AN ABSTRACT OF THE THESIS OF

Adam R. Schwindt for the degree of Master of Science in Fisheries Science presented on December 3, 2002.
Title: Stress Induced Differential Gene Expression in the Brain of Juvenile Steelhead Trout, (*Oncorhynchus mykiss*).

Abstract approved _ Redacted for Privacy _
Carl B. Schreck

Gene expression profiles of tissues and cell-lines can be powerful tools for documenting the genetic response to a particular treatment, such as stressors. However, there is a paucity of information on the genetic stress response in the brain. Therefore, we attempted to profile gene expression in the brain of juvenile steelhead trout (*Oncorhynchus mykiss*) in response to stressors commonly encountered in aquaculture settings and similar to those encountered in hydropower dam mitigation efforts.

We subjected fish to a combined out-of-water and low-water stressor totaling three hours. Plasma stress response factors indicate that fish were undergoing a physiological stress response after 3 hours of continuous stressor. We utilized suppression subtractive hybridization to identify cDNA fragments up- or down-regulated in the brain upon completion of the stressor. Forward and reverse subtractions, and sub-cloning of the purified PCR products yielded 59 clones all of which were sequenced. Sequenced cDNA fragments were subjected to BLASTn and BLASTx searches over the course of one year. Fragments fell into the

following functional categories: those associated with ATP generation, signal transduction, ion transport, translational machinery, DNA packaging and mobilization, cell structure, and cDNA fragments with cryptic function. Of the 59, 12 were selected for further analysis, and 5 were confirmed to be differentially expressed by northern hybridization. The differentially expressed genes included cytochrome b, NADH dehydrogenase subunit 2, ATPsynthase subunit 6, a cDNA fragment with unknown function, and neuron specific gene 1.

Our results present a first attempt to profile gene expression in the brain of fish and demonstrate the power of molecular tools at capturing large amounts of biological information without having to target any one particular gene. A gene expression profile of the brain consequent to stress provides a catalog of responses at a given time point. This catalog can then be used to isolate full-length cDNAs, localize mRNAs in the brain or other tissue, as probes to determine expression patterns and time courses of gene expression in other tissues, and for the quantification of cDNA molecules with real time PCR.

STRESS INDUCED DIFFERENTIAL GENE EXPRESSION IN THE BRAIN OF JUVENILE STEELHEAD TROUT, (*ONCORHYNCHUS MYKISS*)

by
Adam R. Schwindt

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Dean of the Graduate School

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

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Adam R. Schwindt, Author

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CONTRIBUTION OF AUTHORS

Drs. Alonso and Leong provided expertise in molecular biological techniques, interpretation of results from BLAST searches and northern hybridizations, and review of the manuscript. Dr. Contreras-Sánchez assisted with experimental design, protocol, and RNA extraction. Dr. Leong also provided molecular biology laboratory space.

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DEDICATION

This thesis is dedicated to my parents, Rick and Lorie Schwindt,

for instilling the value of education

in their sons at an early age.

STRESS INDUCED DIFFERENTIAL GENE EXPRESSION IN THE BRAIN OF JUVENILE STEELHEAD TROUT, *ONCORHYNCHUS MYKISS*

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Wilfrido Contreras-Sánchez, and Carl B. Schreck

ABSTRACT

We utilized suppression subtractive hybridization to identify cDNA fragments expressed in the brain of juvenile steelhead trout (*Oncorhynchus mykiss*) responding to a combined out-of-water and low-water stressor. The fish exhibited elevated plasma cortisol, glucose, and lactate after subjection to three hours of continuous stressor indicating a physiological stress response. Forward and reverse subtractions, and sub-cloning of the purified PCR products yielded 59 clones all of which were sequenced. The sequenced cDNA fragments fell into the following categories: those associated with ATP generation, signal transduction, ion transport, translational machinery, DNA packaging and mobilization, cell structure, and cDNA fragments with cryptic function. Of the 59, 12 were selected for further analysis, and five were confirmed differentially expressed by northern hybridization.

Keywords: stress; brain; gene expression; trout; suppression subtractive hybridization

INTRODUCTION

The vertebrate brain displays complex inter- and intra-cellular interactions between neuro-active substances in response to stimuli resulting in gene expression (Greengard, 2001). Stressors, factors inducing perceived fear or harm (Schreck, 1981), are one group of stimuli that affect gene expression (Gracey et al., 2001; Shi et al., 2001; Xie et al., 2002); however, details of gene expression consequent to stress in the fish brain remain limited. Previous work focused on documented stress-related genes (Ando et al., 1999; Bernier et al., 1999). However recently developed molecular tools allow the simultaneous capture of numerous genes unique to a particular treatment, providing a profile of gene expression during stress. We utilized suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) to profile gene expression in the brain associated with the stress response to address the following question: "What genes are up- and down-regulated by stress in the juvenile steelhead trout (*Oncorhynchus mykiss*) brain?"

Genes coding for the adrenocorticotropic peptides including corticotropin releasing hormone (CRH) and urotensin I (UI), as well as, arginine vasotocin (AVT) and isotocin (IST) are expressed in the hypothalamus (Lederis et al., 1994; Ando et al., 1999). Adrenocorticotropin (ACTH) stimulates cortisol release, the primary stress steroid in fishes. CRH immunoreactive neurons are found in many brain regions (Lederis et al., 1994), indicating that some CRH functions are not related to ACTH release. For example CRH injection into the chinook brain (*O.*

tshawytscha) induces locomotor activity (Clements et al., 2002). CRH expression is regulated by cortisol in the goldfish (*Carasius auratus*) brain (Bernier et al., 1999) and, may also self-regulate via the CRH receptor (Lederis et al., 1994). Furthermore, in mammalian hypothalamic neurons, CRH and vasopressin (AVT in fish) mRNA levels change through time in response to stress (Kovacs et al., 1996). These pathways, as well as those induced by the catecholamines, comprise the hypothalamo-pituitary-interrenal axis (HPI-axis) (Wendelaar Bonga, 1997) and likely induce a host of other biochemical processes leading to gene expression.

Genes not necessarily associated with stress, but essential for the cellular response to many insults, include those responsible for ATP production and signal transduction, (Gracey et al., 2001; Shi et al., 2001; Xie et al., 2002). ATP production is essential for fueling neural communication (Soengas and Aldegunde, 2002), and the stress response is a metabolically costly process (Mommsen et al., 1999). Therefore, the detection of genes associated with oxidative phosphorylation (Xie et al., 2002), and glycolysis (Gracey et al., 2001) is likely. By-products of oxidative phosphorylation include reactive oxygen species (ROS) (Mathews et al., 2000). Reactive oxygen species garner attention for their purported role in neurodegeneration and association with ß-amyloid plaques (Christen, 2000), the latter of which were reported in senescent kokanee salmon (*O. nerka kennerlyi*) brains (Maldonado et al., 2000) and in Alzheimer's disease victims. Plaque localization occurred in areas with high densities of glucocorticoid receptors

providing a possible role for stress in neuron death (Carruth et al., 2000; Maldonado et al., 2000).

Signal transduction is the neuronal response after neurotransmitters or hormones bind receptors in the target cell. One neuronal response, gene expression, is necessary for long term changes in neural circuitry such as those involved in learning and memory (Greengard, 2001). Amnesia was reported in goldfish (*Carasius aurata*) exposed to protein biosynthesis inhibitors and an isolation stressor (Laudien et al., 1986) signifying the role of stress and protein expression in fish memory formation. Gene expression is controlled, in part, by protein kinases and protein phosphatases activated by second messengers (Greengard, 2001), and over time, aberrations in kinase or phosphatase activity are linked with neurodegeneration (Wagey and Krieger, 1998). The goal for this study was to identify and confirm differential gene expression consequent to stress in the fish brain. This profile of stress-induced gene expression represents the cumulative genetic response of the brain, and may provide clues about cognitive capacity or neurodegeneration resulting from stress.

MATERIALS AND METHODS

We obtained steelhead trout (*Oncorhynchus mykiss*) parr from the Oregon Department of Fish and Wildlife's Alsea River Hatchery, Alsea, OR, on 14-July-

2000. We reared fish in approximately 1.0 m diameter tanks with ~12.8 °C aerated, pathogen free well-water in a flow-through design. Fish experienced a natural photoperiod in our rearing facility at Oregon State University's (OSU) Fish Performance and Genetics Laboratory, Corvallis, OR, in accordance with the OSU Institutional Animal Care and Use Committee guidelines. We fed the fish Bio-Oregon™ semi-moist pellet at ~2% body-weight/day. Prior to the onset of experiments we withheld food for 48 to 72 hours to minimize stomach contents and the potential stress associated with feeding. At the time of the experiment, fork length averaged 18.20 cm (range 13.40-20.30 cm) and mass averaged 66.80 g (range 24.60-91.50 g).

Protocol: experiment 1

On 15-January-2001, at 08:00 we captured 30 fish by dip-net from a single stock tank (0-hour) and euthanized them by anesthetic overdose, a process shown by Barton et al. (1980), to have no effect on plasma cortisol. From the same stock tank as the 0-hour, we captured three groups of fish (9 or 10 fish per group), suspended them in air for 30 seconds, and submersed them for three hours in three tanks with low-water (3-hour). We defined low-water as submersion deep enough to cover the dorsal fin in 0.5 m diameter tanks. This treatment induces a plasma cortisol response in salmonids (Barton et al., 1985; Patino et al., 1987). Upon the

completion of the stressor, we euthanized the treatment fish using the same protocol as the 0-hour group.

At the time of the experiment, there were no papers reporting a time-course for gene expression in the fish brain. However, hypoxic rats displayed immediate early gene (IEG) induction in the forebrain from one to three hours (Gubits et al., 1993). IEGs represent neural activation in response to many factors including stressors (Bozas et al., 1997). Therefore, we erred on the latter portion of this time-frame given that trout are poikilothermic and physiological processes are governed by the Q_{10}-Effect. Also Ando et al. (1999) detected CRH mRNA after three hours of stressing rainbow trout (nonanadromous *O. mykiss*).

Tissue collection and processing

We sampled blood by vacutainer from the dorsal vessel and placed the fish on ice. Within minutes of bleeding we removed brains, and froze them in RNase free cryopreservation vials with liquid N_2. Blood samples were centrifuged and plasma stored at -80 °C. Total RNA was extracted from brains within five to seven hours following euthanasia; however, RNA from one 3-hour triplicate sample remained on liquid N_2 overnight and was not extracted until the following day. We kept total RNA from each triplicate separate pending the outcome of cortisol analyses. After demonstration that the triplicates reacted similarly to the stressor,

we pooled total RNA. Therefore, upon polyA$^+$-RNA selection, up to 30 fish were represented in an effort to account for individual variability in gene expression. We performed a preliminary experiment to determine the quantity of RNA and polyA$^+$-RNA available per brain to arrive at a sample number sufficient for the completion of SSH, northern hybridization, and to allow for a margin of error.

Plasma cortisol was used as an indicator of the primary stress response, while glucose and lactate were used as indicators of the secondary response (Mazeaud et al., 1977). Plasma cortisol was measured by radioimmunoassay similar to Redding et al. (1984). Plasma glucose was assayed as described by Wedemeyer and Yasutake (1989) and plasma lactate was quantified using the method reported by Passoneau (1974).

We extracted total RNA with RNA Stat-60™ RNA isolation reagent (TEL-TEST, Inc., Friendswood, TX) according to manufacturer's protocol and stored the precipitated RNA at -80 °C. This method is based on the acid guanidinium thiocyanate-phenol-chloroform extraction reported by Chomczynski and Sacchi (1987). We pooled total RNA from each triplicate sample prior to polyA$^+$-selection using either the Oligotex™ mRNA spin-columns (Qiagen, Inc. Valencia, CA) or the PolyATtract® mRNA isolation system (Promega Corporation, Madison, WI). Both kits yielded high-quality mRNA (1-4 kb) upon electrophoretic separation (1.2% agarose/0.5μg ethidium bromide buffered with 1X TAE). Immediately after polyA$^+$-selection, cDNAs were reverse transcribed using the CLONTECH PCR-select™ cDNA Subtraction Kit (CLONTECH Laboratories, Palo Alto, CA)

according to manufacturer's protocol. After construction of double-stranded cDNA, we stored the samples at -20 °C until proceeding with SSH.

Suppression subtractive hybridization

We utilized SSH to identify cDNA fragments up or down-regulated during stress by performing forward and reverse subtractions on mRNA isolated from the 0-hour and 3-hour fish. The forward subtraction identifies cDNAs differentially expressed in the stressed fish while the reverse subtraction identifies cDNAs differentially expressed in the control fish. The cDNA fragments present in the control library but not in the stressed library were interpreted as genes putatively down-regulated by stress. The cDNA fragments present in the stressed library but not in the control library were interpreted as genes putatively up-regulated by stress. Our rationale for this interpretation is based on the fact that all fish were obtained from the same broodstock and originated from one rearing tank. Therefore, prior to the initiation of the experiment, the potential for gene expression was identical between 0-hour and 3-hour groups.

According to protocol, cDNA from the 3-hour group (tester for the forward subtraction / driver for the reverse subtraction) was *Rsa* I digested, divided into two separate pools, and ligated to one of two adapter sequences (tester only). Identical side-by-side steps were performed for the 0-hour group (driver for the forward

subtraction / tester for the reverse subtraction). For the forward and reverse subtractions, tester and driver populations were mixed and underwent two hybridizations completing the subtraction of genes common to tester and driver. Then, primary (27 cycles: 94 °C 30 seconds, 66 °C 30 seconds, 72 °C 1.5 minutes) and nested PCR steps (12 cycles: 94 °C 30 seconds, 68 °C 30 seconds, 72 °C 1.5 minutes) (Advantage® cDNA polymerase mix, CLONTECH laboratories) were performed amplifying cDNAs unique to the tester populations while suppressing the cDNAs common to both populations.

We gel-purified (2% agarose/0.5μg ethidium bromide buffered with 1X TAE) nested PCR products (from above), extracted the cDNA using the QIAquick® (Qiagen, Inc) gel extraction kit, and sub-cloned the PCR products with the TOPO TA Cloning® kit using the pCR®2.1-TOPO® vector (Invitrogen Corporation, Carlsbad, CA). Briefly, we transfected the PCR products according to protocol, but extended the reaction time to 25 minutes. We transformed our vectors into the One Shot® (Invitrogen Corporation) chemically competent *E. coli* according to protocol, but extended the incubation time on ice to 20 minutes. We plated cells on prewarmed LB-ampicillin (50 μg/ml) agar (LB-amp) plates and grew colonies overnight. Putative transformants (blue/white screening) were subjected to PCR (94 °C 1 minute jump start followed by 27 cycles of 94 °C 30 seconds; 55°C 1 minute; 72 °C 3 minutes) (2X PCR Master Mix, Fermentas, Inc. Hanover, MD) using M13 forward and reverse primers. Transformants were re-grown and archived on LB-amp plates. PCR products were gel-purified and extracted as described previously.

Purified PCR products were sequenced (Davis Sequencing, LLC, Davis, California) by AP Biotech® DYEnamic ET Terminator cycle sequencing chemistry using ABI PRISM® 3700 DNA sequencers. Upon reception of sequences, and after BLAST queries, positive transformants from archive plates were re-grown in liquid LB-amp media, infused with 40% glycerol, and stored at -80 °C completing the construction of the putative subtracted libraries.

