NOAA
National Marine Fisheries Service

Fishery Bulletin
established 1881

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Evaluation of nucleic acids and plasma IGF1 levels for estimating short-term responses of postsmolt Atlantic salmon (*Salmo salar*) to food availability

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Abstract—We evaluated 4 potential indices obtained by nonlethal sampling for use in determining nutritional state and short-term growth rate in postsmolt Atlantic salmon (*Salmo salar*): the ratio of RNA to DNA, both RNA and DNA normalized to protein, and plasma levels of insulin-like growth factor 1 (IGF1). Fish reared in the laboratory for 27 days were fed, fasted, or refed. Short-term growth rates (7 to 23 day intervals) were calculated on a wet-weight basis. RNA/DNA values were highly correlated to growth rates, responded rapidly to changes in food availability and were the best able to consistently distinguish between the fasted and fed treatments. RNA/protein values were also well correlated with growth rate; however, within any one sampling day, feeding groups could not be differentiated with this index. DNA/protein increased during fasting but was neither strongly correlated with growth rate nor an accurate discriminator of nutritional state. IGF1 values were positively correlated with growth rates and responded rapidly with refeeding but changed little during the 3 weeks of fasting—a result that may have been influenced by sampling serially. We propose that RNA/DNA is a useful nonlethal technique for estimating recent growth rates and for identifying the nutritional condition of individual postsmolt Atlantic salmon exposed to short-term changes in food availability.

Manuscript submitted 15 May 2015.
Manuscript accepted 31 March 2016.
Fish. Bull. 114:288–301 (2106).
Online publication date: 3 May 2016.
doi: 10.7755/FB.114.3.3

The ability to measure the growth rate of a fish can be a powerful tool for evaluating the survival potential of an individual. The ability to assess the nutritional state of a fish, whether the animal is feeding or fasting and for how long the fish has been in that state (hours to days to weeks) is also highly desirable information because variation in nutritive state leads to variation in growth rate. Currently, there are a limited number of nonlethal techniques available for estimating growth rates or nutritional state (or both) in field-caught juvenile fish. Longitudinal cohort analysis is a direct approach to assessing changes in size (growth); however obtaining multiple samples of the same cohort in the field can be challenging. Biochemical indices that indirectly yield estimates of growth rate or nutritional state have the advantage of providing estimates within a single sampling. This point estimate allows an investigation of the connectivity between nutritional state and environmental parameters on relevant temporal and spatial scales.

In this study we evaluated 4 potential biochemical indices of short-term growth-rate or nutritional state in postsmolt Atlantic salmon (*Salmo salar*): the ratio of RNA to DNA (RNA/DNA), both RNA and DNA on a protein basis (RNA/pro and DNA/pro, respectively) and circulating plasma insulin-like growth factor 1 (IGF1). Considerable effort has been directed toward hatchery-based restoration of Atlantic salmon to 8 rivers in Maine, where the population has been listed as endangered since 2009 under the United States Endangered Species Act (Federal Register, 2009). Restoration managers require tools to both assess whether hatchery-reared fish are thriving in the natural environment and to assess the condition of native postsmolts. Identifying a minimally invasive, nonlethal method to provide an index of growth rate or nutritional state in postsmolt Atlan-

tic salmon would allow restoration managers to evaluate the condition of field-captured fish.

Since their first application in the 1970s, RNA-based indices have been used to determine the nutritional state and growth rates of larval and juvenile fish in both the laboratory and field (Bulow, 1970; Buckley, 1979; Buckley et al., 1999; Gwak and Tanaka, 2001; Vasconcelos et al., 2009; Ciotti et al., 2010; among many other studies). Juvenile fish grow rapidly through accretion of protein, and the amount of RNA in a cell is a measure of the capacity of a cell to synthesize protein (Millward et al., 1973). MacLean et al. (2008) evaluated 4 tissues in Atlantic salmon postsmolts and determined that RNA/DNA values from muscle tissue were those that were the most highly correlated with growth rate, and that muscle tissue samples could be obtained by nonlethal means with a biopsy punch. DNA/protein has been shown to increase during fasting (Bulow, 1970; Mathers et al., 1993; Fukuda et al., 2001) and thus could provide useful information about the nutritional state of a fish. Circulating plasma insulin-like growth factor 1 (IGF1) is a polypeptide that is involved in a number of regulatory processes, including differentiation and proliferation of cells. The preponderance of evidence indicates a significant relation between growth rates and the plasma level of IGF1 in fish within some constraints (see review by Beckman, 2011). Pierce et al. (2001) have shown that blood can be drawn by nonlethal means to obtain samples for this index. In most studies of juvenile fish, sampling has been too infrequent to establish the response time of nucleic-acid–based indices or of IGF1 to food variability. Because our field recaptures of hatchery-reared postsmolts occur 2 to 3 weeks after their release, we designed our experiment to focus on nutritive responses to short-term changes in food availability rather than to longer term changes. Results presented here are part of a larger laboratory study designed to evaluate a variety of nonlethal techniques for detecting short-term changes in the nutritional status of postsmolt Atlantic salmon. Results regarding proximate body composition, Fulton's K, and bioelectrical impedance analysis (BIA) of the same individuals reported in the present study can be found in Caldarone et al. (2012).

Materials and methods

Smolts used in this study were progeny of field-caught Atlantic salmon from the Penobscot River, Maine. They had been spawned at Craig Brook National Fish Hatchery, East Orland, Maine, and reared at the Green Lake National Fish Hatchery, Ellsworth, Maine, for 13–15 months. In 2008, 80 randomly selected smolts (52–113 g, 16–21 cm) were anesthetized in buffered tricaine methane sulfonate (MS-222, 150 mg/L) and were implanted intramuscularly with a passive integrated transponder tag (PIT tag, Biomark, Boise, ID[1]) to permit identification of individuals. The smolts were then returned to the hatchery tank to allow time for full recovery, resumption of feeding, and removal of any tagging-related mortalities (5 fish). Twenty-five days later the fish were transported to the University of Rhode Island's Blount Aquarium facility in Narragansett, Rhode Island, where they were randomly placed in two aerated, flow-through tanks (360-L capacity) initially filled with freshwater trucked from the hatchery. Over a period of 5 to 6 hours, freshwater was gradually replaced with sand-filtered seawater (10°C, 30 ppt). During the next 3 weeks, while the fish were recovering from the transfer and acclimating to seawater, the water temperature was gradually raised to 12°C. During this period fish were fed to satiation twice per day with a commercial feed (Corey Optimum Hatchery Feed for Salmonids, Corey Nutrition Co., Fredericton, NB, Canada), supplemented with freeze-dried krill (*Euphausia pacifica*, Aquatic Eco-Systems, Inc., Apopka, FL). Twenty-five days after the initial transfer to seawater, when the now postsmolts appeared to be acclimated and feeding well, the experiment commenced (day 0).

Throughout the experiment, water temperature in each flow-through tank was recorded hourly with an HOBO® data logger (Onset Computer Corp., Bourne, MA), and ammonia levels and salinity were tested weekly. Water temperatures averaged 12.0°C, standard deviation (SD)=0.2; salinity averaged 31 ppt, SD=1; and the photoperiod was 15 hours of light to 9 hours of dark. Part (two-thirds) of each tank surface was covered with black plastic to provide a low-light refuge, and the remaining third was exposed to overhead fluorescent lighting that was covered with red plastic to better mimic natural light.

