Domestication and growth hormone transgenesis cause similar changes in gene expression in coho salmon (*Oncorhynchus kisutch*)

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Domestication has been extensively used in agricultural animals to modify phenotypes such as growth rate. More recently, transgenesis of growth factor genes [primarily growth hormone (GH)] has also been explored as a rapid approach to accelerating performance of agricultural species. Growth rates of many fishes respond dramatically to GH gene transgenesis, whereas genetic engineering of domestic mammalian livestock has resulted in relatively modest gains. The most dramatic effects of GH transgenesis in fish have been seen in relatively wild strains that have undergone little or no selection for enhanced growth, whereas genetic modification of livestock necessarily has been performed in highly domesticated strains that already possess very rapid growth. Such fast-growing domesticates may be refractory to further stimulation if the same regulatory pathways are being exploited by both genetic approaches. By directly comparing gene expression in wild-type, domestic, and GH transgenic strains of coho salmon, we have found that domestication and GH transgenesis are modifying similar genetic pathways. Genes in many different physiological pathways show modified expression in domestic and GH transgenic strains relative to wild-type, but effects are strongly correlated. Genes specifically involved in growth regulation (IGF1, GHR, IGF-II, THR) are also concordantly regulated in domestic and transgenic fish, and both strains show elevated levels of circulating IGF1. Muscle expression of GH in nontransgenic strains was found to be elevated in domesticated fish relative to wild type, providing a possible mechanism for growth enhancement. These data have implications for genetic improvement of existing domesticated species and risk assessment and regulation of emerging transgenic strains.

transgenic | GH | selection | livestock | risk

Altering plant and animal species for human benefit has been a hallmark of man's 10,000-year agronomic history (1), resulting in strains highly specialized for food production or with culturally desirable features (e.g., food species, beasts of burden, hunting dogs and birds, ornamental species). The process of domestication occurs through gradual selection of polygenic variation that adapts organisms to anthropogenic conditions or modifies them to desired traits [such as enhanced growth rate or yield (2)]. Understanding how domesticated organisms have been transformed from wild type is useful both from genetic and evolutionary perspectives, and provides fundamental practical information for future enhancement of agricultural strains through traditional breeding and transgenic methods. Genetic changes that have occurred during domestication of plants are being revealed by gene mapping and genomic analyses (3), and whereas the specific genetic and physiological bases for enhanced phenotypes seen in domesticated vertebrates are more obscure, significant advancements are emerging (4).

Rapid growth rate is one trait often associated with domesticated vertebrates as this phenotype confers significant benefit to agriculture (2). Many fish species and strains are capable of being greatly growth stimulated by growth hormone (GH) treatment/transgenesis, domestication or selective breeding (5–9). Selection programs have found gains in fish can be very high (7–10% per generation) (7), presumably because wild fish strains still possess a large amount of natural allelic and phenotypic variation available for selection. Indeed, fast growth rate in fish domesticates occurs primarily via additive genetic changes, implying contribution of many polygenic loci throughout the genome (8–11). Although separate domestication events appear to influence expression of similar genes (12), specific loci altering the domestication processes are unknown.

Transgenesis provides a comparatively new and more rapid strategy to introduce targeted genetic variation that also can cause remarkable alterations in phenotype. For example, transgenic mice overexpressing growth hormone (GH) show dramatic (2-fold) growth enhancement (13), a result that spawned a great deal of similar research in agricultural mammals and fish. Many fish species used in aquaculture have been found to strongly respond to GH transgenesis (e.g., body sizes increasing up to 35- to 37-fold for mud loach and coho salmon) (5, 14–19), whereas domesticated agricultural mammals engineered with growth factor transgenes have shown only small enhancements of growth rate and some abnormalities (20–24). Pursel *et al.* (23) suggested this modest response in domesticated animals may be due to their long prior selection for maximum growth rate that limits further responses to GH. Testing this hypothesis in mammals is difficult as representative wild-type progenitor strains are in most cases no longer accessible.

