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EFFECTS OF BOVINE GROWTH HORMONE ON THE RESISTANCE OF RAINBOW TROUT (*Oncorhynchus mykiss*) TO VIBRIOSIS

by

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B.Sc., Simon Fraser University, 1993

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Biological Sciences

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SIMON FRASER UNIVERSITY

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0-612-37547-1

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ABSTRACT

A study was undertaken to evaluate the consequence of bovine growth hormone treatment on the resistance of rainbow trout to vibriosis. Juvenile rainbow trout (Oncorhynchus mykiss, 6 months post-hatch, 10-15 g average weight depending on the experiment) were treated with a sustained release growth hormone preparation (10 μg hormone g^{-1} body weight in 0.1 mL sesame oil). Growth hormone-treated fish were, depending on the experiment, about 28 to 32% heavier than control fish at 21 days post-treatment. At day 21 the fish were either bath challenged with a virulent strain of Vibrio anguillarum or sampled to obtain blood. Rainbow trout treated with growth hormone had elevated ($p=0.06$) mortality from acute induced vibriosis over a two-week period (98% total mortality) as compared with control, oil-injected fish (88% total mortality). Evaluation of blood samples for differential blood cell counts, hematocrit, leukocrit and lysozyme activity showed no significant differences between growth hormone-treated and control fish. Dip vaccination against vibriosis indicated no impact on the growth performance of vaccinated fish compared with sham-vaccinated fish. The results are discussed in terms of the multiplicity of growth hormone action in salmonids and the variable life history requirements of fish for growth hormone depending on seasonal and environmental factors. An appendix is included which describes preliminary experiments on the effects of growth

hormone treatment on the feeding and growth performance of rainbow trout under different photoperiod conditions. These preliminary experiments support the discussion provided for the main thesis.

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INTRODUCTION

General background

Research into fish growth promoting technologies has
occurred for many years. As a result, numerous experimental
techniques exist that accelerate the growth of cultured fish,
usually by manipulation of the brain neuroendocrine-growth
hormone (GH or somatotropin)-insulin-like growth factor axis.
These techniques include: application of recombinant and
native growth hormones (Gill et al., 1985; Schulte et al.,
1989; Foster et al., 1991), feeding or injection treatment of
goldfish with apomorphine, a dopamine receptor agonist (Wong
et al., 1993), injection treatment of coho salmon with
placental lactogen, a mammalian hormone belonging to the
growth hormone family (Devlin et al., 1994a), injection
treatment of chinook salmon with mouse monoclonal antibodies
raised against the growth hormone release inhibitor
somatostatin (Mayer et al., 1994), steroid and thyroid
hormone treatment in salmonids (Higgs et al., 1977; Donaldson
et al., 1979), mammalian IGF-I treatment in coho salmon
(McCormick et al., 1992), and the development of transgenic
fish species incorporating non-homologous (and homologous) GH
genes (for example see McLean and Donaldson, 1993; Devlin et
al., 1994b).

In juvenile fish, growth rates often increase after
periods of growth retardation (i.e.,compensatory growth)

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(Weatherley and Gill, 1981; Quinton and Blake, 1990). They can also be increased through photoperiodic manipulations (Clarke et al., 1989 and Steffansson et al., 1991) and through various endocrine treatments as stated above.

Growth hormone treatment appears to be one of the most promising ways for enhancing fish growth under aquaculture conditions. This is not solely because of the growth stimulatory effects observed with somatotropin, but also because recombinant versions of GH are becoming available at low cost.

A variety of methods have been used in the delivery of exogenous GH to fish (see McLean and Donaldson , 1993 for a review). It is usually administered by intraperitoneal (IP) injection (see Duan and Hirano, 1991 for a range of other injection methods). However, GH can also be applied by immersion treatment of the animals (Schulte et al., 1989), by addition to the feed (McLean et al., 1993; Moriyama et al., 1993; Tsai et al., 1994) and by implantation of GH-containing cholesterol pellets (Cravedi et al., 1995) or polymer-encapsulated, sustained-release hormone pellets (McLean et al., 1994).

IP injection of fish with recombinant bovine growth hormone (rbGH) is a common experimental approach. However, since handling and injection of individual fish is time-consuming and stressful to fish, IP injection of exogenous GH can be impractical for commercial aquaculture.

Nonetheless, it is an acceptable technique under experimental conditions and has been shown to enhance the growth performance of a variety of fish species. Markert et al. (1977) showed that weekly injections of bovine growth hormone at 10 µg g^{-1} body weight in yearling coho salmon (Oncorhynchus kisutch) increased appetite, enhanced growth, and improved food and protein conversion. Johnsson and Bjornsson (1994) showed that mammalian GH treatment in juvenile rainbow trout (Oncorhynchus mykiss) resulted in increased growth rates, increased dominance and increased feeding motivation, which may elevate aggression levels.

Biological functions of Growth Hormone in fish

GH belongs to a family of proteins that includes prolactin, placental lactogen, and somatolactin. It is believed that gene duplication events that occurred over the past 350 million years gave rise to these functionally distinct hormones in vertebrates (Miller and Eberhardt, 1983). Even though teleost growth hormones are only approximately 35-40% similar to mammalian growth hormones (McLean and Donaldson 1993) many higher vertebrate GH proteins can be successfully used to enhance the growth performance of fish. Salmonids, including rainbow trout, are tetraploid animals (Sumpter, 1992). As a result of a presumed gene duplication, two distinct GH genes code for two GHs in salmonids (Devlin, 1993). As is similar with other

salmonids, in sockeye (O. nerka) the two genes encode proteins of 210 amino acids. The coding sequences have diverged approximately 18% in their noncoding regions (Devlin, 1993).

In salmonids, GH has functions in the regulation of several physiological processes including osmoregulation (reviewed by Sakamoto et al., 1993), smoltification (Komourdjian et al., 1976; Sweeting et al., 1985), somatic growth (Markert et al., 1977) and reproduction and sexual maturation (reviewed by Le Gac et al., 1993). Note that in winter flounder (Pseudopleuronectes americanus), Idler et al.(1989) have demonstrated that GH (derived from chum salmon pituitary extracts) is a major factor regulating seasonal plasma antifreeze protein synthesis.

GH is a single-chain polypeptide hormone with a molecular weight usually ranging from 20,000-22,000 dalton, depending on vertebrate origin or engineered analog (Down et al., 1989). Bovine somatotropin (bST) has 190 or 191 amino acids, and it can have either of two different amino acids (leucine or valine) at position number 126 in the protein sequence (Wood et al., 1989; Baumann, 1992). In other words, four different variants of bST are produced naturally. Recombinant bovine somatotropins usually contain up to eight additional amino acids at the NH$_2$-terminus of the molecule, depending on the manufacturing process (Juskevich and Guyer, 1990; Baumann, 1992).

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GH secretion and function in fish is best understood from research with cyprinids (notably carp and goldfish) and salmonids. The endocrinology of growth and GH action in fish has recently been reviewed by Peter and Marchant (1995).

Teleostean growth hormone is under multifactorial neuroendocrine control and is secreted by somatotrophic cells of the anterior pituitary gland. The neuropeptide somatostatin is the primary inhibitor of GH secretion (Cook and Peter, 1984; Peter and Marchant, 1995). Known stimulators of growth hormone secretion are GH-releasing factor, gonadotropin-releasing factor, dopamine, neuropeptide Y, thyrotropin-releasing factor and cholecystokinin (see Peter and Marchant, 1995 and Trudeau et al., 1996 for reviews). Following release from the anterior pituitary (or following treatment with exogenous somatotropin), GH stimulates body growth by (i) direct action on receptor sites in the liver, gills, intestine, kidney and gonads, and (ii) by the stimulation of production of insulin-like growth factors or somatomedins (IGFs) (Peter and Marchant, 1995). The primary target organ for GH is the liver, which has a high number of growth hormone receptors (Zhang and Marchant, 1996). The liver also contains the highest concentrations of IGFs, which, when bound to specific binding proteins, appear to travel in the blood and function, in part, in cartilage and/or bone growth (Peter and Marchant, 1995; Duan and Hirano, 1991). GH receptor numbers are influenced by various hormones (including GH) and by nutritional status. For

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example, fasting in growth-stunted pre-smolt coho salmon acclimated to seawater conditions appears to reduce the available number of hepatic GH binding sites (Gray et al., 1992).

GH application in salmonids results in improved food conversion and higher growth rates. It has been noted that: (1) GH treatment can have a variable impact on condition factor; and (2) the magnitude of growth acceleration can depend on the hormone dosage applied (for example Komourdjian et al., 1976; Agellon et al., 1988).

As described previously, exogenous GH increases the efficiency of food conversion in fish (Markert et al., 1977). This effect may be related to the fact that GH stimulates intestinal amino acid transport and intestinal mass (Collie and Stevens, 1985; Sun and Farmanfarmaian, 1992). Increased food conversion efficiency may also be related to the stimulatory influence of GH on lipid breakdown (O'Connor, 1993), thereby sparing amino acids for protein accretion. Within 6 h of bovine GH administration (2 μg g^{-1} body weight) in rainbow trout, the rate of protein synthesis in muscle tissue was twice that of control fish. It is not known whether this effect was related to a direct metabolic insulin-like action of GH or to an indirect effect from IGF stimulation (Fauconneau et al., 1996). Foster et al. (1991) demonstrated that pituitary-derived ovine GH (20 μg g^{-1} body weight) stimulated higher retention of ingested nitrogen and increased protein synthesis rates in rainbow trout.