Feeding treatments and sampling schedule

Five fish were randomly selected on day 0 from the acclimation tanks, sacrificed, and sampled to provide baseline biochemical data. The remaining 70 postsmolts were subdivided into 3 feeding treatments (tanks): fed, fasted, and fasted then refed. The purpose of the different feeding regimens was to produce fish growing at a range of rates, not to test the effect of ration on growth rate. By measuring and sampling tagged fish we were able to assess the relation of the biochemical indices to growth rate on an individual basis.

The fed treatment (n=24) was fed ad libitum, the fasted treatment (n=24) received no food, and the refed treatment (n=22) received no food for 11 days followed by feeding for 16 days. Before being placed in 360-L flow-through treatment tanks on day 0, all individuals were anesthetized with buffered MS-222 (150 mg/L) in chilled (12°C) seawater, blotted dry, measured for initial weight (wet weight, WW_{init}, nearest 0.1 g) and for fork length (FL, nearest 0.1 cm), examined for any gross external abnormalities, and their PIT tag number

[1] Mention of trade names or commercial companies is for identification purposes only and does not imply endorsement by the National Marine Fisheries Service, NOAA.

Table 1

Sampling schedule for postsmolt Atlantic salmon (*Salmo salar*) reared in the laboratory at 12ºC under 3 feeding regimens (fed; fasted; fasted then refed) in order to determine the response time to varyhing food availability. Condition indices obtained by nonlethal sampling techniques (RNA/DNA; RNA/protein; DNA/protein; IGF1) and wet-weight–based growth rate were determined for each fish. The refed group was fasted for 11 days and then fed for 16 days. Numbers listed are number of fish sampled.

	Sampling day and feeding regimen												
		Day 0			Day 3			Day 7			Day 11		
	Baseline	Fed	Fasted	Refed	Fed	Fasted	Refed	Fed	Fasted	Refed	Fed	Fasted	Refed
Length, weight	5	24	24	22	4	4	0	4	4	0	4	4	22
Muscle plugs for nucleic acid and protein	5	0	24	0	4	4	0	4	4	0	4	4	22
Blood sample for IGF1 measurement	5	0	24	0	4	4	0	4	4	0	4	4	22
		Day 15			Day 19			Day 23			Day 27		
		Fed	Fasted	Refed	Fed	Fasted	Refed	Fed	Fasted	Refed	Fed	Fasted	Refed
Length, weight		4	4	5	4	4	5	4	4	5	0	0	7
Muscle plugs for nucleic acid and protein		4	4	5	4	4	5	4	4	5	0	0	7
Blood sample for IGF1 measurement		4	4	5	4	4	5	4	4	5	0	0	7

was recorded. Additionally, 2 muscle plugs and a blood sample were obtained from fish assigned to the fasting treatment in order to track individual response times to fasting. Fish in the group of refed postsmolts were again weighed and measured and then sampled on day 11 to obtain individual baseline fasting values before fish were refed. Total time needed for the biochemical sampling was less than 1 minute per fish. From day 3 onward, 5 fish from the fasted and fed treatments were sampled and sacrificed every 4 days for 20 days. From day 15 onward, 5 fish from the refed treatment were sampled and sacrificed every 4 days until day 27 when 7 fish were sacrificed (Table 1). Final wet weight, FL, 2 muscle plugs for biochemical analysis, and a blood sample for IGF1 determination were obtained from all sacrificed fish. All fish were also sampled for bioelectrical impedance analysis (BIA) indices and proximate body composition (see Caldarone et al., 2012). All aspects of this experiment were conducted in accordance with guidelines established by the Institutional Animal Care and Use Committee (IACUC) at the University of Rhode Island

Sampling protocol and biochemical analyses

IGF1 Blood samples (0.3 mL) for IGF1 analysis were obtained from the caudal vein using a sterile heparinized syringe (23-ga×25-mm needle). Samples were immediately transferred to a microfuge vial, stored on wet ice for < 0.5 hour, and centrifuged at 5000×g for 5 minutes. Plasma was removed by pipet, transferred to a 1.5 mL microfuge vial and stored at –80˚C until further analysis. Samples were analyzed at the National Marine Fisheries Service, Northwest Fisheries Science Center by using an immunoassay to measure the concentration of IGF1. Briefly, IGF1 was isolated from plasma by acid-ethanol extraction, and measured by TRF immunoassay by using a modification of the methods described by Small and Peterson (2005). Each sample was analyzed in duplicate, and samples with low (<30%) or high (> 85%) binding, as well as those with a coefficient of variation exceeding 10%, were reanalyzed or excluded. IGF1 values are reported as ng/mL plasma.

Nucleic acids and protein A 2-mm diameter biopsy punch (MacLean et al., 2008) was used to remove 2 muscle samples for analysis of nucleic acids and protein. Samples were taken from the epaxial muscle between the lateral line and dorsal fin. Each muscle plug was immediately placed in a microfuge vial, stored on wet ice for <0.5 hour and then transferred to –80°C until analysis. Nucleic acid levels were measured by using a 2-enzyme (RNase, DNase) ethidium bromide fluorometric microplate method. On the day of analysis, each muscle plug was transferred to a cold glass slide and any fat layer, skin, or blood was removed. The top 2 mm of the muscle plug was transferred to

a microfuge vial containing 150 µL 1% N-lauroylsarcosine. The vial was placed in an ice slurry and the sample was sonicated for three 5-second pulses followed by 45 minutes of vortexing at room temperature. From that point onward, we followed the protocol of Caldarone et al.[2] for nucleic acid analysis. Results from duplicate plugs were averaged. The ratio of the slope of the DNA standards to the slope of the RNA standards was mean=2.5, SD=0.05 (n=7 microplates). This value can be used to convert the reported RNA/DNA data for direct comparison with other published studies (Caldarone et al., 2006). The remaining extract was stored frozen and later analyzed for protein content by using a bicinchoninic-acid–based assay adapted for a microplate format (Caldarone, 2005). Nucleic acids (µg) are expressed as a RNA to DNA ratio (RNA/DNA; µg/µg), RNA to protein ratio (RNA/pro; µg/mg) and DNA to protein ratio (DNA/pro; µg/mg).

Calculations of growth rates Individual instantaneous weight-based growth rates (per d) were calculated with the following formula:

$$growth\ rate\ (G) = (\ln WW_{t2} - \ln WW_{t1})/(t_2 - t_1) \quad \text{(Ricker, 1979)}, \qquad (1)$$

where WW = the wet weight of an individual at time t (day).

For comparison with previously published data, growth rates were converted to specific growth rates (% per d) with the following formula:

$$SGR = 100(e^G - 1). \qquad (2)$$

Growth rates for fish in the fed and fasted treatments were calculated from day 0 until the day the fish were sacrificed. Growth rates were calculated from day 0 until day 11 for the fasted portion of the refed treatment fish; for the fed portion of the refed treatment fish, growth rates were calculated from the first day of refeeding (day 11) until the day the fish were sacrificed. Because there is inherent variability in measuring the wet weight of fish, coupled with small changes in weight over short time intervals, growth rates from time intervals <4 days were not included in any of the statistical analyses.

