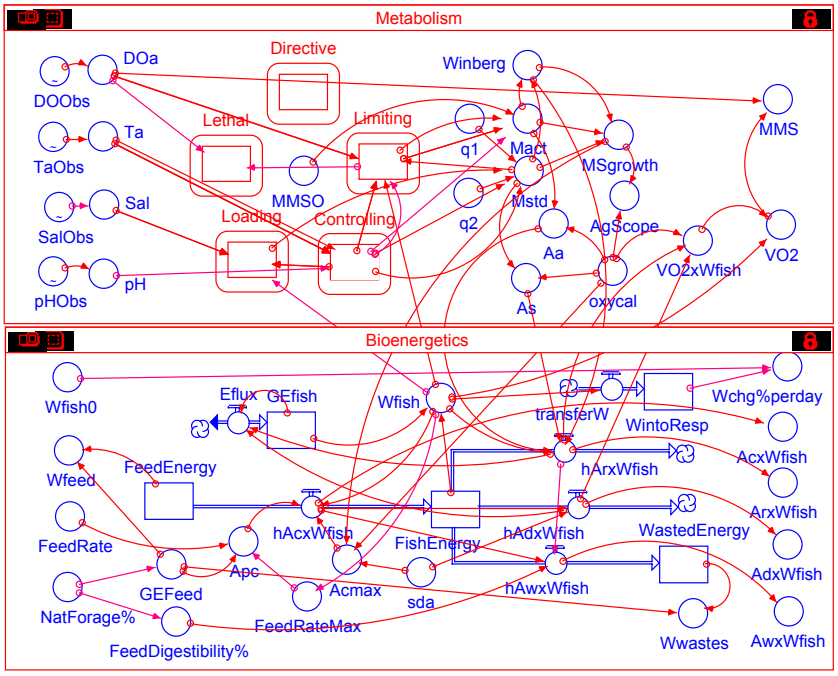
# *Developments in Understanding Fish Growth*





**Brian Small**

**Don M^ac^Kinlay**



*International Congress on the Biology of Fish*
*University of British Columbia, Vancouver, CANADA*

# *Developments in*

# *Understanding Fish Growth*

SYMPOSIUM PROCEEDINGS

**Brian Small**

**Don M^ac^Kinlay**

*International Congress on the Biology of Fish*
*University of British Columbia, Vancouver, CANADA*

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# PREFACE

Organismal growth is a complex process. This symposium is meant to focus attention on how different tissues and associated pathways act and interact to regulate growth of the organism, in this case fish. We know that fish growth is mediated by a variety of factors through autocrine, paracrine, and endocrine mechanisms. While growth is allometric, different tissues growing at different rates, tissues as diverse as brain, liver and muscle contribute factors to a network of signals that regulate organismal growth. Fundamental and applied aspects of how elements of growth regulation fit into the framework of fish growth are explored.

Symposium Organizers:

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Don MacKinlay
Congress Chair

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**ENDOCRINE CONTROL OF GROWTH: A WHOLE-FISH**

**PERSPECTIVE AND EVOLUTIONARY CONSIDERATIONS**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

The major endocrine axis controlling growth in vertebrates consists of pituitary growth hormone (GH), insulin-like growth factor-I (IGF-I) and IGF binding proteins (IGFBPs). Most reviews of this system depict a hierarchical organization (figure 1), in which GH released by the pituitary stimulates hepatic production of IGF-I. IGF-I circulates in the blood bound to the IGFBPs with approximately 1% or less of the IGF-I in the free form (not bound to IGFBPs). IGF-I is a potent mitogenic factor and also maintains cell number by reducing apoptosis. Comprehensive reviews of this axis in fish are available (Duan, 1997; Moriyama, 2000, Kelly et al. 2000). The conventional depiction of the GH-IGF axis (figure 1) gives the impression that there is a top-down hierarchy of events for endocrine control of growth; GH stimulates IGF and then they both enhance growth. Although this depiction of the GH-IGF axis has heuristic value, it does not give an accurate impression of the operation of the axis and may lead to an overemphasis of the role of GH production in controlling growth. The aim of this review is to present different perspectives of the GH-IGF axis and speculate on the importance of its constituents in controlling growth.

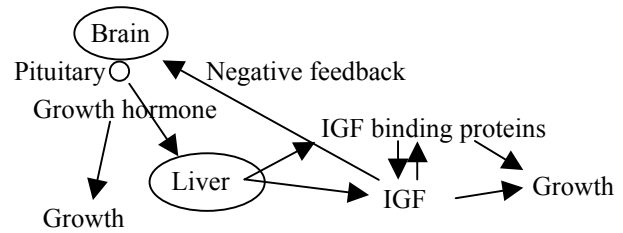


Figure 1. Conventional depiction of the GH-IGF axis.

**A whole-fish perspective**

It is well established that the primary determinants of fish growth are nutrition (ration and energy content) and temperature. Secondary influences on growth include photoperiod in temperate fishes, stress, and reproductive status, among others. For nutritional effects, experiments on fish involving either feeding and fasting or nutritional modification by changing ration or dietary protein level reveal generally consistent effects on the GH-IGF axis. Fasting causes a decline in plasma IGF level and an increase in plasma GH. Dietary modification shows similar effects, for example, Perez-Sanchez et al. (1995; see reviews cited for refs) showed that feeding gilthead seabream isocaloric diets with varying protein content (35, 45 and 55% protein) resulted in graded increases in plasma IGF and graded decreases in plasma GH. The decrease in plasma GH in fasting or dietary protein restriction seems paradoxical, since it may be expected that plasma growth hormone should increase in nutritional states of enhanced growth. However, the enhanced IGF production (plasma IGF levels) during enhanced growth is due, at least in part, to an increase in GHR in the liver. Dietary protein increases plasma amino acids, which enhance hepatic GHR. The increased hepatic GHR makes the liver more sensitive to the stimulatory effects of GH despite decreased total plasma GH. High plasma IGF reduces pituitary release of GH by negative feedback to the brain hypothalamus and pituitary. The decline in plasma GH in response to fasting has been seen almost universally in vertebrates (except in some rodents). The results suggest that GH production may be reduced in high nutritional state. The influence of nutrition on the GH-IGF axis is shown in Figure 2.

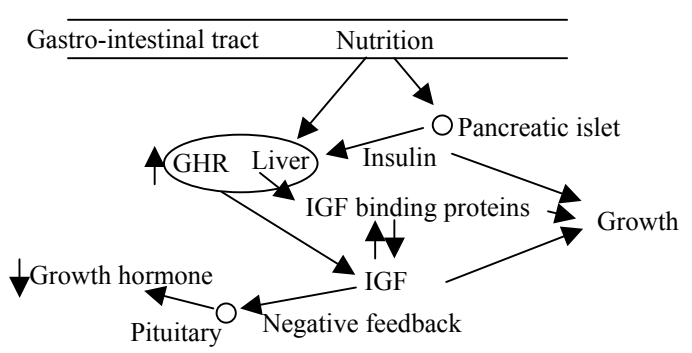


Figure 2. Nutritional effect on GH-IGF Axis.

**What are the relative roles of GH and IGF in controlling growth?**

Often cited evidence for the importance of GH in growth is from experiments with hypophysectomy and hormone replacement, in which replacement with GH has been found to be more effective than replacement with IGF alone. Additional evidence comes from GH transgenics, which show substantially enhanced growth. Although these experiments are informative, hypophysectomized and transgenic animals are extreme manipulations. Recent studies of hormone and hormone receptor knock-outs in mice, although still artificial, may be more informative of how the axis works, at least in mice. Lupu et al., (2001) have made GHR, IGF-I and double mutant knock-outs and compared resultaning growth inhibition. They estimated that the GH-IGF axis accounts for 83% of total body growth, with IGF alone accounting for 35%; GH and IGF together accounting for 34%, and GH alone accounting for 14%. In hamsters, which are insensitive to GH, the contribution of GH to total body growth is zero. The IGF-independent contribution of GH to growth of fish is not known. However, GH clearly has a role in regulation of seasonal growth. In salmonids, it is well-established that increasing photoperiod enhances GH release. In addition, GH or GHR deficiencies would be excellent candidates for a role in dwarf forms of fishes, which is a largely unexplored endocrine topic in fish.

**Evolutionary considerations**

A better understanding of the evolution of the GH-IGF axis may shed light on its functional aspects. The evolution of IGF-I as a separate entity from insulin appears to have occurred in tunicates (McRory and Sherwood, 1997). Since there is no evidence for GH in the suggested pituitary precursor, Hatchek's pit of Branchiostoma, then pitutiaty control of IGF may have first occurred in the vertebrate agnathans. The IGFBPs may have first occurred in agnathans, as well, although the earlier evolution of IGFBPs is attractive in view of their association with Hox genes, which began proliferating in cephalochordates. It could be speculated that there may have been a need for control of axial skeletal growth apart from softer tissue tissue growth for the insulin-IGF dichotomy. Alternatively, separation of tissue hypertrophy (insulin) from hyperplasia (IGF) could also have been adpative. The evolution of IGF-II presumably from IGF gene duplication appears to have occurred in elasmobranchs, which coincides with the appearance of vertebrate viviparity. This last step may have allowed separate control of embryonic and fetal growth.

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# INTRACELLULAR INTEGRATION OF MULTIFACTORIAL NEUROENDOCRINE REGULATION OF GOLDFISH SOMATOTROPE FUNCTIONS

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**Abstract**

Cellular functions of somatotropes are controlled by multiple stimulatory and inhibitory neuroendocrine factors. In goldfish, known stimulatory regulators for growth hormone release include two endogeneous gonadotropin-releasing hormones (GnRHs), dopamine (DA), and pituitary adenylate cyclase-activating polypeptide (PACAP). Inhibitory regulators include somatostatin, of which three endogenous isoforms are known. We have used primary cultures of goldfish somatotropes as a study model to investigate how agonist- and function-specificity can be mediated at the level of intracellular signal transduction. Extracellular $Ca^{2+}$ entry, mobilization of $Ca^{2+}$ from TMB8-sensitive intracellular stores, and activation of calmodulin kinase are all involved in the GH release responses to the two endogenous GnRH forms, DA and PACAP. In contrast, cAMP/PKA-dependent pathways selectively mediate DA and PACAP stimulation of GH release, whereas $Na^{+}/H^{+}$ antiport and PKC-dependent mechanisms participate in GnRH-induced GH secretion. How GnRH actions on hormone secretion are coupled to the nitric oxide and cGMP signalling cascades also differ from those of DA and PACAP. Similarly, the

differential ability of three endogeneous somatostatin isoforms to inhibit basal and stimulated GH release is reflected in their selective ability to modulate the GH response to individual signalling pathways. Other pharmacological studies reveal the presence of several intracellular $Ca^{2+}$ compartments. Selective manipulation of these stores differentially modulates basal GH secretion, cellular contents and mRNA levels, as well as the release responses to the two GnRHs. The multiplicity of signal transduction cascades and the complexity of $Ca^{2+}$ stores provide the basis upon which functional and ligand specificity of multiple neuroendocrine signals may be integrated at the intracellular level. Together, these mechanisms may allow for the selective control of somatotrope functions appropriate for a particular situation in growth and metabolism.

## Introduction

Growth hormone (GH) is a key regulator of growth, metabolism, and reproduction in teleost fishes (Peter and Chang, 1999). In goldfish, both stimulatory (gonadotropin-releasing hormone (GnRH), dopamine (DA), GHRH, thyrotropin releasing hormone, pituitary adenylate cyclase activating polypeptide (PACAP), neuropeptide Y (NPY), bombesin, cholecystokinin (CCK), galanin, activin, and inhibin) and inhibitory (somatostatin (SS), serotonin (5HT), and norepinephrine (NE)) neuroendocrine regulators control GH secretion at the level of the pituitary (Peter and Chang, 1999; Chang et al., 2000). In addition to having a number of neurosecretory factors, diversity in ligand isoforms, receptors and post-receptor signalling allow selective modulation of the pathway from synthesis to secretion of hormone.

**Diversity of peptides and receptors subtypes**

In goldfish, two isoforms of GnRH (salmon (sGnRH) and chicken-II (cGnRH-II)) play a critical role in GH secretion (reviewed in Chang et al., 2000). Moreover, two receptor isoforms for the goldfish GnRH receptor have been recently cloned and shown to be present in goldfish pituitary (Yu et al., 1998; Illing et al., 1999). Both receptors recognize both endogenous GnRH's. However, the partially selective nature of their signal transduction cascades in somatotropes suggests that there must either be some selectivity in receptor-mediated G-protein activation or that a third pituitary cGnRH-II-specific receptor, which has been found among other vertebrates, may exist in goldfish.

The somatostatin (SS) system also exhibits diversity in both peptides and receptors (Lin et al., 2001; reviewed in Lin and Peter, 2001). Three forms of brain SS mRNA ($SS_{14}$, $[Pro^2]SS_{14}$ and $gfSS_{28}$) have been detected in the hypothalamus. Three SS receptor types ($Sst_1$, $Sst_2$, $Sst_5$) are expressed in goldfish pituitary and exhibit selectivity for the SS isoforms. For example, $SS_{14}$ and $[Pro^2]$ $SS_{14}$, but not $gfSS_{28}$ appear to activate $Sst_2$ receptors (Lin et al. 2000). Moreover, $gfSS_{28}$ appears to be slightly more potent in binding $Sst_5$ receptors than either $SS_{14}$ or $[Pro^2]SS_{14}$ (Lin et al., 2001). This variable affinity of receptors for the different SS isoforms clearly offers mechanisms of selectivity within the SS regulatory system (see below).

**Divergence of signal transduction pathways**

GH secretion in goldfish is under the control of two minimally overlapping signalling cascades, which display selective activation by specific neuroendocrine regulators. Divergences between these pathways are outlined in Figure 1. For example, the two endogenous GnRH's (sGnRH & cGnRH-II) utilize the protein kinase C (PKC) pathway and display a dependence on the $Na^+/H^+$ antiporter. In contrast, both DA-D1 and PACAP-PVR1 receptors activate the adenylate cyclase-cyclic AMP (cAMP)-protein kinase A (PKA) system, as well as arachadonic acid signalling (Chang et al., 2000). In addition, extracellular $Ca^{2+}$ entry through voltage-sensitive $Ca^{2+}$ channels (VSCC's) and calmodulin kinase activation appear to play a role in both GnRH/PKC and DA/PACAP/cAMP signalling. However, whether the link between these two signalling pathways, VSCC's, and secretion is direct or indirect (i.e. through $Ca^{2+}$ stores) remains to be explored.

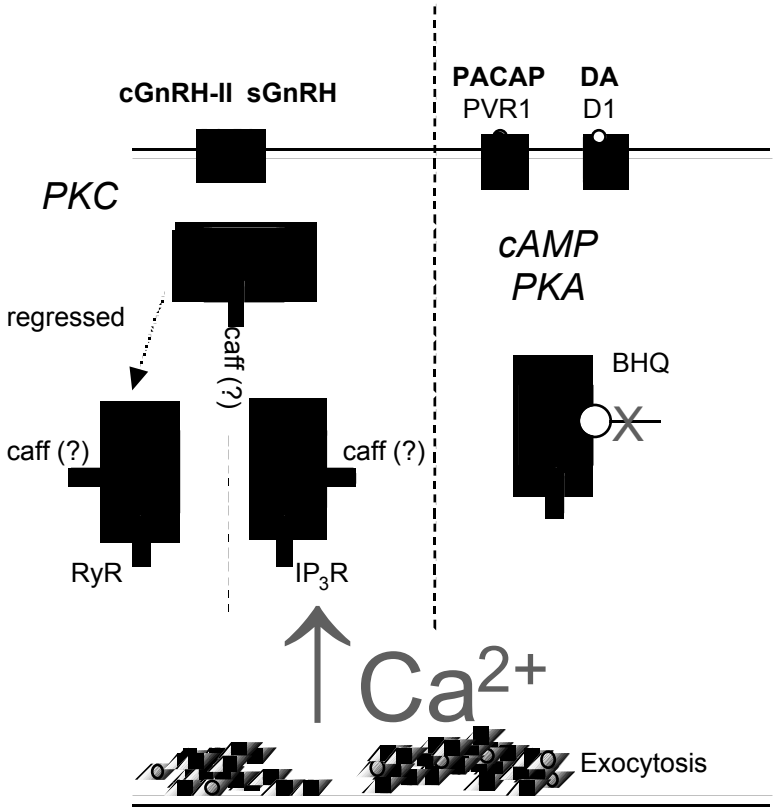


Figure 1 – Divergence in $Ca^{2+}$ stores and major signalling cascades for stimulated growth hormone release in goldfish.

Recently, we have demonstrated the presence of a number of pharmacologically distinct intracellular $Ca^{2+}$ stores, which differentially participate in mediating the secretory response elicited by neuroendocrine regulators (Johnson and Chang, 2000). The involvement of $IP_3$ $Ca^{2+}$-release channels in secretion stimulated by sGnRH, but not cGnRH-II, suggests divergence in pathways between the two GnRH's (Johnson and Chang, 2002). The participation of caffeine-sensitive stores in GnRH, but not DA, action on GH secretion also suggests that there is differential coupling of PKC and cAMP signalling cascades to pharmacologically distinct intracellular $Ca^{2+}$ stores (Wong et al., 2001).

Further divergence among pathways, has been shown by using classical inhibitors of SERC ATPases (e.g., thapsigargin and BHQ). In particular, BHQ-sensitive stores do not appear to be involved in GnRH-evoked release (Johnson and Chang, 2000), which is in contrast with their participation in both PACAP- and DA-stimulated release (unpublished observations). However, there is some shared pharmacology between the two stores. In particular, the general intracellular $Ca^{2+}$ channel blocker, TMB-8 appears to inhibit both GnRH and PACAP/DA signalling.

The nitric oxide synthase (NOS)/cGMP signalling pathway also appears to be involved in GnRH signalling yet plays only a minor role in DA signalling (Uretsky and Chang, 2000). Moreover, NOS signalling may be even less involved in PACAP stimulation of GH secretion (unpublished observations).