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Growth Hormone, the Immune System, and Animal Health

Our current understanding of the potential health consequences of exogenous GH treatments is largely limited to mammalian studies. Some studies have indicated that side effects can occur from treatments with exogenous GH or transgenic GH. Such side effects may take the form of gastric ulcerations, degenerative joint disease, glomerular sclerosis and acromegalic arthropathy (a form of osteoarthritis) (reviewed by McLean et al., 1994). However, with respect to lactating dairy cows treated with recombinant bovine growth hormone to enhance milk production, no validated adverse health effects have been reported (Baumann, 1992). Indeed, a more rapid recovery from experimentally induced mastitis in hormone-treated dairy cows was demonstrated in one study (Burvenich et al., 1989). Similarly, the survival rate following Salmonella typhimurium challenge in hypophysectomized rats was significantly increased by GH administration (Saito et al., 1996). Increased physiological stress in GH-treated cows has not, it appears, been demonstrated. Nonetheless, subtle health consequences due to GH therapy may exist, and examination would require large numbers of animals treated under a range of environmental and management conditions (Eppard et al., 1987).

Our understanding of the physiological role of GH (endogenous or exogenous) in relation to immune performance

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in vertebrates is incomplete. However, GH may well be an
important regulator of immune functions because it is present
in the circulation of animals in virtually all normal
physiological and developmental states (Nicoll, 1993).
Indeed, it is becoming increasingly evident that hormones
such as GH not only affect their classical target organs, but
that they also act in a pleiotropic manner, altering
functional activities of leukocytes as well (Kelley, 1990).
In mammals, it is generally accepted that GH functions in the
hematopoietic system for differentiation and function of
erythroid, myeloid and lymphoid cells (see Calduch-Giner,
1995 and Kelley, 1990 for reviews). GH appears to act as a
cytokine and has been included as a member in the helical
cytokine family (Sprang and Bazan, 1993). GH augments
antibody synthesis, cytolytic activity of T lymphocytes,
natural killer cell activity and differentiation of
neutrophils (see Kelley, 1990 for review). Both GH and IGF-
1 enhance granulopoiesis (Saito et al., 1996). Immunocytes
have been demonstrated to synthesize GH and to bear receptors
for its releasing factor (Saito et al., 1996). GH treatment
in mammals results in a priming of macrophages for superoxide
anion release both in vitro and in vivo (Edwards et al.,
1988; Fu et al., 1991; Saito et al., 1996;). GH is also
necessary for the growth of the thymus gland, which is now
known to be responsible for gene rearrangement of specific
antigen receptors on T lymphocytes (Kelley, 1990).

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Growth Hormone and Fish Health

With respect to fishes, which have been experimentally treated with GH in one form or other for about 50 years, health-related effects have rarely been reported. Cravedi et al. (1995) provided evidence that ovine GH treatment of rainbow trout significantly decreased the level of hepatic cytochrome P450 and the activities of cytochrome P450 dependent monooxygenases. Agellon et al. (1988) reported behavioral and morphological changes among rainbow trout treated with dosages of 2 μg g^{-1} body weight of recombinant rainbow trout GH from bacterial extracts. These changes, however, subsided following final treatment. The authors suggested an unknown interaction of recombinant GH, which was altered from the mature natural form by 11 amino acid residues at the NH2-terminus, with other extract components. Kayes (1977) reported high mortalities among hypophysectomized black bullheads treated with bovine GH at 10 μg g^{-1} body weight. Although an allergic response to bovine GH was suggested, no hormone-specific antibodies were detected in the serum of moribund fish. McLean et al. (1994) reported on the effects of sustained-release polymer encapsulated recombinant porcine somatotropin upon the growth performance of coho salmon. Although no adverse side effects from such polymer pellet treatment was noted, examination of retrieved pellets upon evisceration showed tissue envelopment

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of GH-containing pellets, and no such envelopment for placebo pellets. The most prominent envelopment coincided with pellets that had the greatest GH release rate. An immunological response to porcine recombinant somatotropin was suggested by the authors. Devlin et al. (1995) reported on the transmission and increased severity of phenotypic effects of an antifreeze/GH gene construct in coho salmon F1 progeny. The phenotypic effect was displayed as a distinct green coloration of alevins prior to feeding and a progressive overgrowth of cartilage in the cranial and opercular regions of juvenile fish. Overexpression of GH genes and acceleration of normal pigment ontogeny were suggested as reasons for these effects.

Studies investigating the influence of exogenous GH on immune functions in fish have shown results similar to those reported for mammals. Sakai et al. (1995) have reported finding a dose-dependent enhancement of chemiluminescent response of rainbow trout (52.3±6.7 g) kidney phagocytic cells five days after intraperitoneal injection with chum salmon GH (10 µg GH/fish). Sakai et al. (1996) also reported in vitro activation of rainbow trout phagocytic cells by chum salmon GH, prolactin and somatolactin. The trout in that study had a mean weight of 50 g and the isolated phagocytic cells were exposed to 100 ng GH, prolactin or somatolactin per mL medium overnight at 18 ˚C. Similarly, five days after intraperitoneal injection of rainbow trout (100g mean weight) with 1 µg chum salmon GH per fish, Kajita, et al. (1992)

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described enhancement of non-specific cytotoxic activity of leukocytes. After four weeks of treatment with 1.8 μg or 0.35 μg bovine GH g^{-1} body weight per week equivalent dose (delivered by cholesterol pellet), neutrophil respiratory burst activity was also shown to be elevated in female rainbow trout (size-selected to 167±11 mm) (Kitlen et al., 1997). Specific receptors for GH and the influence of GH on leukocyte growth has been demonstrated in red sea bream (Calduch-Giner et al., 1995). In addition, in vitro phagocytic index is also increased following GH treatment in red sea bream (Calduch-Giner et al., 1997).

Finally, plasma GH concentrations of rainbow trout fasted for six weeks were significantly higher than in animals which were fed. Acute handling stress decreased the plasma GH levels in both groups (Farbridge and Leatherland, 1991). Similarly, Barrett (1988) showed that long-term starvation, transfer from freshwater to seawater and sustained exercise increased plasma GH levels in juvenile salmonids. These increases in plasma GH in response to physical exercise and environmental stress apparently are intended to mobilize energy reserves (notably body lipid). Farrell et al. (1997) show that GH transgenic coho salmon display poorer swimming performance than similar-sized control fish. This finding indicates a physiological cost associated with enhanced body growth.

Purpose

In view of the potential significance of GH use in salmonid aquaculture (given the cost of fish feeds and lengthy production cycles), it is important to know the influence of exogenous GH on other systems of a fish's internal environment. Apart from the physiological aspects of growth and growth enhancement, the effects, if any, on the immune system of fish should be considered. Clearly, any immune impairment due to GH treatment could result in faster growing, but more disease-prone animals. With this in mind, the question of whether treatment with exogenous GH influences the disease resistance of fish undergoing accelerated growth formed the basis of this research. Specifically, selected aspects of immune performance of juvenile rainbow trout undergoing accelerated growth following recombinant bovine growth hormone treatment were evaluated. The null hypothesis was that no significant difference exists between the disease resistance of hormone-treated and control animals.

MATERIALS AND METHODS

A slow-release recombinant bovine growth hormone (rbGH) formulation was used in this study. Elsewhere applied to dairy cattle for the enhanced production of cow's milk, the recombinant hormone product (courtesy Monsanto Company, see below) was a sterile suspension of rbGH in sesame oil. Due to its slow-release, it was possible to treat fish once to accelerate growth through a 21-day period. The length of the treatment period was designed to achieve significantly heavier fish at the end of the 21-day period, while allowing enough time (14 days) for the fish to develop an immune response following vaccination on the seventh day (Part B).

General procedure

The research design involved two parts (A and B) as outlined in Fig. 1. Part A is concerned with an evaluation of the relative survival or mortality of hormone-treated fish compared with control fish following a bacterial disease challenge. Part B is concerned with evaluating a variety of blood factors in hormone-treated and control fish. On day 0, early spring-hatched, 8-12 g juvenile rainbow trout (Oncorhynchus mykiss) were given intraperitoneal (IP) injections of rbGH suspended in sesame oil (GH+oil) or

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Figure 1. Schematic representation of the research design. Please see the text for a complete explanation. Briefly, the research was composed of two portions, Parts A and B. For both parts fish were handled in a similar manner through a 21-day growth period. On day 21 fish were either bath-challenged with a bacterial fish pathogen (Part A), or they were sacrificed (Parts B) to obtain blood samples for differential blood cell counts, lysozyme assays, hematocrit and leukocrit evaluations.