Using default parameters, we queried sequenced PCR products with the BLASTN (nucleotide) and BLASTX (6-frame translated nucleotide) algorithms (Altschul et al., 1990) on three occasions over the course of one year to account for new submissions. E-values <0.01 were used to initially screen for significant identities. The identities were then subjected to the following criteria to determine the likelihood that the identified gene or peptide matched our PCR product: 1) significant identities resulting from BLASTN and BLASTX queries in one or more species were considered *likely* candidates for the identified gene and protein; 2) significant identities in one or more species resulting from BLASTX queries but no corresponding match in the nucleotide database were *moderate* candidates for the identified protein; 3) identities resulting from BLASTN queries, in one or more species, and residing in the mRNA or coding sequence (CDS) of the published gene were *possible* candidates for the identified gene; 4) and all other identities were considered *unlikely* candidates, including matches with 5' and 3' untranslated regions, and other untranslated regions such as introns.

We recognize that identities considered to be *unlikely* candidates may be undescribed genes or represent mRNA that was captured during post-transcriptional modifications. Alternatively, the cDNAs generated by SSH are too short to yield significant identity unless the gene is well characterized in the lower vertebrates (Gracey et al., 2001). Futhermore, without the complete molecular characterization and expression of each gene and protein, we cannot definitively corroborate the results of the BLAST searches. Despite these limitations, our criteria for determining the likelihood that significant identities are indeed the gene or peptide returned from database searches provides a thorough screening of the sequences. Furthermore, our results present an initial step for capturing differential gene expression in the fish brain consequent to stress by creating subtracted cDNA libraries.

Protocol: experiment 2

We obtained steelhead trout parr from the Alsea River hatchery in 08-2001, and reared the fish as in the first experiment. These fish were the same stock as the first experiment but were derived from the subsequent brood year. We conducted the second experiment on 24-February-2002 at 08:00 using the same treatment as the first experiment; however this experiment was not run in triplicate and consisted of ten fish per group. We did not triplicate tanks to limit killing additional

animals and the protocol was validated in the first experiment. At the time of the experiment, fork length averaged 22.07 cm (range 19.80-25.60 cm) and mass averaged 124.30 g (range 83.80-198.00 g). Upon completion of the experiment, we collected blood, then removed and froze the brains in liquid N_2. Total RNA was extracted within five to seven hours of euthanasia, plasma stress indices assayed, and mRNA isolated and subjected to northern hybridization.

Northern hybridization: experiments 1 & 2

To confirm (or refute) the purported differential expression of the subtracted libraries we performed northern hybridization on mRNA from the first and second experiments. One (experiment one) or two μg (experiment two) of mRNA were size-fractionated at 80 V for 1.5 hours in denaturing formaldehyde 1% agarose according to standard procedures (Sambrook et al., 1989). We used twice as much mRNA for experiment two to increase the chance of visualization of rare transcripts. The mRNA for experiment one was limiting hence the use of one μg. Prior to RNA transfer, gels and nylon membranes were washed in DEPC-H_2O and 20X SSC. Messenger RNA was transferred to nylon membranes (Gene Screen Plus NEN® Research Products, Boston, MA) by upward capillary method overnight (Sambrook et al., 1989). The membranes were UV cross-linked at 1200 μJ with a UV Stratalinker (Stratagene, Inc. La Jolla, CA) and immediately subjected to

northern hybridization, or stored at room temperature (22 °C) under vacuum wrapped in aluminum foil for up to seven days. Northern analysis was performed with the North2South® Direct HRP Labeling and Detection Kit (Pierce Endogen, Rockford, IL). Probes, purified PCR products of the subtracted libraries, were hybridized to the membrane for three hours. Membranes were exposed to film for five seconds to 20 minutes to obtain the desired exposure. The mRNA sizes were determined by establishing a linear relationship between the $\log_e$ transformed distance migrated of standards and the known size of the standards [6.5 kb, 4.9 kb, 3.6 kb, 2.6 kb, 1.9 kb, 1.4 kb, 955 bp, 623 bp, and 281 bp (Promega, Corp. Madison, WI)] (Sambrook et al., 1989). The mRNA sizes were then predicted based on distance migrated against known marker RNA.
(Sambrook et al., 1989). Probes were stripped up to three times in 0.5% SDS at 60 °C for 1 hour, transferred to 0.1% SDS at room temperature for 10 minutes, and stored overnight at 4 °C in 2X SSC/0.1% SDS. We tested the efficacy of the stripping protocol by immersing stripped membranes in the chemiluminescent solution provided in the kit and exposing the membranes to film for 30 minutes after which no flourescence was visualized.

We used ImageQuaNT™ software (Molecular Dynamics, Piscataway, NJ) to determine pixel density of individual bands using the area quantification method. Over-expression was determined by dividing the densities of the 3-hour group by the densities of the 0-hour group for the putative up-regulated genes to obtain X-

fold expression. The reverse procedure was performed for the putative down-regulated genes.

Statistical analyses

To test for significant differences in plasma stress indices within the 0- and 3-hour triplicate tanks from the first experiment, one-way ANOVA and the Kruskal-Wallis statistic were used. The Kruskal-Wallis statistic was used for groups with unequal sample size and variance. After pooling the data, means were compared by the Mann-Whitney test suitable for non-parametric data. The Mann-Whitney test was also used for experiment two data. Statistical significance was set at 0.05 and p-values are two-tailed.

RESULTS

We pooled data from the triplicate tanks in the first experiment upon demonstration of no statistical difference between triplicates ($p>0.05$) for plasma cortisol, glucose, and lactate. In the first experiment, pooled plasma cortisol, glucose, and lactate were significantly elevated at three hours compared to the 0-hour fish (Figure 1). In the second experiment, mean plasma cortisol and glucose

from the 3-hour fish differed significantly from the 0-hour (Figure 2a, b). However, two 0-hour fish displayed cortisol values greater then 70 ng/ml signifying a plasma stress response. Mean plasma lactate was not different between the 0-hour and 3-hour fish (Figure 2c).

Figure 1: Box and Whisker Plots of Plasma Stress Indicators from Experiment 1. Boxes represent middle 50% of data, the upper and lower whiskers are > ~25% and < ~25% of the data respectively, the line through the box is the median, and individual points are extreme values. The Mann-Whitney test was used to compare means. Data are mean ± S.E.M. followed by sample size. Zero hour plasma cortisol (12.88 ng/ml ± 2.19; N=30) was different from three hour (118.3 ng/ml ± 7.14; N=28). Plasma glucose zero hour (77.46 mg/dl ± 1.55; N=30) was different from three hour (246.80 mg/dl ± 8.33;N=28).Plasma lactate zero hour (39.39 mg/dl ± 1.98; N=30) was different from three hour (103.40 mg/dl ± 7.09; N=28). *, $p<0.0001$.

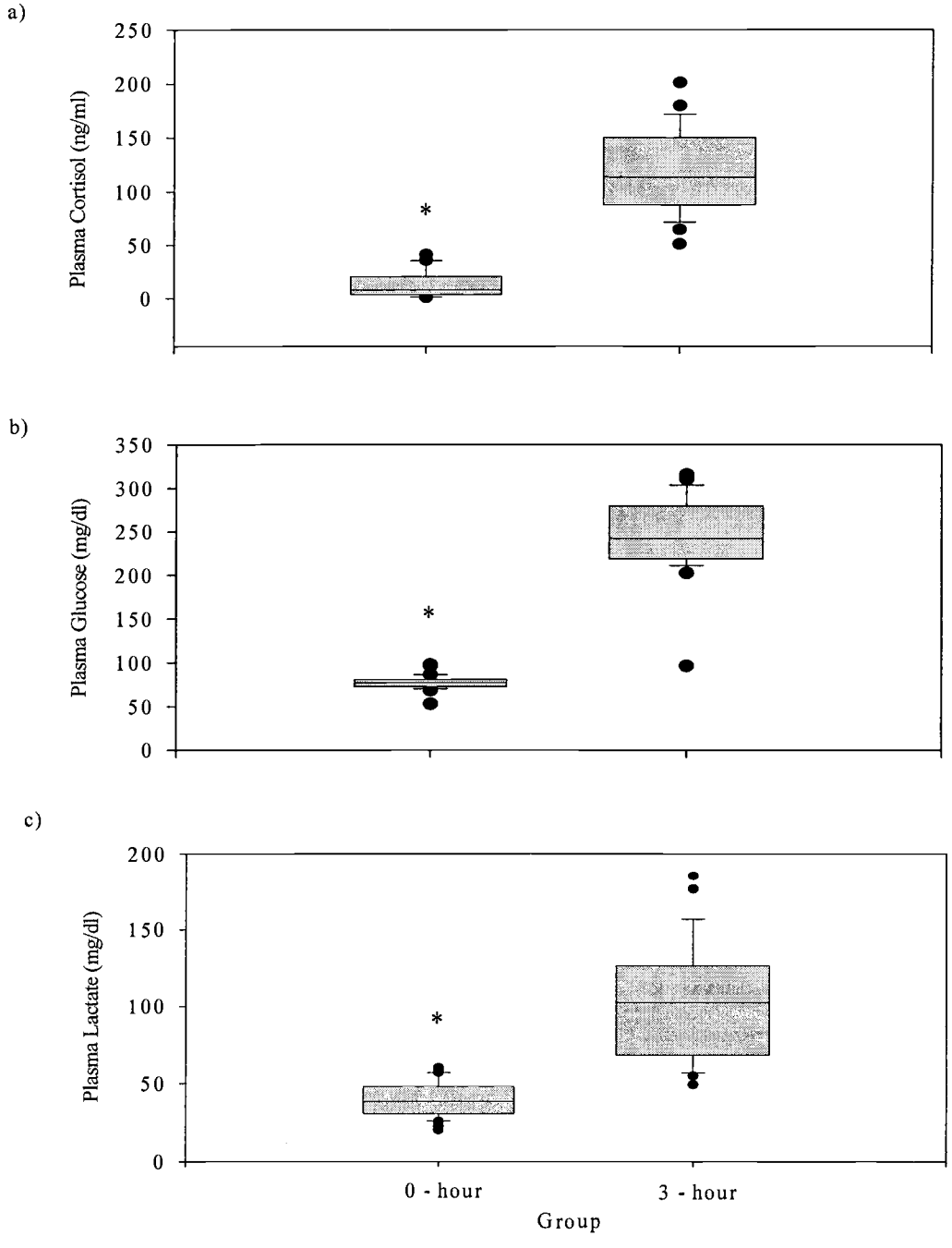


Figure 1: Box and Whisker Plots of Stress Indicators from Experiment 1.

Figure 2: Box and Whisker Plots of Plasma Stress Indicators from Experiment 2. Boxes represent middle 50% of data, the upper and lower whiskers are > ~25% and < ~25% of the data respectively, the line through the box is the median, and individual points are extreme values. The Mann-Whitney test was used to compare means. Data are mean ± S.E.M. followed by sample size. Zero hour cortisol (18.68 ng/ml ± 10.58; N=10) was different from three hour (156.40 ng/ml ± 9.74; N=10). Zero hour glucose (82.70 ng/ml ± 1.93 N=10) was different from three hour (236.10 ng/ml ± 9.19 N=10). Zero hour lactate (18.64 mg/dl ± 0.67 N=10) was not different from three hour (44.49 mg/dl ± 9.97 N=10). *, $p<0.0001$.

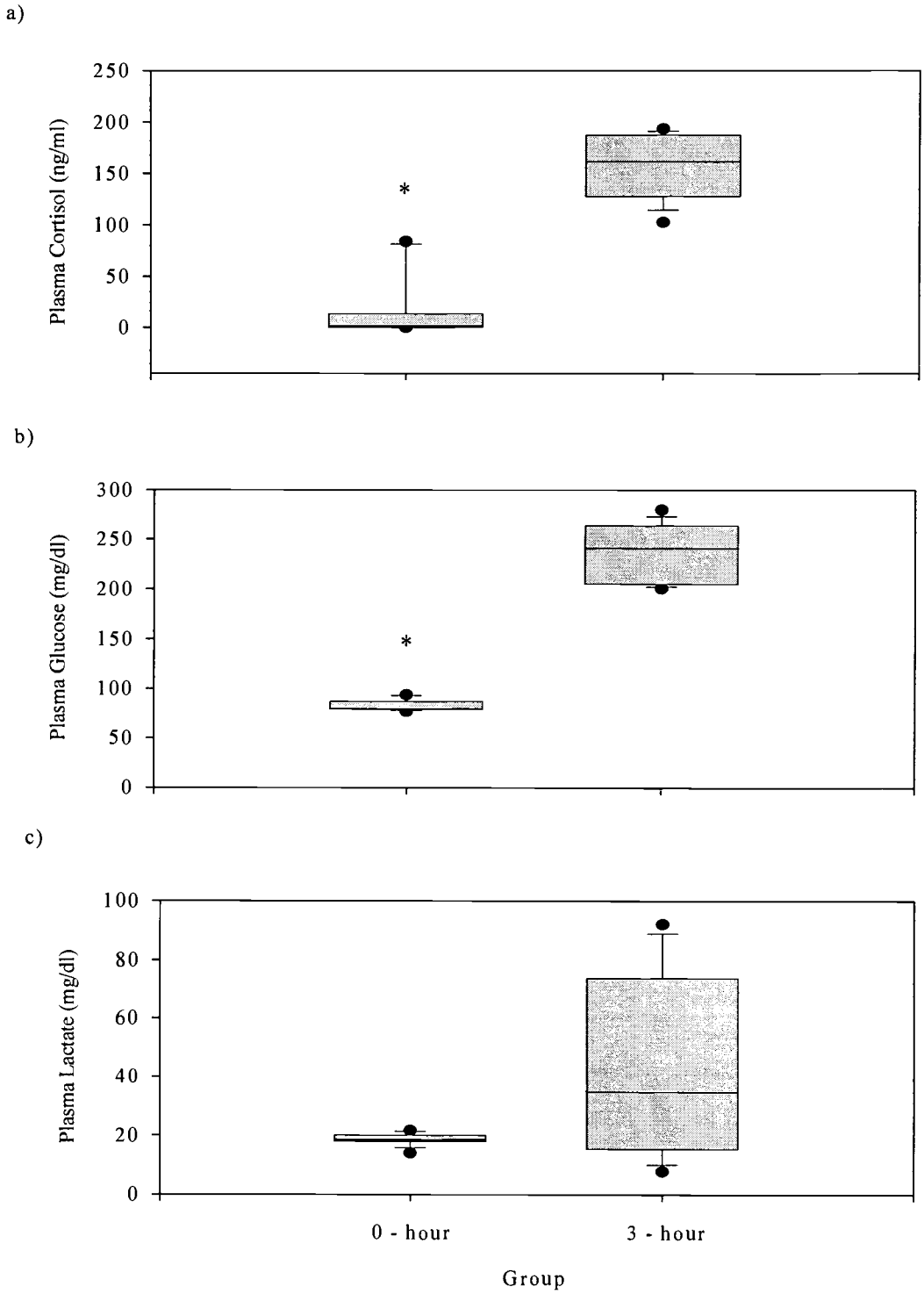


Figure 2: Box and Whisker Plots of Plasma Stress Indicators from Experiment 2.

