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Genetic selection and molecular analysis of domesticated rainbow trout for enhanced growth on alternative diet sources

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Synopsis

Real-time polymerase chain reaction was used to monitor the expression of specific genes and confirm that an alteration in level of expression correlates with changes in diet, metabolism, or immune response. To test these probe and primer sets for their ability to monitor changes in gene expression, groups of fish were experimentally challenged either via daylength, vitamin availability, or with pathogenic microorganisms. The probes and primers sets were then used to analyze expression levels of specific gene products from isolated RNA. The expression of certain genes such as myosin in muscle correlated significantly with protein intake and seasonal daylength. Other genes involved with growth and metabolism such as insulin-like growth factor and pyruvate kinase showed lower levels of significance in correlating with planes of nutrition and varied with diet and treatment. However differences for pyruvate kinase were found in early tests done with animals receiving a vitamin-reduced diet. For factors relating immunological status after infection with microbiological pathogens, several factors such as MX-1 and CD-8 correlated with dose of the viral pathogen infectious hematopoeitic necrosis virus. Association of pathogen dose with immunological expression level was much less pronounced when fish were tested with bacterial microorganisms. This work will aid in evaluating the effects of diet on fish health and nutrient utilization and for evaluating the effects of selection of trout strains.

Introduction

Aquaculture is a rapidly developing agricultural economic resource with room for great expansion. Of increasing importance for the continued growth of the salmonid aquaculture industry is the replacement of fish meal with alternative protein sources (Hardy & Roberts 1998). Cereal grains and their products are promising candidates as renewable replacements for fish meal. Replacement of protein in fish diets with that of cereal grains would significantly reduce our dependence on fish protein sources, and also reduce the content of enriching nutrients, such as phosphorus in facility effluents.

At the Hagerman Fish Culture Experiment Station (HFCES), work has progressed in the study of diets containing cereal grains for rainbow trout (Pfeffer & Henrichfreise 1994, Sugiura et al. 1999, Arndt et al. 1999). Studies have shown that partial replacement of fishmeal with cereal grain proteins can be effective (Gomes et al. 1995, Skonberg et al. 1998), but that high replacement levels reduce growth rates and negatively affect the health of fish (Burrells et al. 1999). These negative effects include loss of appetite, intestinal damage, weakened immune function leading to disease outbreaks, and noticeable physical alterations (splotchy discoloration and fin erosion) (Ketola 1983, Rumsey et al. 1994, Gomes et al. 1995). Before new

diet formulations can be widely recommended, they must be evaluated for their effects on fish physiology and health.

In order to achieve the maximal benefit of alternative diets, it is necessary to screen and genetically select for fish from existing strains that flourish on these diets. We have begun a genetic enhancement program for the selection of rainbow trout families fed a 40% barley replacement diet, based upon fish weight gain and feed conversion. Selection for growth has been shown to be a highly heritable quantitative trait (Fishback et al. 2002). However, selection for growth alone may lead to undesirable phenotypic side effects. For example selection for large, fast growing fish may result in fish that produce and store fat instead of muscle (Kause et al. 2002). Factors related to growth and growth control have also been linked to the immune system of trout (Yada et al. 2001). Opportunities now exist to identify the genes that play a role in the growth process. Molecular examination of genes involved with such quantitative genetic traits as growth can steer a genetic enhancement program toward a more clearly defined phenotype. Depending upon the contribution of the gene in question for the trait under selection, knowledge of the profile of that particular gene's expression pattern may lead to more rapid phenotypic gains when selection emphasis is placed on that specific gene, rather than on growth alone.