Reference wild-type strains of fish are available for comparison with domesticated and transgenic strains. Such comparisons allow identification of the genetic and physiological processes involved in the domestication process and provide a model to determine whether modern approaches (such as GH transgenesis) modify similar regulatory pathways as traditional domestication. Previous research has separately found differences in expression profiles for GH transgenic vs. wild, and for domestic vs. wild strains, however, these studies were performed among different fish species and some were assessed at the whole organism (larvae) levels, precluding direct comparisons of these data (12, 25, 26). To allow direct assessment of these genetic processes, the present study simultaneously examines, in liver and muscle, gene expression in wild-type, GH transgenic, and domesticated coho salmon (*Oncorhynchus kisutch*), using a ≈16,000 (16K) salmonid cDNA microarray chip (27).

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Data deposition: The The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE13846).

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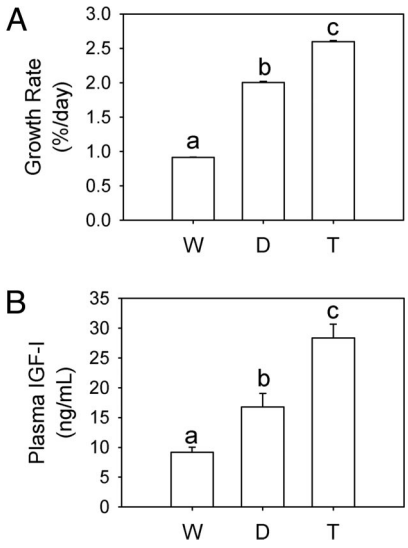

Fig. 1. Growth rates and hormone profiles of wild-type (W), domesticated (D), and GH transgenic (T) salmon. (*A*) Specific growth rates (SGR). (*B*) Plasma IGF1 levels. $n = 10$ per genotype. Letters above bars denote significant differences among groups (1-way ANOVA, $P < 0.05$). Error bars represent standard SEM.

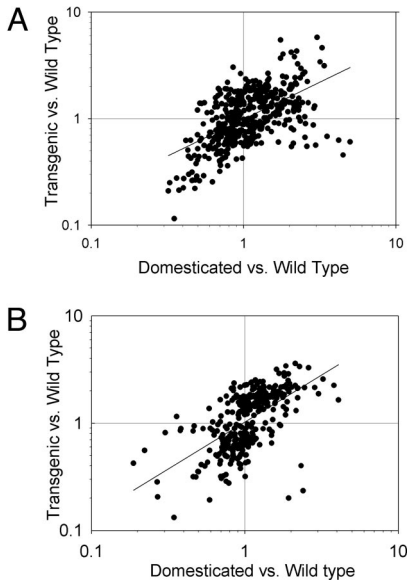

Fig. 2. Spearman rank order correlations between domesticated/wild type gene expression ratio vs. transgenic/wild type gene expression ratio. (*A*) muscle. $n = 456$; $r = 0.555$; $P < 0.001$. (*B*) liver. $n = 321$; $r = 0.640$; $P < 0.001$. Genes with identical IDs but different Accession numbers (Table S1 and Table S2) were counted only once, and gene expression ratios averaged (Table S3, Table S4, Table S5, Table S6, Table S7, Table S8, Tables S9, and Table S10) for correlations. Linear ($\log_{10}$–$\log_{10}$) regression lines are shown.

Results

The domesticated strain used in the present study is of moderately recent origin (mid 1980s) with ≈12 generations of selection in commercial aquaculture, whereas the GH transgenic strain has undergone no directed selection and has been maintained by backcrossing to the progenitor wild strain (28). The transgenic strain is expressing GH in many extrapituitary tissues (from a salmon metallothionein-B promoter/full-length GH gene fusion construct) and shows a high degree of growth stimulation (Fig. 1*A*) relative to wild type (5, 28, 29), whereas the domesticated strain also displayed enhanced growth rates (8), but less than that observed in the transgenic strain (Fig. 1*A*).