Suppression subtractive hybridization and sequence analysis

Screening of the putative subtracted libraries yielded 59 clones, of which 54 were positive for sub-cloned PCR products. Twenty four clones were putatively up-regulated and 30 were putatively down-regulated. Sequenced clones were examined, poor sequence trimmed, and vector sequences removed using the BioEdit sequence alignment editor available at: http://www.mbio.ncsu.edu/BioEdit/bioedit.html. Fragment length ranged from 95-630 bp, with most of the sequences in 350-550 bp range (Table 1).

Although we obtained purified PCR products greater then 600 bp, as evidenced by size fractionation in agarose gel, sequencing of the entire product was incomplete due likely to the (in)efficiency of single-pass sequencing. Five of the clones yielded very short sequences (<20 bp) and were likely false positives for transfection. All told, clones with vector sequence at the 5' and 3' ends likely represent the entire length of the inserted PCR product, while clones with vector sequence only at the 5' end were partially sequenced, for whatever reason.

E-values (the probability of a chance identity) ranged from insignificant ($E > 10^{-2}$; 17 clones) to moderately significant ($E = 10^{-3} - 10^{-5}$; 8 clones) to highly significant ($E < 10^{-6}$; 24 clones). Five clones returned no significant identity using default parameters. The cDNA fragments (clones) returning significant identity to known genes or proteins fall into the following functional categories: a) ATP generation; b) signal transduction; c) ion transport; d) translational machinery; e)

DNA packaging and mobilization; f) cell structure, and g) cDNAs with cryptic function (Table 1). Additionally, clone 12R0A showed no significant identity with any GenBank sequence using the default parameters, but is included in the Table because northern hybridization confirmed that this clone was differentially expressed. Two clones returned significant identity to segments of human chromosomes one and two, and five clones returned significant identity with repetitive elements, such as microsatellite DNA (Appendix Table 1). Clones, including those showing insignificant identity or no significant identity, are shown in Appendix Table 1.

On two occasions, the initial search returned different identities than subsequent searches. Clones 17F3A and 24F3A showed significant identity with insulin-like growth factor-I on the initial search but inducible nitric oxide synthase (iNOS) showed greater identity on subsequent searches, following the recent characterization of iNOS in rainbow trout (Wang et al., 2001). In either case, the region of identity between the reported gene and our gene fragment did not reside in the coding region, and were therefore considered *unlikely* matches despite significant identity. For iNOS, significant identity resided in the 3' untranslated region, possibly resulting from capture during post-transcriptional modification. Clone 19F3A showed no significant identity on the initial search but moderate significance to mucin-I on subsequent queries using BLASTX and was therefore considered a *moderate* match (Table 1).

Table 1: cDNA Fragments Generated by SSH Showing Identity to Known Genes and Peptides. Abbreviations: Reg. column: Reg. = Regulation; u = up-regulated; d = down-regulated. Id (%) / E column: Id (%) percent identity to identified gene and/or peptide. E = E - value (probability of chance identity, similar to p-values). The "Likelihood" column indicates that the named gene or peptide represents a true match based on the criteria described in the "Materials and Methods" section. The papers cited in the "References" column either characterized the gene and/or describe the function of the protein. N/A indicates unknown parameter.

Reg.	Identification / Accession #	Species (* teleost)	Function	Clone (bp)	Id (%) / E	Likelihood	References
cDNA fragments related to ATP generation							
u	Cytochrome b gene / AY150301	*Oncorhynchus mykiss* *	Electron acceptor in the	1F3A (327)	97 / e-158	Likely	Benzi et al., 1991
	Cytochrome b protein	*Oncorhynchus mykiss* *	respiratory chain		89 / 5e-47		Benzi et al., 1992
u	NADH DH subunit 2 / AY150300	*Scomberomorus brasiliensis* *	Donates electrons	30F3A (416)	84 / 1e-17	Likely	Sriram et al., 1997
	NADH DH subunit 2 protein	*Oncorhynchus mykiss* *	to coenzyme Q		90 / 9e-61		
d	ATP synthase subunit 6 / AY150302	*Oncorhynchus mykiss* *	F1/F0 ATP	8R0A (598)	90 / 3e-52	Likely	Mathews et al., 2000
	ATP synthase subunit 6 protein	*Oncorhynchus mykiss* *	synthase complex		77 / 1e-11		
d	Cytosolic Malate DH mRNA / AF543536	*Syphraena idiastes* *	Interconverts malate	34R0A (416)	89 / 5e-87	Likely	Lin et al., 2002
	Cytosolic Malate DH protein	*Syphraena idiastes* *	and oxaloacetate		97 / 3e-44		Mathews et al., 2000
cDNA fragments related to signal transduction							
u	iNOS/NOS2 gene / BU993921	*Oncorhynchus mykiss* *	Chemical messenger	17F3A (576)	87 / 5e-35	Unlikely	Wang et al., 2001
u	iNOS/NOS2 gene / BU993922	*Oncorhynchus mykiss* *	Chemical messenger	24F3A (470)	87 / 4e-35	Unlikely	Wang et al., 2001
u	Mucin – 1 / CA502727	*Homo sapiens*	Tumor suppression	19F3A (336)	44 / 6e-4	Moderate	Unpublished
d	Phytanoyl-CoA hydroxylase mRNA / AF543537	*Homo sapiens*	Cerebellar ataxia	31R0A (95)	91 / 5e-9	Likely	Lee et al., 2000
	Phytanoyl-CoA hydroxylase interacting protein	*Homo sapiens*	and nerve deafness		89 / 5e-3		
d	Atrial natriuretic peptide mRNA / BU993923	*Salmo salar* *	Vasodilation	27R0A (577)	86 / 2e-15	Possible	Majalahti et al., 2000
d	Neuron specific gene 1 / AF543538	*Mus musculus*	Receptor cycling in	14R0A (581)	89 / 3e-9	Likely	Saberan et al., 1998
	Neuron specific protein 1	*Homo sapiens*	neuroendocrine tissue		57 / 7e-12		Steiner et al., 2002
d	Tyrosine phosphatase mRNA / BU993924	*Macaca fascicularis*	Cell differentiation	32R0A (401)	96 / 6e-3	Possible	Diamond et al., 1994

Table 1: (Continued)

Reg.	Identification / Accession #	Species	Function	Clone (bp)	Id (%) / E	Likelihood	References
cDNA fragments associated with ion transport							
u	V-type ATPase B subunit mRNA / BU993925	*Oncorhynchus mykiss* *	$Na^+/K^+/H^+$ exchange	26F3A (197)	99 / 1e-107	Possible	Perry et al., 2000
d	Na/K ATPase A1B1 subunit mRNA / BU993926	*Danio rerio* *	Age related isoform	7R0A (357)	81 / 9e-15	Possible	Unpublished
cDNA fragments associated with translational machinery							
d	40S ribosomal protein S7 mRNA / AF543540	*Ictalurus punctatus* *	Ribosome subunit	4R0A (132)	87 / 5e-29	Likely	Unpublished
	40S ribosomal protein S7 protein	*Ictalurus punctatus* *	assembly		95 / 1e-17		Unpublished
d	40S ribosomal protein S5 mRNA / AF543539	*Ictalurus punctatus* *	Ribosome subunit	11R0A (496)	86 / 1e-99	Likely	Unpublished
	40S ribosomal protein S5 protein	*Ictalurus punctatus* *	assembly		99 / 1e-63		Unpublished
d	18S rRNA gene / BU993927	*Oncorhynchus mykiss* *	Ribosome component	24R0A (429)	99 / 0.00	Possible	Unpublished
cDNA fragments associated with DNA packaging and mobilization							
u	Tc1-like transposon gene / AF545041	*Chionodraco hamatus* *	Gene without fixed	23F3A (595)	82 / 7e-19	Likely	Lam et al., 1996
	Transposase protein	*Pleuronectes platessa* *	location in genome		42 / 5e-34		
d	Histone H1-0 mRNA / AY150299	*Rattus norvegicus*	DNA binding protein;	29R0A (585)	91 / 2e-3	Likely	Martinez et al., 1995
	Histone H1-0 protein	*Xenopus laevis*	Chromatin precursor		64 / 1e-42		Mathews et al., 2000
d	Chromosomal HMG-14 mRNA / BU993928	*Gallus gallus*	Like housekeeping gene	36R0A (603)	92 / 1e-14	Possible	Landsman et al., 1989

Table 1: (Continued)

cDNA fragments associated with cell structure							
Reg.	Identification / Accession #	Species	Function	Clone (bp)	Id (%) / E	Likelihood	References
d	ß - actin mRNA / AF550583	*Oncorhynchus mykiss* *	Cytoskeleton	33R0A (168)	96 / 7e-87	Likely	Unpublished
	ß - actin protein	*Oncorhynchus mykiss* *	component		100 / 2e-26		
d	α - tubulin mRNA / AY150303	*Oncorhynchus keta* *	Cytoskeleton	35R0A (578)	91 / 0.00	Likely	Coe et al., 1992
	α - tubulin protein	*Oncorhynchus keta* *	component		96 / 1e-107		
cDNA fragments with cryptic function							
u	ORF Mus musculus (251aa) / CA502726	*Mus musculus*	N/A	10F3A (428)	28 / 9e-3	Moderate	Unpublished
d	Stress-related mRNA / BU993929	*Oncorhynchus mykiss*	N/A	12R0A (546)	No identity	N/A	Unpublished

Northern analysis

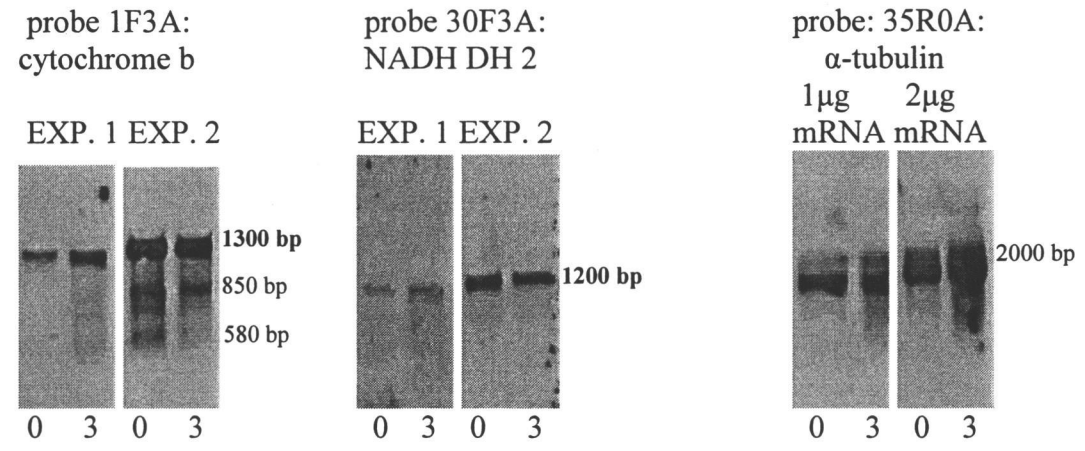
Twelve clones were chosen for subsequent analysis by northern hybridization. Seven clones yielded no signal. Four clones were confirmed differentially expressed from the first experiment while one was confirmed differentially expressed from the second (Figure 3). Probing mRNA from the first experiment, cytochrome b (cytb) (clone 1F3A) and NADH dehydrogenase subunit 2 (NADH DH2) (clone 30F3A) were confirmed up-regulated 3.4X and 1.6X respectively (Figure 3a). Figure 3b is a blot of probe α-tubulin (35R0A) shown for reference. From the second experiment, ATP synthase 6 (ATP6) (clone 8R0A) was confirmed down-regulated 1.2X (Figure 3c). Clones 12R0A (unknown cDNA) and 14R0A [neuron specific gene 1 (NSG1)] were confirmed down-regulated 2.4X and 1.6X respectively from the first experiment (Figure 3c). In no instance were we able to confirm differential regulation of the same gene in both experiments.

The clones confirmed differentially expressed by northern hybridization were also compared across various taxa using ClustalW multiple alignment of nucleotide and deduced amino acid sequences. ClustalW alignments are based on an algorithm similar to that employed in the BLAST searches. The result is a consensus sequence as well as color shading of similar regions. The results from these manipulations are presented in Appendix 2 Figures 1-10.

Figure 3: Northern Hybridization of 1 μg or 2 μg of mRNA Isolated from the First and Second Experiments. Probes are HRP labeled purified secondary PCR products generated by SSH (see Materials and Methods). Blots were stripped and re-probed up to 3 times. The blot on the left of each pair of blots is from the first experiment, and the blot on the right is from the second. b) is blot with probe 35R0A believed to be constitutively expressed shown for reference. To determine fold induction/repression, 3:0 and 0:3 ratios of pixel density were calculated for the up- and down-regulated genes respectively. In a) and c) the band in bold is closest to the published mRNA size and was used for the comparison. For probe 12R0A the bands giving the most intense signal were compared and were likely the most abundant mRNA species. mRNA sizes were determined by establishing a linear relationship between the $\log_e$ transformed distance migrated of standards and the known size of the standards [6.5 kb, 4.9 kb, 3.6 kb, 2.6 kb, 1.9 kb, 1.4 kb, 955 bp, 623 bp, and 281 bp (Promega, Corp. Madison, WI)] (Sambrook et al., 1989). The mRNA sizes were then predicted based on distance migrated against known marker RNA.

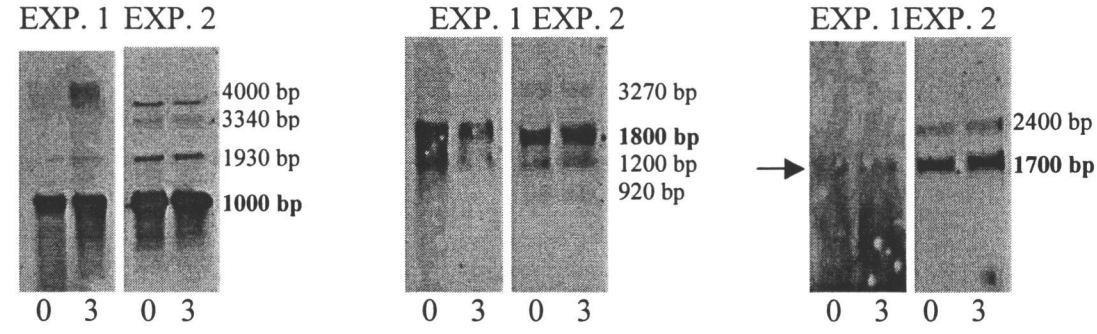
a) Up-regulated cDNAs

b) Equal mRNA loading



probe/ clone	band (bp)	EXP. 1 3:0	EXP. 2 3:0
1F3A	1300	**3.4X**	0.96X
30F3A	1200	**1.6X**	0.80X

c) Down-regulated cDNAs



probe/ clone	band (bp)	EXP. 1 0:3	EXP. 2 0:3
8R0A	1000	0.64X	**1.2X**
12R0A	1800	**2.4X**	0.71X
14R0A	1700	**1.6X**	0.86X

Figure 3: Northern Hybridization of 1 μg or 2 μg of mRNA Isolated from the First and Second Experiments.

