Peak 9 and Peak 12 appear to slowly increase in concentration over the time-course of the study. p values are less than .05 and are given in Table 3.

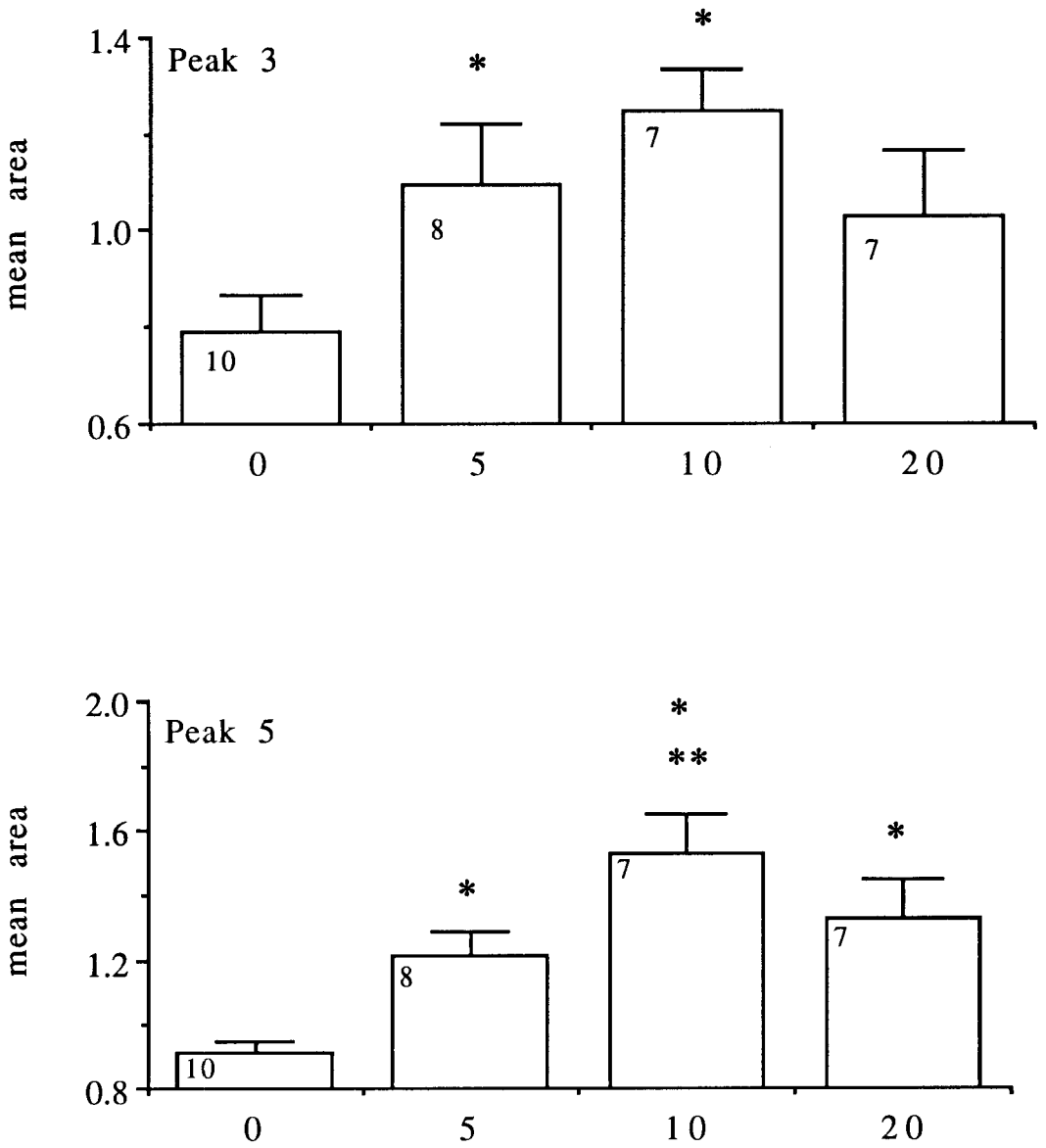

Peak 3
mean area
1.4
1.0
0.6
*
*
10
8
7
7
0
5
10
20
Peak 5
mean area
2.0
1.6
1.2
0.8
*
**
*
*
10
8
7
7
0
5
10
20
Time (minutes after initiation of stress)