Gene expression levels among multiple individuals (matched for size and developmental stage) for each genotype were determined relative to a common wild-type RNA pool, and then subsequently assessed for intergenotype differences in expression for each sequence on the microarray. For known genes on the microarray, expression effects were largely tissue specific, with 94.4% and 86.0% (for domesticated and transgenic fish, respectively) being significantly changed only in liver or only in muscle tissue (Table S1 and Table S2). Overall, more genes (χ^2; $P < 0.001$; common gene IDs pooled, plus unknown genes; Table S1 and Table S2) were differentially expressed between transgenic and wild salmon (521 genes including 106 unknown) than between domesticated and wild salmon (348 genes including 77 unknown). This effect on gene expression in transgenic fish was primarily due to a differential response in liver (271 for transgenic vs. 78 in domestic, χ^2, $P < 0.001$), compared with muscle (320 for transgenics vs. 278 genes for domestic, χ^2, $P = 0.247$). These unequal expression effects were bidirectional in liver, with more genes being either down-regulated or up-regulated in transgenic than domestic fish (χ^2, $P < 0.001$; up-regulated genes: $n = 159$ for transgenic and 40 for domestic; down-regulated genes: $n = 135$ for transgenic and 40 for domestic), whereas no difference in numbers of dysregulated genes was seen between genotypes in muscle (up-regulated genes: $n = 161$ for transgenic and 134 for domestic; down-regulated genes: $n = 165$ for transgenic and 148 for domestic).

As expected, average expression of genes in wild type did not differ greatly from the wild-type RNA pool and were close to unity for both muscle (1.053 ± 0.006) and liver (1.025 ± 0.006). In contrast, average gene expression in domesticated (1.185 ± 0.030 and 1.248 ± 0.099 for muscle and liver, respectively) and GH transgenic strains (1.205 ± 0.035 and 1.311 ± 0.055 for muscle and liver, respectively) were both elevated and differed significantly from wild-type expression (muscle: $P < 0.001$ for both genotypes; liver: $P = 0.017$ for domestic-wild comparison, $P = 0.006$ for transgenic-wild comparison; 1-way ANOVA plus Student Newman Keuls posthoc test; $n = 435$ muscle, 312 for liver). Higher variability seen in the domesticated and transgenic strains than wild-type also reflects effects on gene expression in these 2 growth-enhanced genotypes. The average magnitude of up-regulation or down-regulation (relative to wild type) did not differ in liver between strains (1.937 ± 0.081 vs. 1.855 ± 0.098 for up-regulated genes in transgenic and domesticated fish, respectively; 0.609 ± 0.016 vs. 0.623 ± 0.033 for down-regulated genes in transgenic and domesticated fish, respectively; t tests; $P > 0.05$). For muscle, the expression level of up-regulated genes relative to wild type in transgenic (1.895 ± 0.0580; $n = 161$) and domesticated (1.915 ± 0.058; $n = 134$) fish did not differ, whereas down-regulated genes in muscle were affected more strongly in transgenic (0.622 ± 0.0156; $n = 165$) than domesticated salmon (0.702 ±0.0128; $n = 148$; t test, $P < 0.001$).

A major objective of the present study was to examine whether genes are regulated in discordant or coordinated ways between GH transgenic and domesticated strains. Correlation analysis was performed between transgenic/wild vs. domesticated/wild expression states for all genes found to differ statistically from wild-type salmon. For both muscle ($n = 456$) and liver ($n = 321$), a highly significant positive correlation (Spearman correlation analysis, $P < 0.001$) was observed (Fig. 2 *A* and *B*). More genes in both tissues were affected (χ^2, $P < 0.001$; Table 1) in a corresponding manner (i.e., up in both domesticated and transgenic, or down in both domesticated and transgenic) than showed a discordant expression pattern (up in domesticated and down in transgenic, or down in domesticated and up in transgenic). Thus, for muscle and liver tissue, respectively 68.2% and

Table 1. Number of genes in muscle (top) and liver (bottom) significantly up-regulated (Up) and down-regulated (Down) in domesticated or GH transgenic salmon relative to wild type

Tissue	Transgenic	Domesticated Up	Domesticated Down	*P*
Muscle	Up	164 (57)	79 (4)	<0.001 (<0.001)
	Down	66 (13)	147 (47)	
Liver	Up	135 (49)	38 (6)	<0.001 (<0.001)
	Down	39 (6)	109 (36)	

Numbers in parentheses represent those significant genes that differ by >2-fold in either genotype relative to wild type. Genes with identical IDs but different accession numbers (Table S1 and Table S2) were counted only once.