DISCUSSION

Sub-cloning of PCR products yielded 54 cDNAs from the brains of stressed and unstressed fish, 22 of which showed identity with known genes or proteins. Northern hybridization confirmed the up or down-regulation of five cDNA fragments demonstrating the effect of stress on genes associated with ATP generation, signal transduction, and a cDNA fragment with unknown function. The varying degrees of identity between sequences analyzed by the ClustalW program indicate the relative genetic homology across vertebrate taxa. Unfortunately we were unable to repeat patterns of gene expression in another cohort due possibly to differences in developmental state or other variation between groups. There is also the possibility of stressed individuals in the control group from the second experiment.

The 22 clones returning insignificant or no significant identity were most likely due to the fact that SSH generates short cDNA fragments (sequence data not shown), and unless the genes are well characterized in lower vertebrates, or highly conserved, 350 to 550 bp segments are too short to return significant identities (Gracey et al., 2001). The fact that three clones returned different identities on the first and subsequent BLAST queries demonstrates the importance of conducting numerous BLAST searches over time.

Genes associated with ATP generation

SSH yielded subtracted cDNA fragments that identifed with mitochondrial genes in the respiratory chain (RC), including two confirmed up-regulated genes: cytochrome b (clone 1F3A) and NADH DH 2 (ubiquinone oxidoreductase) (clone 30F3A); and, one confirmed down-regulated mitochondrial gene, ATP synthase 6 (clone 8R0A). The mitochondrial genome consists of six groups of genes: 1) rRNA genes; 2) complex I genes (NADH dehydrogenase); 3) complex III genes (oxidoreductases); 4) complex IV genes (oxidases); 5) complex V genes (ATP synthase); and 6) tRNA genes (Mathews et al., 2000; Calabrese et al., 2001). All complex II genes and several genes of the other complexes are nuclear in origin (Calabrese et al., 2001).

Cytochrome b, part of complex III (Calabrese et al., 2001) and an electron acceptor in the RC (Mathews et al., 2000), was upregulated in 8-72 hour hypoxia exposed goby (*Gillichthys mirabilis*) muscle but not liver (Gracey et al., 2001) demonstrating tissue specific activity of the gene. Our finding of 3.4X upregulation of cytochrome b at three hours of stress is the earliest known induction in the fish brain. After 18 hours of peroxidative stress in the forebrain of rats, (Benzi et al., 1991) reported slight but significant increases in cytochrome b populations. Explanation for increased production of electron acceptors such as cytochrome b likely points to altered redox balance resulting in incomplete reduction of O_2 to H_2O and subsequent generation of ROS (Benzi et al., 1991; Benzi et al., 1992). In

times of high electron flux through the RC that may occur as ATP demand increases, disruption of redox balance ensues. For example, electrically stimulated bullfrog (*Rana catesbeiana*) brains yielded marked increases in reduced cytochrome b as measured by spectrophotometry due, presumably, to the increased activation of NA^+/K^+ ATPase pumps, or the persistence of excitatory neurotransmitters in neurons and glial cells (Kanno et al., 1985). However, Wagner et al., (1990) reported no change in cytochrome b redox states in anoxic cats (*Felis domesticus*).

Redox balance can be altered manually by disrupting the various steps in the RC. Using cytochrome oxidase inhibitors in rat brains, Piantadosi (1989), reported spectrophotometric absorbance consistent with the reduced form of cytochrome b. It appears experimental manipulation of oxidases in the RC produces similar affects to neural activity stimulated by electrical fields as indicated in the previous paragraph. At this time it is impossible to correlate changes in gene expression [our results and (Gracey et al., 2001)] with redox state (Kanno et al., 1985; Piantadosi, 1989), but we conjecture that the (in)capacity to shuttle electrons is linked to the expression of electron acceptors.

Initial electron donation in the RC derives from NADH DH (complex I) which contains at least 25 separate polypeptide chains, and is responsible for reducing coenzyme Q, the first of the electron acceptors in the RC (Mathews et al., 2000). Several subunits of the NADH DH are differentially regulated by various treatments (Cai and Storey, 1996; Shi et al., 2001; Xie et al., 2002). However,

Wagner et al., (1990) reported no change in total NADH DH activity in anoxic cats. We report a 1.6X induction of NADH DH subunit 2 at three hours of stress while NADH DH subunit 5 was up-regulated 2.7X during one to five hours of anoxia in turtle (*Trachemys scripta elegans*) hearts but minimally induced in the brain (Cai and Storey, 1996). Resulting from dopaminergic neurotoxicity in methamphetamine exposed laboratory mice, NADH DH subunit 4 was down-regulated 2.13X at 12 and 24 hours (Xie et al., 2002). Furthermore, NADH DH was down-regulated in laboratory rat cerebral glial cells after 20 hours of dopamine incubation (Shi et al., 2001). In mice exposed to a toxin affecting dopamine neurons, NADH DH initially increased in the striatum from two to eight hours, then declined at 18 hours, declined in the midbrain, and remained unchanged in the cortex showing differential regulation depending on brain region and time (Sriram et al., 1997). At least two possible explanations for the above phenomena exist: 1) NADH DH subunit activation follows a temporal pattern, rising from one to eight hours but depleting from 12 to 24 hours, independent of the treatment; or 2) dopamine-induced neurotoxicity may down-regulate NADH DH while handling and low-water stressor (stressor used in this study) or hypoxia may up-regulate NADH DH. Regardless, our treatments altered the expression of NADH DH, a mitochondrial complex I gene, signifying a direct affect of a short-term stressor on the mitochondria.

The RC terminates at the F_1/F_0-ATP synthase (complex V), the membrane bound proton-driven motor responsible for the synthesis of ATP resulting from

tightly coupled ATP ↔ ADP + P_i reactions (Pedersen and Amzel, 1993; Mathews et al., 2000). Regulation of ATP synthase is rather variable being up or down-regulated depending on the treatment. For example, transcriptional activation of ATP subunit 6 follows after an 18-hour incubation of cortical and glial rat cells in lipopolysaccharide and interferon-γ, however enzymatic activity remained unchanged despite dramatic ATP reductions (Nicoletti et al., 1998). Experimental manipulation of glycemia prior to anoxia exposure in cats increased ATP synthase activity in normoglycemic cats compared to control and hyperglycemic cats (Wagner et al., 1990). Administration of various glucocorticoids to isolated rat brain mitochondria resulted in marked declines in ATP synthase activity (Morin et al., 2000). Our report of 1.2X down-regulation of ATP subunit 6 parallels this finding as plasma cortisol was elevated at three hours in fish from experiment two. In tilapia (*Tilapia mossambica*) exposed to methyl parathion, total ATPases were significantly reduced compared to control in gill and liver, but were not different in brain and muscle tissues (Rao and Rao, 1984). However, these researchers did not isolate ATP synthases from total ATPases masking possible differences in the ATP synthase pools. All of the above experiments expose the organism to some stressor; however, the differential activation of the ATP synthases depending on cell, tissue, or species highlights the complexity of ATP synthesis regulation.

Regulation of mitochondrial gene expression is controlled by constitutively expressed nuclear transcriptional factors that are (de)activated depending on relevant stimuli (Calabrese et al., 2001). Synergy between nuclear and

mitochondrial gene transcription exists to maintain levels of gene products essential for proper RC function (Calabrese et al., 2001). Our treatments induced and suppressed transcription of mitochondrial genes associated with ATP generation, demonstrating the transcriptional synergy necessary for energy production.

The simultaneous up- and down-regulation of genes involved in ATP generation is documented in pharmacology experiments (Shi et al., 2001) or in experiments using long-term hypoxia (Gracey et al., 2001) as the stressor. However, our short-term handling and low-water treatment altered oxidative phosphorylation intermediates despite the fact that O_2 was not likely a limiting factor, since the experimental tanks were circulated with flow through aerated water. It is of note that up to 10 fish were held in 20 L buckets of water for up to five minutes during the random assignment of fish to their tanks. We recognize the possibility of a somewhat hypoxic environment which may have contributed an additive affect to the 30 second out-of-water handling. Additionally, we found significantly higher plasma lactate in the stressed fish (experiment 1) indicating the utilization of glycolysis for ATP production suggesting that O_2 may have been limiting. In summary our results indicate a degree of metabolic load induced by handling and low-water similar to patterns seen in other tissues and organisms.

Genes associated with signal transduction

We detected genes involved in signal transduction such as the confirmed down-regulation of a neuron specific gene 1 (NSG1) localized in the glial stacks of neuroendocrine tissues (Saberan-Djoneidi et al., 1995), olfactory epithelium, and spermatocytes (Saberan-Djoneidi et al., 1998). Saberan-Djoneidi et al. (1995), hypothesized NSG1 to be associated with interneuronal substance trafficking. Other evidence for the functionality of NSG1 derive from a hydrophobic region showing identity with receptor-like phosphatases, and immuno-staining shows cytoplasmic and nuclear localization, the latter of which may be developmentally regulated (Carlock et al., 1996).

Recently, Steiner et al. (2002) reported that NSG1, when localized in endosomes, modulates neuronal transferrin (TF) receptor cycling, and is strongly expressed during neuronal synaptic maturation. Additionally, NSG1 positive endosomes modulate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor cycling, after N-methyl-D-aspartate receptor stimulation (Steiner et al., 2002). Briefly, endosomes are organelles that degrade or recycle membrane bound proteins. Altogether, up-regulation of the NSG1 increases the rate of TF and AMPA receptor cycling, while down-regulation slows the cycling process (Steiner et al., 2002). Furthermore, because the TF and AMPA receptors are unrelated, NSG1 likely mediates the recycling of numerous receptors (Steiner et al., 2002).

Down-regulation of NSG1 in our libraries indicates that neuronal receptor cycling may be attenuated during stress.

Over half of the genes associated with signal transduction were down-regulated by stress, including a tyrosine phosphatase associated with cell growth and proliferation (Diamond et al., 1994). Furthermore, we detected two ribosomal proteins and the 18S rRNA gene in our down-regulated library signifying the suppressive effect of stress on protein synthesis. Down-regulation of genes associated with protein translation and cell growth may represent energy reallocation measures necessary to cope with the stressor (Gracey et al., 2001). Down-regulation of ribosomal proteins also occurs in hypoxia exposed gobies (Gracey et al., 2001) and yeast (*Saccharomyces cerevisiae*) (Eisen et al., 1998).

General comments

The elevation in plasma cortisol likely induces gene transcription mediated by the action of glucocorticoid receptors in the brain (Mendelson, 1992; Teitsma et al., 1997; Carruth et al., 2000). However we did not detect any genes classically associated with the stress response, such as the glucocorticoid receptor (Teitsma et al., 1997) or CRH (Ando et al., 1999; Bernier et al., 1999). One possible explanation is that classically stress-related genes are present in low copies compared to other genes. A recent study profiling the gene expression of the human

hypothalamo-pituitary-adrenal axis (HPI-axis in fish) reported that only 11.8% of the cDNAs sequenced from the hypothalamus were related to the endocrine system (Hu et al., 2000), let alone stress. The remaining genes, associated with metabolism, gene and protein expression, and signal transduction, represented 16.5%, 16.4%, and 18.7%, respectively, of the cDNAs sequenced (Hu et al., 2000).

Only 1:20,000 mRNAs in a white sucker (*Catastomus commersoni*) pre-optic area cDNA library is CRH and visualization by northern hybridization required 10 μg of mRNA (Morley et al., 1991) indicating very low abundance. Low transcript abundance may also explain the absence of signal from seven of our clones in northern hybridization. Our cloning procedure involved the transfection of a pool of PCR products from the entire brain; therefore, the more numerous the product, the more likely transfection occurred, despite the fact that SSH normalized for rare transcripts. Our results do not ignore the role of the HPI-axis in modulation of the stress response; rather, they highlight the host of other biochemical processes occurring in the brain likely induced by the up-regulation of the HPI-axis. Other potential reasons for the absence of genes associated with the HPI- axis include: inappropriate time frame, the initial stored peptides or receptors were not depleted at three hours of stress, or activation of classically known stress-factors is controlled at the protein level.

We were unable to repeat patterns of gene expression in experiment two possibly attributed to several factors including developmental state, rearing conditions, or stressed control fish. The rearing tanks utilized in the second

experiment were larger (3.0 m diameter) than the first (1.0 m diameter). This larger tank may have allowed for better growing conditions and accelerated the development of the fish. Furthermore, the fish from the second experiment were one month older then fish in the first experiment and may have started smoltification, a process known to alter physiology and morphology in preparation for ocean life. Wagner et al. (1963) reported seaward migration in the same stock of fish used in our experiments in late April with downstream movement correlated with size. Therefore, it is conceivable that the fish from experiment two, being sampled in February, and larger then experiment one fish, were smolts. Also, two unstressed fish (0-hour) from experiment two displayed elevated cortisol (nearly 80 ng/ml see Figure 2a), which is known to rise during smoltification (Barton et al., 1985), albeit not to the levels that we detected. An alternative explanation is that control fish from experiment two were stressed, displaying cortisol values comparable to 3-hour fish, and possibly explaining the similarities in gene expression between the 0- and 3-hour fish. However, differences in brain metabolic gene expression are documented between parr and smolt (Hardiman et al., 1994). If the differences in gene expression between the two experiments are indeed developmentally influenced, then exciting research possibilities exist investigating the differential response to stress in parr and smolts as demonstrated by Barton et al., (1985) in coho salmon (*O. kisutch*).

Our results present a first attempt to profile gene expression in the brain of steelhead trout. We detected differentially regulated mitochondrial and nuclear

genes (although some to a slight degree), genes that have not been reported in teleosts, and genes that are well characterized for rainbow trout. The SSH technique, we believe, is a valuable tool to begin delineating the stress response at the gene level, yet is limited by false positives, yields relatively short cDNAs (300-600 bp), and captures only a "snapshot" of the brain, or other tissue, during stress. Regardless, we present a select profile of stress induced gene expression in the brains of trout, and we were able to confirm the differential expression of genes associated with oxidative phosphorylation, signal transduction, and a gene with unidentified function. Further research is needed to more completely characterize differentially regulated genes, localize these genes in the brain, and present a more complete time course of their expression.

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GENERAL CONCLUSIONS

This thesis illustrates a new method to investigate the stress response byallowing one to detect genes known to be expressed during stress, as well as capturing genes not normally associated with stress. Herein lies the power, because the identification of genes not known to be associated with the stress response provides information about the global response of the tissue. This global response is essentially an expression profile of the tissue responding to a particular treatment. The gene expression profile provides an initial library of information from which hypothesis driven experiments can be conducted. That is, now that we know what is *there*, we can ask questions and design experiments to figure out what it *does*.

Creating subtracted libraries has become rather "cookbook" with the advent of commercially available kits such as the Clontech kit we used. The utility of kits are that they allow researchers who don't have extensive training in molecular biology to obtain genetic information quickly, and contribute that information to public databases such as GenBank, or the Expressed Sequence Tag database (dbEST). This is especially important for organisms for which there is little genetic sequence information.