Divergence of signalling pathways is also present among inhibitors of GH release. For example, NE appears to inhibit cAMP signalling (Yunker et al., 2000), whereas 5HT does not (unpublished observations). Similarly, whereas both $SS_{14}$ (Kwong and Chang, 1997) and $[Pro^2]SS_{14}$ abolish cAMP-induced GH secretion, $gfSS_{28}$ has no effect on cAMP-induced GH release. Such specificity provides a mechanism for selective inhibition of individual GH releasers. Indeed, $gfSS_{28}$ appears to be less effective in inhibiting endogenous secretagogues that are cAMP-dependent than are other SS isoforms (unpublished observations). Suprisingly, neither NE nor $SS_{14}$, both of which inhibit basal and stimulated secretion, do not reduce resting $[Ca^{2+}]_i$ (Yunker et al., 2000; Yunker and Chang, 2001). This would suggest that stimulated and basal secretion are quite different in their reliance on $[Ca^{2+}]_i$ and signalling cascades.

**Control of synthesis**

Exploration of the control of GH mRNA expression by a variety of groups has revealed that GH mRNA levels are subject to a variety of very specific control mechanisms which are not necessarily linked, one-to-one, with hormone secretion. For example, SS, which is a potent inhibitor of secretion, does not alter GH mRNA in tilapia and trout (Melamed et al., 1998). Similarly, we have recently shown that perturbations in $Ca^{2+}$ homeostasis appear to dissociate secretion of GH from synthesis of GH (Johnson et al., 2002). Activation of VSCC's stimulates GH secretion but is accompanied by a reduction in GH mRNA. However, activation of ryanodine receptors rapidly stimulates GH release and increases $[Ca^{2+}]_i$, but does not affect GH mRNA levels. Conversely, selective inhibition of thapsigargin-sensitive $Ca^{2+}$ ATPases elevates intracellular $Ca^{2+}$, does not affect GH mRNA, yet inhibits short-term secretion. Moreover, cellular GH protein content can be differentially affected by manipulations of ryanodine- and thapsigargin-sensitive stores, without altering GH mRNA levels (Johnson et al., 2002).

**Seasonal variation in GH release**

The regulation of GH release is subject to tremendous seasonal variation (Figure 2) displaying differential activity throughout the animal's reproductive cycle (Peter and Chang, 1999; unpublished observations). DA, CCK, and GHRH are most effective in sexually regressed animals whereas PACAP acquires prominence during stages of sexual recrudescence. Finally, during latter stages of recrudescence, maturation and spawning, GnRH, TRH, and NPY are at their most effective. Similarly, the inhibitory tone of SS, which is prominent among sexually regressed fish, decreases during recrudescence out of phase with NE, which is most effective in sexually mature animals. These seasonal variations in the control of GH release are paralleled by variations in intracellular $Ca^{2+}$ stores (Johnson and Chang, 2002). In particular, ryanodine-sensitive stores, which selectively participate in cGnRH-II signalling, appear to be present only among somatotropes from sexually regressed fish.

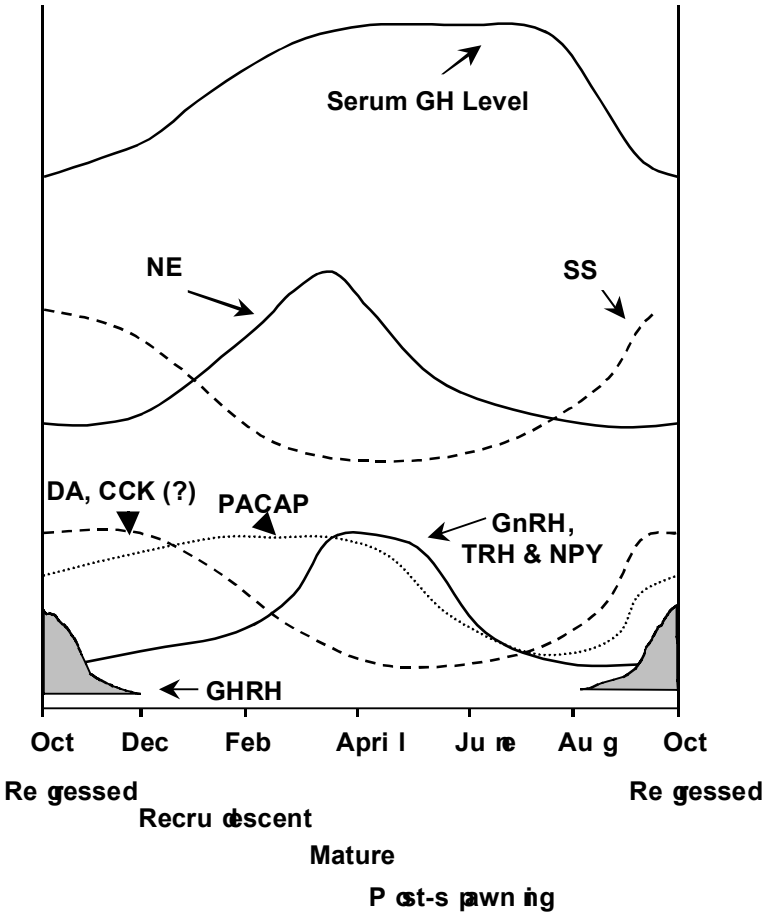


Figure 2 – Seasonal variations in neuroendocrine regulation of growth hormone release in goldfish.

**Conclusions**

Goldfish somatotropes are clearly subject to a variety of neuroendocrine modulators, which provide considerable complexity in regulation of cell function. While there is evidence to support the presence of somatotropes that

respond to multiple stimulatory regulators, we cannot discount the possibility that there may be somatotropes that are responsive to only a select few regulators. Nonetheless, the framework of transmitters and regulatory peptides, receptors and intracellular signalling pathways, including $Ca^{2+}$ stores, provides highly selective regulation of cellular function. As a result, basal GH release, stimulated GH secretion, GH protein synthesis and GH mRNA expression, can be individually fine-tuned by neuroendocrine regulators to meet the continually changing demands for growth and metabolism throughout the reproductive cycle of the animal.

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# DISPARATE MECHANISMS OF GROWTH AND OSMOREGULATION IN COMMERCIALLY IMPORTANT TELEOSTS

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

## Introduction

Recent studies have shown that the pituitary hormones, growth hormone (GH), prolactin (PRL), and the insulin-like growth factors, play well-defined roles in teleost osmoregulation and growth. However, there is a large degree of functional overlap among these hormones. As a consequence, it is often difficult to study the endocrine regulation of a single physiological process without considering how these hormones simultaneously influence other physiological sectors. Adding to this complexity are environmental, physiological, nutritional and social factors that influence levels of these hormones in fish. The idea that a single hormone (e.g., PRL) can regulate multiple physiological pathways suggests that alterations in its regulation may impact these pathways in unpredictable ways. For example, changes in aspects of water quality (salinity), during critical life-history stages, can alter levels of GH and PRL thus affecting growth and development (e.g., stunting in salmonids) in economically-important teleosts.

The endocrine regulation of growth and adaptation in yellow perch (*Perca flavescens*) and channel catfish (*Ictalurus punctatus*) are poorly understood and efforts in our laboratory are thusly focused on characterizing these endocrine pathways. Presently, we are developing the endocrine tools needed to study the estrogen-dependent (Malison *et al.*, 1985), sexually-dimorphic growth patterns (females grow faster and larger than males) seen in yellow perch. As for channel catfish, we (Tang *et al.*, 2001) have recently shown that GH and PRL mRNA levels change in predictable ways when exposed to different salinities. However, we have not been able to show that GH has any hypoosmoregulatory actions in this teleost (Eckert *et al.*, 2001; Tang *et al.*, 2001). Consequently, the

significance of elevated GH mRNA levels (osmoregulatory actions of GH) following transfer to 8 ppt saline water (Tang *et al.*, 2001) is unknown. Further still, the growth-promoting actions of heterologous GH in channel catfish are not consistent with those reported in other teleosts (Wilson *et al.*, 1988). Against this background, we are studying the endocrine physiology of growth and adaptation in both species. Herein, we report preliminary findings in channel catfish.

**Methods:**

***In vivo Experiments:***

Animals were held in flow-through fresh water and given intraperitoneal injections of vehicle (corn oil) alone or vehicle containing the GH secretagogues KP-102 (D-Ala-D-*B*-Nal-Ala-Trp-D-Phe-Lys-$NH_2$) (Kaken Pharmaceuticals, Tokyo, Japan) or $bGHRH_{1-29}$-amide (Sigma Chemical, St. Louis, MO) at a dose of 100 ng/g bw. Recombinant bGH (bovine GH) and bPL (bovine placental lactogen) (Monsanto, St. Louis, MO) were administered at a dose of 1μg/g bw. Injections were given every 6 days for 18 days.

Seven days after the third injection, ½ of the animals were sampled for plasma. Remaining animals were transferred to 15 ppt seawater (SW) over the course of forty-eight hours by diluting natural SW with fresh municipal water. Animals were sampled for plasma twenty-four hours after the adjustment to 15 ppt SW. Plasma osmolality was determined using a vapor pressure osmometer (Wescor, Logan, Utah), whereas plasma GH levels were determined using a newly developed, homologous, ELISA.

**Results and Discussion:**

The effects of rbGH and rbPL, KP-102 and bGHRH on the growth, osmoregulatory and endocrine physiology of channel catfish were examined. We found that rbGH and rbPL significantly elevated body weight when compared with controls, whereas the GH secretagogues appeared to reduce body weight (data not shown). The osmoregulatory response of treated channel catfish (in 15 ppt SW) was unexpected (Figure 1). Compared with control values, all the treatment groups displayed significant increases in plasma osmolality that are consistent with the $Na^+$-retaining actions of PRL. At the time of this study, we did not know whether GHRH/KP-102 treatment would affect GH levels in catfish, thus these results were difficult to interpret. Recently, we have developed a homologous ELISA (with collaborators) to measure plasma GH levels in channel catfish. Our findings demonstrate that transfer of catfish to

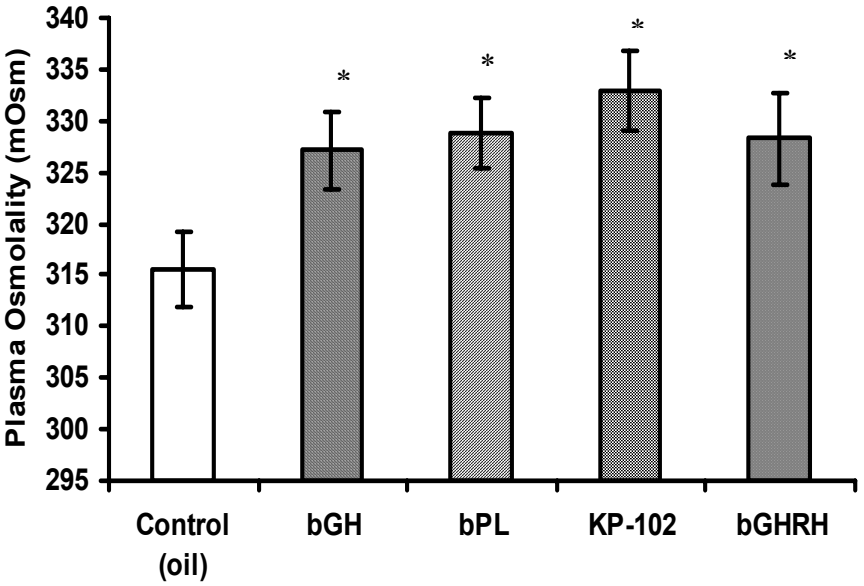


**Figure 1:** Effects of hormone treatment on plasma osmolality in channel catfish transferred to 15 ppt seawater. Values are means ± SEM (n = 20/treatment). *P < 0.05 compared with control value (Fisher's Protected Least Significant Difference Test).

15 ppt SW elicits an increase in plasma GH levels that are further augmented (in FW or 15 ppt SW) by treatment with the GH secretagogues (Figure 2). In this study, plasma GH levels and plasma osmolality were elevated suggesting that native and/or heterologous GH may bind to the catfish PRL receptor, thus eliciting a PRL-like response. While this hypothesis is untested, it is somewhat analogous to findings wherein the variant form of tilapia prolactin ($tPRL_{177}$) was shown to bind to the tilapia GH receptor (Shepherd *et al.*, 1997). This possibility, and others, will be examined in future studies.

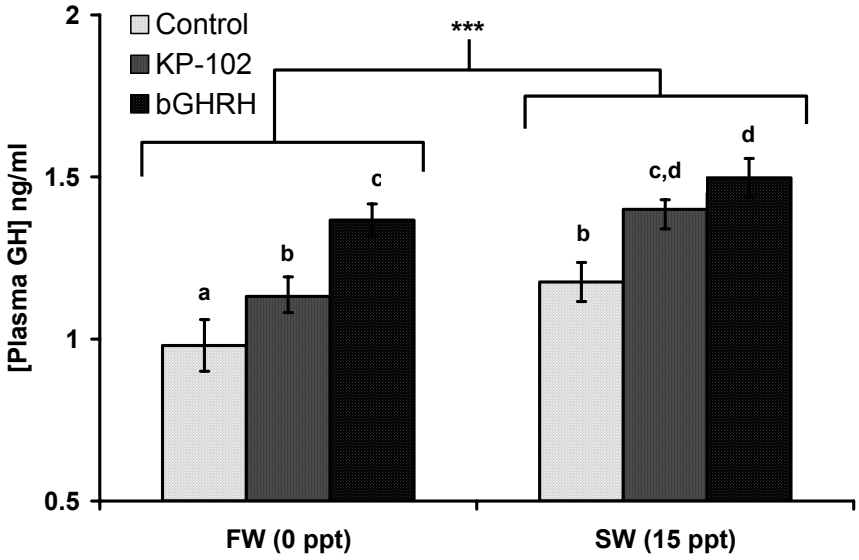


**Figure 2:** Effects of environmental salinity and GH secretagogues (KP-102 and bGHRH) on plasma GH levels in channel catfish. Plasma GH levels were determined using a homologous ELISA. Values are means ± SEM (n = 20/treatment). ***P<0.005, FW vs. 15 ppt SW groups (two-way ANOVA). Groups with different letters are significantly (P<0.05) different (Fisher's Protected Least Significant Difference Test).

**Acknowledgements:**

We thank Steve Eckert, Steven Shimoda, Dr. E.G. Grau, Dr. T. Hirano, Dr. S. Moriyama, Dr. J. Silverstein and Dr. B. Small for their assistance. We also wish to thank Kaken Pharmaceuticals (Tokyo, Japan) for their gift of KP-102. This work was supported by grants from the USDA/NRICGP (97-352206-5094 and 2002-35206-11629) and the USGS/KWRRI (01HQGR0133) to B.S.S. The views expressed herein are those of the author and do not necessarily reflect the views of the U.S. Government.

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# PATTERNS OF GROWTH AND NUTRIENT DEPOSITION IN BROOK TROUT (*SALVELINUS FONTINALIS)*, LAKE TROUT (*SALVELINUS NAMAYCUSH*) AND THEIR HYBRID, SPLAKE (*S. FONTINALIS* X *S. NAMAYCUSH*) AS A FUNCTION OF TEMPERATURE

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

## Introduction

Brook trout (*Salvelinus fontinalis*), lake trout (*Salvelinus namaycush*) and their hybrid, F1 splake (*Salvelinus namaycush* X *Salvelinus fontinalis*) account for 80-90% of total fish stocked in lake rehabilitation efforts of the Ontario Ministry of Natural Resources (OMNR). Yet, little information exists in the literature on the patterns of growth and carcass composition for these species (Sadler *et al*., 1986). Such information could be useful for the development of bioenergetics, nutrient-flow, feed requirement, and waste output models for these fish species. These models can allow fish culturists to estimate such factors as time to stocking size, feed requirement, standing biomass, waste output, etc. There have been many attempts to mathematically describe growth of other fish species using a large diversity of approaches and concepts (Iwama and Tautz, 1981). These attempts, however, are often made without reference to water temperatures. Fish are poikilotherms and as such, water temperature is the major factor regulating their metabolic rate and energy expenditure. Fish growth tends to increase as temperature increases within an optimum range of temperatures (Sadler *et al*., 1986). Brook trout and lake trout have different thermal optima for growth which, may result in significantly different patterns of growth at

different temperatures. F1 splake offers an opportunity to compare the effects of temperature on growth of a hybrid with its parents. Water temperature vary greatly between the fish culture stations of the OMNR contributing to a wide range of growth rates observed for the species cultured.

The objectives of this study were to examine 1) growth of juvenile brook trout, lake trout and splake 2) the effect of water temperature on the growth of the three species 3) the pattern and cost of nutrient deposition in the three species as a function of temperature.

**Materials and Methods**

Triplicate groups (100 fish per group) of each species, initial body weight 2.5, 3.3 and 4.1g for lake, brook and splake respectively, were raised at 5, 10 and 15$^{o}$C over a 16-week period. Fish were fed three times per day to near-satiation with MNR98HS starter diet (ca. 50% protein, 17% lipid). Temperature was monitored continuously; feed intake was measured weekly and weight gains every 28 days. Carcass samples, taken at 0, 8 and 16 weeks, were analysed for moisture, crude protein, lipid and ash contents.

**Results**

Live weight gain increased as temperature increased for all species. Brook trout weight gain was highest, splake intermediate and lake trout lowest at each temperature (Table 1). In all cases the hybrid, F1 splake, performed intermediately between its parents.

Table 1. Weight gain (g/fish) of brook trout, lake trout and splake at each temperature

| | 5$^{o}$C | 10$^{o}$C | 15$^{o}$C |
|---|---|---|---|
| Brook trout | 8.3 ± 0.1 | 19.7 ± 0.4 | 38.3 ± 0.6 |
| Splake | 4.7 ± 0.2 | 12.7 ± 0.3 | 18.7 ± 1.1 |
| Lake trout | 3.8 ± 0.2 | 8.8 ± 0.17 | 12.2 ± 0.5 |

Iwama and Tautz (1981) showed, for rainbow trout, that a cubic root transformation of live weight resulted in a linear function whose slope increased linearly with increasing temperature. This observation resulted in the development of a growth model known as the Thermal-unit Growth Coefficient (TGC = 100 x (Final body weight$^{1/3}$ - Initial body weight$^{1/3}$)/ Sum (Days * Temp

(°C)). Results indicate that brook trout growth at 5, 10 and 15°C was well described by the TGC model. However, for lake trout and splake, the slope of the curve decreases between 10 and 15°C and was not well described by the TGC model. Modification of the TGC model is needed to better describe the growth of these species at different temperatures.