Part A

Timeline:	day 0 -------------------------------> day 21 --------------------> day 35

*Groups:			
GH + oil fish	GH-treat	disease challenge	end
oil fish	sham-treat	disease challenge	end

*four replicates per group; 15 fish per replicate

Part B

Timeline:	day 0 ----------> day 7 --------------------> day 21

*Groups:			
GH + oil fish	GH-treat	dip-vaccinate	sampling
oil fish	sham-treat	dip-vaccinate	sampling
GH + oil fish	GH-treat	sham-vaccinate	sampling
oil fish	sham-treat	sham-vaccinate	sampling

*two replicates per group; 10 fish per replicate

control sesame oil (oil). For Part A, fish were grown for 21 days and then bath-challenged with a virulent live culture of _Vibrio anguillarum_, a piscine bacterial pathogen. For Part B on day 7, fish from all experimental groups were either immersion-vaccinated using a bivalent vibriosis vaccine, or sham-vaccinated to evaluate the effects of vaccination during the growth period. At the end of the 21-day growth period the fish were sacrificed to obtain blood samples for the preparation of blood smears, lysozyme assays, leukocrits and hematocrits.

Preparation of Growth Hormone for injection

Recombinant bovine growth hormone (500 mg Monsanto Co., Lot 91F 18/10) was diluted in sesame oil (Sigma) to obtain an estimated hormone stock concentration of 50 mg GH mL^{-1} sesame oil. This stock suspension was maintained at 4 °C. A working suspension was made by further dilution of stock material: for a 0.1 mL injectable volume of GH + oil per fish, the concentration of GH was adjusted to render approximately 10 μg hormone g^{-1} fish body weight. For example, for fish with an average weight of 10 g, 0.2 mL stock (10 mg GH) diluted with 9.8 mL sesame oil provides an estimated 10 μg GH g^{-1} fish in 0.1 mL. IP injection of viscous GH + oil or control sesame oil into fish was achieved using disposable 27G 1/2" Beckton Dickinson needles fastened to 1 mL interchangeable Luerlock glass syringes.

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Maintenance and feeding of experimental animals

All experimental animals used for this research were commercial rainbow trout obtained in June/July 1997 at 10 °C water temperature from Spring Valley Trout Farm, Langley, BC. In the farm building, fish were exposed to subdued incandescent light during the daytime.

Juvenile rainbow trout (8-12 g average weight) were acclimated for at least two weeks to dechlorinated municipal water conditions at the Alcan Aquatic Research Centre, Simon Fraser University (T = 15 °C ± 0.5 °C). The fish were held in a 500 L circular holding tank, with aeration and continuous flow-through water supply. Photoperiod was adjusted to 9hLight:15hDark (08:00-17:00), and the light intensity from overhead fluorescent light was measured directly above the water as 10-15 lux. Fish were fed sparingly at about 1% biomass every other day during the acclimation period. For Parts A and B (see above) fish were kept in 77 L circular tanks with continuous flow-through water supply and aeration. Photoperiod and light intensity in the 77 L tanks was provided in a manner unchanged from those conditions given in the 500 L acclimation tank. All fish were fed once daily to satiation during the 21-day growth period. Fish were considered satiated when they either ignored or accepted and spat out any offered feed pellets (Moore-Clark dry extruded fish feed, 2 mm size). Daily feed intake for each experimental group was monitored through the 21-day growth period.

Handling of experimental animals

On day 0 of the 21-day growth period, fish were anesthetized with 2-phenoxyethanol (98%, Aldrich) at an approximate final concentration of 1:7000. Fish were then individually graded on the basis of weight, IP injected either with 0.1 mL of a 10 μg g^{-1} body weight GH in sesame oil suspension or 0.1 mL control sesame oil and assigned to appropriate experimental tanks where they were allowed to recover.

On day 7 for Part B, half of all experimental groups of fish were immersion vaccinated for 20 sec using a bivalent Vibrio anguillarum 775 / Vibrio ordalli MT615 commercial vaccine (Microvib™, Microtek Int. Ltd., Saanichton, BC) according to manufacturer's instructions. The other half of experimental fish were sham-vaccinated using the same treatment sequence as the vaccinated fish.

On day 21, fish from all experimental groups were either bath-challenged with a virulent live culture of Vibrio anguillarum (see Part A below) or sacrificed to obtain blood samples for further processing (see Part B). For Parts A and B, all fish were individually weighed prior to challenge or sacrifice.

Part A

Challenge procedure

The validity of a bacterial challenge in fish as an indicator of prior stress is reviewed by Wedemeyer et al.(1990). Vibrio anguillarum MT513 (courtesy Microtek Int. Ltd., Saanichton, BC) was grown in a culture medium of trypticase soy broth (TSB, BDH Mikrobiologie) plus 1.5% NaCl. One small drop of Antifoam B Emulsion (Sigma) was added to every 100 mL of culture broth to prevent excessive foaming. Culturing took place with continuous shaking and aeration at room temperature for 10-11 h. Sample volumes of the culture were periodically removed aseptically and diluted 10-fold in fresh, sterile culture broth and the optical density determined at 650 nm using a Bausch & Lomb Spectronic 21 spectrophotometer. Immediately after the original broth culture had surpassed $OD_{650nm}=2.2$, the culture was placed on ice and diluted with fresh sterile culture medium to a final $OD_{650nm}=2.2$.

Bath challenges were performed by mixing 5.0 mL of culture in 100 L of aerated fish tank water containing 0.9% added NaCl. The challenge water was then dispensed in equal volumes into eight large buckets. Aeration was provided and fish (n=15) were exposed for 30 min. A water sample taken directly from the 100 L challenge water was serially diluted in peptone saline to determine cell forming units (cfu) per mL by the drop plate method using TSA + 1.5% NaCl. The original challenge concentration of V. anguillarum was

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estimated to have been 8×10^5 cells mL^{-1} water. Mortalities due to vibriosis among challenged fish were tallied for 14 days post-challenge. Approximately 10% of moribund or dead fish were necropsied to confirm the cause of disease or death due to vibriosis. In all cases *Vibrio* sp. was isolated from necropsied animals and presumptively identified on the basis of colony morphology and Gram-stain characteristics (Gram-negative, slightly curved rods, 0.4 to 0.6 μm x 1.2 to 2 μm).

Part B

Collection of blood

      Whole blood was collected from the severed tail of each fish. Blood was drawn into heparinized microhematocrit capillary tubes to evaluate the ratios of packed white and red blood cell volumes. Several µL of blood were also immediately placed on blood smear slides. Blood smears were made and allowed to air dry. The remaining blood for each fish was collected in 1.5 mL capped microcentrifuge tubes and allowed to coagulate. Serum samples were obtained after centrifugation at 11,000 rpm for 3 min (Eppendorf 5415 C centrifuge) and frozen at -20°C.

Differential blood cell counts

      Air-dried blood smears were fixed in 95% ethanol for five min and then stained for 30 sec in undiluted Modified Giemsa stain (Accustain™, Sigma). Stained slides were evaluated under oil immersion to determine the relative % representation of different white blood cell types among leukocytes. Each slide was evaluated for a total of about 100 cells. These cells were observed in an average of about 70 fields. Identification and classification of fish leukocytes and recogniton of cell and smear artifacts were based on details provided by Ellis (1977), Rowley (1990) and Yasutake and Wales (1983).

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Leukocrit and hematocrit

Heparinized microhematocrit capillary tubes filled with fish blood were centrifuged for three minutes at 3000 rpm. Packed cell volumes of red (hematocrit) and white (leukocrit) blood cells were measured and expressed as a percentage in total blood (Siwicki and Anderson, 1993).

Lysozyme assay

Lysozyme activity in trout serum was assessed using the modification of the lysoplate method described by Yousif et al. (1991). Briefly, 10 μL volumes of serum samples were placed into wells (3.5 mm diameter x 4 mm deep) cut into 0.5% agarose (Type 1, Sigma) in 15 cm diameter petri plates. The agarose contained 0.06 M pH 6.0 phosphate buffer, 0.02 M NaCl and 0.6 mg mL^{-1} Micrococcus lysodeikticus (freeze dried, Sigma). Before the 0.5% agarose was added, the solution was brought to a brief boil to ensure that all M. lysodeikticus cells were killed. Each agarose plate was supplied with a positive control well containing 10 μL of 1 mg mL^{-1} hen egg white lysozyme (20,000 units mg^{-1}, Sigma) in 0.06 M pH 6.0 phosphate buffer plus 0.02 M NaCl. This control well (referred to as plate control from now on) was included to verify homogeneity of agarose plate evaluations during the assay. After incubation for 17 h at room temperature in a humid chamber, the diameters (mm) of zones of M. lysodeikticus lysis were measured.

Statistical analyses

Experimental units (i.e., individual tanks of n=10 fish) were run with replication for all fish groups being tested. For the challenge experiment, experimental groups were divided into four different tanks per group (n=15). In the case of differential blood cell counts, data were evaluated using two-factor ANOVA with replication (Microsoft® Excel version 5.0). All other results were evaluated by two-factor ANOVA with replication as above and also pooled as permitted by Student's t-test and then compared by two-factor ANOVA without replication. Other statistical analyses were accomplished using Student t-test comparisons. Mortality data obtained in Part A were analyzed for statistical significance using Fisher's exact test – the total chi-square method for replicate samples with evaluation of heterogeneity chi-square as described by Ostle and Mensing (1975). Significance levels for all tests were set at p=0.05. Power analysis was performed using JMP version 3.1.5 statistical software.