Data analysis To examine the effect of the feeding treatment and sampling day on growth rate, RNA/DNA, RNA/pro, DNA/pro and IGF1, a 2-way multivariate analysis of covariance (MANCOVA) for unbalanced design was used with WW_{init} as the covariate. When interactions were significant, feeding treatment was nested in day, and follow-up comparisons were examined by using Tukey's HSD multiple range test. Linear growth rate models with all combinations of the 4 biochemical variables plus WW_{init} were constructed. Akaike's information criterion for small sample sizes (AICc; Wagenmakers and Farrell, 2004) was used to select the best candidate model from the 31 models tested. Because of high collinearity of RNA/DNA with the other 3 biochemical indices, all combinations of models without the RNA/DNA term were also investigated. To examine the response of the 4 biochemical indices and growth rate in individual fish to food withdrawal or introduction, paired initial and final data from individual fish from both the starved and refed fish were analyzed by using a repeated measure t-test.

Within each feeding group, a Dunnett 2-tailed t-test with WW_{init} as a covariate was used to detect changes in growth rate and the 4 biochemical variables compared with those in a control. Day 0 values were specified as the control for the biochemical variables for both fasted and fed treatments. The average growth rate of all fish from the time they were tagged until day 0 (50 days) was used as the control growth-rate value with the understanding that growth rates during this time would have been less than optimal. For the refed treatment, values for day 11 (day they were refed) were used as the control for all variables. An ANCOVA was used to test whether the slope of the relation of growth rate to the measured biochemical indices was significantly different between the fed and refed groups. All statistical analyses were carried out with SAS software, vers. 9.3 (Statistical Analysis Software Inst., Inc., Cary, NC) with a significance level set at $P \leq 0.05$.

Results

At the start of the experiment (day 0) ~78% of the fish exhibited frayed and ~12% exhibited eroded dorsal and pectoral fins. The frequency and severity of these conditions did not change throughout the experiment and there were no mortalities during the study. All fish appeared to be immature and sex was not a significant factor in any of the statistical analyses. On day 0, WW_{init} ranged from 43 to 132 g and FL from 18 to 23 cm. Initial size distributions (WW_{init}) between feeding treatments were not significantly different (fed mean=76 g, SD=12; fasted mean=75 g, SD=13; fasted then refed mean=80 g, SD=4).

Weight-based growth rates of the fish responded quickly to changes in food availability. Fasted fish lost weight beginning on day 7 and by day 11 their growth rates were statistically significantly less than the continually fed fish (Fig. 1A). Negative growth rates of the fasted fish remained constant throughout the experiment (Dunnett, P=0.747), whereas fed fish growth rates increased in relation to day 0 rates (Dunnett, P<0.0001). On day 19, fed fish had faster growth rates than refed fish, whereas the relation was reversed on day 23. Refed fish grew significantly faster than fasted fish beginning 4 days after refeeding (day 15). During the experiment we noted that the feeding intensity of the salmon visibly decreased when the total num-

[2] Caldarone, E. M., M. Wagner. J. St. Onge-Burns, and L. J. Buckley. 2001. Protocol and guide for estimating nucleic acids in larval fish using a fluorescence microplate reader. Northeast Fish. Sci. Cent. Ref. Doc. 01-11, 22 p. [Available at website]

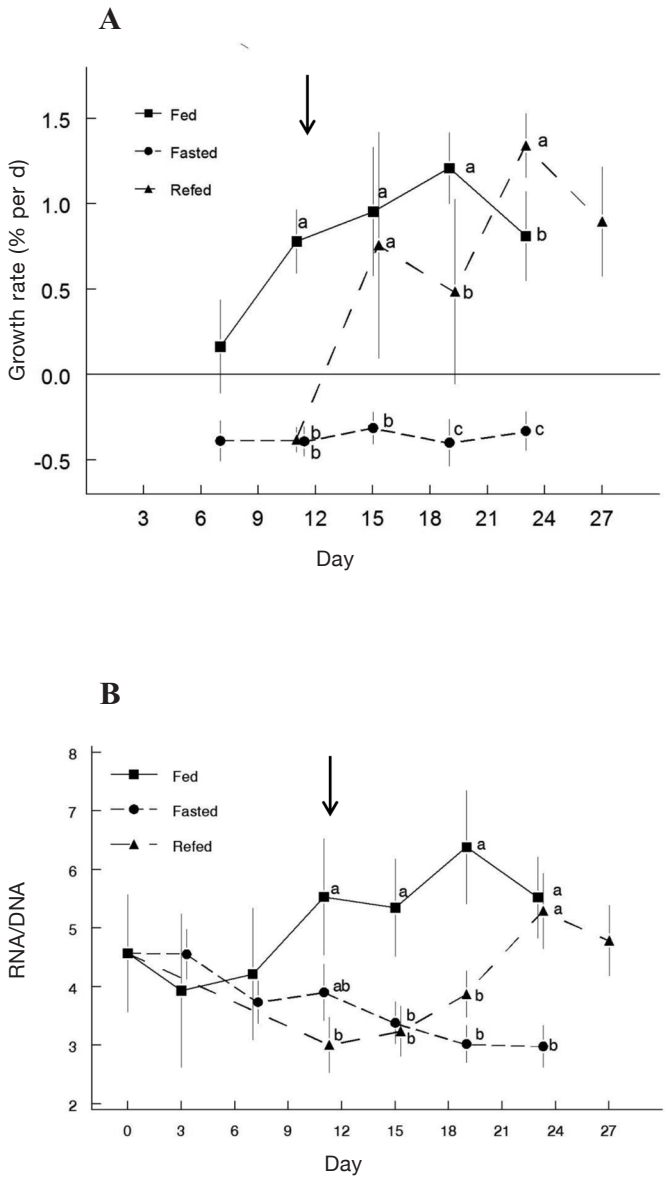

Figure 1

(**A**) Specific growth rates (% wet weight per d); and (**B**) RNA/DNA values of postsmolt Atlantic salmon (*Salmo salar*) reared in the laboratory at 12°C. Fish were either fed ad libitum, fasted, or refed (fed after 11 days of fasting). Values are mean values (±standard deviation [SD]) for each sampling day. Growth rates for fish in the fed and fasted treatments were calculated from day 0 until the day they were sacrificed. Growth rates for the refed treatment were calculated from the first day of refeeding (day 11) until the day fish were sacrificed. Within a sampling day, food treatments showing a common superscript, or without superscripts, do not differ significantly (Tukey's HSD multiple range tests). Where error bars would overlap, data are offset to facilitate viewing. The arrow indicates the day when food was introduced to the refed group. For fed and fasted fish, n=4 for each sampling day. For refed fish, n=22 for day 11, n=5 for days 15, 19, and 23, and n=7 for day 27.

ber of fish in the tanks fell below 8 individuals because of sampling. This observation was confirmed by the decrease in average growth rates for fish sampled on the last day of both the fed (day 23) and refed (day 27) treatments (Fig. 1A).