In our studies, the initial selection of rainbow trout families and individuals within families was based on weight gain and feed conversion ratio (FCR). Subsequently we expect to evaluate selected families by quantifying the expression of genes related to the phenotype under selection. Identification and analyses of the expression of appropriate genes related to growth, health, and nutrient utilization have been undertaken to determine methods and techniques to analyze gene expression related to physical characteristics (Overturf & Hardy 2001). One method is through the use of fluorescent-labeled probes and real-time PCR. Similar to reverse-transcribed polymerase chain reaction (RT-PCR) for quantification, this method uses two primers, one that acts to prime the reverse transcription reaction for the generation of complimentary DNA (cDNA) and also functions in concert with the other primer during the polymerase chain reaction (PCR) amplification steps. This standard method of quantitative PCR has been used in measuring TGF-beta mRNA in teleost fish (Harms et al. 2000). Typically, in quantitative PCR, an aliquot of the reaction product is run on agarose gels and the gel is analyzed by an external imaging method that compares the intensity of stained or radioactively labeled bands to that of a standard. Recently this method has been improved to provide an internal measurement of expansion of individual PCR product during the log phase of amplification for each individual reaction. This is accomplished by the addition of a sequence-specific, complementary fluorescent-labeled reporter probe that binds to the region being amplified between the two PCR primers. Upon cleavage of the sequence-bound probe by the 5′ nuclease activity of the *Taq* polymerase, the fluorescent reporter component of the probe is released from a terminal-quenching dye attached to the probe, emitting a detectable fluorescent signal. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence (Lakowicz 1983). Normalization of the amplification reactions is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of a passive reference dye that is included in all experimental and control reactions.

Prior work from this laboratory has demonstrated that myosin expression correlates directly with nutritional intake in rainbow trout (Overturf & Hardy 2001). The expression of this gene is now being studied in selected lines of rainbow trout to determine if it can be used for the analysis of growth as related to muscle deposition and feed usage throughout the year. This same myosin probe was tested on steelhead reared in outdoor raceways under seasonal photoperiod to determine if a relationship exists between day length and myosin expression in fish fed at a constant level.

We are also initiating efforts to develop other probe and primer sets that can be used in genetic enhancement programs to evaluate and select for rainbow trout that grow on alternative protein sources. Specific probes for immune factors have been developed to generate an expression profile of 'healthy' fish and to look at the fish response on varied formulated diets. Here we report on the ability of the recently designed probes to measure changes in expression occurring in animals exposed to different disease-causing microorganisms. These probes might also be used for the selection of disease resistant stocks of trout.

Probe and primer sequences have also been generated to amplify gene sequences for trout metabolic and growth factors. These types of factors should prove useful in monitoring the effectiveness of different

diet formulations, and lead to the evaluation and identification of metabolic enzymes or pathways that are specific for energy or nutrient utilization. Knowledge regarding the stimulation of growth factor expression will be useful in selecting for fish that grow rapidly while converting energy to muscle instead of depositing it as fat. With an understanding of gene expression levels and their relationships to changes for trait attributes using real-time PCR to quantify expression of specific genes can be used to enhance selection for specialized phenotypes in rainbow trout.

Materials and methods

Care and feeding of the trout strains

Steelhead myosin study. Steelhead, *Oncorhynchus mykiss*, (80 g) were reared at the U.S. Fish and Wildlife National Hatchery in Hagerman, Idaho in outdoor cement raceways receiving a constant flow (1 212 gal min^{-1}) of 58°C water during the entire trial from September to March. They were fed a standard commercial trout feed containing 44% crude protein and 16% fat (Nelson and Sons, Inc., Murray, UT) at a constant feed rate per average weight of fish each day. Steelhead were sampled every 2 weeks for 7 months (five fish for each sample time point).

Myosin strain study. After known crosses (four strains, 2 × 2 factorial crosses) were performed, 16 families of rainbow trout were obtained from suppliers as eggs or swim-up young and reared to 3.0 g prior to the study. Each family for each strain was placed in triplicate 145 l tanks at a density of 100 fish per tank. When the fish reached 100 g, they were transferred to 450 l tanks for the remainder of the study. The fish were randomly assigned to tanks to avoid confounding environmental effects on growth. The fish were reared in pathogen-free, 14.5°C spring water at a flow rate of 4–8 l min^{-1} per tank, depending on the size of the tank. Photoperiod was controlled by timers at 14 h light and 10 h of dark. Control fish families (three tanks of 100 randomly selected fish for each strain from all generated families of that strain) were reared on a commercial trout feed containing 44% crude protein and 16% fat (Nelson and Sons, Inc., Murray, UT). For experimental selected trout strain and family studies analyzed, the standard diet was modified to contain 40% Waxbar barley. Each group of fish was counted and bulk weighed every 2 weeks, and weight gain and FCR were calculated for a 4-month period. After 4 months, the top 10% of fish from the two highest performing families of each strain, determined by a combination of best average weight gain and FCR levels, and the bottom 15% of fish from the poorest performing families were kept for further studies. Samples of fish were overdosed with MS-222 (tricaine methanosulfonate, Argent Laboratories, Redmond, WA) and RNA was extracted from tissue.