Carcass analysis (Table 2) showed an effect of final body weight on carcass contents. Accordingly, analysis was performed using final body weight as a covariate. An effect of species ($P<0.05$) and temperature ($P<0.06$) on moisture contents was evident. The relative percentage of which, decreased with increasing temperature for all species. Trends in crude protein contents were shown to be species specific, only lake trout showed an increase in crude protein with increase in temperature. Trends in lipid contents were also shown to be species specific. A linear increase of lipid contents with increasing temperature was evident for brook trout ($P< 0.05$) but no such trends were evident for lake trout or splake. No species or temperature trends were evident for ash contents.

Table 2. Final carcass composition, percentage of live weight, of brook trout lake trout and splake at each temperature

| | | 5 °C | 10 °C | 15 °C |
|---|---|---|---|---|
| Brook | $H_20$ | 77.0 ± 0.2 | 75.9 ± 0.1 | 74.9 ± 0.2 |
| | CP | 14.5 ± 0.0 | 14.9 ± 0.1 | 15.3 ± 0.0 |
| | LIPID | 5.9 ± 0.2 | 6.7 ± 0.1 | 7.4 ± 0.2 |
| | ASH | 2.0 ± 0.0 | 2.0 ± 0.0 | 2.1 ± 0.0 |
| Lake | $H_20$ | 80.1 ± 0.1 | 78.7 ± 0.1 | 79.4 ± 0.1 |
| | CP | 15.4 ± 0.1 | 15.9 ± 0.2 | 15.0 ± 0.1 |
| | LIPID | 2.7 ± 0.1 | 3.4 ± 0.1 | 4.6 ± 0.1 |
| | ASH | 2.1 ± 0.0 | 2.1 ± 0.0 | 2.2 ± 0.0 |
| Splake | $H_20$ | 78.9 ± 0.1 | 77.6 ± 0.2 | 76.5 ± 0.3 |
| | CP | 15.3 ± 0.1 | 15.0 ± 0.2 | 16.3 ± 0.1 |
| | LIPID | 3.8 ± 0.1 | 4.7 ± 0.2 | 5.6 ± 0.3 |
| | ASH | 2.1 ± 0.0 | 2.1 ± 0.0 | 2.1 ± 0.1 |

The information gathered from this project will now be used to develop models of nutrient deposition and to extend the applicability of the bioenergetics, feed requirement, and waste outputs models of Cho (1992) and Cho and Bureau (1998) to these fish species.

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# THE SEX RATIOS AND GROWTH STRATEGIES OF WILD AND CAPTIVE JAPANESE EELS *Anguilla japonica*

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**Abstract**

The role of gender and sexual differences in the growth histories of Japanese eel *Anguilla japonica* were linked with respect to the sex ratio and growth rate of wild eels collected from Taiwanese rivers. The sex ratio of wild eels was compared with that of eels semi-intensively cultured in a pond and intensively cultured in an aquarium. The sex ratio of wild eels from a low-density river habitat was dominated by females (86.4% of sex-determined eels), slightly dominated by males (57.1%) in a cultured pond, and dominated by males (90.0%) in an aquarium. This evidence supported the hypothesis that the sex of eels is determined by population density. The parameters of the von Bertalanffy growth equation demonstrated that males grew faster to reach a smaller asymptotic length than did females. We propose that the variation in eel sex ratio interacts with sex-linked differences in growth strategy to play an important role in density-dependent population regulation.

**Keyword**: Japanese eel (*Anguilla japonica*); sex ratio; growth strategy

## Introduction

The Japanese eel, *Anguilla japonica* Temminck and Schlegel, is a catadromous fish (Ege, 1939). The population is panmictic (Sang et al., 1994), spawning west of the Mariana Islands (Tsukamoto, 1992) and growing in brackish estuaries and freshwater rivers and lakes in Taiwan, China, Korea and Japan in northeastern Asia (Tesch, 1977). The eel enters coastal estuaries and freshwaters as a sexually undifferentiated elver, which feeds and grows as a yellow eel for 5-8 years until metamorphosing from yellow to silver eel (Tzeng et al., 2000a). After maturation, eels migrate back to their birthplace to spawn and die (Tesch, 1977). Japanese, European (*A. anguilla*) and American (*A. rostrata*) eels have been reported to have ZW sex chromosomes (Passakas and Klekowski, 1972; Park and Grimm, 1981). However, further karyotype studies failed to identify heteromorphic sex chromosomes (Sola et al., 1980; Wiberg, 1983). Sexual differentiation of the eel occurs during the yellow phase. Ovaries are found in European eels of 22-30 cm in length, possibly derived from undifferentiated gonads or from the Syrski organ, a gonad containing both early testes and oogonia, and spermatogonium B-type testes are observed in eels longer than 30 cm (Colombo and Grandi, 1996). Elvers of undifferentiated sex could be feminized by treatment with estrodiol-17β (Degani and Kushnirov, 1992; Chiba et al., 1993), indicating that the sex of the eel is labile. A long period of undifferentiated status and influenceable sex indicates that the sex determination mechanisms of the eel are complicated and unique.

In a natural environment, eels show a variable sex ratio, ranging from almost all males to predominantly female (Matsui, 1972; Parsons et al., 1977; Tesch, 1977; Jessop, 1987; Tzeng et al., 1995; Oliveira, 1997; 1999; Oliveira et al., 2001). Parsons et al. (1977) demonstrated that the proportion of migrating male silver eels markedly increased at higher elver restocking densities in a lough with low eel density. Oliveira et al. (2001) found that the percentage of lake area in a river significantly affected the sex ratio of the eel, i.e., eels migrating from lacustrine habitats of the river were mostly females, but eels migrating from fluvial habitats were mostly males. Whether the sex determination and differentiation of eels is dependent upon genotype or phenotype (environmental) is controversial. Two main hypotheses have been proposed to explain the sexual variability throughout the eel's distribution. First, if eel sex determination is genotype-dependent, then the unequal distributions of the sexes in the rivers might be the result of different habitat choice, with females preferring to migrate upstream and males preferring to stay in estuaries (Bertin, 1956; Tesch, 1977), or perhaps females prefer a habitat suitable for faster growth while males prefer a slow-growth habitat (Helfman et al., 1987). Second, if eel sex determination is phenotype-dependent, then the habitat in which they grow will affect their sex. Most studies favor the environmental sex-determination hypothesis, i.e., at high population densities,

males dominate the sex ratio and at low population densities females dominate (Colombo et al., 1984; Krueger and Oliveira, 1999). In addition, male eels have been hypothesized to maximize evolutionary fitness by time-minimizing in the growth-phase, yellow eel stage, whereas females use a size-maximizing strategy that spends more time to gain a larger size to increase fecundity (Helfman et al., 1987). A sex ratio that varies with population density is consistent with different growth rates for each sex.

The relationship between the variability in eel sex ratio and their growth strategy was evaluated by comparing the sex ratios at different population densities of wild and captive eels and by comparing the growth rates of each sex in relation to sex ratio.

**Materials and Methods**

Wild Japanese eels were collected by eel trap in the down stream of the Kaoping River of southwest Taiwan (120□50' E and 22□40' N) between November 1998 and November 2001. Cultured eels were obtained from a semi-intensively cultured pond in the Lukang area of central Taiwan (n= 49), and from an indoor aquarium at the Taiwan Fisheries Research Institute (TFRI) (n=40, 600 days old). Total length (±1 mm) and weight (±0.1g) of the eels were measured. The density of wild eels in the river (*d*) was estimated by the formula:

$$d = N/A$$

$$A = \pi \square (S/2)^2 \qquad (1)$$

where *N* is the number of the eels caught by an eel trap and *A* is the area of the fishing zone. *S* is the distance between two eel traps, approximately 10 m, while the density of captive eels was the number of reared eels divided by the bottom area of the pond and aquarium.

The sex of each eel was determined by gonad histology. The eel gonads were fixed in Bouin's solution, dehydrated in an ethanol series and embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin. The developmental stages of ovaries and testes were classified according to the methods of Yamamoto et al. (1974) and Miura et al. (1991), respectively. The sex ratios of the eels in relation to length, month and environmental history pattern were tested with Chi-square analysis of Contingency Tables (Mason et al., 1994). The sex ratios of wild eels from the Kaoping River, Taiwan and reared eels from Lukang and TFRI were compared with those of previous studies of wild eels from the Tanshui River north Taiwan and reared eels from Japan. (Tzeng et al., 1995; Satoh and Nimura, 1991; 1992).

The largest pair of otoliths (sagitta) of the eel was extracted, dried at 60° C for 10 min and embedded in a silicon rubber mould with petropoxy 154 (Palouse Petro Products, U.S.A.). Each otolith was ground and polished from the proximal side with sandpaper until the primordium was revealed and coated with carbon. Strontium (Sr) and calcium (Ca) concentrations in otolith were measured with a wavelength-dispersive X-ray spectrometer (Shimadzu-ARL-EMX-SM7). The measurement procedures for Sr and Ca were similar to those of previous studies (Tzeng et al., 1997; 2002). The Sr/Ca ratios in otolith were used to reconstruct the previous migratory and environmental history of each sex so as to evaluate whether the sex ratio of the eel changed with habitat.

After microchemistry analysis, the otolith was etched with EDTA to reveal the annulus for age determination (Tzeng et al., 1994; 2002b). The radii from the primordium to the glass eel mark ($r_o$), annuli ($r_n$) and the otolith edge ($R$) were measured to estimate the total lengths at the glass eel stage ($l_o$) and at the annulus formation period ($l_n$) by the Dahl-Lea formula (Francis, 1990):

$$l_n = r_n L R^{-1} \qquad (2)$$

where, $L$ is the total length of the silver eel at capture. The mean $l_n$ was used to estimate the growth parameters ($K$, growth coefficient; $L_\infty$, asymptotic length) of the von Bertalanffy growth equation by Ford-Walford plot (Ricker, 1958). The growth parameters $K$ and $L_\infty$ for males and females were compared for eels collected from the Kaoping River and for those from previous studies of the Pearl River of South China and Japan (Tzeng et al., 2000a, 2002).

The wild eels were divided by skin coloration pattern into yellow and silver stages: 1) yellow stage: the eel was white/gray on the pectoral fins, green/gray on the back and yellow/white on the belly; 2) silver stage: the eel was countershaded, with dark pigmentation on the back and pectoral fins, and silver/bronze on the belly. Then the mean absolute growth rate ($G$) of the silver eels was calculated according to the formula:

$$G = (L - l_o)\, t^{-1} \qquad (3)$$

where $l_o$ is the total length at the glass eel stage as estimated from $r_o$ and $t$ is the age of silver eels estimated from counts of the otolith annuli. The effect of growth rate on the age and size of the eel at metamorphosis from the yellow to the migratory silver eel stage was examined by calculating the regressions of age and length on growth rate for each sex of the silver eels collected from the Kaoping River of Taiwan and from China and Japan (Tzeng et al., 2000a; 2002). The significant differences in mean length and age of the silver eels between the sexes were examined using one-way analysis of variance (ANOVA) and Tukey's

pairwise comparison of means or Kruskal-Wallis ANOVA on ranks with Dunn's pairwise comparison of mean ranks if the data were not normally distributed with equal variance (Mason et al., 1994).

**Results**

1. Comparison of the sex ratio of wild and cultured eels

The sex of most Japanese eels longer than 250 mm was readily determined. The density of eels in the sampling area of the Kaoping River was approximately 0.02±0.01 eels/m$^2$. Females comprised 87.1% of the eels for which sex was determined. The sex ratio did not change with growth or season because the sex ratio was not significantly related to eel total length (Wild: $\chi^2$=3.469, $p$=0.483, Fig. 1; Captivity: $\chi^2$=0.484, $p$=0.785, Fig. 2) or to seasonal occurrence ($\chi^2$=7.782, $p$=0.051, Fig. 3). The sample size of the sex ratio for the environmental history comparison was too small to calculate the $\chi^2$ but the proportion of females was larger than for males in all the cases (Fig. 4). Thus, females, irrespective of eel developmental stage seasonal occurrence or habitat choice, dominated the sex ratios of wild eels from the Kaoping River. Thus, females, irrespective of eel developmental stage, seasonal occurrence or habitat choice, dominated the sex ratios of wild eels from the Kaoping River.

In contrast, the sex ratio of eels reared at a density of approximately 20.0 eels/m$^2$ in a semi-intensively cultured pond in the Lukang area was slightly dominated by males (more than 50% the sex-determined eels) (Fig2, Table1).

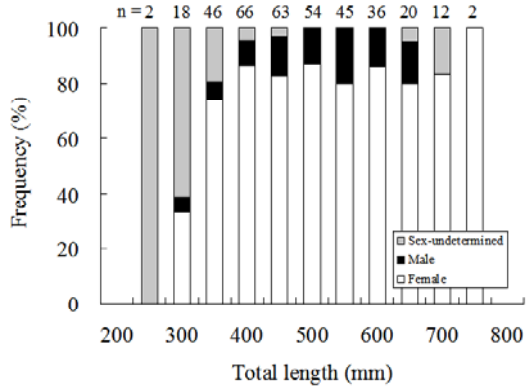


**Figure 1. The sex ratio in relation to total length of**

**Japanese eels in the Kaoping River.**

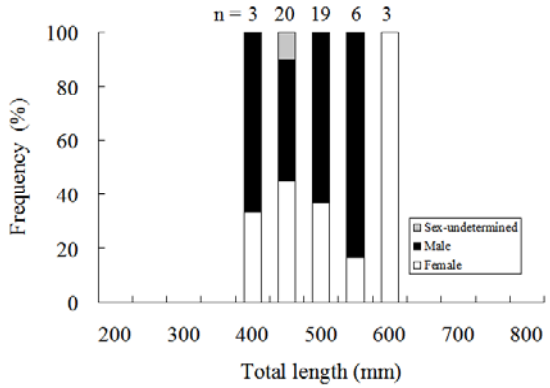


**Figure 2. The sex ratio in relation to total length of Japanese eels in a semi-intensively cultured pond in Lukang.**

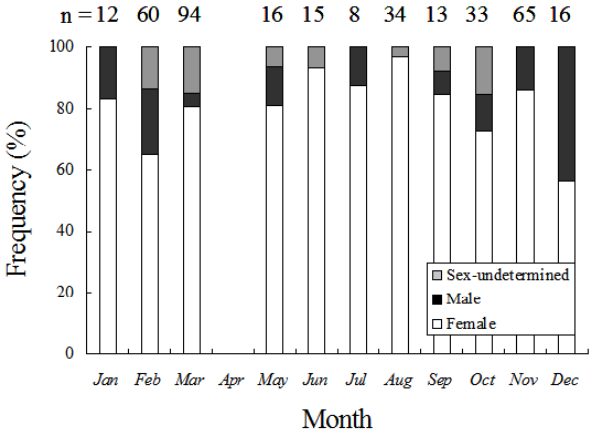


**Figure 3. The sex ratio, by month, of Japanese eels in the Kaoping River.**

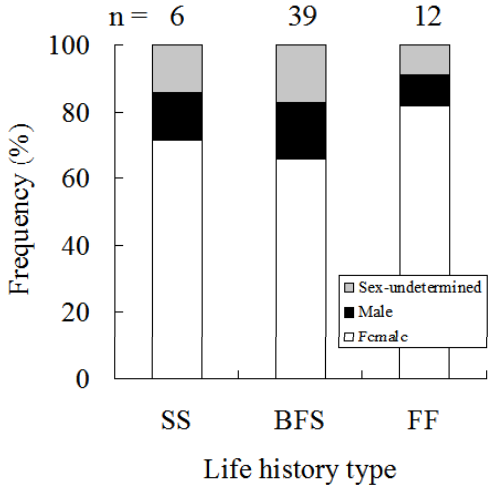

**Figure 4. The sex ratio of Japanese eels with different environmental histories in the Kaoping River. SS: seawater, BFS: between fresh water and seawater, FF: freshwater.**

Table 1. Sex ratios of *Anguilla japonica* in the wild and in captivity.

| Habitat | *n* | Stocking density (No. $m^{-2}$) | Sex ratio (%) | |
|---|---|---|---|---|
| | | | Male | Female |
| Wild (TanShui River) | 97 | 0.12±0.01 | 7.22 | 92.78 |
| Wild (KaoPing River) | 346 | 0.02±0.01 | 13.6 | 86.4 |
| Cultured pond (Lukang) | 49 | 20.0 | 57.1 | 42.9 |
| Aquarium (Japan) | 84 | 34.4 | 89.3 | 10.7 |
| Aquarium (TFRI) | 40 | 50.0 | 90.0 | 10.0 |

The sex ratio of eels reared at a density of 50.0 eels/$m^2$ in an intensive aquarium at the TFRI was almost all males (90%) (Table 1), while females were dominant in the low-density Tanshui River of north Taiwan (Table 1, Tzeng et al., 1995), and males were dominant in a high-density aquarium in Japan (Table 1, Satoh and Nimura, 1991; 1992).

The proportion of females was negatively correlated with the stocking density of both wild and captive eels ($R^2$=0.99, Fig. 5). Thus, the sex ratios of Japanese eels were predominately female at low densities and predominately male at high densities.