After 11 days, RNA/DNA values were significantly greater in fed fish than in the fasted portion of the refed fish (Fig. 1B), and from day 15 onward, significantly greater than values for fish in the fasted group. Twelve days after refeeding, RNA/DNA values of the refed group were greater than the fasted fish and equal to the continually fed fish (day 23, Fig. 1B). Mean RNA/DNA values in fed and refed fish increased in relation to their mean start values (Dunnett, P=0.011, P<0.0001 respectively), whereas mean RNA/DNA values for fasted fish decreased beginning 15 days after food was withheld (Dunnett, P=0.003). Repeated measurements from individuals in the fasted group exhibited an overall significant decrease in RNA/DNA from their start values (Student's paired t test P<0.0001, Fig. 2A). Repeated measurements of refed fish exhibited an overall significant increase from their individual start values (Student's paired t-test P<0.0001, Fig. 2B). RNA/DNA values were highly positively correlated with growth rates in the fed group, refed group, and the all-data-combined group, but not in the fasted group (Table 2).

Beginning on day 11, RNA/pro values were generally greater in the fed fish than in the fish in the fasted treatment (Fig. 3A). In fed fish, mean RNA/pro values did not change from mean initial values (Dunnett P=0.391), whereas fasted fish showed modest decreases with a significant Dunnett value (P=0.009), primarily driven by 1 fish on day 19 (Fig. 3A). Beginning 8 days after refeeding (day 19), mean RNA/pro values of refed fish increased from the mean start value (Dunnett P<0.0001). The same pattern was seen in paired data from individuals; repeated measurements from fasted fish exhibited an overall significant decrease from their start values (Student's paired t-test P=0.002), whereas values of refed fish increased (Student's paired t-test P<0.0001) (plots not shown). RNA/pro values were positively correlated with growth rates in the fed group, refed group, and the all-data-combined group, but not in the fasted group (Table 2).

The DNA/pro ratio of fasted and refed fish was higher (smaller cells) than in fed fish, and the main effect of feeding treatment on DNA/pro was statistically significant (Fig. 3B); but, because of high daily variability, coupled with a small sample size, most Tukey-Kramer post-hoc comparisons of feeding treatment within a sam-

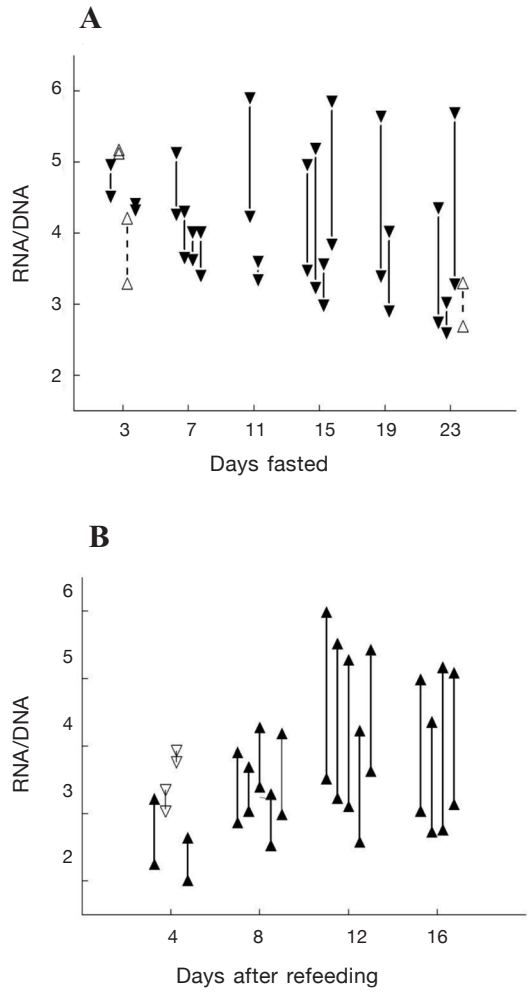

Figure 2

Each arrow represents changes in RNA/DNA values of individual laboratory-reared postsmolt Atlantic salmon (*Salmo salar*) that were either (**A**) fasted or (**B**) refed (fed after 11 days of fasting). Direction of the triangle is the direction of change in the values from (**A**) day 0 start values (when fish were fed) through the number of days (23 d) that fish were fasted; and (**B**) day 11 values (start of fasting) through number of days (16 days) that fish were refed after fasting. The open and closed triangles differentiate between the two different directions of change.

ple day were not statistically significant. Within each food treatment, DNA/pro did not change from mean initial values (Dunnett P=0.295, 0.090, 0.071 fasted, fed, refed, respectively). However, repeated measurements of individuals indicated that DNA/pro in individual fish increased during fasting (Student's repeated measure t-test P=0.033) (plot not shown). DNA/pro values were not correlated with growth rates in the 3 individual food treatment groups but had a significant, although low, negative correlation when all three groups were combined (Table 2).

Table 2

Pearson product-moment correlations (r) between the instantaneous weight-based growth rate (per d) of postsmolt Atlantic salmon (*Salmo salar*) and the ratio of RNA:DNA (RNA/DNA, µg/µg), RNA and DNA on a protein basis (RNA/pro, DNA/pro, respectively, µg/mg), and circulating plasma insulin-like growth factor 1 (IGF1, ng/mL). Fish were either fed or fasted for 23 days, or refed (fed for 16 days after 11 days of fasting). For each feeding treatment, boldface type highlights the highest significant correlation with growth rate. *P<0.05, **P<0.005, ***P<0.0001. n=number of fish sampled.

Variable	Fed	Fasted	Refed	All data
n	19	19	17	55
RNA/DNA	**0.882*****	–0.295	**0.853*****	**0.832*****
RNA/pro	0.765***	–0.190	0.666**	0.727***
DNA/pro	–0.354	0.090	–0.238	–0.507***
IGF1	0.502*	–0.403	0.543*	0.661***

Within a sample day, IGF1 values between feeding treatments were not statistically significantly different (Fig. 3C). As with DNA/protein measurements, high variance (SD), coupled with a small sample size, yielded very low statistical power to detect differences between treatments. Beginning on day 15 and continuing until the conclusion of the experiment, mean IGF1 values in fed fish were significantly greater than day-0 mean values (Dunnett P<0.0001). Fasted fish showed no change in IGF1 with time, either on a daily mean (Dunnett P=0.722) or on an individual basis (Student's repeated measure t-test P=0.065, Fig. 4A). Mean IGF1 values of refed fish increased 12 days after food was introduced (day 23, Dunnett P<0.0001), and repeated measurements of individuals indicated that final IGF1 values in refed fish were greater than their start values (Student's repeated measure t-test P=0.0001, Fig. 4B). IGF1 values were positively and significantly correlated with growth rate**s** in the fed group, refed group, and the all-data-combined group (Fig. 5, Table 2).