Part A

Challenge experiment

The average weights of rainbow trout on days 0 and 21 for treated (GH + sesame oil) and control (sesame oil) groups are shown in Table 1. On day 21, hormone-treated fish were significantly heavier ($p<0.001$) than control fish. The percent weight increase of hormone-treated and control fish was 252% and 196%, respectively. GH-treated fish were on average about 32% heavier than control fish at the end of the growth period.

Frequently, GH-injected fish displayed what appeared to be intraspecific agressive behavior where fish would be seen to chase and nip one another. These observations were made during feeding times. A similar 'chase and nip' behavior was not observed among control fish.

The pooled results of the challenge trials are summarized in Table 2. GH-injected fish that were bath challenged with Vibrio anguillarum MT513 on day 21 showed total mortalities that were not significantly greater ($p=0.06$) as compared with control fish. The % cumulative mortality rate for experimental groups is shown in Figure 2. The first mortalities were noted on the second day after the challenge. Mortalities peaked over the next one to two days

and then decreased. Expressed in mean number of days to death, GH-treated fish had a similar onset of mortalitiy as control animals (see Table 2). The total mortality for hormone-treated and control fish from all experimental units 14 days post-challenge was 98 and 88%, respectively.

Table 1. Average weights of rainbow trout on days 0 and 21.
Fish were injected either with growth hormone + sesame oil
(treatment, indicated as GH + oil) or sesame oil only
(control, indicated as oil) on day 0. The growth period was
21-days. Values are shown as averages of four experimental
units (n = 15 each) ± SE. NS (not statistically
significant).

Category	GH + oil (treatment)	oil (control)	probability
avg. fish weight on day 0 (g)	11.21 ± 0.27	10.92 ± 0.44	NS
avg. fish weight on day 21 (g)	28.23 ± 0.94	21.45 ± 0.81	$p < 0.001$
% weight gain	252	196	

Table 2. Total mortalities and survival from four

experimental units (n = 15 each) of rainbow trout following a

bath challenge on day 21 with _Vibrio anguillarum_ MT513.

Values, where applicable, are shown as averages ± SE. NS

(not statistically significant). GH + oil = growth hormone +

sesame oil (treatment) and oil = sesame oil (control).

Category	GH + oil (treatment)	oil (control)	probability
total mortalities	59	53	
total survivors	1	7	p = 0.06
total % mortality	98	88	
total % survival	2	12	
mean # of days to death	2.68 ± 0.15	2.80 ± 0.10	NS

Figure 2. Cumulative % mortality over the 14-day post-challenge period (days 21 - 35) for growth hormone + sesame oil (treatment, indicated as GH + oil) and sesame oil (control, indicated as oil) groups. (Y-bars indicate the cumulative % mortality range for four tanks of n = 15 fish each.)

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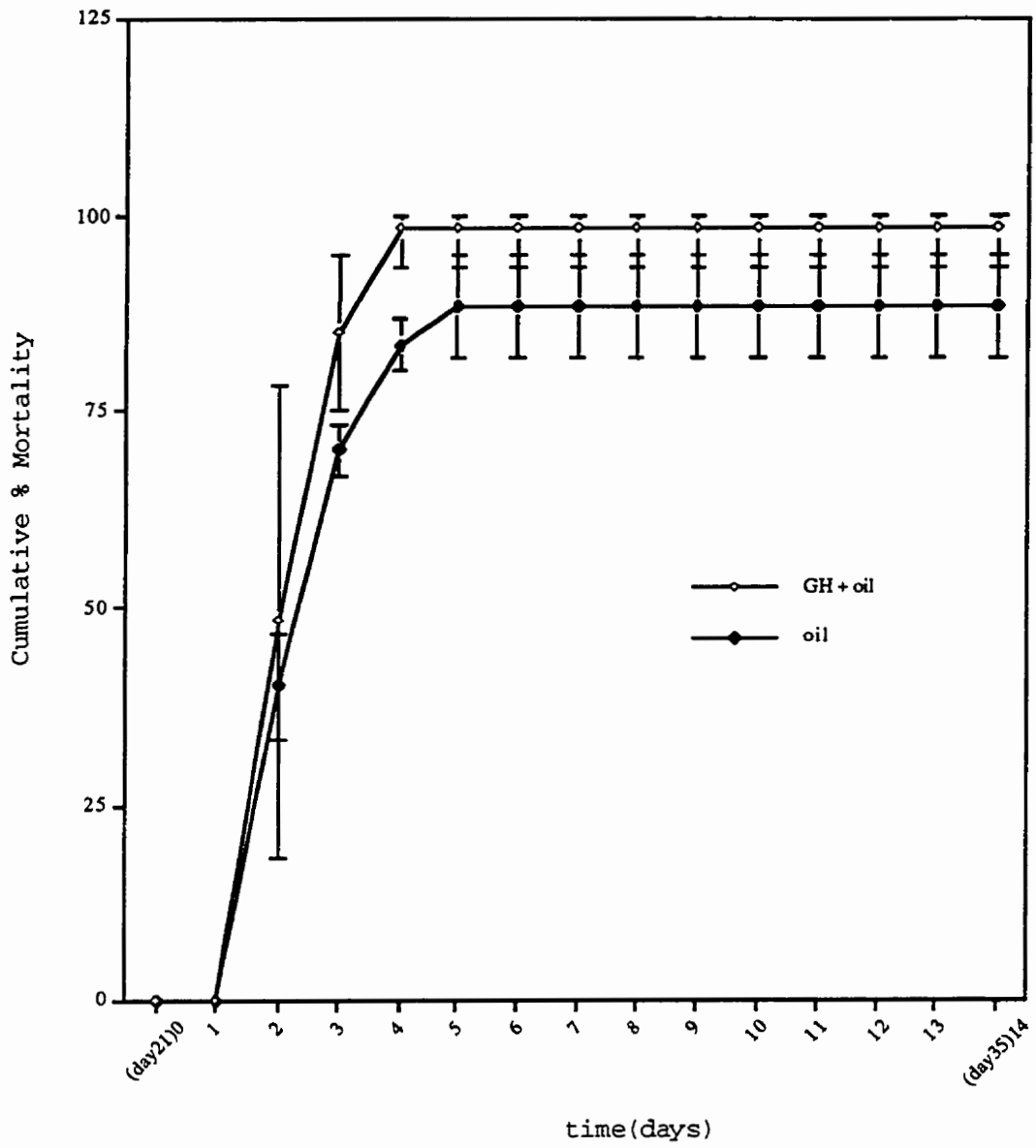
Part B

For the rainbow trout used in Part B, the average weights of fish on days 0 and 21 for treated (GH + oil) and control (oil) groups are indicated in Table 3. On day 21, hormone-treated fish were significantly heavier than control fish as tabulated. The percent weight increases of hormone-treated and control fish, whether vaccinated or sham-vaccinated, were 274 to 275% and 213 to 221%, respectively. GH-treated fish were on average about 28% heavier than control fish at the end of the growth period.

Differential blood cell counts

The relative percent occurrence of different white blood cell species observed in whole blood smears is summarized in Table 4. With the exception of significantly more monocytes for growth hormone-treated fish as compared to controls, no statisticcally significant differences in leukocyte species were observed. Statistical power with least significant number is shown in Table 4.

Lysozyme activity

The results for the lysozyme activity assays using the lysoplate method are summarized in Table 5. Sera from all experimental animals showed the amount of lysis of Micrococcus lysodeikticus on 0.5% agarose plates. No significant differences in lysozyme activity were observed.

(Statistical power = 0.21; least significant number = 256.)
The plate control samples applied to each plate averaged 7.18
mm ± 0.09.

Hematocrit

Results for the blood hematocrit measurements are
summarized in Table 6. A significant difference ($p < 0.05$) in
hematocrit values was observed in a comparison of pooled
vaccinated GH-treated fish with pooled vaccinated control
fish. No significant differences were observed among other
hematocrit measurements. (Statistical power = 0.62; least
significant number = 83.)

Leukocrit

Results for the blood leukocrit measurements are
summarized in Table 7. The results indicate a significantly
elevated ($p < 0.05$) leukocrit from pooled vaccinated fish as
compared to pooled sham-vaccinated fish. No significant
differences were observed among other leukocrit measurements.
(Statistical power = 0.19; least significant number = 303.)

Table 3. Mean weights of rainbow trout on days 0 and 21.
Fish were injected either with growth hormone + sesame oil
(treated) or sesame oil only (control) on day 0. On day 7
fish were dip-vaccinated or sham-vaccinated. The growth
period was 21 days. Values are shown as averages ± SE. NS
(not statistically significant). Probability results refer
to the indicated columns (A,B,C,D,a,b,c and d) in
superscripts. : = compared with; two adjoining superscript
letters represent their additive effect; NS = not
statistically significant; LSN = least significant number.