Immunology factor study. Specific-pathogen-free (SPF) rainbow trout (mean initial weight, 14.0 g) were supplied by Clear Springs Foods, Inc. (Buhl, ID). Fish were held in indoor research 378 l tanks supplied with SPF 15°C spring water treated by ultraviolet light. During the pathogen infection phase of these studies the fish were transferred to 19 l tanks for use in challenge studies. Fish were fed 1% body weight per day of a pelleted trout feed (Clear Springs Foods) while being held in the large tanks, and *ad libitum* after pathogen infection.

Vitamin deficiency study. A standard fish meal, fish oil, and ground wheat diet (Table 1) was manufactured by compression pelleting at the HFCES (Hagerman, Id.). Fish oil and ground wheat levels were varied to maintain isocaloric equilibrium within the diets. Four experimental diets were produced, varying only in the level of vitamin premix (Table 1). The vitamin premix was made to provide the NRC (1993) requirements when added at 4% to the diet. This vitamin premix was fed at 1%, 2%, 4%, and 8% of the diet to provide 25%, 50%, 100%, and 200% of the NRC requirements for rainbow trout. Each diet was fed to three replicate tanks of trout, starting weight 10 g, and the arrangement of the diets among tanks was according to a completely randomized design. Every 4 weeks fish were bulk weighed and two fish from each tank were removed for mRNA expression analysis. Fish were handled and treated in accordance with the guidelines approved by the Animal Care and Use Committee of the University of Idaho.

Bacterial pathogen infection

A virulent strain of *Flavobacterium psychrophilum* (CSF-259-93) was used. The isolate was cultured in tryptone yeast extract salts (TYES) broth and maintained at 15°C (0.4% tryptone, 0.04% yeast extract,

Table 1. Composition of diets fed to rainbow trout for 12 weeks in the vitamin deficiency study.

Ingredients	Diet 1	Diet 2	Diet 3	Diet 4
Fish meal (LT aqua-grade)	600	600	600	600
Ground wheat	259	249	229	189
Fish oil	123	130	137	144
Ascorbic acid (phosphate ester) 35% active	1.0	1.0	1.0	1.0
Choline chloride (50% dry)	6	6	6	6
Trace mineral premix	1	1	1	1
Vitamin premix*	10	20	40	80

Vitamin	Dose (per kg/feed)
A	2 500 IU
D	2 400 IU
E	50 IU
K	10 g
Thiamin	1 g
Riboflavin	4 g
Pyridoxine	3 g
Pantothenic acid	20 g
Niacin	10 g
Biotin	0.150 g
Folic acid	1 g
Vitamin B_{12}	0.01 g

*Vitamin premix formula for purified diets.

0.05% calcium chloride, 0.05% magnesium sulfate, and pH 7.2). For challenge trials, *F. psychrophilum* was grown on TYES agar at 15°C for 72 h. Bacteria was harvested by gentle swabbing with a cotton applicator stick and re-suspended in 0.85% saline to an optical density (OD) of 0.2, 0.4, and 0.6 at 525 nm. Twenty rainbow trout were inoculated subcutaneously with 25 µl of each OD suspension, or 0.85% saline alone as a negative control. Plate counts were done on TYES agar at the time of subcutaneous injection of the bacteria.

A virulent strain of *Aeromonas salmonicida* was also tested in rainbow trout. Stock 1% (w/v) bacterial suspensions were prepared in 0.5% (w/v) tryptone after growth on TS agar plates. Twenty rainbow trout were inoculated intraperitoneally with 100 µl of a 1 : 10, 1 : 100, and 1 : 1 000 dilutions of the 1% stocks, or tryptone alone as a negative control. Plate counts were done on TS agar at the time of subcutaneous injection of the bacteria.

Virus infection

A virulent strain of infectious hematopoietic necrosis virus (IHNV; CSF-220-90) was propagated in EPC cells using standard methods (LaPatra et al. 1994). Twenty rainbow trout were inoculated intraperitoneally with 100 µl of a 1 : 10 000, 1 : 100 000, and 1 : 1 000 000 dilutions of the stock virus. Concentrations of viruses that were inoculated or detected in rainbow trout tissues after infection were determined by plaque assay as previously described (LaPatra et al. 1994).