Figure 7A

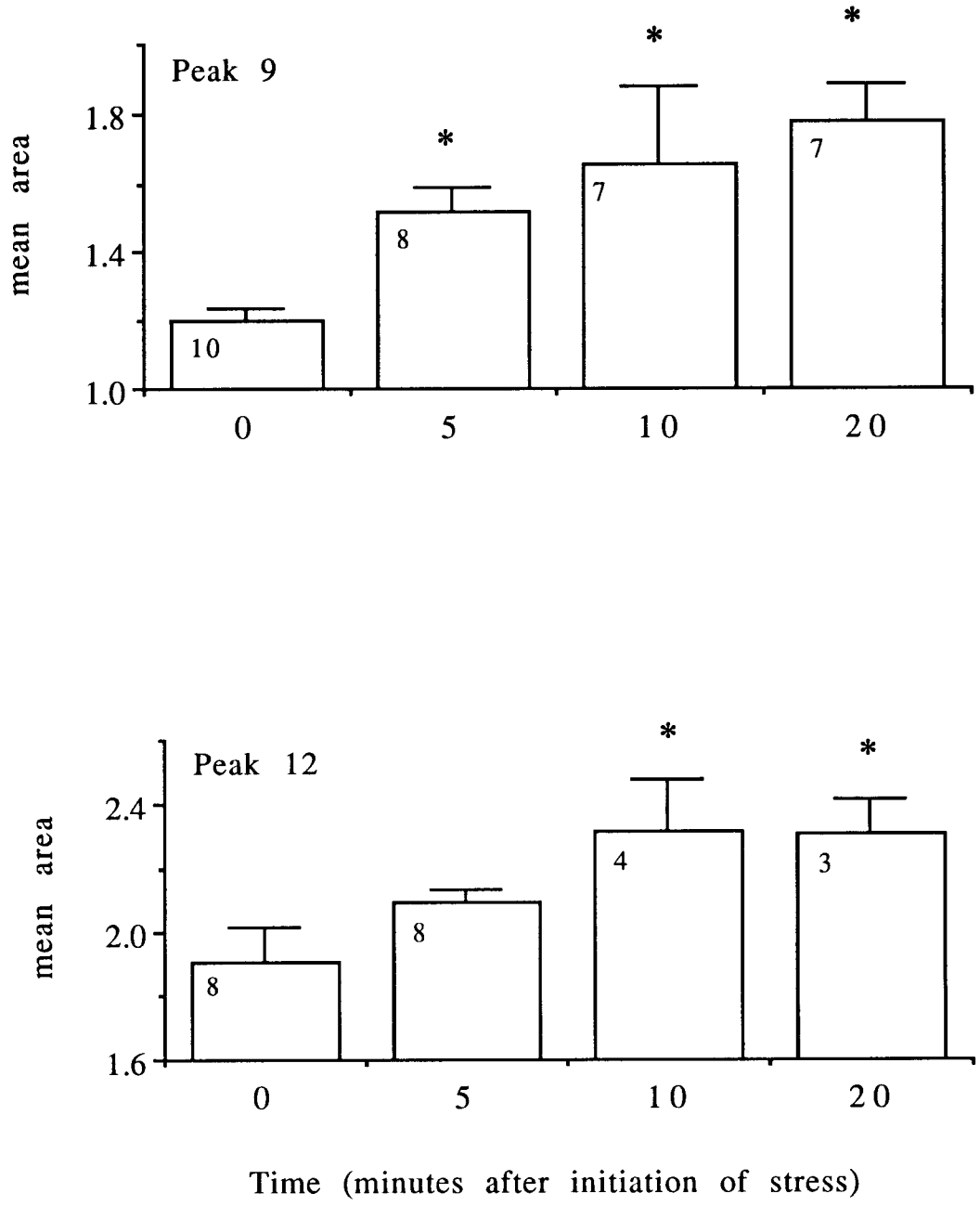

Peak 9
mean area
1.8
1.4
1.0
10
8
7
7
*
*
*
0
5
10
20
Peak 12
mean area
2.4
2.0
1.6
8
8
4
3
*
*
0
5
10
20
Time (minutes after initiation of stress)