Category	A	a	B	b	C	c	D	d	probability
	GH + oil vacc. (n=20)		oil vacc. (n=20)		GH + oil sham (n=20)		oil sham (n=20)		
avg. fish weight on day 0 (g)	15.24±0.40 (n=10) 15.38±0.39 (n=10)	15.31±0.27	15.19±0.23 (n=10) 15.41±0.40 (n=10)	15.30±0.23	14.90±0.33 (n=10) 15.25±0.36 (n=10)	15.07±0.24	14.98±0.31 (n=10) 14.83±0.40 (n=10)	14.91±0.25	NS[a:b] NS[c:d] NS[a:c] NS[b:d] NS[ab:cd] NS[ac:bd] NS[AB:CD] NS[AC:BD]
avg. fish weight on day 21 (g)	42.37±1.66 (n=10) 41.58±1.09 (n=10)	41.97±0.97	34.57±0.94 (n=10) 30.51±1.06 (n=10)	32.54±0.83	40.75±0.91 (n=10) 42.27±1.33 (n=10)	41.51±0.80	33.04±1.19 (n=10) 32.81±1.07 (n=10)	32.93±0.72	p<0.001[a:b] p<0.001[c:d] NS[a:c] NS[b:d] p<0.05[ac:bd] NS[ab:cd] p<0.01[AC:BD] NS[AB:CD]
% weight gain		274		213		275		221	

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Table 4. Relative percent occurrence of different white blood cells in blood smears taken on day 21 for four different experimental groups. Values are averages ± SE. Probability results refer to the indicated columns (A,B,C,D,a,b,c and d) in superscripts. : = compared with; two adjoining superscript letters represent their additive effect; NS = not statistically significant; LSN = least significant number.

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Category	A GH + oil vacc.	a	B oil vacc.	b	C GH + oil sham	c	D oil sham	d	probability
relative % lymphocytes	77.54±3.93 (n=10) 74.37±4.02 (n=10)	75.96±1.59	77.49±3.87 (n=10) 74.87±3.58 (n=10)	76.18±1.31	81.01±4.43 (n=10) 75.97±4.52 (n=10)	78.49±2.52	77.23±3.78 (n=10) 81.62±3.62 (n=10)	79.43±2.20	NS[AB.CD] NS[AC.BD]
relative % thrombocytes	20.67±4.14 (n=10) 23.54±3.60 (n=10	22.11±1.44	21.94±3.64 (n=10) 23.11±3.57 (n=10)	22.53±0.59	14.81±4.12 (n=10) 21.55±3.98 (n=10)	18.19±3.37	21.30±3.90 (n=10) 17.34±4.12 (n=10)	19.32±1.98	NS[AB.CD] NS[AC.BD]
relative % neutrophils	1.59±0.91 (n=10) 1.99±0.73 (n=10)	1.79±0.20	0.58±0.20 (n=10) 1.92±0.51 (n=10)	1.25±0.67	3.98±1.32 (n=10) 2.32±1.01 (n=10)	3.15±0.83	1.45±0.71 (n=10) 0.97±0.45 (n=10)	1.21±0.24	NS[AB.CD] NS[AC.BD]
relative % monocytes	0.20±0.23 (n=10) 0.10±0.09 (n=10)	0.15±0.05	0.00±0.00 (n=10) 0.09±0.10 (n=10)	0.05±0.05	0.20±0.13 (n=10) 0.16±0.15 (n=10)	0.18±0.02	0.02±0.10 (n=10) 0.07±0.06 (n=10)	0.05±0.03	NS[AB.CD] $p<0.05$[AC.BD]

Table 5. Diameters of lytic zones (mm) surrounding agarose

plate wells inoculated with 10 μL trout serum samples or 10

μL of 1000 μg hen egg white lysozyme mL^{-1} buffer (plate

control). Values are averages ± SE. Probability results

refer to the indicated columns (A,B,C,D,a,b,c and d) in

superscripts. : = compared with; two adjoining superscript

letters represent their additive effect; NS = not

statistically significant.

Category	A	a	B	b	C	c	D	d	plate control	prob.
	GH + oil vacc. (n=19)		oil vacc. (n=20)		GH + oil sham (n=18)		oil sham (n=20)			
average diameter (mm) of lytic zone	10.02 ±0.35 (n=9) / 10.18 ±0.26 (n=10)	10.10±0.22	10.48 ±0.17 (n=10) / 10.27 ±0.20 (n=10)	10.38±0.13	10.64 ±0.28 (n=9) / 10.25 ±0.20 (n=9)	10.44±0.17	10.15 ±0.21 (n=10) / 10.43 ±0.17 (n=10)	10.29±0.13	7.18±0.09	NS^a,b NS^a,d NS^a,c NS^b,d NS^b,c,d NS^c,d,c,d NS^a,b,c,d NS^c,b,d

Table 6. Hematocrit values on day 21 for hormone-treated and
control fish, with and without vaccination. Values are
averages ± SE. Probability results refer to the indicated
columns (A,B,C,D,a,b,c and d) in superscripts. : = compared
with; two adjoining superscript letters represent their
additive effect; NS = not statistically significant.

Category	A	a	B	b	C	c	D	d	probability
	GII + oil vacc. (n=20)		oil vacc. (n=20)		GII + oil sham (n=20)		oil sham (n=20)		
hematocrit (%)	42.09±1.60 (n=10)	40.23±1.23	36.26±1.24 (n=10)	36.10±1.14	38.97±1.93 (n=10)	39.80±1.36	39.34±1.59 (n=10)	38.52±0.91	p<0.05[a,b] NS[c,d] NS[a,c] NS[b,d] NS[ab,cd] NS[ac,bd] NS[ab,cd] NS[ac,bd]
	38.37±1.45 (n=10)		35.95±1.98 (n=10)		40.62±2.00 (n=10)		37.70±0.91 (n=10)		

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Table 7. Leukocrit values on day 21 for hormone-treated and
control fish, with and without vaccination. Values are
averages ± SE. Probability results refer to the indicated
columns (A,B,C,D,a,b,c and d) in superscripts. : = compared
with; two adjoining superscript letters represent thier
additive effect; NS = not statistically significant.

Category	A	a	B	b	C	c	D	d	probability
	GII + oil vacc. (n=20)		oil vacc. (n=20)		GII + oil sham (n=20)		oil sham (n=20)		
leukocrit (%)	0.86±0.11 (n=10) 1.08±0.13 (n=10	0.97±0.09	1.04±0.16 (n=10) 0.82±0.11 (n=10)	0.93±0.10	0.90±0.09 (n=10) 0.79±0.12 (n=10)	0.85±0.08	0.74±0.15 (n=10) 0.85±0.17 (n=10)	0.79±0.11	NS[a:b] NS[c:d] NS[a:c] NS[b:d] p<0.05[Ab:cd] NS[aa:bj] NS[Ab:CD] NS[AC:BD]

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DISCUSSION

Juvenile rainbow trout treated with growth hormone were approximately 32% (Part A) and 28% (Part B) heavier than control sesame oil-injected fish after three weeks of growth (Tables 1 and 3). These results demonstrate the ability of this hormone to enhance the growth of cultured finfish, with specific reference to rainbow trout.

It is difficult to directly relate these observed increases in the growth rates of rainbow trout to other reported data on growth enhancement in other fish. This is because of the wide range of growth hormone types and their concentrations, treatment frequencies, hormone vehicles, and application methods that have been employed previously (see the Introduction for referenced literature). In addition, fish growth itself is dependent on a number of factors including age and species of fish, ambient temperature, feed quality and ration size, loading density, nutritional status and seasonality (see Sumpter, 1992 for a review of this subject). The Appendix provides a further discussion of this topic.

At $p \leq 0.05$, mortalities due to vibriosis in growth hormone-treated fish were similar, although at $p = 0.06$ a minor difference was noted between the two groups of fish (Table 2). Since the disease challenge was set at a very high lethal dose of _V. anguillarum_, it appears that the hypothesis as stated in the introduction is correct. That

is, in the case of vibriosis, the resistance to the disease is unaffected by growth hormone treatment as employed in these experiments. To achieve a more precise conclusion, however, a lower lethal dose (LD50 as the experimental end point) would be desirable (Sprague 1990). At the higher lethal dose, the concentration-effect relationship displays greater variability.

The statistical power associated with Part B results is very low. Therefore, any conclusions drawn from these results (discussed below) are necessarily limited.

Exogenous GH did not have an impact on serum lysozyme levels in treated fish as compared to untreated fish when evaluated by the lysoplate technique (Table 5).

Lysozyme activity was also not found to be elevated for vaccinated fish (Table 5), which one might expect. A reason for this may be that the lysozyme response was determined two weeks following vaccination. It is possible that any previous response had subsided in the two-week time period. Also, a subtle response may have occurred but was not detected by the assays employed. Nevertheless, it should be noted that Kitlen et al. (1997) give a preliminary report from unpublished observations that GH treatment of rainbow trout induced elevated serum lysozyme levels. The authors, however, did not indicate methodology or specify experimental conditions.

Apart from a significantly higher (p<0.05) hematocrit for vaccinated GH-treated fish compared to vaccinated control

fish (pooled data), blood hematocrits were found not to be significantly different between hormone-treated and control trout, regardless of vaccination procedure (Table 6). This finding was also noted by Komourdjian et al. (1978) who reported that porcine GH treatment in rainbow trout had no influence on hematocrit values. Unfortunately, these authors did not provide further details. Further work would be useful to determine if hematocrit levels in GH-treated rainbow trout are affected during the two-week post-treatment period.

The challenge results presented here appear to contradict those of Sakai et al. (1997). These authors reported an increase in the mean number of days to death (6.9 days) due to vibriosis by intraperitoneal injection in GH-treated rainbow trout as compared to control fish (4.1 days). Further, the LD50 of V. anguillarum was 7.6 x 10^4 cfu (recombinant chum salmon GH-treated) and 7.6 x 10^3 cfu (bovine serum albumin-treated control). That is, these authors reported an apparent protective effect of exogenous growth hormone against vibriosis in this salmonid.