Tissue sampling – At 1 and 5 day post-infection the kidneys, spleens, and livers of five fish were sampled for pathogen detection. Tissue samples were processed using standard methods (Drolet et al. 1994).

RNA isolation from tissues

Either muscle removed from the left mid section or tissue isolated from the brain, spleen, and kidney of freshly sacrificed control (sham infected) or experimental fish was immediately placed in TRIZol or frozen in liquid nitrogen in 2 ml cryo-vials or in aluminum foil. If the isolation was not performed onsite, the tissue samples were transported to the HFCES on dry ice where they were kept at −80°C until the time of isolation. Within a week the frozen tissue samples were thawed and total RNA was isolated using the TRIZol extraction method (GIBCO, Lifetechnologies, Grand Island, New York). The quantity and purity of the RNA was determined by analysis on a spectrophotometer at 260 nM.

In vitro *transcription of standard mRNAs*

PCR primers with *BamHI* 3′ and *EcoRI* 5′ restriction sites were engineered to amplify a region slightly larger than the amplicon used for sequence detection for each of the immunological sequences as standards. The PCR primers used for amplification of control standards are as follows: IL-8 (interleukin-8), accession #AJ279069 5′ forward primer 161–181 and 3′ reverse primer 314–335; CD-8 (cell surface antigen) accession #AF178054 5′forward primer 413–433 and 3′ reverse primer 504–524; C-3 (complement component C3) accession #L24433 5′ forward primer 2022–2054 and 3′ reverse primer 2136–2156R; MX-1 (interferon induced protein) accession #U30253 5′ forward primer 964–986 and 3′ reverse primer 1139–1160;

pyruvate kinase accession #AF246146 5′ forward primer 37–56 and 3′ reverse primer 323–342; ILGF-II (insulin-like growth factor-II) accession #M95184 5′ forward primer 366–386 3′ reverse primer 526–547; and the control β-actin accession #AF254414 5′ forward primer 353–373 and 3′ reverse primer 480–500. After PCR the amplified DNA was gel isolated and restriction digested with *BamHI* and *EcoRI* and cloned into a similarly digested pBluescript plasmid (Stratagene, La Jolla, CA). From these clones control standards were generated by *in vitro* transcription off the T7 priming site using the Riboprobe *in vitro* transcription system (Promega, Madison, WI). The myosin probe and primer sets have been previously described (Overturf & Hardy 2001).

Transcripts were run on formaldehyde/MOPS gels to ensure the presence of a single band corresponding to the correct size. Quantification of isolated transcripts was performed by two methods, using a spectrophotometer (Eppendorf, Westbury, NY) and fluorometer (Turner Designs, Sunnyvale, CA) with RiboGreen (Molecular Probes, Eugene, OR). These transcripts were then used as quantitative standards in the analysis of experimental samples. Standardization of samples and determination of copy number has been previously reported (Overturf & Hardy 2001, Overturf et al. 2001).

Sequence detection

After quantification, 75 ng of total isolated RNA from each sample were added to a microcentrifuge tube containing the following; 1X TaqMan buffer, 3 mM MnOAc, 0.3 mM dNTPs except dTTP, 0.6 mM dUTP, 0.3 μM forward primer, 0.3 μM reverse primer, 0.2 μM FAM-6 (6-carboxyfluorescein) labeled probe, 5 units *rTH* DNA polymerase (reverse transcriptase and DNA polymerase), and 0.5 units AmpErase UNG enzyme (to prevent reamplification cDNA amplified products) (PE Biosystems, Foster City, CA). The primers and fluorescent-labeled probes used for reverse transcription of mRNA and then in the subsequent sequence detection reaction are as follows: IL-8 forward primer 189–208, reverse primer 287–311, and the probe 209–237; CD-8 forward primer 445–469, reverse primer 498–518, and the probe 472–499; C-3 forward primer 2060–2084, reverse primer 2124–2143, and the probe 2085–2109; MX-1 forward primer 1044–1064, reverse primer 1105–1127, and the probe 1079–1096; ILGF-II forward primer 421–444, reverse primer 495–514, and the probe 461–485; pyruvate kinase forward primer 97–122, reverse primer 182–205, and the probe 164–183; and the housekeeping control probe and primer set β-actin was forward primer 372–390, reverse primer 428–451R, and the probe 399–319. All probes were labeled with the fluorescent tag 6-FAM (PE Biosystems, Foster City, CA). Fifty microliters from each sample was then pipeted into individual wells of a 96 well optical plate, capped, and placed into an ABI-Prism model 7700 sequence detector. To ensure consistency of reagent handling between each reaction all liquid manipulation was done on a Qiagen 8000 liquid handling robot (Valencia, CA). A serial dilution of six duplicate standards was run with each probe for quantification. RT-PCR conditions were as follows; 2 min @ 50°C, 30 min @ 60°C, 5 min @ 95°C and then 40 cycles of PCR consisting of 20 s @ 92°C followed by 1 min @ 62°C. The fluorescence output for each cycle of the polymerase reaction was measured and downloaded to a Macintosh G3 computer after completion of the run. Accumulated data were analyzed using the computer program Sequence Detector version 1.7 (Applied Biosystems, Foster City, CA).