A plot of growth rate vs. RNA/DNA by food treatment revealed a difference in the relation of RNA/DNA to growth rate between the fed and refed groups (Fig. 6). ANCOVA results confirmed that the slopes of the regression lines of the two feeding treatments were the same but the intercept of the refed data was significantly greater. Slopes and intercepts of the regressions between the other 3 biochemical measures and growth rate did not differ between the fed and refed groups. Linear growth models containing all combinations of WW_{init}, RNA/pro, DNA/pro, RNA/DNA, and IGF1 were examined (31 models). On the basis of AICc values, the best candidate model for predicting growth rate in-

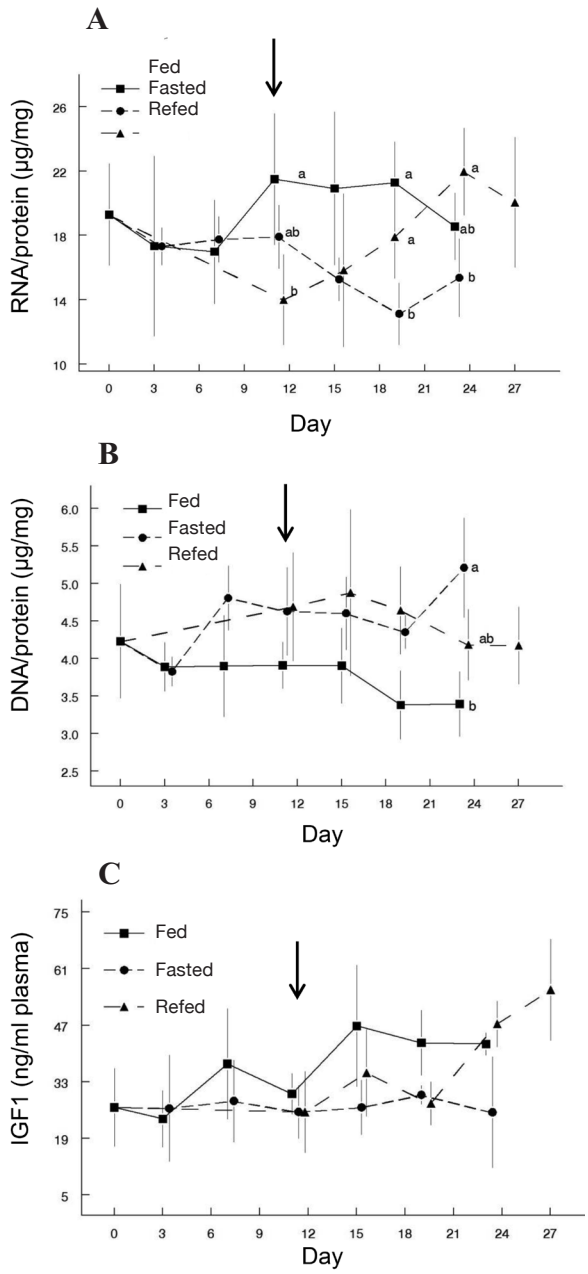

Figure 3

(**A**) RNA/protein (µg/mg); (**B**) DNA/protein (µg/mg) and (**C**) IGF1 (ng/mL plasma) values of postsmolt Atlantic salmon (*Salmo salar*) reared in the laboratory at 12°C. Values are mean values (±standard deviation [SD]) for each sampling day. Fish were either fed ad libitum, fasted, or refed (fed after 11 days of fasting). Within a sampling day, food treatments showing a common superscript, or without superscripts, do not differ significantly (Tukey's HSD multiple range tests). Where error bars would overlap, data are offset to facilitate viewing. The arrow indicates the day when food was introduced to the refed group. For fed and fasted fish, n=4 for each sampling day. For refed fish, n=22 for day 11, n=5 for days 15, 19, and 23 and n=7 for day 27.

cluded RNA/DNA and IGF1 (Table 3). Because RNA/DNA was highly correlated with the other biochemical terms, we also tested all combinations of the remaining 4 terms after eliminating RNA/DNA (15 models). The best candidate for a growth rate model from this grouping included RNA/pro, DNA/pro, and IGF1, and was essentially identical in predictive capability and mathematically equivalent to the model containing RNA/DNA and IGF1 (Table 3).

Summary

RNA/DNA ratios and recent growth rates of fed fish increased throughout the study and were highly correlated. Fed and fasted feeding treatments could be differentiated by RNA/DNA values after 11 days of fasting, and on an individual basis, significant decreases in RNA/DNA values were observed after 7 days of fasting. Growth rates and RNA/DNA values of previously fasted fish increased rapidly beginning 4 days after refeeding. The intercept from regressing growth rate on RNA/DNA was greater in refed fish than in continually fed fish, whereas the slopes were parallel. In the fasted group, the rate of weight loss remained fairly constant throughout the experiment and RNA/DNA values decreased.

In all 3 feeding treatments, RNA/pro values showed similar trends to those of RNA/DNA values; however, there was less statistical differentiation between the feeding treatments on most sampling days. Overall, fasted fish had significantly greater DNA/pro values (smaller cells) than fed fish, yet feeding treatments within a day could not be distinguished on this basis. Mean IGF1 values increased in fed fish but remained constant in fasted fish owing to inconsistent individual responses to fasting. Based on repeated measurements of individuals, IGF1 values responded rapidly (4 days) to refeeding. Owing to high daily variability, feeding treatments within a sampling day could not be distinguished with this index. A positive and significant relation was found between IGF1 and growth rate.

Of the 31 models tested, the best-fit growth rate model included RNA/DNA and IGF1 with a coefficient of determination (r^2) =0.73.

Discussion

The goal of our research and its companion study (Caldarone et al., 2012) was to identify physiological indices that could be obtained by nonlethal means and that would respond rapidly to short-term changes (4–23 days) in the nutritional state (feeding and fasting) of individual postsmolt salmon. The response time of an index allows investigators to match the index to environmental parameters on relevant temporal and spatial scales. Of the four indices we measured in this study, RNA/DNA was the most highly correlated with short-term growth rate. In sexually immature fish, an index that reflects protein production should be appro-

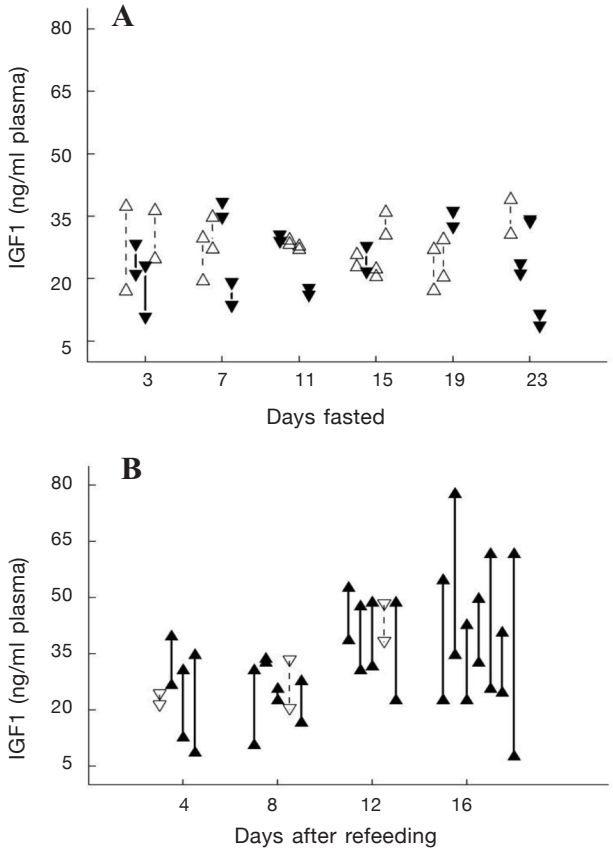

Figure 4

Each arrow represents changes in IGF1 values (ng/mL plasma) of individual laboratory-reared postsmolt Atlantic salmon (*Salmo salar*) that were either (**A**) fasted or (**B**) refed (fed after 11 days of fasting). Direction of the triangle is the direction of change in the values from (**A**) day 0 start values (when fish were fed) through the number of days (23 d) that fish were fasted; and (**B**) day 11 values (start of fasting) through number of days (16 days) that fish were refed after fasting. The open and closed triangles differentiate between the two different directions of change.