Subtractive hybridization is not without limits. Short sequences and false positives are two drawbacks that limit the amount of information to be obtained. Half of the sequences we obtained showed no identity, identity with genomic DNA

submitted during genome sequencing projects, or with repetitive elements. This is most likely due to the short sequences generated by subtractive hybridization (see Discussion). False positives are also a problem and much time can be spent on northern blots before marked changes in expression are found. Although this drawback can be remedied by performing differential screening of the subtracted libraries (recommended by Clontech) it requires the use of ^{32}P-labeled probes.

Despite the drawbacks, we believe that the technological explosion in biochemistry will only increase our understanding of organismal responses to various stimuli. For example, cDNA microarrays allow even more extensive profiling of gene expression with enough resolution to include numerous subtracted libraries and thousands of genes. Gene expression profiles tell nothing about the function of a particular gene, they describe the cumulative genetic response of the animal responding to a given treatment. A gene expression profile is akin to a brain map, which, without, one would not describe the function of the hypothalamus.

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APPENDICES

APPENDIX 1

Table 1: cDNA Fragments with Unknown Function or Insignificant Identity. Id (%) indicates the percent identity with the sequence named in the Identification column. "E" is the E-value. Unpublished in the status column indicates sequences have not been submitted to any database. N/A = not available. Identification information obtained from the nucleotide database (BLASTn). Sequence identity information from the protein database (BLASTx) always returned less significant E-values then the nucleotide database searches and are therefore not reported.

Up-regulated cDNA fragments with unknown function or insignificant identity				
Clone (bp)	Putative Identification	Species (* teleost)	Id (%) / E	Status
2F3A (280)	Clone RP11-102K13 chromosome 1	*Homo sapiens*	100 / 1e-3	Unpublished
3F3A (271)	Clone RP11-803N5 chromosome 2	*Homo sapiens*	100 / 1.6e-2	Unpublished
4F3A (610)	Clone ssa34NUIG microsatelite	*Salmo salar* *	94 / 2e-3	Unpublished
5F3A (619)	BAC clone RP11-466K9	*Homo sapiens*	100 / 5.3e-1	Unpublished
7F3A (610)	Clone BHMS413 microsatelite	*Salmo salar* *	92 / 4e-73	Unpublished
8F3A (605)	Clone BHMS413 microsatelite	*Salmo salar* *	87 / 4e-45	Unpublished
12F3A (630)	Clone CTD-2583A14 chromosome 19	*Homo sapiens*	96 / 1.5e-1	Unpublished
13F3A (615)	Clone RP11-632P5 chromosome 2	*Homo sapiens*	100 / 5.7e-1	Unpublished
16F3A (613)	BAC clone RP11-466K9 chromosome 2	*Homo sapiens*	100 / 5.7e-11	Unpublished
20F3A (577)	Clone RP11-466I1 chromosome 11	*Homo sapiens*	100 / 5.6e-1	Unpublished
21F3A (593)	Rsal30 microsatelite	*Salmo salar* *	94 / 6e-4	Unpublished
22F3A (575)	Clone BHMS137 microsatelite	*Salmo salar* *	90 / 2e-25	Unpublished
25F3A (466)	Clone RP23-476D16 chromosome X	*Homo sapiens*	100 / 4.5e-1	Unpublished
28F3A (287)	Nuclear factor kappa-B gene, exon 5	*Homo sapiens*	100 / 7e-2	Unpublished
29F3A (570)	Clone BHMS137 microsatelite	*Salmo salar* *	90 / 2e-25	Unpublished
31F3A (584)	No significant identity w/ default parameters	N/A	N/A	Unpublished

Table 1: (Continued)

Down-regulated cDNA fragments with unknown function or insignificant identity				
Clone	Putative Identification	Species (* teleost)	Id (%) / E	Status
1R0A (550)	No significant identity w/ default parameters	N/A	N/A	Unpublished
2R0A (591)	Polymorphic marker	*Rattus norvegicus*	93 / 3.7e-2	Unpublished
5R0A (592)	No significant identity w/ default parameters	N/A	N/A	Unpublished
6R0A (164)	Clone RP11-384K20 chromosome 2	*Homo sapiens*	100 / 5.8e-1	Unpublished
9R0A (520)	BAC clone RP11-19M18 chromosome 2	*Homo sapiens*	96 / 5e-1	Unpublished
13R0A (241)	BAC clone RP11-111J6 chromosome 2	*Homo sapiens*	100 / 8.8e-1	Unpublished
19R0A (599)	BAC clone CTA-315H11	*Homo sapiens*	95 / 2.0	Unpublished
20R0A (600)	BAC clone RP11-564A4 chromosome 9	*Homo sapiens*	100 / 9e-3	Unpublished
21R0A (577)	PAC clone RP5-1164F5 from 7q11.2-q22	*Homo sapiens*	100 / 1.4e-1	Unpublished
22R0A (206)	Hae021 microsatelite	*Salmo salar* *	90 / 2.0	Unpublished
23R0A (213)	Clone RP11-477C23 chromosome 1	*Homo sapiens*	100 / 6e-3	Unpublished
25R0A (609)	No significant identity w/ default parameters	N/A	N/A	Unpublished
26R0A (345)	BAC clone RP11-731119 chromosome 2	*Homo sapiens*	96 / 2.1e-2	Unpublished
28R0A (618)	Immunoglobin heavy chain variable region	*Homo sapiens*	100 / 3e-2	Unpublished
30R0A (602)	Arabidopsis thaliana BAC T26D22	*Arabidopsis thaliana*	100 / 4e-2	Unpublished

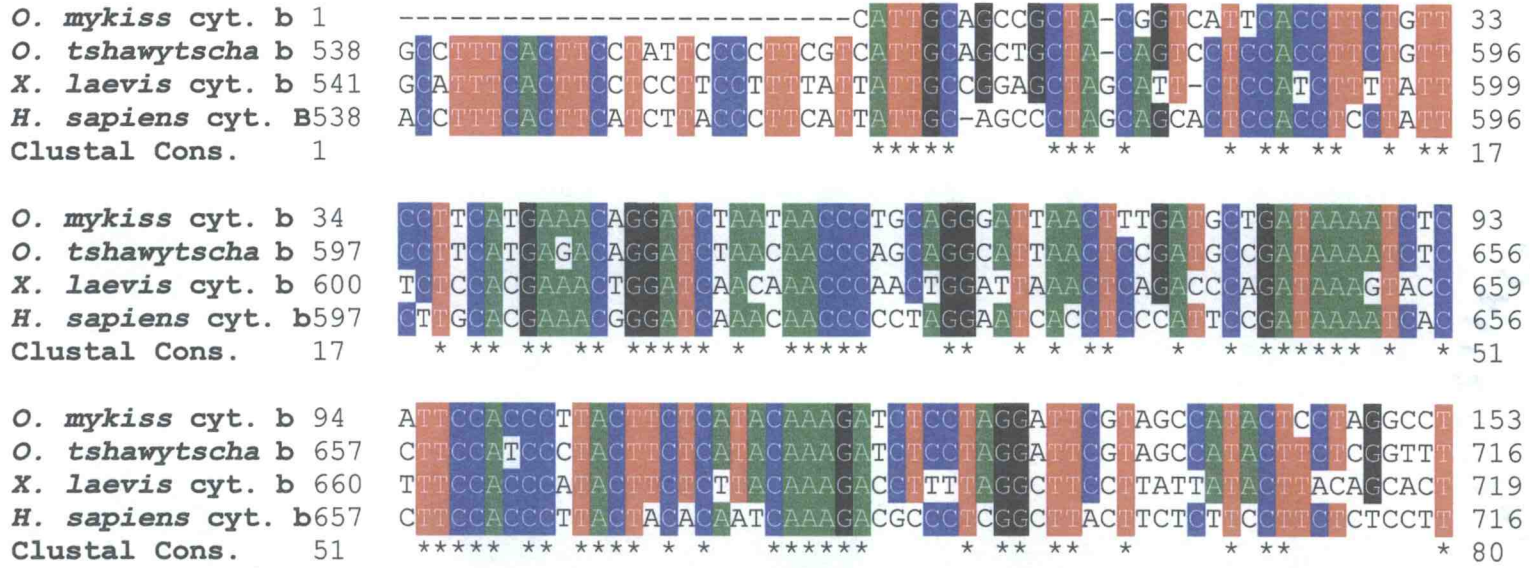


Figure 1: Multiple Alignment (ClustalW, boot-strapped 1000x) of Nucleotides from *Oncorhynchus mykiss*, *O. tshawytscha*, *Xenopus laevis*, and *Homo sapiens* Cytochrome b. For all Appendix 2 figures, the *O. mykiss* sequences were those identified by suppression subtractive hybridization during the present study. Since the *O. mykiss* sequences are only portions of the entire cDNA, only regions of identity between all species are shown. That is, the sequence preceding and following region of identity with *O. mykiss* is not shown for the other species. Numbers preceding and following each line of sequence indicate nucleotide (or amino acid) number. Dashes indicate gaps in sequence. Clustal Cons. is the consensus sequence between all species.

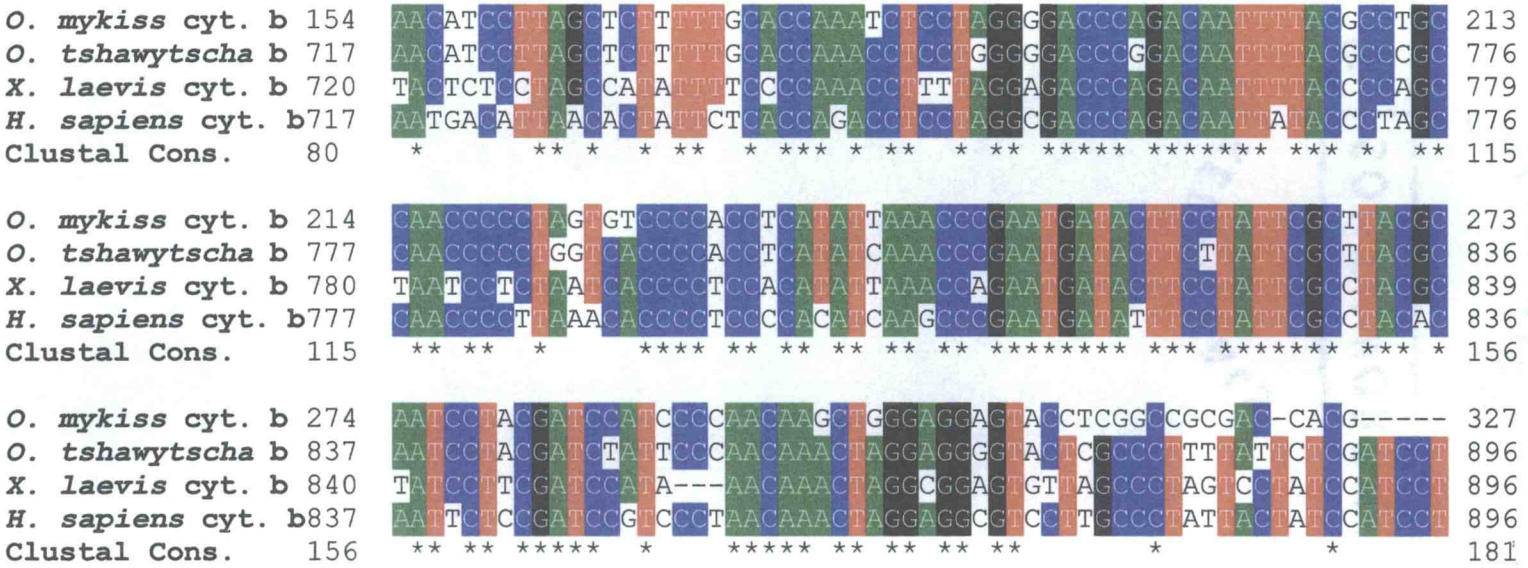


Figure 1: (Continued)

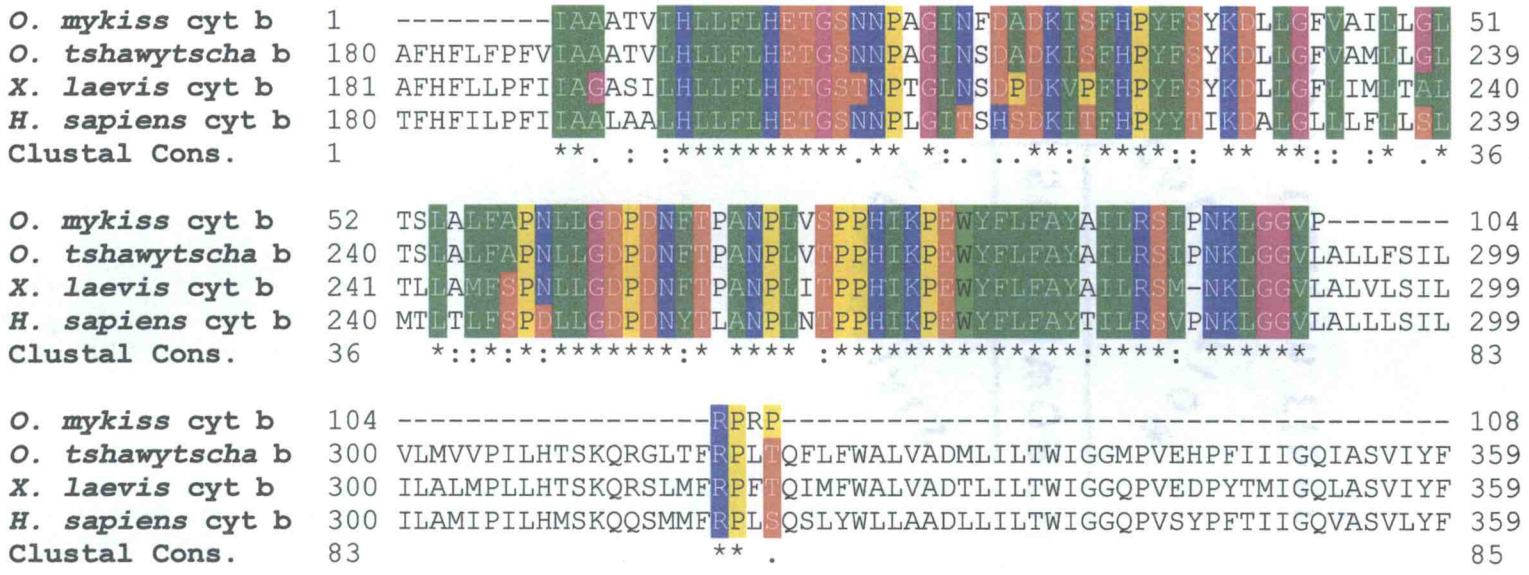


Figure 2: Multiple Alignment of Deduced Amino Acid Sequences from *O. mykiss*, *O. tshawytscha*, *X. laevis*, and *H. sapiens* Cytochrome b. See Figure 1 caption for more complete description of figure components.