Data analysis

Average fish weight gain and FCRs were calculated monthly to determine effects of dietary changes. Statistical significance was calculated for the standardized expression levels between experimental groups. Data were transformed as necessary and analyzed using Student's t-tests, correlation or statistical analysis of variance using Sigma Stat 2.0 (Jandel Scientific, San Rafael, CA). A significance level of $P < 0.05$ was used and tank mean values were considered units of observation for statistical analysis of growth performance data and expression studies.

Results

Myosin expression relative to daylength and growth

From September to March, steelhead trout held in outdoor raceways were sampled every 2 weeks for muscle myosin expression levels. A significant correlation ($r = 0.8$) was found between the level of myosin expression and day length. The lowest

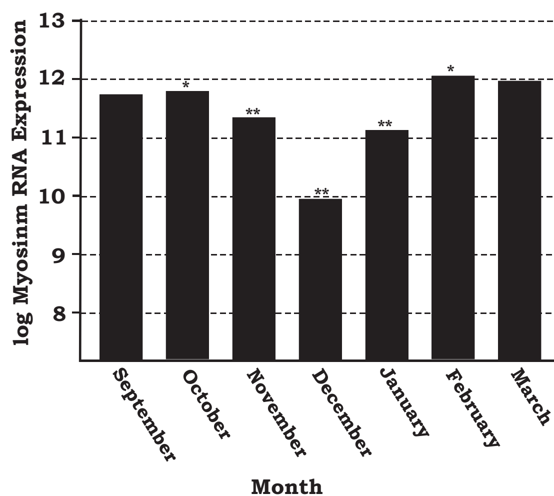

Figure 1. Analysis of myosin expression in families from four different trout strains reared under selection for weight and FCR for 7 months. *Significantly different from the next following month for October or the preceding month for February ($p < 0.05$). **Significantly different from the preceding and following month ($P < 0.05$).

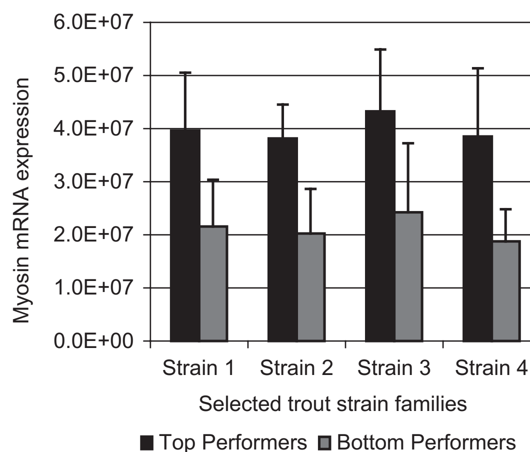

Figure 2. Myosin expression levels in muscle RNA from steelhead maintained on constant feed reared in outdoor raceways. *Significant difference between top and bottom performers for each family ($p < 0.05$).

myosin expression levels occurred during the month of December (Figure 1).

Myosin expression was measured in families from four strains of trout fed a diet containing 40% barley. Higher levels of myosin expression were observed in the top 10% from the highest performing families than from their smaller counterparts (Figure 2) ($p < 0.05$).