As explained previously (see Introduction), GH appears to have a stimulatory function on several immune responses of finfish. For example, chum salmon GH injection into rainbow trout activates natural killer cells (Kajita et al., 1992) and macrophages (Sakai et al., 1995). However, evidence for a stimulatory role of GH on immune functions as discussed in this study was not found.

With respect to leukocrit examinations (Table 7), these were elevated for vaccinated treated and control fish as compared to sham-vaccinated fish (p<0.05, pooled data). Because of the low statistical power, however, there is insufficient evidence to draw reliable conclusions from these data. Nonetheless, an elevated leukocrit two weeks after vaccination may have been present and would have been related to the vaccine - primarily its lipopolysaccharide component acting as an immunostimulant (Velji et al., 1992) - with a resultant increase in white blood cell concentrations.

More subtle responses than those discussed above in a comparison of treated and control fish, whether vaccinated or not may also exist, however a clearer determination of this would depend on an expanded body of sampling evidence. Further work in this area should be compared to literature reports for a stimulatory role of GH in fish immune cell development and proliferation (see Calduch-Giner et al., 1995 and Kelley, 1990 for reviews).

The increased leukocrit values which occurred following vaccination could not be related to an elevated appearance of a specific group of leukocytes (Table 4). Except for an increased relative monocyte presence for growth hormone-treated fish, all other comparisons showed an isometric increase in leukocyte species. Other subtleties in relative blood cell presence may well exist but were not observed in this study.

Evidence for a stress response in the GH-treated fish in
this study may have come from observations of agressive
behavior of the treated animals. Agressive behavior of
hormone-treated fish was observed during feeding times with
fish in Part A. This was not the case in Part B. This
observation suggests a difference existed between the fish
evaluated in the two parts. It is possible that agressive
behavior itself could be viewed as a performance indicator of
stress. Aspects concerning physiological stress and behavior
have been reviewed by numerous authors (Riley, 1981;
Laudenslager, 1983; Schreck, 1990).

The relationship between physiological stress and the
immune system in fish is incompletely understood.
Environmental stressors, especially temperature, can affect
immune functions (Avtalion, 1981) as can some physiological
responses to stress (Barton and Schreck, 1987). The stress
hormone, cortisol, can have immunosuppressive functions;
however, the sensitivity of the salmonid immune system to
cortisol is seasonally dependent (Maule et al., 1993 and
1996). Generally, published reports indicate that stress in
fish can be linked to immunosuppression (for example Espelid
et al., 1996; Schreck, 1996). However, since not all tests
of fish immunity are equally effective in evaluating a given
stressor, and since a wide range of assays - some testing the
same response in multiple ways - is desirable, misleading or
difficult-to-interpret statistical results can be obtained

(Maule et al., 1989; Siwicki and Anderson, 1993; Pegg et al., 1995).

Growth rates are rarely maximal in juvenile fish (Johnsson and Bjornsson, 1994). The question thus arises whether unusually high growth rates have deleterious consequences on the health of the fish, despite the realization of an otherwise unutilized growth potential. Are there trade-offs between growth and fish health?

According to Pyke et al.(1977) it is unlikely that the energetic cost of synthesizing and secreting more GH could outweigh the positive influence of the hormone on reproductive success and fitness. However, while faster growth from GH treatment can improve appetite, it can also increase aggression levels (Johnsson and Bjornsson, 1994). Trade-offs to increased growth rates may include greater risks of predation in an effort to obtain more food (Lima and Dill, 1990), or an elevated probability of developmental errors and a cost for investment in maintenance and repair (Sibly and Calow, 1986). Farrell et al.(1997) demonstrate that growth-enhanced transgenic coho salmon can have significantly poorer-than-expected swimming performance (critical swimming speed). This indicates a physiological cost associated with enhanced body growth.

While these factors may all play a role in retaining sub-maximal growth rates in juvenile salmonids, other arguments that are more specifically related to the numerous roles of GH should be considered.

As expressed in the Introduction, GH plays a role in several aspects of salmonid biology (for a review see Bjornsson 1997), including somatic growth, osmoregulation, smoltification, the development and activity of immune cells, maturation and reproduction. Indeed, normal hormone levels are known to fluctuate on a daily basis (see Bates et al., 1989). Seasonal variations also exist. For example, hormone levels are elevated in juvenile coho salmon at the time of smoltification (Sweeting et al., 1985).

It may be said, therefore, that natural GH levels, much like growth rates, are rarely maximal. Indeed, hormone levels appear to be optimally adjusted according to life history requirements. Therefore it is conceivable that abnormally high levels of GH in young salmon or rainbow trout, whether transgenically expressed or exogenously applied, can have undesirable physiological costs (see Devlin et al., 1994b and 1995). For example, it has been noted during some of the experiments presented in this study that application of exogenous GH to juvenile rainbow trout can lead to an accentuated stress response in the fish following sudden alterations in ambient light conditions (see Appendix). Seasonal GH requirements and the physiological response to excess GH may depend on specific environmental cues (notably light) and the entrainment of a circannual endogenous rythm.

To explain the minor elevated challenge mortalities and agressive fish behavior in Part A, a stress response has been

suggested. This response relates to an environmental or seasonal variable that is linked, via an endogenous rythm, to the physiology of GH action. If so, then this would help explain the conflicting evidence reported by (Sakai et al., 1997) that GH treatment in rainbow trout was protective against vibriosis. With respect to that study one should point out that: (1) firstly, the challenge procedure by intraperitoneal injection commenced 5 days after GH treatment at 18°C (not 21 days after treatment and at 15°C) and (2) secondly, prior to treatment, the fish had been maintained for two weeks at 14°C in outdoor tanks (not indoor tanks under artificial environmental conditions). Further details regarding seasonality and environmental conditions were not provided.

This study did not indicate an influence of immersion vaccination against vibriosis on the growth performance of rainbow trout. However, injection vaccination against vibriosis has been reported to result in depressed growth rates in GH-treated and control rainbow trout; it is not known whether a bacterial or adjuvant component of the vaccine was responsible for the observed growth depression (Kitlen et al., 1997). Also see Sawyer and Strout (1977) and Lillehaug (1991) for further results concerning the effects of vibriosis vaccination on fish growth.

Finally, there are a number of assumptions and limitations associated with the results and interpretation of this research. These are listed as follows:

1. Biological activity of rbGH was confirmed on the basis of accelerated weight increases in rainbow trout. It was assumed that the rbGH was still active at day 21. Evidence in support of this assumption has been gathered by Garber et al. (1995).

2. The possibility exists (although only theoretical as there is no data to indicate that this is the case) that using heterologous rbGH in salmonids could elicit unexpected responses by cross-reacting with receptors of GH-related hormones such as prolactin and somatolactin (Bjornsson, 1997).

3. It was assumed that a valid comparison could be made between test animals which were injected with GH suspended in sesame oil and control animals that were injected with sesame oil only. Biological side effects, if any, due to sesame oil or the combination of GH and sesame oil are unknown. Nonetheless, a possible adjuvant-effect due to the sesame may have occurred. Any such influence, however, was assumed to be equivalent for both test and control fish.

4. This study did not investigate the performance of non-injected ('normal') fish with respect to oil injected animals.

5. Uncertainties in the interpretation of test results (Part B) exist as the result of low replication and, therefore, limited statistical power.

6. The effects of GH on the disease resistance of rainbow trout were studied using vibriosis or vaccination against vibriosis as a model. The assumption is made that other bacterial fish pathogens generate a similar response pattern in fish.

7. Experimental findings were limited to juvenile rainbow trout (6 months old, 10-15 g average weight) at a temperature of 15°C.

CONCLUSION

Considering the complex function of GH in salmonid
biology, the results expressed in this work should be viewed
with reference to the seasonal and environmental conditions
under which the data were collected (ie. early to mid summer
at 15°C with a photoperiod of 9h Dark:15h Light from 08:00-
17:00 at 10-15 lux). In addition, pre-experimental
maintenance and rearing conditions, feeding regime, and the
length and nature of the acclimation period should also be
considered.

While GH treatment was not found to have a significant
impact on several blood factors, a possible negative effect
on disease resistance to vibriosis was noted. Overall, GH
treatment appeared to have a rather benign influence on those
factors which were measured in this study.

Further research in this area should include an
assessment of the health impact (stress/immunology) of GH
treatment in rainbow trout (and other salmonids) under
different environmental conditions. Seasonal GH requirements
and the physiological response to excess GH may depend on (1)
specific environmental cues and (2) the entrainment of a
circannual endogenous rythm. It is important to consider the
potential for a competing function of exogenous GH in the
overall health performance of salmonids. Previous work
indicating a stimulatory role of GH on immune functions in

fish include Calduch-Giner et al.(1995 and 1997), Kitlen et al.(1997), Sakai et al.(1995, 1996, 1997).