priate for estimating growth. At this stage, juvenile fish are directing their energy toward increasing their size to enable them to better escape predators and search for and capture prey (Shulman and Love, 1999). RNA based indices have consistently been shown to be well correlated with both weight-based and protein-based recent growth rates in multiple species of juvenile fish (Arndt et al., 1994; Peck et al., 2003; Stierhoff et al., 2009; Ciotti et al., 2010). RNA/DNA values of our fish responded quickly to changes in food availability. On the basis of repeated measurements of individuals, one-half of the refed fish sampled exhibited increases in RNA/DNA 4 days after food re-introduction, and RNA/DNA values in all refed fish increased by the 8th day. Other researchers have reported statistically significant increases in RNA/DNA and RNA concentration in fish within 1–4 days after being refed (Malloy and Targett, 1994; Stierhoff et al., 2009; Ciotti et al., 2010). The differing response times to refeeding is most likely linked to varying lengths of time fasted before food was re-introduced, to sizes of the fish, and to developmental stage or species. Our fish lost weight after 7 days of fasting and repeated measurements of individuals indicated that RNA/DNA values also decreased within this time frame. Decreases in RNA/DNA and RNA concentration have been observed after 1–14 days in a variety of juvenile fish (Loughna and Goldspink, 1984; Lowery and Somero, 1990; Arndt et al., 1996; Stierhoff et al., 2009; Ciotti et al., 2010). A differing response time of RNA/DNA to fasting is most likely due to temperature, species, developmental stage, and amount of fat stored (i.e., resistance to fasting). For example, Arndt et al.'s (1996) Atlantic salmon fry were much smaller than our postsmolts, averaged a weight loss rate 10× faster (–4.3% vs. –0.36%), and their RNA/DNA values decreased in approximately 1/2 the time compared with that of our postsmolts. But in all instances, response time of RNA/DNA to fasting has been observed over a time period of days to two weeks. This relatively rapid response of RNA/DNA values to short-term changes in food availability would allow researchers to investigate linkages between environmental variables and nutritional status of postsmolt Atlantic salmon on ecologically relevant scales.

The rate of protein accumulation is the difference between the rate of degradation and the rate of protein synthesis, and the rate of protein synthesis is dependent not only on RNA concentration but also its activity (rate of translation) and efficiency, among other factors (see review by Fraser and Rogers, 2007). Our fasted fish lost weight at a fairly constant rate throughout the experiment; however, a wide range of RNA/DNA values (4 to 2.5, Fig. 6) were associated with this negative growth rate and there was a noticeable trend toward lesser values as fasting days increased. These results indicate that, at least initially, the observed weight loss was either due to protein degradation rates increasing or translation rates decreasing (or both) before RNA concentrations decreased. An initial decrease in translation rates preceding a decrease in ribosomal number has been observed in fasting fish (Loughna and Goldspink, 1984; Lowery and Somero, 1990). During a previous study of Atlantic salmon postsmolt (MacLean et al., 2008), we observed a similar range of RNA/DNA values (4 to 2) associated with negative growth rates (Fig. 7). Because both studies were conducted at comparable temperatures and nucleic acids were analyzed with identical methodologies, RNA/DNA values from the two studies can be combined. On the basis of the data from both studies, we propose an RNA/DNA value of 3.0 as a conservative cutoff for distinguishing between positive and negative growth rates in juvenile

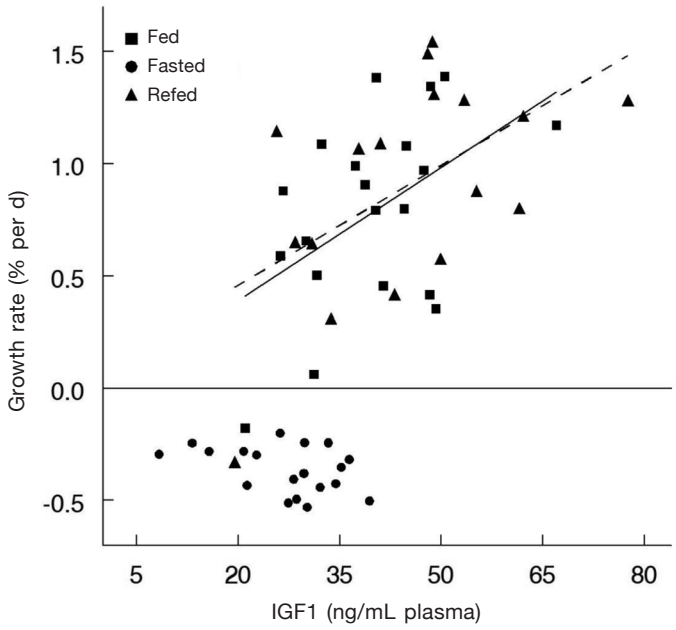

Figure 5

Growth rate (% wet weight per d) versus IGF1 for postsmolt Atlantic salmon (*Salmo salar*) reared in the laboratory at 12°C and sampled throughout a 27-day period. Fish were fed ad libitum, fasted, or refed (fed after 11 days of fasting). Regression lines represent fed (—) and refed (- -) fish.

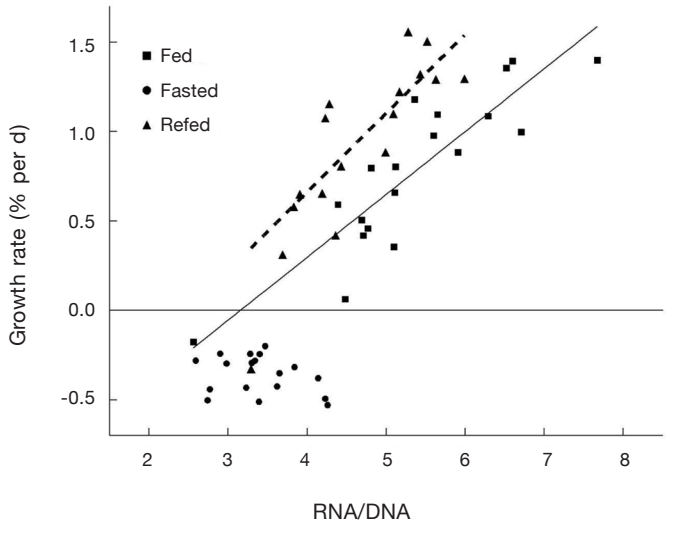

Figure 6

Specific growth rate (% wet weight per d) versus RNA/DNA for postsmolt Atlantic salmon (*Salmo salar*) reared in the laboratory at 12°C and sampled throughout a 27-day period. Fish were fed ad libitum, fasted, or refed (fed after 11 days of fasting). Regression lines represent fed (—) and refed (- -) fish.

Atlantic salmon residing at temperatures near 12°C.