76.0% of genes showed parallel responses between transgenic and domesticated strains, with 85.9% and 87.6% showing parallel effects for genes altered >2-fold from wild type. A similar overall pattern of correlation is also apparent when all genes on the microarray (27) (i.e., including those not significantly different from wild type) were examined (Spearman correlation, $P < 0.001$).

Analyses of the functional annotations of GH transgenic and domestication-responsive genes revealed that a broad range of physiological functions were affected by domestication and transgenesis in both tissues, with major influences on stress/immune functions, cell and tissue structure, energy production, and protein synthesis (Fig. 3*A* and *B*). In general, most categories of genes showed more concordant than discordant expression effects between domesticated and transgenic fish in both tissues. In liver, carbohydrate and amino acid metabolism effects were completely concordant between transgenic and domesticated strains, whereas in muscle both concordant and discordant effects were observed (strongly so for carbohydrate metabolism). For example, transaldolase mRNA (pentose phosphate shunt) is up-regulated in both muscle and liver (both in microarray and from QPCR data; Fig. 4), presumably to support energy production and DNA synthesis (25). Of the 10 most strongly down-regulated genes in muscle in both transgenic and domesticated fish, 3 are structural (telethonin, kinesin, myosin) and 3 are involved in proteolysis or protein synthesis (cathepsin, hsp30, 12S rRNA). Myosin and β-actin both showed reduced mRNA levels in muscle by Q-PCR analysis, whereas a QPCR normalizer gene (ubiquitin, stably transcribed in all individuals tested) was unaffected (Fig. 4*A*). The 10 most strongly up-regulated genes in both genotypes in muscle include a mixture of functions (lipid metabolism/transport, chromosome structure, proteolysis/protein trafficking, amino acid metabolism, stress/immune function, unknown). For liver, the 10 most strongly up-regulated and down-regulated genes included functions involving transcription/mRNA processing, amino acid metabolism, respiration, stress/immune function, lipid metabolism/transport, organismal physiology, brain/neuron function, cell/tissue structure/matrix, and carbohydrate metabolism. For example, mRNA for iron-sulfur cluster assembly enzyme (important for mitochondrial function) assessed by QPCR (Fig. 4) shows the same direction of effects as seen by microarray analysis.