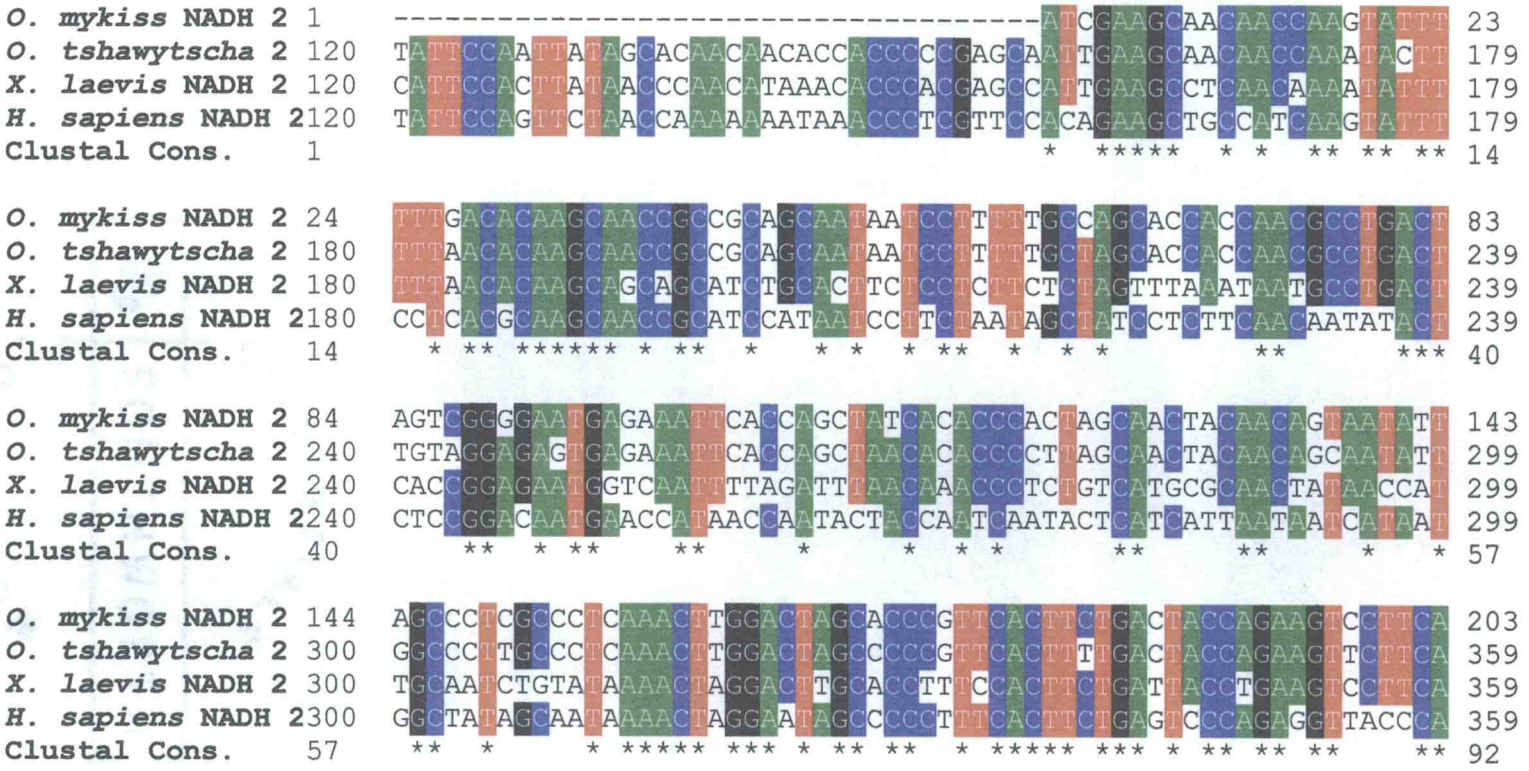
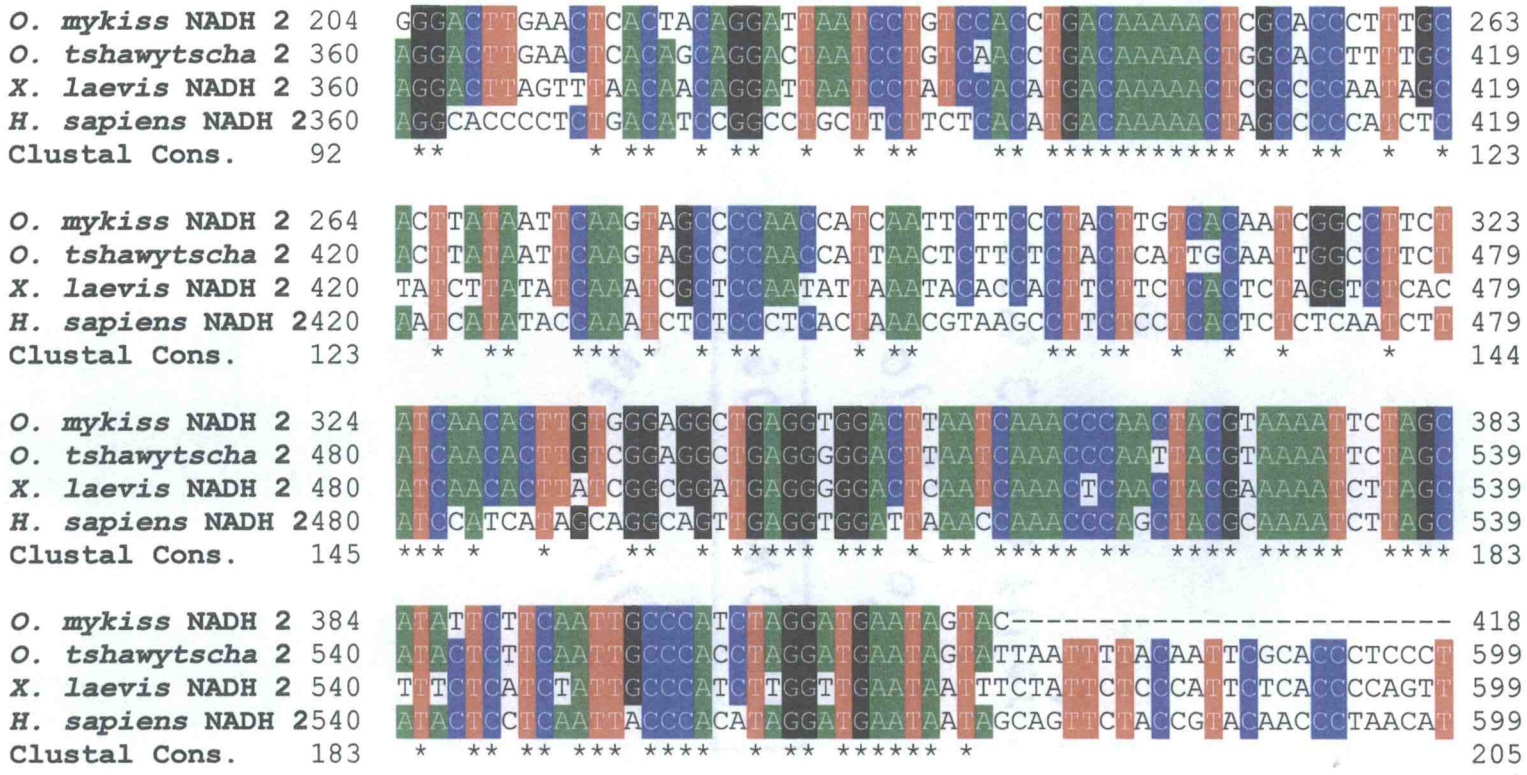


Figure 3: Multiple Alignment of Nucleotides from *O. mykiss*, *O. tshawytscha*, *X. laevis*, and *H. sapiens* NADH Dehydrogenase Subunit 2 (NADH2). See Figure 1 caption for more complete description of figure components.



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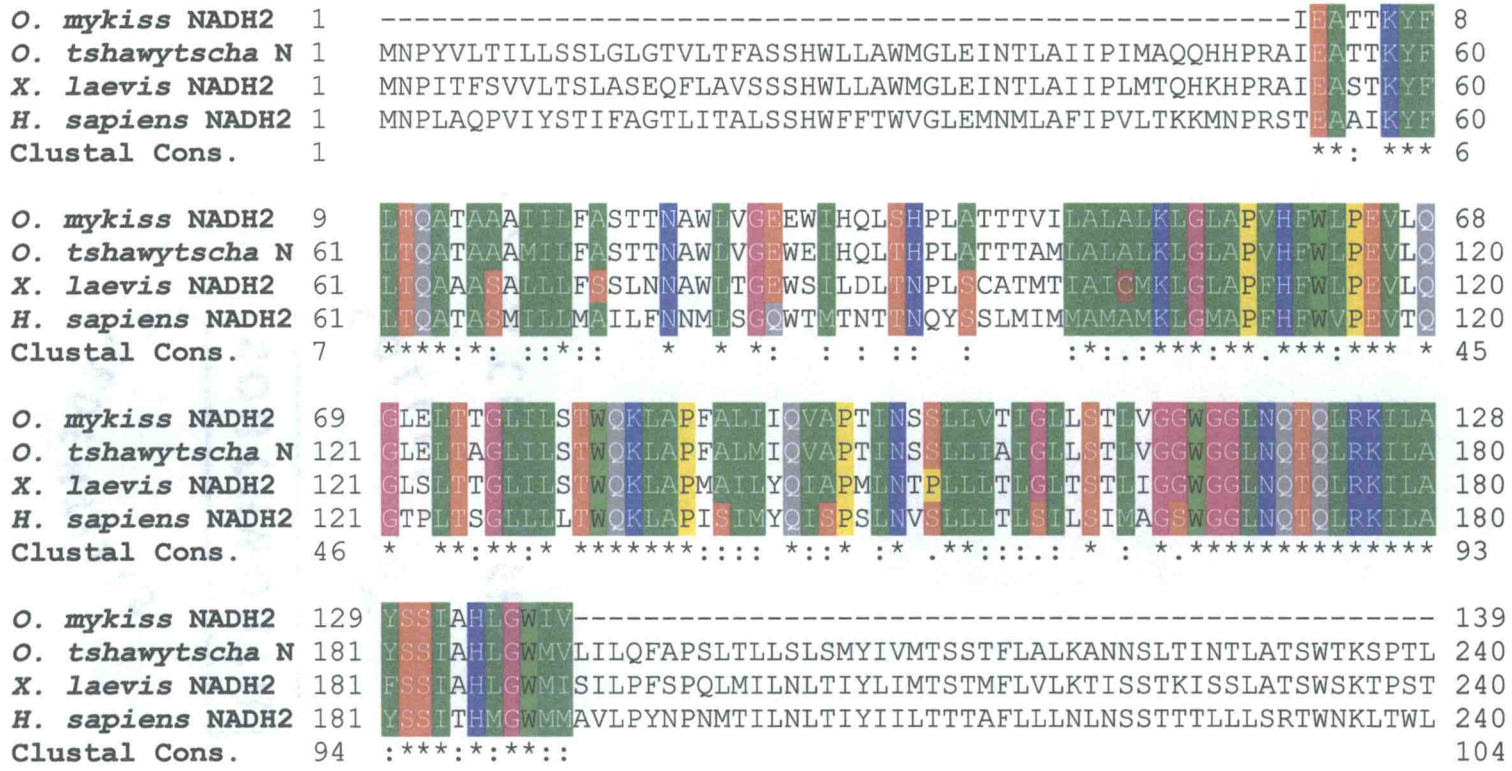


Figure 4: Multiple Alignment of Deduced Amino Acid Sequences from *O. mykiss*, *O. tshawytscha*, *X. laevis*, and *H. sapiens* NADH2. See Figure 1 caption for more complete description of figure components.

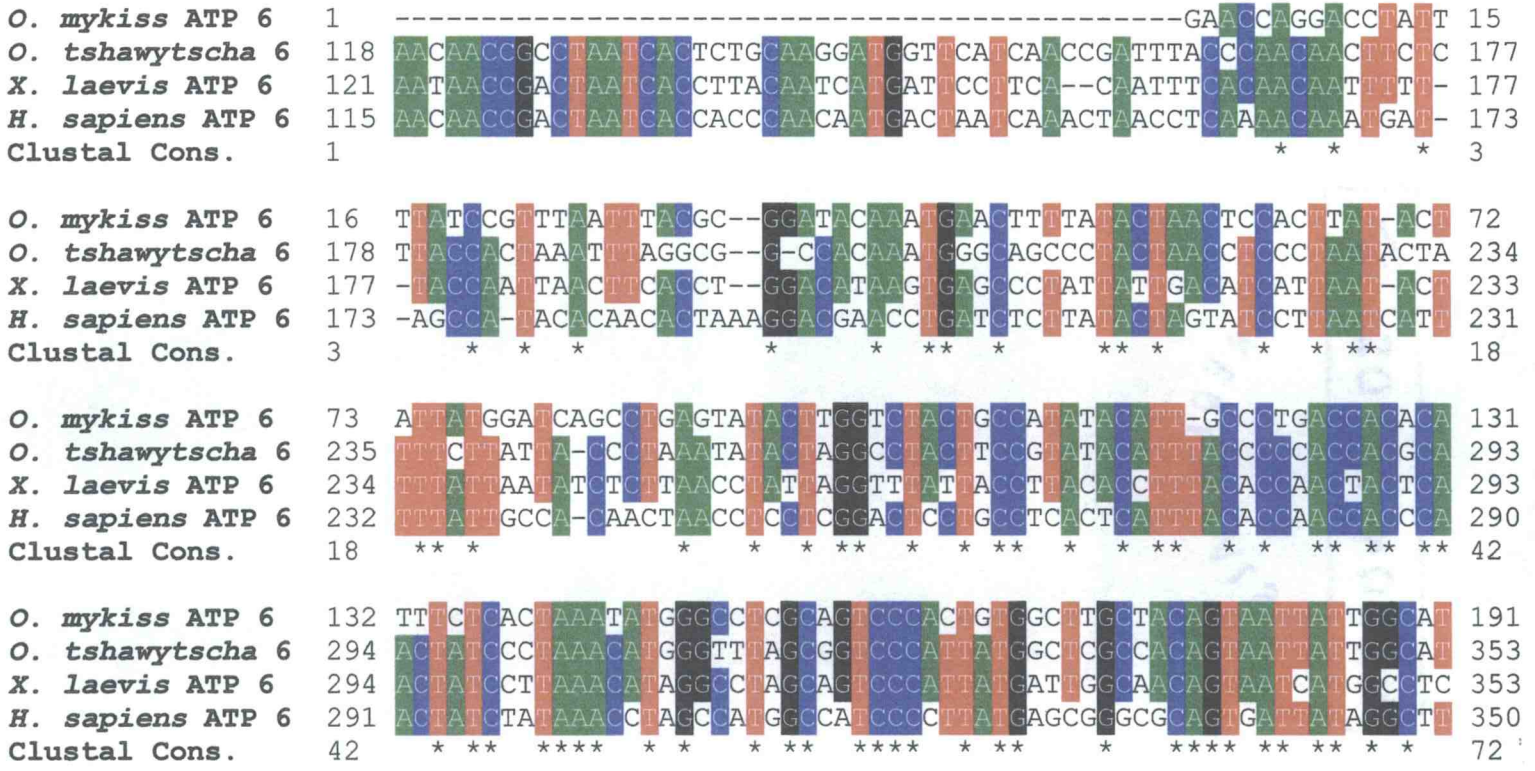
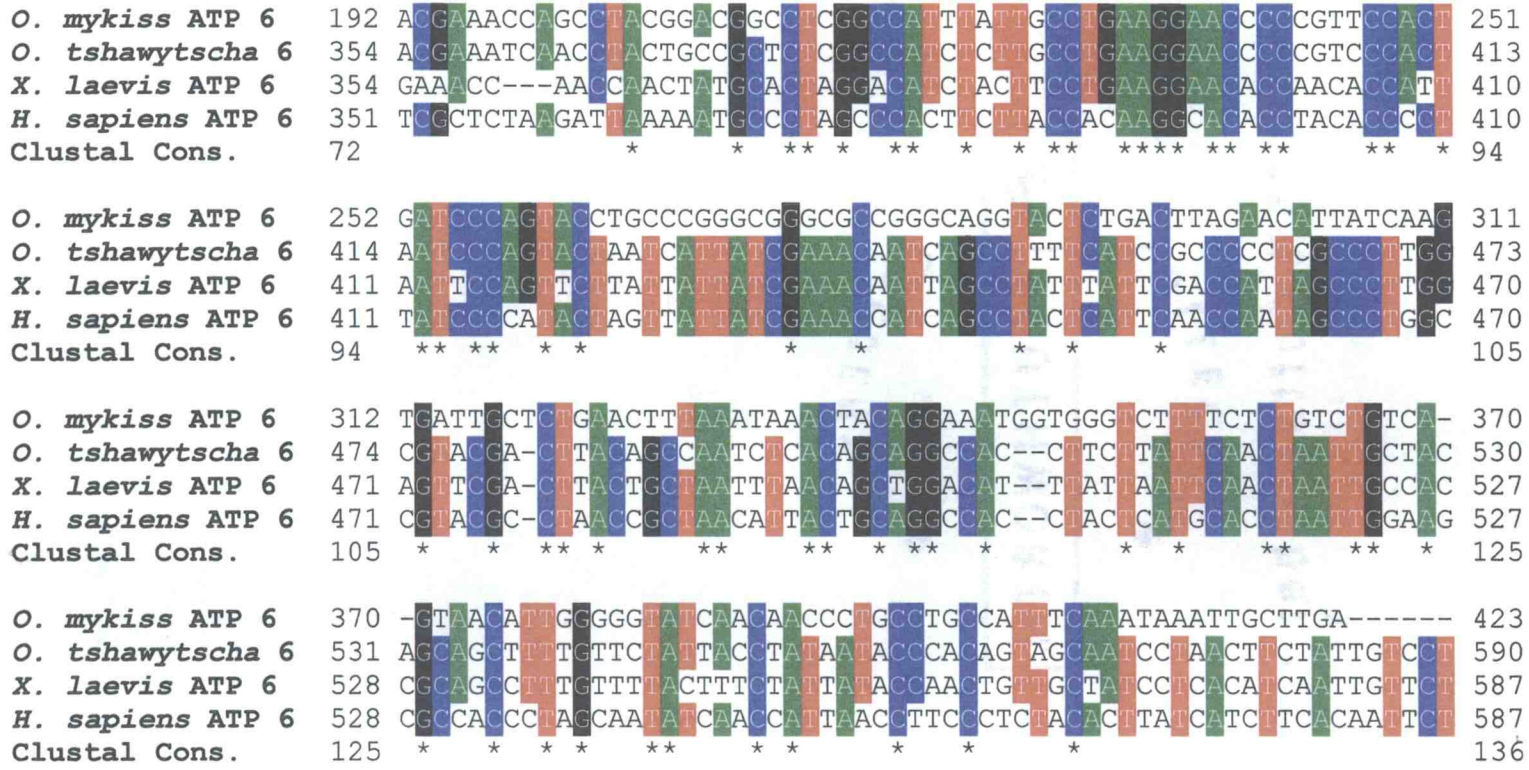