Figure 7B

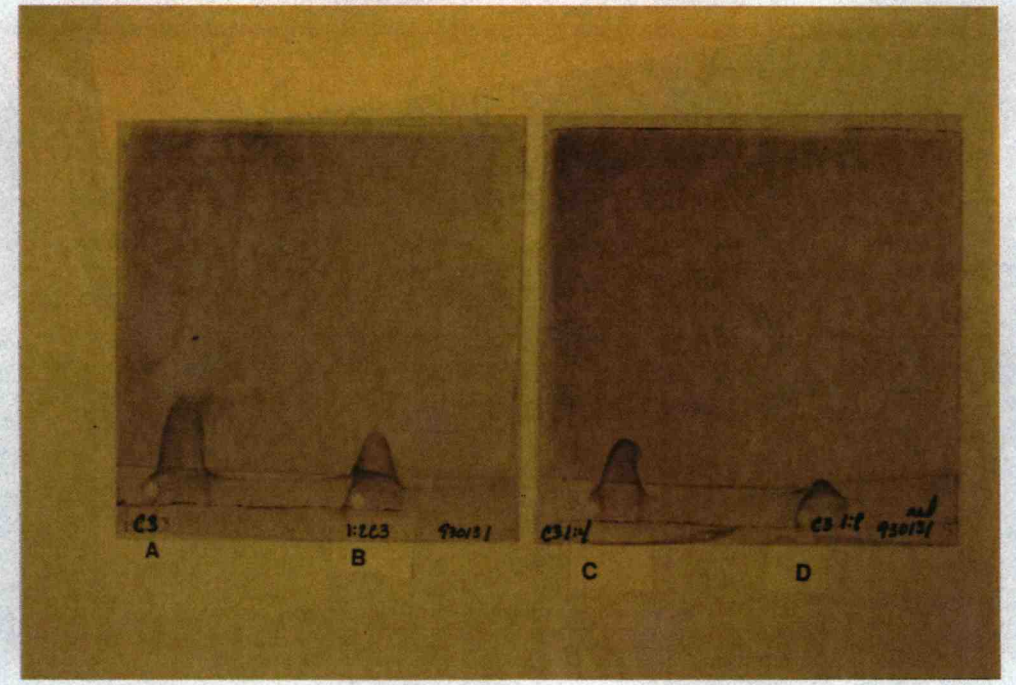
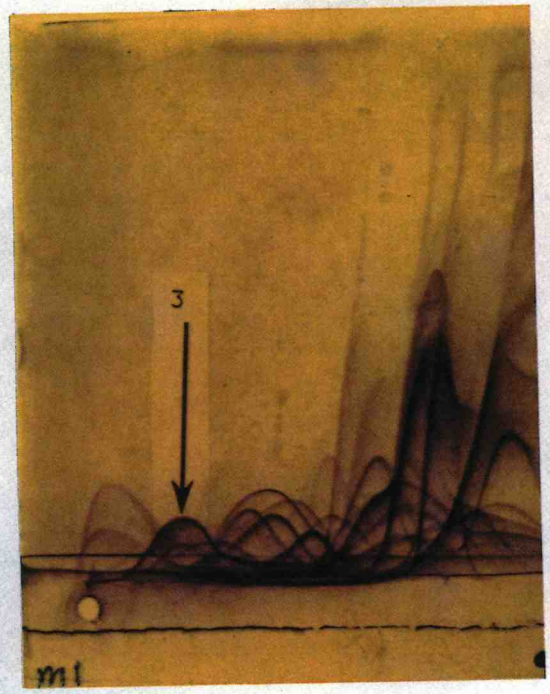
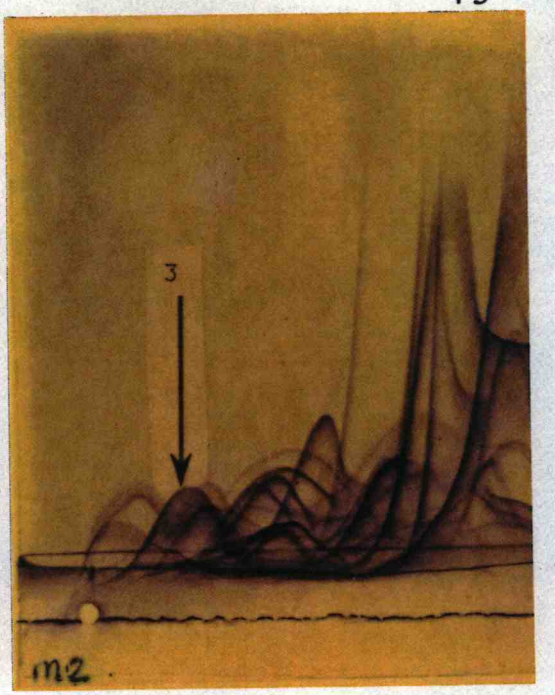


Figure 8. CIEP gels of purified trout Complement component C3. Quantities loaded were 10µl each: A: 1.32 mg/mL. B. 0.66 mg/mL. C. 0.33 mg/mL. D. 0.165 mg/mL.