Expression of immunological factors

To determine the effectiveness of the probes and primer sets used to analyze health and immunological status, initial investigations were carried out on fish infected with pathogenic microorganisms. Groups of fish received three different doses of bacterial pathogens or virus; their organs (spleen, kidney, and liver) were harvested 1 and 5 days post-infection to determine the presence of pathogens and expression levels of immunofactors. *F. psychrofilum* infection resulted in elevated changes of IL-8 and CD-8 in the spleen at 5 days post-infection and a slight elevation with a low dose for C-3 at 1 day post-infection. No significant changes for C-3, MX-1, IL-8, and CD-8 were detected in the liver. At 5 days post-infection significant increases were found in the kidney for the expression of CD-8, IL-8, and C-3 in animals that received the mid-range dose of *F. psychrofilum* (data not shown).

Fish exposed to *A. salmonicida* showed elevation in the spleen for MX-1 at 1 day post-infection but not at 5 days. In the spleen there was an increase in the expression of IL-8 with dose after 1 day post-infection ($r = 0.71$). However, only at the high dose of *A. salmonicida* was the expression level significantly higher than that of control animals. This trend was also observed with the 5 days post-infected fish; unfortunately none of the high level dosed fish survived to the 5 days post-infection sampling. MX-1 levels were elevated at 1 day post-infection in the spleen but not at 5 days post-infection. In the liver, levels of C-3 and IL-8 were significantly increased in fish receiving the high dose of *A. salmonicida* at 1 day post-infection but not after 5 days, again because none of these fish survived (data not shown). The kidneys of fish infected with *A. salmonicida* showed elevated levels of IL-8 at high pathogen dose 1 day after infection and elevated levels of C-3 at 5 days after infection at all doses. However, there was no correlation between dose and expression level (Figure 3a). CD-8 levels were elevated at 1 day post-infection for the mid- and high-dosed animals, while MX-1 expression appeared to be elevated for only mid-dosed fish at this time.

Infectious hematopoietic necrosis virus infected fish showed elevated expression for CD-8 and IL-8 in the spleen at 1 day post-infection and CD-8 was correlative with pathogen dose ($r = 0.88$). At 5 days

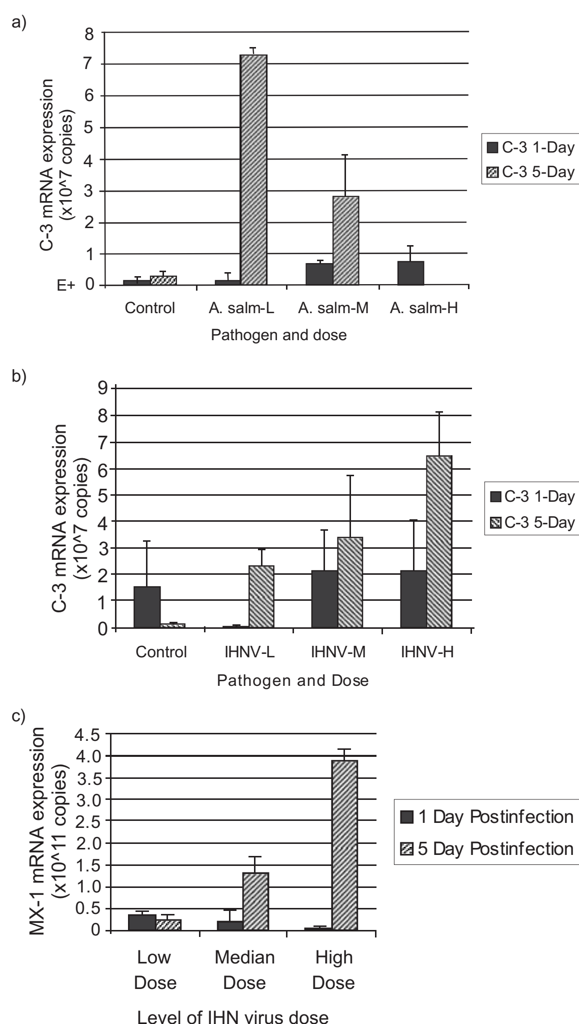

Figure 3. Expression levels of immunological factors from injected fish. (a) Expression level of C-3 from the kidney of fish infected with *A. salmonicida*. (b) Expression level of C-3 from the spleen of fish infected with *IHNV*. (c) Expression level of MX-1 from the liver of fish infected with *IHNV*.