Figure 5: Multiple Alignment of Nucleotides from *O. mykiss*, *O. tshawytscha*, *X. laevis*, and *H. sapiens* ATPase Subunit 6. See Figure 1 caption for more complete description of figure components.



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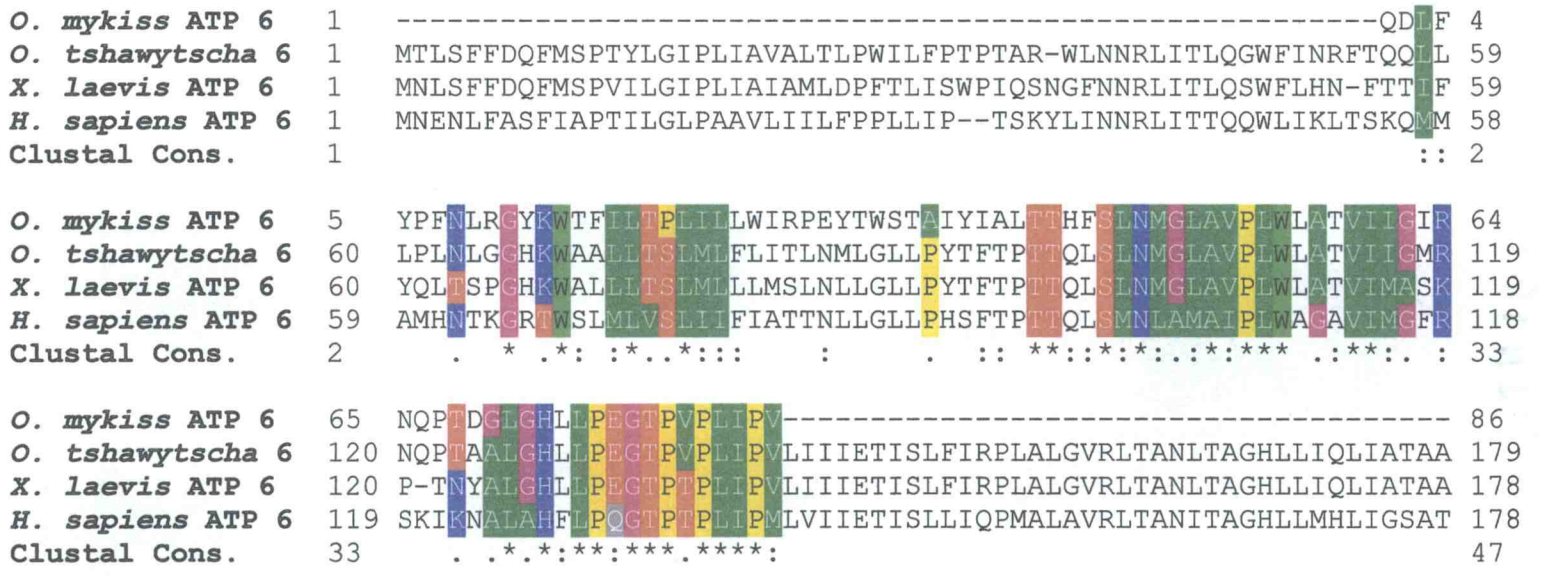


Figure 6: Multiple Alignment of Deduced Amino Acid Sequences from *O. mykiss*, *O. tshawytscha*, *X. laevis*, and *H. sapiens* ATPase Subunit 6. See Figure 1 caption for more complete description of figure components.

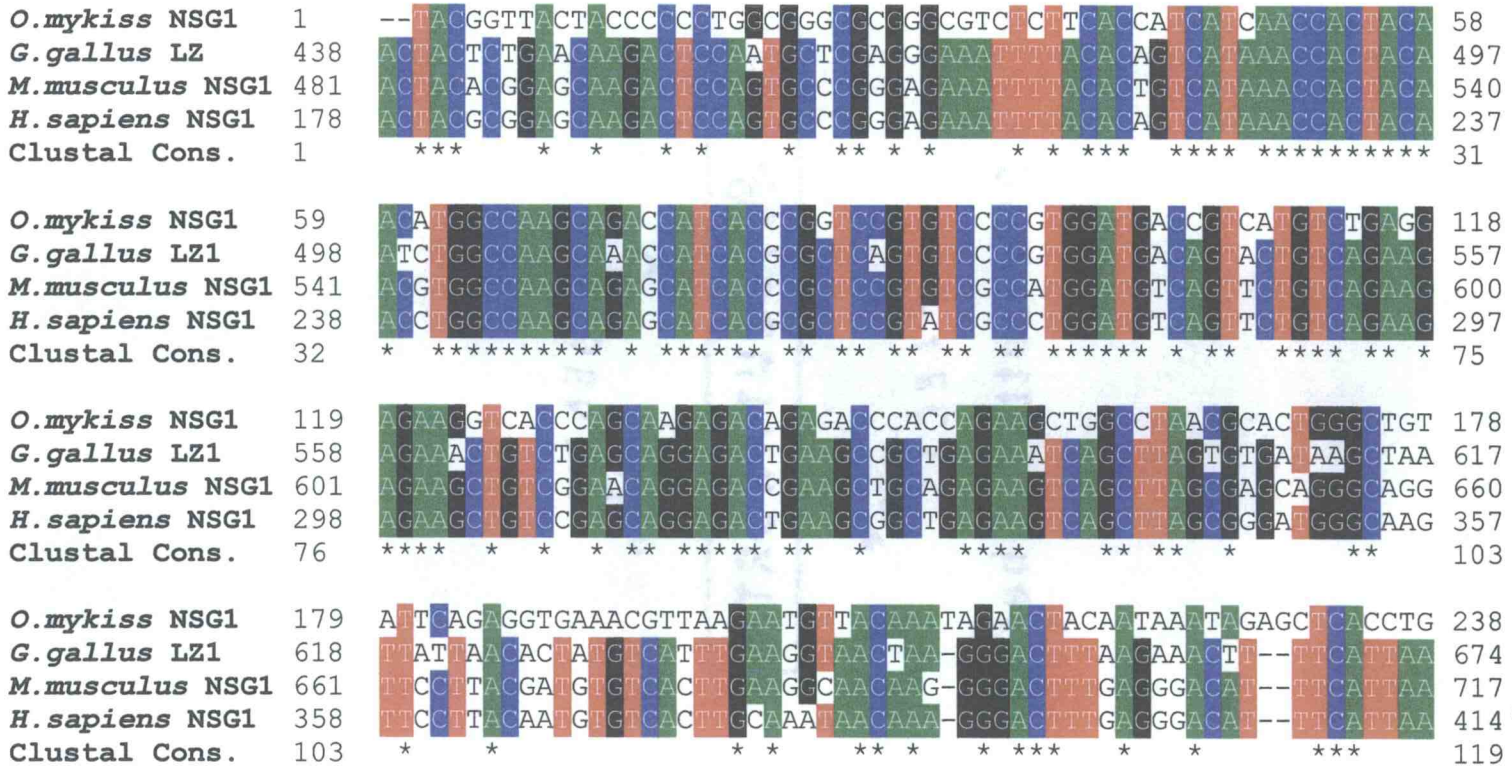
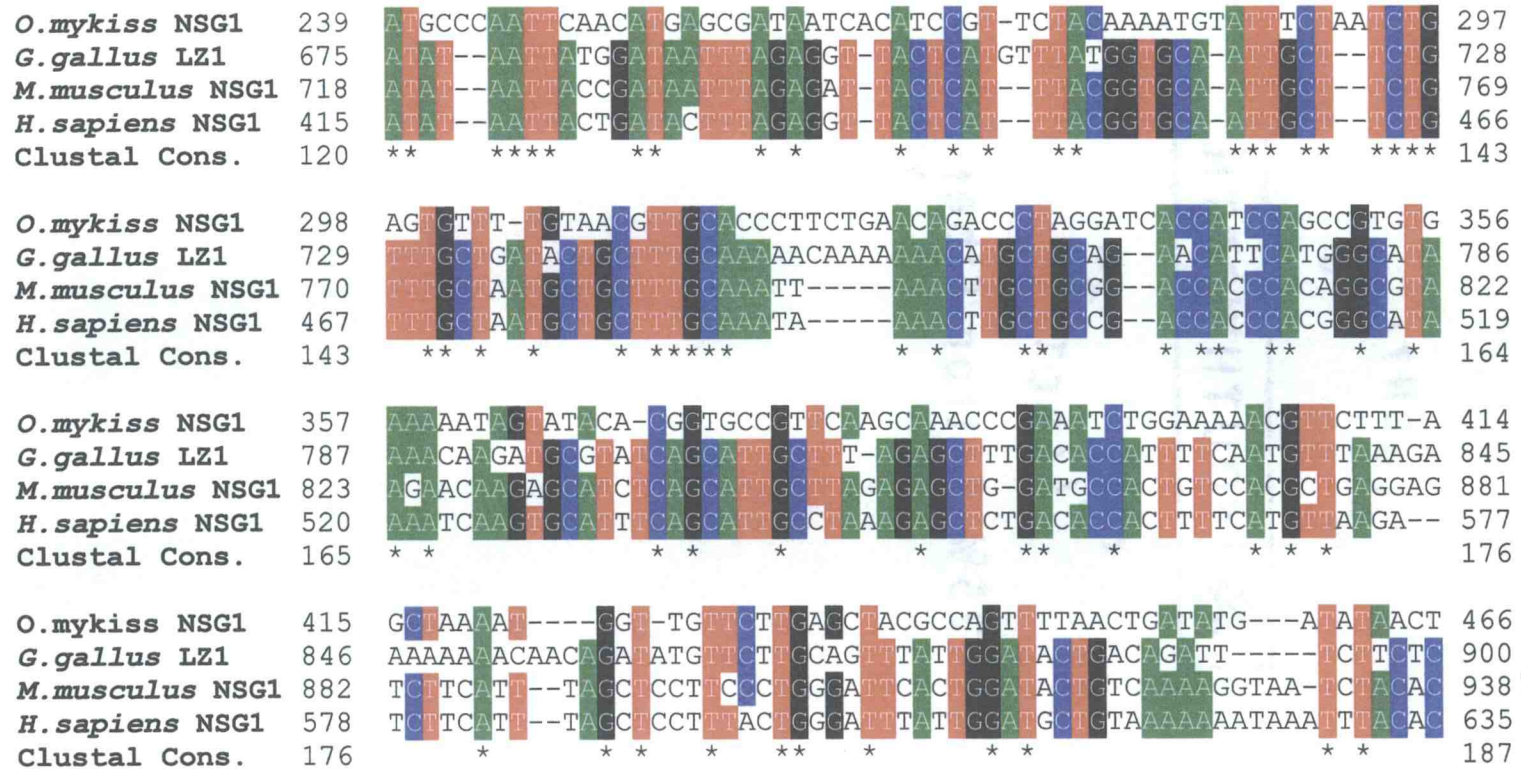


Figure 7: Multiple Alignment of Nucleotides from *O. mykiss*, *Mus musculus*, and *H. sapiens* Neuron Specific Gene 1 (NSG1) and a Gene Related to Brain Development (LZ1) from *Gallus gallus.* See Figure 1 caption for more complete description of figure components.