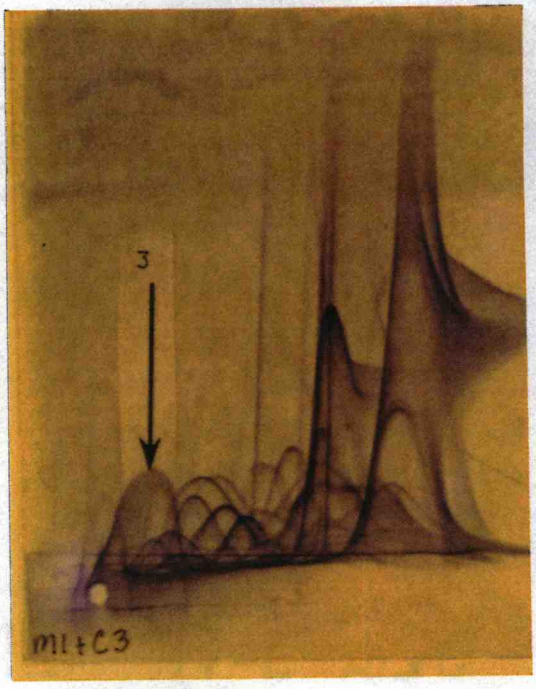
Figure 9. Photographs of CIEP gels used to identify C3 as Peak 3. A. Fish M1 (resting); note the size and location of Peak 3. B. Fish M2 (5 minute stress pool). C. Fish M1 plus 5 μL purified trout C3. Note that the change in the size of Peak 3 between resting and stressed samples (A and B) is similar to change in area seen upon addition of purified C3 (C).