Changes in RNA translation rates also may have influenced the relation of RNA/DNA to growth rate in the refed fish. Refed fish accumulated more protein per unit of RNA than their continually fed counterparts as evidenced by the significantly greater regression intercept (Fig. 6). This increase in protein accumulation could be due to an increase in translation rate or to a decrease in protein degradation rate (or to both). Researchers who have observed an increase in protein accumulation per unit of RNA have attributed it to a translation increase (McMillan and Houlihan, 1988, 1989; Miglavs and Jobling, 1989) although direct published evidence for choosing between the two pathways is scarce. Because traditional estimates of nucleic-acid–based growth-rates do not include a translation rate estimate, field estimates of RNA/DNA–based growth rates will not be exact. Nonetheless, by combining our refed with the fed and fasted data sets we obtained a significant regression between RNA/DNA and growth rate (r^2=0.692, P<0.0001) that can be used to coarsely estimate field growth rates at temperatures near 12°C. Because temperature affects protein degradation and translation rates (Buckley et al., 1999; McCarthy et al., 1999; Ciotti et al., 2010), a model incorporating an interaction term of temperature with RNA/DNA would need to be constructed before growth rates at different temperatures could be estimated.

RNA/pro In our study, RNA/pro values were less correlated with growth rate than the RNA/DNA values, although the relation of both indices to growth rate was similar, i.e., there was a high correlation with positive growth rates but a range of values associated with a constant negative growth rate. Unlike RNA/DNA, RNA/pro from both the fed and refed groups had the same relation to positive growth rates which could be advantageous for estimating field growth rates where the past feeding history of the animal is unknown. Repeated measurements of individuals indicated that RNA/pro responded quickly to food withdrawal (7 days) and reintroduction (4 days), but within any one-day values were variable, resulting in sampling days where the feeding groups could not be differentiated with this index. The positive and negative response of growth rate, RNA/DNA, and RNA/pro in individual fish to the positive and negative changes in food availability, respectively, and the high correlation

Table 3

Coefficients and Akaike's second-order information criterion for small sample sizes (AICc) for the top candidate regression models for growth rate of postsmolt Atlantic salmon (*Salmo salar*) reared at 12ºC under 3 feeding regimens in order to generate a range of nutritional condition and growth rates. RNA/DNA (µg/µg); IGF1=circulating plasma insulin-like growth factor 1 (ng/mL); RNA/pro=RNA/protein (µg/mg); DNA/pro=DNA/protein (µg/mg); ΔAICc=difference in AICc values with respect to the best candidate model. For all models $P<0.0001$. r^2=coefficient of determination.

Dependent variable	*n*	Model	r^2	AICc	ΔAICc
All variables tested					
Growth rate (per d)	53	–0.0181+0.0040(RNA/DNA)+0.0001(IGF1)	0.733	–547.81	0
Growth rate (per d)	53	–0.0173+0.0048(RNA/DNA)	0.686	–542.39	5.42
RNA/DNA not included					
Growth rate (per d)	53	-0.0045+0.0011(RNA/pro)-0.0036(DNA/pro)+0.0001(IGF1)	0.738	–546.53	1.28
Growth rate (per d)	53	0.0001+0.0013(RNA/pro)-0.0045(DNA/pro)	0.712	–543.82	3.99

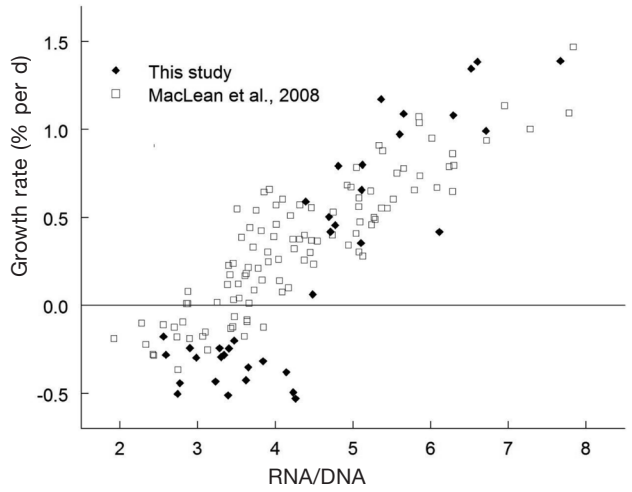

Figure 7

Specific growth rate (% wet weight per d) versus RNA/DNA for postsmolt Atlantic salmon (*Salmo salar*) reared in the laboratory. ♦ denotes data from fed and fasted fish held at 12° C and sampled throughout a 27-day period (present study). ❑ denotes data from the study of MacLean et al. (2008) in which postsmolts were sampled at the end of 30 days at a final water temperature of 12.8°C. In both experiments nucleic acid values were determined by the same method.

of RNA/DNA and RNA/pro to fed and refed growth rates are evidence that the relations between RNA indices and growth rate were not altered by repeated sampling of individual fish.

How best to standardize RNA values (RNA/DNA vs. RNA/pro) is not clear and may depend upon the developmental stage of the fish. Fish muscle is unique in that it increases in size throughout the life of a fish owing to both hyperplasia (increase in cell number) and hypertrophy (increase in cell size) (Weatherly et al., 1988; Higgins and Thorpe, 1990; Koumans et al., 1993; Mommsen, 2001). Hyperplastic muscle growth is accomplished by fusion of myosatellite cells, resulting in a brief initial increase in DNA per cell followed by a nearly constant amount of DNA per cell. In contrast, muscle growth through hypertrophy produces multiple nuclei per cell and often multiple copies of DNA per nucleus (polyploidy) resulting in a variable amount of DNA per cell (Jimenez and Kinsey, 2012). Higgins and Thorpe (1990) investigated muscle growth in Atlantic salmon and concluded that juvenile Atlantic salmon (<15 cm) increased muscle mass by hyperplasia, whereas hypertrophy was more important in autumn and winter when growth of the salmon was slow. Weatherly et al. (1988) concluded that in fish smaller than approximately 44% of their maximum size, most fish muscle growth was due to hyperplasia. Our fish were recent postsmolts, approximately 23% of their maximum size, and most likely increasing their muscle size predominantly through hyperplasia. In our study, RNA/DNA performed better than RNA/pro for indicating short-term growth. In general, RNA/DNA may be the better indicator of growth rate during larval and juvenile stages when a fish is growing rapidly by increasing cell numbers. Until the relation of RNA to DNA in polyploidy cells is better known, RNA/pro may be the preferred indicator of growth rate in older fish where growth by hypertrophy predominates. In adults, however, RNA-based indices may not be an appropriate index of condition or growth rate. In the adult stage, protein synthesis is directed more toward protein turnover rather than protein accretion. Additionally, fat retention or gonad development may be the driving force behind weight-specific growth. This increase in nonprotein mass would cause an uncoupling of the relation of RNA-mass to growth rate. Because the turnover rate of RNAs (mRNA, tRNA, rRNA) ranges from minutes to a few days (see Fraser and Rogers, 2007), RNA-based indices would be most useful for estimating recent growth rates and current nutritional state and would

not be an appropriate index to measure growth rates over long periods of time (months).