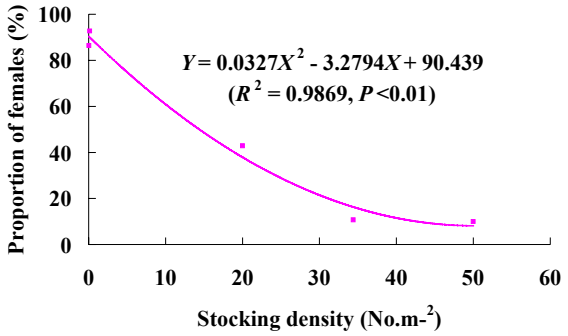


**Figure 5. The relationship between proportions of females and eel density.**

Table 2. Significant test of the difference in mean length and age of wild silver Japanese eels from Taiwan, China and Japan. C.V. (%) is the coefficient of variation.

| Location | Sex | *n* | Length (mm) | | | Age (yr) | | |
|---|---|---|---|---|---|---|---|---|
| | | | mean±SD | range | C.V. | mean±SD | range | C.V. |
| Taiwan | Male (M) | 26 | 53.82±8.01 | 42.1-67.5 | 14.88 | 5.35±1.67 | 3□9 | 31.27 |
| | Female (F) | 14 | 63.30±4.91 | 47.1-70.4 | 7.76 | 7.00±1.30 | 5□9 | 18.58 |
| | Difference between sexes (Probability) | | F>M (*P*<0.001) | | | F>M (*P*=0.003) | | |
| China | Male (M) | 32 | 48.34±4.37 | 42.0-59.0 | 9.04 | 6.38±1.62 | 4□10 | 25.39 |
| | Female (F) | 30 | 61.73±3.98 | 50.5-70.5 | 6.45 | 8.30±1.62 | 5□10 | 19.52 |
| | Difference between sexes (Probability) | | F>M (*P*<0.001) | | | F>M (*P*<0.001) | | |
| Japan | Male (M) | 40 | 52.54±7.17 | 43.0-72.2 | 13.65 | 6.10±1.52 | 3□10 | 24.85 |
| | Female (F) | 39 | 74.51±8.37 | 62.0-96.0 | 11.24 | 7.67±2.32 | 3□15 | 30.27 |
| | Difference between sexes (Probability) | | F>M (*P*<0.001) | | | F>M (*P*<0.001) | | |

2. Difference in growth strategies between sexes

The mean lengths of silver eels were significantly smaller for males than females from the Kaoping River of Taiwan and from China and Japan ($P<0.001$) (Table 2). This is probably because males have a higher von Bertalanffy growth coefficient ($K$) than do females. Thus, male eels grow faster to reach an asymptotic length ($L_\infty$) that is smaller than for female eels because the relationship between $K$ and $L_\infty$ was inversely related between the sexes (Table 3).

Table 3. Parameters of the von-Bertalanffy growth equation for Japanese eel *Anguilla japonica* from Taiwan, China, and Japan.

| Location | Sex | $n$ | $K$ | $L_\infty$ | $t_0$ |
|---|---|---|---|---|---|
| Taiwan | Male | 26 | 0.28 | 59.43 | -0.08 |
| | Female | 14 | 0.17 | 79.79 | -0.22 |
| China | Male | 31 | 0.24 | 53.74 | -0.22 |
| | Female | 29 | 0.15 | 76.02 | -0.43 |
| Japan | Male | 40 | 0.28 | 56.43 | -0.26 |
| | Female | 39 | 0.23 | 80.02 | -0.28 |

The regressions of length on growth rate were not significant for eels of either sex from Japan, Taiwan and China except the male of Japan (Fig 6). In contrast, the ages of both sexes were all significantly negatively correlated with their mean growth rates, irrespective of location (Fig.7). This indicated that the total lengths of silver eels did not change with growth rate, but the ages of silver eels changed with growth rate for both sexes. The coefficient of variation of the age of silver eels (18.58-31.27%) was approximately three-fold greater than that for the length of the eel (6.45-14.88%) (Table2). In other words, the length of the eel at metamorphosis from the yellow to silver eel stage was constant irrespective of the great variability in growth rates. However, the age at metamorphosis varied greatly with growth rate, with faster growing eels metamorphosing earlier than slower growing eels. The age of silver eels at metamorphosis from the yellow stage was more susceptible to influence by growth rate than was length.

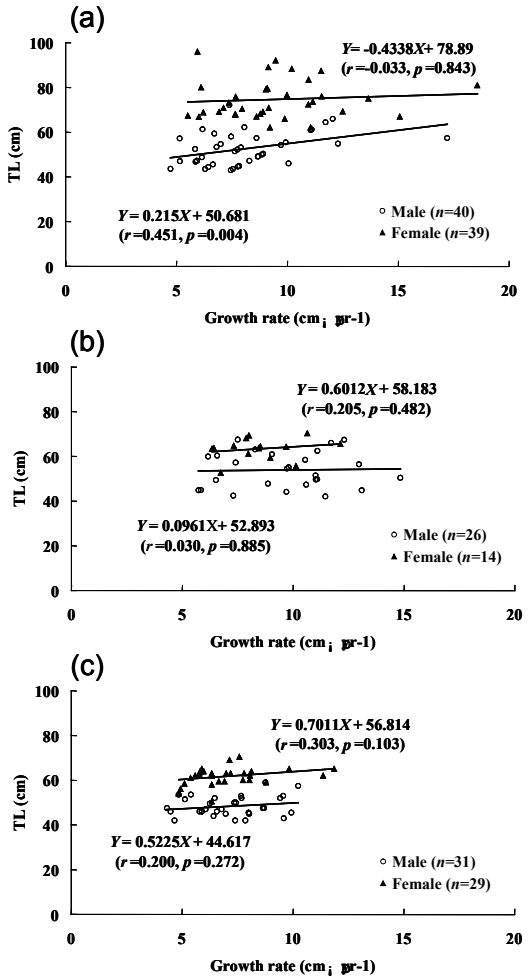


**Figure 6. The total length relative to growth rate, by sex, of silver-phase Japanese eels from Japan (a), Taiwan (b) and China (c).**

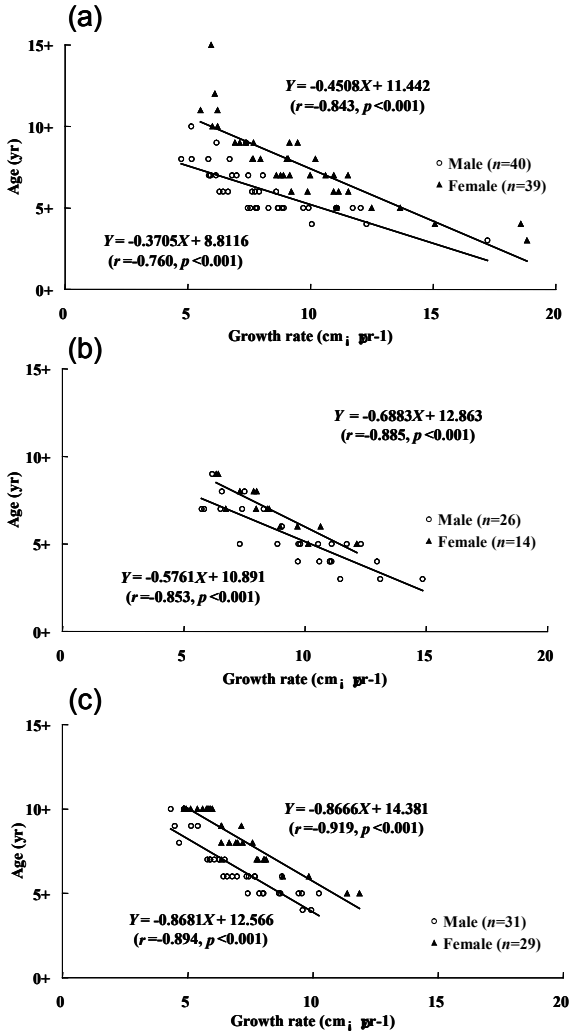


**Figure 7. The age relative to growth rate, by sex, of silver-phase Japanese eels from Japan (a), Taiwan (b) and China (c).**

## Discussion

The sex ratio of Japanese eels at high stocking densities was dominated by males, as was also found for American and European eels (Parsons et al., 1977; Jessop, 1987; Beullens et al., 1997; Roncarati et al., 1997; Oliveira, 1997, 1999; Oliveira et al., 2001). If the sex determination of the eel is genotypically determined, then unequal sex distributions for eels in a river should result from different habitat preferences by each sex. Both sexes may have different hydrotaxis and migratory behavior with females prefering lacustrine habitats while males prefer fluvial habitats (Bertin, 1956; Todd, 1980; Haraldstad et al., 1985; Helfman et al., 1987; Krueger and Oliveira, 1999). However, we found that wild eels in the Kaoping River were mostly female, irrespective of their environmental history (Fig. 4). This indicated that there was no difference in marine or freshwater habitat preference between sexes. Furthermore, captivity limits eel migration and thus differences between the sexes in behaviour should not influence the sex ratio. Thus, the hypothesis that habitat choice was the proximate cause of the varied sex distributions in rivers may not be true.

Most studies agreed that population density is probably the principal factor determining the sex of the eel, with habitats having high densities of eels dominated by males and low density habitats dominated by females (Tesch, 1977; Krueger and Oliveira, 1999; Oliveira et al., 2001). Elver restocking experiments further support this conclusion (e.g. Parsons et al., 1997; Roncarati et al., 1997). Degani and Kushnirov (1992) demonstrated that European eels maintained at higher densities produced more males than those maintained at lower densities. Our data indicated that females predominated in the low density habitats in the rivers of Taiwan and males predominated in a crowded aquarium (Figs. 1-4, Table 1). Thus, eel sex determination may be due primarily to environmental factors, particularly density. Alternatively, the sex of elvers in high-density captivity could be feminized by the treatment of estradiol-17β (Degani and Kushnirov, 1992; Satoh et al., 1992; Chiba et al., 1993). We do not know if pollutants in the Kaoping River and estuary contained environmental hormone analogues capable of influencing the gender of eels.

In Taiwan, Japanese eel elvers in the estuary were overexploited for aquaculture (Tzeng, 1984; 1997). Consequently, the eel population density in the river was very low (Table 1). Thus, the high proportion of female eels in the rivers of Taiwan may support the density-dependent environmental sex determination hypothesis (Colombo et al., 1984; Krueger and Oliveira, 1999). How the signal of population density switches on the process of sex differentiation in the eel is unknown. However, it is clear that the variation in sex ratio, with high-density stocks dominated by males and low-density stocks dominated by females, is

advantageous for eel population growth.

Males at the metamorphosis from the yellow to the migratory silver eel stage are smaller and younger than females, i.e., the male eels exhibit a time-minimizing growth strategy by maturing as soon as possible, while females postpone maturation with a size-maximizing growth strategy to attain higher fecundity (Helfman et al., 1987; Vøllestad and Johnson, 1986; Larsson et al., 1990; Vøllestad, 1992). Thus, density-dependent sex determination is a very important mechanism for eel population regulation. In high-density habitats, food resources are limited and the earlier differentiation of males may promote an earlier spawning migration. This could reduce intraspecific competition and population mortality. In contrast, in low-density stocks, food resources are relatively plentiful and eels differentiating into females would have enough time to fully utilize the resources and grow larger to achieve higher fecundity and increased reproductive success. This could help in eel population recovery.

In conclusion, eel population density is probably a trigger for sex differentiation and the variation in sex ratio in conjunction with different growth strategies for each sex serves to maximize the evolutionary fitness of the eel. However, the mechanism of how population density generates the signal to begin sexual differentiation of the eel requires further study.

**Acknowledgments**

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# INFLUENCE OF ENERGY INTAKE ON THE SOMATOTROPIC AXIS IN ARCTIC CHARR

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

## Introduction

The starvation-induced elevations of plasma growth hormone (GH), in the absence of any growth promoting effects of GH, has been observed in mammals as well as in fishes (Farbridge and Leatherland, 1992;Thissen et al., 1994). It appears that an insensitivity to GH results in decreased expression of hepatic insulin-like growth factor-I (IGF-I) leading to low plasma IGF-I levels (Duan and Plisetskaya, 1993). It has been suggested that a lack of negative feedback by IGF-I allows plasma GH to become elevated.

This study was undertaken to investigate the nutritional control of the GH / IGF-I axis in Arctic charr (*Salvelinus alpinus*), by examining the effect of food intake on both in vivo plasma hormone concentrations and in vitro control of GH secretion by SRIF-14 and IGF-I.

## Materials and methods

Arctic charr (*Salvelinus alpinus*) of the Labrador strain were obtained from the Alma Aquaculture Research Station, Alma, ON and were randomly assigned to one of three ration groups (0, 0.35, and 0.7 % $BW \bullet d^{-1}$, 3 replicate tanks each). At five weeks the fish were euthanized, blood was collected in heparinized tubes, and

pituitary glands were removed for use in pituitary cultures.

Pituitary glands were separated into equal halves and the hemipituitaries were cultured in medium 199 for 4 hours at 10°C to establish basal release. This was followed by an 18 hour incubation with or without human recombinant IGF-I (GroPep Pty. Ltd., Adelaide, Australia) or somatostatin-14 (Sigma Chemical Co., St. Louis, MO) at concentrations of 0.01 nM and 1 nM.

GH in plasma and pituitary gland culture medium was measured using a double antibody enzyme-linked immunosorbent assay (ELISA). Plasma IGF-I was measured using a fish-specific radioimmunoassay (GroPep Pty. Ltd., Adelaide, Australia).

**Results**

Average final body weight (ave BW) increased significantly with increasing ration. Thermal-unit growth coefficients (TGC) were significantly higher for fish fed the 0.7 % BW•$d^{-1}$ and 0.35 % BW•$d^{-1}$, as compared to the group fed 0 % BW•$d^{-1}$. Feed conversion efficiencies (FCE) were not significantly different between groups fed 0.7 % BW•$d^{-1}$ and 0.35 % BW•$d^{-1}$ (table 1).

Table 1. Average body weights, thermal-unit growth coefficients (TGC), feed conversion efficiencies (FCE), and plasma growth hormone (GH) concentrations for Arctic charr fed three different rations (0, 0.35, and 0.7 % BW•$d^{-1}$) for 5 weeks. Data presented as mean ± SEM. Means with different superscripts are significantly different (α=0.05).

| ration (%BW•$d^{-1}$) | ave BW (g) | TGC | FCE | GH (ng•$ml^{-1}$) |
|---|---|---|---|---|
| 0 | 494.9 ± 3.6 $^{a}$ | 0.010 ± 0.01 $^{a}$ | 0 $^{a}$ | 26.4 ± 5.9 $^{a}$ |
| 0.35 | 561.9 ± 5.7 $^{b}$ | 0.150 ± 0.02 $^{b}$ | 1.3 ± 0.1 $^{b}$ | 47.8 ± 9.3 $^{ab}$ |
| 0.7 | 629.1 ± 17.2 $^{c}$ | 0.204 ± 0.01 $^{b}$ | 1.7 ± 0.2 $^{b}$ | 59.6 ± 10.2 $^{b}$ |

Plasma GH concentrations were significantly higher in fish fed full ration as compared to those that were starved, however there was no significant difference between fish fed 0.35 % BW•$d^{-1}$ and either those that were starved, or fed 0.7 % BW•$d^{-1}$.

Arctic charr hemipituitaries exposed to doses of 0.01 nM or 1 nM hIGF-I show a significant inhibition of GH release, except at a dose of 0.01 nM in fish fed 0 % BW•$d^{-1}$ . Hemipituitaries exposed to SRIF-14 display a dose dependent inhibition of GH release from 0.01 nM to 1 nM. No significant differences were observed between treatment groups with respect to hemipituitary responses to either hIGF-I or SRIF-14 (figure 1).

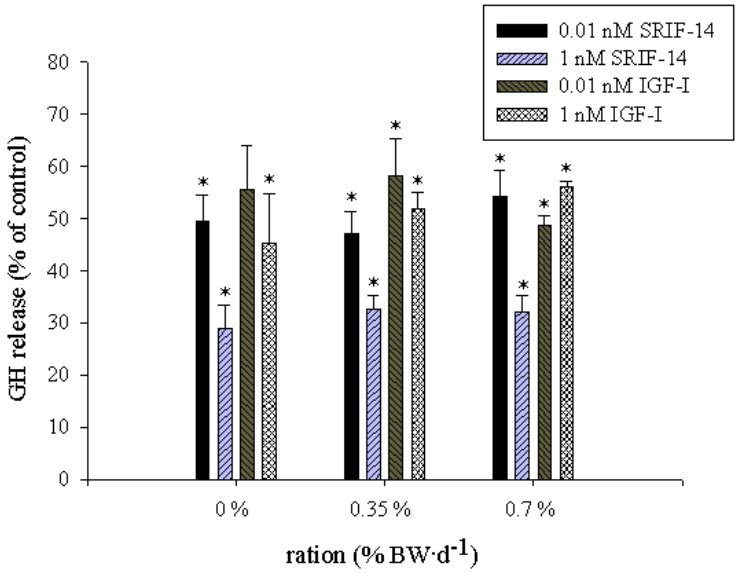


Figure 1. Percent growth hormone release relative to control of Arctic charr hemipituitaries exposed to SRIF-14 and IGF-I at doses of 0.01 nM and 1 nM for 18 hours in static culture. Asterisks indicate values significantly different from control ($\alpha$=0.05).

**Discussion**

The differences observed in average body weights and TGC indicate that the different rations achieved different rates of growth. The lack of significant difference in TGC between groups fed 0.7 % BW•$d^{-1}$ and 0.35 % BW•$d^{-1}$ may be explained by the trend for a higher FCE in fish fed 0.7 % BW•$d^{-1}$, indicating that this group may have been slightly overfed.

The higher plasma GH levels in groups fed a ration of 0.7 % BW•$d^{-1}$ was unexpected. These data will be considered with regard to parallel measurements of plasma IGF-I concentrations.

This study confirms that hIGF-I is a potent inhibitor of GH release from the Arctic charr pituitary gland. However, SRIF-14 resulted in a higher maximum inhibition of GH release (31.7% of control) as compared to IGF-I (52.9%) at the doses used. The lack of dose-dependent inhibition by IGF-I may indicate that maximal inhibition of GH release was achieved with all doses used.

The lack of treatment (ration) effect indicates that changes in pituitary sensitivity to either SRIF-14 or IGF-I in response to ration level cannot account for differences observed in plasma GH concentrations. These results will be compared to data collected from a pituitary perifusion system to investigate whether short-term (secretory) patterns of GH release from Arctic charr pituitaries in response to SRIF-14 and IGF-I reflect those seen in long-term pituitary culture.

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# PACAP RELATED GHRHLP: JUST HOW MUCH LIKE GHRH IS IT?

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

## Introduction

Pituitary growth hormone (GH) secretion in fish is generally poorly understood. While it is becoming increasing clear that the GH axis is controlled by a complex regulatory system involving multiple stimulatory and inhibitory factors, pituitary GH secretion has traditionally been accepted to be under the dual hypothalamic control of growth hormone releasing hormone (GHRH) and somatostatin (SRIF). GHRH interacts with SRIF to regulate GH release from somatotroph cells in the anterior pituitary through the dual antagonistic regulation of adenylate cyclase and $Ca^{+2}$ influx. Although a single hypophysiotropic hormone with equipotent activity to mammalian GHRH and representive of all teleost species has not been reported, a peptide with deduced sequence similarities has been reported for many fish species. This GHRH-like peptide (GHRHLP) is encoded by the same gene as another hormone, pituitary adenylate cylase-activating polypeptide (PACAP). In mammals, PACAP-related peptide (PRP) occupies the same upstream position on the PACAP gene, and GHRH is encoded by a separate gene on a separate chromosome.