A



B



C

Figure 9

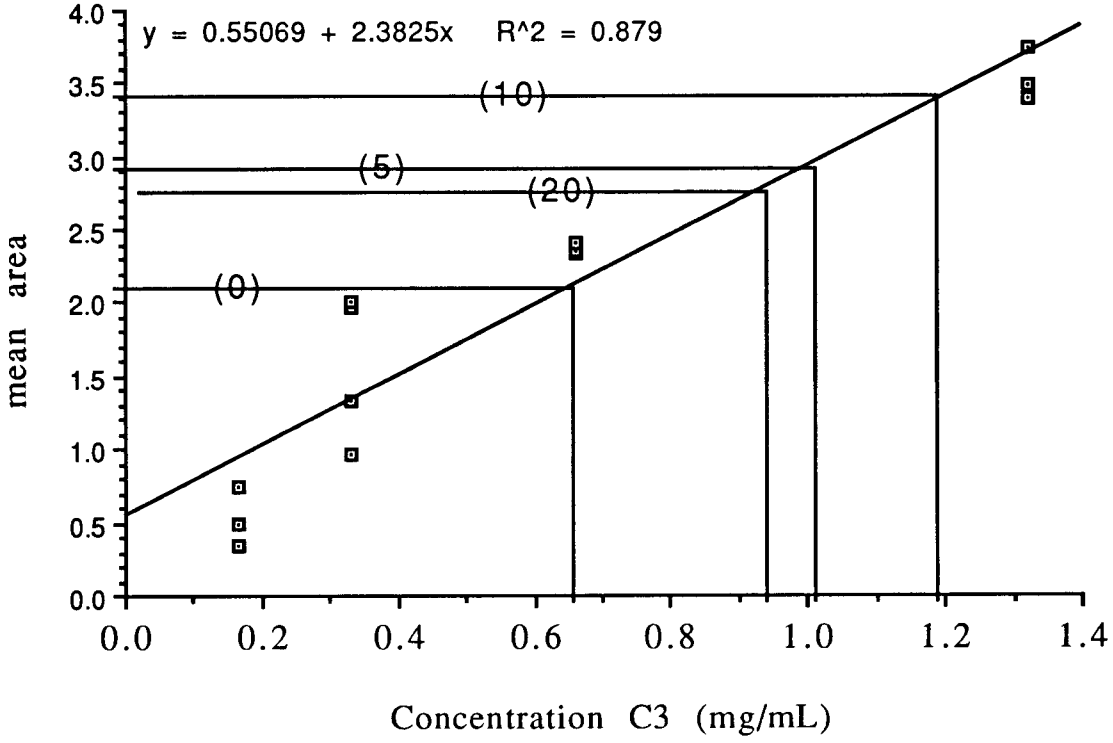


Figure 10. Correlation of C3 concentration and area under the curve. Using the standard line obtained by the best fit to the areas under the peak of known concentrations of C3, mean concentrations of C3 in resting and stressed pools of rainbow trout plasma were calculated by back transformation of the mean of Peak 3 for each group. By this analysis, resting trout had approximately 0.65 mg C3/ml (0); fish from the 5 minute stress group had approximately 1.02 mg C3/mL (5); fish from the 10 minute stress group had approximately 1.195 mg C3/mL (10), and the fish from the 20 minute stress group had approximately 0.95 mg C3/mL (20).

sample as it was located in an area containing many other peaks (data not shown).

Purified Trout Ig was analyzed in the same manner, however, there was no peak visualized in the sample containing purified trout Ig. In an effort to discern the reason for this, an agarose gel was run in the first dimension only, and a trough parallel to the separated antigens was filled with rabbit anti-serum to rainbow trout plasma. The anti-serum was allowed to diffuse overnight, then the gel was rinsed, dried, and stained with CBB as in CIEP. Numerous precipitin lines were visible in samples containing whole rainbow trout plasma; however, no precipitin lines were visible in samples containing purified trout Ig.

In an attempt to identify CRP by the loss of its peak in CIEP, 15μL rabbit-anti-trout CRP, kindly provided by H. Kodama (University of Osaka Prefecture, Osaka, Japan), was incubated with 15μL rainbow trout plasma for 1 hour at room temperature. The sample was spun at 3000 rpm for 5 minutes and the supernatant run by CIEP. A plasma sample containing PBS instead of anti-CRP was run at the same time. No significant differences were seen between the two gels (data not shown).

Antigen detection on nitrocellulose

Salmonid plasma and trout C3 were electrophoretically separated in an agarose gel in the first dimension. The antigens were transferred to a nitrocellulose membrane, which was probed with monoclonal anti-rainbow trout C3 (Figure 11). The mobility of the most reactive component is similar to C3 identified by CIEP (Figure

9). In a follow-up experiment, serial dilutions of purified C3 and plasma samples were directly blotted onto nitrocellulose, using a Bio-Rad dot-blot template, and probed using the same protocol (Figure 12). The level of C3 in the 10 minute sample is similar to the amount of C3 in the purified sample, about 1.3 mg/mL.

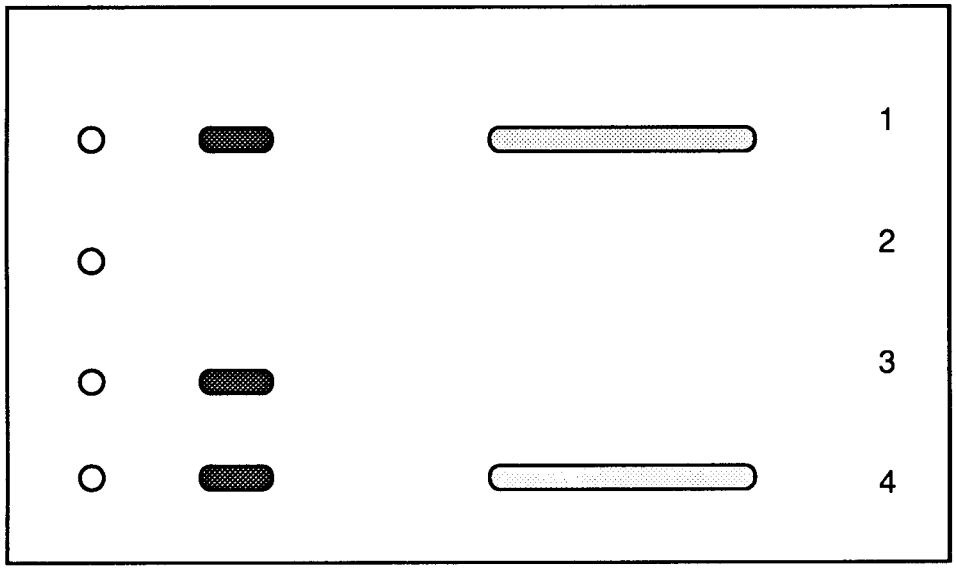


Figure 11. Pattern obtained after antigens were separated in the first dimension in agarose, transferred onto nitrocellulose, and probed with monoclonal anti-C3. The contents of the rows are:

1. Rainbow trout plasma
2. Rabbit antiserum to rainbow trout (negative control)
3. Purified Trout C3.
4. Coho serum.

The darkest area, near the origin is in the same area of the gel as Peak 3 in CIEP.