DNA/pro Some studies have reported an increase in DNA/pro (or its equivalent DNA/dry weight) during starvation, presumably due to muscle protein being used as an energy source while DNA content remained stable (Fukuda et al., 2001; Mathers et al., 1993; Malzahn et al., 2003). Our results are consistent with this observation with our fasted fish having significantly more DNA per unit of protein than both the fed and refed groups. However, DNA/pro was not strongly correlated with growth rate and feeding treatments within a day could not be distinguished on the basis of this index. Given the complex relation between DNA concentration and hyperplasia and hypertrophy, DNA/pro would not be a good potential physiological index of short-term changes in growth rate or nutritional state in juvenile salmon.

IGF1 IGF1 is an essential component in the endocrine system that regulates growth. Because of this attribute, experiments have been conducted to investigate the endocrine response of the coupled growth hormone and IGF1 systems (GH-IGF1) to nutritional state to help understand how that system regulates growth. Numerous studies have reported decreases in IGF1 in fish fasted for 4 or more weeks (Moriyama et al., 1994; Larsen et al., 2001; Picha et al., 2008) but few studies have investigated its use to assess the nutritional condition of fish over short time periods. Shimizu et al. (2009) reported a decline in IGF1 levels in coho salmon (*Oncorhynchus kisutch*) after 1 week of fasting and a statistically significant decrease after 3 weeks, whereas IGF1 levels in young Chinook salmon (*Oncorhynchus tshawytscha*) decreased significantly 6 days after fasting (Pierce et al., 2005). Based on repeated measurements of individuals in our study, IGF1 values in our fasting fish changed little throughout the 3 weeks. Different resistances to fasting may explain the different results. Of the aforementioned studies, Chinook salmon had low fat levels (3–5% by weight) and had the greatest rate of weight loss. Fat content of our Atlantic salmon was 7–8%; their body composition remained relatively stable throughout the experiment and there was only a small loss of fat in the fasted treatment (Caldarone et al., 2012). Based on repeated measurements of individuals, IGF1 levels in our fish responded rapidly to refeeding; values increased 4 days after the fish were refed. Immature rainbow trout (*Oncorhynchus mykiss*) that had been fasted for 4 weeks also exhibited increases in IGF1 levels 4 days after they were refed (Gabillard et al., 2006), whereas Atlantic salmon smolts after 15 days of fasting showed no change 7 days after being refed (Wilkinson et al., 2006). Further research is needed to determine factors affecting the response time of IGF1 to changes in food availability.

High variability in both the fasted and fed groups, coupled with a small sample size, hampered detection of statistically significant differences in IGF1 between our food treatments. Researchers have suggested that large differences in growth rate or a large sample size may be needed to use IGF1 levels to separate fish by nutritional condition (Beckman et al., 2004a, 2004b) (also see below with regard to serial sampling, acute stress, and IGF1 levels).

A significant linear relation has been observed between circulating plasma IGF1 levels and growth rate in a variety of salmonids (Pierce et al., 2001; Beckman et al., 2004a; Dyer et al., 2004). Beckman (2011) stated a number of caveats for the use of IGF1 as a growth index; in particular, do not compare fish in differing stages of maturation, be aware of issues which may be introduced by rapid changes in temperature, and be aware of potential difficulties which may be introduced by acute stress. Indeed in some studies, nonsignificant relations between IGF1 and growth have been reported (Silverstein et al., 1998; Andrews et al., 2011, in large but not small juvenile lingcod; Beckman et al., 2004b, in juvenile coho salmon soon after transfer to cool water, but not fish maintained in warm water nor fish acclimated to cool water). In our study the relation between IGF1 levels and growth rate was positive and significant but not highly correlative. It is possible that the IGF1 values were affected by our serial sampling protocol (multiple nonlethal blood draws). Pierce et al. (2001) compared IGF1 and growth relations between terminally and serially sampled juvenile coho salmon and found a large decrease in the correlation coefficient (r=0.78 vs r=0.51) between the two protocols. The correlation between IGF1 and growth in the present work (r=0.66) was more in line with Pierce's serially sampled values than with the more highly correlated responses found in studies with terminally sampled values (see Beckman, 2011).

The best candidate model for estimating growth rate did contain both IGF1 and RNA/DNA terms. These two indices reflect differing aspects of the physiology of growth. As part of the GH-IGF1 endocrine system, IGF1 levels reflect a specific stimulus for cellular growth, whereas RNA/DNA is a measure of a cell's capacity for growth. Thus the two measures together would reflect both upstream regulation of cellular growth and downstream response to that regulation.

Circulating levels of plasma IGF1 in fish are regulated by a suite of at least 6 different IGF binding proteins (Duan, 2002). These binding proteins themselves are differentially regulated by nutritional state as well as other factors, perhaps including stress (Kelley et al., 2001). The circulating level of IGF1 in the blood generally is determined by the most abundant binding protein (IGFBP2b), which itself is regulated by nutritional states (Shimizu et al., 2009; Kawaguchi et al., 2013). The establishment of methods to measure IGF binding proteins in fish blood is still on-going (Shimizu et al., 2011a, 2011b) and our understanding of the factors that regulate the abundance of different binding proteins and how they affect IGF1 levels is quite incomplete. We do not have the technical capacity to determine whether or not the current results (IGF1 and

fasting, IGF1 and growth, IGF1 and refeeding) were due to the induction of different suites of binding proteins and how these proteins may have affected circulating IGF1 levels differently from those of other studies. Shimizu et al. (2009) demonstrated a significant increase in 41kDa IGFBP of fasting fish in response to refeeding; perhaps the IGF1 increase we observed in our individual refed fish was modulated by changes in circulating IGFBP levels. The fact that IGF1 levels of fasted fish did increase in response to refeeding and that the overall relation of IGF1 to growth was positive and significant gives us confidence that IGF1 responds to changes in nutrition and growth. The use of IGF1 measures has been demonstrated in several ecological studies of juvenile salmonids where differences in IGF1 levels have been observed in fish reared in different areas (Bond et al., 2014; Daly et al., 2014; Ferriss et al., 2014). However, we suggest that the specific response of IGF1 and IGFBPs to fasting, feeding, and growth, in combination with acute or chronic stress, should be investigated.

Of the indices reported here (RNA/DNA, RNA/pro, DNA/pro, IGF1) and in Caldarone et al. (2012) (Fulton's K, BIA), RNA/DNA was the most suited for estimating recent growth rates and identifying the nutritional condition of our individual postsmolt Atlantic salmon exposed to short-term changes in food availability. Removing muscle samples with a biopsy punch for RNA/DNA analysis did not result in any mortalities and did not appear to inhibit growth of the fish, as evidenced by the rapid increase in growth rates of refed fish soon after a muscle sample was taken. The short response time of RNA/DNA (4–8 days) in individual fish to both positive and negative changes in food availability would allow researchers to investigate linkages between environmental variables and nutritional state on ecologically relevant scales. With the addition of a temperature calibration, estimates of growth rates in the field at a variety of temperatures could be calculated with RNA/DNA values

Acknowledgments

The authors would like to thank E. Baker, and K. Fredrick for assistance in aquarium setup and temperature control, M. Prezioso and J. St. Onge-Burns for help in rearing the salmon, K. Cooper for running the IGF1 analyses, and the University of Rhode Island Graduate School of Oceanography for the use of Blount Aquarium.

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