The lack of conclusive data concerning the neuroendocrine regulation of GH release in fish is a problem derived from many different factors. The high degree of variability in GHRHLP regulation of GH which exists in the literature for fish may be attributed to evolutionary diversity, source of GHRHLP, lack of a true GHRH in fish, or perhaps autonomous basal release of GH in some fish species. This paper will describe similarities and differences in the cDNA and derived amino acid sequences of published GHRHLPs, the implications of such, and the actions of GHRHLP's and other potential secretagogues on GH secretion in fish.

## Discussion

Vaughan et al. (1992) were the first to isolate and fully characterize a GHRH-like peptide from a fish. The native peptide, isolated from carp (*Cyprinus carpio*) hypothalamus, shares structural similarity to members of the glucagon-secretin superfamily and stimulates GH release from goldfish (*Carassius auratus*) pituitary cells in a dose dependent manor. A few years later the prepro-PACAP gene was isolated and sequenced from sockeye salmon (*Oncorhynchus nerka*) (Parker et al., 1993). At that time, Parker et al. (1993) identified an upstream coding area on the prepro-PACAP gene which potentially codes for a 45 amino acid peptide sharing 91% amino acid sequence identity with carp GHRHLP. However, Parker et al. (1997) were unable to demonstrate consistent GH stimulation in salmon pituitary cell cultures with the salmon GHRHLP, suggesting the possibility of a more effective GH secretagogue, of which a cDNA sequence has yet to be identified. While the native carp GHRHLP sequence has been available for a decade, isolation of a cDNA encoding carp GHRHLP has not been forthcoming.

Characterization of the channel catfish (*Ictalurus punctatus*) prepro-PACAP gene suggests processing in a manner similar to all species studied to date (Small and Nonneman, 2001). While the processing of PACAP is clear, processing of the upstream GHRHLP is less clear. Channel catfish GHRHLP is proceeded by a threonine with a dibasic Arg-Arg site three amino acids up stream, and followed by a Lys-Arg site at the C-terminus. In the rat, cleavage at three dibasic sites initially generates a large intermediate PRP precursor and PACAP-38. Cleavage at a single arginine, which might correspond to $Arg^{113}$ in channel catfish, followed by carboxypeptidase hydrolysis generates rat PRP, not GHRH (Rouillé et al., 1995). Similar processing of the catfish GHRHLP/PACAP precursor would yield a 32 amino acid PRP-like peptide rather than the putative 45 amino acid GHRHLP.

The stimulatory effect of synthetic PACAP-related GHRHLPs on GH release in various fish species is inconsistent. PACAP itself appears to stimulate GH release, *in vitro,* more consistently (Parker et al., 1997). The stimulatory effects of other neuropeptides (i.e. TRH, CRH, GnRH, carp GHRHLP, human GHRH) on GH release is also inconsistent across species. In a comprehensive study to determine the role of various neuropeptides and signal transduction effectors on GH release from turbot (*Psetta maxima)* and eel (*Anguilla anguilla*) somatotropes, Rousseau et al. (2001) demonstrated species-specific regulation and the likely autonomy of GH release.

The question remains. What is the basic neuroendocrine control of GH release in teleosts? The answer remains unclear; however, PACAP-related GHRHLP does

not appear to share the same function as mammalian GHRH.

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# THYROID HORMONES AND GROWTH IN WARM WATER TELEOSTS: THE CHANNEL CATFISH AND THE RED DRUM

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

Substantial data exist demonstrating that activation of thyroid hormone production accompanies periods of active growth in fish. Whereas this suggests that thyroid hormones function to promote growth in fish, clear evidence for their direct role in the cellular processes of fish growth remains elusive (Leatherland, 1994). Our objective has been to develop model warm water species in which to study the regulation and action of thyroid hormones during periods of rapid growth, in comparison to the extensive literature for cold water salmonids. We sought species which are used in aquaculture, but also exhibit rapid juvenile growth, elevated thyroid hormone levels, and the opportunity for controlled studies in the laboratory and under natural or semi-natural conditions. These traits are evident in two warm water teleost species, the channel catfish (*Ictalurus punctatus*), and the red drum (*Sciaenops ocellatus*). Whereas channel catfish are a fresh water siluriform species with extensive information on nutritive and environmental requirements for growth, red drum are an estuarine perciform species for which basic information on aquacultural requirements is currently accumulating.

In both species (red drum captured in the wild or channel catfish held in outdoor aquaculture ponds), the circulating thyroid hormones thyroxine (T4) and 3,5,3Õ-triiodothyronine (T3) reach highest levels in the summer, when water temperatures are maximal and animals are feeding and growing most actively. As with other fish species (but not mammals, birds, or reptiles), circulating T3 is often found in equivalent, or greater, concentrations than T4 (MacKenzie *et al*.,

1989). This appears to be due to a combination of active peripheral deiodination of T4, enterohepatic cycling of T3, and enhanced T3 plasma protein binding. We have found that circulating T3 levels more accurately reflect the anabolic state of the fish, as they can be elevated (up to a maximal level) by increases in diet quantity or quality (MacKenzie *et al*., 1993). These elevations of T3 are not consistently associated with increases in the activity of the primary hepatic outer ring deiodinase (ORD), but may reflect instead differences in blood protein thyroid hormone binding capacity between different nutritional states. Although experimental elevations of circulating T3 are capable of altering body composition and activity of the pituitary-thyroid axis, direct physiological actions of circulating T3 in these species has yet to be established.

In contrast to T3, T4 levels do not consistently reflect anabolic state. This may be due in part to the timing of blood sampling. We have found daily cycles in circulating levels of T4 in both species, with significant elevations in the late afternoon. In the red drum, these daily T4 cycles appear to be driven by a circadian oscillator, as they are maintained under constant illumination conditions (Leiner and MacKenzie, 2001). In both species, food deprivation results in a significant reduction in T4, suggesting that the amplitude of the oscillations is regulated by nutritional state.

These results suggest that the elevated levels of thyroid hormones that are observed during active periods of feeding and growth are due to both central mechanisms, via the hypothalamic-pituitary-thyroid axis to establish T4 oscillations, and peripheral mechanisms, through regulation of deiodination and transport of both T3 and T4. The basal production of thyroid hormones can thus be tuned to nutritive state, and possibly also growth status, via regulatory feedback of nutrients or hormones at both central and peripheral locations.

**Acknowledgements**

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# GROWTH RATE AND MUSCLE COMPOSITION OF THREE DIFFERENT GROUPS OF FARMED ATLANTIC SALMON (*SALMO SALAR*).

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

Norwegian salmon farming industry use a variety of smolt production strategies which allow them to release smolts to seawater almost the whole year around. Fish that are released at spring and autumn will display a very different growth pattern during the first year in seawater, due to different water temperature and photoperiod. This paper will focus on the different growth pattern after release to sea water, and how the growth pattern influences on chemical composition of the muscle.

Farmed Atlantic salmon from three different smolt production strategies commonly used today in the Norwegian aquaculture industry, where sampled during the sea water growth periode with regular measurement of muscle composition (lipid, protein and water content) throughout the sea water production periode ( approximatly 2 year). Group 1 were one year old, large grading smolt, tranferred to sea water in April after 16 months in freshwater. Group 2 were one year old, middle grading smolt, transferres to seawater in June after 16 months in fresh water. Group 3 were under yearling, large grading smolt, transferred to seawater in september after 9 months in freshwater.

The thermal growth coefficient (TGC) was used to assess growth, because TGC incorporates both fish size and temperature effects in the growth trajectory. TGC was calculated as $(W_2^{1/3} - W_1^{1/3})(\sum T)^{-1}$, where $W_2$ is the final weight, $W_1$ is the initial weight and $\sum T$ is the sum of day degrees.

The fillet was weighted, homogenized and then analysed for chemical composition of lipid, protein, and water.

Results :

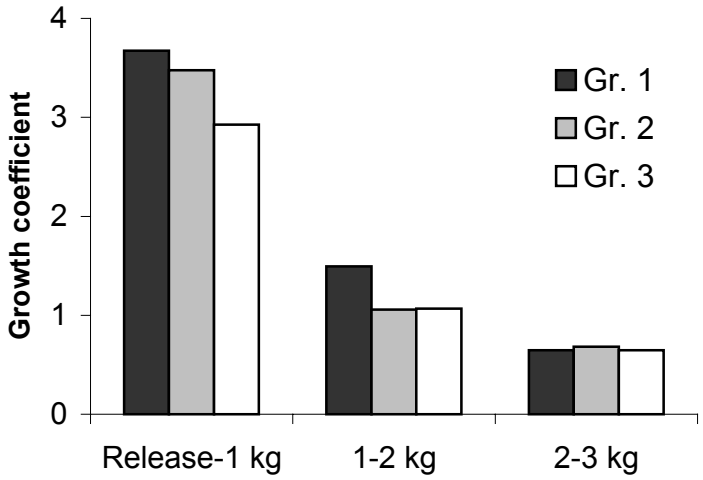


Fig. 1. Thermal growth coefficient (TGC) of the three salmon groups during the growth period in sea.

Figure 1 show the thermal growth coefficient (TGC) during the sea water growth periode. Group 1 and 2 have about the same TGC during the first growth periode in seawater. Group 3 have lower TGC during the first months in seawater, until it reaches 1 kg. From one to two kg group 1 have the highest TGC comparing to the other two groups. From 2 –3 kg the three groups have the same TGC.

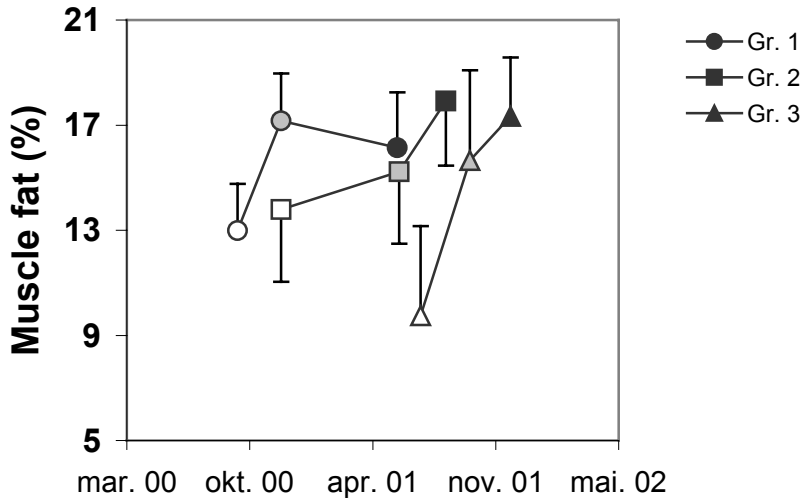


Fig. 2. Lipid content in the muscle at three different size classes indicated with different colour of the symbols. White symbols ca 1 kg, grey symbols ca 2 kg and black symbols ca 3 kg. The figure also indicates what time of the year the different salmon groups reaches the different size classes.

Muscle fat content of the different groups of salmon at different size classes are shown in figure 2. Group 3 have lower muscle fat content than the other two groups at the size of one kg. At 2 and 3 kg there where no significant different between the groups.

So even if the growth pattern and espesially the fat content is different in the first period, the final fat at ca 3 kg content is similar and indipendent of the season.

Further results will be presented and discussed in the presentation.

# EVALUATING EFFECTS OF NUTRITION ON FISH GROWTH AND HEALTH WITH THE USE MOLECULAR PROBES.

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

The continued selection and genetic improvement of fish stocks requires novel techniques that allow a more precise understanding of the cellular and physiological aspects that underlie traits of interest. While sequence mapping and cellular studies have greatly improved our understanding of cellular mechanisms and transcriptional changes involved with certain physical characteristics in humans and some importantly cultured domesticated animals, this type of knowledge in aquaculture is woefully lacking. To gain a more precise understanding of the specific genes that are involved with useful quantitative traits, studies have begun that analyze gene expression levels in the tissues of animals under different experimental conditions. Some studies have shown gene expression to correlate reasonably well with protein and enzymatic levels for some proteins (Rocha et al., 1996). In order to gain a better understanding of specific genes that could be involved in selectable traits of interest, specific genes were selected to monitor their expression level changes under varied experimental conditions. The genetic traits of interest that are currently under study at the Hagerman Fish Culture Experiment Station are growth, on formulated fish diets using alternative protein sources to fish meal, disease resistance, and health issues related to different diets and environmental conditions. Utilizing knowledge gained from previous work in fish and other animals, and the availability of sequence, certain transcribed genes were chosen for analysis using real-time PCR. Probes and primer pairs, such as myosin and ILGF-II, were designed and tested for growth analysis, pyruvate kinase and lactate dehydrogenase, for analysis of metabolic enzymes, and tumor necrosis factor (TNF), CD-8, interferon (MX-1), lysozyme, complement factor C3, interleukin-8 (IL-8), natural resistance associated macrophage protein-alpha (Nramp-alpha) and natural killer cell enhancement factor (NKEF), as immunological and health related indicators.

Using the myosin probe, myosin expression levels in fish on varied planes of nutrition was studied (Overturf & Hardy, 2001). Groups of fish were reared for 2 months on the following diet regimes; diet 1-100% (fed to satiation), diet-2 75% of diet 1, diet-3 50% of diet 1, diet-4 25% of diet 1, and diet-5 was held off feed during the trial. After only two weeks it was noted that myosin expression levels correlated with the nutritional plane of the groups of fish and this correlation became stronger as the trial continued (r=0.98)(Figure 1). This

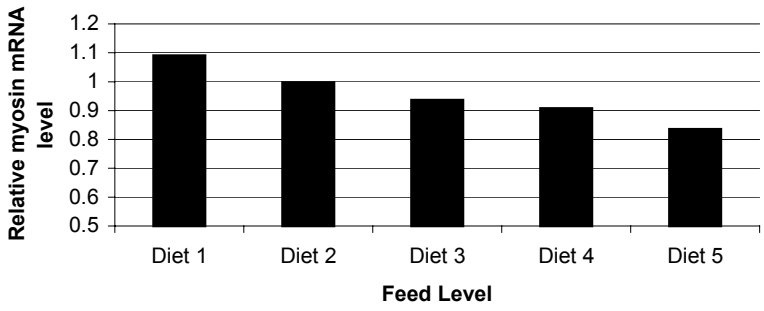


**Figure 1. Relative myosin mRNA expression level from the muscle of groups of fish fed at reducing nutritional levels.**

suggests that myosin expression level, and hence muscle deposition, are directly related to the nutritional intake of the animal and that this method would be effective in monitoring nutrient quality of diets. For studies looking at metabolic enzyme mRNA expression levels, individual groups of fish were reared on complete and serial limiting vitamin levels, as these reduced levels have demonstrated adverse effects for fish growth and health (Halver, 2002). Examination of the expression levels for ILGF-II and pyruvate kinase found that after only 3 sampling periods (every 3 weeks) there were significant differences in the level of pyruvate kinase expression correlating with the vitamin level for each diet (r=0.98) (Figure 2). There seemed to be no correlative response of ILGF-II to varying vitamin level at this point in the study.

Another problem that has become associated with diet changes in fish, especially the use of high level replacement of fish meal with plant material as an alternative protein source, is irritation of the intestinal tract that seems to be related to immune function (Rumsey et al., 1995). As an initial assessment of probes and primers generated for analysis of aspects related to the natural and adaptive immune system, the expression level of specific immune factors was

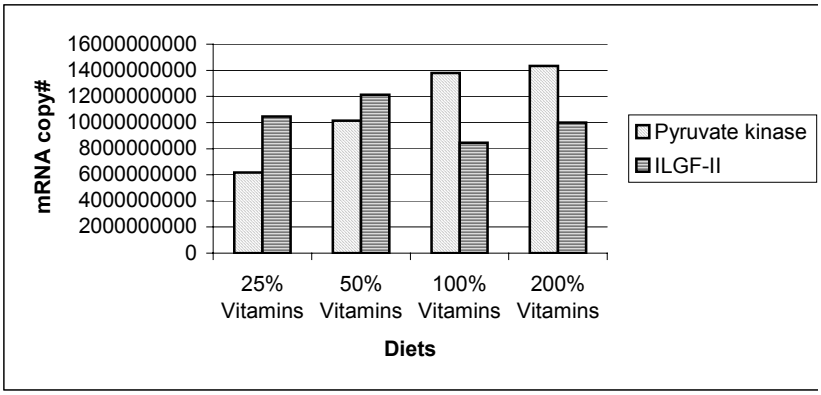


**Figure 2. Expression level of pyruvate kinase and ILGF-II mRNA in the livers of groups of fish fed diets containing required vitamin C levels or**

studied in fish that were exposed to bacterial and viral pathogens. Individual groups of fish received three different dosage levels of either *Aeromonas salmonicida*, *Flavobacterium psychrofilum*, or *infectious haematopoietic necrosis virus* (IHNV) and the levels of the immune factors were determined at 1 and 5 days post-infection in the liver, spleen and head kidney of infected fish. It was found that *F. psychrofilum* did not demonstrate any major effects on the level of most of the immune factors involved in this study. *A. salmonicida* showed changes typically in the spleen and liver for factors such as tumor necrosis factor, IL-8, and CD-8. IHNV showed highly correlative expression levels for MX-1, C-3, CD-8, and TNF in the liver and somewhat in the spleen.

With these and other probe and primer sets that are being developed it is anticipated that a better understanding of the nutritional aspects related to fish health can be assessed and ultimately utilized for selection of improved stocks for aquaculture.

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## CHARACTERIZATION OF MYOSTATIN IN BROOK TROUT

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**EXTENDED ABSTRACT ONLY-DO NOT CITE**

Myostatin (MSTN), a member of the Transforming Growth Factor-β (TGF-β) superfamily, has been established as a regulator of development and growth in several vertebrates. MSTN, originally termed growth and differentiation factor - 8 (GDF-8), is a negative regulator of muscle mass and thus has the potential to be an important factor in the production of farmed animals. A dramatic increase in muscle mass has been observed in mutant mice and artificially selected bovine species such as the Belgian Blue (Lee and McPherron, 1999). To understand the role that MSTN could have in the growth and development of fish, the present study characterized MSTN expression in the brook trout.