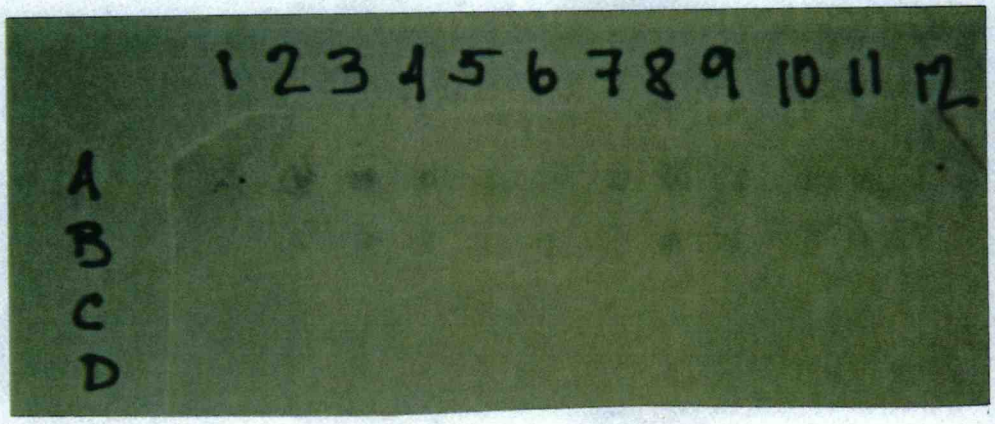


Figure 12. Dot Blot analysis of rainbow trout plasma probed with monoclonal antibody specific for trout C3. The columns contain:

1. no samples

2, 3. Purified C3

4,5. Plasma from resting fish N1

6,7. Plasma from fish M2in 5 minute stress pool.

8,9. Plasma from fish J2b in 10 minute stress pool.

10,11. Plasma from fish P2b in 20 minute stress pool.

12. no sample

Each spot contains 5 1 µl drops of sample. Row A contains purified, undiluted samples. Row B. contains 1:5 dilutions in PBS of samples.

Table 3. Summary of results. Mean values of plasma cortisol, glucose, and peak areas in resting and stressed samples. Confidence intervals (95%) and p values are also given. * indicates a significant difference from resting values.

TABLE 3	0 min. resting	n	5 min. stress	n	10 min. stress	n	20 min. stress	n	p
mean cortisol (ng/mL)	4.03077	13	78.92*	10	119.075*	8	165.666*	3	<.0001
upper 95% limit	24.757		102.552		145.496		208.812		
lower 95% limit	-16.697		55.288		92.653		122.52		
mean glucose (mg/dL)	69.53846	13	79.7	10	92*	8	116*	3	<.0001
upper 95% limit	77.302		88.5519		101.896		132.16132		
lower 95% limit	61.77481		70.848		82.1032		99.838		
mean Peak 3	0.788	10	1.095 *	8	1.247 *	7	1.029	7	0.0289
upper 95% limit	0.983		1.313		1.479		1.262		
lower 95% limit	0.593		0.878		1.014		0.954669		
mean Peak 5	0.915	10	1.217 *	8	1.529 *	7	1.329 *	7	0.0001
upper 95% limit	1.069		1.39		1.714		1.514		
lower 95% limit	0.759		1.043		1.343		1.143		
mean Peak 9	1.199	10	1.521 *	8	1.655 *	7	1.780 *	7	0.0062
upper 95% limit	1.411		1.758		1.908		2.032		
lower 95% limit	0.988		1.285		1.403		1.527		
mean Peak 12	1.907	8	2.095	8	2.314 *	4	2.304 *	3	0.0424
upper 95% limit	2.09		2.278		2.573		2.603		
lower 95% limit	1.724		1.912		2.056		2.006		