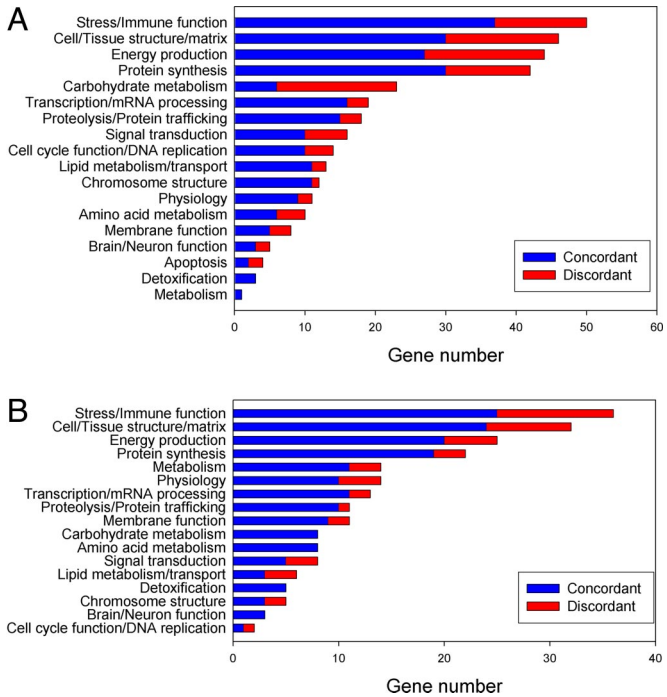

Fig. 3. Comparison of genes affected by domestication and GH transgenesis among different functional pathways. (*A*) muscle. (*B*) liver. Blue bars represent genes showing concordant responses in expression between domesticated and GH transgenic salmon. Red bars represent discordant responses.

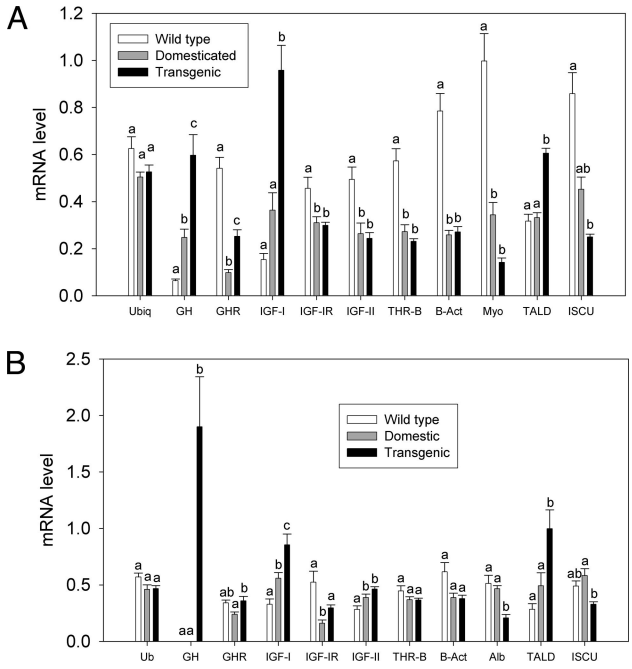

Fig. 4. Levels of mRNA for specific growth-related and control genes in muscle (*A*) and liver (*B*). Letters above bars represent statistical significance among groups with a single gene (1-way ANOVA, $P < 0.05$). See Table S11 for gene abbreviations. Error bars represent SEM

Because domestication and GH transgenesis in salmonids strongly influence growth rate, we also examined the expression of a subset of growth-related loci by QPCR analysis. Levels of mRNA for GH in liver were undetectable in wild-type and domesticated fish, but were enhanced in transgenic strains as expected from extrapituitary expression of GH from the transgene (Fig. 4*B*). In muscle, transgenic salmon also expressed elevated levels of GH mRNA as expected, but both wild-type and domesticated nontransgenic salmon also possessed detectable GH mRNA in this tissue. Surprisingly, the level of muscle GH mRNA in domesticated salmon was >3-fold greater than in wild type (Fig. 4*A*).

IGF1, a major downstream gene responsive to GH and involved in mediating growth, showed elevated liver and muscle mRNA levels in both domesticated and transgenic salmon (Fig.

4). Plasma levels of IGF1 hormone were also found to closely parallel growth rates observed among the 3 strains (Fig. 1*B*). IGF-II, the role of which in mediating growth in salmon is not currently clear, showed a reduction in mRNA levels in muscle and slightly elevated levels in liver. Levels of receptor mRNAs for GH, IGF1, and thyroid hormone were either unaffected or reduced in domesticated and transgenic strains (Fig. 4).

Discussion

Major effects of GH transgenesis and domestication on energy metabolism of carbohydrates, lipids, and protein have been observed, as were effects on protein synthesis, stress and immune function, and cellular structure. These findings are consistent with previous data showing alterations in many processes including nutritional requirements, energetics, muscle fibre structure, and cartilage deposition in transgenic fish (25, 30–35). Complex effects on metabolism and GH receptor expression have also been observed in liver and muscle in rainbow trout treated with GH protein (36).