post-infection, elevated C-3 levels correlated with dose ($r = 0.91$) (Figure 3b). In the liver C-3, CD-8, and MX-1 were all elevated in a manner that correlated with dose at 5 days post-infection, $r = 0.865$, 0.94, and 0.98, respectively. Expression of MX-1 was greatly increased at all doses over controls (Figure 3c). Within the kidney MX-1 was elevated at 5 days post-infection with a median dose of virus, and C-3 and IL-8 appeared to also be elevated at 5 days but only significantly in animals that received the lowest dose of virus.

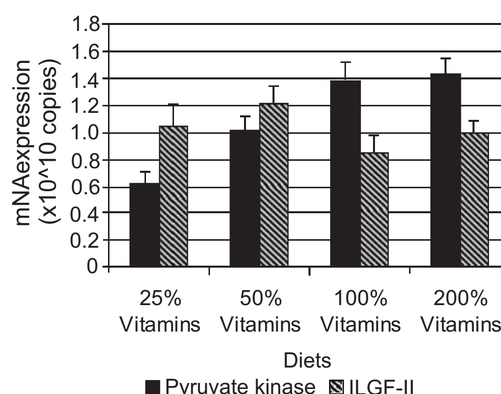

Figure 4. Expression levels of pyruvate kinase and ILGF-II in fish maintained on four diets varying in vitamin concentrations.

Expression of metabolic factors in fish on a vitamin limited diet

Groups of fish fed diets containing 200%, 100%, 50%, and 25% of the recommended vitamin levels for rainbow trout were tested for expression of pyruvate kinase and ILGF-II in their livers. The fish had been fed the diets for 12 weeks and sampled three times during the study (once every 4 weeks). Figure 4 shows the results from fish sampled at 8 and 12 weeks during the experiment. Pyruvate kinase displayed a positive correlation with dietary vitamin level ($r = 0.89$) from 25% to 100%; expression differed significantly at these values ($p < 0.05$). Increasing the vitamin level to 200% did not significantly increase ($p > 0.1$) pyruvate kinase expression. ILGF-II mRNA levels in fish were not significantly different between any of the vitamin diets.

Discussion

These results demonstrate that changes in mRNA transcript levels are measurable and correlate with alterations in the diet, environment, or health of the fish. Previous research examining myosin expression showed that it was highly correlated with fish growth and energy intake (Overturf & Hardy 2001). Federal and state hatcheries raising steelhead for mitigation are concerned with the size and health of smolts at the time of release. Strict feeding regimes are used to produce fish of a specific size for a specified release date. To gain a better understanding of feed utilization in steelhead during hatchery rearing, myosin expression

was monitored in a group of fish maintained on a constant feeding regime from young until they were released, a period of 8 months. Even though these fish were fed at a constant rate, their level of myosin expression significantly decreased with declining day length during the late fall and increased after the winter solstice. Wild fish grow when food is plentiful during the warmer months, and accumulate energy stores to survive during the winter months when food is scarce (Rand-Weaver et al. 1995). Hence, during the fall and winter months of short day length, hatchery fish with continued access to feed accumulate fat instead of producing somatic tissue. These findings suggest that it might be possible to select for fish that express myosin at continual high levels even with decreased daylength. Commercial hatcheries would benefit from fish stocks that grow more evenly or continually year-round.

Myosin expression was also examined in families of trout that had been positively and negatively selected for growth. In the early stages of our genetic selection program, families generated by 2×2 factorial crosses from four different strains were placed under selection for growth based upon weight gain and FCRs. In all strains, myosin was expressed at higher levels in fish from families that exhibited more rapid weight gain and lower FCRs. Monitoring myosin expression might prove useful in determining the potential of different diets for growth when weight differences or other physical determinants between control and experimental groups is not significant. For selection programs seeking strains with enhanced growth, incorporating selection for individuals that express higher myosin on the same diet may select for fish with a higher propensity to produce muscle instead of fat.