Discussion

Cortisol and Glucose

The mean cortisol value for resting fish in this study was 4.03 ng/mL. Levels less than 10 ng/mL in trout are considered resting (Schreck, personal communication). Cortisol increased to nearly 80 ng/mL within 5 minutes, and continued to increase throughout the time course of this study. This is consistent with earlier reports (Singley and Chavin, 1975; Strange et al., 1977; Barton and Iwama, 1991; Waring et al., 1992; Pottinger and Moran, 1993). Cortisol released from interrenal tissue into the blood stream of teleost fish is generally regarded as having an effect on intermediary metabolism to maintain homeostasis. Most studies of the immunological consequences of stress in fish have dealt with the effects of long term, repeated, or chronic stress, and have found immunosuppresive effects, usually as a result of suppression of specific antibody production (Robertson et al., 1963, Ellsaesser and Clem, 1986). Elevations in cortisol, along with increases in glucose, were noted often at the earliest time measured, which was usually hours or days after the initiation of stress (reviewed by Barton and Iwama, 1991).

This study confirms a rapid elevation of plasma cortisol. It is not known whether the rapid rise in cortisol is partly responsible for the increases in plasma proteins shown in this study, although the recent identification of a receptor for cortisol on plasma membranes of amphibians (Orchinik, 1991) suggests that it is possible. The signal required to release proteins obviously acts quickly. Effects of cortisol

characteristically require longer response times (Barton and Iwama, 1991). Therefore, catecholamines or other fast-acting hormomes such as interleukins or insulin all acting in concert may be more likely to effect these quick changes in plasma protein concentrations.

Plasma glucose levels were significantly higher than resting values within 10 minutes of stress in this study. Rises in plasma glucose levels may be induced by the actions of catecholamines mobilizing hepatic glycogen reserves (reviewed by Thomas, 1990). Injections of ACTH in atlantic salmon did not lead to increases of plasma glucose (Nichols and Weisbart, 1984). In brown trout, rises of plasma glucose were not significantly higher until 4 hour after a handling and capture stress (Melotti et al., 1991). In golden perch, glucose levels were significantly higher than controls after 3 hours following repeated capture and anesthesia (Braley and Anderson, 1992). A report by Ramos and Smith (1978) described increased plasma glucose within minutes of stress. Concentrations of plasma glucose of red drum increased within 4 minutes as a result of stress during bleeding (Robertson, 1988). Plasma glucose provides a source of energy for any cell in the body. The loss of glycogen granules in liver cells of goldfish within minutes (Chavin, 1973) may be a primary source of the plasma glucose released upon stress. Rapid rises in levels of glucose are part of the "fight or flight" response. Energy requirements of cells such as macrophages, neutrophils and hepatocytes that produce numerous plasma proteins can be fufilled quickly by glucose present in the plasma released from hepatocytes, macrophages or other cells.

CIEP

Plasma protein concentrations increased from resting levels in four of twelve protein peaks analyzed by CIEP. Numerous reports (Ramos and Smith, 1978; Gordon, 1985; Koj, 1985; Schreiber, 1987; Baumann, 1988; Kodama et al., 1989a,b; Congleton and Wagner, 1991) show increases of acute-phase plasma proteins within hours or days of a stressor. No published data showing increases of plasma proteins within minutes of acute stress in teleosts or mammals has been found. Increased concentrations of innate proteins that play a role in protection of the host follow Selye's proposed General Adaptation Syndrome.

There appear to be two different kinetics of concentration changes in these four peaks. Peak 3 and Peak 5 appear to increase in concentration up to 10 minutes after stress, and then begin to decrease in concentration (Figure 7A). Peak 9 and Peak 12 appear to slowly increase over time as a result of the handling stress (Figure 7B).

The electrophoretic mobility of C3 observed in this study was consistent with that reported by Perrier and Perrier (1984). The availability of a mouse monoclonal anti-trout C3 provided a means to confirm the identification made with the purified protein. The resulting nitrocellulose membrane (Figure 11) provided this confirmation. It showed that purified C3 moved in generally the same position as peak 3 in CIEP. In the dot blot analysis, serial dilutions of C3 and individual rainbow trout plasma probed with monoclonal anti-C3 revealed the same pattern of concentrations: plasma from fish stressed 10 minutes had higher C3 concentrations

than resting fish (Figure 12). Increases of C3 within minutes of a stressor could help identify any invading microorganism for destruction and clearance by macrophages, and help maintain homeostasis by marking damaged host tissue for removal in the same manner.