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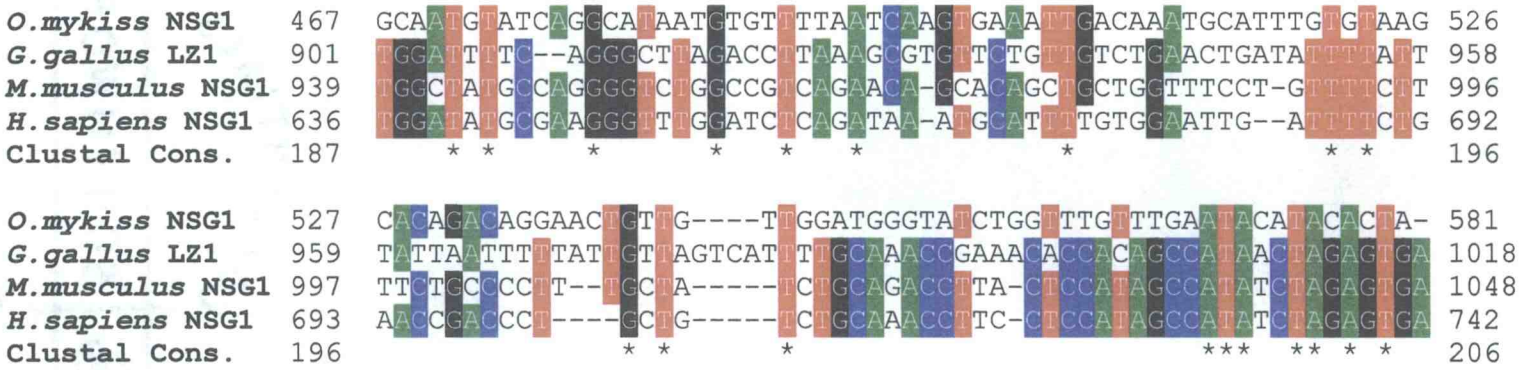

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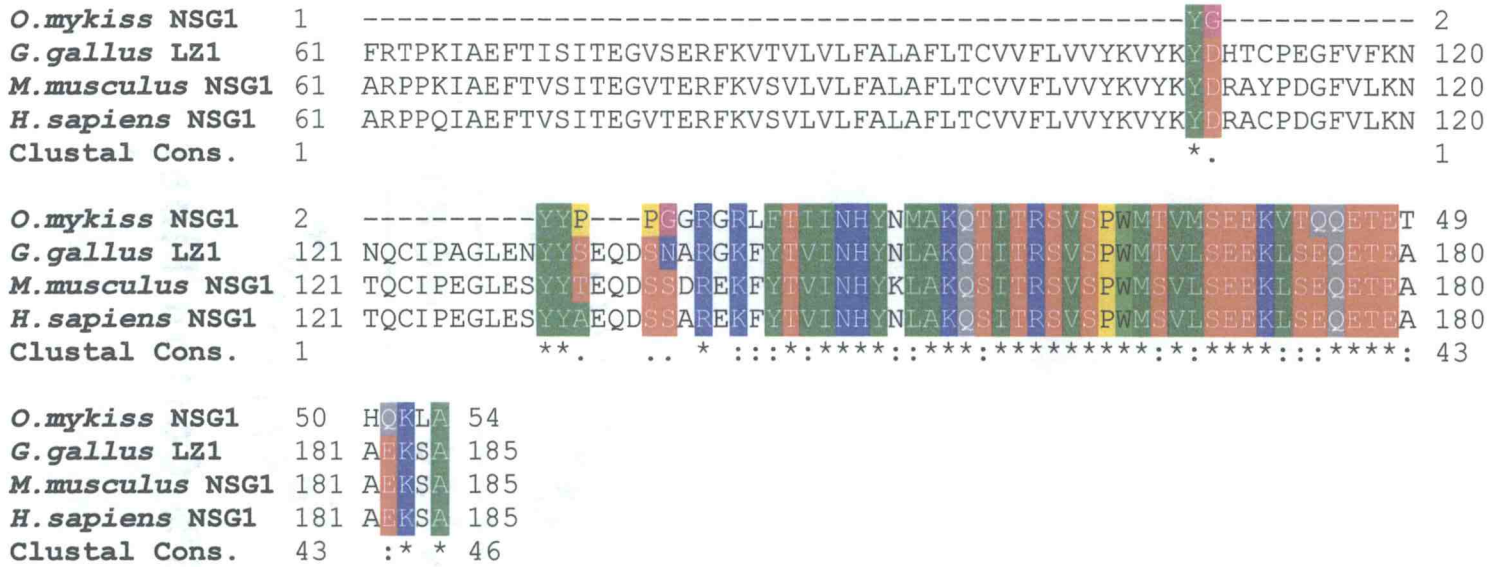


Figure 8: Multiple Alignment of Deduced Amino Acid Sequences from *O. mykiss*, *M. musculus*, and *H. sapiens* NSG1 and a Gene Related to Brain Development (LZ1) from *G. gallus.* See Figure 1 caption for more complete description of figure components.

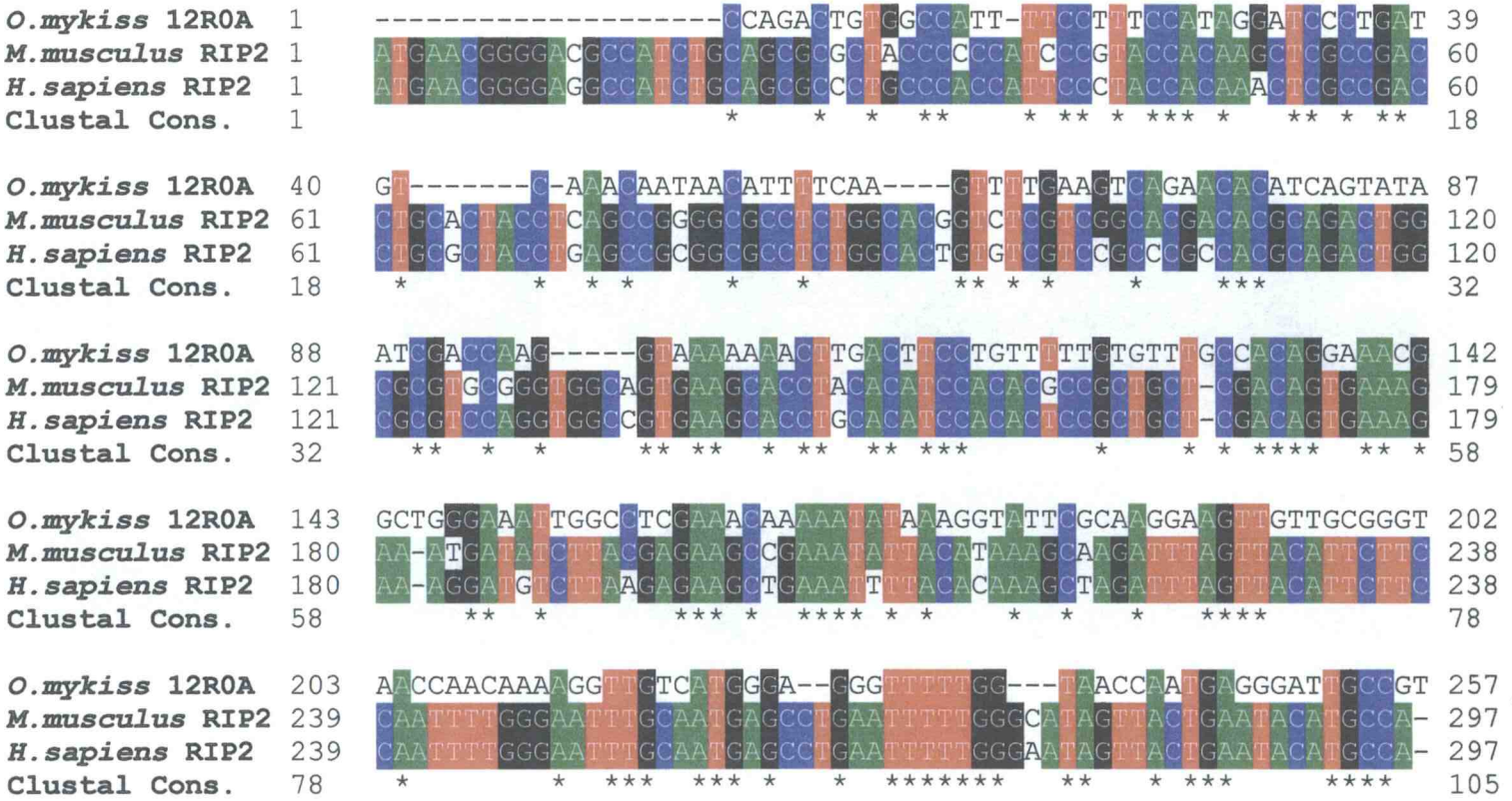
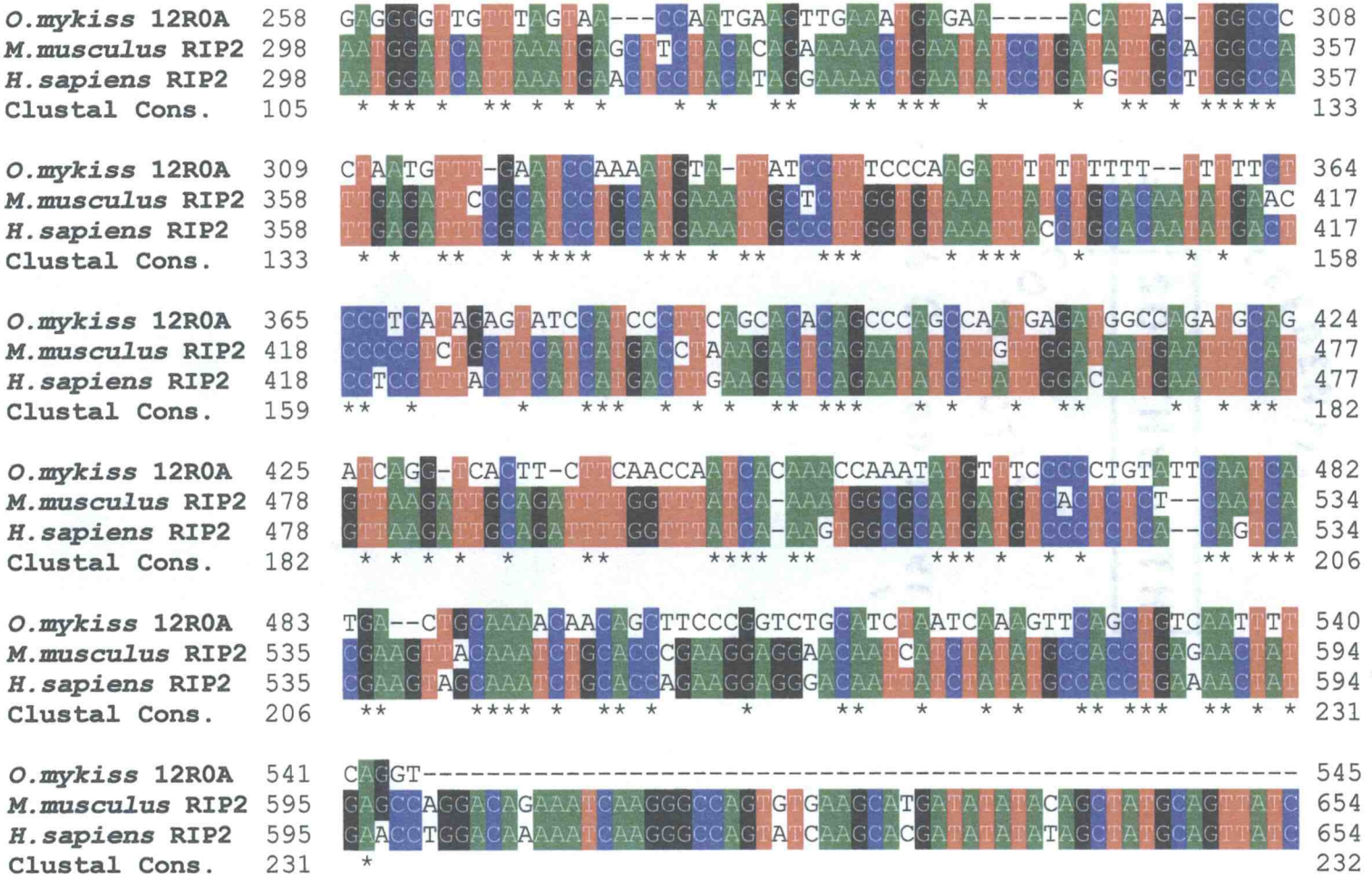


Figure 9: Multiple Alignment of Nucleotides from *O. mykiss* Unidentified cDNA (clone 12R0A) with *H. sapiens* and *M. musculus* Receptor Interacting Protein 2 (RIP2). BLASTX search results indicated that RIP2 most significantly identified with *O. mykiss* clone 12R0A. However, the E-value did not meet the author's criteria for significant identity. See Figure 1 caption for more complete description of figure components.



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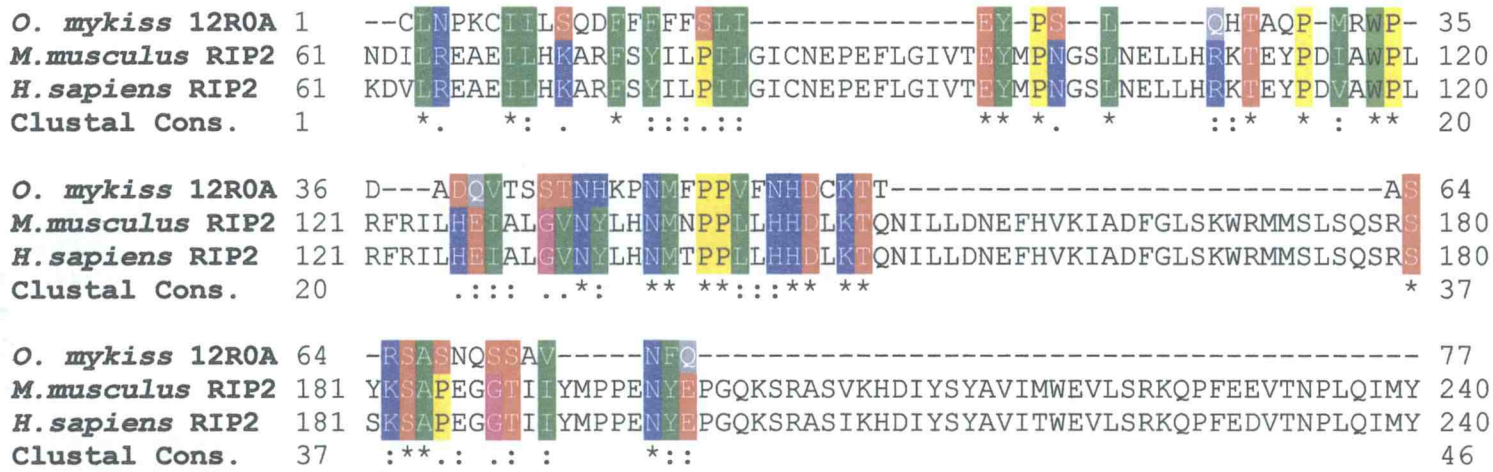

Figure 10: Multiple Alignment of Amino Acids from unknown *O. mykiss* cDNA (12R0A) and RIP2 from *H. sapiens* and *M. musculus*. See Figure 1 caption for more complete description of figure components.