The present study revealed that effects on gene expression in domesticated and GH transgenic coho salmon strains, relative to wild type, are occurring largely in parallel ways. Microarray analyses showed that the majority of genes that showed changes were affected in concordant ways (for both up-regulated and down-regulated genes) in transgenic and domesticated strains. Further, genes specifically associated with the growth-regulation pathway (GH receptor, IGF1, and IGF1 receptor, thyroid hormone receptor, IGF-II) analyzed by QPCR also showed parallel effects between strains in all cases. Such concordant regulation in genes arising from these 2 distinct genetic processes implies that they share modification of regulatory pathways controlling the expression of genes involved in growth. Gene expression effects in GH transgenic animals clearly arise from elevated extrapituitary expression of GH and its consequent effects on downstream GH-responsive genes and physiologies. Parallel effects on gene expression in domesticated fish strongly suggests that the same downstream pathways are also being affected.

Domestication and directed selection can have very strong effects transforming phenotype from wild type. For example, in rainbow trout, selection underway for over a century has generated domesticated strains with growth rates similar to very fast-growing GH transgenic strains (16), both of which are very growth enhanced relative to wild type. The GH pathway has been implicated in mediating enhanced growth rate in the domesticated strain because treatment of wild and domesticated strains with GH (by transgenesis and by GH protein injection) revealed that the slow-growing wild strain showed much greater growth stimulation than the fast-growing domesticated strain (16). Further, GH-induced abnormalities (e.g., analogous to acromegaly) were induced in domesticated but not wild-type fish (16), suggesting that the former already possessed elevated levels of, or sensitivity to, GH. GH treatments have also been found to be more pronounced in slow-growing than fast-growing strains of channel catfish and Atlantic salmon (16, 37, 38). In these cases, GH no longer appears to be fully rate limiting, but where domestication is incomplete (e.g., coho salmon), GH transgenesis and domestication were found to act additively (16). These data together suggest that fast-growing strains may already have up-regulated GH and/or its downstream pathways such that further stimulation with this hormone is dampened relative to unselected strains. In mice, although effects of GH protein treatment were found to be similar in fast-growing and slow-growing strains (39), GH transgenesis had a greater effect in slow-growing strains (40, 41). Collectively, these phenotypic data suggest that domestication in vertebrates may be using the same genetic and physiological pathways regulated by the GH endocrine axis.

Growth-axis endocrine changes associated with domestication have been investigated in a number of vertebrate species, but results have varied. Circulating levels of GH were elevated in domesticated pigs relative to wild boars in some but not all studies, and IGF1 levels were not found in all studies to correlate with faster growth between boars and pigs or among domesticated strains (42, 43). In domestic dogs, elevated GH (during early life) and IGF1 are associated with large dog breeds (44, 45), and the IGF1 locus has been associated with variation influencing body size (46). Extensive work in domestic chickens and turkeys has found GH and IGF1 to be variably correlated with growth rate or to have an ability to further stimulate organismal or cellular growth (47–50). In fish, fast-growing strains of rainbow trout have not been found to possess elevated plasma or pituitary GH or plasma IGF1 (51), and fast-growing Atlantic salmon showed no change in IGF1 but pituitary and plasma GH were elevated when examined across stages (52). Domesticated rainbow trout and coho salmon have been recently shown to possess elevated circulating hormone and gene expression levels of GH and IGF1 (53), consistent with their enhanced growth rate and the findings of the present study. Despite these variable observations, these data together suggest that the GH/IGF1 axis could be playing a role in some cases during domestication of fish and other vertebrates. The present comparison study also implicates common downstream regulation of cellular and physiological pathways between GH transgenic and domesticated strains.