Quantification of C3 in mouse acute-phase sera revealed a pattern of kinetics that may be different from that shown here (Taktak and Stenning, 1991). Their study measured C3 concentrations at 0, 2, 4, 6, 24, 48 and 72 hours after injection of endotoxin. Concentrations of C3 were higher at 0 time, than 2, 4, or 6 hours later, and were not significantly higher than resting until 24 hours after the injection. It is possible that the mice had already released presynthesized stores of C3 at the 0 time point and the increase seen at 24 hours was due to activation of transcription known to occur after stressors of this type. This study reveals increases in the concentration of C3 within 5 minutes of stress.

Some plasma proteins other than C3 also increased in concentration. The location of CRP in CIEP should have been evident by the lack of a peak in the gel of the immunoprecipitated plasma as compared to the control gel. This was unsuccessful. It appeared that there were more peaks in the sample containing anti-CRP than in the control. Problems encountered attempting to identify Ig have not been resolved. Peak 12 has mobolity similar to albumin, a known acute-phase reactant in mammals. Positive identification awaits further analysis.

Fewer peaks were measured in gels containing stressed fish plasma than resting samples (Table 2). This could have been a result

of concentrations of antigens increasing beyond the precipitating capacities of antibody present in the gel, causing an incomplete precipitation line. Incomplete lines were not measured. For that reason, actual increases in concentrations for some proteins after stress may be even higher than those recorded here.

Crossed immunoelectrophoresis has provided evidence of the increase in concentration of four plasma proteins of rainbow trout after acute handling stress. It seems reasonable to conclude that the handling stress resulted in the release of plasma proteins, and that at least one of them, C3, is an opsonin that would enhance innate immunity by identifying non-self for destruction by lysis or phagocytosis. It has also been shown that plasma cortisol concentrations increased within 5 minutes of a handling stress, and continued to rise for at least 20 minutes. Plasma glucose levels increased significantly within 10 minutes of an acute handling stress.

Acute stress is virtually inescapable. The "fight or flight" response as typified by the stress-induced release of catecholamines and cortisol could include the release of plasma proteins from hepatocytes and macrophages either directly or through the actions of interleukins and other cytokines. These plasma proteins could function as opsonins and mediators of the acute-phase response. In this way, the host defense system could exploit the existence of conserved epitopes in stress proteins to respond immediately to antigenically diverse pathogens and host cellular changes, producing an initial defense that need not await the development of immunity to novel antigens (Young and Elliot, 1989; Young, 1990; Murray and Young, 1992). Intuitively, a rapid adaptive response makes sense.

The General Adaptation Syndrome suggests that an animal responds to stress with hormonal changes that should help it adapt to a stressful situation. The potential result of many stressors is damage and injury. The release of plasma proteins that help identify foreign or damaged tissue after damage or injury is in agreement with this theory of adaptation. Many animals lack specific immunity and obviously are able to respond and survive using only natural or innate defense mechanisms.

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APPENDIX

Appendix

CIEP

Tris-Barbital Buffer pH 8.6 (5X)

132.9 g Tris base
67.2 g Barbital
1.95 g Sodium Azide
1.59 g Calcium Lactate

Dissolve ingredients in 3 liters distilled water. For use, 600 mL of 5X solution is added to 2.4 liters distilled water. Adjust to pH 8.6.

Phosphate Buffered Saline (PBS) pH 7.4

8 g Sodium Chloride
0.2 g Potassium Chloride
1.15 g Sodium Phosphate (dibasic)
0.2 g Potassium Phosphate

Dissolve the salts in 1 liter distilled water and autoclave. Store at 4°C until use.

Coomassie Brilliant Blue Stain

5 g Coomassie Brilliant Blue R-250
450 mL 95% Ethanol
100 mL glacial acetic acid
450 mL distilled water

Destain

450 mL 95% Ethanol
100 mL glacial acetic acid
450 mL distilled water

CBB was removed from destain with activated charcoal so the destain could be reused.

Dot Blot

Tris Buffered Saline (TBS) pH 7.5

4.84 g Tris Base
58.48 g Sodium Chloride

Dissolve salts in 2 liters deionized distilled water. Adjust pH to 7.5 with HCl.

Blocking solution
Dissolve 3 g gelatin in 100 mL. TBS at 37°C and stored at room temperature with 0.01% Thimerosal.

Antibody Buffer
Dissolve 2 g gelatin in 200 mL TBS at 37°C and store at room temperature with 0.01% Thimerosal.

HRP Color Development Reagent
60 mg HRP color development reagent (Bio-Rad) was added to 20 mL ice cold methanol. Add 600 µl 3% hydrogen peroxide to 100 mL TBS and combine with the methanol just prior to use. Color was allowed to develop 5-45 minutes.