Appendix

Preliminary results on the effects of growth hormone in sesame oil on feeding and growth performance of rainbow trout (Oncorhynchus mykiss) under different photoperiod conditions

Abstract

Juvenile rainbow trout were intraperitoneally injected with 10 µg g^{-1} body weight of a sustained-release rbGH formulation or an equivalent volume of sesame oil (control) and their feeding and growth performance was evaluated over a 21-day period. In experiment A, fish were maintained under continuous light conditions following treatment, while in experiment B the photoperiod remained unaltered from pre-treatment conditions (9 h L : 15 h D). Weight increases for hormone-treated fish in experiments A and B were, respectively, 12.1% and 27.3% greater than control-injected fish. This corresponded to a greater weight increase ($p<0.05$) for treated fish in experiment B (260%±9) than A (213%±3). During days 15-20 of the growth period, hormone-treated fish had a significantly higher ADFI (average daily feed intake) than control fish for both expts. A and B. A compensatory feeding response was noted. For this interval (days 15-20) the percent difference in ADFI between hormone-treated and control fish was significantly higher in expt. B than expt. A. It is concluded that the combined effect of photoperiod and exogenous GH can lead to a suppression of appetite in rainbow trout. This appetite suppression may be a result of an accentuated stress response and leads to reduced potential growth enhancement - despite increased food conversion. Generalizations must be made with caution, however. The results are discussed with reference to the complex relationship existing between light and endogenous GH in salmonid life history.

key words: growth hormone, sesame oil, compensatory growth, feeding, appetite, photoperiod, stress, smoltification

Introduction

Although GH administration has been shown to consistently enhance growth rates in fish, reports on the effects of hormone treatment on feed intake in fish have been variable. Markert et al. (1977) found no effect on feed consumption in coho salmon, while Agellon (1988) and Johnsson and Bjornsson (1994) describe increased feed consumption and appetite in rainbow trout. Garber et al. (1995), on the other hand, showed that hormone-injected rainbow trout had a 44.8% improvement in average daily weight gain in the first two weeks following treatment, even though the same fish, during the same time frame, exhibited a 17.6% reduction of feed intake compared to control animals. A satisfactory explanation for this latter finding was not provided by these authors.

The objective of this preliminary study was to examine the growth and feeding performance of juvenile rainbow trout injected with a slow-release rbGH formulation under two different photoperiod conditions.

Materials and Methods

Experimental animals

Juvenile rainbow trout averaging approximately 12 g each were obtained from a commercial hatchery (Spring Valley

Hatchery, Langley, BC, Canada) in June/July 1997. At no time were the fish exposed to natural photoperiod conditions. Fish were acclimated to local dechlorinated municipal water conditions (Alcan Aquatic Research Center, Simon Fraser University, BC, Canada) in a 500 L circular tank with constant flow-through water supply and aeration for at least two weeks before treatment. The water temperature was maintained at 15.5 ± 1 °C and the photoperiod was set at 9 h L:15 h D using overhead fluorescent lights (approx. 10-15 lux at water surface). Fish were fed sparingly at about 1% biomass every other day during the acclimation period.

Preparation of growth hormone for injection

Recombinant bovine growth hormone (500 mg Monsanto Co., Lot 91F 18/10) was diluted in sesame oil (Sigma) to obtain an estimated hormone stock concentration of 50 mg GH mL^{-1} sesame oil. This stock suspension was maintained at 4 °C. A working suspension was made by further dilution of stock material: for a 0.1 mL injectable volume of GH + oil per fish, the concentration of GH was adjusted to render approximately 10 μg hormone g^{-1} fish body weight. IP injection of 0.1 mL volumes of viscous GH+oil or control sesame oil into fish was achieved using disposable 27G 1/2″ Beckton Dickinson needles fastened to 1 mL interchangeable Luerlock glass syringes.

Experimental treatments

The two growth performance experiments are described as experiment A and experiment B. Experiment B was performed first, leaving fish that were too small behind for use in experiment A. Experiment A followed about three weeks later. In both experiments fish were handled and maintained in the same manner through a 21-day growth period, except that in experiment A the photoperiod was abruptly changed following treatment to provide continuous light conditions (24 h L:0 h D), while for the duration of experiment B the photoperiod remained unaltered (9 h L:15 h D).

On day 0, experimental animals were anaesthetized (2-phenoxyethanol, 1:7000), individually weighed to the nearest 0.01 g and injected with 10 μg rbGH g^{-1} body weight as described above. Control fish received an equivalent injectable volume of hormone carrier (0.1 mL sesame oil). Following injection, fish were transferred to 77 L circular tanks supplied with constant flow-through water and aeration. On day 21, the growth experiments were terminated and all fish were again individually weighed. Feeding of fish occurred once daily, to satiation, beginning on day 1 and ending on day 20. Fish were considered satiated when they either ignored or accepted and spat out any offered feed pellets (Moore-Clark dry extruded fish feed, 2 mm size).

Analytical methods

Statistical work was accomplished using 2-tailed Student t-test analysis, with statistical significance set at p=0.05. Power analysis was performed using JMP version 3.1.5 statistical software. Weight specific growth rates (SGR) were expressed as % body weight / day and determined according to the formula SGR=$[lnW_f-lnW_o]/[T_f-T_o]$x100, where T_f and T_o are day 21 and day 0 of the growth period, respectively, and where W_f and W_o are the natural logarithms of weight at the end and beginning of the growth period, respectively. Average food conversion (FC) per experimental group for the growth period was calculated according to the formula FC=F/G, where G is total grams weight gain per group and F is total grams of feed ingested per group. Average daily feed intake per experimental group (ADFI) was calculated for three time intervals: days 1-7, days 8-14, and days 15-20.

Results

Effects of rbGH on growth performance and feeding are summarized in Table 1 (experiment A) and Table 2 (experiment B). Statistical comparisons of experiments A and B are described below. The final weights of hormone-treated fish in experiments A and B were, respectively, 12.1% and 27.3% greater than control fish. This relates to a greater weight increase (p<0.05) for treated fish in experiment B (260%±9)

than A (213%±3). The weight increase for control fish between the two experiments was not significant (p=0.06): control fish in experiment A and B grew, respectively, 187%±1 and 199%±3.

During the interval days 1-7, there was a significant difference in the relative ADFI of treated fish over control fish between the two experiments (A: -30.2%±0.03, B: 14.5%±0.01, p<0.01).

For the interval days 8-14 there was a significant difference in the relative increase in ADFI for treated over control fish between the two experiments (A: 3.4%±0.01, B: 12.5%±0.02, p<0.05).

During the interval days 15-20, hormone-treated fish of experiment A and experiment B, respectively, had ADFIs that were significantly greater than control fish by p<0.05 and p<0.001. This relates to a significant difference in the relative increase in ADFI for treated fish over control fish between the two experiments (A: 14.8%±0.03, B: 33.5%±0.01, p<0.05).

For the interval days 1-20 there was a significant difference in the relative increase in ADFI for treated over control fish between the two experiments (A: 1.3%±0.01, B: 22.2%±0.01, p<0.01).

In both experiments, the FC for hormone-treated fish was significantly better compared to control fish (p<0.01). Between treated fish in the two experiments, the FC values were significantly better for experiment B than A (p<0.01).

Between control fish in the two experiments, the FC values were significantly better for experiment B than A (p<0.05). There was no significant difference in the improvement in FC for treated over control fish between the two experiments.

In experiment A, SGR was not significantly different between treated and control fish (p=0.07). In experiment B, SGR was significantly greater for hormone-treated fish (p<0.05) than control fish. Between treated fish in the two experiments, the SGR values were significantly higher for experiment B than A (p<0.05). Between control fish in the two experiments, the SGR values were not significantly different. The SGR improvement for treated fish over control fish in the two experiments was significantly different (A: 20.6%±0.01, B: 40.5%±0.01, p<0.01).

Between control fish in the two experiments, the ADFI improvement between the intervals days 1-7 and days 15-20 was significantly different (A: 192%±7, B: 269±8, p<0.05).

Discussion

An increased food conversion efficiency in fish receiving exogenous growth hormone has been reported for many years and the results from experiments A and B confirm previous findings. Improved food conversion may be related to a stimulatory effect of GH on intestinal amino acid transport and intestinal mass (Collie and Stevens, 1985; Sun and Farmanfarmaian, 1992). Increased food conversion

efficiency may also be related to the stimulatory influence of GH on lipid breakdown (O'Connor, 1993), thereby sparing amino acids for protein accretion. This suggests that accelerated body growth in the presence of exogenous growth hormone does not require a concomitant increase in feed intake. Indeed, Garber et al. (1995) show that accelerated growth is possible within 14 days of sustained release hormone treatment, despite a significant reduction in feed intake compared to control fish. Although the present results are not significant for experiment A (but note the low statistical power and see Garber et al., 1995, for significant results), an early, temporary reduction in feed intake for GH-treated fish compared with control fish (days 1-7) may have been present. Indeed, a comparison between experiments A and B for this time interval supports this interpretation.

Control fish in experiment B showed a better food conversion and ADFI increase than control fish in experiment A. It is unclear from this study whether this difference stemmed from the environmental light manipulation, or from a difference in the nutritional status of the fish prior to experimentation, or both.