### Methods

*Sampling*
Male and Female brook trout were sampled in April (1 yr old), July (1.5 yr old), and October (2 yr old). For the analysis of brook trout embryos, eggs were fertilized and raised in Heath incubators for 12 weeks. Samples were taken 10 times throughout the period from fertilization to swim-up.

*mRNA and RNA Analysis*
The full-length MSTN cDNA clone observed in brain and muscle tissue (b/m MSTN) obtained from cDNA library screening and the brook trout cDNA fragment from ovarian tissue (ov BT MSTN), were used to probe brook trout mRNA tissue blots. Total RNA samples were analyzed quantitatively using Taqman probes designed to hybridize to brook trout b/m MSTN, ov MSTN, and 18S RNA. Quantitative RT-PCR (Thermoscript One-Step System - Invitrogen) was used with the Opticon Continuous Fluorescence Detection System (MJ Research).

*Protein Analysis*
Recombinant MSTN protein was produced using the region coding for the mature peptide bp 794-1120 (Accession# AF247650). This 326 bp fragment obtained from PCR was directly inserted into the pBAD TOPO/*Lac*Z vector and transformed into TOP10 *E. coli* cells (Invitrogen). Recombinant protein was produced, purified using His-Bind Resin chromatography (Novagen), and used to produce a polyclonal antibody in rabbits. Protein (10 ug) from adult muscle tissue or whole embryos was transferred to membranes, hybridized with antibody, and quantified by Western blotting.

**Results**

On Northern blots, a 2.7 kb transcript was observed in red muscle when probed with the b/m BT MSTN cDNA (Figure 1). A less abundant transcript was present in brain (Figure 1). When the full-length b/m BT MSTN cDNA was used as a probe with ovarian tissue taken at ovulation, no hybridization was observed. On Northern blots of ovarian tissue taken at different reproductive stages and probed with the ov BT MSTN cDNA fragment, increased transcript levels were observed during ovulation in several individuals (Roberts and Goetz, 2001)

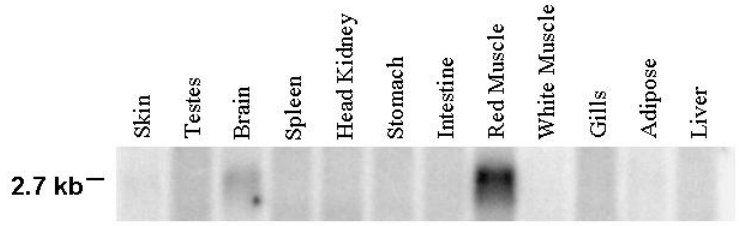


Fig. 1: Northern blot of mRNA (0.5 ug/lane) from brook trout tissues

Quantitative RT-PCR data were similar to the results of Northern analysis with red muscle generally having higher levels of b/m MSTN compared with white muscle (Figure 2). The difference in amounts of b/m MSTN RNA among red and white muscle varied, with the greatest difference observed in the fall. Levels of ov MSTN RNA were observed in all muscle tissue samples taken, though the levels were less than b/m MTSN RNA.

No b/m MSTN was detected in any of the embryos sampled. Levels of ov MSTN were observed in all embryo samples except for unfertilized eggs and only occasionally in eggs 2 days after fertilization. Levels of ov MSTN were comparable to levels observed in adult muscle tissue.

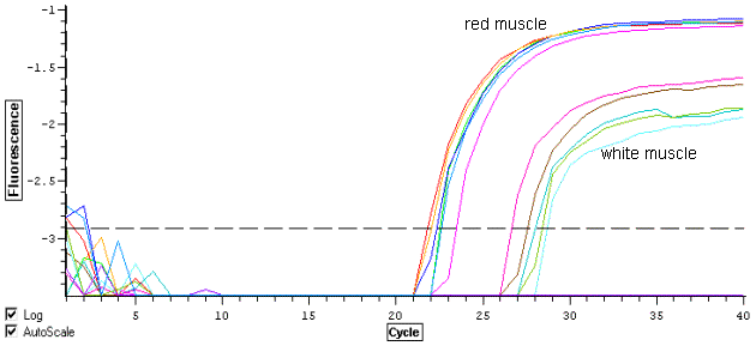


Fig 2. Representative b/m MSTN quantitative real time RT-PCR data from female brook trout red (n=5) and white (n=5) muscle tissue sampled in October.

The MSTN antibody recognized a single immunoreactive band of ~50 kDa. Protein expression was similar to RT-PCR data in that higher levels of an immunoreactive protein were observed in red muscle with the greatest difference seen in the Fall. In general, males had slightly higher levels of protein in both red and white muscle. In brook trout embryos, expression of immunoreactive MSTN protein was evident 45 days after fertilization.

**Conclusions**

These data are consistent with investigations characterizing MSTN in other teleosts in which multiple isoforms of MSTN were reported. This study demonstrates the presence of MSTN RNA and immunoreactive protein in several life stages of brook trout and the differential expression among muscle type, gender, and season. One explanation for the difference in MSTN expression in adult fish could be related to the reproductive status, as gonad development has been show to influence somatic growth rates. Quantitative RT-

PCR analysis also demonstrated that both MSTN isoforms are found in adult muscle tissue. Therefore, the previously named "ov MSTN" is actually expressed in multiple tissues. The presence of ov MSTN in brook trout embryos could be the result of a maternal transfer of MSTN from the ovary to the developing larvae. The absence of b/m MSTN RNA in larval fish could indicate a functional role for this isoform as a negative regulator of muscle mass since muscle increases during this stage of development.

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# MOLECULAR CLONING OF GROWTH HORMONE RECEPTOR IN MASU SALMON

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

## Introduction

Growth hormone (GH) plays many important roles in somatic growth, maturation, seawater adaptation and immunity in fish. The actions of GH are initiated from interaction by its binding to GH receptor (GHR) on the cell membrane of various target tissues. Association of GH with GHR triggers a phosphorylation cascade for signaling and gene expression, which results in biological actions of GH. GH receptor cDNAs have been cloned in mammals, avian species (Argestinger and Carter-Su, 1996), *Xenopus laevis* (Huang and Brown, 2000) and soft-shelled turtle (Zhang et al., 2000). The GHR is a member of cytokine receptor superfamily and has the following common features: three pairs of cysteine residues and a YGEFS motif in the extracellular domain, a single transmembrane region, and proline-rich box 1 and box 2 in the cytoplasmic region. Recently GHR cDNA of teleosts have been cloned in goldfish (Lee et al., 2001) and turbot (Calduch-Giner et al., 2001). The common features of GHR were also conserved in teleosts.

To understand physiological roles of GH, two types of GHR-like cDNA (msGHR1 and msGHR2) were isolated from the liver of masu salmon (*Oncorhynchus masou*). Further, tissue distribution of two msGHRs was also confirmed.

## Materials and Methods

Total RNA was obtained from liver of masu salmon to enable cDNA cloning of msGHRs. The msGHRs cDNA fragments were amplified by RT-PCR using degenerate primers designed from conserved regions of GHR in other

species. The cloning of msGHR1 cDNA was cloned from λgt10 cDNA library of masu salmon liver. The lacking open reading regions of msGHR1 cDNA and the full length of msGHR2 cDNA were obtained using 5'- and 3'-RACE PCRs.

The cDNAs of several types of tissues of masu salmon were prepared to confirm the tissue distribution of two GHRs by PCR using msGHRs cDNA specific primers. As a control of reverse transcription, β-actin primers were used in PCR.

**Results**

The cDNAs of msGHR1 and msGHR2 encoded 594 and 657 amino acids and showed 55.7 % homology. The deduced amino acid sequence of msGHR2 had three pairs of cysteine residues but msGHR1 had only two pairs. Except this point, these deduced amino acid sequences conserved the following specific features of GHR; YGEFS motif in extracellular domain, a single transmembrane region, and box 1 and box 2 domains in the cytoplasmic region.

The deduced amino acid sequences of the msGHR1 and msGHR2 were 39.3 % and 52.5 % with goldfish GHR, and 37.1 and 53.7 with turbot GHR, respectively. The amino acid sequences of extracellular domain showed relatively high homology as follows; 51 % and 59.6 % identical with goldfish, and 40 % and 59.6 % identical with turbot. The overall homologies between msGHRs and other vertebrate GHRs were relatively low (under 35 %).

The expression of both GHRs was observed in almost of all types of tissue (brain, pit, gill, heart, kidney, spleen, liver, muscle, testis and ovary; Fig.1). The primers of GHRs were very specific. In all samples, β-actin was amplified by PCR.

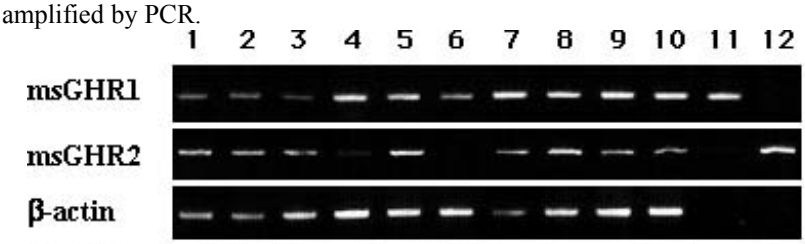


Fig.1 Tissue distributions of msGHR1 and msGHR2 transcripts determined by RT-PCR. 1:brain; 2:pituitary; 3:gill; 4: heart; 5: kidney; 6: spleen; 7: liver; 8: muscle; 9: testis; 10:ovary; 11: msGHR1 cDNA;12: msGHR2 cDNA

## Discussion

In this study, two types of GHR-like cDNAs (msGHR1 and msGHR2) were obtained from the liver of masu salmon. The deduced amino acid sequence of msGHR2 showed the common feature of GHR, especially having YGEFS motif, which is a specific characteristic of GHR. Although the putative msGHR1 proteins lacked one pair of cysteine residues in extracellular domain, other common features of GHR were confirmed. Further the deduced amino acid sequences of two msGHRs showed high homology with other teleosts GHRs. These results suggested that isolated two GHR-like cDNAs were functional GHR genes of masu salmon. However it is necessary to confirm whether their recombinant proteins can bind GH in the future.

The result of RT-PCR showed that both msGHRs expressed in various types of tissues like other vertebrates GHRs.

## Acknowledgments

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# WHAT WE KNOW AND DON'T YET KNOW ABOUT THE GROWTH-MODULATING ROLES OF INSULIN-LIKE GROWTH FACTOR-BINDING PROTEINS (IGFBPs) IN FISHES

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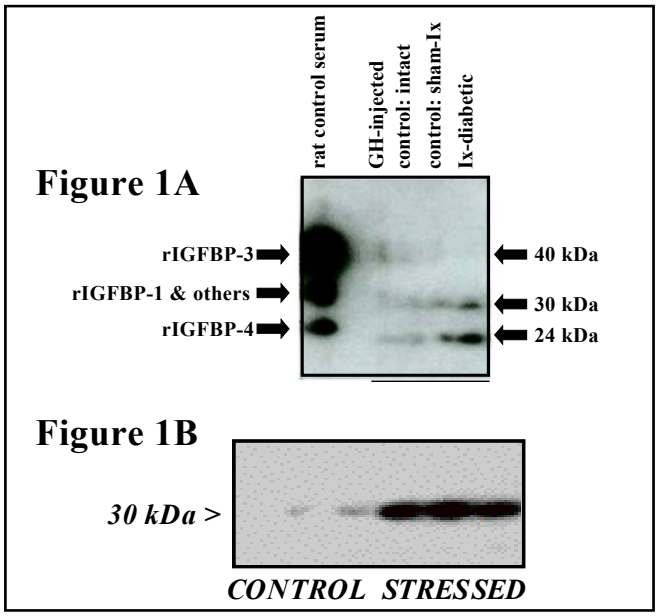
IGFBPs, unrelated to IGF receptors, are derived from a superfamily of cysteine-rich growth factors (Kelley et al., 2002). Having a central position between IGF ligand and receptor, IGFBPs are 'growth integrators', having important influences on the distribution and bioavailability of IGF in the cell and physiological environments. IGFBPs exhibit an array of specialized properties, including interaction with proteins that post-translationally modify them, cell membrane association, direct IGF-independent cellular actions, and intracellular nuclear actions (Mohan and Baylink, 2002). Up to 6 IGFBP genes are expressed in a given tissue or cell type, with a diversity of factors regulating their individual expression, meaning that different physiological circumstances will result in a characteristic local IGF bioactivity.

Mammalian IGFBP-3 carries >90% of serum IGFs, and a ~140 kDa 'ternary' complex of IGFBP-3 (45 kDa), IGF (7 kDa), and a bound 'acid-labile subunit' (ALS; 90 kDa) predominates the serum IGF-binding capacity of mammals. Growth hormone (GH) stimulates the production of all three (IGF-I, IGFBP-3, ALS) and their levels in the circulation are positively correlated with growth rate. The large size of the IGFBP-3 ternary complex functions to maintain a substantial, functionally important reservoir of IGF within the blood circulation. In fishes, however, it has not been possible to demonstrate any large IGFBP

complex in serum, while the levels of all IGFBPs are much lower than those of mammals (Figure 1A compares IGFBPs present in equal volumes (4 µl) of serum from rat versus the goby *Gillichthys mirabilis*). Indeed, sera from all fish species tested to date exhibit similarly low relative levels of IGFBPs, as well as lower IGF-I concentrations (Kelley et al., 2002).

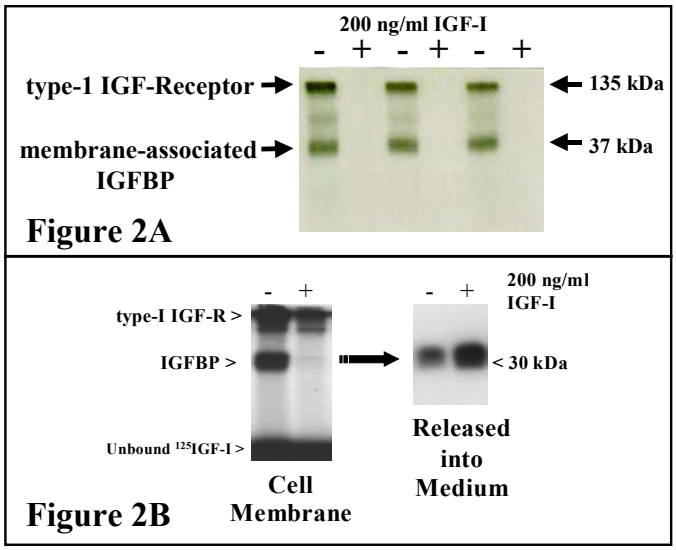



Thus, at least under basal physiological conditions, IGFBPs and IGFs are far less prominent features of fish serum as compared with that of mammals. However, if provoked physiologically (e.g., hormone injection or stress induction), levels of one or more particular IGFBPs will be substantially altered. As shown in Figure 1A, low levels of the 40 kDa IGFBP are seen in control gobies, but are increased after injection of growth hormone (GH). Alternatively, the 30 and 24 kDa IGFBPs are up-regulated under catabolic circumstances, when body growth is inhibited (e.g., diabetic fish, Fig. 1A). Based on such findings, we propose that in fishes, enhancement (or inhibition) of growth is somewhat like "filling an empty glass", in that the serum requires "addition" of growth factors (IGF & IGFBP) from low basal levels, in contrast to mammals that maintain a standing reservoir of these growth factors.

In catabolic fish (induced by stress, fasting, or diabetes), levels of a 30 kDa IGFBP (& 24 kDa, IGFBP in some species) are increased in serum in

association with elevated cortisol levels and a suppression of growth (3). In Figure 1B, serum IGFBP in jack mackerel (*Trachurus symmetricus*) is seen to be substantially increased after 60 min of handling stress (along with 8-fold increased serum cortisol). Thus, our findings to date indicate a physiology comparable to that of mammalian IGFBP-1, a key growth inhibitor via its high-affinity binding of IGF (blocks cell receptor binding). The connection between stress and growth inhibition in fishes, via alterations in IGFBPs, has raised an interesting possibility: that measurements of IGFBPs, which require only a few µl of serum, can serve as a useful biomarker of the impacts of stress on growth (Kelley et al., 2001). For instance, the physiological impacts of "catching and releasing", a practice intended to protect fisheries, may well depend on the degree to which stress and IGFBPs are altered, a subject about which very little is known.

It is now understood that IGFBPs are produced by a wide variety of cell types in fishes, as in mammals, and therefore that a "local IGF/IGFBP axis" exists in fish tissues. There are only a very limited number of studies on the properties of IGFBPs at the cellular level using non-mammalian models. This is a critical yet unanswered issue, as the dynamics of IGFBPs in the microenvironment of mammalian cells are essential in IGF-regulated processes. Until recently, it was not known whether IGFBPs from lower vertebrates utilized cell surface association, or whether such properties were important in influencing IGF-mediated growth. We have found that there are apparent cell surface-associated IGFBPs both in goby muscle membrane preparations and in heart cells of green iguana. In Figure 2A, affinity crosslinking of $^{125}$I-IGF-I identifies two binding sites in goby muscle cells, the 135-kDa alpha-subunit of the IGF receptor and a 30-kDa IGFBP. Both sites bind specifically, as excess unlabeled IGF-I competitively inhibits label binding. In monolayer cultures of iguana heart cells (Fig. 2B), a 30 kDa IGFBP is present both as a soluble form in the medium as well as localized to the cell membranes. Interestingly, when IGF-I is added, it results in a rapid, complete release of the IGFBP from the membrane into the medium (compare left and right panels in Fig. 2B). Cells in which membrane-associated IGFBP has been removed experimentally show a greater proliferative responsive to IGF-I, leading to the hypothesis that the IGFBP serves a growth-inhibitory role. Based on our studies to date, it appears that IGFBP membrane association occurs in fish and reptilian cells, with relevance to whether IGF activates its cell receptor to stimulate growth.