Knowledge of the factors involved in the expression of immunological factors can have an impact on a number of issues relating to aquaculture. These include genetic selection for disease resistance and animal health, as well as those that involve stress and effects of the environment and essential dietary nutrient levels. The findings from this study demonstrate that changes in expression of genes involved in immunological responses can be detected. In some instances, such as for IHNV exposure, gene expression levels and pathogen dosage were positively correlated for certain immune related factors. Following IHNV injection, expression of C-3, IL-8, CD-8, and MX-1 increased at 5 days post-infection with a high correlation for almost all tissues analyzed. In contrast, although exposure to bacterial pathogens showed changes in expression level in various tissues for some of the factors, increased microorganism dosage only infrequently correlated with mRNA expression. IL-8 expression in the spleen of fish infected with *A. salmonicida* is an example of a correlative dose-response that occurred after bacterial infection. Except for elevated IL-8 and CD-8 expression with *F. psychrofilum* infection, almost all changes detected with bacterial exposure occurred at 1 day post-infection. This could be due to problems with the injection, dose or condition of the bacteria and thus not a true dose-response. At low pathogen doses certain immune factors may not be activated to combat the pathogen and at higher doses the infection may overwhelm the animal and specific immune responses. Studies are necessary to determine the optimum dose and range of specific bacterial pathogens to generate elevated immune response to increased dosage challenge. Nevertheless, this work demonstrates that immune responses can be measured with gene expression using RT-PCR. Further, under conditions whereupon either a pathogen possesses a strong antigen, or proper dose of pathogen is used, gene expression shows a strong correlation with dose. As our understanding of the fish health and the immune system increases, selection of disease resistant fish based upon evaluation of immune factor expression may become important for stock enhancement programs. The goal at this facility is to attempt to generate a profile of the expression of immune- and health-related genes and use this to evaluate immunostimulatory or antinutritional stress related components in formulated feeds.

Another area of interest for testing the effects of diet on fish is how different protein sources or other dietary components affect metabolic and regulatory growth pathways. Fish were fed diets with reduced level of essential vitamins, and the expression levels of pyruvate kinase and ILGF-II were studied. The expression level of pyruvate kinase correlated well with the expression level of the enzyme, decreasing as the vitamin concentration of the diet was reduced. Increases in vitamin levels over NRC recommended levels did not significantly increase pyruvate kinase gene expression. This work was done on relatively young trout, it will be interesting to see if the same effect occurs in older larger fish. It may be that as vitamins become limiting in the diet, key factors necessary for coenzymes and other cofactors become reduced. This could either alter the kinetics of enzymes in the pathway or trigger

a negative feedback pathway for reducing the rate of transcription of genes involved directly or indirectly with the pathway. Differing vitamin concentrations did not significantly change the expression of ILGF-II. It might be more useful to measure the expression of other factors related to the growth hormone pathway, such as ILGF binding proteins that appear to be more tightly regulated in controlling growth (Moriyama et al. 2000).

Most of the recent work with real-time PCR involving fish or aquaculture has focused on disease detection and analysis of immunological factors related to disease (Overturf et al. 2001). The research presented here demonstrates how the use of real-time PCR can be expanded to evaluate change in expression as a means of quantifying a physiological change that cannot be easily measured by other methods. If expression patterns of specific genes vary in a manner that correlates with experimental treatment, the mRNA expression of that gene can be used to monitor that trait even if the product of the gene itself is not directly involved with the physical or physiological change of interest, but is only indirectly acted upon by the pathway of actual interest. It is possible to speculate about the function of the gene under study but the function cannot be completely understood until research is performed on the translated protein. Nevertheless, this approach can be effective in analyzing and tracking specific traits through populations and during selection.

We have demonstrated that expression of a metabolic enzyme correlates with dietary vitamin level consistent with previous reports showing relationships between vitamin levels and growth and health of fish (Kitamura et al. 1967, Morito et al. 1986, Halver 2002). Analyses of expression of immunological factors are likely to be beneficial in diet studies as it has been previously shown that high protein replacement with some cereal crops can actually be detrimental and generate an intestinal immune response in fish (Rumsey et al. 1995). Analysis of expression of immunological factors that are related to other health related factors, such as a cortisol, will likely become more important for understanding and selecting for growth and health of fish on new diet formulations. In fact there is evidence from other researchers documenting an association between stress resistance, immunity and growth performance (Fevolden et al. 2002). The use of RT-PCR will be of great assistance in evaluating expression changes of genes involved with these factors and determining the interactions between them.

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