The mechanism by which GH axis up-regulation is occurring in domesticated fish is currently not clear. The present study has found that both wild type and domesticated (nontransgenic) salmon express detectable GH mRNA in muscle, consistent with previous observations of extrapituitary expression of GH genes (29, 54). Although expression of GH in muscle in wild-type was found to be only $\approx$1/20 that found in the pituitary gland (54), given the large mass of muscle in fish, this expression could contribute a significant proportion of GH production in the body. Further, domesticated salmon expressed muscle GH at levels $\approx$3-fold higher than wild-type salmon, suggesting that increased expression of GH genes in salmonid muscle may have been selected during domestication and be responsible in part for enhanced growth rate seen in these strains.

The number of genes affected, and magnitude of effects, were greater in transgenic animals than in domesticates, a result consistent with the stronger phenotypic transformation from wild type in transgenic than domesticated salmon (8, 28). Stronger effects on gene expression in transgenic strains may arise from dysregulation of pathways coping with a significant genetic alteration occurring in a single generation, as opposed to domestication, which arises through gradual selection at multiple loci over many generations, allowing maintenance of homeostasis. Genes showing discordant patterns of expression between domesticated and GH transgenic fish are intriguing as they are candidates for pathways causing some of the pleiotropic morphological and physiological abnormalities that have been described in GH transgenic salmon (55) that arise from overexpression of GH and the transgene's inability to respond to normal negative feedback regulatory controls that operate in wild-type fish.

The present findings have significance for experimental approaches designed to modify the phenotype of domesticated organisms that have been previously selected (directly or indirectly) for the trait of interest. If natural allelic variation has been selected during domestication to an extent where genes controlling the phenotype are no longer rate limiting, or where their further expression induces abnormalities (21, 23, 33, 56), then further augmentation of their expression by transgenesis may not yield further beneficial phenotypic change (23). In this case, modification or creation of alterna-

tive pathways, and/or targeting other points in the pathway, which may have become rate limited during domestication, may allow enhancement of phenotype.

The present data also have implications for food safety and environmental risk assessments of transgenic and domesticated organisms (55, 57). In some jurisdictions, genetically modified organisms are regulated based on the process by which they are generated, whereas in other cases regulation is product based (i.e., assessment of the characteristics of the organism rather than of the process by which it was made). Multiple generation fitness information is emerging (11) for domesticated (nontransgenic) strains from natural environments (e.g., fish stocked into natural lakes or used in oceanic net pens) facilitating the use of such strains as comparators for risk assessment of transgenic fish. Such analyses may benefit from studies examining to what extent transgenic and domesticated organisms are being phenotypically altered by substantially similar mechanisms.

Materials and Methods

Strains of coho salmon (*Oncorhynchus kisutch*) analyzed include: (*i*) wild-type salmon from the Chehalis River, (*ii*) a domesticated strain that has been found to possess a higher growth rate than several wild-type coho strains (8), and (*iii*) hemizygous growth hormone transgenic salmon containing a MT-B promoter/GH gene fusion [strain M77, F_6 generation (5, 28)]. Fish were size and developmentally matched in October 2006, at which time RNA was isolated and subjected to microarray analysis as described (25, 27), using 5 individuals per genotype (one slide per individual) against a common wild-type RNA pool. For both liver and muscle tissue (Table S1 and Table S2, respectively), genes with significant differences were determined by comparison of transgenic and wild type or domestic and wild type within the GeneSpring software ($P < 0.05$). Functions for significant genes for discordant and concordantly regulated genes for each tissue are shown in Tables S3 to S10. Functions were assigned using information from GO terms within the Gene Ontology (www.geneontology.org) and UniProt (www.uniprot.org) websites, and by DAVID/EASE analysis (http://david.abcc.ncifcrf.gov/). The data discussed in this publication have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (accession no. GSE13846). Q-PCR analyses were performed on 10 individual fish per genotype using primers (Table S11) designed to conserved regions of salmonid genes. Statistical analyses of Q-PCR data were performed by One-Way ANOVA followed by Student-Newman-Keuls post hoc test. Plasma IGF1 was measured using a kit from GroPep (Adelaide, Australia) as described by the manufacturer, and analyzed using 1-way ANOVA. Further detailed information on experimental procedures is provided in *SI Materials and Methods*.

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