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# CHARACTERIZATION OF A 41-KDA INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN IN SALMON

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Insulin-like growth factor-I (IGF-I) is a potent mitogen that exerts its actions through endocrine, paracrine and autocrine fashions. In mammals, IGF-I is tightly associated with six IGF-binding proteins (IGFBPs). Since affinity of IGFBPs to IGF-I approaches or exceeds that of IGF receptor, IGFBPs are capable of controlling the availability of IGF-I to target tissues and thus act as important modulators of IGF-I actions. Recent findings also indicate IGF-independent actions of IGFBPs in multiple cell types. In mammals, about 80% of circulating IGF-I is bound to IGFBP-3. IGFBP-3 is a N-glycosylated protein with molecular mass of 41 kDa and its production is regulated by growth hormone (GH) and nutritional status. In salmon, a similar-sized (41 kDa) IGFBP has been found in serum. As in mammalian IGFBP-3, salmon 41-kDa IGFBP is up-regulated by GH treatment and down-regulated by fasting, suggesting that it is a homolog of IGFBP-3 (Shimizu et al., 1999). This IGFBP, however, has not been characterized due to lack of availability of intact protein. We have recently purified the 41-kDa IGFBP from serum of chinook salmon (*Oncorhynchus tshawytscha*). This study aimed to characterize the 41 kDa IGFBP.

The 41-kDa IGFBP was purified from serum by IGF-I affinity chromatography followed by reverse-phase chromatography. Purified IGFBP appeared as doublet bands at 43 and 41 kDa on electrophoresis and those bands were N-glycosylated (Figure 1). Since purified IGFBP gave a single N-terminal amino acid sequence, the doublet bands presumably

represent different glycosylated forms of IGFBP as is the case for mammalian IGFBP-3. The IGFBP family has cysteine rich domains in both N and C-terminal regions, which are essential for high-affinity IGF binding. Partial amino acid sequencing of the purified protein revealed that the N-terminal cysteine rich domain is conserved in the 41-kDa IGFBP. Although the 41-kDa IGFBP showed highest amino acid sequence homology with zebrafish IGFBP-2 (Duan et al., 1997), the highly conserved nature of the N-terminus makes it impossible to identify the type of IGFBP. However, based on physiological responses, molecular weight and type of glycosylation, we assume that this IGFBP is salmon IGFBP-3.

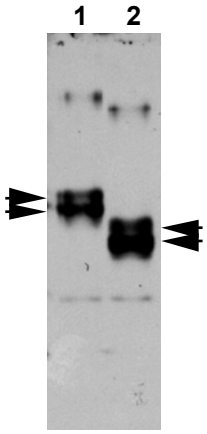


Figure 1. Western ligand blotting of purified 41-kDa IGFBP before (1) and after (2) enzymatic digestion. Forty nanograms of purified 41-kDa IGFBP was digested with glycopeptidase F under non-reducing conditions and subjected to Western ligand blotting using digoxygenin-labeled hIGF-I. Arrowheads indicate migration positions of 41-kDa IGFBP doublet bands.

On binding assay using $^{125}$I-salmon IGF-I, purified 41-kDa IGFBP specifically bound salmon IGF-I, human IGF-I and human IGF-II, but neither Long R$^3$IGF-I nor salmon insulin (Figure 2). No binding with Long R$^3$IGF-I, which has little binding ability to mammalian IGFBPs, indicates that binding characteristic of the salmon IGFBP is similar to those of mammalian IGFBPs. Affinity constant (Ka) of 41-kDa IGFBP for salmon IGF-I was calculated as 4 nM$^{-1}$ from a Scatchard plot derived from the competitive binding curve. The Ka value is comparable to those of fish type I IGF receptor preparations (Ka = 2.3-7.7 nM$^{-1}$; Navarro et al., 1999). This

finding supports the concept that fish IGFBP is capable of modulating bioactivity of IGF-I in target tissues by competing with type I IGF receptor.

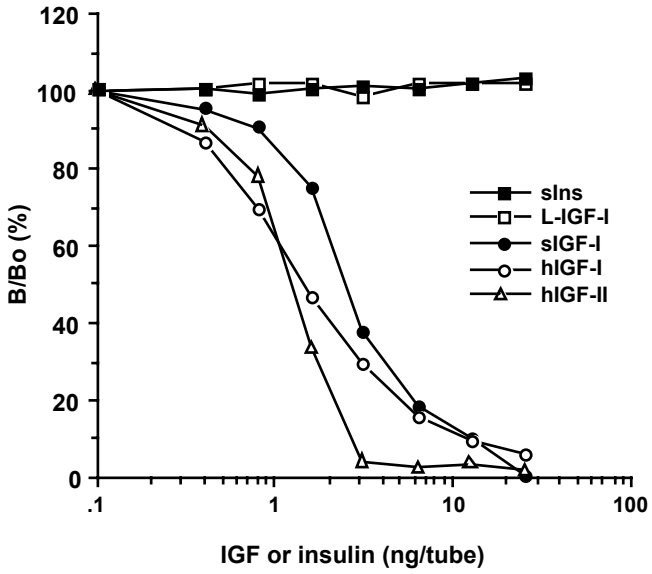


Figure 2. Binding specificity of 41-kDa IGFBP to IGFs and insulin. Ten nanograms of purified 41-kDa IGFBP was incubated with 15,000 cpm $^{125}$I-salmon IGF-I and various amounts of unlabeled salmon IGF-I (sIGF I), human IGF-I (hIGF-I), human IGF-II (hIGF-II), Long R$^{3}$IGF-I ( L IGF-I), or salmon insulin (sIns). Binding (B/Bo) was expressed as a percentage of specific binding. All values are means of triplicate.

**Acknowledgment**

This work was supported by a grant from the U.S. Dept. Agriculture, NRICGP, Animal Growth and Nutrients Utilization Program.

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# NOVEL BIOLOGICAL ACTIVITIES OF THE E-PEPTIDE

# OF FISH PRO-IGF-I

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

E-peptides of pro-insulin-like growth factor-I (pro-IGF-I) are proteolytically cleaved from the pro-hormone after translation, and have long been regarded as biologically inactive. Tian *et al.* (1999) recently demonstrated that recombinant rainbow trout pro-IGF-I E-peptides (rtEa-2-, rtEa-3- and rtEa-4-peptide), like hIGF-I, exhibited a dose-dependent mitogenic activity in several non-transformed mammalian cell lines.

We showed recently that treatment of established human and fish cancer cells (MCF-7; HT-29; HepG2, ZR-75-1, SK-N-F1 and HC) and retroviral transformed human embryonic kidney cells (293GP) with recombinant rtEa-2- or rtEa-4-, but not rtEa-3-peptide, resulted in a dose-dependent induction of morphological change and enhanced cell attachment Chen *et al*., 2002).

The Ea-4-peptide-induced morphological changes were sensitive to treatment with -amanitin or cycloheximide, known inhibitors of RNA and protein synthesis. The *in vitro* colony formation activity of established human tumor cells (HT-29 and MDA-MB-231) was greatly reduced or diminished by treatment with the rtEa-4-peptide.

Both morphological change and reduction of colony formation activity in MDA-MB-231 cells were also observed following transfection with an Ea-4 transgene

construct. Furthermore, the invasive activity of HT1080 cells, known invasive cancer cells, was reduced 3-4 fold by treatment with the rtEa-4-peptide.

Results of transgenic studies revealed that transfer of a transgene expressing rtEa4-peptide into newly fertilized eggs of medaka resulted development of heart and blood vessel abnormality. These results suggest that rtEa4 might possess antiangiogenic activity. The antiangiogenic activity of rtEa4-peptide was further confirmed by an assay conducted on chicken Chorio-Allantoic Membrane (CAM).

As shown in Figures 1 and 2, rtEa4-peptide caused a dose-response dependent reduction of blood vessel density development on the chicken CAM. These results suggest that Ea-4-peptide of rainbow trout pro-IGF-I possesses novel biological activities controlling malignant properties of cancer cells *in vitro*. These novel biological activities include: mitogenic activity in NIH3T3 cells, induction of morphological changes in cancer cells, reduction of colony formation and invasive activities and antiangiogenic activity

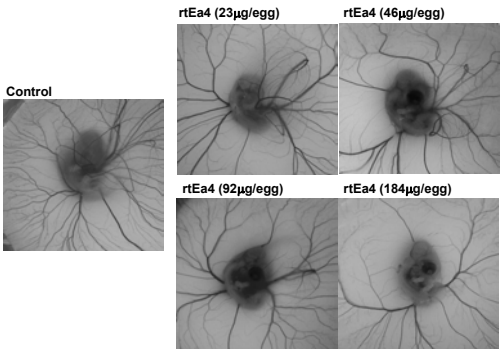


Figure 1: Effect of rtEa4-peptide on blood vessel density of the Chorio-Allatoic Membrane of cghicken embryos. Day 3 chicken embryos were placed in egg-cups, treated with various amounts of rtEa4-peptide, and then incubated at 37$^{o}$C for an additional four days. At the end of incubation, the embryos were photographed with a digital camera.

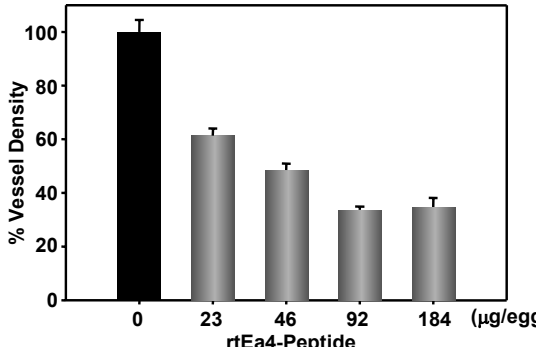


Figure 2: Dose-dependent effect of rtEa4-peptide on blood vessel density development on the chicken Chorio-Allantoic Membrane.

**Acknowledgement**

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# THE RELATIONSHIP BETWEEN PLASMA IGF-I AND GROWTH IN POST-SMOLT COHO SALMON: AFFECTS OF SHORT-TERM VARIATION IN FEEDING RATE AND TEMPERATURE.

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Growth is mediated by the endocrine system; consequently, endocrine correlates of growth may provide a valuable tool for assessing relative growth rate. Growth hormone, insulin-like growth factor-I, insulin, thyroid hormones and anabolic steroids have all been demonstrated to either stimulate or maintain high growth rates. However, many of these hormones have proved ineffective at providing an endocrine correlate of growth. Mammalian models have shown that IGF-I is a primary stimulus for growth at the cellular level. A homologous RIA for fish IGF-I has been established in the last decade. Establishing basic endocrine and physiological relations of IGF-I have provided the primary focus for most piscine work to date, including a major focus on the suite of IGF-I binding proteins found in fish. However, one might also take a more organismal view, and examine if circulating IGF-I levels provides a signal of an animal's recent growth history. With this goal in mind, we have conducted several experiments examining the circumstances in which total plasma IGF-I might exhibit a significant relation with growth rate.

In one study we examined the effects of fluctuating feeding rates on IGF-I vs. growth relations over two week intervals. In June 2000, approximately 300 yearling coho salmon were individually PIT-tagged and placed in four 1.3m circular tanks and fed a maintenance ration for four weeks. Subsequently, fish in two tanks were fed at a 2% body weight/day ration with the remainder were fed 1%. After four weeks feeding rates were again bifurcated, with one tank from each ration level shifted to a 0.5% body weight/day ration for four

additional weeks. Finally, all tanks were restored to their original ration level for a further two weeks. All fish were measured at two week intervals. A sub-sample of 12 fish/tank was obtained at two week intervals, plasma collected, and plasma IGF-I was measured by RIA (Shimizu et al.). Plasma IGF-I was significantly correlated with growth rate over each two week interval ($r^2$>0.4). This relation was not constant. The slope of the growth vs. IGF-I relation increased significantly in September. Samples from male salmon which were obviously maturing (enlarged testis) had significantly higher IGF-I levels than non-maturing fish. The IGF-I levels found in these fish did not relate to growth rate and the samples were removed from the analysis.

In a second study we investigated the effects of different rearing temperatures on the IGF-I vs. growth relation. In June 2001, 280 yearling coho salmon were selected from a group of previously PIT-tagged (January 2001) fish. These fish were placed in four 1.3m circular tanks and fed a ration of 1% body weight/day. After 1 week the water temperature in two tanks was reduced from 11$^{o}$C to 6.5$^{o}$C. Subsequently feeding rates were 1.5 and 1% body weight/day in the 11$^{o}$C tanks and 1 and 0.5% body weight/day in the 6.5$^{o}$C tanks. All fish were measured at two week intervals over the next two months. A sub-sample of 10 fish/tank was obtained at two week intervals, plasma collected, and plasma IGF-I was measured by RIA. There was a significant, positive relation between growth and plasma IGF-I for the fish held at 11$^{o}$C on all four sampling dates ($r^2$>0.4). For fish held at 6.5$^{o}$C, there was no relation between growth and IGF-I on the first two sampling dates after the temperature decrease. Subsequent samples revealed a similar relation between IGF-I and growth for fish held at both 11 and 6.5 $^{o}$C (no significant difference between slope and intercept).

These experiments show that a significant relation between short-term growth rate (two weeks) and plasma IGF-I exists. Short-term variations in feeding rate did not appear to alter this relation. Maturing males had IGF-I levels significantly higher than non-maturing fish and IGF-I levels did not correlate with growth in these animals. The slope of the IGF-I vs. growth relation increased significantly in September (as compared to June, July, and August). Finally, a decrease in rearing temperature disturbed the growth vs. IGF-I relation for a period of about a month. After this month, fish reared at 6.5 and 11.0 $^{o}$C displayed similar growth vs. IGF-I relations. In summary, these experiments suggest that plasma IGF-I may be used as an estimator of short-term growth rates in post-smolt coho salmon.

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# DEVELOPMENT AND VALIDATION OF A TAQMAN QUANTITATIVE RT-PCR ASSAY FOR SALMONID IGF-I MESSENGER RNA

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

The mitogenic peptide insulin-like growth factor-I (IGF-I) is a key regulator of growth. Circulating IGF-I is produced by the liver, under regulation by growth hormone (GH) and nutritional status. The recently developed TaqMan technology (Applied Biosystems, Foster City CA; henceforth ABI) uses cleavage of florescent DNA probes to monitor the accumulation of specific PCR products in real time. When combined with a reverse transcription (RT) step, TaqMan assays enable quantification of the amounts of mRNA species present in biological samples. Unlike Northern blotting or RNase protection assays, TaqMan RT-PCR assays are high throughput and have a large range of detection (Giulietti et al., 2001). We report here the development of a TaqMan RT-PCR assay for salmonid IGF-I.

TaqMan assays require normalization of target gene expression levels to the level of an invariant "housekeeping gene." We sequenced a fragment of the salmonid acidic ribosomal phosphoprotein P0 (ARP) cDNA for use as a housekeeping gene. ARP is a ubiquitously expressed structural component of the ribosome. Primers were designed based on ARP sequences available in zebrafish and catfish. PCR on chinook and coho salmon cDNA template yielded a ~450 basepair (bp) product, which was then sequenced. Liver and

pituitary template from different coho salmon gave identical sequence. Two single bp differences between coho and chinook salmon were third bases in synonymous codons.

TaqMan PCR primers and probes were designed to standard cycling conditions using the Primer Express program according to manufacturers guidelines (Table 1). The IGF-I amplicon is in the leader peptide region of the gene, and thus should detect all splicing variants of IGF-I mRNA (Duguay et al., 1994). Each assay was designed so that a primer crosses a known (IGF-I) or predicted (ARP) intron/exon boundary, in order to prevent amplification of genomic DNA, as was confirmed. RT reactions used random hexamer primers and Gibco SuperScript II reverse transcriptase. Real time PCR reactions were run in 96 well format on an ABI 7700 Sequence Detector, using ABI Universal PCR MasterMix reagent. An earlier version of the IGF-I assay showed a systematic bias across the PCR plate, with higher threshold cycle (Ct) numbers in the center of the plate. This bias was not found with the present primers and probe.

Table 1: Design of IGF-I and ARP TaqMan assays. PCR efficiencies (fold increase in DNA/PCR cycle) were calculated as in Pfaffl (2001).

| **Assay** | **Element** | **Sequence (5'-3')** | **Amplicon; Efficiency** |
|---|---|---|---|
| IGF-I | F primer | GATGTCTTCAAGAGTGCGATGTG | 84 bp; |
| | R primer | CGCCGAAGTCAGGGTTAGG | 1.601 |
| | Probe | TCTCCTGTACCCACACCCTCTCACTGCT | |
| ARP | F primer | GAAAATCATCCAATTGCTGGATG | 106 bp; |
| | R primer | CTTCCCACGCAAGGACAGA | 1.783 |
| | Probe | CTATCCCAAATGTTTCATTGTCGGCGC | |

IGF-I expression levels were quantified using an efficiency corrected relative expression method. Efficiencies of the PCR reactions in the IGF-I and ARP assays were calculated based on dilutional series of cDNA from a sample (Table 1). Expression levels of the target gene (IGF-I) were then calculated relative to an arbitrarily selected calibrator sample according to the method of Pfaffl (2001). This method has the advantage of not requiring equal efficiencies of the target and housekeeping gene PCR reactions. We have found that PCR efficiencies of different amplicons are often not the same (Giulietti et al., 2001).

The biological validity of the TaqMan IGF-I mRNA assay was confirmed *in vivo* with a feeding experiment, and *in vitro* using hepatocyte culture. In fasted chinook salmon, hepatic IGF-I expression declined to 48% of fed levels after 22 days, consistent with previous reports using an RNase protection assay (Duan and Plisetskaya, 1993). Incubation of coho salmon primary hepatocytes with GH caused a concentration dependent increase in IGF-I expression (Figure 1), again consistent with previous reports (Duan et al., 1992). In contrast, individual incubations with insulin ($10^{-6}$M), glucagon ($10^{-5}$M), thyroid hormone ($T_3$; $10^{-7}$M), and dexamethasone ($10^{-6}$M) did not change IGF-I expression.

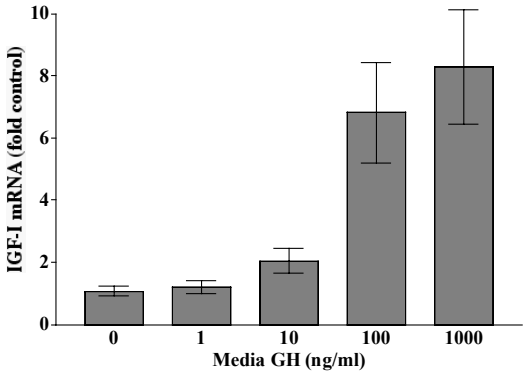


Figure 1. Effect of media GH concentration on IGF-I expression in cultured coho salmon hepatocytes. Cells were harvested by collagenase digestion, plated in RPMI medium, allowed to adhere for 24 hours, and then exposed for three days to the indicated concentration of GH in RPMI.