An evaluation of the continued and overall growth of rainbow trout through to the end of the growth period demonstrates a disparity between the performance of hormone-treated fish in the two experiments. The final growth enhancement of hormone-treated fish over control fish was

much less prominent in experiment A than experiment B. A compensatory feeding response is noted for the time interval days 15-20 for both experiments. However, while both experiments indicate a sudden increase in ADFI in the third week of growth (days 15-20), this increase was more significant for hormone-treated fish in experiment B than experiment A. Compensatory feeding - rather than compensatory growth (see Dobson and Holmes, 1984) - might best be described as a phase of increased appetite, greater than normal or control appetite, following physiological adjustment to utilize excess levels of GH for accelerated body growth. It is not known for how long this feeding response commences. The role of a compensatory growth response resultant from the nutritional status of the fish before experimentation is unclear, however it may have occurred. Although the control fish for both experiments were derived from the same original group, the fish for experiment A comprised the smaller-sized individuals which required more growing time before experimentation.

Feed intake may be influenced by a number of factors, including environmental salinity, temperature, individual species' body and viscera size and rate of digestion (reviewed by Brett, 1979; Sumpter, 1992; Garber et al., 1995). In addition, stress in fish may reduce feeding performance and growth (Adams, 1990; Schreck, 1990; Iwama, 1993). The combined effect of photoperiod and GH has led to a suppression in appetite in this study. It may be

speculated that an initial decrease in feed intake by hormone-treated fish relative to control fish (experiment A) occurred immediately following treatment (days 1-7), which then led to reduced overall growth. This initial decrease in ADFI could be viewed as a performance indicator of physiological stress and be treatment effect-dependent (ie. nature and severity of the initial stressor).

It is worth considering why changes in light regime may result in a stress response in growth hormone-treated salmonids. Although GH research often focuses on the growth stimulating effects of the hormone, it is well recognized that GH also functions in other aspects of salmonid biology, including smoltification, osmoregulation, reproduction and maturation (Hoar, 1988; Bjornsson, 1997). The role of GH as part of a light-pituitary axis in growth and smoltification has long been recognized (Komourdjian et al., 1976). Plasma GH levels increase in smoltifying salmonids following photoperiod increases, but are inhibited under continuous light conditions (for a review see Bjornsson, 1997). Also, photoperiod and daylength changes are believed to play an important role in the entrainment of an endogenous circannual rythm leading to the physiological adjustments necessary for smoltification (Eriksson and Lundqvist, 1982; Duston and Saunders, 1990).

Thus, given the complex seasonal interplay of an endogenous circannual rythm, environmental conditions (notably light), and endogenous GH production, it is not

surprising that sudden alterations in ambient light
conditions can upset the normal physiological functioning of
salmonids, particularly when also exposed to exogenous GH.

The appetite supression and overall reduced growth
performance of hormone-treated fish in experiment A appears
to relate to a physiological response associated with the
sudden processing of changes in two interrelated factors,
photoperiod and GH.

A sudden change in ambient light conditions may itself
be a source of stress for fish (Thrush et al., 1994). Ambient
light changes also have implications for endogenous GH
secretion (Bjornsson, 1997). However, exogenous GH
application can accentuate a possible stress response. This
response is probably controlled at the hypothalamus-pituitary
level. However, generalizations must be made with caution.
The complex nature of this response, considering the role of
an endogenous circannual rythm and the multiplicity of GH
action in salmonid life history, indicates the need for
further research. The combined effects of light
(periodicity, wavelength and intensity) and exogenous GH on
endogenous GH secretion, feed consumption and stress should
be further evaluated.

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Table 1. Experiment A (24 h L:0 h D). Growth and feeding performance of rainbow trout (n=20) treated with a sesame oil-suspended bovine growth hormone formulation. Values are shown as averages ± SE.

	GH+oil (10μg g⁻¹ body weight)	oil	Probability
Day 0 weight (g/fish)	12.37 ± 0.26	12.56 ± 0.32	NS*
Day 21 weight (g/fish)	26.30 ± 1.00	23.47 ± 0.76	p<0.05
% weight gain	212.6	186.9	
ADFI (g feed/group)			
Days 1-7	2.61 ± 0.30	3.39 ± 0.31	NS,p=0.08**
Days 8-14	5.00 ± 0.21	4.84 ± 0.24	NS
Days 15-20	7.43 ± 0.26	6.48 ± 0.37	p<0.05
Days 1-20	4.89 ± 0.34	4.82 ± 0.26	NS
SGR	3.42 ± 0.14	2.84 ± 0.09	NS,p=0.07
FC	0.70 ± 0.01	0.89 ± 0.01	p<0.01

*NS = not significantly different, p>0.05
** statistical power = 0.42

Table 2. Experiment B (9 h L:15 h D). Growth and feeding performance of rainbow trout (n=20) treated with a sesame oil-suspended bovine growth hormone formulation. Values are shown as averages ± SE.

	GH+oil (10μg g⁻¹ body weight)	oil	Probability
Day 0 weight (g/fish)	11.03 ± 0.18	11.33 ± 0.24	NS*
Day 21 weight (g/fish)	28.67 ± 0.57	22.52 ± 0.81	p<0.001
% weight gain	259.9	198.8	
ADFI (g feed/group)			
Days 1-7	2.70 ± 0.18	2.36 ± 0.18	NS
Days 8-14	4.94 ± 0.27	4.40 ± 0.18	NS
Days 15-20	8.47 ± 0.47	6.34 ± 0.30	p<0.001
Days 1-20	5.22 ± 0.41	4.27 ± 0.28	NS,p=0.06
SGR	4.55 ± 0.19	3.24 ± 0.11	p<0.05
FC	0.59 ± 0.01	0.78 ± 0.01	p<0.01

*NS = not significantly different, p>0.05

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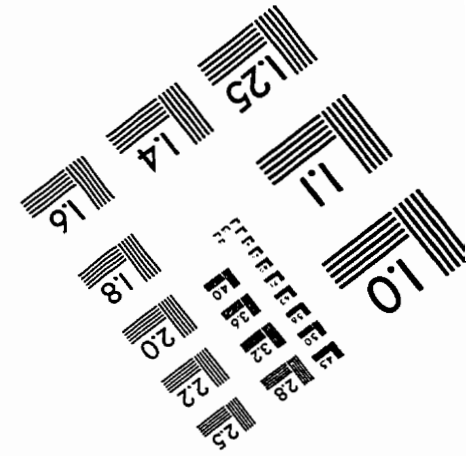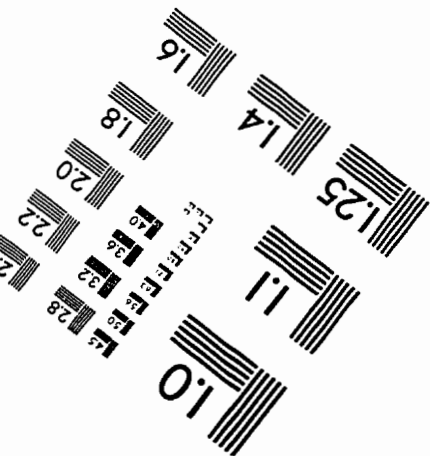
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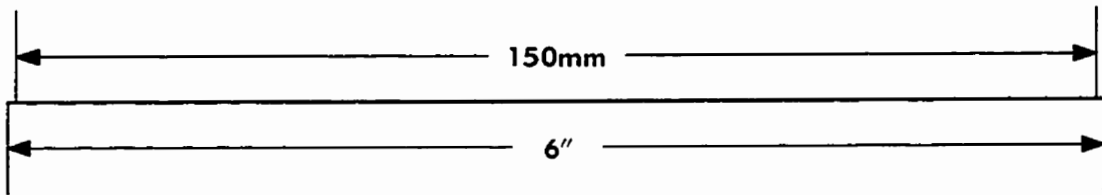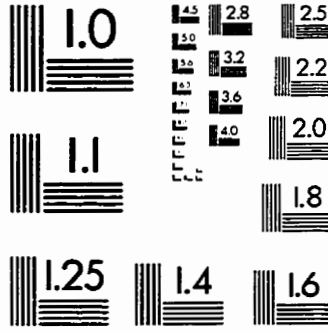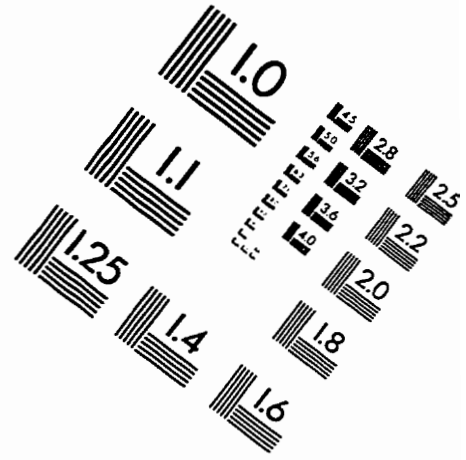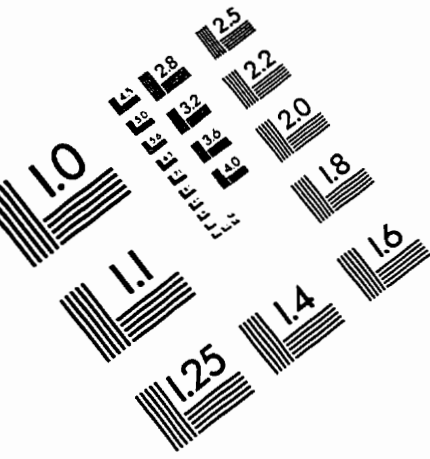
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IMAGE EVALUATION
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