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# FUNCTIONAL CHARACTERIZATION AND PHYSIOLOGICAL REGULATION OF A GLUT4 HOMOLOG IN SALMONIDS

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

In most animal cells, glucose is transported across cell membranes by facilitated diffusion mediated by a family of glucose transporters (GLUTs). Several GLUT isoforms (GLUT1-5, 8-11) have been identified in mammals, with different tissue-specific expression patterns and biochemical properties. In particular, GLUT4 is expressed predominantly in muscle and adipose tissue and is responsible for the stimulation of glucose uptake in these two tissues by insulin. In mammals, insulin rapidly increases the number of GLUT4 molecules at the plasma membrane through its stimulation of the translocation of GLUT4 from intracellular storage sites in both muscle and adipose cells (Kahn 1996). In fish, insulin is also known to increase the in vivo uptake and utilization of glucose in skeletal muscle and to regulate the number of specific insulin receptors in muscle as well as in adipose tissue. Although the direct effects of insulin on glucose uptake by fish muscle or adipose cells are not clearly established, it is possible that insulin could exert its hypoglycemic effect by stimulating glucose uptake mediated by an insulin-sensitive GLUT. In order to address this question, we initiated studies to identify the presence of a GLUT4 homolog in fish (salmonid) muscle and adipose tissue and study its function and regulation.

**Identification of a GLUT4 homolog in fish muscle and adipose tissue**

Initially, we cloned by RT-PCR followed by 5'- and 3'-RACE a GLUT molecule from brown trout (*Salmon trutta*) skeletal muscle with high homology (approximately 80%) with mammalian GLUT4 sequences (Planas et al. 2000). This transporter, which was initially named btGLUT and which encoded for a protein of 503 amino acids, had structural features typical of a

functional GLUT (e.g. 12 transmembrane domains, intracellular amino- and carboxi-termini, one large intracellular loop between TMVI and VII, etc.). In addition, btGLUT showed several GLUT4-specific motifs, known to be important for trafficking in mammalian cells. btGLUT was found to be predominantly expressed in skeletal muscle, kidney and gill, which are tissues with high glucose utilization, and to a lesser extent also in adipose tissue, testis and intestine. Subsequently, we have cloned another GLUT4 homolog by screening a coho salmon (*Oncorhynchus kisutch*) adipose cDNA library with a btGLUT cDNA probe. This molecule has high homology to mammalian GLUT4 (again 80%) and is almost identical (over 95% identity) to btGLUT, indicating that okGLUT4 and btGLUT correspond most likely to the same isoform of transporter (GLUT4) in adipose tissue and muscle, respectively, from two different salmonid species

**Functional characterization of the fish GLUT4 homolog**

In order to study the glucose transport properties of the fish GLUT4 homolog, we have expressed okGLUT4 in *Xenopus* oocytes and have determined its kinetic and biochemical characteristics. The Km or affinity constant for 2-deoxyglucose (2-DG) transport was in the range of 5 to 9 mM measured under zero-*trans* conditions. These values were higher than the value found for GLUT4 in mammals (5 mM) but lower than those for rainbow trout GLUT1 expressed in the same system (8-15 mM) (Teerijoki et al. 2001). Transport of 2-DG by okGLUT4-injected oocytes was estereospecific, and was competed by D-glucose, D-mannose and, to a lower extent, D-galactose and D-fructose. 2-DG transport was inhibitable by cytochalasin B and ethylidene glucose in a concentration-dependent manner. Moreover, glucose transport in oocytes expressing okGLUT4 was significantly stimulated by insulin.

**Regulation of btGLUT expression by blood insulin levels**

In order to study the possible physiological regulation of the expression of btGLUT in fish, we have examined by Northern blot its expression in red and white skeletal muscle from trout in which blood insulin levels had been manipulated experimentally. The expression of btGLUT in red muscle increased significantly when insulin plasma levels were elevated either by insulin (10 μg/100g fish; 24 hours; Fig. 1) or arginine (6.6. μmol/g fish; 24 hours) treatment and decreased significantly when insulin plasma levels were reduced either by fasting (45 days) or by feeding a low-protein, high-carbohydrate diet (2 months). In contrast, the expression of btGLUT in white muscle was not affected by changes in the plasma levels of insulin. Contrary to btGLUT expression, no changes in the expression of OnmyGLUT1, a

rainbow trout GLUT1 homologue, were detected in either red or white muscle in response to alterations in insulin plasma levels. These results strongly suggest that insulin could be regulating the expression of btGLUT in trout red muscle in vivo. It will be important to determine if insulin increases btGLUT mRNA levels because of an increase in the rate of transcription of the btGLUT gene.

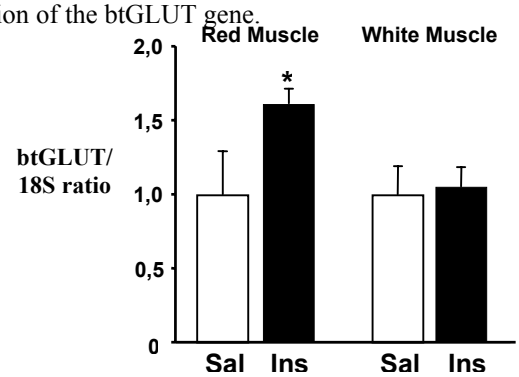


Figure 1. Effects of insulin treatment on btGLUT mRNA expression in red and white muscle. Sal, saline; Ins, insulin.

## Conclusions

Our results indicate that the muscle and adipose tissue of salmonid fish express a structural and functional GLUT4 homolog. We have shown that insulin can stimulate its expression in red skeletal muscle and can cause a modest increase in glucose uptake when the transporter is expressed in Xenopus oocytes. However, the fish GLUT4 homolog has lower affinity for glucose than mammalian GLUT4, which could help explain the lower ability of fish to clear a glucose load, when compared to mammals. Key to our understanding to the function of the fish GLUT4 homolog will be to determine its ability to be recruited to the plasma membrane in response to insulin.

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# ECOPHYS.FISH: A SIMULATION MODEL OF FISH GROWTH IN TIME-VARYING ENVIRONMENTAL REGIMES

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Ecophys.Fish is a model for simulating rates of fish growth in environmental regimes that have simultaneous temporal variation in food, oxygen, temperature, pH, and conductivity/salinity. The purpose of this presentation is to introduce Ecophys.Fish to those who might want to use it as a framework or starting point for applications of their own.

Our model incorporates a quantitatively explicit statement of concepts originally formalized by F.E.J. Fry (Fry 1947, 1971) and subsequently elaborated by Neill and Bryan (1991), among others. Fry's "physiological classification of environment" and "metabolic scope" concept were coupled with conventional bioenergetics to

provide the model's theoretical basis. Jointly, the factors of environment determine the animal's metabolic scope, which is the difference between its active (= maximum aerobic) and standard (= obligatory minimum) metabolic rates; metabolic scope is the animal's capacity to perform useful activities like locomotion, feeding, and the physiological processing of food that leads to growth.

Ecophys.Fish's central (and distinguishing) rule is this: Fish eat all appropriate food encountered or until available digestive or metabolic capacities become insufficient to support the processing of more food; the fish then partitions the consumed food energy and substrates in the usual ways (conventional bioenergetics), between various obligatory activities and growth; if obligatory activities cost more than available metabolic scope, the fish loses mass and energy (but does not die). Time-varying food, oxygen, temperature, pH, and conductivity/salinity are accommodated as limiting, controlling, and loading effects on metabolism and, thus, on metabolic scope. Because the present version of the model lacks explicit treatment of swimming and its metabolic costs, it has been expedient to adopt "Winberg's rule": routine metabolism is twice standard metabolism. This leads to a functional definition of metabolic scope for growth as the active metabolic rate less twice the standard rate.

As implemented in STELLA®, the model has two functional modules, metabolism and bioenergetics (Figure 1). The metabolism module has a STELLA subspace for each of Fry's five classes of environmental factors. (At present, the directive-factor subspace is empty; and, consideration of the lethal-factor subspace is beyond the scope of this presentation.) In the loading-factor subspace, the salinity subroutine computes and returns a standard-metabolism intercept, which is multiplied by a function returned by the temperature subroutine in the controlling-factor subspace, and by $Wfish^{-0.2}$, to give standard metabolic rate. Temperature's controlling effect on standard metabolism is modeled as the product of steady-state and transient-state components, reflecting both the Ahrennius effect and thermal acclimation. Both the temperature and pH subroutines in the controlling-factor subspace produce outputs that control weight-dependent active metabolism. These controlling effects are modeled as interactions with the limiting effect of DO, in the limiting-factor subspace. The residual intercept of active metabolism is MMSO, where MMS is marginal metabolic scope (Neill and Bryan 1991). MMSO we interpret to represent inherent metabolic efficiency of the fish-environment system, after the effects of temperature, pH, DO, conductivity/salinity, and fish size have been taken into account. MMS offers a practical measure of "water quality" from the fish's perspective and is relatively easy to determine, via automated routine respirometry (Neill and Bryan 1991).

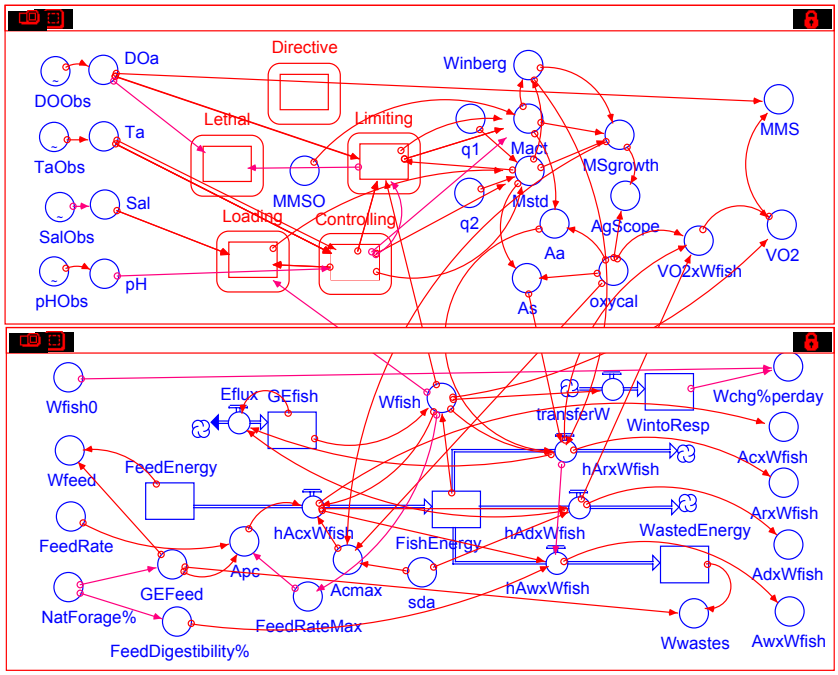

Figure 1. Ecophys.Fish: STELLA model of fish growth in response to ecophysiological factors.

The bioenergetics module reflects conventional "rules of thumb" (Warren and Davis 1967; Brett and Groves 1979), with the above-mentioned addition of a component for metabolic limitation of food intake. Consumed energy is converted to new fish biomass as a residual, after expenditures for standard and routine-activity components of metabolism, SDA (0.15*food energy, for most foods), and wastes (0.15 to 0.35*food energy). Our modeled fish conserves body form, by reducing and increasing caloric density of its tissues (within species-specific ranges) during times of food-energy limitation and surplus, respectively.

The model's inputs are time series of temperature, pH, DO, conductivity/salinity, food availability and energy content, and initial size of fish. Outputs are food consumption, oxygen consumption, waste production, energy-density of fish

biomass, and growth.

Indirectly, the output is a measure of relative fitness of the fish-environment system to support fish growth.

Two variants of the STELLA simulation model represent red drum (*Sciaenops ocellatus*) and bluegill (*Lepomis macrochirus*). Ecophys.Fish had its beginnings in lab experiments with juvenile red drum, at Texas A&M; these experiments enabled definition of functions and their parameterization, leading to a working model that accurately simulated growth of red drum in various pond and estuary trials with caged fish (Figure 2). Subsequently, Ecophys.Fish was converted to simulate growth rates

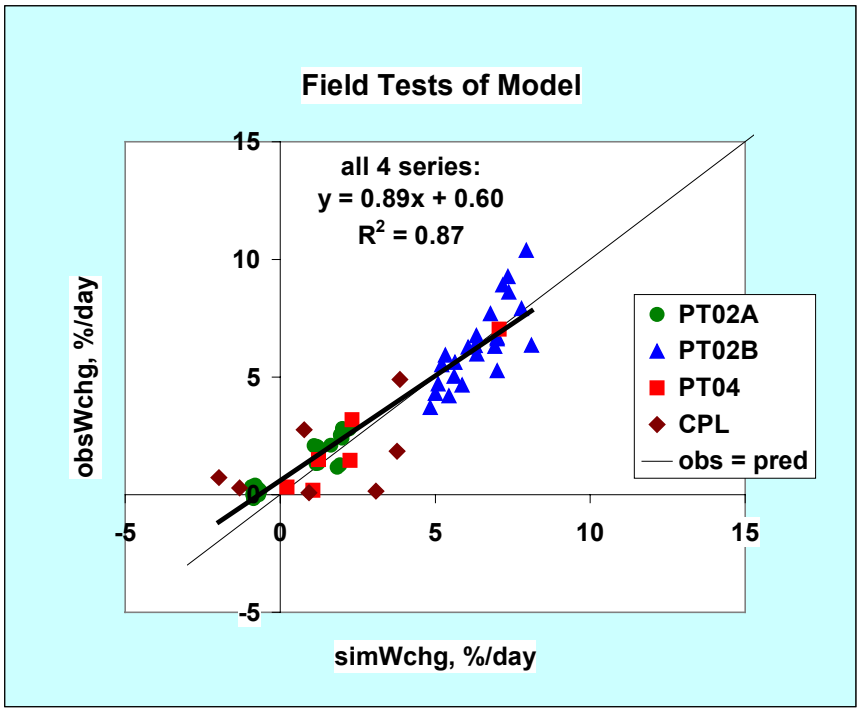

Figure 2. Observed versus simulated growth rates of caged red drum, in four field tests of model.

of caged bluegill involved in stream ecoassays. The latter work confirmed the utility of automated routine respirometry for estimating the model's MMSO parameter empirically.

We have gained confidence in the model to the degree that we now have come to consider deviations between observed and simulated results not so much as evidence of model flaws, as a source of insight into the biological and ecological processes involved in fish growth.

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**EFFECT OF COPPER SULFATE ON IONS BALANCE AND GROWTH IN TILAPIA LARVAE (*Oreochromis mossambicus*)**

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**EXTENDED ABSTRACT ONLY- DO NOT CITE**

The newly-hatched tilapia larvae were exposed to sub-lethal concentration $Cu^{2+}$ (0, 30, 50 and 100 μg/L) and lethal concentration $Cu^{2+}$ (0, 200 and 400 μg/L) for 24-96 h. Following the larval development, a whole-body $Cu^{2+}$ concentration was dramatically increased and the amount of $Cu^{2+}$ accumulation indicated a dose-response relationship with the ambient level of $Cu^{2+}$ when the $Cu^{2+}$ concentration was lower than 400 μg/L. Furthermore, the $Cu^{2+}$ accumulation was significantly increased following the $Cu^{2+}$ exposure time until 96 h. $Cu^{2+}$ in the medium revealed a dose-response effect on the $Na^{+}$, $K^{+}$, and $Ca^{+}$ contents in larvae. Changes of the $Na^{+}$ and $K^{+}$ contents were statistically significant when larvae exposure with higher than 100 μg/L $Cu^{2+}$ and exposure time over 72 h. Calcium contents also appeared to decrease significantly in tilapia, that exposure with higher than 200 μg/L $Cu^{2+}$ for 24 h. However, $Na^{+}$ and $K^{+}$ content caused significant increase of 17-23% in larval as compared to the control when larvae exposure to low concentration of $Cu^{2+}$ (30-50 μg/L) for 24-72 h. Copper did not cause any significant effect on body $Cl^{-}$ and osmolality content in tilapia larvae as compared to the control. However, there was a restored phenomenon in the larvae exposure 100 μg/L $Cu^{2+}$ between 24 and 72 h. The water content did not significantly decrease at any dose of $Cu^{2+}$ exposure between 24 and 72 h, but it appeared statistically significant decreasing until the larvae exposure $Cu^{2+}$ for 96 h. The growth of tilapia larvae was significantly suppressed when they were exposed to 100 μg/L $Cu^{2+}$ over 72 h. These results suggested that the sensitivity to $Cu^{2+}$ in larval development was more evident than others stage. Furthermore, the

larvae were able to restore or regulate ions and osmolality when they were exposed to low dose of $Cu^{2+}$ or at short time.

Table 1. Comparison of whole-body $Na^{+}$ contents (μg/larva) of new-hatching larvae exposed to different levels of waterborne $Cu^{2+}$ for 24 to 96 hr.

| Brood1 | | | | |
|---|---|---|---|---|
| Exposure Time (hr) | Concentration of Copper (μg/L) | | | |
| | 0 | 30 | 50 | 100 |
| 24 | 1.85±0.02 [a] | 1.85 | 2.17±0.30 [b] | 1.89±0.40 [a] |
| 72 | 1.92±0.24 [a] | 2.43±0.39 [a] | 1.76±0.20 [a] | 1.84±0.32 [a] |
| 96 | 6.93±0.42 [a] | 4.77±0.42 [b] | 3.08±0.62 [b] | 3.13±0.58 [b] |

| Brood2 | | | |
|---|---|---|---|
| Exposure Time(hr) | Concentration of Copper (μg/L) | | |
| | 0 | 200 | 400 |
| 24 | 3.63±0.15 [a] | 2.43±0.16 [b] | 1.81±0.19 [b] |
| 72 | 4.51±0.21 [a] | 2.02±0.25 [b] | 1.58±0.21 [b] |
| 96 | 5.17±0.11 [a] | 1.92±0.43 [b] | ----- |

Mean±SD (n=4-6) is indicated
---- No data available due to the larvae died
Different superscribed letters indicate significant difference ($p$<0.05) among treatments (ANOVA analysis with Tukey's comparison were using)

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